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The role of gut microbiota in animal gastrointestinal diseases

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The role of gut microbiota in animal gastrointestinal diseases

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Editorial: The role of gut microbiota in animal gastrointestinal diseases

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Editorial on the Research Topic
The role of gut microbiota in animal gastrointestinal diseases

The gut microbiota is a complex micro-ecological system that harbors a diverse range of bacteria, fungi, and viruses (Li et al., 2023; Perez, 2021). Recently, it has received significant attention due to its crucial role in host health and physiological functions. Numerous studies have demonstrated that the gut microbiota is closely associated with the host's nutrient metabolism, digestion, absorption, and the maturation of the immune system (Ansaldo et al., 2019; Wang et al., 2024). Furthermore, research has highlighted its vigorous contributions to intestinal barrier integrity, bone development, epithelial cell differentiation, disease prevention and management (Yuan et al., 2024). As an essential biochemical convertor, the gut microbiota can utilize the food and nutrients ingested by the host to produce metabolites that benefit the host's health, including short-chain fatty acids, antimicrobial peptides, and vitamins (Liu et al., 2019; Marra et al., 2021). These metabolites are critical for maintaining host health and support various physiological functions. However, the gut microbiota is sensitive to alterations induced by a range of external factors. For instance, age, gender, and species are important internal factors that influence the composition and structure of the gut microbiota (Lee et al., 2023). Additionally, external factors such as heavy metals, antibiotics, and pesticides can markedly disrupt the gut microbiota composition, leading to dysbiosis (Kaur and Rawal, 2023; Li et al, 2024). Research has indicated that dysbiosis is closely associated with the development of various diseases, particularly intestinal disorders such as diarrhea, colitis, and colorectal cancer (Wang et al., 2023). Moreover, dysbiosis of the gut microbiota in the intestine, releases metabolites from certain pathogens, such as lipopolysaccharides which can breach the intestinal barrier and jeopardize host health, contributing to the onset of various diseases (Shen et al., 2021). Therefore, maintaining the stability of the gut microbiota is essential for host health. Given the significant role of the gut microbiota in gastrointestinal diseases, an increasing number of studies are dedicated to exploring the potential relationships between

the gut microbiota and the gastrointestinal tract. This Research Topic includes some articles covering all the above aspects.

Rumen acidosis is one of the most prevalent gastrointestinal diseases affecting beef cattle, significantly threatening their health and growth performance, and posing a substantial risk to the beef cattle industry. Despite its importance, relatively few studies examine the potential relationship between rumen acidosis and gut microbiota. Wu et al. conducted a high-throughput sequencing study involving 8 healthy calves and 8 calves diagnosed with rumen acidosis, revealing that rumen acidosis can induce alterations in the composition and diversity of the gut microbiota in calves. Specifically, rumen acidosis was found to affect 70 bacterial genera, with 47 exhibiting increased abundance and 23 showing decreased abundance. Notably, the levels of certain beneficial bacteria, such as Prevotella, Succinivibrio, and Succinivibrionaceae, decreased significantly. These substantial changes in intestinal composition and abundance may serve as critical driving factors for the development of rumen acidosis.

Yaks are an indigenous breed inhabiting the Qinghai-Tibet Plateau, exhibiting strong adaptability to the high-altitude hypoxic environment. Additionally, yaks serve as a vital means of transportation for the residents of the Qinghai-Tibet Plateau and provide milk, meat products, and leather. However, yaks are susceptible to diarrhea caused by *Escherichia coli*, which can lead to significant economic losses and health issues. Diarrhea is one of the primary causes of reduced productivity and mortality in ruminants and is considered a major factor impeding the development of animal husbandry in various countries. Early surveys indicated that diarrhea affects nearly all ruminants, particularly in newborn goats, sheep, cattle, and yaks, whose gastrointestinal tracts are not yet fully developed, resulting in the death of approximately half of these animals. The gut microbiota of ruminants contains a diverse array of beneficial microorganisms, including lactic acid bacteria, bifidobacteria, and lactococci. These probiotics have demonstrated a significant role in managing gastrointestinal diseases in the ruminants. Zhang et al. found that lactic acid bacteria can reduce the translocation rate of pathogens and enhance the intestinal barrier, thereby alleviating diseases caused by *E. coli* in yaks. Consequently, lactic acid bacteria may serve as a promising therapeutic option for treating *E. coli* infections in yaks.

Factors such as transportation, vaccinations, and heat stress can negatively impact calves, resulting in decreased immunity and growth performance, as well as increased morbidity and mortality. The gut microbiota plays a crucial role in host disease resistance, immune system maturation, and metabolism. Furthermore, the gut microbiota is closely associated with host digestion and feed conversion rates. Therefore, enhancing and maintaining the gut microbiota of calves is essential for their health and growth. Yang et al. investigated the effects of supplementation with Shen Qi Bu Qi Powder (SQBQP) on serum biochemistry, antioxidants, gastrointestinal flora, and metabolism in calves. The results indicated that SQBQP supplementation enhanced the growth performance, antioxidant capacity, and digestive enzyme contents in calves. Additionally, SQBQP supplementation improved the gut microbiota and metabolism of calves. This study demonstrates that in light of ongoing demands to reduce antibiotic use, the development of Chinese veterinary compounds aimed at improving the gut microbiota structure of calves is vital for their health and growth.

In recent years, the advancement of science and technology, coupled with the faster turnover of electronic products, has led to explosive growth in industrial production, significantly enhancing people's quality of life. However, a substantial portion of industrial heavy metal products remains ineffectively recyclable, posing serious threats to the surrounding environment as well as the health of both animals and plants. Furthermore, heavy metals can accumulate in plants and subsequently transfer to other animals and humans through the food chain, presenting a considerable risk to public health and food safety. The intestine serves as the primary pathway for heavy metals to enter the host, indicating that both the intestine and gut microbiota are inevitably affected by these metals. Yang et al. discovered that zinc can induce kidney and intestinal damage, disrupt intestinal barrier function, and cause imbalances in gut microbiota in piglets. Additionally, this study demonstrated that disorders in gut microbiota, which are central to the 'gut-kidney' axis, play a significant role in promoting zinc-induced nephrotoxicity. However, supplementation with hesperidin has been shown to mitigate the adverse effects of zinc on piglet health by modulating gut microbiota.

Author contributions

AL: Writing – original draft, Writing – review & editing. FK: Writing – review & editing. JL: Writing – review & editing. FL: Writing – review & editing. Y-FC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could potentially create a conflict of interest.

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References

Ansaldo, E., Slayden, L. C., Ching, K. L., Koch, M. A., Wolf, N. K., Plichta, D. R., et al. (2019). Akkermansia muciniphila induces intestinal adaptive immune responses during homeostasis. *Science.* 364, 1179–1184. doi: 10.1126/science.aaw7479

Kaur, R., and Rawal, R. (2023). Influence of heavy metal exposure on gut microbiota: recent advances. *J. Biochem. Mol. Toxicol.* 37, e23485. doi: 10.1002/jbt.23485

Lee, S. Y., Kim, J. H., Lee, D. Y., and Hur, S. J. (2023). Characterization of gut microbiota in mouse models of aging and sarcopenia. *Microbiol. Res.* 275, 127462. doi: 10.1016/j.micres.2023.127462

Li, A., Liu, F., Si, W., Wang, Y., Wang, D., Yuan, Z., et al. (2024). Pesticide butachlor exposure perturbs gut microbial homeostasis. *Ecotoxicol. Environ. Saf.* 281, 116646. doi: 10.1016/j.ecoenv.2024.116646

Li, A., Wang, Y., Kulyar, M. F., Iqbal, M., Lai, R., Zhu, H., et al. (2023). Environmental microplastic exposure decreases antioxidant ability and perturbs gut microbial homeostasis and metabolism in chickens. *Sci. Total Environ.* 856, 159089. doi: 10.1016/j.scitotenv.2022.159089

Liu, C. S., Liang, X., Wei, X. H., Jin, Z., Chen, F. L., Tang, Q. F., et al. (2019). Gegen qinlian decoction treats diarrhea in piglets by modulating gut microbiota and short-chain fatty acids. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00825

Marra, A., Hanson, M. A., Kondo, S., Erkosar, B., and Lemaitre, B. (2021). Drosophila antimicrobial peptides and lysozymes regulate gut microbiota composition and abundance. *Mbio.* 12, e82421. doi: 10.1128/mBio.00824-21

Perez, J. C. (2021). Fungi of the human gut microbiota: roles and significance. *Int. J. Med. Microbiol.* 311, 151490. doi: 10.1016/j.ijmm.2021.151490

Shen, B., Wang, J., Guo, Y., Gu, T., Shen, Z., Zhou, C., et al. (2021). Dextran sulfate sodium salt-induced colitis aggravates gut microbiota dysbiosis and liver injury in mice with non-alcoholic steatohepatitis. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.756299

Wang, L., Nabi, F., Zhang, X., Zhou, G., Shah, Q. A., Li, S., et al (2024). Effects of lactobacillus plantarum on broiler health: integrated microbial and metabolomics analysis. *Probiotics Antimicrob. Proteins.* 1-19. doi: 10.1007/s12602-024-10336-x

Wang, M., Zheng, H., Wang, S., Luo, H., Li, Z., Song, X., et al. (2023). Comparative analysis of changes in diarrhea and gut microbiota in beigang pigs. *Microb. Pathog.* 185, 106441. doi: 10.1016/j.micpath.2023.106441

Yuan, X., Liu, J., Nie, C., Ma, Q., Wang, C., Liu, H., et al. (2024). Comparative study of the effects of dietary-free and -bound nepsilon-carboxymethyllysine on gut microbiota and intestinal barrier. *J. Agric. Food. Chem.* 72, 5014–5025. doi: 10.1021/acs.jafc.3c09395

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Comparative analysis of the intestinal microbiota of black–necked cranes (*Grus nigricollis*) in different wintering areas

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Fecal microbiota is essential for host health because it increases digestive effectiveness. The crane species *Grus nigricollis* (*G. nigricollis*) is considered to be near threatened. The fecal microbial composition of crane is less understood, particularly in the Tibet, China. This study was performed to investigate the differences in fecal microbial composition and diversity of crane in different wintering areas using third-generation single-molecule real-time sequencing technology in the Tibet, China. According to the findings, 20 samples were used to generate 936 bacterial amplicon sequence variants (ASVs) and 1,800 fungal ASVs, only 4 bacterial ASVs and 20 fungal ASVs were shared in four distinct locations. Firmicutes were the dominant bacterial phylum in all samples, and Ascomycota and Basidiomycota were the dominant fungal phylum. At the genus level, *Lactobacillus* was the dominant genus in Linzhi City (LZ), Shannan City (SN), and Lasa City (LS), whereas Megamonas was the dominant genus in Rikaze City (RKZ). *Naganishia* and *Mycosphaerella* were the dominant fungal genera in SN and RKZ. *Mycosphaerella* and *Tausonia* were the dominant fungal genera in LZ. *Naganishia* and *Fusarium* were the dominant fungal genera in LS. And the fecal microbial composition varied between the four groups, as shown by the underweighted pair-group method with arithmetic means and principal coordinates analysis. This study offers a theoretical basis for understanding the fecal microbial composition of crane.

KEYWORDS

fecal microbiota, Grus nigricollis, Tibet, bacteria, fungi

Introduction

The black-necked crane (*Grus nigricollis* [*G. nigricollis*]) is a unique, rare crane species and a Class I protected animal in China. There are 15 species of crane in the world, but only the black-necked crane is a plateau crane. The northern and western parts of the Qinghai–Tibet Plateau are its breeding areas. The main wintering areas are the Yarlung Zangbo River valley,

the southern Himalayan slope, and the Yunnan Guizhou Plateau. Black-necked cranes can only reproduce and spend the winter in Tibet. Therefore, protecting black-necked cranes in Tibet is essential to their continued existence.

Animal gut microbiota are critical in individual nutrient absorption, metabolic regulation, and immune function. They also play a crucial role in maintaining the organism's health and adaptive evolution (Waite and Taylor, 2015; Gao et al., 2016; Li et al., 2018). Birds serve as a critical environmental indicator organism with their diverse genetic makeup and species diversity. The study of the composition and function of the intestinal microbiota of birds has increased exponentially in recent years due to the development of molecular biology techniques and growing interest in the bird population (Dong et al., 2021; Wang et al., 2021). However, due to the limitations of sampling challenges and low DNA extraction amounts in field conditions, the research was primarily focused on poultry, and there were relatively few studies on the intestinal microbiota of wild birds (Gao et al., 2016). The physiological activities of birds are subject to more substantial selective pressure due to their complex life history characteristics, diverse feeding habits, mating system, physiological characteristics, flying life, long-distance migration, etc., which complicate changes in the intestinal microbiota. The species, environment, life-cycle stage, digestive tract region, and other factors impact the composition and diversity of the intestinal microbiota of birds (Sun et al., 2022). The formation of the intestinal microbiota in birds is mainly influenced by food composition (Youngblut et al., 2018). The composition and essential functions of enterobacteria have been reported to play an important role in maintaining host homeostasis. However, the intestinal microbiota of the black-necked crane in the Tibet Autonomous Region is unknown. Here, we used a third-generation single-molecule real-time sequencing technology to characterize the fecal microbial community and compare the differences in fecal microbial composition and diversity of cranes living in four different geographical regions in Tibet, China.

Materials and methods

Sample collection

Fecal samples were collected from cranes living in four different regions: Linzhi City (LZ), Lasa City (LS), Shannan City (SN), and Rikaze City (RKZ), Tibet, China. The geographical sources and sample codes are shown in Table 1. At each sample location, 20 fresh fecal samples were collected in the morning and thoroughly mixed to form a single composite sample to ensure the experiment was representative. Using sterile disposable forceps to remove the surface, only the middle portions of the fecal samples were collected to avoid contamination from the ground. The samples were then stored in 15 mL sterile centrifuge tubes. The samples were immediately placed in a −20°C portable freezer and stored at −80°C for long-term preservation.

Fecal-sample DNA extraction, amplification, and MiSeq sequencing

According to the manufacturer's instructions, the total DNA was extracted using an OMEGA-soil kit (Omega Bio-Tek, United States, Cat. # D5625-01). The purity and concentration of DNA were assessed using a NanoDrop ND-2000 ultraviolet spectrophotometer (Thermo Scientific, Wilmington, United States), and the DNA quality was determined using 0.8% agarose gel electrophoresis. The bacterial 16S rRNA gene sequence was amplified using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′) (Yang et al., 2018) and the fungal internal transcribed spacer (ITS) sequence was amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) (Shi et al., 2021). A total volume of 25 μL was used for the polymerase chain reaction (PCR), which contained 12.5 μL of 2X Taq PCR MasterMix, 1 μL of forward and reverse primers (10 mM), 1 μL of DNA template, and 9.5 μL of distilled deionized water. The following conditions were used for the PCR amplification: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s; 72°C for 10 min. The electrophoresis of 1% agarose gel was used to monitor the PCR product, which was purified using the MinElute PCR purification kit (Qiagen) and quantified using the Qubit fluorometer (Invitrogen, Waltham, MA, USA). The purified amplicons were sequenced using a PacBio-based single-molecule real-time (SMRT) sequencing platform (Pacific Biosciences, Menlo Park, CA, USA) from Personal Biotechnology Co., Ltd. (Shanghai, China).

TABLE 1 Geographical sources and sample codes.

Sampling Site	Linzhi City	Lasa City	Shannan City	Rikaze City
Habitat type	Nunja	Nunja	Nunja	Nunja
Latitude	29°21′42.7″N	29°51′31.4″N	29°17′22.5″N	29°19′40.3″N
Longitude	94°25′28.5″E	91°21′36.5″E	91°7′4.1″E	88°49′23.1″E
Altitude (m)	2 917	3 705	3 560	3 813
Annual average temperature (°C)	9.30	2.90	8.60	6.30
Annual average precipitation (mm)	652.60	491.00	356.60	400.00
Main food	Grain residues	Grain residues	Grain residues and insects	Plant roots, stems, leaves, and insects

Data processing and analysis

The PacBio Sequel II platform generated the raw sequencing reads. The high-quality sequences were obtained through denoising and filtering using the DADA2 package (version 1.16) in R (version 4.0.3) and Vsearch software (version 2.13.4) (Callahan et al., 2016; Rognes et al., 2016). After quality was controlled, the optimized sequences were transferred into QIIME2 software (version 2019.10) for additional downstream analysis (Bolyen et al., 2019). Amplicon sequence variants (ASVs) were clustered using Uparse software (version 7.1) and a cutoff of >97% (Edgar, 2013). Then, using the Greengenes database (Release 13.8, http://greengenes.secondgenome.com/) for the bacterial community (Quast et al., 2013) and the UNITE database (Release 8.0, https://unite.ut.ee/) for the fungal community (Koljalg et al., 2013), the ASVs were annotated and assigned taxonomies by QIIME2 software (version 2019.10).

Statistical analysis

The Chao1 index, Shannon index, and Simpson index were among the alpha diversity indices of microbiota calculated by QIIME2 and the ggplot2 package of the R project. Alpha diversity indices between certain groups were evaluated using the Kruskal–Wallis test with multiple testing corrections. Venn diagram of software R drew Venn diagrams based on the ASVs sequence to display the number of standard and unique ASVs. A principal component analysis based on Bray–Curtis distance was generated using QIIME2 and the R project ape package to analyze community dissimilarity. Heatmap and hierarchical clustering were sketched using R tools, and the linear discriminant analysis (LDA) effect size was used to assess the specific species differences (Segata et al., 2011).

Results

Sequencing-data analysis

Crane fecal samples yielded 46,460 effective bacterial sequences after quality control, ranging from 9,925 to 13,778 per sample. The length of each effective sequence ranged from 1,385 bp to 1,819 bp, with an average length of 1,476 bp (Table 2). All samples found 60,979 effective fungal sequences, ranging from 12,089 bp to 22,959 bp per sample. The length of each effective sequence ranged from 654 bp to 2,800 bp long, with an average length of 1,209 bp (Table 2). Sequencing coverage and rarefaction curves were used to assess the sequencing depth. The rarefaction curves analysis showed that the sob index reached saturation with increasing sequencing depth, reflecting the actual number of observed species in the samples (Figure S1), indicating that the sequencing data was sufficient and could reflect the existing microbial community in the samples. The sequencing coverage was >99% per sample. After classification matching, 936 bacterial ASVs and 1,800 fungal ASVs were obtained from all samples, with the LZ, SN, LS, and RKZ groups generating 178, 121, 402, and 235 bacterial ASVs, respectively, and 621, 326, 495, and 358 fungal ASVs, respectively. Only 20 fungal ASVs were shared, according to 1.11% of the total fungal ASVs, and only four bacterial ASVs were shared in four distinct locations, according to 0.43% of the total bacterial ASVs, based on the Venn diagram at the ASV level (Figures 1A, B). These findings showed a significant difference in the microbial community composition in the feces of cranes from four different regions.

Alpha diversity analysis

The alpha diversity was calculated by QIIME2 and R project ggplot2 package, including Chao1 and Shannon index, to analyze further the changes in the fecal microbial community of cranes living in different habitats. The results revealed a significant difference between the groups, with LS and RKZ having higher community richness (Chao1 index) of bacterial species and SN having the lowest (Figure 2A). When comparing the bacterial community diversity of the three groups, RKZ exhibited the highest Shannon index. (Figure 2B). LS had higher community richness (Chao1 index) of fungal species than SN, which was significantly different (p = 0.017). However, no significant differences existed among the other habitats (Figure 2C). The Shannon index for the fungal community diversity in LZ was highest, and that in RKZ was lowest, significantly different between LZ and RKZ (p = 0.017). Nevertheless, other habitats had no significant difference (Figure 2D). In conclusion, the alpha diversity in the fecal microbial community of cranes inhabiting four geographical regions differed.

TABLE 2 Amplicon sequence variants and related sequence indexes in the fecal samples of crane.

Samples	Effective sequence number		Average length/bp		Sequencing coverage/%		Number of ASVs	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
LZ	9 925	12 089	1 476	1 209	99.98	99.92	178	621
SN	12 433	13 707	1 476	1 209	99.99	99.93	121	326
LS	10 324	12 224	1 476	1 209	99.91	99.77	402	495
RKZ	13 778	22 959	1 476	1 209	99.98	99.82	235	358

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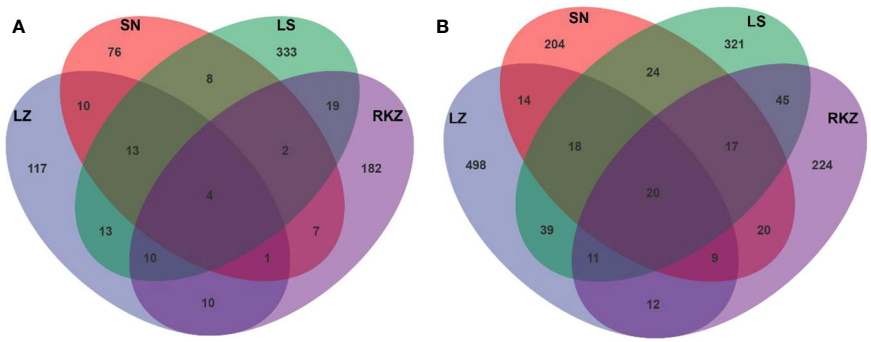

FIGURE 1
Venn diagrams of bacterial **(A)** and fungal **(B)** amplicon sequence variants detected in the fecal samples of crane.

The fecal microbial community composition

The bacterial ASVs were classified into 10 phyla, 22 classes, 39 orders, 109 families, 109 genera, and 129 species across all fecal samples. The fungal ASVs were classified into 9 phyla, 22 classes, 43 orders, 82 families, 125 genera, and 183 species. The fecal microbial composition was analyzed at the phylum and the genus levels. The dominant bacterial phyla in the four groups of the crane were Firmicutes (89.11% in LZ, 99.47% in SN, 87.35% in LS, and 72.56% in RKZ), Proteobacteria (6.37%), and Streptophyta (3.83%) were the second dominant phyla in LZ; Proteobacteria (10.79%) was the second dominant phyla in LS; Proteobacteria (19.04%), Fusobacteria (7.33%), and Tenericutes (1.04%) were the second dominant phyla in RKZ. Except for Firmicutes, no phyla demonstrated an average relative abundance >1% in SN. The findings showed that the bacterial communities in feces from different geographical regions differed (Figure 3A). At the genus level, 15 bacterial genera showed an average relative abundance >1% in all fecal samples, namely *Enterococcus*, *Lactobacillus*, *Clostridium*, unclassified_f_Lachnospiraceae, unclassified_f_Peptostreptococcaceae, *Faecalibacterium*, *Megamonas*, *Fusobacterium*, unclassified_o_Burkholderiales, *Campylobacter*, *Anaerobiospirillum*, *Escherichia*, *Rahnella*, *Serratia*, and *Pseudomonas*. *Lactobacillus* was the dominant genus in LZ (44.90%), SN (96.72%), and LS (67.69%), whereas *Megamonas* was the dominant genus in RKZ (49.84%). Meanwhile, the composition and relative abundance of the bacterial communities differed between groups (Figure 3B). The dominant fungal phyla in the four groups (LZ, SN, LS, and RKZ) of the crane were Ascomycota (23.85%, 31.29%, 34.53%, and 29.83%, respectively) and Basidiomycota (12.35%, 44.29%, 25.29%, and 24.23%, respectively) (Figure 4A). As shown in Figure 4B, at the genus level, 18 fungal genera showed an average relative abundance >1% in the fecal sample, namely *Mycosphaerella*, unclassified_Didymellaceae, *Alternaria*, *Exophiala*, *Botrytis*, *Debaryomyces*, *Dipodascus*, *Fusarium*, *Cephalotrichum*, *Lacrymaria*, *Rhodotorula*, *Sporobolomyces*, *Tausonia*, *Filobasidium*, *Naganishia*, *Saitozyma*, *Sporisorium*, and *Mortierella*. *Naganishia* (38.22% and 6.84%) and *Mycosphaerella* (25.16% and 24.78%) were the dominant fungal genera in SN and RKZ. *Mycosphaerella* (9.54%) and *Tausonia* (5.63%) were the dominant fungal genera in LZ. *Naganishia* (20.80%) and *Fusarium* (16.19%) were the dominant fungal genera in LS. The

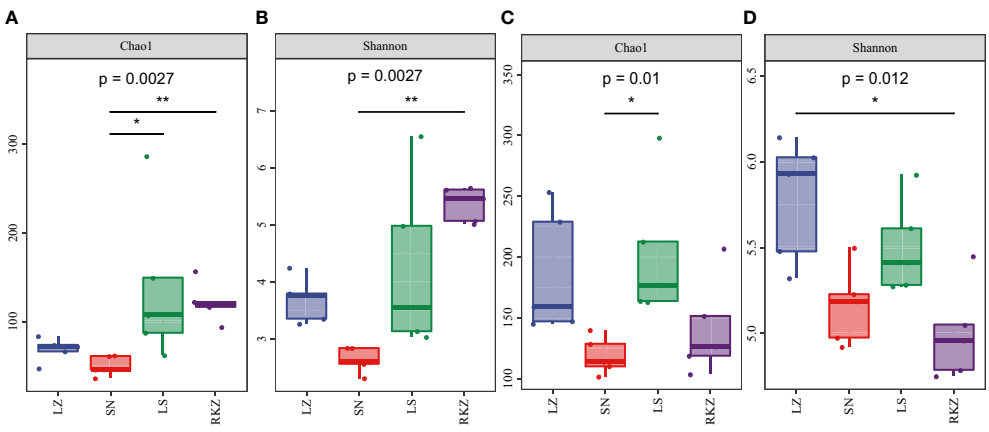

FIGURE 2
Alpha diversity indexes of the bacterial **(A, B)** and the fungal **(C, D)** communities in the fecal samples of crane. * represents $P < 0.05$ and ** represents $P < 0.01$.

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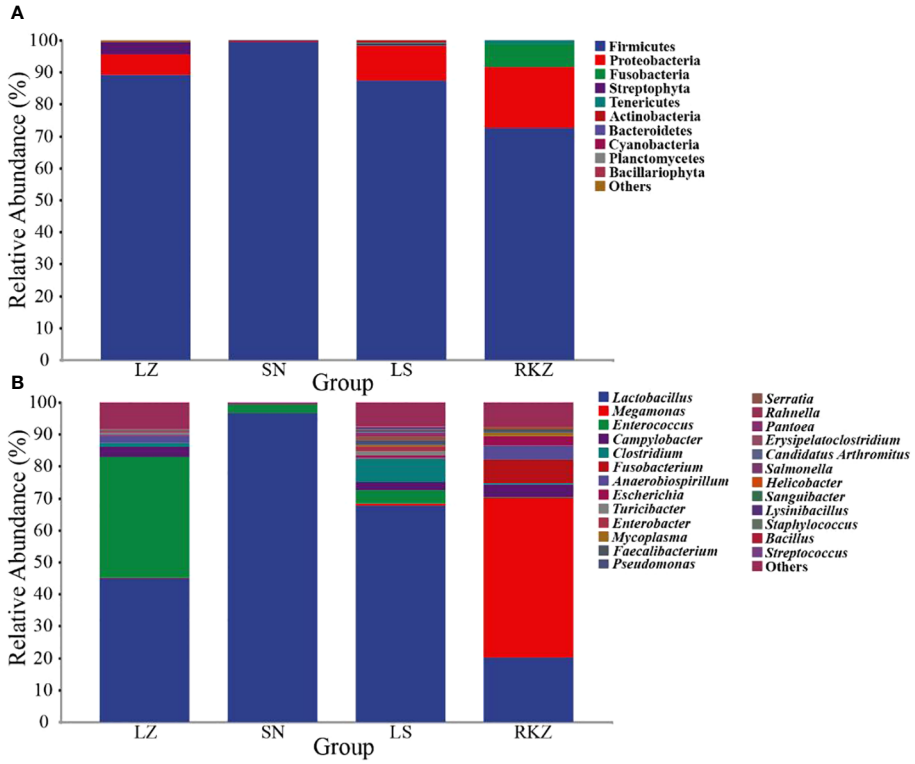

FIGURE 3
Composition of the bacterial community in the fecal samples of the crane at the phylum **(A)** and the genus **(B)** levels.

findings showed differences in the group composition and relative abundance of the dominating fungal genera. Further analysis of the heatmap of microbial community compositions at the species level (Figure 5) indicated that *Campylobacter canadensis*, *Enterococcus faecium*, *Lactobacillus aviaries*, *Fusobacterium mortiferum*, *Escherichia coli*, *Turicibacter* sp. H121, and *Rahnella aquatilis* were the dominant bacterial species, with 20 fungal species showing an average relative abundance >1% in all samples, for example, *Mycosphaerella tassiana*, *Naganishia albidosimilis*, and *Naganishia adeliensis*. However, the dominant microbial species per sample was different.

Beta diversity analysis

The beta diversity analysis, which included hierarchical clustering based on an unweighted pair-group method with arithmetic means at the genus level and principal coordinates analysis (PCoA) based on Bray-Curtis distances, was conducted to explore further the dissimilarity in fecal microbial community composition among various groups. The feces from the same group were significantly clustered, according to the results of the hierarchical clustering analysis, suggesting that the composition of the fecal microbial community was very similar. Comparatively, the bacterial community of all samples was divided into three groups, with the SN and LS constituting the first cluster, LZ the second cluster, and RKZ the third cluster (Figure 6A). This was in contrast to the fungal community, where the RKZ and SN constituted the first cluster, LS the second cluster, and LZ the third cluster (Figure 6B). The PCoA was used to analyze and further display the statistical difference. As shown in Figure 7A, at the genus level, the different explaining rate of PCoA1 and PCoA2 was 54.3% and 20.1%, respectively, with a sum of 74.4% (Figure 7A), which separated the bacterial samples into three groups that matched the results of hierarchical clustering analysis. The different explaining rate of PCoA1 and PCoA2 in the fungal samples was 31.3% and 24.6%, respectively, with a sum of 55.9% (Figure 7B) that separated the fungal samples into four groups. The results of PCoA were similar to hierarchical clustering analysis. These results suggested that various groups had diverse fecal microbial compositions.

Differences in fecal microbial communities between different groups

Linear discriminant analysis effect size (LEfSe), based on an LDA score >4.0, was performed to visualize the significant difference in fecal microbes of cranes between four geographical regions (Figure 8). We screened the biomarker taxa among different groups (Figure 9). According to the LEfSe analysis plot, 37 bacterial characteristics were significantly other among the LZ, SN, LS, and RKZ groups. The four bacterial characteristics (*Enterococcaceae*, *Enterococcus*, *Streptophyta*, and *Viridiplantae*) were richer in the LZ group. *Firmicutes*, *Bacilli,*

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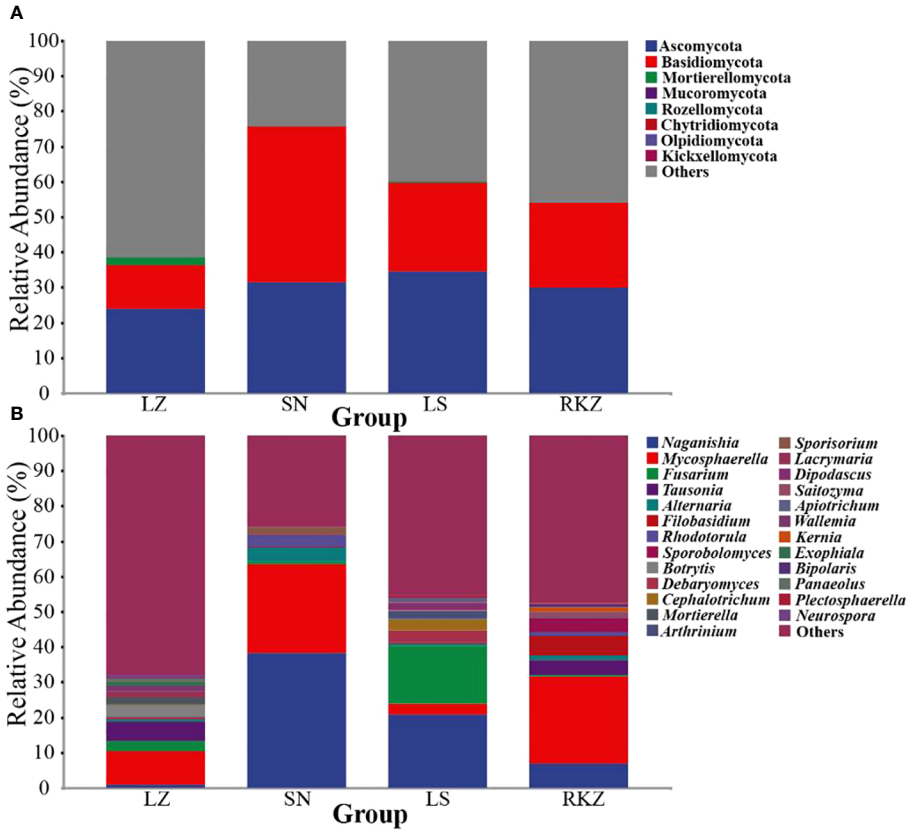

FIGURE 4
Composition of the fungal community in the fecal samples of the crane at the phylum **(A)** and the genus **(B)** levels.

Lactobacillales, *Lactobacillaceae*, and *Lactobacillus*, were revealed to be five bacterial characteristics significantly richer in the SN group. In the LS group, the bacteria *Clostridia, Gammaproteobacteria, Clostridiales, Clostridiaceae, Enterobacteriaceae, Yersiniaceae*, and *Clostridium* had significantly higher abundance than other groups. A unique flora (21 characteristics) was seen in the RKZ group, including *Negativicutes, Selenomonadales, Selenomonadaceae*, and *Megamonas* (Figure 8A, Figure 9A). Eight different fungal characteristics were found in the LZ group, including *Leotiomycetes, Agaricomycetes, Eurotiomycetes, Helotiales, Agaricales, Sclerotiniaceae*, *Tausonia*, and *Botrytis*. Fourteen fungal biomarker taxa were found in the SN group, including *Naganishia*, *Mycosphaerella*, *Rhodotorula*, *Alternaria*, and *Sporisorium*. In comparison to other groups, the LS group had considerably higher abundance values for *Sordariomycetes, Saccharomycetes, Hypocreales, Saccharomycetales, Xylariales, Nectriaceae, Debaryomycetaceae, Apiosporaceae, Fusarium,*

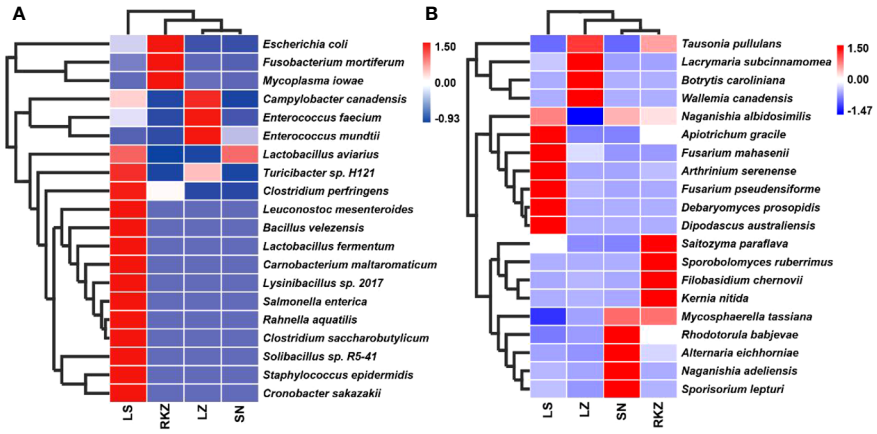

FIGURE 5
Composition of the bacterial **(A)** and the fungal **(B)** communities in the fecal samples of crane at the species levels.

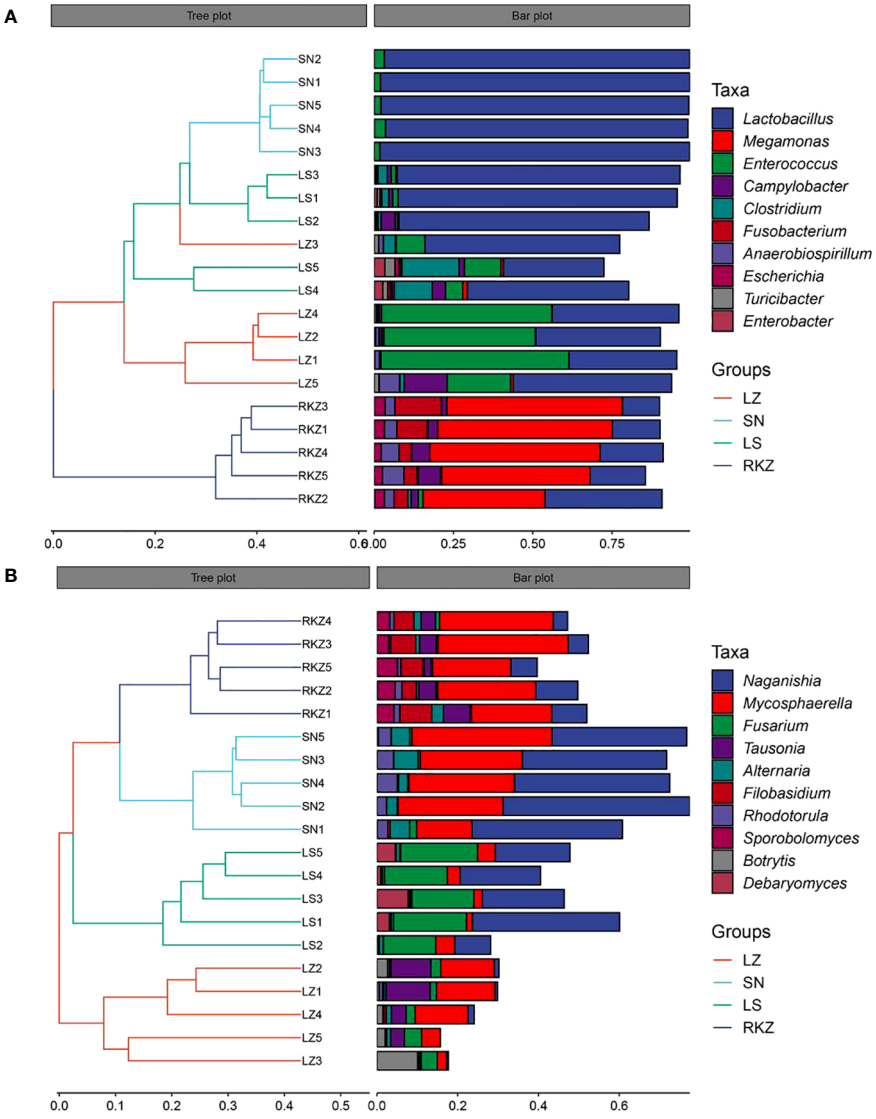

FIGURE 6
Hierarchical clustering of the fecal bacterial **(A)** and fungal **(B)** based on the unweighted pair-group method with arithmetic means at the genus level.

Debaryomyces, Cephalotrichum, and *Arthrinium*. *Microbotryomycetes, Sporidiobolales, Sporidiobolaceae, Filobasidium*, and *Sporobolomyces* were the fungal biomarkers in the RKZ group (Figure 7B, Figure 9B).

Discussion

The IUCN Red List of endangered species classifies the black-necked crane (*G. nigricollis*), one of the 15 crane species found around the world that spends its entire life on the plateau (Liu et al., 2012; Jia et al., 2019), as near endangered. As a flagship species and environmental indicator, crane contributes significantly to preserving the biodiversity of plateau ecosystems (Hou et al., 2021). The primary research areas are population size, distribution, habitat selection, and crane conservation (Kuang et al., 2010; Yang and Zhang, 2014; Li et al., 2022). However, little is understood about the fecal microbiota of cranes. The fecal microbiota plays crucial functions in the host's health by increasing digestive efficiency, maintaining homeostasis, regulating metabolism, and providing immunological protection (Waite and Taylor, 2015; Gao et al., 2016; Li et al., 2018). Here, the third-generation SMRT sequencing technology was used to analyze and compare the fecal microbial community of cranes raised in four different geographical regions. The goal was to provide a starting point for future research with the aim of disease prevention and species protection.

This study analyzed and compared crane fecal bacterial and fungal communities for the first time. The results showed that there were variations in the fecal microbiota. For example, there was no similar trend between the bacterial and fungal communities, and cranes' fecal microbial richness and diversity differed significantly. Previous studies have shown that diet is a crucial determinant of the fecal microbial community (Sun et al., 2022). Black-necked cranes in the LS and RKZ

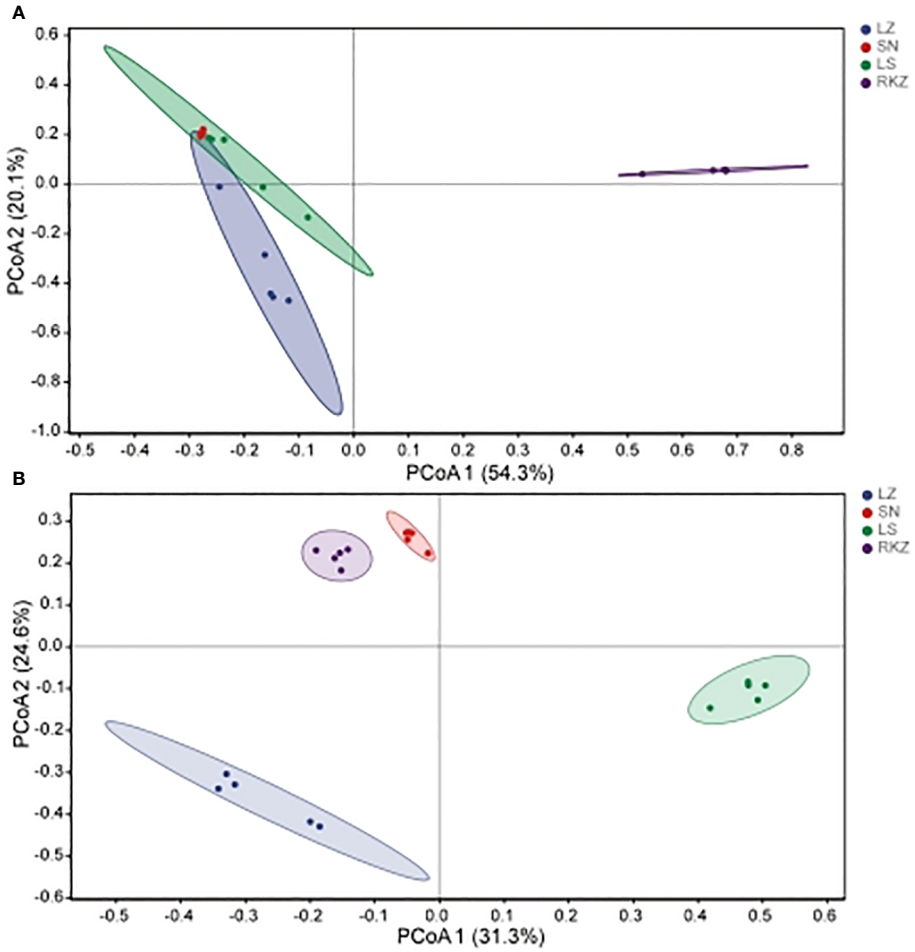

FIGURE 7
Principal component analysis of the bacterial **(A)** and the fungal **(B)** communities in the fecal samples of crane.

were fed potato, barley roots, and *Potentilla anserina L.* in the SN. In contrast, black-necked cranes in the SN mostly foraged *Cyperus rotundus L.*, *Potentilla anserina L.*, and other herbs. Different types of foods impacted how the fecal microbiota colonized (Sun et al., 2022). Meanwhile, some research also showed that long-distance migration primarily affects the bird's fecal microbiota due to the changes in the habitat environment in the migratory process (Phillips et al., 2018; Wu et al., 2018; San Juan et al., 2020). Other wild animals showed similar results (Barelli et al., 2015; Tzeng et al., 2015; Price et al., 2017). The crane that inhabits the Qinghai–Tibet plateau migrated from several breeding areas to wintering areas. Dietary composition and living environment may have contributed to the changes in fecal microbial composition found in cranes from four different geographical regions.

The wild crane was thought to have a distinct intestinal microbiota because of the varied living environments in the various wintering areas. Only four bacterial and 20 fungal ASVs are shared by four different regions. The results matched PCoA. Distinct crane groups had varying fecal microbial compositions. In addition to food resources, other factors such as altitude, age, sex, and other environmental factors may also contribute to these differences in beta diversity. A few studies found that fecal microbial composition was associated with altitude (Zhang et al., 2016; Li et al., 2018). However, the fecal microbial composition changes in cranes were not correlated with changes in the corresponding altitudes. Due to individual differences, the studies found that the age of the host had a significant impact on fecal microbiota (Bibbo et al., 2016). Meanwhile, sex differences have an impact on intestinal microflora (Capunitan et al., 2020). The fecal microbial communities could be influenced by environmental factors such as climate, fauna, flora, and the host's age and sex (Song et al., 2020). Therefore, multiple factors contributed to the variations in the fecal microbiota of cranes found in different geographical regions.

Twenty fecal samples were used in our study, identifying 10 bacterial phyla and 9 fungal phyla. The bacterial phyla were primarily made up of Firmicutes and Proteobacteria, and this result was consistent with earlier studies on other birds, including the *bar-headed goose* (Dong et al., 2021), *Whooper Swan* (Wang et al., 2021), *Great Bustard, Common Coot*, and *Common Crane* (Lu et al., 2022). Animals have a large distribution of Firmicutes, essential to hosts in sustaining energy metabolism (Kaoutari et al., 2013) and as a gauge of fecal health (Ley et al., 2006). Crops, vegetation, and animal debris comprised most black-necked crane diets in the overwintering region (Dong et al., 2016). Therefore, the greater relative abundance of Firmicutes may aid in the ability of black-necked cranes to digest and absorb food nutrients and withstand the harsh environment. According to *hooded cranes*,

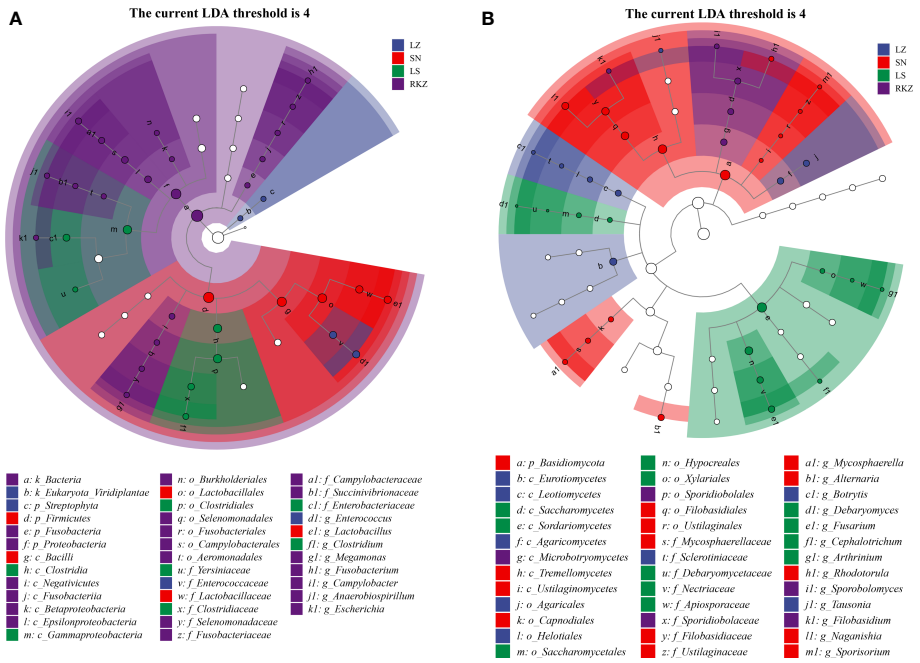

FIGURE 8
Linear discriminant analysis effect size analysis showing the fecal bacterial **(A)** and fungal **(B)** biomarkers with significant differences in the fecal samples of crane (linear discriminant analysis score >4.0).

Ascomycota and Basidiomycota comprised the most fungal species (Wu et al., 2022). To break down complex polysaccharides and enhance the ability of the host to absorb nutrients, several Ascomycota members secret large cellulase and hemicellulase (Linton, 2020; Sun et al., 2022). Maintaining fecal ecological balance and function benefits from the abundance of Ascomycota and Basidiomycota (Li et al., 2021). These dominant phyla play vital roles for cranes in maintaining energy metabolism. Regarding genera, the intestinal microbiota of black-necked cranes comprised 15 dominant bacterial genera and 18 dominant fungal genera. A higher percentage of these were *Lactobacillus*, *Megamonas*, and *Enterococcus*, which were widely distributed in other birds and were influential in regulating intestinal health, metabolic capacity, and antimicrobial activity (Gao et al., 2021; Zhao et al., 2022). Remarkably, we also discovered several genera that are opportunistically pathogenic, including *Pseudomonas*, *Pantoe*, *Escherichia*, *Burkholderia*, *Helicobacter*, *Fusarium*, and *Rhodotorula* (Xiang et al., 2019; Wang et al., 2020; Wu et al., 2022). Overall, the data suggested that monitoring the changes in pathogenic species was important for conserving endangered species. We conducted an LEfSe analysis taking into account the different overwintering regions and found a few unique flora at the genus level, such as *Enterococcus*, *Tausonia*, and *Botrytis* in the LZ group; *Lactobacillus*, *Naganishia*, *Mycosphaerella*, *Rhodotorula*, *Alternaria*, and *Sporisorium* in the SN group; *Clostridium, Fusarium, Debaryomyces, Cephalotrichum*, and *Arthrinium* in the LS group; and *Megamonas*, *Fusobacterium*, *Anaerobiospirillum*, *Campylobacter*, *Escherichia*, *Filobasidium*, and *Sporobolomyces* in the RKZ group. Probiotics such as *Enterococcus*, *Megamonas*, *Clostridium*, and *Lactobacillus* have been proven in studies

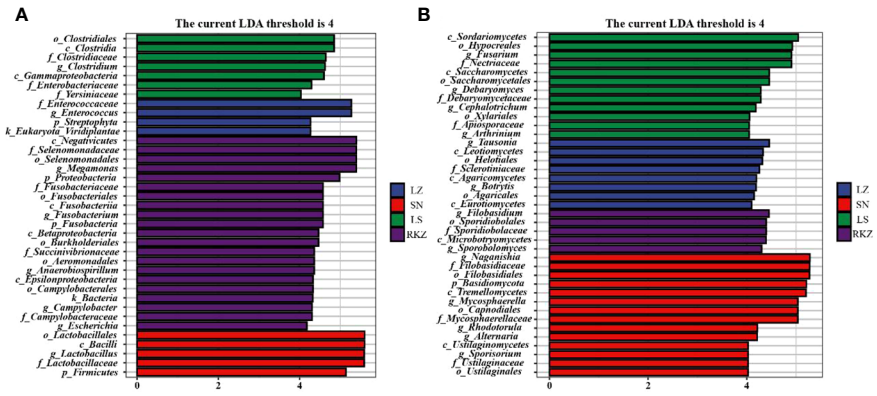

FIGURE 9
The plot from linear discriminant analysis effect size analysis on the fecal bacterial **(A)** and fungal **(B)** biomarkers of crane living in different geographical regions (linear discriminant analysis score >4.0).

to enhance intestinal microenvironment, metabolic capacity, and antimicrobial activity (Yang et al., 2012; Gao et al., 2021; Zhao et al., 2022). However, *Fusarium* and *Rhodotorula* may be harmful to cranes (Wu et al., 2022). It was shown that intestinal micro-ecology modifies environmental changes (such as diet and altitude) by regulating flora abundance, allowing the host to better adapt to the environment. In this study, 129 bacterial species and 183 fungal species were found, including dominant bacterial species, such as *Campylobacter canadensis*, *Enterococcus faecium*, *Lactobacillus aviaries*, *Fusobacterium mortiferum*, *Escherichia coli*, *Turicibacter* sp. H121, and *Rahnella aquatilis* and dominant fungal species, such as *Mycosphaerella tassiana*, *Naganishia albidosimilis*, and *Naganishia adeliensis*. However, the dominant microbial species per sample was different. Traditional culture methods further explored the function of these species in cranes.

In summary, we first analyzed the intestinal microbial community of cranes using third-generation SMRT sequencing technology. We found the difference in fecal microbiota in cranes in different geographical regions. Factors such as diet, climate, and flora may drive this difference. The community composition of the intestinal microbiota in cranes was accurately analyzed in this study, providing a theoretical basis for identifying and applying functional probiotics, disease prevention, and species protection.

Data availability statement

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

Ethics statement

The animal study was approved by The Northeast Forestry University Animal Care Committee reviewed and approved the animal work. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZBW designed the conception and design of the research. ZBW, and EHZ collected the samples. ZBW, EHZ, CZ, KR, PS, SM, YT, and JJW performed analysis and interpretation of data and statistical analysis. ZBW and EHZ wrote the first draft of the manuscript. JZM revised the manuscript for important intellectual content. All authors have read and approved the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1302785/full#supplementary-material

References

Barelli, C., Albanese, D., Donati, C., Pindo, M., Dallago, C., Rovero, F., et al. (2015). Habitat fragmentation is associated with fecal microbiota diversity of an endangered primate: implications for conservation. *Sci. Rep.* 5, 14862. doi: 10.1038/srep14862

Bibbo, S., Ianiro, G., Giorgio, V., Scaldaferri, F., Masucci, L., Gasbarrini, A., et al. (2016). The role of diet on fecal microbiota composition. *Eur. Rev. Med. Pharmacol.Sci* 20, 4742–4749.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N., Abnet, C. C., AlGhalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/NMETH.3869

Capunitan, D. C., Johnson, O., Terrill, R. S., and Hird, S. M. (2020). Evolutionary signal in the fecal microbiomes of 74 bird species from Equatorial Guinea. *Mol. Ecol.* 29, 829–847. doi: 10.1111/mec.15354

Dong, H. Y., Lu, G. Y., Zhong, X. Y., and Yang, X. J. (2016). Winter diet and food selection of the black-necked crane *Grus nigricollis* in Dashanbao, Yunnan, China. *PeerJ.* 4, e1968. doi: 10.7717/peerj.1968

Dong, S. X., Xu, S. J., Zhang, J., Hussain, R., Lu, H., Ye, Y. R., et al. (2021). First report of fecal microflora of wild bar-headed goose in Tibet Plateau. *Front. Vet. Sci.* 8. doi: 10.3389/fvets.2021.791461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996. doi: 10.1038/nmeth.2604

Gao, L., Liu, L., Du, C., and Hou, Q. C. (2021). Comparative analysis of fecal bacterial microbiota of six bird species. *Front. Vet. Sci.* 8. doi: 10.3389/fvets.2021.791287

Gao, G. L., Zhao, X. Z., Li, Q., He, C., Zhao, W. J., Liu, S. Y., et al. (2016). Genome and metagenome analyses reveal adaptive evolution of the host and interaction with the fecal microbiota in the goose. *Microbiota goose. Sci. Rep.* 6 (1), 32961. doi: 10.1038/srep32961

Hou, M. J., Bao, X. K., Ge, J., and Liang, T. G. (2021). Land cover pattern and habitat suitability on the global largest breeding sites for Black-necked Cranes. *J. Clean. Prod.* 322, 128968. doi: 10.1016/j.jclepro.2021.128968

Jia, R., Ma, T., Zhang, F. J., Zhang, G. G., Liu, D. P., and Lu, J. (2019). Population dynamics and habitat use of the Black-necked Crane (*Grus nigricollis*) in the Yarlung Tsangpo River basin, Tibet, China. *Avian Res.* 10, 366–373. doi:

Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D., and Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human fecal microbiota. *Nat. Rev. Microbiol.* 11, 497–504. doi: 10.1038/nrmicro3050

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecology.* 22, 5271–5277. doi: 10.1111/mec.12481

Kuang, F. L., Zhuoma, C., Li, J. C., Le, Y., and Ning, L. (2010). Nest site characteristics and foraging habitat selection of breeding black-necked cranes in bange, Tibet autonomous region, China. *J. Northeast Forestry University.* 38 (11), 89–92. doi: 10.3724/SP.J.1011.2010.01138

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: human fecal microbes associated with obesity. *Nature.* 444, 1022–1023. doi: 10.1038/4441022a

Li, A., Liu, B., Li, F., He, Y., Wang, L., Fakhar-E-Alam, K. M., et al. (2021). Integrated bacterial and fungal diversity analysis reveals the fecal microbial alterations in diarrheic giraffes. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.712092

Li, H., Qu, J., Li, T., Wirth, S., Zhang, Y., Zhao, X., et al. (2018). Diet simplifcation selects for high fecal microbial diversity and strong fermenting ability in high-altitude pikas. *Appl. Microbiol. Biotechnol.* 102, 6739–6751. doi: 10.1007/s00253-018-9097-z

Li, Y. P., Yang, H. M., Xu, L., Wang, Z. Y., Zhao, Y., and Chen, X. S. (2018). Effects of dietary fiber levels on cecal microbiota composition in geese. *Australas. J. Anim. Sci.* 31 (8), 1285–1290. doi: 10.5713/ajas.17.0915

Li, M. M., Zhou, H. M., Bai, J., Zhang, T. X., Liu, Y. X., and Ran, J. H. (2022). *Grus nigricollis* distribution of breeding population and predicting future habitat under climate change of Black-Necked Crane (Przevalski, 1876) in Shaluli Mountains. *Anim. (Basel).* 12 (19), 2594. doi: 10.3390/ani12192594

Linton, S. M. (2020). Review: The structure and function of cellulase (endo-β-1, 4-glucanase) and hemicellulase (β-1, 3-glucanase and endo-β-1, 4-mannase) enzymes in invertebrates that consume materials ranging from microbes, algae to leaf litter. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 240, 110354. doi: 10.1016/j.cbpb.2019.110354

Liu, Q., Li, F. S., Buzzard, P., Qian, F. W., Zhang, F., Zhao, J. L., et al. (2012). Migration routes and new breeding areas of black-necked cranes. *Wilson J. Ornithol.* 124, 704–712. doi: 10.2307/23324519

Lu, Z. Y., Li, S. S., Wang, M., Wang, C., Meng, D. R., and Liu, J. Z. (2022). Comparative analysis of the fecal microbiota of three sympatric terrestrial wild bird species overwintering in farmland habitats. *Front. Microbiol.N.* 13. doi: 10.3389/fmicb.2022.905668

Phillips, J. N., Berlow, M., and Derryberry, E. P. (2018). The effects of landscape urbanization on the fecal microbiome: an exploration into the fecal of urban and rural White-Crowned Sparrows. *Front. Ecol. Evol.* 6. doi: 10.3389/fevo.2018.00148

Price, J. T., Paladino, F. V., Lamont, M. M., Witherington, B. E., Bates, S. T., and Soule, T. (2017). Characterization of the juvenile green turtle (Chelonia mydas) microbiome throughout an ontogenetic shift from pelagic to neritic habitats. *PloS One* 12, e0177642. doi: 10.1371/journal.pone.0177642

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). Vsearch: a versatile open source tool for metagenomics. *Peerj.* 4 (10), e2584. doi: 10.7717/PEERJ.2584

San Juan, P. A., Hendershot, J. N., Daily, G. C., and Fukami, T. (2020). Land-use change has host-specific influences on avian fecal microbiomes. *ISME J.* 14, 318–321. doi: 10.1038/s41396-019-0535-4

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Shi, X. L., Zhao, X. H., Ren, J. Y., Dong, J. L., Zhang, H., Dong, Q. Q., et al. (2021). Influence of peanut, sorghum, and soil salinity on microbial community composition in interspecific interaction zone. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.678250

Song, M., Chan, A. T., and Sun, J. (2020). Influence of the fecal microbiome diet, and environment on risk of colorectal cancer. *gastroenterology.* 158, 322–340. doi: 10.1053/j.gastro.2019.06.048

Sun, F. F., Chen, J. F., Liu, K., Tang, M. Z., and Yang, Y. W. (2022). The avian gut microbiota: Diversity, influencing factors, and future directions. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.934272

Sun, Y., Liu, X., Sun, L., Men, M. Q., Wang, B., Deng, L. T., et al. (2022). Microecological insight to fungal structure and key fungal communities regulating nitrogen transformation based on spatial heterogeneity during cow manure composting by multi-angle and multi-aspect analyses. *Waste Manage.* 142, 132–142. doi: 10.1016/j.wasman.2022.02.013

Tzeng, T. D., Pao, Y. Y., Chen, P. C., Weng, F. C. H., Jean, W. D., and Wang, D. (2015). Effects of host phylogeny and habitats on fecal microbiomes of oriental river prawn (*Macrobrachium nipponense*). *PloS One* 10, e0132860. doi: 10.1371/journal.pone.0132860

Waite, D. W., and Taylor, M. W. (2015). Exploring the avian gut microbiota: current trends and future directions. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00673

Wang, W., Huang, S., Yang, L., and Zhang, G. (2021). Comparative analysis of the fecal bacterial microbiota of wintering Whooper Swans (Cygnus Cygnus). *Front. Vet. Sci.* 8. doi: 10.3389/fvets.2021.670645

Wang, W., Wang, F., Li, L., Wang, A., Sharshov, K., Druzyaka, A, et al. (2020). Characterization of the gut microbiome of black-necked cranes (*Grus nigricollis*) in six wintering areas in China. *Arch. Microbiol.* 202 (5), 983–993. doi: 10.1007/s00203-019-01802-0

Wu, Y. N., Li, Z. H., Zhao, J. R., Chen, Z., and Xiang, X. J. (2022). Significant differences in intestinal fungal community of hooded cranes along the wintering periods. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.991998

Wu, Y. N., Yang, Y. Z., Cao, L., Yin, H. Q., Xu, M. Y., Wang, Z. J., et al. (2018). Habitat environments impacted the fecal microbiome of long-distance migratory swan geese but central species conserved. *Sci. Rep.* 8, 13314. doi: 10.1038/s41598-018-31731-9

Xiang, X. J., Zhang, F. L., Fu, R., Yan, S. F., and Zhou, L. Z. (2019). Significant differences in bacterial and potentially pathogenic communities between sympatric hooded crane and greater white-fronted goose. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00163

Yang, C. M., Cao, G. T., Ferket, P. R., Liu, T. T., Zhou, L., Zhang, L., et al. (2012). Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. *Poult Sci.* 91 (9), 2121–2129. doi: 10.3382/ps.2011-02131

Yang, Y., Li, Y., and Liang, Z. (2018). Assessment of bacterial communities in skin ulceration in cultured sea cucumber apostichopus japonicus (selenka). *Int. Aquat. Res.* 10 (3), 275–282. doi: 10.1007/s40071-018-0205-6

Yang, F., and Zhang, Y. Q. (2014). Quantities and distribution of the black-necked crane (*Grus nigricollis*) and other large waterfowl on the yunnan and guizhou plateau. *Zoological Res.* 35, 80–84. doi: 10.13918/j.issn.2095-8137.2014.s1.0080

Youngblut, N. D., Reischer, G. H., Walters, W., Schuster, N., Walzer, C., Stalder, G., et al. (2018). Host diet and evolutionary history explain different aspects of fecal microbiome diversity among vertebrate clades. *Nat. Commun.* 10 (1), 2200. doi: 10.1101/484006

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zhao, C. Q., Liu, L., Gao, L., and Bai, L. G. (2022). A comprehensive comparison of fecal microbiota in three ecological bird groups of raptors, waders, and waterfowl. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.919111

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Microbial interventions in yak colibacillosis: Lactobacillus-mediated regulation of intestinal barrier

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Introduction: The etiology of Escherichia coli in yaks, along with its drug resistance, results in economic losses within the yak breeding industry. The utilization of lactic acid bacteria treatment has emerged as a viable alternative to antibiotics in managing colibacillosis.

Methods: To elucidate the therapeutic mechanisms of Lactobacillus against Escherichia coli-induced intestinal barrier damage in yaks, we employed yak epithelial cells as the experimental model and established a monolayer epithelial barrier using Transwell. The study encompassed four groups: a control group, a model group (exposed to E. coli O78), a low-dose Lactobacillus group (*E. coli* O78 + 1×10^5CFU LAB), and a high-dose Lactobacillus group (*E. coli* O78 + 1×10^7CFU LAB). Various techniques, including transmembrane resistance measurement, CFU counting, RT-qPCR, and Western Blot, were employed to assess indicators related to cell barrier permeability and tight junction integrity.

Results: In the Model group, Escherichia coli O78 significantly compromised the permeability and tight junction integrity of the yak epithelial barrier. It resulted in decreased transmembrane resistance, elevated FD4 flux, and bacterial translocation. Furthermore, it downregulated the mRNA and protein expression of MUC2, Occludin, and ZO-1, while upregulating the mRNA expression and protein expression of FABP2 and Zonulin, thereby impairing intestinal barrier function. Contrastingly, Lactobacillus exhibited a remarkable protective effect. It substantially increased transmembrane resistance, mitigated FD4 flux, and reduced bacterial translocation. Moreover, it significantly upregulated the mRNA and protein expression of MUC2, Occludin, and ZO-1, while downregulating the mRNA and protein expression of FABP2 and Zonulin. Notably, high-dose LAB demonstrated superior regulatory effects compared to the low-dose LAB group.

Discussion: In conclusion, our findings suggest that Lactobacillus holds promise in treating yak colibacillosis by enhancing mucin and tight junction protein expression. Furthermore, we propose that Lactobacillus achieves these effects through the regulation of Zonulin.

KEYWORDS

yak, Lactobacillus, Zonulin, intestinal barrier, tight junction

1 Introduction

Yak, a cattle species indigenous to the Qinghai-Tibet Plateau, holds a predominant position in Xizang's animal husbandry, constituting a substantial portion of the region's livestock (Liu et al., 2023). Yak colibacillosis, an infectious disease triggered by Escherichia coli (*E. coli*) infection, manifests with typical symptoms such as high fever and diarrhea. It exhibits a high incidence and mortality rate, particularly affecting calf yaks and female yaks. The associated diarrhea poses a significant challenge in the yak breeding industry in recent years. (Rehman et al., 2017). The gastrointestinal tract of adult ruminants undergoes colonization by diverse microorganisms, comprising bacteria, archaea, viruses, protozoa and fungi, culminating in the formation of intestinal microbial barriers (Furman et al., 2020). The colonization of microorganisms in young individuals exerts a lasting influence on the rumen microbial population (Guo et al., 2020). In the case of female yaks, the intestinal microflora they carry is transmitted to newborn calves, influencing the establishment of the calves' intestinal microbiota. Genomic analysis of fecal samples from pre-weaning calves and lactating cows reveals distinctions in the fecal bacterial community, underscoring the impact of age on microbial composition (Haley et al., 2020), Utilizing 16SrRNAsequencing to investigate the gastrointestinal microflora of goats, it was observed that young goats exhibited heightened sensitivity to changes in gastrointestinal flora, rendering them more susceptible to diarrhea symptoms (Wang et al., 2018). This indicates that environmental bacteria acquired by calves have the potential to disrupt intestinal microflora, leading to imbalances and subsequent diarrhea. Gastrointestinal microflora play a crucial role in protecting hosts from external threats and diseases through various mechanisms while also providing nitrogen sources for ruminants. Disturbances in this symbiotic relationship can result in gastrointestinal diseases such as acidosis, nutritional poisoning, abdominal distension and diarrhea (Belanche et al., 2021). Presently, the conventional approach to treating yak colibacillosis involves antibiotic therapy, which, over an extended period, has given rise to concerns related to drug residues and the disruption of intestinal flora (Jia et al., 2018; Dong et al., 2020). Given the intricate physiological structure of the gastrointestinal system in ruminants, solely relying on antibiotic therapy proves insufficient in addressing gastrointestinal flora-related ailments. The escalation in the detection of drug-resistant strains of *E. coli* in recent years has further compounded the challenges associated with preventing and treating yak colibacillosis.

The intestinal barrier serves as a defensive structure that demarcates sterile tissue *in vivo* from the microbiota *in vitro*. Its core mechanism lies in selective permeability (Felipe-López et al., 2023; Rogers et al., 2023). The transport of intestinal molecules from the intestinal lumen to the lamina propria involves two types of mechanisms: membrane receptor-mediated transcellular transport, including Transcellular transport, and Taracellular transport through the intercellular space regulated by Tight Junction (TJ) (Mowat et al., 2004). The Tight Junction of intestinal epithelial cells stands as the primary structure that constitutes the epithelial barrier. Its principal function is to maintain the surface polarity of intestinal epithelial cells, reversibly preventing the diffusion of macromolecules and microorganisms both inside and outside the epithelium (Kucharzik et al., 2001; Yu and Yang, 2009). Numerous proteins contribute to the integrity of the intestinal barrier and tight junction structure. Mucins MUC1 and MUC2 can adhere to pathogenic bacteria, safeguarding the intestinal epithelial mucosa (Cox et al., 2023). Occludin, the first integral membrane protein discovered in tight junction fibers, influences the permeability of the intestinal epithelial cell barrier, modulating the entry of macromolecules (McCarthy et al., 1996; Mazzon et al., 2002). FABP2 expression is inversely correlated with intestinal functional integrity, and its release is regulated by an imbalance in intestinal microbiota (Guerrant et al., 2016; Dutta et al., 2019; Stevens et al., 2018). The Zonula Occludens (ZO) protein family, comprising ZO-1, ZO-2 and ZO-3, acts as the scaffold protein of TJ and belongs to the membrane-associated guanosine kinase-like protein (Maguk) family (González-Mariscal et al., 2000). The double knockout of genes and proteins of ZO-1 and ZO-2 entirely prevents the formation of TJ chains, eliminating the selective permeability of the epithelial barrier (Umeda et al., 2006). Zonulin, the precursor protein of Haptoglobin-2 (pre-Haptoglobin-2), serves as the sole known regulator and marker of intestinal tight junctions. Its upregulation often signifies the disruption of tight junction structures and increased intestinal permeability (Fasano et al., 2000; Clemente et al., 2003; Tripathi et al., 2009; Fasano, 2012). Research indicates that exposure of the small intestine to Gram-negative bacteria induces the secretion of Zonulin, resulting in decreased transmembrane resistance of the mammalian small intestine and enterocyte monolayer. This downregulates the mRNA expression of intestinal tight junction-related proteins, leading to intestinal permeability increased and impaired intestinal barrier function (El Asmar et al., 2002; He et al., 2022). When the intestinal cell barrier is compromised, it becomes more susceptible to invasion by pathogenic microorganisms, fostering the colonization of pathogenic bacteria and triggering inflammation. This vulnerability is a direct contributor to the diarrhea associated with yak colibacillosis.

Lactobacillus (LAB) stands as a prevalent intestinal probiotic in mammals. exerting its influence through the regulation of host intestinal ecology and the intricate interplay among microbial populations (Huang et al., 2022). Existing research underscores the capacity of probiotics, including LAB, to mitigate intestinal inflammatory damage, uphold the integrity of tight junction proteins, diminish intestinal permeability, and fortify overall intestinal barrier function (Embleton and Berrington, 2013; Liu et al., 2013; Orlando et al., 2014). LAB has demonstrated efficacy in attenuating cell apoptosis and modulating the transcription of immune response genes induced by lipopolysaccharide (LPS). Additionally, it down-regulate the expression of genes associated with inflammatory responses (Vale et al., 2023). Notably, LAB strains isolated from newborn calf feces exhibit the potential for co-agglutinate with *E. coli*, thereby conferring resistance against intestinal damage caused by the latter. (Chouraddi et al., 2023). Moreover, probiotic interventions, characterized by a natural symbiosis between probiotics and their host, circumvent concerns related to drug resistance, drug residues, and aberrant host

immunity. As such, this approach emerges as a secure and environmentally sustainable means of addressing colibacillosis in yaks (Bernard, 2023; Zaib et al., 2023).

Despite the wealth of literature documenting the preventive and therapeutic efficacy of LAB in intestinal diseases, limited attention has been directed toward its application in preventing and treating colibacillosis in yaks. Consequently, the present study employs an *in vitro* culture model utilizing yak epithelial cells to investigate LAB's protective effects on the intestinal tract, particularly its resistance to *E. coli* O78. The study seeks to ascertain whether the observed effects are intricately linked to the regulation of the Zonulin protein. By doing so, it aspires to furnish both theoretical and technical underpinnings for the application of LAB in the treatment of yak colibacillosis.

2 Materials and methods

2.1 Preparation of pathogenic *E. coli* and LAB

Pathogenic *E. coli* O78 was sourced from diarrhea-afflicted yaks in Linzhi City, Xizang Autonomous Region, and preserved by the Clinical key Laboratory of the School of Animal Science, Tibet Agricultural and Animal Husbandry University. The bacteria strains were reanimated, inoculated on nutrient Agar, and cultured at 37°C for 24 hours. Single colonies were then transferred to nutritious broth and cultured overnight in a shaker incubator at 37°C. Strain detection employed Eosin-Methylene Blue Agar, and the colony-forming unit (CFU) counting method determined the required strain concentration.

Lactobacillus yoelii Lac-2, isolated from yaks, was also preserved by the Clinical key Laboratory of the School of Animal Science, Tibet Agricultural and Animal Husbandry University. *In vitro* bacteriostatic tests demonstrated the strain's efficacy against *E. coli*, Salmonella, and Staphylococci (Wu et al., 2018). Lactobacillus was reanimated, inoculated on MRS Agar medium, and a single colony was selected and inoculated into MRS liquid medium at 37°C for 24 hours. Strain concentration was determined using the CFU counting method.

2.2 Cell culture and grouping

Yak intestinal epithelial cells were isolated from 30-60-day-old yak fetuses obtained from Linzhi slaughterhouse. Intestinal tissues were dissected into $1mm^3$ and washed with aseptic PBS. The yak intestinal epithelial cells were cultured with DMEM-F12 (Invitrogen, CA, USA) supplemented with 10%FBS (PAN, Adenbach, Germany) in 5%CO_2 and 37°C incubators.

ABC staining kit (SA1002, Boster, Wuhan, China) was employed to detect the binding of Anti-Cytokeratin 18 antibody (BB12213553, Bioss, Beijing, China). Post-digestion, the cells were cultured on cell slides in a six-well plate. The cells were fixed with pre-cooled acetone at 4°C for 10 minutes, washed thrice with PBS, treated with 3% H2O2 deionized water for 10 minutes, again washed thrice with PBS, and then incubated with 5% BSA blocking solution dropwise at 37°C for 30 minutes followed by spin drying. CK18 keratin antibody was added overnight at 4°C, rinsed thrice with PBS, followed by the addition of biotin-labeled goat antibody IgG and incubation at 37°C for 30 minutes. After rinsing thrice with PBS, SABC was added and incubated at 37°C for 30 minutes, followed by thrice rinsing with PBS. The chromogenic solution DAB was added, and color development was monitored under a microscope (AE31E Trinocular 100W, Motic, China). After completion of color development, slides were rinsed with tap water and sealed with neutral gum.

Post-trypsin digestion, the cells were transferred to a 0.4 μm pore diameter PC membrane Transwell chamber for culture, with a cell count of 1×10^5 cells per well. After 2 days, the medium was replaced, and cells were cultured for an additional 12 hours. The groups were divided into four: Control group (Control, normal culture), Model group (Model,1×10^5CFU *E. coli* for 4 hours), Low Dose Lactobacillus group (LLAB, 1×10^5CFU *E. coli* and 1×10^5CFU LAB for 4 hours), High Dose Lactobacillus group (HLAB,1×10^5CFU *E. coli* and 1×10^7CFU LAB for 4 hours).

2.3 Detection of epithelial cell barrier permeability

The integrity of epithelial cell barrier was assessed using transmembrane resistance (TEER), FITC-D4 flux and bacterial translocation. Transmembrane resistance was measured with an electrical resistance system (Millipore, MA, USA). After adding FITC-D4 for 2 hours to the upper chamber of each group, the fluorescence value of the culture medium in the lower chamber was measured. After 4 hours, the lower chamber culture medium was collected for colony-forming unit calculation (CFU) to quantify bacterial translocation.

2.4 Measurement of mRNA expression of MUCIN and TJ proteins

The mRNA expression levels of MUC1, MUC2, Zonulin, FABP2, Occludin, ZO-1 were determined using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Total RNA was extracted using the RNA-easy Isolation Reagent kit (R701, Vazyme, Nanjing, China), and its purity, integrity and concentration were measured by NanoDrop (Thermo Fisher Scientific, Wilmington, NC, USA). Complementary DNA synthesis was performed from 1 μg of total RNA using a first-strand cDNA synthesis kit (K1622, Thermo Fisher Scientific, Wilmington, NC, USA) following the kit protocol. Primers were synthesized by Tsingke Biotech (Tsingke, Beijing, China), and the primer sequences are shown in Table 1. Fluorescence (SYBR Green Master Mix, A25742, Thermo Fisher Scientific, Wilmington, NC, USA) was measured using ABI Prism 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The cycle conditions are 95°C for 3 min and 40 cycles of 95°C for 10 s, and 60°C for 30 s. Using GAPDH as the internal reference, the relative expression of gene mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

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TABLE 1 Real-time PCR Primer.

Primer name	Primer sequence	Product length
MUC1-F	TTGCGCTGGCCATCATCTAT	
MUC1-R	AAGTGGCTGCCAGGTTTGTA	237
MUC2-F	AAGCAGACCTGCCTGAAGAC	
MUC2-R	CAGGTTCACCGTCTGCTCAT	108
FABP2-F	AGACCATGGCGTTTGATGGT	
FABP2-R	TTCAGTTCCATCTGCGAGGC	239
Occludin-F	GTTTGGTTCGCTGGGAGAAGA	
Occludin-R	CATTGGTCGAACGTGCATCTC	200
zonulin-F	CCAAGTACCAGGACGACACC	
zonulin-R	ACCATACTCAGCCACAGCAC	131
ZO1-F	GACCATCGTCTGCGCTATGA	
ZO1-R	CTCTGGTGAGCACGGATTGT	565
GAPDH-F	ACAGTCAAGGCAGAGAACGG	
GAPDH-R	CTCGTGGTTCACACCCATCA	240

2.5 Measurement of protein expression of MUCIN and TJ proteins

Cells in each group were washed with PBS, lysed with RIPA Lysis Buffer (BL504A, Labgic, Beijing, China) on ice for 30 minutes, and centrifuge at 4°C, 12000 rpm for 10 minutes to collect the supernatant. The protein concentration and quality are determined by NanoDrop, then 5 × Loading Buffer was added to prepare the protein lysate. The total protein of each group was separated by 10% SDS-PAGE, the protein was transferred to NC membrane using the wet transfer method, and sealed with 5% skim milk on the shaker for 1 h. After the first antibody (Zonulin, A1571, Abclonal, China; Occludin, A12621, Abclonal, China; MUC1, A21726, Abclonal, China; MUC2, A14659, Abclonal, China; FABP2, A1621, Abclonal, China; ZO-1, A0659, Abclonal, China; β-Actin, AC026, Abclonal, China) was incubated for 2 hours, the membrane was washed with 1 × TBST for 5 times and then washed again for 5 times with 1 × TBST after the second antibody (HRP Goat Anti Rabbit IgG H+L, AS014, Abclonal, China) was incubated for 1 h. The gray value of the bands was obtained by the chemiluminescence method, and β-actin was used as the internal reference. The gray value of the bands was analyzed by ImageJ software for standardized processing.

2.6 Statistical analysis of data

The experiment was repeated more than 3 times in each group, and the data was expressed as mean ± standard deviation. GraphPadPrism9.0 was used to analyze the one-way ANOVA of all data, and then Bonferroni test was used to compare the mean of each group. $P<0.05$, it is considered that the difference is statistically significant.

3 Results

3.1 Epithelial cell identification

Yak intestinal epithelial cells isolated using the tissue block culture method are depicted in Figure 1. staining for CK18 with the ABC kit revealed nuclei of the epithelial cells unstained, while the surrounding nuclei were stained brown, confirming the cultured cells from yak small intestine tissue blocks as epithelial cells.

3.2 Detection of epithelial cell barrier function

The transmembrane resistance of Model group began to decrease at 1 h, reaching about 50% of the control group. The difference was significant at 2 h and 3 h ($P<0.05$). The transmembrane resistance of LLAB and HLAB groups remained at normal levels at all times, showing no significant difference compared with control group($P>0.05$) (Figure 2A). Throughout the duration, the bacterial translocation in the Model group was over twice that of the LLAB and HLAB groups, increasing with time. LAB addition reduced bacterial translocation, with HLAB showing slightly lower levels than LLAB (Figure 2B). At 4 h, the FD4 permeability of Model group was significantly higher than that of Control group ($P<0.05$), while the LLAB and HLAB groups was exhibited significantly lower permeability than the Model group ($P<0.05$) (Figure 2C). Overall, indicators of intestinal cell barrier permeability suggest that LAB addition has a positive therapeutic effect on barrier function.

3.3 mRNA expression of mucin and tight junction protein

After RT-qPCR of mucin and tight junction protein in each group, MUC1 exhibited no significant change. The mRNA levels of MUC2 and ZO-1 in Model group decreased significantly, with Occludin mRNA levels showing an significant decrease. Zonulin and FABP2 mRNA levels increased significantly. LAB addition demonstrated a therapeutic effect on the abnormal expression of these genes caused by *E. coli*, except MUC1. Both low-dose and high-dose LAB significantly decreased the mRNA expression level of Zonulin in Model group. Compared with Model group, the expression levels of FABP2 and ZO-1 mRNA in HLAB group were significantly decreased ($P<0.0l$) (Figure 3).

3.4 Expression of mucin and tight junction protein

Results of Western blotting for MUCIN and TJ proteins are shown in Figure 4. Consistent with mRNA expression, there was no difference in MUC1 protein among the groups ($P>0.05$) (Figure 4C). In Model group, the addition of *E. coli* significantly decreased the expression of MUC2, ZO-1 and Occludin protein ($P<0.05$), and up-regulated the expression of Zonulin and FABP2

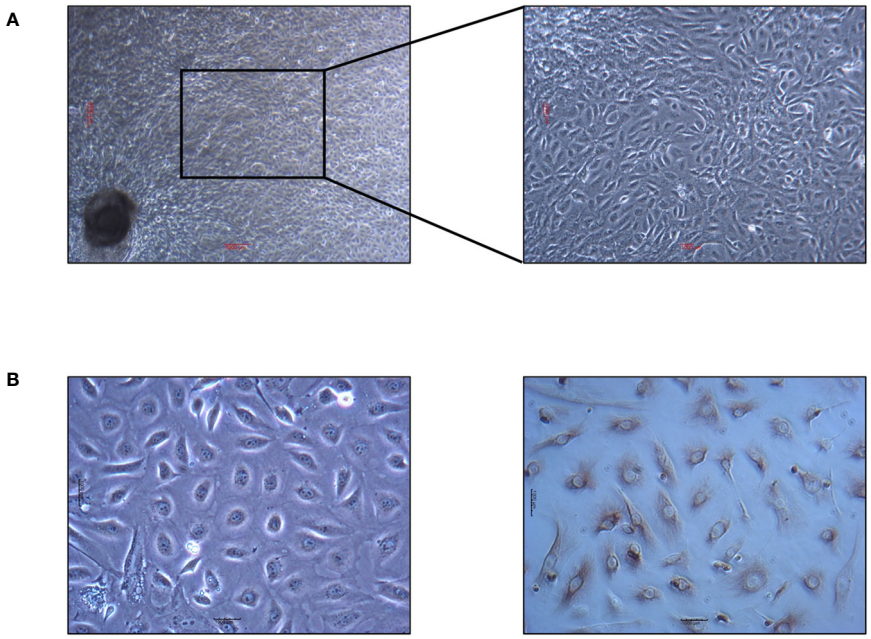

FIGURE 1
Yak intestinal epithelial cells cultured by tissue block culture method and identification results of keratin staining. **(A)** Primary yak small intestinal epithelial cells. The scale in the figure is 5000μm. **(B)** Identification results of keratin CK18 by ABC staining. The scale in the figure is 1000μm.

protein(P<0.05) (Figures 4E, F), aligning with RT-qPCR results. In LLAB and HLAB groups, the abnormal expression of these proteins was restored by LAB addition, with ZO-1 and Occludin proteins exhibiting LAB dose dependence. LAB protected TJ protein and skeleton (Figures 4G, H). For MUC2, the addition of LAB enhanced the stability of the mucous layer of intestinal cells, rendering it ineffective for *E. coli* to disrupt the mucous layer (Figure 4D).

4 Discussion

In this experiment, we employed *in vitro* cultured yak intestinal cells to simulate the impact of pathogenic *E. coli* infection on intestinal barrier function and studied the therapeutic effect of adding LAB to the intestinal barrier. The decrease in TEER caused by *E. coli* infection indicates insufficient intestinal

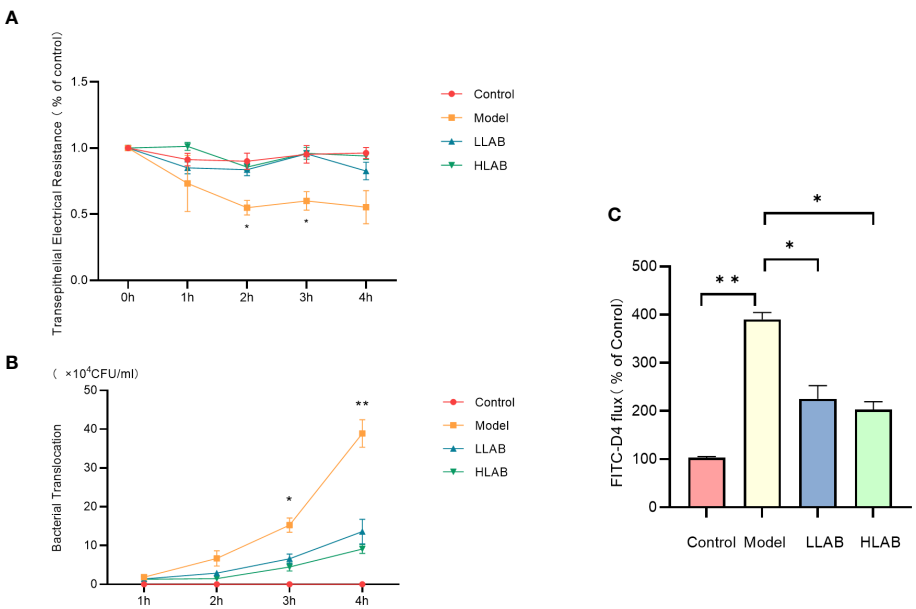

FIGURE 2
Effect of LAB on barrier function of epithelial cells infected with *E coli* O78. **(A)** Transmembrane resistance (TEER) statistical analysis. **(B)** Bacterial translocation statistical analysis. **(C)** FD4 through statistical analysis. * P<0.05, ** P<0.01.

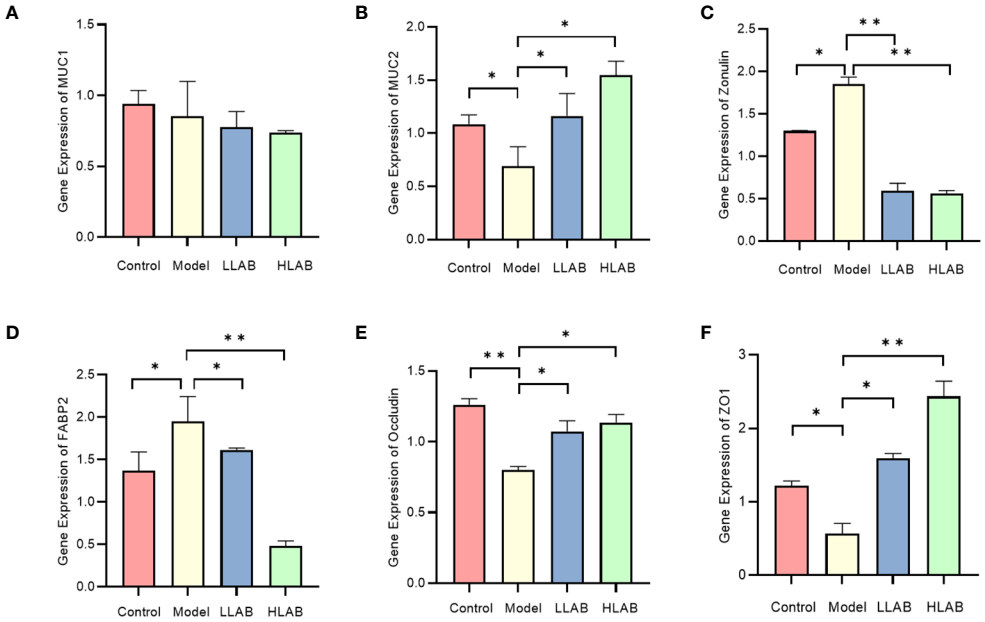

FIGURE 3
The relative expression level of mucin and tight junction protein mRNA. **(A–F)** Statistical analysis of relative expression level of MUC1, MUC2, Zonulin, FABP2, Occludin, ZO-1 mRNA. * $P<0.05$, ** $P<0.01$.

epithelium to maintain normal barrier function, with subsequent damage to the tight junction structure between epithelial cells, as confirmed by subsequent TJ protein detection. The increase in FD4 permeability signifies an increase in intestinal paracellular transport volume. Correspondingly, bacterial translocation increases, causing the loss of selective permeability in epithelial cells (González-Quilen et al., 2021). The addition of LAB restored normal transmembrane resistance, weakened FD4 flux, and reduced the ability of pathogenic bacteria to breach the cellular barrier. This aligns with previous studies, demonstrating LAB's positive impact on barrier function (González-Orozco et al., 2023; Song et al., 2023). To elucidate the mechanism of LAB, we further explored the relationship between LAB and TJ structure and the intestinal mucus layer.

The intestinal tract comprises a physical barrier composed of the TJ structure and a chemical barrier formed by the mucus layer.

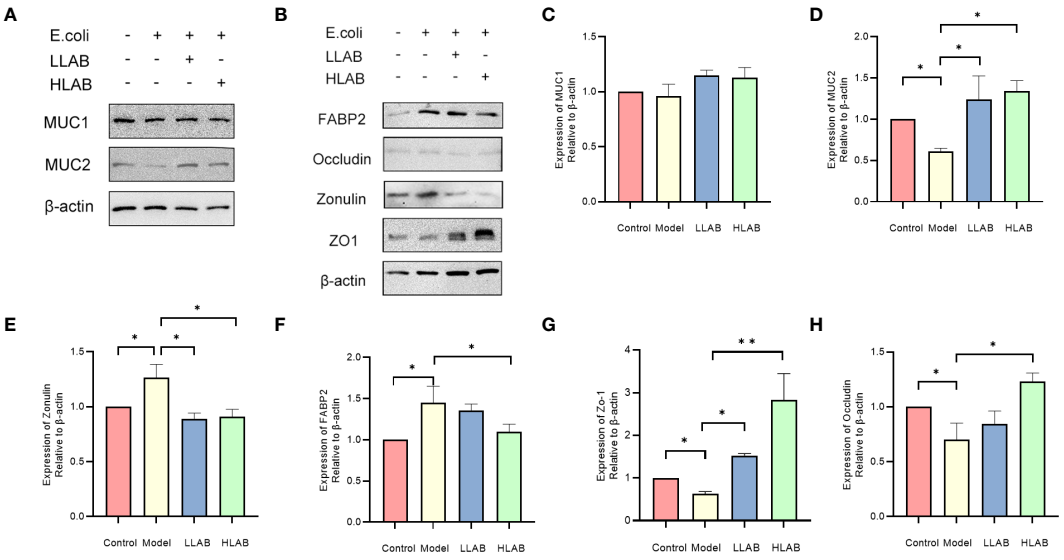

FIGURE 4
Statistical analysis of the expression of mucin and tight junction associated protein in intestinal epithelial cells. **(A)** Chemiluminescence gray image of mucin MUC1 and MCU2. **(B)** Chemiluminescence gray image of FABP2, Occludin, Zonulin, ZO-1 proteins. **(C–H)** Statistical analysis of protein expression levels of MUC1, MUC2, Zonulin, FABP2 and ZO-1,Occludin. * $P<0.05$, ** $P<0.01$.

The former is regulated by TJ proteins, and the latter by MUCIN proteins (Halpern and Denning, 2015). Pathogenic bacteria infection down-regulated ZO-1 and Occludin expression. ZO-1, located at the top of epithelial cells, is a crucial constituent protein of the tight junction structure (Pizzuti et al., 2004; Strauss et al., 2021). We speculate that pathogenic *E. coli* led to ZO-1 protein destruction, resulting in cytoskeleton rearrangement and tight junction structure decomposition. This is generally associated with ZO-1 and myosin 1C phosphorylation (Sturgeon et al., 2017; Pederzolli et al., 2018; Zeng et al., 2023). Similarly, Occludin detection results showed a decrease in Occludin expression caused by pathogenic *E. coli*, leading to the translocation of Occludin in the TJ structure and increased intestinal barrier permeability (Mazzon et al., 2002). After LAB treatment, the expression of ZO-1 and Occludin proteins returned to normal, indicating that LAB can help intestinal epithelial cells resist the destruction of tight junction structure caused by *E. coli*. We also observed changes in FABP2 levels in different treatment groups. The up-regulation of FABP2 expression, negatively correlated with intestinal functional integrity, occurred when *E. coli* infected epithelial cells (Guerrant et al., 2016; Dutta et al., 2019; Bruce et al., 2018). After LAB addition, FABP2 expression returned to control group levels, suggesting that LAB positively affects abnormal FABP2 expression caused by *E. coli* infection.

Zonulin, considered a tight junction regulator, is associated with conditions such as diabetes and bipolar disorder (Craig Sturgeon and Fasano, 2016; Wood Heickman et al., 2020). We detected Zonulin expression in different treatment groups, noting that *E. coli* treatment resulted in Zonulin upregulation, generally considered a sign of intestinal tight junction destruction (Asbjornsdottir et al., 2023; Zhao et al., 2023). This aligns with the down-regulation of tight junction-related proteins ZO-1 and Occludin observed previously. Studies have shown that Zonulin up-regulation-induced changes in tight junction permeability are part of autologous maintenance of intestinal homeostasis, including specific immune response regulation and increased intestinal permeability (Fasano and Shea-Donohue, 2005; Sturgeon and Fasano, 2016; Sturgeon et al., 2017). Zonulin and tight junction protein levels returned to normal in the LAB treatment group, suggesting that LAB may reduce Zonulin's down-regulation on related tight junction proteins and protect the intestinal barrier by affecting the up-regulation of intestinal Zonulin expression caused by Gram-negative bacteria (Serek and Oleksy-Wawrzyniak, 2021). Further research is needed to determine whether LAB directly affects Zonulin expression to regulate tight junction protein expression.

Mucin MUC1 and MUC2 present in the intestinal mucous layer can adhere to pathogenic bacteria, protecting the intestinal epithelial mucosa (Cox et al., 2023). Our results showed that after *E. coli* infection, MUC2 protein was significantly down-regulated, weakening intestinal adhesion and pathogen resistance. However, its function recovered significantly after Lactobacillus treatment, proving that Lactobacillus restored mucous layer function, increased mucin expression, and Lactobacillus extracellular polysaccharides (LAB-EPS) exhibited anti-inflammatory and antibacterial activities. This inhibits *E. coli* growth in the intestinal tract, improving intestinal flora balance and immune function (Wang et al., 2023; Yang et al., 2023a; Yang et al., 2023b). However, there is no difference in the expression of MUC1 among different treatment groups, necessitating further studies to determine whether *E. coli* no effect on intestinal MUC1.

In summary, Lactobacillus can protect intestinal barrier function, improving the down-regulation of tight junction proteins caused by pathogens, balancing the intestinal flora structure, maintaining normal intestinal permeability. Its function may stem from Lactobacillus's regulation of Zonulin protein, affecting the overall TJ structure. This study provides new theoretical support for probiotic treatment of yak colibacillosis, potentially contributing to the economic benefits of the yak breeding industry. Future research will explore whether LAB can still regulate TJ protein after Zonulin gene knockout.

5 Conclusion

LAB can help intestinal epithelial cells resist the destruction of the intestinal barrier caused by *E. coli*. This therapeutic mechanism involves the regulation of MUC2 protein by LAB, enhancing the adhesion function of the intestinal mucous layer. Additionally, LAB regulates TJ-related proteins, enhancing the stability of the TJ between intestinal epithelial cells. The function may also be attributed to the regulation of Zonulin protein by LAB, affecting the overall TJ structure. This study offers new theoretical support for probiotic treatment of yak colibacillosis, with potential contributions to the economic benefits of the yak breeding industry. Future investigations will further verify LAB's regulatory role in TJ proteins after Zonulin gene knockout.

Data availability statement

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

Ethics statement

The requirement of ethical approval was waived by Tibet Agriculture and Animal Husbandry University for the studies involving animals because of sampling of slaughtered yaks from the slaughterhouse of Linzhi City. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

JZ: Data curation, Methodology, Writing – original draft, Writing – review & editing. XR: Methodology, Writing – review

& editing. SW: Methodology, Writing – review & editing. RL: Methodology, Writing – review & editing. BS: Methodology, Writing – review & editing. HD: Writing – review & editing, Methodology. QW: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

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

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References

Asbjornsdottir, B., Sigurdsson, S., Miranda-Ribera, A., Fiorentino, M., Konno, T., Lan, J., et al. (2023). Evaluating prophylactic effect of bovine colostrum on intestinal barrier function in zonulin transgenic mice: A transcriptomic study. *Int. J. Mol. Sci.* 24 (19), 147305. doi: 10.3390/ijms241914730

Belanche, A., Patra, A. K., Morgavi, D. P., Suen, G., Newbold, C., and Yáñez-Ruiz., D. R. (2021). Editorial: gut microbiome modulation in ruminants: enhancing advantages and minimizing drawbacks. *Front. Microbiol.* 11. doi: 10.3389/fmicb.2020.622002

Bernard, N. J. (2023). Probiotics boost immunotherapy. *Nat. Immunol.* 24 (5), 732. doi: 10.1038/s41590-023-01512-2

Chouraddi, R., Kumar, S., Kumar, B., Bhatia, M., Varada, V. V., Tyagi, N., et al. (2023). Techno-functional characterization of fecal lactobacilli isolates of Bos indicus calves for probiotic properties. *Vet. Res. Commun.* 47, 1285–302. doi: 10.1007/s11259-023-10077-2

Clemente, M., De Virgiliis, S., Kang, J. S., Macatagney, R., Musu, M. P., Di Pierro, M. R., et al. (2003). Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut* 52 (2), 218–2235. doi: 10.1136/gut.52.2.218

Cox, K. E., Liu, S., Lwin, T. M., Hoffman, R. M., Batra, S. K., and Bouvet, M. (2023). The mucin family of proteins: candidates as potential biomarkers for colon cancer. *Cancers* 15 (5), 14915. doi: 10.3390/cancers15051491

Dong, H., Liu, B., Li, A., Iqbal, M., Mehmood, K., Jamil, T., et al. (2020). Microbiome analysis reveals the attenuation effect of lactobacillus from yaks on diarrhea via modulation of gut microbiota. *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.610781

Dutta, D., Methe, B., Amar, S., Morris, A., and Lim, S. H. (2019). Intestinal injury and gut permeability in sickle cell disease. *J. Trans. Med.* 17 (1), 1835. doi: 10.1186/s12967-019-1938-8

El Asmar, R., Panigrahi, P., Bamford, P., Berti, I., Not, T., Coppa, G. V., et al. (2002). Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology* 123 (5), 1607–16155. doi: 10.1053/gast.2002.36578

Embleton, N., and Berrington, J. E. (2013). Probiotics reduce the risk of necrotising enterocolitis (NEC) in preterm infants. *BMJ Evidence-Based Med.* 18 (6), 219–2205. doi: 10.1053/gast.2002.36578

Fasano, A. (2012). Intestinal permeability and its regulation by zonulin: diagnostic and therapeutic implications. *Clin. Gastroenterol. Hepatol.* 10 (10), 1096–1100. doi: 10.1016/j.cgh.2012.08.012

Fasano, A., Not, T., Wang, W., Uzzau, S., Berti, I., Tommasini, A., et al. (2000). Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 355(9214), 1518–1519. doi: 10.1016/s0140-6736(00)02169-3

Fasano, A., and Shea-Donohue, T. (2005). Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat. Clin. Pract. Gastroenterol. Hepatol.* 2 (9), 416–422. doi: 10.1038/ncpgasthep0259

Felipe-López, A., Hansmeier, N., Danzer, C., and Hensel, M. (2023). Manipulation of microvillar proteins during Salmonella enterica invasion results in brush border effacement and actin remodeling. *Front. Cell. Infect. Microbiol.* 13. doi: 10.3389/fcimb.2023.1137062

Furman, O., Shenhav, L., Sasson, G., Kokou, F., Honig, H., Jacoby, S., et al. (2020). Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat. Commun.* 11 (1), 19045. doi: 10.1038/s41467-020-15652-8

González-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Semin. Cell Dev. Biol.* 11 (4), 315–324. doi: 10.1006/scdb.2000.0178

González-Orozco, B. D., Kosmerl, E., Jiménez-Flores, R., and Alvarez, V. B. (2023). Enhanced probiotic potential of Lactobacillus kefiranofaciens OSU-BDGOA1 through co-culture with Kluyveromyces marxianus bdgo-ym6. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1236634

González-Quilen, C., Grau-Bové, C., Jorba-Martín, R., Caro-Tarragó, A., Pinent, M., Ardévol, A., et al. (2021). Protective properties of grape-seed proanthocyanidins in human ex vivo acute colonic dysfunction induced by dextran sodium sulfate. *Eur. J. Nutr.* 60 (1), 79–885. doi: 10.1007/s00394-020-02222-3

Guerrant, R. L., Leite, A. M., Pinkerton, R., Medeiros, P. H., Cavalcante, P. A., DeBoer, M., et al. (2016). Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PloS One* 11 (9), e0158772. doi: 10.1371/journal.pone.0158772

Guo, C. Y., Ji, S. K., Yan, H., Wang, Y. J., Liu, J. J., Cao, Z. J., et al. (2020). Dynamic change of the gastrointestinal bacterial ecology in cows from birth to adulthood. *Microbiologyopen* 9 (11), e1119. doi: 10.1002/mbo3.1119

Haley, B. J., Kim, S. W., Salaheen, S., Hovingh, E., and Van Kessel, J. A. S. (2020). Differences in the microbial community and resistome structures of feces from preweaned calves and lactating dairy cows in commercial dairy herds. *Foodborne Pathog. Dis.* 17 (8), 494–503. doi: 10.1089/fpd.2019.2768

Halpern, M. D., and Denning, P. W. (2015). The role of intestinal epithelial barrier function in the development of NEC. *Tissue Barriers* 3 (1-2), e1000707. doi: 10.1080/21688370.2014.1000707

He, L., Wang, C., Simujide, H., Aricha, H., Zhang, J., Liu, B., et al. (2022). Effect of early pathogenic escherichia coli infection on the intestinal barrier and immune function in newborn calves. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.818276

Huang, R., Wu, F., Zhou, Q., Wei, W., Yue, J., Xiao, B., et al. (2022). Lactobacillus and intestinal diseases: Mechanisms of action and clinical applications. *Microbiol. Res.* 260, 127019. doi: 10.1016/j.micres.2022.127019

Jia, Z., Chen, A., Bao, F., He, M., Gao, S., Xu, J., et al. (2018). Effect of nisin on microbiome-brain-gut axis neurochemicals by Escherichia coli-induced diarrhea in mice. *Microbial. Pathogen.* 119, 65–71. doi: 10.1016/j.micpath.2018.04.005

Kucharzik, T., Walsh, S. V., Chen, J., Parkos, C. A., and Nusrat, A. (2001). Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *Am. J. Pathol.* 159 (6), 2001–2009. doi: 10.1016/s0002-9440(10)63051-9

Liu, X., Gao, J., Liu, S., Cheng, Y., Hao, L., Liu, S., et al. (2023). The uniqueness and superiority of energy utilization in yaks compared with cattle in the highlands: A review. *Anim. Nutr.* 12, 138–144. doi: 10.1016/j.aninu.2022.09.011

Liu, Z.-H., Huang, M.-J., Zhang, X.-W., Wang, L., Huang, N.-Q., Peng, H., et al. (2013). The effects of perioperative probiotic treatment on serum zonulin concentration and subsequent postoperative infectious complications after colorectal cancer surgery: a double-center and double-blind randomized clinical trial. *Am. J. Clin. Nutr.* 97 (1), 117–1265. doi: 10.3945/ajcn.112.040949

Mazzon, E., Sturniolo, G. C., Puzzolo, D., Frisina, N., and Fries., W. (2002). Effect of stress on the paracellular barrier in the rat ileum. *Gut* 51 (4), 507–5135. doi: 10.1136/gut.51.4.507

McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S., Rogers, R. A., et al. (1996). Occludin is a functional component of the tight junction. *J. Cell Sci.* 109 (Pt 9), 2287–2298. doi: 10.1242/jcs.109.9.2287

Mowat, A. M., Millington, O. R., and Chirdo., F. G. (2004). Anatomical and cellular basis of immunity and tolerance in the intestine. *J. Pediatr. Gastroenterol. Nutr.* 39, S723–S724. doi: 10.1097/00005176-200406003-00003

Orlando, A., Linsalata, M., Notarnicola, M., Tutino, V., and Russo, F. (2014). Lactobacillus GG restoration of the gliadin induced epithelial barrier disruption: the role of cellular polyamines. *BMC Microbiol.* 14 (1), 1–125. doi: 10.1186/1471-2180-14-19

Pederzolli, R.-L. A., Van Kessel, A. G., Campbell, J., Hendrick, S., Wood, K. M., and Penner, G. B. (2018). Effect of ruminal acidosis and short-term low feed intake on indicators of gastrointestinal barrier function in Holstein steers. *J. Anim. Sci.* 96 (1), 108–1255. doi: 10.1093/jas/skx049

Pizzuti, D., Bortolami, M., Mazzon, E., Buda, A., Guariso, G., D'Odorico, A., et al. (2004). Transcriptional downregulation of tight junction protein ZO-1 in active coeliac disease is reversed after a gluten-free diet. *Dig Liver Dis.* 36 (5), 337–341. doi: 10.1016/j.dld.2004.01.013

Rehman, M. U., Zhang, H., Iqbal, M. K., Nabi, F., Huang, S., Lan, Y., et al. (2017). Antibiotic Resistance of Escherichia coli in Free-Ranging Yaks (Bos grunniens) from Tibetan Plateau, China. *Pakistan Vet. J.* 37 (2), 139-144.

Rogers, A. P., Mileto, S. J., and Lyras, D. (2023). Impact of enteric bacterial infections at and beyond the epithelial barrier. *Nat. Rev. Microbiol.* 21 (4), 260–274. doi: 10.1038/s41579-022-00794-x

Serek, P., and Oleksy-Wawrzyniak, M. (2021). The effect of bacterial infections, probiotics and zonulin on intestinal barrier integrity. *Int. J. Mol. Sci.* 22 (21). doi: 10.3390/ijms222111359

Song, R., McAlpine, W., Fond, A. M., Nair-Gill, E., Choi, J. H., Nyström, E. E. L., et al. (2023). Trans-Golgi protein TVP23B regulates host-microbe interactions via Paneth cell homeostasis and Goblet cell glycosylation. *Nat. Commun.* 14 (1), 36525. doi: 10.1038/s41467-023-39398-1

Stevens, B. R., Goel, R., Kim, S., Richards, E. M., Holbert, R. C., Pepine Carl, J., et al. (2018). Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. *Gut* 67 (8), 15555. doi: 10.1136/gutjnl-2017-314759

Strauss, R. E., Mezache, L., Veeraraghavan, R., and Gourdie, R. G. (2021). The cx43 carboxyl-terminal mimetic peptide αCT1 protects endothelial barrier function in a ZO1 binding-competent manner. *Biomolecules* 11 (8), 11925. doi: 10.3390/biom11081192

Sturgeon, C., and Fasano, A. (2016). Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. *Tissue barriers* 4 (4), e12513845. doi: 10.1080/21688370.2016.1251384

Sturgeon, C., Lan, J., and Fasano, A. (2017). Zonulin transgenic mice show altered gut permeability and increased morbidity/mortality in the DSS colitis model. *Ann. N Y Acad. Sci.* 1397 (1), 130–142. doi: 10.1111/nyas.13343

Tripathi, A., Lammers, K. M., Goldblum, S., Shea-Donohue, T., Netzel-Arnett, S., Buzza, M. S., et al. (2009). Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. *Proc. Natl. Acad. Sci.* 106 (39), 16799–168045. doi: 10.1073/pnas.0906773106

Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., et al. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* 126 (4), 741–7545. doi: 10.1016/j.cell.2006.06.043

Vale, G. C., Mota, B. I., Ando-Suguimoto, E. S., and Mayer, M. P. A. (2023). Lactobacilli probiotics modulate antibacterial response gene transcription of dendritic cells challenged with LPS. *Probio. Antimicrobial. Proteins*. doi: 10.1007/s12602-023-10043-z

Wang, L., Lin, Z., Ali, M., Zhu, X., Zhang, Y., Li, S., et al. (2023). Effects of lactic acid bacteria isolated from Tibetan chickens on the growth performance and gut microbiota of broiler. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1171074

Wang, Y., Zhang, H., Zhu, L., Xu, Y., Liu, N., Sun, X., et al. (2018). Dynamic distribution of gut microbiota in goats at different ages and health states. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02509

Wood Heickman, L. K., DeBoer, M. D., and Fasano, A. (2020). Zonulin as a potential putative biomarker of risk for shared type 1 diabetes and celiac disease autoimmunity. *Diabetes Metab. Res. Rev.* 36 (5), e3309. doi: 10.1002/dmrr.3309

Wu, Q., Zhang, H., Mehmood, K., Li, K., Chang, Z., Jiang, X., et al. (2018). Biological characteristics and phylogenetic analysis of lactic acid bacteria isolated from free-range yaks (Bos grunniens) in Qinghai-Tibet plateau. *Int. J. Agric. Biol.* 20, 902–906. doi: 10.17957/IJAB/15.0596

Yang, C., Zhu, J., Bai, J., Zhang, J., Wu, Z., Li, X., et al. (2023a). Effects of lactobacillus on the differentiation of intestinal mucosa immune cells and the composition of gut microbiota in soybean-sensitized mice. *Foods* 12 (3), 6275. doi: 10.3390/foods12030627

Yang, S., Xu, X., Peng, Q., Ma, L., Qiao, Y., and Shi, B. (2023b). Exopolysaccharides from lactic acid bacteria, as an alternative to antibiotics, on regulation of intestinal health and the immune system. *Anim. Nutr.* 13, 78–89. doi: 10.1016/j.aninu.2023.02.004

Yu, Q.-H., and Yang, Q. (2009). Diversity of tight junctions (TJs) between gastrointestinal epithelial cells and their function in maintaining the mucosal barrier. *Cell Biol. Int.* 33 (1), 78–825. doi: 10.1016/j.cellbi.2008.09.007

Zaib, S., Hayat, A., and Khan, I. (2023). Probiotics and their beneficial health effects. *Mini Rev. med. Chem.* 24 (1), 110–125. doi: 10.2174/1389557523666230608163823

Zeng, J., Li, X., Yin, L., Chen, T., and Hou, J. (2023). Porphyromonas gingivalis infection causes umbilical vein endothelial barrier dysfunction in *vitro* by down-regulating ZO-1, occludin and VE-cadherin expression. *Nan Fang yi ke da xue xue bao= J. South. Med. Univ.* 43 (2), 287–293. doi: 10.12122/j.issn.1673-4254.2023.02.18

Zhao, X., Zhang, T., Zheng, Y., Zhao, Z., Ding, W., Zhang, Z., et al. (2023). Gut microbiota from short-chain chlorinated paraffin-exposed mice promotes astrocyte activation by disrupting the intestinal tight junction via zonulin upregulation. *J. Agric. Food Chem.* 71 (21), 8192–82025. doi: 10.1021/acs.jafc.3c01058

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Correlation of caecal microbiome endotoxins genes and intestinal immune cells in *Eimeria tenella* infection based on bioinformatics

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Introduction: The infection with *Eimeria tenella* (ET) can elicit expression of various intestinal immune cells, incite inflammation, disrupt intestinal homeostasis, and facilitate co-infection with diverse bacteria. However, the reciprocal interaction between intestinal immune cells and intestinal flora in the progression of ET-infection remains unclear.

Objective: The aim of this study was to investigate the correlation between cecal microbial endotoxin (CME)-related genes and intestinal immunity in ET-infection, with subsequent identification of hub potential biomarker and immunotherapy target.

Methods: Differential expression genes (DEGs) within ET-infection and hub genes related to CME were identified through GSE39602 dataset based on bioinformatic methods and Protein-protein interaction (PPI) network analysis. Moreover, immune infiltration was analyzed by CIBERSORT method. Subsequently, comprehensive functional enrichment analyses employing Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis along with Gene Ontology (GO), gene set enrichment analysis (GSEA), and gene set variation analysis (GSVA) were performed.

Results: A total of 1089 DEGs and 25 hub genes were identified and CXCR4 was ultimately identified as a essential CME related potential biomarker and immunotherapy target in the ET-infection. Furthermore, activated natural killer cells, M0 macrophages, M2 macrophages, and T regulatory cells were identified as expressed intestinal immune cells. The functional enrichment analysis revealed that both DEGs and hub genes were significantly enriched in immune-related signaling pathways.

Conclusion: CXCR4 was identified as a pivotal CME-related potential biomarker and immunotherapy target for expression of intestinal immune cells during ET-infection. These findings have significant implications in elucidating the intricate interplay among ET-infection, CME, and intestinal immunity.

KEYWORDS

Eimeria tenella, intestinal immune cells, cecal microbial, endotoxins, GEO datasets

1 Introduction

Coccidiosis, an acute epidemic intestinal disease of poultry, is caused by the parasitic protozoan genus *Eimeria*. Among these species, *Eimeria tenella* (ET) exhibits the highest pathogenicity (Blake et al., 2015). The proliferation of ET in cecal epithelial cells induces acute inflammation, while concurrently promoting local recruitment of T cells, natural killer (NK) cells, and macrophages (Lillehoj and Trout, 1996). During ET-infection, cecal tissue exhibited increased levels of various interleukins (IL), interferon (IFN), transforming growth factor β (TGF-β) 1-4, tumor necrosis factor α (TNF-α), and TNF superfamily 15 (TNFSF15) (Laurent et al., 2001; Hong et al., 2006; Kim et al., 2019a). Immunity plays a pivotal role in the pathogenesis of ET-infection, and Nod-like receptors (NLRs) have been demonstrated to be associated with diverse protozoan infections (Gurung and Kanneganti, 2016). The IFN-γ-mediated T helper (Th) 1 cells response was initially considered as the primary immune response to coccidiosis, while Th17 and T regulatory (Treg) cells also play a crucial role in maintaining intestinal homeostasis (Kim et al., 2019a). However, the correlation between intestinal immune cells and ET-infection remains unclear.

The cecum harbors the highest abundance and diversity of bacteria within the gastrointestinal tract, and alterations in both microbial diversity and composition have been observed as a consequence of ET-infection (Wei et al., 2013; Sergeant et al., 2014; Qi et al., 2019). Additionally, coccidia infection induces an increase in intestinal permeability, often resulting in secondary bacterial infection (Pham et al., 2021). Consequently, this leads to dysbiosis of the intestinal microbiota and exacerbates infections caused by *Clostridium perfringens*, *Salmonella enterica serovars* Enteritis, or Typhimurium (Arakawa et al., 1981; Qin et al., 1995; Moore, 2016). The alteration of intestinal microflora can promote the proliferation and infection of ET, primarily attributed to the facilitation of acute inflammatory response by microflora (Gaboriaud et al., 2020; Tomal et al., 2023a). The presence of endotoxins can activate macrophages and neutrophils, thereby concurrently inducing the expression of TNF-α and IL-1. Among these cytokines, the upregulation of TNF-α may plays a pivotal role in facilitating ET-infection (Zhang et al., 1995; Stephens and Mythen, 2000). Consequently, genes associated with caecal microbiome endotoxins (CME) possess the potential to modulate ET-infection by exerting influence on host immunity. The infection of ET generally leads to expression of intestinal immune cells and disruption of the cecal flora balance, thereby facilitating secondary bacterial and pathogen infections. However, the intricate interplay between cecal flora, immunity, and ET-infection remains poorly elucidated, with the core genes involved yet to be identified.

Weighted gene co-expression network analysis (WGCNA) is a systems biology method employed to characterize patterns of gene associations across different samples. It can be utilized for the identification of gene sets exhibiting high covariation and for pinpointing potential biomarker genes or therapeutic targets based on the endogeneity of the gene set and its association with the phenotype (Lin et al., 2021). Instead of solely focusing on DEGs, WGCNA leverages information from thousands or nearly 10,000 of the most diverse genes or all genes to identify gene sets of interest and conduct significant association analysis with phenotypes. The primary objective is to maximize the utilization of available information, while the secondary goal is to transform the associations between numerous genes and phenotypes into associations between a select few gene sets and phenotypes, thereby addressing the issue of multiple hypothesis testing and correction (Nangraj et al., 2020). Least Absolute Shrinkage and Selection Operator (LASSO) is a linear regression technique that utilizes L1 regularization. By incorporating L1 regularization, specific learned features are assigned zero weights, thereby achieving the objective of sparsity and feature selection. The fundamental concept underlying the Lasso method is to minimize the sum of squared residuals while constraining the absolute sum of regression coefficients below a constant value. This enables the generation of precisely equal-to-zero regression coefficients and facilitates obtaining an interpretable model (Tibshirani, 1996). By utilizing the WGCNA and LASSO algorithms, we have gained valuable insights into the fundamental modules and biomarkers associated with gene expression, thereby enhancing the scientific rigor of our study.

In this study, the GSE39602 dataset was obtained from the GEO database. The Linear models for microarray data (LIMMA), WGCNA, and LASSO algorithms and Protein-protein interaction (PPI) were employed to identify differentially expressed genes (DEGs) and hub genes, while the CIBERSORT algorithms were utilized for the screening of activated NK cells, M0 macrophages,

M2 macrophages, and Treg cells which were expressed in ET-infection. The functional enrichment analysis revealed a significant enrichment of both DEGs and hub genes in immune-related signaling pathways. The core potential biomarker and immunotherapy target of CME associated with the expression of four intestinal immune cells in ET-infection was ultimately identified as CXCR4, providing valuable references into the relationship and functionality of CME-related genes with intestinal immune cells during ET-infection.

2 Materials and methods

2.1 Acquisition of data sources and identification of differentially expressed genes (DEGs)

The related ET-infection datasets were obtained from the NCBI GEO common repository (http://www.ncbi.nlm.nih.gov/geo) (Barrett et al., 2013) by employing the search term "coccidiosis". Further refinement was carried out based on sequencing type (array analysis), animal species (Gallus gallus), and sample source (tissue). GSE39602 was generated utilizing the GPL3213 platform and consisted of three ET-infected cecum samples along with three control cecum samples. The genes associated with coccidiosis were retrieved from the CTD database (https://ctdbase.org/) (Davis et al., 2023) using "coccidiosis" as the search query. Furthermore, the genes related to endotoxins were obtained from the CTD database by employing the search term "endotoxins".

LIMMA is a differential expression screening approach based on generalized linear models (Ritchie et al., 2015). In this study, we utilized the R software package limma (version 3.40.6) to perform differential analysis and identify genes that exhibit differential expression between various comparison groups and control groups. Specifically, after obtaining the expression profile dataset, we initially applied log2 transformation to the data and then conducted multiple linear regression using the lmFit function. Subsequently, we employed the eBays function to compute moderated t-statistics, moderated F-statistics, and log-odds of differential expression through empirical Bayes moderation of standard errors towards a common value. The identification of DEGs was accomplished through a meticulous investigation employing the limma test, which necessitated a stringent p value threshold of 0.05 and a log2 fold change ≥1.

2.2 WGCNA and module identification

Modules were obtained by WGCNA using the expression matrix of GSE39602. Initially, Pearson's correlation matrices and average linkage method were applied to all pairwise genes. Then, a weighted adjacency matrix was created using a power function: A_mn=|C_mn|^β (where C_mn represents Pearson's correlation between Gene_m and Gene_n; A_mn denotes adjacency between Gene m and Gene n). The parameter β served as a soft-thresholding parameter that emphasized strong correlations while penalizing weak ones. After selecting a power value of 9, the adjacency matrix was transformed into a topological overlap matrix (TOM), which measured the network connectivity of each gene by summing its adjacencies with all other genes in the network generation. The corresponding dissimilarity (1-TOM) was calculated accordingly. To classify genes with similar expression profiles into modules, average linkage hierarchical clustering based on TOM-based dissimilarity measure was performed with a minimum module size of 30 for gene groups in dendrogram analysis while maintaining sensitivity level at three during this process. Furthermore,dissimilarity for module eigenGenes was computed to analyze modules in more detail,and an appropriate cut line for merging certain modules was determined. Finally, the hub modules and module-hub genes are obtained.

2.3 Functional enrichment analysis of biological variables

The Kyoto Encyclopedia of Genes and Genomes (KEGG) rest API (https://www.kegg.jp/kegg/rest/keggapi.html) is utilized for gene set function analysis enrichment, obtaining the latest KEGG pathway gene annotation as the background to map genes in the collection. Enrichment analysis was conducted using clusterProfiler package (version 3.14.3) in R software to obtain gene set enrichment results. A minimum of 5 and a maximum of 5000 genes were considered for each gene set, with a P value threshold of <0.05 and a false discovery rate (FDR) threshold of <0.25, indicating statistical significance.

For the functional enrichment analysis of gene sets, we utilized the Gene Ontology (GO) annotation from the R software package org.Hs.eg.db (version 3.1.0) as the background to map genes into the reference set. Subsequently, we performed enrichment analysis using clusterProfiler (version 3.14.3), an R software package. The minimum gene set size was set to 5, while the maximum gene set size was limited to 5000. Statistical significance was determined by a P-value threshold of <0.05 and FDR below <0.25.

Gene Set Enrichment Analysis (GSEA) is employed to assess the distribution pattern of genes within a predefined gene set in a ranked gene list based on their phenotypic relevance, facilitating the evaluation of their contribution to the phenotype. For GSEA, we performed the analysis using GSEA software (version 3.0) obtained from the GSEA website (http://software.broadinstitute.org/gsea/index.jsp) (Subramanian et al., 2005). The sample was stratified into two groups based on Comment information for GSE39602, and Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/downloads.jsp) (Liberzon et al., 2011) was utilized to download the c5.Go.Bp.V7.4.Symbols GMT collections for gene expression profile and phenotypic grouping analysis. We set a minimum gene set size of 5 and a maximum gene set size of 5000, performed 1000 resampling iterations, considered P value <0.05 and FDR <0.25 as statistically significant requirement.

The Gene Set Variation Analysis (GSVA) is an algorithm employed in the GSEA, facilitating unsupervised classification of samples based on alterations in pathway activity by incorporating gene expression and multiple pathway information from the outset. The R packages from GSVA (version 1.40.1) (Hanzelmann et al.,

2013) were employed for GSVA. To compute the enrichment score of each sample in the gene set, we utilized Hanzelmann et al's method (Hanzelmann et al., 2013) and Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/downloads.jsp) (Liberzon et al., 2011) to download the c5.Go.V7.4.Symbols.GMT collections for predefining the gene rank. A minimum gene set size of 5 and a maximum gene set size of 5000 were defined to assess relevant pathways and molecular mechanisms, followed by calculation of the enrichment score for each sample in each gene set. Finally, an enrichment score matrix was obtained.

2.4 Acquisition of hub genes and Lasso analysis

The intersection of DEGs, coccidiosis-related genes, CME related genes and module-hub genes was obtained by Venny 2.1.0, namely hub genes. Moreover, we employed the R software package glmnet to integrate survival time, survival state, and hub genes expression data and performed regression analysis using the lasso-cox method. Furthermore, we implemented a 3-fold cross-validation approach to obtain the optimal model. The Lambda value was set at 0.012. The area under curve (AUC) was calculated using the R software package pROC (version 1.17.0.1) through Receiver operating characteristic (ROC) analysis.

2.5 Construction of Protein-Protein interaction (PPI) network

The information on the protein interaction network for DEGs and hub genes was obtained from the STRING database (https://cn.string-db.org/) (Szklarczyk et al., 2016), respectively. The screening criteria for organisms are set to "Gallus gallus," with a minimum required interaction score of "medium confidence (0.4)". PPI information was imported into Cytoscape 3.10.1 for visualization. To identify hub targets, three Cytoscape plug-ins were integrated for data analysis, with the specific coefficients outlined as follows. According to the Centiscape 2.2 plug-in, betweenness centrality (BC), closeness centrality (CC), and degree values were utilized for an in-depth analysis of node properties within the interaction network. Target nodes were selected based on their degree values, BC, and CC, which exceeded the corresponding median values observed in the PPI network. According to the MCODE plug-in, strongly connected regions were identified using the following cut-off values: degree cutoff = 2, node score cutoff = 0.2, k-core = 2, and maximum depth = 100. The CytoHubba plug-in enables the identification of top10 node genes in ten different ways, allowing for the selection of key gene intersections.

2.6 Identification and correlation of immune infiltrating cells in ET-infection

The CIBERSORT algorithm employs linear support vector regression to deconvolute gene expression profiles, utilizing RNA sequencing data for estimating the abundance of intestinal immune cells in a given sample. In this study, we utilized the CIBERSORT algorithm within R software to calculate the proportions of 8 distinct immune cell types across various ET-infection samples present in GSE39602. Additionally, we visually depicted the composition of intestinal immune cells using box plots. Statistical significance was determined by conducting T-tests and variance tests to assess differences in immune cell proportions, with a significance threshold set at $P < 0.05$.

3 Results

3.1 DEGs in ET-infection and functional analysis

The GSE39602 dataset was retrieved from the GEO database for analysis. A total of 1089 DEGs were identified, including 800 up-regulated genes and 289 down-regulated genes. These DEGs are visually depicted using volcano plots (Figure 1A) and heat maps (Figure 1B). The DEGs were found to be significantly enriched in immune related pathways including the T cell receptor signaling pathway, TH17 cell differentiation, B cell receptor signaling pathway, T cell activation, immune response and immune system process based on KEGG (Figure 1C) and GO (Figure 1D) analysis. To corroborate the findings of KEGG and GO analysis, GSEA analysis was performed on GSE39602, with a specific focus on immune-related signaling pathways encompassing T cell receptor signaling, B cell receptor signaling, natural killer cell-mediated cytotoxicity, and B cell apoptotic processes (Figures 1E–H).

3.2 Construction of WGCNA network in the GSE39602 dataset

The soft-power thresholds were determined based on the scale independence (Figure 2A) and mean connectivity (Figure 2B). Furthermore, it was observed that the samples displayed clustering behavior (Figure 2C). Additionally, a total of 9 co-expression modules were obtained by merging modules with distances less than 0.25. Moreover, module feature vector clustering (Figure 2D) and gene clustering (Figure 2E) were performed. According to the analysis results, a relatively high degree of independence is observed between modules in terms of gene expression. Figure 2F illustrates the analysis conducted on significant modules during ET-infection. We aimed to identify associations with the highest level of significance following ET-infection and module correlation. The findings revealed that module ivory exhibited the most substantial association with ET-infection (Figure 2F). Furthermore, there was a significant correlation between the mean value of ivory modules and gene significance (GS) (Figure 2G). We computed the correlation between the module feature vector and gene expression to derive the module membership (MM). By applying a cut-off criterion ($|MM| > 0.8$), we identified 2491 genes exhibiting high connectivity within the clinically significant module, thus designating them as module-hub genes.

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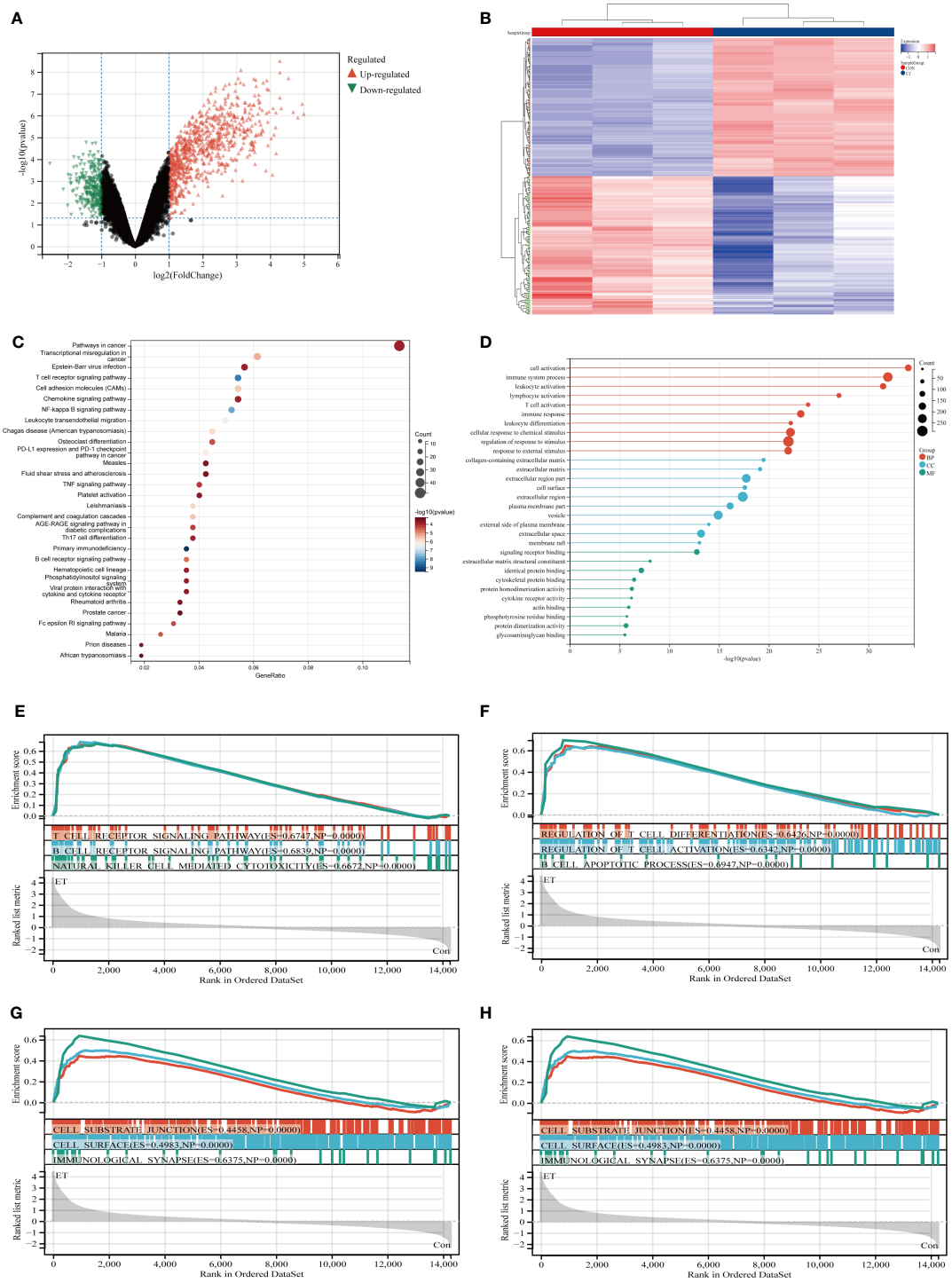

FIGURE 1
Identification and functional enrichment analysis of DEGs from GSE39602 dataset. **(A)** Volcanic maps of DEGs; **(B)** Heat maps of DEGs; **(C)** Top 30 KEGG pathways of DEGs; **(D)** Top 10 BP terms, CC terms, and MF terms of GO analysis in DEGs; **(E–H)** GSEA analysis of DEGs. CON, the control group; ET, *Eimeria tenella*; DEGs, differentially expressed genes.

3.3 Functional analysis of the ivory module

The heat map depicted the expression patterns of module-hub genes across different groups (Figure 3A). Furthermore, KEGG and GO analyses revealed that these module-hub genes were predominantly enriched in immune-related pathways, including the T cell receptor signaling pathway, TNF signaling pathway, Th17 cell differentiation, B cell receptor signaling pathway, cellular activation, immune system processes, and other relevant pathways (Figures 3B, C).

We further employed GSVA to analyze module-hub genes and validate the functional significance of the ivory module by assessing their enrichment in KEGG gene sets and GO gene sets. The volcano

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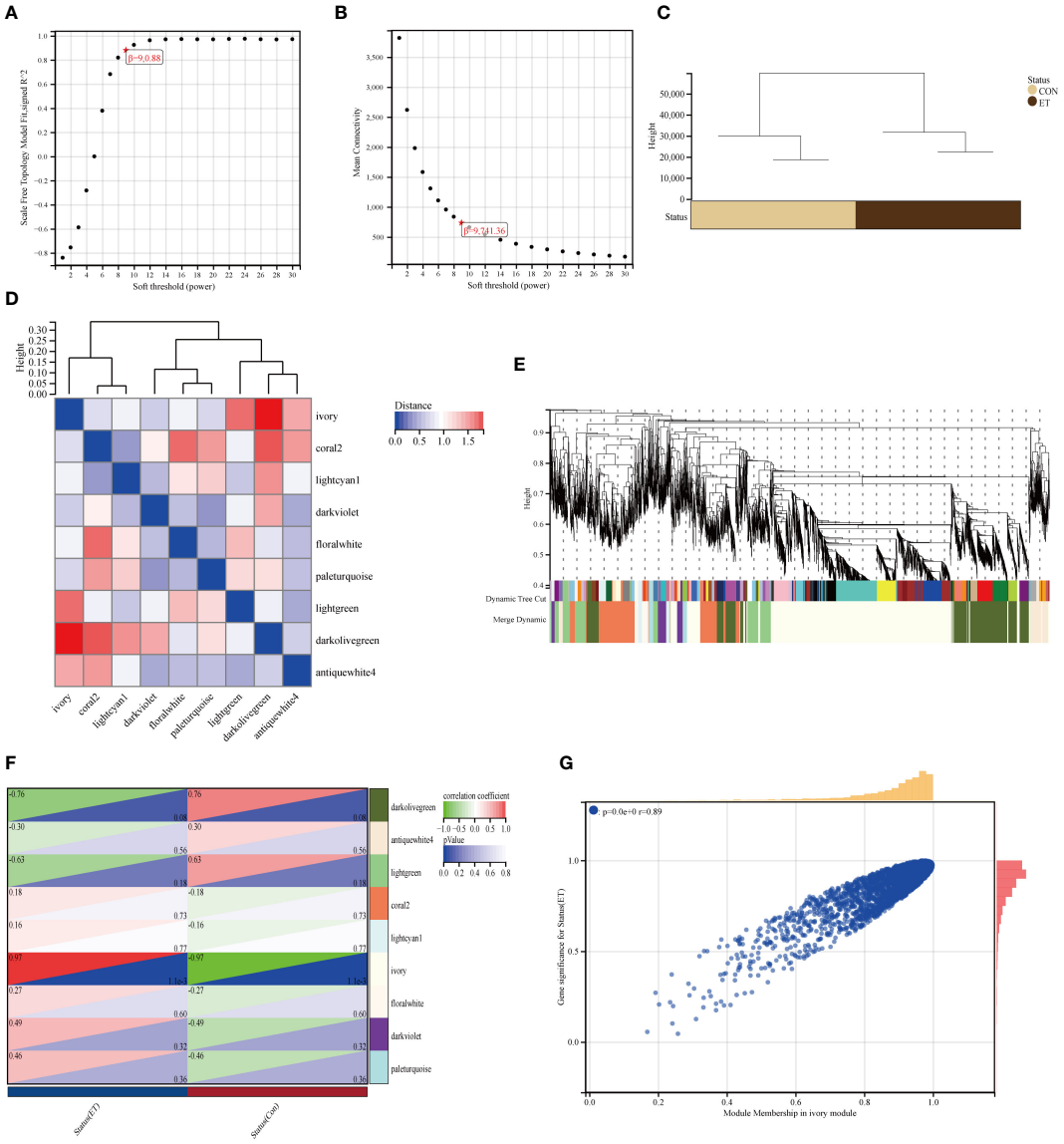

FIGURE 2
Construction of the expression network of ET-infection in the GSE39602 dataset. **(A, B)** The fitting index of network topology with scale-free characteristics is obtained by conducting soft threshold power analysis; **(C)** Sample clustering; **(D)** The heat map depicts the topological overlap matrix (TOM) of genes selected for weighted coexpression network analysis; **(E)** The coexpression clusters, along with their corresponding color allocation, are identified through hierarchical cluster analysis. Each color represents a distinct module within the gene co-expression network constructed using WGCNA; **(F)** The relationship of Groups and 9 modules; **(G)** The scatter plot demonstrates the correlation between MM and GS within the ivory module. CON, the control group; ET, *Eimeria tenella*.

map and heat map revealed that within these gene sets, module-hub genes were predominantly enriched in crucial pathways such as T cell receptor signaling pathway, nod-like receptor signaling pathway, toll-like receptor signaling pathway, leukocyte migration involved in inflammatory response, mature B cell differentiation involved in immune response, and mature B cell differentiation (Figures 3D–G).

3.4 Identification and functional analysis of hub genes associated with CME and acquisition of biomarkers

Venny diagram illustrates that the hub genes consist of a total of 25 genes (Figure 4A). The heat map demonstrates the expression levels of each individual hub gene across different groups (Figure 4B), while the box plot analysis reveals variations in expression for each hub gene (Figure 4C). The findings indicate significant differences in the expression patterns of all hub genes, except COL5A1 and CCL20, when compared to the control group. Moreover, KEGG and GO analyses revealed that the hub genes were predominantly enriched in pathways associated with Th17 cell differentiation, Th1 and Th2 cell differentiation, TNF signaling pathway, immune system processes, and immune responses (Figures 4D, E).

The LASSO algorithm was employed to identify four potential biomarkers, namely ANXA5, CXCR4, ZBTB16, and VIM, out of 25 hub genes that exert a significant impact on CME related ET-

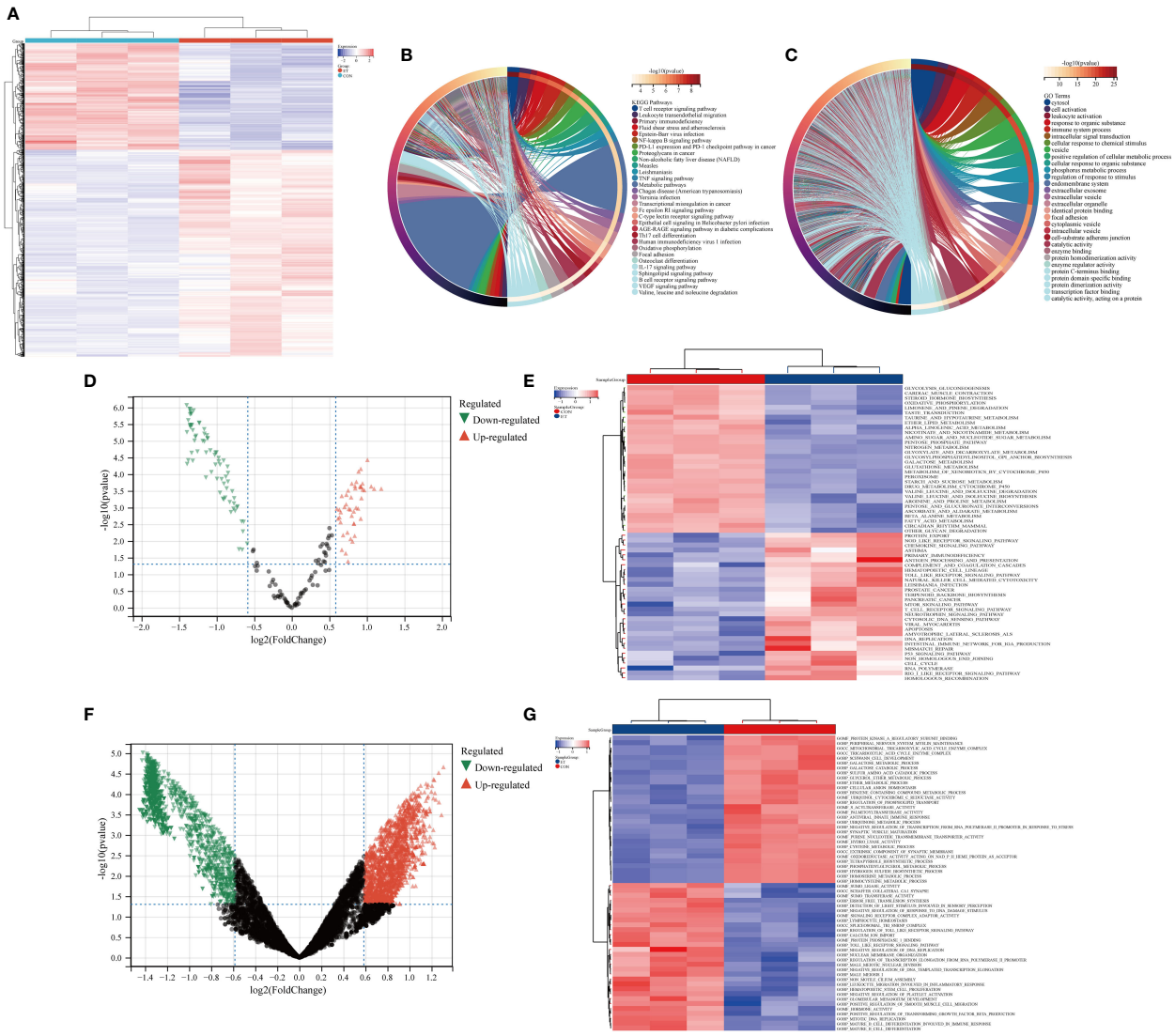

FIGURE 3
Functional enrichment analysis of ivory module genes. **(A)** Heat maps of ivory module genes; **(B)** Top 30 KEGG pathways of ivory module genes; **(C)** Top 30 GO analysis of ivory module genes; **(D, E)** GSVA analysis of ivory module genes based on KEGG gene sets; **(F, G)** GSVA analysis of ivory module genes based on GO gene sets. CON, the control group; ET, *Eimeria tenella*.

infection. The optimal model was constructed using the following formula: RiskScore=0.0030*ANXA5 + 0.0004*CXCR4 + 0.0003*ZBTB16 + 0.0001*VIM (Figures 4F, G). Furthermore, ROC analysis was utilized to evaluate the predictive performance of the constructed model, demonstrating its efficacy in outcome prediction (Figure 4H).

3.5 Construction of PPI network of DEGs and hub genes

To comprehensively investigate the role of proteins in bacterial endotoxin-related ET-infection, we conducted a comparative analysis of protein interactions between DEGs and hub genes using Centiscape 2.2, CytoHubba, and MCODE plug-ins. The results of the PPI network analysis revealed CD4, PTPRC, IL1B, PTPN6, LCP2, KLHL6, BLK, MYO1F, RGS18, and PLEK as identified hub DEGs (Figures 5A–C). Furthermore, the PPI network demonstrated that IL1B, ANXA5, CXCR4, MMP9, CD44, and BCL2 are crucial hub genes (Figures 5D–F). Moreover, combined with the results from the LASSO algorithm analysis, these findings highlight ANXA5 and CXCR4 as core potential biomarkers in CME related ET-infection.

3.6 Relationship between ET-infection and intestinal immune cells

The immune tumor biology computational tool IOBR (Zeng et al., 2021) was employed in this study to analyze the expression profile and calculate the scores of eight immunoinfiltrating cell types in each sample using the CIBERSORT method (Newman et al., 2015), which was implemented through R software package

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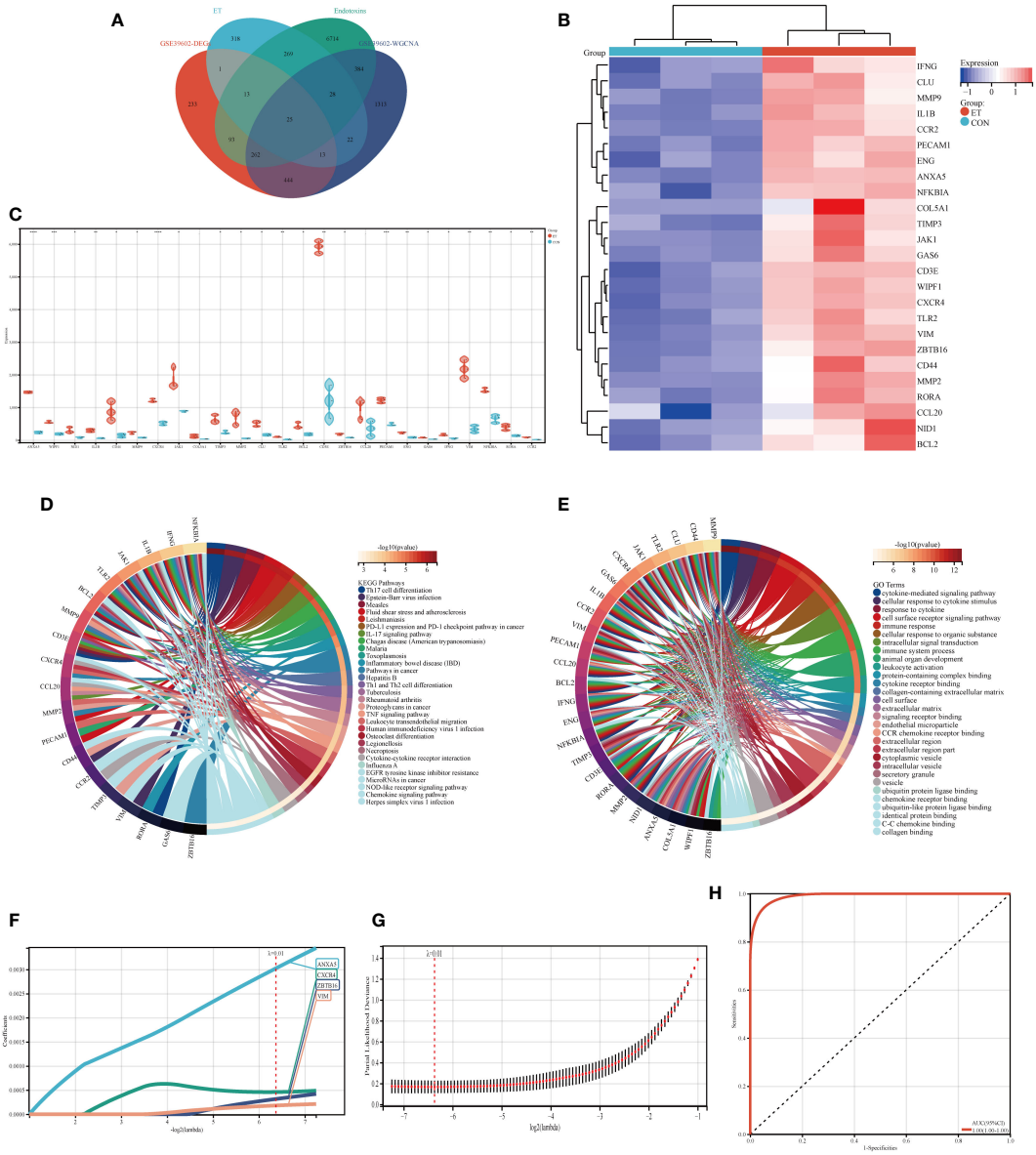

FIGURE 4
Identification of hub genes and potential biomarkers. **(A)** Intersection gene of DEGs, ET, Endotoxins, and ivory module gene; **(B)** Heat maps of hub genes; **(C)** The expression of hub genes in GSE39602; **(D)** Top 30 KEGG pathways of hub genes; **(E)** Top 30 GO analysis of hub genes; **(F, G)** Screening of potential biomarkers from hub genes using the LASSO algorithm; **(H)** Receiver operating characteristic (ROC) curve of predicted risk scores in ET-infection. CON, the control group; ET, *Eimeria tenella*; DEGs, differentially expressed genes. * P<0.05; ** P<0.01; *** P<0.005; **** P<0.001.

IOBR. The present study investigated the infiltration and correlation of intestinal immune cells (Figures 6A, B), expression of intestinal immune cells between ET and CON groups (Figure 6C), as well as the association between intestinal immune cells and hub genes (Figure 6D). Spearman's method was employed for visualizing the results of correlation analysis. The Treg cells are associated with both the T gamma delta cells and plasma cells, while the activated NK cells are linked to the resting NK cells, T gamma delta cells, Treg cells, and plasma cells (Figure 6B). Additionally, M0 macrophages are correlated with activated NK cells, T CD4 memory activated cells, and plasma cells (Figure 6B). Furthermore, there is a relationship between M0 macrophages and M0 macrophages as well as activated NK cells, T gamma delta cells, Treg cells and plasma cells (Figure 6B). Significant differences were observed between the ET and CON groups in terms of activated NK cells, M0 macrophages, M2 macrophages, and Treg cells,. Specifically, enhanced expression of Treg cells and M2 macrophages was noted in the ET group, while activated NK cells and M0 macrophages were suppressed (Figure 6C). Moreover, these four cell types exhibit a close association with the expression of hub genes, including CCR2, VIM, WIPF1, GLU, TLR2, CXCR4, and CD3E (-log10(P Value > 2) (Figure 6D). The core potential biomarker and the CME-related gene CXCR4 were ultimately identified, which is implicated in immune cell expression.

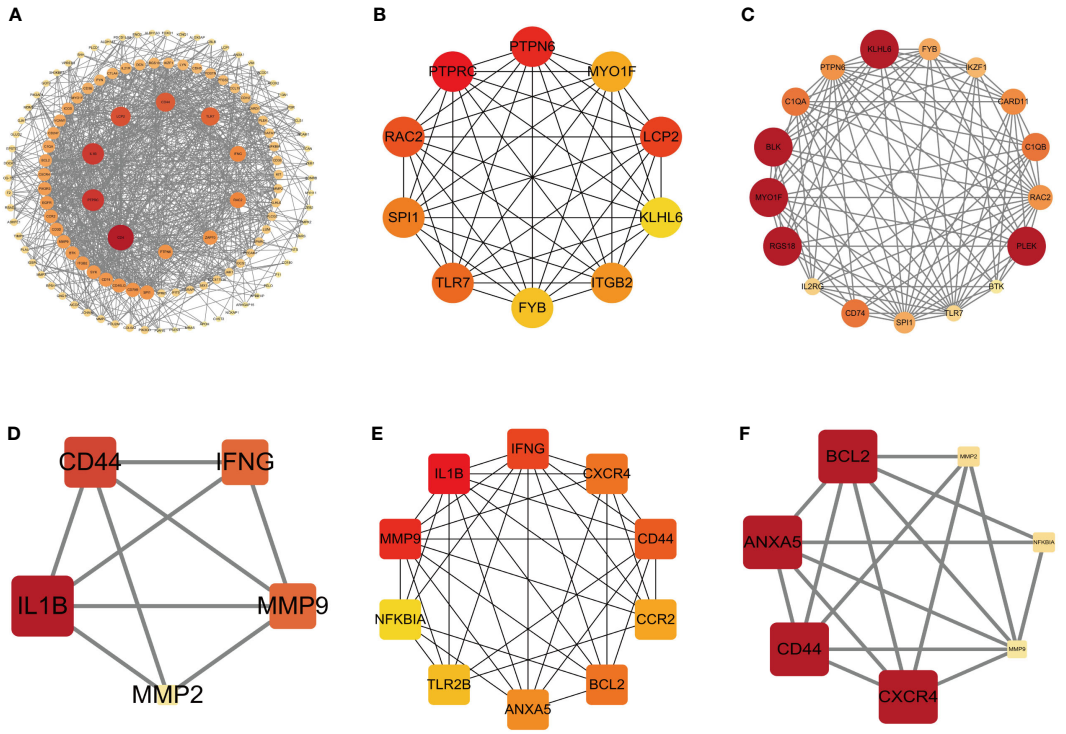

FIGURE 5
PPI network of DEGs and hub genes. **(A)** PPI network of DEGs based on Centiscape 2.2 plug-in. **(B)** Top 10 DEGs explored by CytoHubba plug-in. **(C)** PPI network of DEGs based on clustery analysis using the MCODE plug-in (score: 12.5). **(D)** PPI network of hub genes based on Centiscape 2.2 plug-in. **(E)** Top 10 hub genes explored by CytoHubba plug-in. **(F)** PPI network of hub genes based on clustery analysis using the MCODE plug-in (score: 5.667). The node color of Cluster was from pale yellow to red, and the corresponding score gradually larger. DEGs, differentially expressed genes.

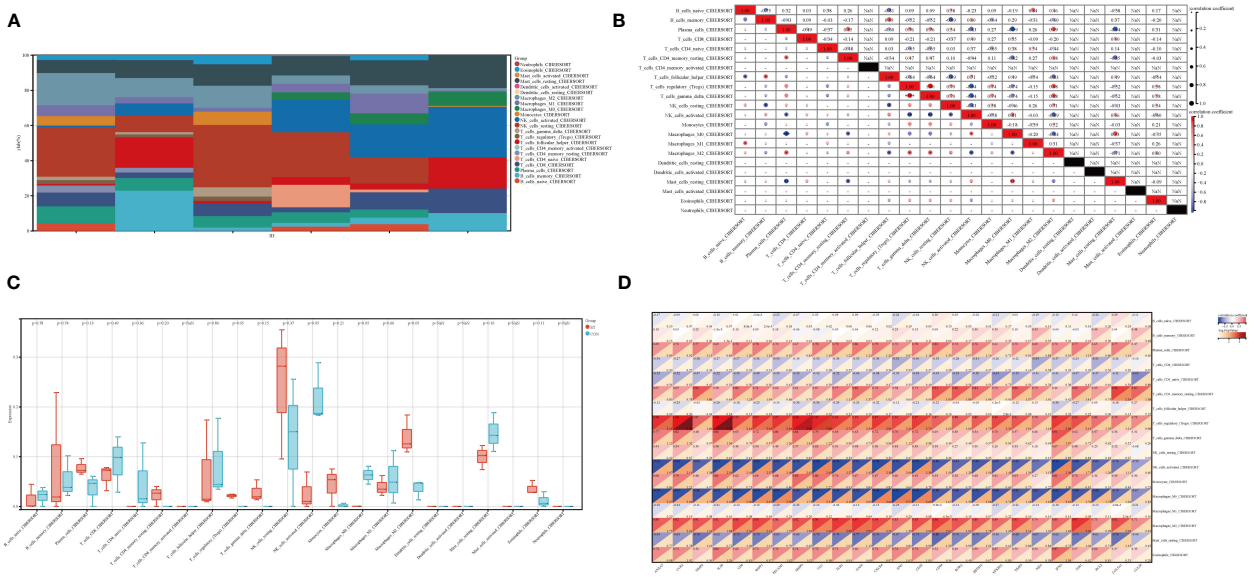

FIGURE 6
Immune characteristics in ET-infection. **(A)** The superposition diagram depicts the distribution of immune cell composition in the CON and ET groups, wherein each color corresponds to a distinct immune cell subtype. The horizontal axis represents different samples; **(B)** Correlation between different immune cells in ET-infection; **(C)** Expression variations among distinct immune cells in ET-infection; **(D)** The correlation between immune cells and hub genes in ET-infection. CON, the control group; ET, *Eimeria tenella*.

4 Discussion

The infection of ET can modify the diversity and composition of cecum flora. In comparison to the control group, there was a significant reduction in populations of *Proteobacteria*, *Enterococcus*, *Incertae*, *Escherichia–Shigella*, *Lactobacillus*, *Faecalibacterium*, and *Roseburia*. Conversely, populations of *Alistipes*, *Prevotella pectinovora*, *Clostridium*, *Lysinibacillus Escherichia*, *Bacteroidales*, and *Rikenella* increased significantly (Huang et al., 2018; Zhou et al., 2020; Yu et al., 2023). These findings indicate the presence of a diverse array of pathogenic bacteria in the upregulated flora during ET-infection. It is imperative to investigate the impact of up-regulation of pathogenic flora on ET-infection, and functional analysis of DEGs, module-hub genes, and hub genes revealed their significant enrichment in immune-related pathways. Therefore, this study further investigates the impact of caecal microbiome alterations on ET-infection by examining the correlation between CME-related genes and ET-infection as well as intestinal immune cells. We have identified a set of 25 CME related hub genes and subsequently determined that CXCR4 functions as a pivotal potential biomarker and immunotherapy target significantly associated with expression of intestinal immune cells. Therefore, we postulated that alterations in the caecal microbiome during ET-infection may lead to subsequent modulation of CXCR4 expression and immune cell activity, ultimately influencing the course of ET-infection.

The immune regulation of ET heavily relies on the induction of adaptive immunity through auxiliary Th1 responses and IFN-γ production (Hunter and Sibley, 2012). Furthermore, IL-17 production and its associated inflammation are also stimulated, in addition to promoting a Th1 response (Drinkall et al., 2017; Raballah et al., 2017). Whereas, the production of IL-17 appears to contribute to the pathological mechanisms associated with Apicomplexan infection (Ivanova et al., 2019). The immune response to infection of Apicomplexan involves the participation of diverse innate immune cell populations, including granulocytes, monocytes, macrophages, dendritic cells, and mast cells (Iwasaki and Akashi, 2007). These cellular components mount a reaction against infectious opportunistic lymphocyte populations through the production of chemokines and cytokines while presenting antigens. NK cells, classified as Group I innate lymphoid cells (ILCs), possess cytotoxic capabilities that distinguish them from other Group ILCs (Spits et al., 2013; Cortez and Colonna, 2016). The NK cells collaborate with the ILC2 and ILC3 cell populations to generate heightened levels of IFN-γ and TNF-α in response to IL-12, IL-15 and IL-18 stimulation induced by Th1 cells (Cortez and Colonna, 2016). The role of NK cells in ET-infection remains elusive. Previous studies have demonstrated that NK cells do not exert a protective role in Eimeria infection in mice (Smith et al., 1994). Moreover, following infection, the activity of NK cells in the spleen and intestine initially declines but subsequently recovers in chickens (Lillehoj, 1989), which may be associated with the observed decline of NK cells expression reported in this study. Furthermore, this study unveils a downregulation of NK cell expression during ET-infection, which is associated with the expression of CME-related genes including CCR2, VIM, WIPF1, GLU, TLR2, CXCR4 and CD3E. Consequently, it is postulated that perturbations in the intestinal microbiota caused by ET-infection may impede NK cell expression through the modulation of CME-related genes thereby facilitating ET-infection.

Treg cells, a subset of T cells involved in immunosuppression, comprise CD4+CD25+ T cells that suppressing activated immune cells in avian species (Shanmugasundaram and Selvaraj, 2011). Treg cells possess the capability to secrete a substantial quantity of IL-10, TGF-β, CTLA-4, and LAG-3 (Selvaraj, 2013), with particular emphasis on the pivotal role played by IL-10 in evading the host immune response. Treg cells possess the capacity to suppress Th17 cells, thereby attenuating tissue damage caused by ET-infection (Kim et al., 2019b). However, an alternative hypothesis suggests that coccidial parasites have evolved to induce IL-10 expression in Treg cells, promoting chicken invasion and survival through inhibition of the IFN-γ-associated Th1 response (Kim et al., 2019a). Therefore, it is essential to investigate the role of Treg cells in the pathogenesis of ET-infection. The findings in this study demonstrate that Treg cells are also upregulated during ET-infection, which correlates with the differential expression of numerous CME-related genes in these cells. We propose that during ET-infection, changes in the cecal microbiota induce endotoxin-mediated activation of Treg cells, leading to their upregulation. Consequently, this may result in an elevation of IL-10 expression, thereby suppressing host immunity and facilitating ET-infection.

The occurrence of acute inflammation is the primary etiological factor underlying cecum injury subsequent to ET-infection, and this process is characterized by the infiltration of inflammatory cells and the release of pro-inflammatory mediators, including IFN-γ, IL-1β, IL-6, and IL-17 (Laurent et al., 2001; Hong et al., 2006). Macrophages, as components of the innate immune system, are capable of inducing the production of IL-1β, IL-6, and IL-17, in addition to various chemokines subsequent to ET-infection. This process facilitates the initiation and progression of inflammation. Alterations in the cecal microbiota have been demonstrated to play a pivotal role in macrophage recruitment. However, ET-infection alone is insufficient for fulfilling this function. Moreover, the microbiota can stimulate the expression of pro-inflammatory genes, thereby contributing to the development of cecal lesions (Tomal et al., 2023b). The presence of endotoxins and IFN-γ has been demonstrated to induce the polarization of M0 macrophages towards the M1 phenotype, while IL-4 and IL-13 drive the polarization of M1 macrophages into the M2 macrophages (Popena et al., 2018). Moreover, M2 macrophages possess the capability to secrete pro-inflammatory cytokines such as IL-1β and TNF-α, which contribute to the progression of inflammation (Popena et al., 2018). It is noteworthy that in this study, we observed an association between CME-associated genes and the downregulation of M0 macrophages as well as the upregulation of M2 macrophages. Therefore, our hypothesis posits that CME may potentially exacerbate inflammation by modulating the polarization

of M0 macrophages towards an M2 macrophages, thereby facilitating ET-infection.

In summary, based on bioinformatics, this study revealed that CXCR4 associated with CME exert a regulatory influence on the activity of NK cells, M0 macrophages, M2 macrophages, and Treg cells during ET-infection, thereby impacting the progression of ET-infection. Therefore, CXCR4 is expected to emerge as a potential biomarker and immunotherapy target for elucidating the impact of caecal microbiome on ET-infection. These findings provide valuable insights into the involvement of cecal flora in ET-infection.

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.

Author contributions

MH: Data curation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. JL: Resources, Software, Writing – original draft, Writing – review & editing. YW: Methodology, Validation, Writing – original draft. JzL: Data curation, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

References

Arakawa, A., Baba, E., and Fukata, T. (1981). Eimeria tenella infection enhances Salmonella typhimurium infection in chickens. *Poult. Sci.* 60, 2203–2209. doi: 10.3382/ps.0602203

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., et al. (2013). NCBI GEO: archive for functional genomics data sets–update. *Nucleic Acids Res.* 41, D991–D995. doi: 10.1093/nar/gks1193

Blake, D. P., Clark, E. L., Macdonald, S. E., Thenmozhi, V., Kundu, K., Garg, R., et al. (2015). Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. *Proc. Natl. Acad. Sci.* 112, E5343–E5350. doi: 10.1073/pnas.1506468112

Cortez, V. S., and Colonna, M. (2016). Diversity and function of group 1 innate lymphoid cells. *Immunol. Lett.* 179, 19–24. doi: 10.1016/j.imlet.2016.07.005

Davis, A. P., Wiegers, T. C., Johnson, R. J., Sciaky, D., Wiegers, J., and Mattingly, C. J. (2023). Comparative toxicogenomics database (CTD): update 2023. *Nucleic Acids Res.* 51, D1257–D1262. doi: 10.1093/nar/gkac833

Drinkall, E., Wass, M. J., Coffey, T. J., and Flynn, R. J. (2017). A rapid IL-17 response to Cryptosporidium parvum in the bovine intestine. *Vet. Immunol. Immunopathol.* 191, 1–4. doi: 10.1016/j.vetimm.2017.07.009

Gaboriaud, P., Sadrin, G., Guitton, E., Fort, G., Niepceron, A., Lallier, N., et al. (2020). The absence of gut microbiota alters the development of the apicomplexan parasite eimeria tenella. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.632556

Gurung, P., and Kanneganti, T. D. (2016). Immune responses against protozoan parasites: a focus on the emerging role of Nod-like receptors. *Cell. Mol. Life Sci.* 73, 3035–3051. doi: 10.1007/s00018-016-2212-3

Hanzelmann, S., Castelo, R., and Guinney, J. (2013). GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinf.* 14, 7. doi: 10.1186/1471-2105-14-7

Hong, Y. H., Lillehoj, H. S., Lee, S. H., Dalloul, R. A., and Lillehoj, E. P. (2006). Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. *Vet. Immunol. Immunopathol.* 114, 209–223. doi: 10.1016/j.vetimm.2006.07.007

Huang, G., Tang, X., Bi, F., Hao, Z., Han, Z., Suo, J., et al. (2018). Eimeria tenella infection perturbs the chicken gut microbiota from the onset of oocyst shedding. *Vet. Parasitol.* 258, 30–37. doi: 10.1016/j.vetpar.2018.06.005

Hunter, C. A., and Sibley, L. D. (2012). Modulation of innate immunity by Toxoplasma gondii virulence effectors. *Nat. Rev. Microbiol.* 10, 766–778. doi: 10.1038/nrmicro2858

Ivanova, D. L., Denton, S. L., Fettel, K. D., Sondgeroth, K. S., Munoz, G. J., Bangoura, B., et al. (2019). Innate lymphoid cells in protection, pathology, and adaptive immunity during apicomplexan infection. *Front. Immunol.* 10. doi: 10.3389/fimmu.2019.00196

Iwasaki, H., and Akashi, K. (2007). Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 26, 726–740. doi: 10.1016/j.immuni.2007.06.004

Kim, W. H., Chaudhari, A. A., and Lillehoj, H. S. (2019a). Involvement of T cell immunity in avian coccidiosis. *Front. Immunol.* 10. doi: 10.3389/fimmu.2019.02732

Kim, W. H., Lillehoj, H. S., and Min, W. (2019b). Indole treatment alleviates intestinal tissue damage induced by chicken coccidiosis through activation of the aryl hydrocarbon receptor. *Front. Immunol.* 10, 560. doi: 10.3389/fimmu.2019.00560

Laurent, F., Mancassola, R., Lacroix, S., Menezes, R., and Naciri, M. (2001). Analysis of chicken mucosal immune response to Eimeria tenella and Eimeria maxima infection by quantitative reverse transcription-PCR. *Infect. Immun.* 69, 2527–2534. doi: 10.1128/IAI.69.4.2527-2534.2001

Liberzon, A., Subramanian, A., Pinchback, R., Thorvaldsdottir, H., Tamayo, P., and Mesirov, J. P. (2011). Molecular signatures database (MSigDB) 3.0. *Bioinformatics* 27, 1739–1740. doi: 10.1093/bioinformatics/btr260

Lillehoj, H. S. (1989). Intestinal intraepithelial and splenic natural killer cell responses to eimerian infections in inbred chickens. *Infect. Immun.* 57, 1879–1884. doi: 10.1128/iai.57.7.1879-1884.1989

Lillehoj, H. S., and Trout, J. M. (1996). Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. *Clin. Microbiol. Rev.* 9, 349–360. doi: 10.1128/CMR.9.3.349

Lin, W., Wang, Y., Chen, Y., Wang, Q., Gu, Z., and Zhu, Y. (2021). Role of calcium signaling pathway-related gene regulatory networks in ischemic stroke based on

multiple WGCNA and single-cell analysis. *Oxid. Med. Cell. Longev.* 2021, 8060477. doi: 10.1155/2021/8060477

Moore, R. J. (2016). Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol.* 45, 275–281. doi: 10.1080/03079457.2016.1150587

Nangraj, A. S., Selvaraj, G., Kaliamurthi, S., Kaushik, A. C., Cho, W. C., and Wei, D. Q. (2020). Integrated PPI- and WGCNA-retrieval of hub gene signatures shared between Barrett's esophagus and esophageal adenocarcinoma. *Front. Pharmacol.* 11. doi: 10.3389/fphar.2020.00881

Newman, A. M., Liu, C. L., Green, M. R., Gentles, A. J., Feng, W., Xu, Y., et al. (2015). Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods* 12, 453–457. doi: 10.1038/nmeth.3337

Pham, H. H. S., Matsubayashi, M., Tsuji, N., and Hatabu, T. (2021). Relationship between Eimeria tenella associated-early clinical signs and molecular changes in the intestinal barrier function. *Vet. Immunol. Immunopathol.* 240, 110321. doi: 10.1016/j.vetimm.2021.110321

Popena, I., Abols, A., Saulite, L., Pleiko, K., Zandberga, E., Jekabsons, K., et al. (2018). Effect of colorectal cancer-derived extracellular vesicles on the immunophenotype and cytokine secretion profile of monocytes and macrophages. *Cell Commun. Signal.* 16, 17. doi: 10.1186/s12964-018-0229-y

Qi, Z., Shi, S., Tu, J., and Li, S. (2019). Comparative metagenomic sequencing analysis of cecum microbiotal diversity and function in broilers and layers. *3 Biotech.* 9, 1–10. doi: 10.1007/s13205-019-1834-1

Qin, Z. R., Fukata, T., Baba, E., and Arakawa, A. (1995). Effect of Eimeria tenella infection on Salmonella enteritidis infection in chickens. *Poult. Sci.* 74, 1–7. doi: 10.3382/ps.0740001

Raballah, E., Kempaiah, P., Karim, Z., Orinda, G. O., Otieno, M. F., Perkins, D. J., et al. (2017). CD4 T-cell expression of IFN-gamma and IL-17 in pediatric malarial anemia. *PloS One* 12, e0175864. doi: 10.1371/journal.pone.0175864

Ritchie, M. E., Phipson, B., Wu, D. I., Hu, Y., Law, C. W., Shi, W., et al. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43, e47–e47. doi: 10.1093/nar/gkv007

Selvaraj, R. K. (2013). Avian CD4+ CD25+ regulatory T cells: Properties and therapeutic applications. *Dev. Comp. Immunol.* 41, 397–402. doi: 10.1016/j.dci.2013.04.018

Sergeant, M. J., Constantinidou, C., Cogan, T. A., Bedford, M. R., Penn, C. W., and Pallen, M. J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. *PloS One* 9, e91941. doi: 10.1371/journal.pone.0091941

Shanmugasundaram, R., and Selvaraj, R. K. (2011). Regulatory T cell properties of chicken CD4+ CD25+ cells. *J. Immunol.* 186, 1997–2002. doi: 10.4049/jimmunol.1002040

Smith, A. L., Rose, M. E., and Wakelin, D. (1994). The role of natural killer cells in resistance to coccidiosis: investigations in a murine model. *Clin. Exp. Immunol.* 97, 273–279. doi: 10.1111/j.1365-2249.1994.tb06080.x

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., et al. (2013). Innate lymphoid cells—a proposal for uniform nomenclature. *Nat. Rev. Immunol.* 13, 145–149. doi: 10.1038/nri3365

Stephens, R., and Mythen, M. (2000). Endotoxin immunization. *Intensive Care Med.* 26 Suppl 1, S129–S136. doi: 10.1007/s001340051130

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* 102, 15545–15550. doi: 10.1073/pnas.0506580102

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., et al. (2016). The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.* 45(D1): D362–D368. doi: 10.1093/nar/gkw937

Tibshirani, R. (1996). Regression shrinkage and selection via the lasso. *J. R. Stat. Soc. Ser. B: Stat. Method.* 58, 267–288. doi: 10.1111/j.2517-6161.1996.tb02080.x

Tomal, F., Sadrin, G., Gaboriaud, P., Guitton, E., Sedano, L., Lallier, N., et al. (2023a). The caecal microbiota promotes the acute inflammatory response and the loss of the intestinal barrier integrity during severe Eimeria tenella infection. *Front. Cell. Infect. Microbiol.* 13. doi: 10.3389/fcimb.2023.1250080

Tomal, F., Sausset, A., Le Vern, Y., Sedano, L., Techer, C., Lacroix-Lamande, S., et al. (2023b). Microbiota promotes recruitment and pro-inflammatory response of caecal macrophages during E. tenella infection. *Gut Pathog.* 15, 65. doi: 10.1186/s13099-023-00591-8

Wei, S., Morrison, M., and Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poult. Sci.* 92, 671–683. doi: 10.3382/ps.2012-02822

Yu, H., Wang, Q., Tang, J., Dong, L., Dai, G., Zhang, T., et al. (2023). Comprehensive analysis of gut microbiome and host transcriptome in chickens after Eimeria tenella infection. *Front. Cell. Infect. Microbiol.* 13. doi: 10.3389/fcimb.2023.1191939

Zeng, D., Ye, Z., Shen, R., Yu, G., Wu, J., Xiong, Y., et al. (2021). IOBR: multi-omics immuno-oncology biological research to decode tumor microenvironment and signatures. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.687975

Zhang, S., Lillehoj, H. S., and Ruff, M. D. (1995). Chicken tumor necrosis-like factor.: 1. in *vitro* production by macrophages stimulated with Eimeria tenella or bacterial lipopolysaccharide. *Poultry Sci.* 74, 1304–1310. doi: 10.3382/ps.0741304

Zhou, B. H., Jia, L. S., Wei, S. S., Ding, H. Y., Yang, J. Y., and Wang, H. W. (2020). Effects of Eimeria tenella infection on the barrier damage and microbiota diversity of chicken cecum. *Poult. Sci.* 99, 1297–1305. doi: 10.1016/j.psj.2019.10.073

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Hesperidin alleviates zinc-induced nephrotoxicity via the gut-kidney axis in swine

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Introduction: Zinc (Zn) is an essential trace element in animals, but excessive intake can lead to renal toxicity damage. Thus, the exploration of effective natural antagonists to reduce the toxicity caused by Zn has become a major scientific problem.

Methods: Here, we found that hesperidin could effectively alleviate the renal toxicity induced by Zn in pigs by using hematoxylin-eosin staining, transmission electron microscope, immunohistochemistry, fluorescence quantitative PCR, and microfloral DNA sequencing.

Results: The results showed that hesperidin could effectively attenuate the pathological injury in kidney, and reduce autophagy and apoptosis induced by Zn, which evidenced by the downregulation of LC3, ATG5, Bak1, Bax, Caspase-3 and upregulation of p62 and Bcl2. Additionally, hesperidin could reverse colon injury and the decrease of ZO-1 protein expression. Interestingly, hesperidin restored the intestinal flora structure disturbed by Zn, and significantly reduced the abundance of Tenericutes (phylum level) and Christensenella (genus level).

Discussion: Thus, altered intestinal flora and intestinal barrier function constitute the gut-kidney axis, which is involved in hesperidin alleviating Zn-induced nephrotoxicity. Our study provides theoretical basis and practical significance of hesperidin for the prevention and treatment of Zn-induced nephrotoxicity through gut-kidney axis.

KEYWORDS

zinc, nephrotoxicity, hesperidin, gut-kidney axis, gut microbiota

1 Introduction

Zinc (Zn) is an essential trace element, which is involved in many important physiological processes (Hutchens et al., 2021). In eukaryotes, Zn plays an important role in biological processes such as energy metabolism, oxidative stress and signal transduction (Kim and Lee, 2021). A number of studies have shown that appropriate amount of Zn added to feed can

improve the growth efficiency of animals (Li et al., 2021c). However, excessive intake of Zn can cause acute and chronic poisoning in animals (Kataba et al., 2021). Studies have pointed out that the toxic effect of Zn is mainly through disrupting the physiological homeostasis of lipid bimolecular structure and changing the structure of glycoprotein on the cell surface, and causing disorders to the structure and function of Zn-containing enzymes such as ATP and nucleotidase, resulting in reduction of ATP synthesis, dysfunction of membrane active transport, organelle edema and other diseases (Alhasawi et al., 2014; Ren et al., 2017). When animals are supplemented with excessive Zn, they will show loss of appetite, sluggish activity, diarrhea, growth and development arrest and other toxic symptoms. Recently, lots of studies have shown that the homeostasis of Zn is related to the occurrence and development of various diseases (Wang et al., 2020).

Hesperidin is a derivative of dihydroflavone and mainly exists in the peel of citrus fruits (Xiong et al., 2019). In recent years, hesperidin has attracted extensive attention in livestock and poultry breeding because of its antioxidant, lipid metabolism regulation, anti-inflammatory and other biological activities (Hager-Theodorides et al., 2021). It has been reported that hesperidin can effectively relieve the symptoms of heat stress in broilers and improve the growth efficiency of the animals, so it is gradually recommended to be used in animal feed as a feed additive (Kamboh et al., 2013; Kouvedaki et al., 2024). Studies have found that hesperidin regulates gut flora to alleviate liver damage in mice (Li et al., 2022). However, there are few studies on the mechanism of hesperidin in toxic diseases and in the regulation of intestinal flora.

The "gut microbiota-gut-kidney axis" theory was first proposed to explain how changes in gut microecology affect chronic kidney disease through regulation of metabolites (Nouri et al., 2022). Recent studies have found that a decrease in the abundance of intestinal probiotics in animal models of chronic kidney disease, accompanied by an increase in the abundance of pathogenic bacteria, which causes dyshomeostasis of intestinal flora, directly destroys intestinal barrier function and leads to bacterial translocation and the accumulation of enterogenous uremic toxins in the blood, thereby activating renal inflammation, oxidative stress and fibrosis pathways (Plata et al., 2019; Li et al., 2023a). In addition, excessive metabolic waste cannot be fully excreted by the kidney and re-enters the intestine, further aggravating the imbalance of intestinal flora and leading to the sustainable development of the disease. Therefore, the discovery or development of effective drugs through the "gut microbiota - enteric-kidney axis" is of great significance for the treatment of kidney diseases (Hsu and Tain, 2022). At present, there are still gaps in the research on the role and mechanism of "gut - kidney axis" in natural compounds on chronic kidney injury caused by heavy metals in animals, which is also the focus of the scientific community in recent years.

Studies have confirmed that high levels of Zn can induce kidney damage (Ratn et al., 2018). At the same time, hesperidin has been shown to have significant effects in maintaining intestinal flora homeostasis and alleviating kidney injury, but its underlying mechanism is still unclear (Mas-Capdevila et al., 2020). In this study, pigs were used as experimental animals, and hesperidin was used for treatment based on the establishment of a pathological injury model of high levels of Zn. Based on the theory of "intestinal microbiota - enteric-kidney axis", changes in intestinal flora structure and metabolites, changes in intestinal barrier function, renal function, and dynamic processes of autophagy and apoptosis were detected by detecting changes in intestinal flora of pigs. Further, the mechanism of "intestinal flora - intestinal renal axis" in hesperidin alleviating kidney injury caused by Zn poisoning was discussed, which laid a foundation for elucidation of the protective mechanism of hesperidin in regulating kidney injury of pigs with high levels of Zn.

2 Materials and methods

2.1 Animal treatment

A total of 80 weaned pigs, one-month-old (approximate 10 kg) were fed with basal mixed ration for one week during adaptive phase (Supplementary Table S1). Pigs were randomly divided into four groups: control (75 mg/kg anhydrous Zn sulfate), Zn group (1500 mg/kg anhydrous Zn sulfate), Zn+hes group (1500 mg/kg anhydrous Zn sulfate + 150 mg/kg hesperidin), and hes group (150 mg/kg hesperidin). The feed was supplemented with anhydrous Zn sulfate and/or hesperidin, and the periods lasted for 40 days. The samples were collected under anesthesia (sodium pentobarbital) on day 40. The experiment was approved from the Animal Care and Use Committee of Chongqing Three Gorges Vocational College.

2.2 Hematoxylin-eosin staining

After deparaffinization and rehydration of the tissue sections, they were stained with hematoxylin and eosin, followed by dehydration with gradient alcohol and transparency with xylene, and finally sealed with neutral resin.

2.3 Ultrastructure observation

The kidney tissues were collected and incubated in osmium tetroxide for 2 h. Then, the samples were performed as previous study (Fang et al., 2021). The ultrathin sections were detected by a HITACHI HT 7800 transmission electron microscope (HITACHI, Japan).

2.4 Immunohistochemical observation

The section preparation, antigen restore, peroxidase clearance and blocking in immunohistochemistry were carried out according to the previous studies (Liao et al., 2020). The primary antibody (ZO-1) was incubated for 16 h, followed by incubation with the secondary antibody conjugated with biotin for 1 h. The slides were observed and photographed under a microscope (Leica, Germany).

2.5 mRNA expression levels analysis

Total RNA was isolated with Trizol reagent (Takara, Japan). Reverse transcription steps of cDNA were carried out by using

BeyoRT™ II cDNA synthesis kit (Beyotime, China). The primers of LC3, p62, ATG5, Bak1, Bax, Bcl2, Caspase-3 and GAPDH were presented in Supplementary Table S2. The RT-qPCR was operated on the Applied Biosystems SimpliAmp PCR System (Thermo Fisher, USA). The results of relative mRNA expressions were shown as $2^{-\triangle\triangle CT}$.

2.6 Microfloral DNA extraction and sequencing

Intestine flora DNA was collected by HiPure Stool DNA Kits (Magen, Guangzhou, China) following the producer's instructions. A set of V3/V4 conserved region was amplified by PCR. The amplified PCR products were extracted by using DNA Gel Extraction Kit (Beyotime, China) for target fragment recovery. Purified amplicons were sequenced according to previous study (Xie et al., 2022). Prediction of flora function was determined on the TaxFun platform (Gene Denovo, China).

2.7 Statistical analysis

GraphPad Prism 8.5 (GraphPad Inc., LaJolla, CA, USA) was used for data statistics. The collected data were expressed as mean ± standard errors and analyzed by one-way analysis of variance (ANOVA). The statistical significance was deemed at $p < 0.05$.

3 Results

3.1 Effects of Zn on the growth performance and renal injury

As shown in Figure 1A, the body weight of pigs in Zn group was decreased compared to control group. Meanwhile, the levels of creatinine and urea nitrogen in serum were remarkably increased under Zn treatment compared to control group ($p<0.05$ or $p<0.01$). Hesperidin increased body weight and significantly decreased creatinine and urea nitrogen levels ($p<0.05$) (Figures 1B, C). Histological observations showed that excessive exposure to Zn caused significant glomerular atrophy compared with control group. However, after hesperidin treatment, the pathological changes were significantly improved (Figure 1D). Through further ultrastructural observation, we found that mitochondria in the kidney exposed to high level of Zn showed vacuolation and ridge breakage, while hesperidin combined with Zn treatment group did not show a large number of damaged mitochondria (Figure 1E).

3.2 Effects of hesperidin on Zn-induced autophagy and apoptosis in kidney

As shown as Figure 2, the mRNA levels of LC3, ATG5, Bak1, Bax, and Caspase-3 were significantly upregulated in Zn group compared to control group ($p<0.01$), and the mRNA expression levels of p62 and Bcl2 were markedly downregulated ($p<0.01$). Additionally, hesperidin could remarkably reduce the mRNA levels of LC3, ATG5, Bak1, Bax, and Caspase-3 and elevate the levels of p62 and Bcl2 under Zn treatment.

3.3 Effects of hesperidin on Zn-induced colonic barrier dysfunction

Here, after excessive Zn intake, the colon mucosa is damaged and the number of glands is significantly reduced (Figure 3A), and the PAS staining also showed that the number of goblet cells was decreased significantly under Zn treatment in contrast to the control group ($p<0.05$) (Figures 3A, B). Whereas, hesperidin could signally relieve the pathological damage of the colon (Figures 3A, B). In addition, the result of immunohistochemistry revealed that the tight junction protein ZO-1 was expressed at a low level under excess Zn treatment ($p<0.01$), and hesperidin can significantly reverse the trend ($p<0.05$) (Figures 3A, C).

3.4 Effects of hesperidin on alpha and beta diversity indices in intestinal microflora under Zn treatment

The alpha diversity of gut flora was analyzed by the Sob, ACE, and chao1. Here, the Sob (Figure 4A), ACE (Figure 4B), and chao1 (Figure 4C) in the control group were higher than that in Zn group, hes group, and Zn+hes group. Additionally, PCoA, PCA, and NMDS plot were used to evaluate Beta-diversity. Different colored spots represented the different groups. The gathered spots meant that the composition of the microbial structure between samples is more similar. In this study, the PCoA, PCA plot, and NMDS showed that each group's plots were independent of the other group (Figures 4D–F).

3.5 Effects of hesperidin on the composition of the gut microbiota under Zn treatment

In the present study, the results of venn diagram showed at the phylum level, a total of 16 OTUs in the control group and the Zn group, and a total of 15 OTUs both in Zn group and Zn+hes group. At the level of genus, a total of 137 OTUs in the control group and the Zn group, and a total of 135 OTUs both in Zn group and Zn +hes group (Figures 5A, B). We found that the Firmicutes is the dominant flora. Compared with the control group, the abundance of Bacteroidetes was increased and Actinobacteria was decreased in the Zn group, while the Zn+hes group showed a reverse trend compared with the Zn group, and the hes group and the control group were close at phylum level (Figures 5C, E). In addition, the abundance of Ruminococcaceae_UCG-014 was increased and Olsenella and Lactobacillus were decreased in the Zn group, while the Zn+hes group showed a reverse trend compared with the Zn

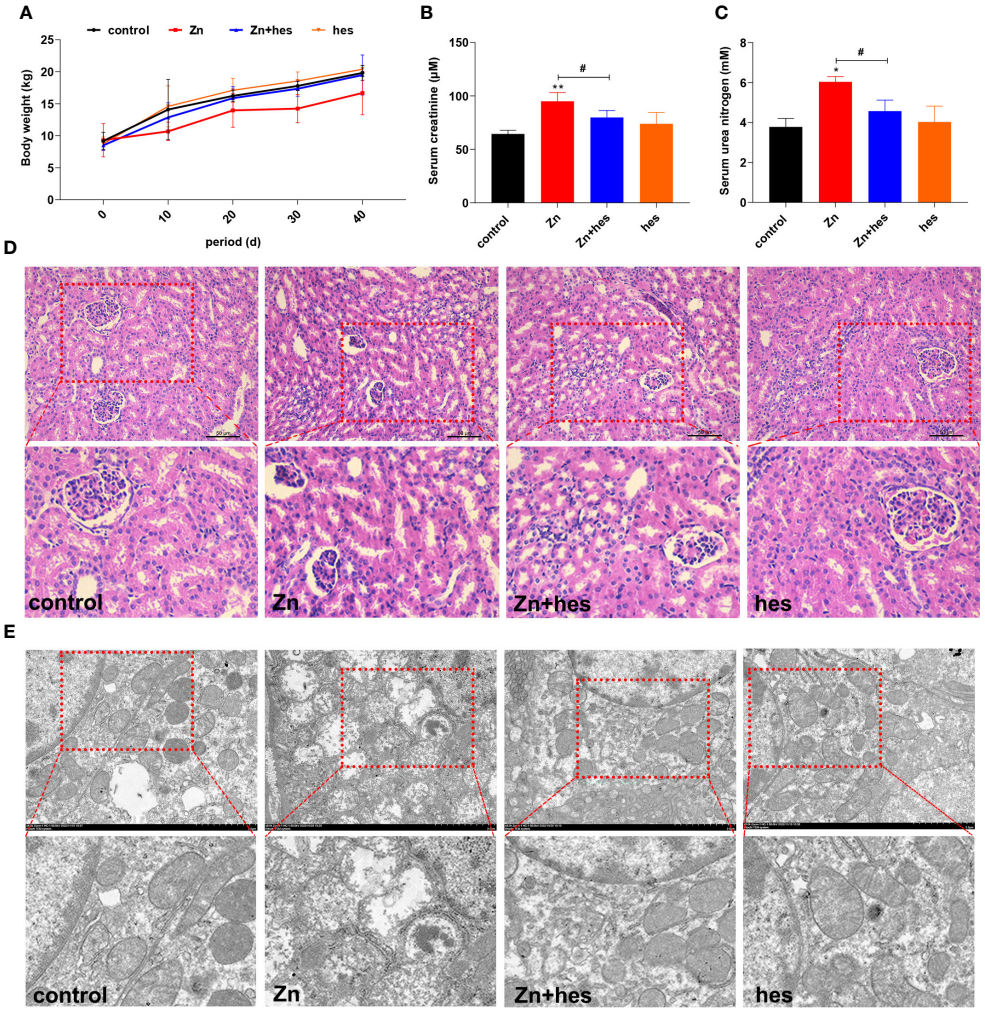

FIGURE 1
Effects of Zn on the growth performance and renal injury in pigs. **(A)** Body weight. **(B)** Serum creatinine. **(C)** Urea nitrogen. **(D)** HE staining. **(E)** TEM observation.

group at genus level (Figures 5D, F). Through the significance analysis, it was found that the abundance of Bacteroidetes and Tenericutes were increased significantly and Proteobacteria was decreased remarkably in Zn group compared to control group at phylum level. Interestingly, hesperidin could markedly decrease the abundance of Tenericutes under Zn treatment (Figures 6A, C). At genus level, the abundance of Rikenellaceae_RC9_gut_group, Ruminococcaceae_UCG-005, and Christensenella were increased remarkably in Zn group compared to control group, and Pseudoramibacter and Dorea were significantly decreased. Meanwhile, hesperidin could decrease the abundance of Christensenella under Zn treatment (Figures 6B, D). Besides that, the function prediction possibly indicated that the differential flora might participate in the cell growth and death, nutrition metabolism, energy metabolism and so on (Figure 6E).

4 Discussion

Zn is an essential trace element involved in a variety of life processes. Dietary supplementation of appropriate amount of Zn can not only improve animal reproductive performance and maintain intestinal microenvironment homeostasis, but also improve antioxidant function and enhance immunity. However, excessive intake of Zn in animals can lead to decreased antioxidant function, resulting in oxidative stress and programmed death (Yang et al., 2022; Li et al., 2023b). It has been proved that the kidney is an important target organ for Zn toxicity, but its pathogenic mechanism is still unclear. The “gut-kidney axis” theory holds that the disturbance of intestinal flora can induce the impairment of intestinal barrier function and systemic micro-inflammatory response, thus inducing the functional impairment of the kidney (Yang et al., 2018). Therefore, through the search for corresponding drugs, through the “entero-renal axis” to treat kidney disease has become a hot research direction. Hesperidin is a kind of natural flavonoid widely found in citrus fruits, which has been found to have antioxidant, anti-inflammatory and other biological activities (Li et al., 2021b). Here, this study identified the mechanism of “intestinal flora - intestinal kidney axis” in hesperidin alleviating kidney injury caused by Zn, and further clarified the pathway of hesperidin in Zn-induced kidney injury.

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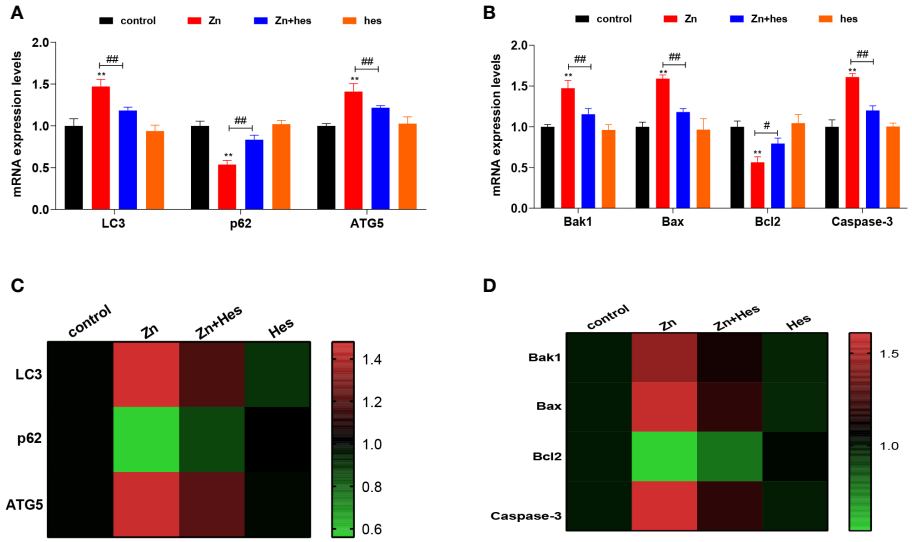

FIGURE 2
Effects of Zn on the autophagy and apoptosis in kidney. **(A)** mRNA levels of LC3, p62, and ATG5. **(B)** mRNA levels of Bak1, Bax, Bcl2, and Caspase-3. **(C)** Heat mat of the autophagy-related genes expression. **(D)** Heat mat of the apoptosis-related genes expression. "*" expressed the statistical difference compared with the control group (*P < 0.05, **P < 0.01 and ***P < 0.001). "#" expressed the statistical difference between the two groups (#P < 0.05, ##P < 0.01 and ###P < 0.001).

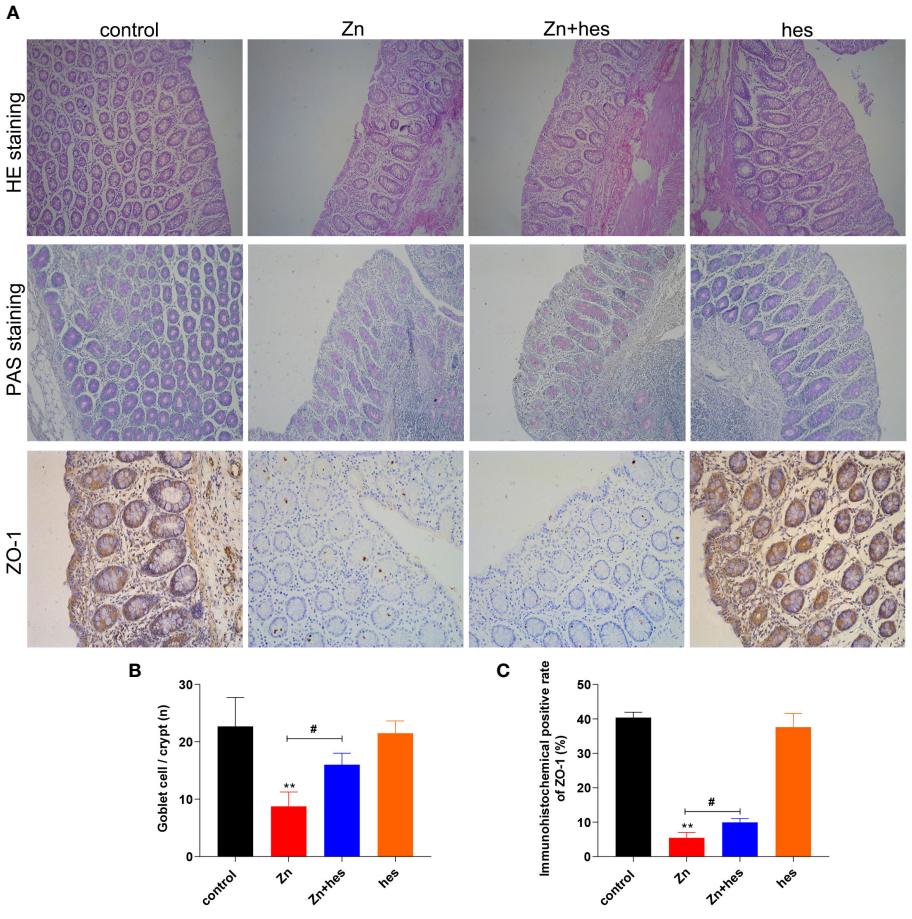

FIGURE 3
Effects of Zn on the barrier function in colon. **(A)** HE staining, PAS staining, and immunofluorescencal detection of ZO-1 in colon. **(B)** The number of goblet cells. **(C)** Immunofluorescence positive rate of ZO-1. "*" expressed the statistical difference compared with the control group (*P < 0.05, **P < 0.01 and ***P < 0.001). "#" expressed the statistical difference between the two groups (#P < 0.05, ##P < 0.01 and ###P < 0.001).

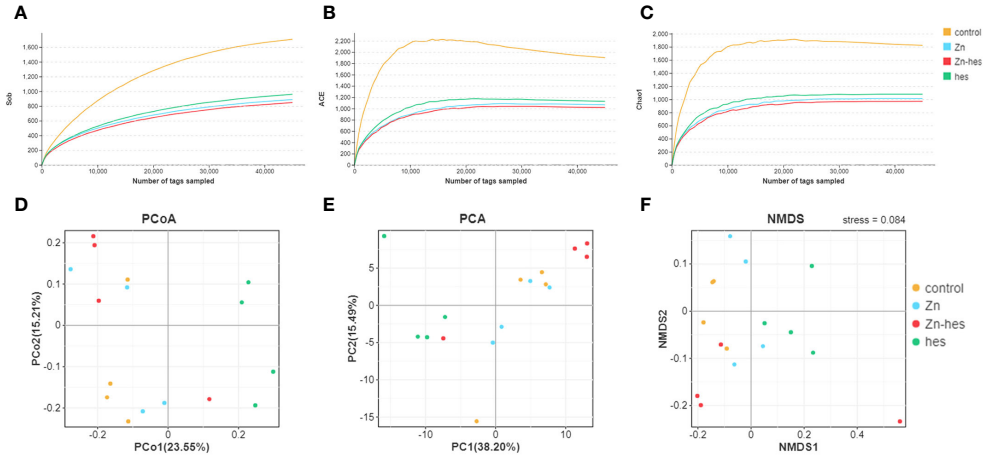

FIGURE 4
Effects of Zn on alpha and beta diversity indices in colonic microflora. **(A–C)** Alpha diversity is analyzed by Sob **(A)**, ACE **(B)**, and chao1 **(C)**. **(D–F)** Beta diversity is analyzed by PCoA **(D)**, PCA **(E)**, and NMDS **(F)**. Each dot represents an individual sample.

A large number of studies have confirmed that cell damage caused by heavy metals is often accompanied by autophagy and apoptosis (Fang et al., 2021; Li et al., 2021a). Autophagy is a biological mechanism widely existing in eukaryotic cells, and it is considered to be an important way for cells to degrade large quantities of senescent proteins and damaged organelles. Autophagy forms an independent double-layer membrane structure, wraps the cytoplasm under the regulation of a variety of proteins, fuses with lysosomes and finally degrades into small molecules, which are released back into the cytoplasm for use (Klionsky et al., 2021). During autophagy, ATG5 promotes the formation of bilayer membrane of autophagosome, recruits LC3 and promotes its transformation from LC3-I to LC3-II. The degradation of p62 is another marker for monitoring autophagy, as p62 can bind to LC3 and be selectively degraded by autophagy (Levine and Kroemer, 2019). In addition, apoptosis is a type of programmed cell death that is highly regulated by multiple genes and characterized by chromatin condensation, DNA cleavage, and the formation of apoptotic bodies. The mechanism of apoptosis is very complex, mainly divided into endogenous and exogenous pathways (D'Arcy, 2019). Among them, most of the research focuses on endogenous apoptosis, and the apoptosis of mitochondrial pathway has also become a research hotspot for most scholars. In most cases, mitochondria-mediated endogenous apoptosis is controlled by the Bcl family, which is composed of pro-apoptotic factors such as Bax and Bak-1 and anti-apoptotic factors Bcl-2 (Yuan et al., 2021). When the apoptotic signal is received, the pro-apoptotic protein will be transferred to the mitochondrial membrane, and the Bcl-2 protein will be further down-regulated, resulting in the permeability of the mitochondrial outer membrane, the reduction of mitochondrial membrane potential, and the release of intermembrane pro-apoptotic substances, which will further promote the shear of Caspase-3, destroy the nuclear structure, and break down the cell into apoptotic bodies (Liao et al., 2019). It has been confirmed that feeding high dose of copper can increase the protein expression levels of LC3-II/LC3-I, ATG5, Bax, and cleaved Caspase-3 (Liao et al., 2020, 2021). Zhang et al. also confirmed that molybdenum and cadmium combined feeding in ducks also led to renal autophagy and apoptosis (Zhang et al., 2022b). In our study, we have found that Zn could induced the high expression level of autophagy and apoptosis-related genes, and hesperidin could reverse these effects, which verified the anti-renal damage effect of hesperidin.

Studies have shown that the toxic effect of heavy metals is caused by the absorption of digestive tract and metabolism in the body (Witkowska et al., 2021). At the same time, intestinal damage may become an important bridge for toxin-induced organ toxicity damage (Liao et al., 2022). Therefore, the evaluation of intestinal damage is also an important indicator for heavy metal poisoning. In this study, we evaluated the intestinal toxicity of Zn by histology and expression of ZO-1 protein. ZO-1 is one of intestinal tight junction proteins, which can maintain the structural integrity of intestinal epithelial cells and the continuity of intestinal mucosal barrier, and plays an important role in cell proliferation, differentiation and growth, regulation of intercellular signal transduction and penetration (Kuo et al., 2022). Here, we found that Zn exposure could cause pathological damage to the colon, and induce the ZO-1 protein expression decreased significantly. Nevertheless, hesperidin treatment could upregulate the ZO-1 expression level, which indicating that hesperidin can repair barrier barriers, improve the structure and function of intestinal epithelium, and regulate intestinal mucosal permeability.

In this study, we have revealed that Zn can cause nephrotoxicity, and the colon also produces pathological damage, and hesperidin can reverse the damage both of them. It has reported that the process of drug alleviating kidney injury may involve the role of the "gut-kidney" axis, especially the function of intestinal flora (Xie et al., 2022). Therefore, we speculate that hesperidin plays a key role in alleviating Zn-induced nephrotoxicity by the gut microbiota. In recent years, with the attention paid to the occurrence of kidney diseases caused by intestinal microecological disorders, the relationship between gut and kidney has gradually become a hot

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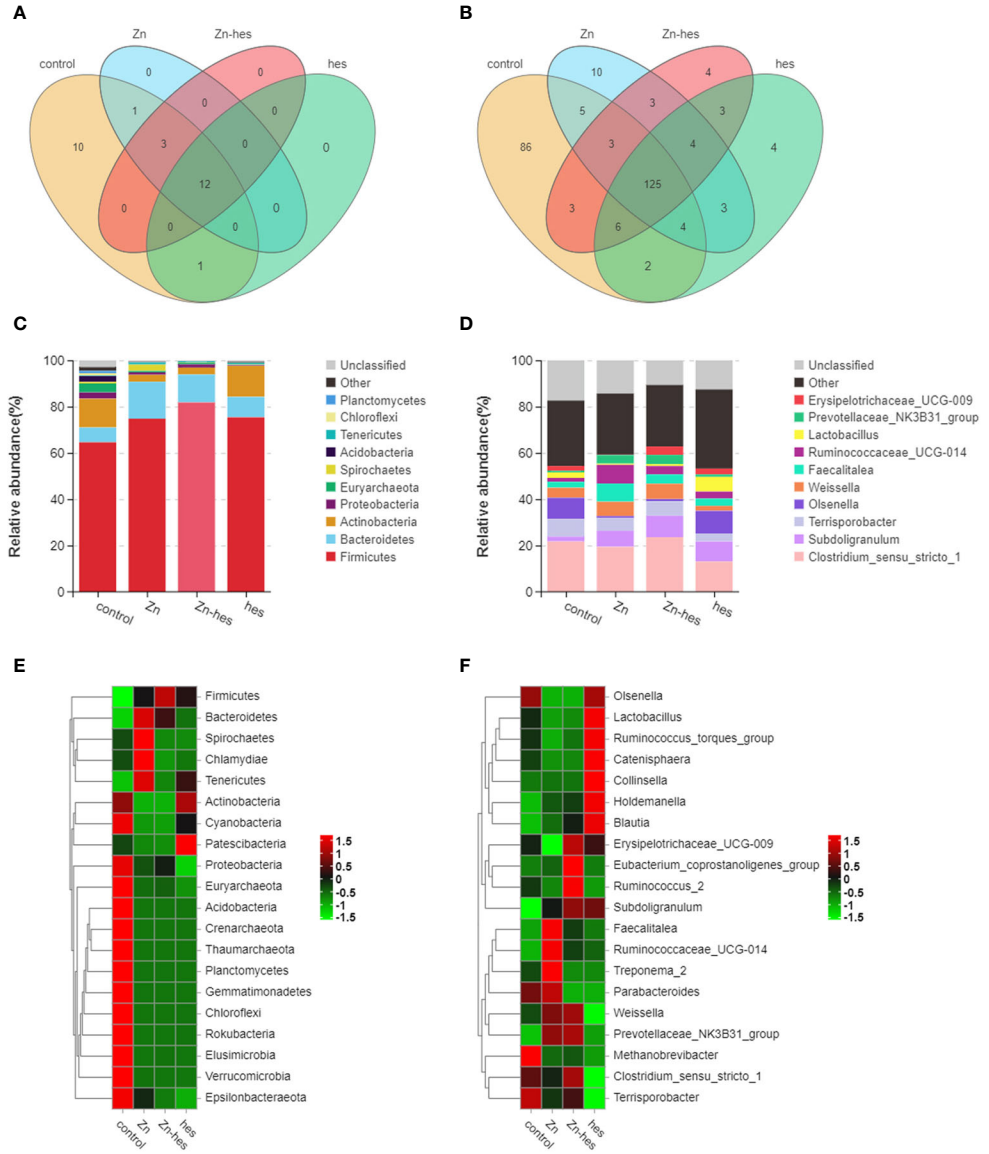

FIGURE 5
Effects of Zn on the changes in intestinal flora. **(A)** Venn diagram of gut flora at the phylum level. **(B)** Venn diagram of gut flora at the genus level. **(C)** Composition of gut flora at the phylum level. **(D)** Composition of gut flora at the genus level. **(E)** Heat map of gut flora at the phylum level. **(F)** Heat map of gut flora at the genus level.

topic of research, and the theory of "gut-kidney axis" has been gradually confirmed (Evenepoel et al., 2017). As reported, 20% of patients with inflammatory bowel disease have mild tubular damage, which may be related to the effects of conventional treatment drugs and inflammatory cytokines. The proposal of the "gut-kidney" axis explores the regulating effect of the gut on the kidney. As an important role of the "gut-kidney" axis, intestinal flora plays an important role, and the disturbance of gut microbiota homeostasis and the intestinal barrier dysfunction are the inducing factors in the occurrence of kidney diseases (Chen et al., 2019). Here, we found that Zn could remarkably increase the abundance of Tenericutes and Christensenella, and hesperidin significantly decrease their abundance. Zhang et al. showed that the Tenericutes was reduced in diabetic nephropathy after Lycoperoside H treatment, which provided an important reference for the involvement of Tenericutes in the regulation of kidney diseases through "gut-kidney" axis (Zhang et al., 2022a). In addition, as a probiotic, Christensenella has been used for the treatment of diabetes, which could enhance the intestinal barrier and reduce intestinal inflammation (Pan et al., 2022). Therefore, hesperidin was found to improve Zn-induced intestinal barrier dysfunction by changing the abundance of Tenericutes and Christensenella, thus alleviating zinc-induced kidney injury via "gut-kidney" axis. In subsequent studies, we can use high-throughput sequencing to screen signal molecules to explore the molecular targets of hesperidin in regulating zinc-induced kidney injury.

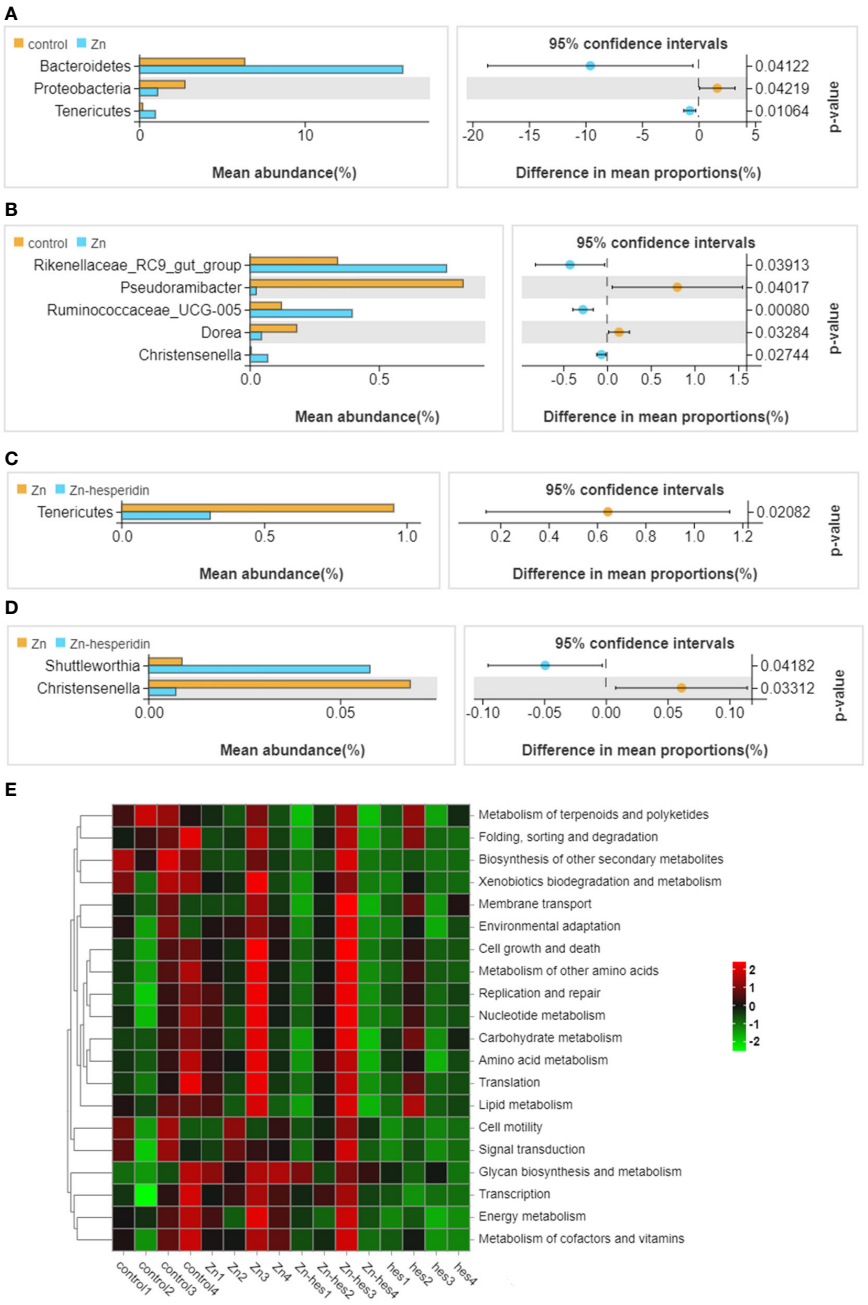

FIGURE 6
Differential flora screening and functional prediction. **(A)** Different bacteria at the phylum level between control group and Zn group. **(B)** Different bacteria at the genus level between control group and Zn group. **(C)** Different bacteria at the phylum level between Zn group and Zn+hes group. **(D)** Different bacteria at the genus level between Zn group and Zn+hes group. **(E)** Function prediction of differential flora.

5 Conclusion

In summary, Zn could induce nephrotoxicity and intestinal damage, and the disordered intestinal flora, as the core of "gut-kidney" axis, is an important pathway to promote Zn-induced nephrotoxicity. Additionally, hesperidin could improve the zinc-induced gut microbiota disorder for alleviating Zn-induced nephrotoxicity *via* "gut-kidney" axis.

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 South China Agricultural University ethics committee. 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

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Conflict of interest

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

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Supplementary material

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References

Alhasawi, A., Auger, C., Appanna, V. P., Chahma, M., and Appanna, V. D. (2014). Zinc toxicity and ATP production in Pseudomonas fluorescens. *J. Appl. Microbiol.* 117, 65–73. doi: 10.1111/jam.12497

Chen, Y. Y., Chen, D. Q., Chen, L., Liu, J. R., Vaziri, N. D., Guo, Y., et al. (2019). Microbiome-metabolome reveals the contribution of gut-kidney axis on kidney disease. *J. Trans. Med.* 17, 5. doi: 10.1186/s12967-018-1756-4

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol. Int.* 43, 582–592. doi: 10.1002/cbin.11137

Evenepoel, P., Poesen, R., and Meijers, B. (2017). The gut-kidney axis. *Pediatr. Nephrol.* 32, 2005–2014. doi: 10.1007/s00467-016-3527-x

Fang, Y., Xing, C., Wang, X., Cao, H., Zhang, C., Guo, X., et al. (2021). Activation of the ROS/HO-1/NQO1 signaling pathway contributes to the copper-induced oxidative stress and autophagy in duck renal tubular epithelial cells. *Sci. OF THE TOTAL Environ.* 757, 143753. doi: 10.1016/j.scitotenv.2020.143753

Hager-Theodorides, A. L., Massouras, T., Simitzis, P. E., Moschou, K., Zoidis, E., Sfakianaki, E., et al. (2021). Hesperidin and naringin improve broiler meat fatty acid profile and modulate the expression of genes involved in fatty acid beta-oxidation and antioxidant defense in a dose dependent manner. *Foods* 10 (4), 739. doi: 10.3390/foods10040739

Hsu, C. N., and Tain, Y. L. (2022). Chronic kidney disease and gut microbiota: what is their connection in early life? *Int. J. Mol. Sci.* 23 (7), 3954. doi: 10.3390/ijms23073954

Hutchens, W. M., Tokach, M. D., Dritz, S. S., Gebhardt, J., Woodworth, J. C., DeRouchey, J. M., et al. (2021). The effects of pharmacological levels of zinc, diet acidification, and dietary crude protein on growth performance in nursery pigs. *J. Anim. Sci.* 99 (10), skab259. doi: 10.1093/jas/skab259

Kamboh, A. A., Hang, S. Q., Bakhetgul, M., and Zhu, W. Y. (2013). Effects of genistein and hesperidin on biomarkers of heat stress in broilers under persistent summer stress. *Poult Sci.* 92, 2411–2418. doi: 10.3382/ps.2012-02960

Kataba, A., Nakayama, S., Yohannes, Y. B., Toyomaki, H., Nakata, H., Ikenaka, Y., et al. (2021). Effects of zinc on tissue uptake and toxicity of lead in Sprague Dawley rat. *J. Of Veterinary Med. Sci.* 83, 1674–1685. doi: 10.1292/jvms.20-0684

Kim, B., and Lee, W. W. (2021). Regulatory role of zinc in immune cell signaling. *Molecules Cells* 44, 335–341. doi: 10.14348/molcells.2021.0061

Klionsky, D. J., Petroni, G., Amaravadi, R. K., Baehrecke, E. H., Ballabio, A., Boya, P., et al. (2021). Autophagy in major human diseases. *EMBO Joournal* 40, e108863. doi: 10.15252/embj.2021108863

Kouvedaki, I., Pappas, A. C., Surai, P. F., and Zoidis, E. (2024). Nutrigenomics of natural antioxidants in broilers. *Antioxidants (Basel)* 13, 270. doi: 10.3390/antiox13030270

Kuo, W. T., Odenwald, M. A., Turner, J. R., and Zuo, L. (2022). Tight junction proteins occludin and ZO-1 as regulators of epithelial proliferation and survival. *Ann. N Y Acad. Sci.* 1514, 21–33. doi: 10.1111/nyas.14798

Levine, B., and Kroemer, G. (2019). Biological functions of autophagy genes: A disease perspective. *Cell* 176, 11–42. doi: 10.1016/j.cell.2018.09.048

Li, Y., Chen, H., Liao, J., Chen, K., Javed, M. T., Qiao, N., et al. (2021a). Long-term copper exposure promotes apoptosis and autophagy by inducing oxidative stress in pig testis. *Environ. Sci. pollut. Res. Int.* 28, 55140–55153. doi: 10.1007/s11356-021-14853-y

Li, J., Wang, T., Liu, P., Yang, F., Wang, X., Zheng, W., et al. (2021b). Hesperetin ameliorates hepatic oxidative stress and inflammation *via* the PI3K/AKT-Nrf2-ARE pathway in oleic acid-induced HepG2 cells and a rat model of high-fat diet-induced NAFLD. *Food Funct.* 12, 3898–3918. doi: 10.1039/D0FO02736G

Li, N., Wang, Y., Wei, P., Min, Y., Yu, M., Zhou, G., et al. (2023a). Causal effects of specific gut microbiota on chronic kidney diseases and renal function-A two-sample mendelian randomization study. *Nutrients* 15 (2), 306. doi: 10.3390/nu15020360

Li, X., Wen, J., Jiao, L., Wang, C., Hong, Q., Feng, J., et al. (2021c). Dietary copper/zinc-loaded montmorillonite improved growth performance and intestinal barrier and changed gut microbiota in weaned piglets. *J. Anim. Physiol. Anim. Nutr. (Berl)* 105, 678–686. doi: 10.1111/jpn.13522

Li, Q., Yang, Q., Guo, P., Feng, Y., Wang, S., Guo, J., et al. (2023b). Mitophagy contributes to zinc-induced ferroptosis in porcine testis cells. *Food Chem. Toxicol.* 179, 113950. doi: 10.1016/j.fct.2023.113950

Li, X., Yao, Y., Wang, Y., Hua, L., Wu, M., Chen, F., et al. (2022). Effect of hesperidin supplementation on liver metabolomics and gut microbiota in a high-fat diet-induced NAFLD mice model. *J. Agric. Food Chem.* 70, 11224–11235. doi: 10.1021/acs.jafc.2c02334

Liao, J., Hu, Z., Li, Q., Li, H., Chen, W., Huo, H., et al. (2022). Endoplasmic reticulum stress contributes to copper-induced pyroptosis *via* regulating the IRE1alpha-XBP1 pathway in pig jejunal epithelial cells. *J. Agric. Food Chem.* 70, 1293–1303. doi: 10.1021/acs.jafc.1c07927

Liao, N. C., Shih, Y. L., Chou, J. S., Chen, K. W., Chen, Y. L., Lee, M. H., et al. (2019). Cardamonin induces cell cycle arrest, apoptosis and alters apoptosis associated gene expression in WEHI-3 mouse leukemia cells. *Am. J. Chin. Med.* 47, 635–656. doi: 10.1142/S0192415X19500332

Liao, J., Yang, F., Bai, Y., Yu, W., Qiao, N., Han, Q., et al. (2021). Metabolomics analysis reveals the effects of copper on mitochondria-mediated apoptosis in kidney of broiler chicken (Gallus gallus). *J. Inorganic Biochem.* 224, 111581. doi: 10.1016/j.jinorgbio.2021.111581

Liao, J., Yang, F., Yu, W., Qiao, N., Zhang, H., Han, Q., et al. (2020). Copper induces energy metabolic dysfunction and AMPK-mTOR pathway-mediated autophagy in kidney of broiler chickens. *Ecotoxicol Environ. Saf.* 206, 111366. doi: 10.1016/j.ecoenv.2020.111366

Mas-Capdevila, A., Teichenne, J., Domenech-Coca, C., Caimari, A., Del, B. J., Escote, X., et al. (2020). Effect of hesperidin on cardiovascular disease risk factors: the role of intestinal microbiota on hesperidin bioavailability. *Nutrients* 12 (5), 1488. doi: 10.3390/nu12051488

Nouri, Z., Zhang, X. Y., Khakisahneh, S., Degen, A. A., and Wang, D. H. (2022). The microbiota-gut-kidney axis mediates host osmoregulation in a small desert mammal. *NPJ Biofilms Microbiomes* 8, 16. doi: 10.1038/s41522-022-00280-5

Pan, T., Zheng, S., Zheng, W., Shi, C., Ning, K., Zhang, Q., et al. (2022). Christensenella regulated by Huang-Qi-Ling-Hua-San is a key factor by which to improve type 2 diabetes. *Front. Microbiol.* 13, 1022403. doi: 10.3389/fmicb.2022.1022403

Plata, C., Cruz, C., Cervantes, L. G., and Ramirez, V. (2019). The gut microbiota and its relationship with chronic kidney disease. *Int. Urol. Nephrol.* 51, 2209–2226. doi: 10.1007/s11255-019-02291-2

Ratn, A., Prasad, R., Awasthi, Y., Kumar, M., Misra, A., and Trivedi, S. P. (2018). Zn (2+) induced molecular responses associated with oxidative stress, DNA damage and histopathological lesions in liver and kidney of the fish, Channa punctatus (Bloch 1793). *Ecotoxicol Environ. Saf.* 151, 10–20. doi: 10.1016/j.ecoenv.2017.12.058

Ren, T., Fu, G. H., Liu, T. F., Hu, K., Li, H. R., Fang, W. H., et al. (2017). Toxicity and accumulation of zinc pyrithione in the liver and kidneys of Carassius auratus gibelio: association with P-glycoprotein expression. *FISH Physiol. Biochem.* 43, 1–9. doi: 10.1007/s10695-016-0262-y

Wang, M., Phadke, M., Packard, D., Yadav, D., and Gorelick, F. (2020). Zinc: Roles in pancreatic physiology and disease. *Pancreatology* 20, 1413–1420. doi: 10.1016/j.pan.2020.08.016

Witkowska, D., Slowik, J., and Chilicka, K. (2021). Heavy metals and human health: possible exposure pathways and the competition for protein binding sites. *Molecules* 26 (19), 6060. doi: 10.3390/molecules26196060

Xie, Y., Hu, X., Li, S., Qiu, Y., Cao, R., Xu, C., et al. (2022). Pharmacological targeting macrophage phenotype *via* gut-kidney axis ameliorates renal fibrosis in mice. *Pharmacol. Res.* 178, 106161. doi: 10.1016/j.phrs.2022.106161

Xiong, H., Wang, J., Ran, Q., Lou, G., Peng, C., Gan, Q., et al. (2019). Hesperidin: A therapeutic agent for obesity. *Drug Des. Devel Ther.* 13, 3855–3866. doi: 10.2147/DDDT

Yang, Q., Fang, Y., Zhang, C., Liu, X., Wu, Y., Zhang, Y., et al. (2022). Exposure to zinc induces lysosomal-mitochondrial axis-mediated apoptosis in PK-15 cells. *Ecotoxicol Environ. Saf.* 241, 113716. doi: 10.1016/j.ecoenv.2022.113716

Yang, T., Richards, E. M., Pepine, C. J., and Raizada, M. K. (2018). The gut microbiota and the brain-gut-kidney axis in hypertension and chronic kidney disease. *Nat. Rev. Nephrol.* 14, 442–456. doi: 10.1038/s41581-018-0018-2

Yuan, Z., Dewson, G., Czabotar, P. E., and Birkinshaw, R. W. (2021). VDAC2 and the BCL-2 family of proteins. *Biochem. Soc. Trans.* 49, 2787–2795. doi: 10.1042/BST20210753

Zhang, Z., Li, Q., Liu, F., and Wang, D. (2022a). Lycoperoside H protects against diabetic nephropathy *via* alteration of gut microbiota and inflammation. *J. Biochem. Mol. Toxicol.* 36, e23216. doi: 10.1002/jbt.23216

Zhang, C., Lin, T., Nie, G., Hu, R., Pi, S., Wei, Z., et al. (2022b). *In vivo* assessment of molybdenum and cadmium co-induce nephrotoxicity *via* causing calcium homeostasis disorder and autophagy in ducks (Anas platyrhyncha). *Ecotoxicol Environ. Saf.* 230, 113099. doi: 10.1016/j.ecoenv.2021.113099

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Research progress in isolation and identification of rumen probiotics

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With the increasing research on the exploitation of rumen microbial resources, rumen probiotics have attracted much attention for their positive contributions in promoting nutrient digestion, inhibiting pathogenic bacteria, and improving production performance. In the past two decades, macrogenomics has provided a rich source of new-generation probiotic candidates, but most of these "dark substances" have not been successfully cultured due to the restrictive growth conditions. However, fueled by high-throughput culture and sorting technologies, it is expected that the potential probiotics in the rumen can be exploited on a large scale, and their potential applications in medicine and agriculture can be explored. In this paper, we review and summarize the classical techniques for isolation and identification of rumen probiotics, introduce the development of droplet-based high-throughput cell culture and single-cell sequencing for microbial culture and identification, and finally introduce promising cultureomics techniques. The aim is to provide technical references for the development of related technologies and microbiological research to promote the further development of the field of rumen microbiology research.

KEYWORDS

rumen probiotics, microorganisms, isolation, culture, identification

1 Introduction

The Food and Agriculture Organization of the United Nations and the World Health Organization defines probiotics as live microorganisms which provide a beneficial effect on the host when ingested in moderation (FAO/WHO, 2001). This definition is widely accepted by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Hill et al., 2014). Probiotics perform many biological functions in the ecosystem, such as aiding in digestion, inhibiting pathogenic bacteria, promoting growth and regulating immunity (Dasriya et al., 2024). The efficacy of probiotics has been demonstrated through *in vivo* experiments. Probiotics' beneficial effects and safety are usually evaluated through *in vivo* experiments, while their beneficial potential and safety are characterized through *in vitro*

studies or animal models (Reid, 2006). Therefore, the first step in evaluating probiotics for food use is *in vitro* studies, followed by double-blind, randomized, placebo-controlled human trials (Fijan, 2014; de Melo Pereira et al., 2018).General *in vitro* study properties of probiotics include resistance to gastric acid, bile acid resistance, adhesion to mucus or human epithelial cells, antimicrobial activity against potentially pathogenic bacteria or fungi, reduction of pathogen adhesion, bile salt hydrolase activity and enhancement of beneficial bacterial viability (Megur et al., 2023; Prakash et al., 2023; Sreepathi et al., 2023).

The rumen is one of the vital nutrient digestive organs in the digestive system of ruminants, and its internal microflora is very rich, including bacteria, fungi, archaea and a small number of phage viruses (Mizrahi et al., 2021). Rumen probiotics are probiotics that grow and multiply in the rumen of ruminants (Kmet et al., 1993) Rumen probiotics have specific functional roles in nutrition, digestion, immunity and health of ruminants. Numerous studies have shown that rumen probiotics can promote nitrite metabolism (Latham et al., 2018; Deng et al., 2021) promote intestinal development (Arshad et al., 2021) reduce methane production (Maake et al., 2021; Pittaluga et al., 2023), Fibre degradation (Chen et al., 2022), inhibition of pathogenic bacteria (Poothong et al., 2024), immunomodulation (Varada et al., 2022) and regulation of rumen acidosis (Lettat et al., 2012).

In recent years, high-throughput sequencing has provided a wealth of information on the composition, host specificity, and spatial and temporal dynamics of rumen-associated microbial communities (Li et al., 2023c). With the development of non-targeted and new high-throughput culture methods, culture genomics platforms using a range of media and high-throughput screening methods offer the potential to bring more "dark matter" into culture (Zhang et al., 2021). The genomics-based approach provides additional insights and suggests new hypotheses for most uncultured organisms (Gutleben et al., 2018). More importantly, with the elucidation of the beneficial mechanisms of some new strains, mining the next generation of candidate probiotics from the gut has become a new research hotspot (Wan et al., 2023). This paper provides an overview of the current applications of rumen probiotics in production, the classical techniques for culturing and identifying rumen probiotics, the culture strategies for cultivating "dark matter" from the rumen, and the technological tools for analyzing the diversity and dynamics of rumen bacteria (Figure 1).

2 Probiotic bacteria in livestock and poultry breeding applications

With the current research background of "forbidden resistance" on the feed side and "antibiotic reduction" and "substitute antibiotic" on the breeding side, probiotics are often used in the most common and widely used feed additives in the market because they can effectively improve the growth performance of livestock, enhance the immunity and regulate the gastrointestinal flora, and they are safe and environmentally friendly (Li et al., 2019; Wang et al., 2019a). Probiotic microecological preparations are prepared by isolating, identifying, and screening probiotics and their

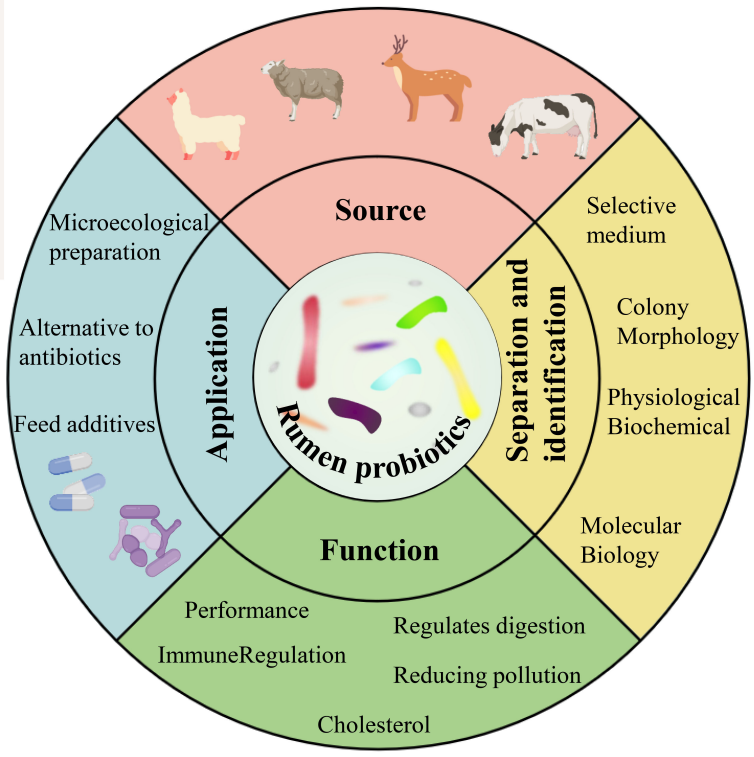

FIGURE 1
Sources, isolation and identification, functions and applications of rumen probiotics (Yuan et al., 2015; Noel et al., 2019; Xiong et al., 2020).

metabolites from different ecosystems and evaluating their safety *in vitro*, and through a series of technological treatments, they are prepared into biological preparations containing rich active bacteria (Xie et al., 2021). Current research has shown that enzyme preparations of cellulolytic bacteria isolated from the rumen of reindeer can increase average milk yield, enhance ciliate abundance, and improve the natural resistance of newborn calves (Litonina et al., 2021); The addition of Lactobacillus casei and Saccharomyces cerevisiae to diets increased the growth performance and apparent digestibility of nutrients, the relative abundance of short-chain fatty acid-producing microbiota, and the total short-chain fatty acid content of fattening pigs (Hassan et al., 2020). A strain of Bacillus denitrificans 79R4 (Paenibacillus 79R4) isolated from the bovine rumen enhanced rumen nitrite detoxification and reduced the risk of methemoglobinemia in cattle due to nitrate addition (Latham et al., 2019).

Other selected probiotic or potential probiotic application studies are shown in Table 1.

TABLE 1 Research on the application of some probiotics in livestock and poultry farming.

Probiotics	Animals	Functionality	Reference
Bacillus paralicheniformis (SN-6)	Simmental beef cattle	Increases body weight, alters metabolomic patterns in Simmental beef cattle, and increases the relative abundance of beneficial bacteria. Enriches intestinal metabolites to maintain intestinal homeostasis. Enhances amino acid metabolism and lipid metabolism pathways	Yang et al., 2022
Lactobacillus plantarum	Ovine	Increased digestibility and reduced methane emissions	Zhang et al., 2022
Lactobacillus acidophilus	Ovine	Reducing Salmonella carriage in sheep	Pepoyan et al., 2020
Bacillus subtilis	Infected computer in a botnet	Improve broiler growth performance and enhance intestinal immunity	Khalifa et al., 2023
Bacillus licheniformis	Ovine	Reducing methane emissions from sheep while increasing ration conversion rates	Deng et al., 2018
Lactobacillus rhamnosus	Piglet	Improves the physical, biological and immune barrier of the intestinal mucosa and benefits the intestinal health of pre-weaned piglets	Wang et al., 2019b
Enterococcus faecalis	Milk cow	Its secretion produces the peptide AS-48, which is used in the prevention and treatment of mastitis in cows	Davidse et al., 2004
Enterococcus faecium	Pigeon	Improve antioxidant performance and immune function of pigeon, promote growth and improve meat quality	Han et al., 2022
Ruminococcus flavefaciens	Lambs	Increases daily lamb weight gain and nutrient digestibility, reduces NH3 -N and methane production, and reduces greenhouse gas emissions	Kumar et al., 2021
Clostridium butyricum	Goats	Improving rumen fermentation and growth performance of goats under heat stress	Cai et al., 2021
Bacillus amyloliquefaciens (H57)	Ovine calf	Affects animal behaviour, promotes digestion and increases body weight	Schofield et al., 2018

(Continued)

3 Isolation of rumen probiotics

The growth of microorganisms depends on various nutrient factors within the medium and various physicochemical factors in the culture environment, which are the primary conditions for the pure culture of microorganisms Ren et al. (2016). Therefore, understanding the nutritional requirements and metabolic properties is very important for the isolation and stable cultivation of microorganisms. The pure culture of rumen microorganisms is an important cornerstone for in-depth research on genes, proteins, and metabolic pathways, as well as a valuable resource for experimental research on microbial traits, enriching reference databases and biological classification frameworks (Stewart et al., 2019). The classical methods for isolation and culture of rumen probiotics include selective isolation and culture and the Heinz rolling tube method, which have inherent drawbacks such as low throughput, labor-intensive and high cost. The development of emerging technologies, such as microfluidic devices, offers great promise for high-throughput cultures (Figure 2).

3.1 Selective isolation culture method

Selective isolation and culture method is to prepare the corresponding selection medium to enrich the target probiotics, which is essentially to eliminate the undesired microbial flora (Bonnet et al., 2019).The media used can be undefined and optimize the media using biochemical studies or one-factor methods according to experimental needs, which can reduce the complex effects between complex components and also allow easier detection of target metabolites (Hayek et al., 2019; Lewis et al., 2021). However, the number of rumen probiotics in the traditional culture strategy is limited, and the growth and metabolic characteristics of many rumen microorganisms are still unclear, which hinders the

TABLE 1 Continued

Probiotics	Animals	Functionality	Reference
Pichia kudriavzevii	Mongolian gazelle	Enhanced acetate-based fermentation for fibre and lipid digestion	Wang et al., 2022

systematic study of rumen microorganisms. Currently, selective culture media among probiotics include Bifidobacterium selective medium, Lactobacillus selective medium, low carbon-resistant selective medium, and sodium carboxymethylcellulose, etc (Kumar et al., 2021; Jaglan et al., 2019; Kuppusamy et al., 2020). The addition of antioxidants (ascorbic acid, glutathione, and uric acid in the culture medium) enhances the culture of anaerobic bacteria (Tidjani Alou et al., 2020); It can also use the rumen fluid to simulate the natural environment of certain bacteria to promote their growth (Bonnet et al., 2019). Recently, strategies combining classical microbiological techniques with macro-barcoding methods have emerged to assess the selective enrichment efficacy of media for specific rumen microorganisms (Botero Rute et al., 2023). In addition, pharmaceuticals can be added to the medium as screening indicators according to experimental needs, e.g. Tyagi et al. (2020) to obtain lactic acid bacteria with conjugated linoleic acid production potential from the rumen fluid of lactating goats, linoleic acid was added to MRS broth medium; Zhong et al. (2023) to isolate novel urea-hydrolyzing bacteria from the rumen fluid of dairy cows, urea and phenol red were used as the screening indexes.

3.2 Hungate's roll-tube technique

This method was proposed by American microbiologist Hungate in 1950 and applied to the isolation and culture of anaerobic bacteria (Hungate and Macy, 1973). After years of practice and improvement has been very mature. The basic steps of this method4~5 mL of dissolved agar culture solution were dispensed into Heinz tubes and inoculated when it cooled down to 43°C, immediately, the tubes were rolled rapidly at low temperatures to solidify the liquid agar. After agar curing, microbial colonies will be produced after several days of incubation under a constant temperature incubator at 39°C. Then, under an anaerobic environment, single colonies on the wall of the rolled tubes will be picked out by inoculation ring and put into fresh medium, and purified strains will be obtained through several times of rolling tubes and isolation of single colonies and the number of viable bacteria in the bacterial liquid will be measured, which will provide a basis for quantitative determination (Cheng et al., 2006; Jing et al., 2011). The Heinz tube rolling method can meet the growth requirements of different cultures, and isolation, purification, and morphological identification can be achieved in one tube. However, different microorganisms have different needs for the optimal medium, resulting in different growth rates, which has a greater impact on quantitative analysis (Guo et al., 2009). Hu et al. (2022) used the roll-tube technique to isolate Streptococcus equinus, Enterococcus avium, and Streptococcus lutetiensis, which have inhibitory effects on Escherichia coli and Staphylococcus aureus, from the rumen juice of Holstein cows as potential probiotics or silage inoculants. Kumar et al. (2021) screened three strains of cellulose-degrading rumen-producing Ruminococcus flavefaciens from buffalo rumen fluid by dispensing carboxymethylcellulose medium in Heinz tubes, and subsequent animal experiments showed that Ruminococcus flavefaciens significantly increased the number and activity of beneficial gut microorganisms and enhanced the digestive function of milk-producing buffaloes.

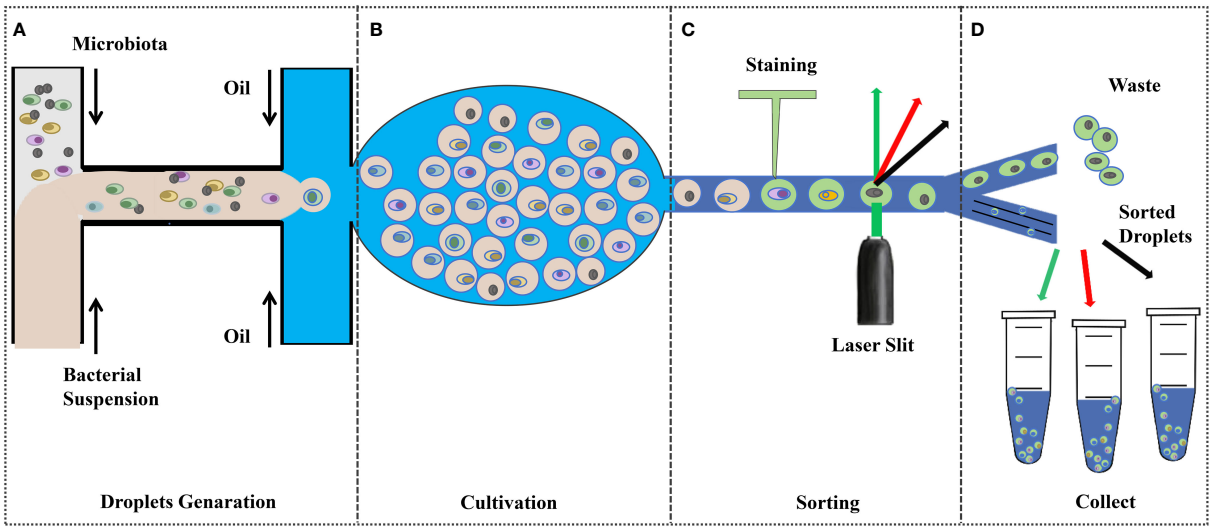

FIGURE 2
Droplet-based high-throughput culture (Tan et al., 2020; Anggraini et al., 2022; Yin et al., 2022; Zhao et al., 2016). **(A)** Pre-treated bacterial suspensions were injected into the microfluidic device to encapsulate the cells in the droplets; **(B)** Incubation under ideal conditions; **(C)**; Fluorescence Signal Dependent Droplet Sorting; and **(D)** collect.

3.3 Droplet-based single-cell cultures

For microbial culture and screening, droplet-based microfluidic methods offer the advantages of single-cell, high-throughput, high-resolution, and low-cost, making them a promising approach for isolating uncultured microorganisms (Huys and Raes, 2018). Droplet-based microfluidics can provide a separate space for cells to divide and block the influence of competitors or predators, precisely control the microenvironment of single-cell cultures, manipulate individual cells through external interventions, and detect single-cell behaviors in real time for enzyme, antibody, or rare-cell screenings, and recover cells from the microfluidic system for a variety of downstream analyses, even after cultivation (Huys and Raes, 2018; Li et al., 2023a).

Microfluidic Stripe Plate (MSP) and SlipChip are two representative microfluidic methods for static droplets (Yu et al., 2022). MSP builds on traditional streak plate technology for high-throughput single-cell analysis and culture using nanolitre droplets that can be manually or robotically streaked onto Petri dishes for single-cell culture, as well as encoding chemical gradients in the droplet array for comprehensive dose-response analysis (Jiang et al., 2016). MSP can restore microbial diversity more than traditional agar plates. Currently, there are examples of applications of the MSP method in culturing and isolating Fastidous bacteria, such as the successful isolation of fluoranthene-degrading Blastococcus from the soil, studies of termite gut microbiota, and isolation of rare deep-sea biosphere members in long cultures (Jiang et al., 2016; Xu et al., 2018a; Zhou et al., 2019a). The SlipChip consists of two glass plates with several pre-filled reagent holes, the top plate is pre-filled with samples, and the bottom plate is pre-filled with reagents. Fluorocarbon compounds act as a lubricant layer between the two plates, and when the two plates slip, the complementary pattern of holes in the plates overlap to form tens of thousands of closed chambers or channels, the top plate sample-containing wells are exposed to the bottom plate reagent wells for reaction (Du et al., 2009). The enclosed microenvironment is particularly suitable for the cultivation of anaerobic microorganisms (Yu et al., 2022). Chen et al. (2019) A SlipChip device for chemotaxis sorting and a microfluidic streak plate device for bacterial culture were newly developed as new pipelines for screening and isolating microbial species that can degrade imidazolidinone as an imidazolidinone degrader.

Recently, Watterson et al. (2020) built a platform consisting of an image processing system and a droplet microfluidic device operating in an anaerobic chamber. This platform sorts slow-growers in microorganisms in droplets by density, speeding up their growth and enriching rare taxa in fecal microbiota samples, and realize high-throughput single-cell cultivation. However, as the vast majority of microorganisms colonizing the gastrointestinal tract are almost exclusively anaerobic, integrating a single-cell isolation platform into a standard anaerobic workstation is costly. Yin et al. (2022) improved a simple droplet-based method for isolation and enrichment of functional gut bacteria by encapsulation of single-cell suspensions, which was accomplished by using diluted bacterial suspensions as the dispersed phase mineral oil as the continuous phase and then transferring to agar plates in an anaerobic chamber for incubation to form discrete single-cell colonies. This method does not require sophisticated instrumentation to sort droplets and therefore can easily be operated in a conventional anaerobic chamber to successfully isolate anaerobic Lactobacilli and Bifidobacteria.

3.4 Droplet-based co-culture techniques

Droplet-based co-culture systems are likewise a promising approach for the discovery of natural microbial products and the isolation of uncultured microorganisms (Jian et al., 2020; Park et al., 2011). The system promotes continuous co-culture of colonies and cells by adjusting the concentration of microbial communities, developing microdroplets with different proportions of cells, simulating microenvironments to meet microbial growth requirements, and securing information exchange between strains (Qi et al., 2023). This process is expected to solve the problem that traditional pure culture methods can interrupt the ecological interactions between microorganisms, and provide a promising way to assess complex microbial communities in more detail (Anggraini et al., 2022; Kim et al., 2023; Baichman-Kass et al., 2023). Hua et al. (2022) created a rapid screening platform for actinomycetes——the whole-cell biosensor and producer co-cultivation-based microfluidic platform for screening actinomycetes (WELCOME), By combining an MphR-based Escherichia coli whole-cell biosensor sensitive to erythromycin with Saccharopolyspora erythraea co-cultivation, they isolated six high production erythromycin-producing strains from industrial strains within a short time. Tan et al. (2020) Complex human faecal samples were dissected into sub-communities for highly parallel co-culture using a droplet microfluidic device. Twenty-two individual droplets with strong bacterial symbiosis were then selected by microfluidics, in which a partial genome of a representative of a new genus of Neisseriaceae was found, highlighting the ability of microfluidic co-cultures to access and study uncharacterized microbial diversity.

4 Identification of rumen probiotics

In the early days, microbiological studies based on morphological features and physiological and biochemical traits provided insights into the microbial world, but today, they can only provide limited resolution (Escobar-Zepeda et al., 2015). Advances in molecular techniques have provided access to the "new uncultured world" of microbial communities. Among these techniques, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in-situ* hybridization (FISH) and rRNA gene cloning and sequencing have had a significant impact (Escobar-Zepeda et al., 2015). However, while they describe

the diversity of microorganisms, amplicon sequencing, macrogenomics, and single-cell genomics are the most widely used techniques for solving environmental microbiological problems (Xu and Zhao, 2018b).

4.1 Morphological identification

Morphological features are the important references for the classification and identification of microorganisms. Identification is carried out by observing both colony morphology and microscopic morphology. Colony morphology includes colony size, shape, surface, texture, transparency, degree of elevation, and medium color (Meruvu and Harsa, 2023). The bacterial morphology reflects the survival value of bacteria for acquiring nutrients, moving, and avoiding predators (Young, 2007). In microscopic morphology, commonly used types of microscopes are ordinary optical microscopes and phase contrast microscopes, for ultra-fine structures and complex structures can use scanning electron microscope and transmission electron microscope observation (Sun and Lin, 2009). With the rapid development of microimaging technologies and microfluidic chips, it becomes feasible to observe microbial germination and growth in real time (Zhou et al., 2019b). Zhang et al. (2019) used a gradient microfluidic chip to observe the morphological changes of various bacteria under the action of antibiotics. The rumen probiotics can also be classified with the help of Gram staining, which provides a reference basis for isolating rumen probiotics (Wang et al., 2021). Han et al. (2018) and Li et al. (2017) used colony morphology and Gram staining techniques for preliminary isolation and identification of probiotic bacteria in the samples, followed by 16SrRNA gene homology analysis to obtain probiotic strains of bovine origin.

4.2 Physiological and biochemical tests

The production and metabolic activities of microorganisms depend on extracellular enzymes to degrade macromolecular substances, the enzyme systems of different microorganisms will show significant differences in metabolic activities, which are confirmed by the changes in the substances in the vicinity of the microbial colonies, providing a basis for the identification and classification of microorganisms (Wu et al., 2018). At present, the physiological and biochemical tests that are more frequently used in identifying probiotics include the gelatin liquefaction test, methyl red test, glycolysis test, indole test, and starch hydrolysis test for the preliminary assessment of probiotics (Lin, 1999). The test is used for the preliminary evaluation of probiotics. However, due to the slow growth of some bacteria and the difficulty of cultivation, they cannot meet the requirements of biochemical reactions for microbial concentration and colony freshness, and the accuracy of biochemical tests for identifying anaerobic microorganisms in the rumen needs to be improved. Abid et al. (2022) in order to screen probiotic strains favorable for milk fermentation, four strains of Lactobacillus spp. were screened from 15 strains showing gas production in Durham tubes. These four probiotic strains were identified morphologically, identified by biochemical tests, and evaluated for their probiotic potential. It was found that Lactobacillus fermentum strains showed significant viability in the presence of pepsin, trypsin, and lysozyme.

4.3 16SrRNA-based amplicon sequencing

The advantage of amplicon sequencing lies in the contrasting biases generated by using only one phylogenetic marker (Escobar-Zepeda et al., 2015). Because 16SrRNAs are ubiquitous in all species and are functionally integral to the core genome, the composition and relative abundance of microbial communities in environmental samples are often investigated by amplifying and sequencing specific regions of the 16SrRNA gene (Daubin et al., 2003; Větrovský and Baldrian, 2013). The 16SrDNA gene sequence is divided into constant and variable regions. The constant region reflects the kinship between species (Head et al., 1998); The variable region reflects the specificity of the species and is used to classify them biologically (Chaudhary et al., 2015). Second-generation sequencing platforms, such as Illumina, can sequence amplicons of up to 600 bp with high precision (Bharti and Grimm, 2021). Third-generation amplicon sequencing platforms such as PacBio and Oxford Nanopore can sequence full-length 16S rRNA genes in a short period of time at the single-molecule level, which reduces the problems of amplification bias and short read lengths and makes it possible to annotate the microbiome at the species and strain level (Abellan-Schneyder et al., 2021). The 16SrRNA homology analysis has been successfully used to construct a gene library of Holstein cow rumen bacteria, which facilitates microbial species analysis (Yang et al., 2010; King et al., 2011). Although the 16SrRNA identification technique reflects the diversity of rumen microbes, it does not have a sufficient resolution at the species level, resulting in a loss of information on low-abundance members of the microbiota and an inability to understand the function of the community (Bowers et al., 2022). Abedini et al. (2023) reported for the first time the isolation of probiotics from camel rumen, preliminary screening of Gram-positive, catalase-negative colonies with white-colored colonies from the contents of camel rumen, identified as Enterococcus faecium96B4 by 16SrRNA, and subsequent evaluation of the probiotic activity and safety evaluation revealed good probiotic potential, reflecting the potential research value of camel rumen as a pristine environment.

4.4 Denaturing gradient gel electrophoresis

DGGE is an electrophoretic technique that separates DNA fragments based on differences in the order of DNA bases and is used to detect nucleic acid mutations and point mutations. Its basic principle is that in DNA molecules under the influence of specific

temperature conditions and chemical denaturants, a region of double-stranded DNA starts to unlink, the unlinking region is related to the order of the base arrangement, and the unlinking can occur with the difference of only one base pair, and the difference of the base sequence of the DNA fragments will be denatured under the corresponding denaturing conditions in the process of the denaturing gradient gel swimming. When the ends of double-stranded DNA molecules are unstranded, their electrophoretic resistance increases greatly and their speed decreases significantly, which leads to the DNA fragments staying in different parts of the gel to achieve separation (Li et al., 2008; Ercolini, 2004). DGGE has the characteristics of good reproducibility, high detection rate, convenience and quickness, etc. It can be applied to the analysis of uncultured microorganisms (McGenity et al., 2010). DGGE was first applied to the structural analysis of rumen microorganisms by Kocherginskaya et al. (2001) analyzed the effect of two diets, hay, and maize, on the structure of the rumen bacterial community of castrated cows and showed that the bacterial populations in the rumen were relatively more diverse and numerous after feeding the maize diet. Through DGGE technology, Yu et al. (2020) found that supplementing Dihydropyridine (DHP) in the diet can promote the growth of Xanthomonadaceae and Xanthomonas and enhance the diversity of ruminal bacteria. Min et al. (2021) explored the effect of supplementing Condensed Tannins (CT) on calf rumen bacterial diversity and methane emissions. DGGE results showed that Firmicutes and Bacteroidetes populations seemed to increase as CT content increased., CT and can exert anti-methanogenic activity by directly inhibiting methanogens or indirectly through rumen fermentation, thereby reducing methane emissions.

4.5 Terminal restriction fragment length polymorphism

T-RFLP is an extension of RFLP. T-RFLP allows culture-independent assessment of subtle genetic differences between strains and provides a molecular approach to the evaluation of microbial community structure and function (Marsh, 1999). The technique uses PCR to amplify small subunit rRNA genes from total community DNA, where one or two primers are labeled with a fluorescent dye, followed by digestion of the PCR product with a restriction endonuclease with a four-base-pair recognition site and determination of the size and relative abundance of fluorescently-labeled T-RFs using a DNA sequencer. Because differences in T-RF size reflect sequence polymorphism, phylogenetically distinct populations of organisms can be resolved (Schütte et al., 2008). Although the use of T-RFLP is declining, it is still the method of choice for community dynamics studies (Prakash et al., 2014). Zhu et al. (2018) combined T-RFLP and clone library analysis to compare changes in rumen bacterial and archaeal communities in response to dietary disturbances before and after low-grain and high-grain production and found significant changes in the relative abundance of methane-producing communities in cows during the transition period, as well as a clear shift in rumen fermentation patterns.

4.6 Fluorescence *in situ* hybridization

FISH was introduced in 1980 (Bauman et al., 1980). FISH uses fluorescein oligonucleotide probes to bind complementarily to specific target nucleic acid sequences and detects the corresponding fluorescent signals for single-cell identification and quantification by fluorescence microscopy, whole-slide images, or flow cytometry (Amann and Fuchs, 2008). FISH has been widely used for the diagnosis of chromosomal aberrations in medicine, the identification of microorganisms in complex samples, and the identification of microorganisms (Ratan et al., 2017)., also provides a basis for *in situ* image-based spatial transcriptomics (Wen et al., 2022; Zhou et al., 2023). With improvements in fluorescence microscopy and fluorescent labeling of various nucleic acid probes, FISH has evolved to be used with other biotechnologies as a rapid and accurate biosensor system (Kuo et al., 2020). FISH combined with Raman spectroscopy can rapidly identify target microorganisms in complex samples by labeling the DNA of specific species (Cui et al., 2022). FISH can also be integrated into microfluidic microarray platforms to speed up the process of colony identification, reduce reagent consumption, and have the potential for automation (Rodrigues et al., 2021). Liu et al. (2009) developed a microfluidic device that integrates FISH identification and droplet-splitting modules for parallel high-throughput single-cell culture and identification. The single-cell droplet was split into two sub-droplets with the aid of a droplet-splitting chip, one of which was added to an agar plate, and the other was subjected to FISH identification, and the droplet that encapsulated the target species was finally selected from the agar plate based on the identification results. Liu et al. (2011) Seamlessly integrated two components, FISH and fluorescence-activated cell sorting (FACS), into a microfluidic device, which forms a hybridization chamber between two photopolymeric membranes in which cells and probes are electrophoretically loaded, incubated, and washed; a downstream cross structure is used to electrically focus the cells into a single flow for FACS analysis, providing a quantitative detection of microbial cells in complex samples automated platform. Batani et al. (2019) developed a new method to isolate live bacteria based only on their 16S rRNA gene sequences (Live-FISH), which combines FISH with FACS to enable the sorting and culturing of live bacteria. With the development of highly specific probes, Live-FISH has greater potential for targeted sorting of target microorganisms. However, FISH requires the rRNA content or the number of microorganisms in the probe target organisms, otherwise the fluorescence signal cannot be detected under the microscope (Hoshino et al., 2008). In addition, the FISH technique suffers from the problem of crosstalk of organic fluorescein excitation light (Waters, 2009). Meanwhile, FISH can only identify a small number of microorganisms with a high degree of certainty in a single experiment, and cannot elucidate the distribution of microorganisms in the overall microecosystem. The development of combinatorial labeling and spectroscopic imaging can increase the microspatial relationships of different species of microorganisms in a single field of view, but it is too costly (Valm et al., 2011).

4.7 Macrogenomics

Automated sequencing of DNA by Sanger sequencing in the late 1970s ushered in the era of genomics Sanger sequencing retrieves up to 96 sequences per run at an average length of 650 bp, which may be sufficient for phylogenetic marker analyses, but the emergence of what is known as next-generation sequencing (NGS) technology has enabled researchers to bypass the cultivation of parallel sequencing of millions of DNA molecules with varying yields and sequence lengths directly after extraction from highly diverse populations, i.e., macrogenome sequencing (Escobar-Zepeda et al., 2015; Hu et al., 2021). It is usually performed using birdshot sequencing methods that are non-discriminatory, allowing for the quantitative assignment of taxonomy and organisms to the species level, and with the help of databases of identified functional genes, such as KEGG, GO, COG, and eggNOG, the understanding of microbial communities in terms of their composition, function, evolution, and interactions in their natural environments, which directly contributes to the flourishing of microbial ecology (Tatusov et al., 1997; Kanehisa et al., 2008; Huerta-Cepas et al., 2016). However, macrogenome sequencing uses environmental DNA samples, which cannot link each functional gene to a specific microbial individual, and for high-diversity samples or low-abundance organisms, it is very difficult to assemble a single discrete genome to capture strain-level variation (Kaster and Sobol, 2020; Daliri et al., 2021). Li et al. (2023c) studied changes in bovine rumen microbes from pre-transport to 1-month post-transport by macrogenomics and found that the abundance of rumen bacteria and archaea was higher on day 16 post-transport than pre-transport, but eukaryotic abundance was highest on day 30 post-transport. Before transport, most bacteria were mainly involved in polysaccharide digestion. On day 4 post-transport, KEGG pathway enrichment was most notable for nucleotide metabolism. On day 16 post-transhipment, energy metabolism and rumen content of MCPs and VFAs increased significantly, but at the same time, energy loss due to methane production (Methanobrevibacter) and pathogenic bacteria (Saccharopolyspora rectivirgula) together induced inflammation and oxidative stress in cattle, which is important for the establishment of new management and nutritional specification strategies.

4.8 Single-cell genomics

Genomics for microorganisms in the environment, macro-genome sequencing describes the full range of genetic information, and single-cell genomics reveals individual genomes, and combining the two can compensate for their respective shortcomings (Nobu et al., 2015; Mende et al., 2016). Sequencing the microbiome at the resolution of individual microorganisms effectively improves the efficiency and accuracy of obtaining genome-wide information from complex microbial communities, and also allows for the study of individual cellular behaviors underlying the complexity of microbial ecosystems (Lloréns-Rico et al., 2022; Madhu et al., 2023). Single-cell isolation is essential to performing high-throughput single-cell genomics workflows (Xu et al., 2018a). Microfluidic devices offer advantages in terms of throughput, affordability, and automation for single-cell capture and retrieval applications (Han et al., 2023). Its subsequent steps include DNA extraction, 16S rRNA gene PCR phylogenetic identification, multiple displacement amplification (MDA), library construction, sequencing, and data analysis (Xu and Zhao, 2018b; Arikawa et al. (2021) developed a framework for integrating single-cell genomics and macrogenomics, integrating single-cell amplified genomes (SAG) and macrogenome assembled genomes (MAG) to reconstruct competent microbial genomes, and achieving high-quality recovery of strain-resolved genomes. Zheng et al. (2022) developed and validated a strain-resolved, high-throughput single-cell sequencing method (Microbe-seq) that uses a microfluidic platform to individually encapsulate microorganisms into droplets where whole-genome amplification and specific barcode coding are performed, followed by sequencing of merged labeled DNA to generate a single amplified genome (SAG), and then finally co-assembling the SAGs of the same bacterial species. SAGs from the same bacterial species are finally co-assembled to achieve single-strain level resolution. However, SAGs can introduce chimeric and biased sequences during genome amplification, leading to sequence incompleteness. To address this problem, Kogawa et al. (2023) developed a single-cell amplified genome long read-length assembly workflow to construct complete circular SAGs (cSAGs).

5 Culturography

Culturomics is a high-throughput method of isolating and culturing bacteria under as many combinations of culture conditions as possible, using a combination of matrix-assisted laser-resolved ionization time-of-flight mass spectrometry (MALDI-TOF MS) or other sequencing technologies for microbial identification (Diakite et al., 2021; Wan et al., 2023; Yu et al., 2023). It is a high-throughput method for bacterial isolation and culture. The technique was first used for the analysis of uncultured microorganisms identified in the human gut, and with the significant expansion of strain databases, many microorganisms previously overlooked or considered unculturable have been brought into the culture (Diakite et al., 2021). Classical cultureomics is an untargeted strategy that includes steps such as sample collection, sample processing, microbial isolation, culture, identification, and preservation (Wan et al., 2023). That is, the treated samples are dispersed and cultured into different media, and then the characteristic colonies are selected for identification using MALDI-TOF MS analysis if the reference profiles are lacking, 16SrRNA sequencing is required for further identification, and if the similarity is < 98.65%, it is considered to be a new species, and then describing new species using taxonomic genomics (Lagier et al., 2018; Peng et al., 2020). In addition, with the use of membrane diffusion culture (Nichols et al., 2010; Chaudhary et al., 2019) microfluidic devices (Anggraini et al., 2022; Luo et al., 2022) FACS, FISH (Waters, 2009) and reverse genomics (Cross et al., 2019), and other high-throughput culture technologies and targeted sorting techniques, the culture results of cultureomics will be greatly enriched (Figure 3). Combined with full-length 16S rRNA gene amplicons and birdshot macro-genome sequencing, culture-

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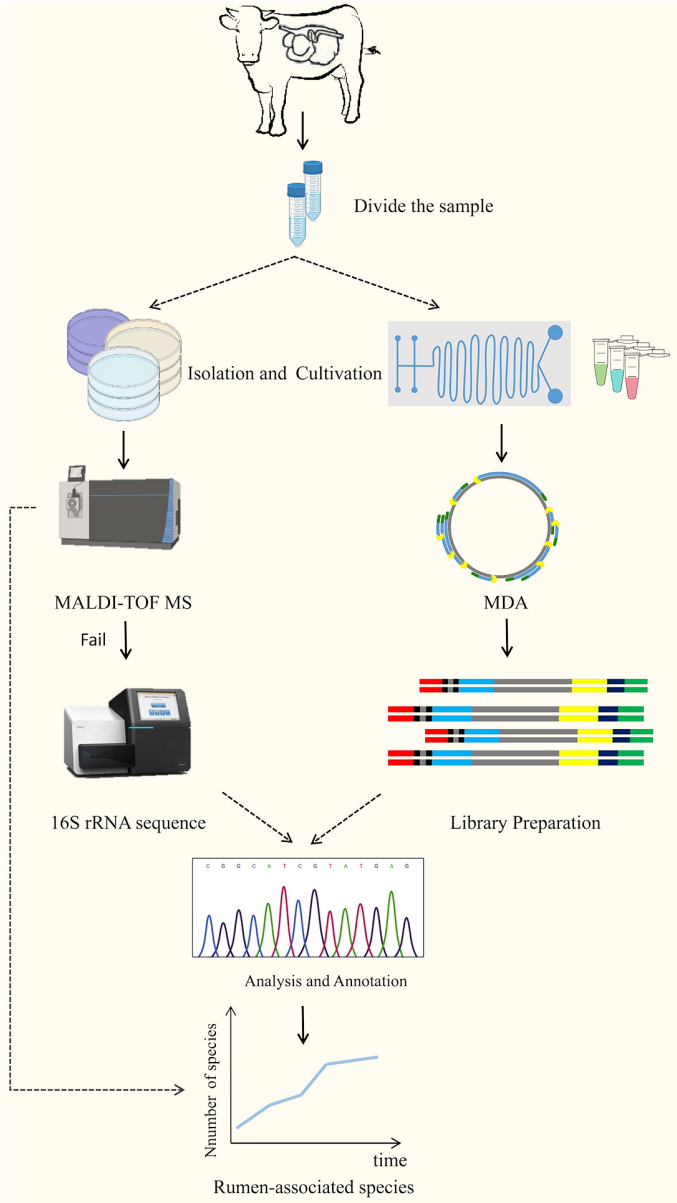

FIGURE 3
The technical route of culturomics (Wan et al., 2023).

based metabonomics (CBM) will deeply explore untapped novel bacterial resources at high resolution (Li et al., 2023b). In addition, information specific to the target microorganisms obtained in advance from literature studies or macrogenomics data can also be used to reverse-guide the isolation and culture of the target microorganisms, providing additional opportunities to obtain pure cultures (Bellais et al., 2022; Liu et al., 2022). Of course, there are still some constraints in cultureomics: it is still at the beginning stage in the cultivation of rumen microorganisms, which is labor-intensive and will result in a waste of manpower and resources (Mordant and Kleiner, 2021); gut-microbiota interactions and symbiotic relationships between microorganisms are still unclear (Yadav et al., 2022).

6 Perspectives and conclusions

The rumen microecological environment is extremely complex, and it is difficult to simulate its physicochemical parameters *in vitro*. Traditional *in vitro* techniques for isolation and culture of rumen probiotics have significant limitations and it is difficult to identify rare microorganisms by general identification techniques. Therefore, it is necessary to introduce new techniques for large-scale mining of probiotics in the rumen. This will help rationally define the beneficial flora, pinpoint microbial-derived metabolites with beneficial effects, and provide technical support for developing novel probiotic formulations. This is of great significance for animal nutrition and health, food safety, and ecological protection.

7 Future Direction

The first key feature of the state-of-the-art technologies presented in this review is high-throughput culture. Traditional laboratory microbiological cultures are usually inefficient and time-consuming, but the development of microfluidic devices has created conditions for high-throughput cultures. Therefore, instrument design, microfluidic chip design, and fabrication have become crucial. The integration of experimental functions such as dilution, separation, single-cell encapsulation, anaerobic incubation, and targeted sorting onto a small chip requires interdisciplinary collaboration, while reagent development, construction of equipment such as anaerobic devices, and sorting and collection devices requires an experienced R&D team. Second, genome sequencing at single-cell resolution is another key feature. Single-cell sequencing can reveal functional information about rare species, help understand inter-microbial or microbe-host interactions, and explore potential metabolic pathways. The development of various histological approaches will also provide information on the rumen microbiome and its metabolites, which will help guide the isolation and culture of target microorganisms. While cultureomics, with its attributes of high-throughput culture and single-cell resolution, will significantly increase the potential for isolating and culturing "dark matter" from the rumen, this endeavor has significant challenges. The adaptation of optimal media for microorganisms is time- and labor-intensive, and the design of co-culture or mono-culture systems may not be able to meet the growth needs of all microorganisms. In addition, targeted sorting devices such as FACS and FISH still suffer from problems such as crosstalk and insufficient number of stains. Overcoming these technological bottlenecks requires the unremitting efforts of the R&D team.

Rumen probiotics from cattle and sheep are relatively well documented, but there are fewer reports on probiotics from antelope, deer, and musk family sources. The rumen microbial composition is very rich and susceptible to factors such as feeding management, geographic location, and significant differences in microbial composition between individuals and species, so gastrointestinal microbiological studies in rare ruminants may yield unexpected findings. In addition, the research and application of rumen probiotics require the establishment of a standard system for probiotic isolation and culture, identification, evaluation of probiotic properties, safety evaluation, and application. Only by ensuring the maximum use of strain resources can the potential of rumen probiotics be better explored, their application in agriculture and industry be improved, and a greater contribution be made to the sustainable development of animal husbandry.

Author contributions

RW: Writing – original draft, Writing – review & editing. PJ: Conceptualization, Investigation, Project administration, Supervision, Writing – review & editing. YH: Data curation, Funding acquisition, Resources, Writing – review & editing. HL: Conceptualization, Investigation, Writing – review & editing. WZ: Data curation, Writing – review & editing. YW: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

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

Publisher's note

References

Abedini, R., Zaghari, G., Jabbari, L., Salekdeh, G. H., and Hashemi, M. (2023). A potential probiotic Enterococcus faecium isolated from camel rumen, fatty acids biotransformation, antilisteria activity and safety assessment. *Int. Dairy J.* 145, 105706. doi: 10.1016/j.idairyj.2023.105706

Abellan-Schneyder, I., MatChado, M. S., Reitmeier, S., Sommer, A., Sewald, Z., Baumbach, J., et al. (2021). Primer, pipelines, parameters: issues in 16S rRNA gene sequencing. *mSphere* 6, e01202–e01220. doi: 10.1128/mSphere.01202-20

Abid, S., Farid, A., Abid, R., Rehman, M. U., Alsanie, W. F., Alhomrani, M., et al. (2022). Identification, Biochemical Characterization, and Safety Attributes of Locally Isolated Lactobacillus fermentum from Bubalus bubalis (buffalo) Milk as a Probiotic. *Microorganisms* 10, 954. doi: 10.3390/microorganisms10050954

Amann, R., and Fuchs, B. M. (2008). Single-cell identification in microbial communities by improved fluorescence in *situ* hybridization techniques. *Nat. Rev. Microbiol.* 6, 339–348. doi: 10.1038/nrmicro1888

Anggraini, D., Ota, N., Shen, Y., Tang, T., Tanaka, Y., Hosokawa, Y., et al. (2022). Recent advances in microfluidic devices for single-cell cultivation: methods and applications. *Lab. chip* 22, 1438–1468. doi: 10.1039/D1LC01030A

Arikawa, K., Ide, K., Kogawa, M., Saeki, T., Yoda, T., Endoh, T., et al. (2021). Recovery of strain-resolved genomes from human microbiome through an integration framework of single-cell genomics and metagenomics. *Microbiome* 9, 202. doi: 10.1186/s40168-021-01152-4

Arshad, M. A., Hassan, F. U., Rehman, M. S., Huws, S. A., Cheng, Y., and Din, A. U. (2021). Gut microbiome colonization and development in neonatal ruminants: Strategies, prospects, and opportunities. *Anim. Nutr. (Zhongguo xu mu shou yi xue hui)* 7 (3), 883–895. doi: 10.1016/j.aninu.2021.03.004

Baichman-Kass, A., Song, T., and Friedman, J. (2023). Competitive interactions between culturable bacteria are highly non-additive. *eLife* 12, e83398. doi: 10.7554/eLife.83398.sa2

Batani, G., Bayer, K., Böge, J., Hentschel, U., and Thomas, T. (2019). Fluorescence in *situ* hybridization (FISH) and cell sorting of living bacteria. *Sci. Rep.* 9, 18618. doi: 10.1038/s41598-019-55049-2

Bauman, J. G., Wiegant, J., Borst, P., and van Duijn, P. (1980). A new method for fluorescence microscopical localization of specific DNA sequences by in *situ* hybridization of fluorochromelabelled RNA. *Exp. Cell Res.* 128, 485–490. doi: 10.1016/0014-4827(80)90087-7

Bellais, S., Nehlich, M., Ania, M., Duquenoy, A., Mazier, W., van den Engh, G., et al. (2022). Species-targeted sorting and cultivation of commensal bacteria from the gut microbiome using flow cytometry under anaerobic conditions. *Microbiome* 10, 24. doi: 10.1186/s40168-021-01206-7

Bharti, R., and Grimm, D. G. (2021). Current challenges and best-practice protocols for microbiome analysis. *Briefings Bioinf.* 22, 178–193. doi: 10.1093/bib/bbz155

Bonnet, M., Lagier, J. C., Raoult, D., and Khelaifia, S. (2019). Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. *New Microbes New Infect.* 34, 100622. doi: 10.1016/j.nmni.2019.100622

Botero Rute, L. M., Caro-Quintero, A., and Acosta-González, A. (2023). Enhancing the conventional culture: the evaluation of several culture media and growth conditions improves the isolation of ruminal bacteria. *Microbial Ecol.* 87, 13. doi: 10.1007/s00248-023-02319-2

Bowers, R. M., Nayfach, S., Schulz, F., Jungbluth, S. P., Ruhl, I. A., Sheremet, A., et al. (2022). Dissecting the dominant hot spring microbial populations based on community-wide sampling at single-cell genomic resolution. *ISME J.* 16, 1337–1347. doi: 10.1038/s41396-021-01178-4

Cai, L., Hartanto, R., Zhang, J., and Qi, D. (2021). Clostridium butyricum improves rumen fermentation and growth performance of heat-stressed goats *in vitro* and *in vivo*. *Animals: an Open Access J. MDPI* 11, 3261. doi: 10.3390/ani11113261

Chaudhary, D. K., Khulan, A., and Kim, J. (2019). Development of a novel cultivation technique for uncultured soil bacteria. *Sci. Rep.* 9, 6666. doi: 10.1038/s41598-019-43182-x

Chaudhary, N., Sharma, A. K., Agarwal, P., Gupta, A., and Sharma, V. K. (2015). 16S classifier: a tool for fast and accurate taxonomic classification of 16S rRNA hypervariable regions in metagenomic datasets. *PloS One* 10, e0116106. doi: 10.1371/journal.pone.0116106

Chen, D., Liu, S. J., and Du, W. (2019). Chemotactic screening of imidazolinone-degrading bacteria by microfluidic SlipChip. *J. Hazard. Mater.* 366, 512–519. doi: 10.1016/j.jhazmat.2018.12.029

Chen, X., Yan, F., Liu, T., Zhang, Y., Li, X., Wang, M., et al. (2022). Ruminal microbiota determines the high-fiber utilization of ruminants: evidence from the ruminal microbiota transplant. *Microbiol. Spectr.* 10, e0044622. doi: 10.1128/spectrum.00446-22

Cheng, Y. M., Deng, W. D., and Mao, H. M. (2006). Research progress on traditional techniques and molecular biological techniques in modern times for rumen microbial diversity. *Prog. Vet. Med.* S1, 51–54. doi: 10.16437/j.cnki.1007-5038.2006.s1.013

Cross, K. L., Campbell, J. H., Balachandran, M., Campbell, A. G., Cooper, C. J., Griffen, A., et al. (2019). Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nat. Biotechnol.* 37, 1314–1321. doi: 10.1038/s41587-019-0260-6

Cui, D., Kong, L., Wang, Y., Zhu, Y., and Zhang, C. (2022). *In situ* identification of environmental microorganisms with Raman spectroscopy. *Environ. Sci. ecotechnology* 11, 100187. doi: 10.1016/j.ese.2022.100187

Daliri, E. B., Ofosu, F. K., Chelliah, R., Lee, B. H., and Oh, D. H. (2021). Challenges and perspective in integrated multi-omics in gut microbiota studies. *Biomolecules* 11, 300. doi: 10.3390/biom11020300

Dasriya, V. L., Samtiya, M., Ranveer, S., Dhillon, H. S., Devi, N., Sharma, V., et al. (2024). Modulation of gut-microbiota through probiotics and dietary interventions to improve host health. *J. Sci. Food Agric.*, 1–17. doi: 10.1002/jsfa.13370

Daubin, V., Moran, N. A., and Ochman, H. (2003). Phylogenetics and the cohesion of bacterial genomes. *Science* 301, 829–832. doi: 10.1126/science.1086568

Davidse, E. K., Balla, E., Holzapfel, W. H., Muller, C. J. C., Cloete, S. W. P., and Dicks, L. M. T. (2004). Peptide AS-48 (Enterococcus faecalis) for the prevention and treatment of mastitis in dairy cows. *Online J. Vet. Res.* 8, 22–32.

de Melo Pereira, G. V., de Oliveira Coelho, B., Magalhães Júnior, A. I., Thomaz-Soccol, V., and Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* 36 (8), 2060–2076. doi: 10.1016/j.biotechadv.2018.09.003

Deng, B., Wang, L., Ma, Q., Yu, T., Liu, D., Dai, Y., et al. (2021). Genomics analysis of bacillus megaterium 1259 as a probiotic and its effects on performance in lactating dairy cows. *Animals: an Open Access J. MDPI* 11, 397. doi: 10.3390/ani11020397

Deng, K. D., Xiao., Y., Ma., T., Tu., Y., Diao., Q. Y., Chen., Y. H., et al. (2018). Ruminal fermentation, nutrient metabolism, and methane emissions of sheep in response to dietary supplementation with Bacillus licheniformis. *Anim. Feed Sci. Technol.* 241, 38–44. doi: 10.1016/j.anifeedsci.2018.04.014

Diakite, A., Dubourg, G., and Raoult, D. (2021). Updating the repertoire of cultured bacteria from the human being. *Microbial pathogenesis* 150, 104698. doi: 10.1016/j.micpath.2020.104698

Du, W., Li, L., Nichols, K. P., and Ismagilov, R. F. (2009). SlipChip. *Lab. chip* 9, 2286–2292. doi: 10.1039/b908978k

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Methods* 56 (3), 297–314. doi: 10.1016/j.mimet.2003.11.006

Escobar-Zepeda, A., Vera-Ponce de León, A., and Sanchez-Flores, A. (2015). The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. *Front. Genet.* 6. doi: 10.3389/fgene.2015.00348

FAO/WHO (2001). *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria*. Food and Agriculture Organization of the United Nations, 1–14.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. *Int. J. Environ. Res. Public Health* 11, 4745–4767. doi: 10.3390/ijerph110504745

Guo, J. T., Wang, J. Q., Wang, J. P., Huo, X. K., Bo, D. P., Wei, H. Y., et al. (2009). Research progress on quantitative method for rumen microorganism. *China Anim. Husbandry Vet. Med.* 36, 19–24.

Gutleben, J., Chaib De Mares, M., van Elsas, J. D., Smidt, H., Overmann, J., and Sipkema, D. (2018). The multi-omics promise in context: from sequence to microbial isolate. *Crit. Rev. Microbiol.* 44, 212–229. doi: 10.1080/1040841X.2017.1332003

Han, Y., Li, X., Guo, P. F., Guan, G. Q., Liu, J. L., Liu, A. H., et al. (2018). lsolation and ldentification of probiotics bacterium in calve faeces. *China Dairy Cattle* 07, 21–24.

Han, X., Xu, X., Yang, C., and Liu, G. (2023). Microfluidic design in single-cell sequencing and application to cancer precision medicine. *Cell Rep. Methods* 3, 100591. doi: 10.1016/j.crmeth.2023.100591

Han, P. M., Zhang, R., Bian, S. X., Li, L., Ni, A. X., Wang, Y. M., et al. (2022). Effects of Enterococcus faeciumand Bacillus subtilis on Growth Performance,Carcass Traits and lmmune Function of Squabs. *Acta Veterinaria Zootechnica Sin.* 53, 3880–3891. doi: 10.5555/20230041782

Hassan, A., Gado, H., Anele, U. Y., Berasain, M. A. M., and Salem, A. Z. M. (2020). Influence of dietary probiotic inclusion on growth performance, nutrient utilization, ruminal fermentation activities and methane production in growing lambs. *Anim. Biotechnol.* 31, 365–372. doi: 10.1080/10495398.2019.1604380

Hayek, S. A., Gyawali, R., Aljaloud, S. O., Krastanov, A., and Ibrahim, S. A. (2019). Cultivation media for lactic acid bacteria used in dairy products. *J. Dairy Res.* 86, 490–502. doi: 10.1017/S002202991900075X

Head, I. M., Saunders, J. R., and Pickup, R. W. (1998). Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecol.* 35, 1–21. doi: 10.1007/s002489900056

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hoshino, T., Yilmaz, L. S., Noguera, D. R., Daims, H., and Wagner, M. (2008). Quantification of target molecules needed to detect microorganisms by fluorescence in *situ* hybridization (FISH) and catalyzed reporter deposition-FISH. *Appl. Environ. Microbiol.* 74, 5068–5077. doi: 10.1128/AEM.00208-08

Hu, G., Jiang, H., Zong, Y., Datsomor, O., Kou, L., An, Y., et al. (2022). Characterization of lactic acid-producing bacteria isolated from rumen: growth, acid and bile salt tolerance, and antimicrobial function. *Fermentation* 8, 385. doi: 10.3390/fermentation8080385

Hu, B., Xu, P., Ma, L., Chen, D., Wang, J., Dai, X., et al. (2021). One cell at a time: droplet-based microbial cultivation, screening and sequencing. *Mar. Life Sci. Technol.* 3, 169–188. doi: 10.1007/s42995-020-00082-8

Hua, E., Zhang, Y., Yun, K., Pan, W., Liu, Y., Li, S., et al. (2022). Whole-cell biosensor and producer co-cultivation-based microfludic platform for screening saccharopolyspora erythraea with hyper erythromycin production. *ACS synthetic Biol.* 11, 2697–2708. doi: 10.1021/acssynbio.2c00102

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., et al. (2016). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.* 44, D286–D293. doi: 10.1093/nar/gkv1248

Hungate, R. E., and Macy, J. (1973). The roll-tube method for cultivation of strict anaerobes. *Bulletins Ecol. Res. committee* 17), 123–126. Available at: http://www.jstor.org/stable/20111550.

Huys, G. R., and Raes, J. (2018). Go with the flow or solitary confinement: a look inside the single-cell toolbox for isolation of rare and uncultured microbes. *Curr. Opin. Microbiol.* 44, 1–8. doi: 10.1016/j.mib.2018.05.002

Jaglan, N., Kumar, S., Choudhury, P. K., Tyagi, B., and Tyagi, A. K. (2019). Isolation, characterization and conjugated linoleic acid production potential of bifidobacterial

isolates from ruminal fluid samples of Murrah buffaloes. *Anaerobe* 56, 40–45. doi: 10.1016/j.anaerobe.2019.02.001

Jian, X., Guo, X., Wang, J., Tan, Z. L., Xing, X. H., Wang, L., et al. (2020). Microbial microdroplet culture system (MMC): An integrated platform for automated, high-throughput microbial cultivation and adaptive evolution. *Biotechnol. Bioeng.* 117, 1724–1737. doi: 10.1002/bit.27327

Jiang, C. Y., Dong, L., Zhao, J. K., Hu, X., Shen, C., Qiao, Y., et al. (2016). High-throughput single-cell cultivation on microfluidic streak plates. *Appl. Environ. Microbiol.* 82, 2210–2218. doi: 10.1128/AEM.03588-15

Jing, Y. Q., Song, E. L., Yang, W. R., Liu, G. F., Tan, X. W., and Wan, F. C. (2011). Progress on quantification methods of bovine rumen microbes. *Prog. Vet. Med.* 32, 93–96. doi: 10.16437/j.cnki.1007-5038.2011.07.002

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, D480–D484. doi: 10.1093/nar/gkm882

Kaster, A. K., and Sobol, M. S. (2020). Microbial single-cell omics: the crux of the matter. *Appl. Microbiol. Biotechnol.* 104, 8209–8220. doi: 10.1007/s00253-020-10844-0

Khalifa, A., Ibrahim, H. M., and Sheikh, A. (2023). Bacillus subtilis PM5 from Camel Milk Boosts Chicken Immunity and Abrogates Salmonella entertitidis Infections. *Microorganisms* 11, 1719. doi: 10.3390/microorganisms11071719

Kim, S., Moon, J. H., and Jung, G. Y. (2023). Recent progress in the development of droplet-based microfluidic technologies for phenotypic screening using cell-cell interactions. *Biotechnol. Bioprocess Eng.* 28 (6), 929–935. doi: 10.1007/s12257-022-0081-1

King, E. E., Smith, R. P., St-Pierre, B., and Wright, A. D. (2011). Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. *Appl. Environ. Microbiol.* 77, 5682–5687. doi: 10.1128/AEM.05130-11

Kmet, V., Flint, H. J., and Wallace, R. J. (1993). Probiotics and manipulation of rumen development and function. *Archiv fur Tierernahrung* 44, 1–10. doi: 10.1080/17450399309386053

Kocherginskaya, S. A., Aminov, R. I., and White, B. A. (2001). Analysis of the Rumen Bacterial Diversity under two Different Diet Conditions using Denaturing Gradient Gel Electrophoresis, Random Sequencing, and Statistical Ecology Approaches. *Anaerobe* 7, 1–16. doi: 10.1006/anae.2001.0378

Kogawa, M., Nishikawa, Y., Saeki, T., Yoda, T., Arikawa, K., Takeyama, H., et al. (2023). Revealing within-species diversity in uncultured human gut bacteria with single-cell long-read sequencing. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1133917

Kumar, B., Kumar, D., Mahesh, M. S., and Sheel, R. (2021). Effect of oral dosing of live and autoclaved culture of Ruminococcus flavefaciens FD-1 on rumen bacterial and fungal populations in Murrah buffaloes. *Indian J. Dairy Sci.* 74 (5), 458–461. doi: 10.33785/IJDS.2021.v74i05.014

Kuo, J. T., Chang, L. L., Yen, C. Y., Tsai, T. H., Chang, Y. C., Huang, Y. T., et al. (2020). Development of fluorescence *in situ* hybridization as a rapid, accurate method for detecting coliforms in water samples. *Biosensors* 11 (1), 8. doi: 10.3390/bios11010008

Kuppusamy, P., Kim, D., Soundharrajan, I., Park, H. S., Jung, J. S., Yang, S. H., et al. (2020). Low-carbohydrate tolerant LAB strains identified from rumen fluid: investigation of probiotic activity and legume silage fermentation. *Microorganisms* 8, 1044. doi: 10.3390/microorganisms8071044

Lagier, J. C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., et al. (2018). Culturing the human microbiota and culturomics. *Nat. Rev. Microbiol.* 16, 540–550. doi: 10.1038/s41579-018-0041-0

Latham, E. A., Pinchak, W. E., Trachsel, J., Allen, H. K., Callaway, T. R., Nisbet, D. J., et al. (2018). Isolation, characterization and strain selection of a Paenibacillus species for use as a probiotic to aid in ruminal methane mitigation, nitrate/nitrite detoxification and food safety. *Bioresource Technol.* 263, 358–364. doi: 10.1016/j.biortech.2018.04.116

Latham, E. A., Pinchak, W. E., Trachsel, J., Allen, H. K., Callaway, T. R., Nisbet, D. J., et al. (2019). Paenibacillus 79R4, a potential rumen probiotic to enhance nitrite detoxification and methane mitigation in nitrate-treated ruminants. *Sci. total Environ.* 671, 324–328. doi: 10.1016/j.scitotenv.2019.03.390

Lettat, A., Nozière, P., Silberberg, M., Morgavi, D. P., Berger, C., and Martin, C. (2012). Rumen microbial and fermentation characteristics are affected differently by bacterial probiotic supplementation during induced lactic and subacute acidosis in sheep. *BMC Microbiol.* 12, 142. doi: 10.1186/1471-2180-12-142

Lewis, W. H., Tahon, G., Geesink, P., Sousa, D. Z., and Ettema, T. J. G. (2021). Innovations to culturing the uncultured microbial majority. *Nat. Rev. Microbiol.* 19, 225–240. doi: 10.1038/s41579-020-00458-8

Li, Q. L., Li, Y. P., and Wang, S. Z. (2008). The Application of DGGE technology in polymorphism analysis of rumen microorganisms. *China Cattle Sci.* 01, 12–14. doi: 10.3969/j.issn.1673-1182.2008.03.024

Li, S., Lian, W. H., Han, J. R., Ali, M., Lin, Z. L., Liu, Y. H., et al. (2023b). Capturing the microbial dark matter in desert soils using culturomics-based metagenomics and high-resolution analysis. *NPJ biofilms microbiomes* 9 (1), 67. doi: 10.1038/s41522-023-00439-8

Li, B., Ma, X., Cheng, J., Tian, T., Guo, J., Wang, Y., et al. (2023a). Droplets microfluidics platform-A tool for single cell research. *Front. Bioeng. Biotechnol.* 11. doi: 10.3389/fbioe.2023.1121870

Li, Y., Mao, K., Zang, Y., Lu, G., Qiu, Q., Ouyang, K., et al. (2023c). Revealing the developmental characterization of rumen microbiome and its host in newly received cattle during receiving period contributes to formulating precise nutritional strategies. *Microbiome* 11, 238. doi: 10.1186/s40168-023-01682-z

Li, Q. F., Zhou, Z. H., and Zie, Y. X. (2019). Application of Micro-ecological agents in ruminant Feed. *Anim. Husbandry Feed Sci.* 40, 29–30. doi: 10.5555/20193267181

Li, L. P., Zhu, H. J., Jing, X. Q., Huang, S., and Qu, L. (2017). lsolation and identification of four bovine-derived probiotics. *Heilongjiang Anim. Sci. Vet. Med.* 23, 69–71, 294. doi: 10.13881/j.cnki.hljxmsy.2017.2156

Lin, D. W. (1999). *Taxonomic identification and experimental methods of lactic acid bacteria*. (Beijing: China Light Industry Press).

Litonina, A. S., Smirnova, Y. M., Platonov, A. V., Laptev, G. Y., Dunyashev, T. P., and Butakova, M. V. (2021). Application of enzyme probiotic drug developed based on microorganisms of the rumen of reindeer (Rangifer tarandus) in feeding cows. *Regul. Mech. Biosyst.* 12, 109–115. doi: 10.15421/022117

Liu, W., Kim, H. J., Lucchetta, E. M., Du, W., and Ismagilov, R. F. (2009). Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistrode and stochastic confinement. *Lab. chip* 9, 2153–2162. doi: 10.1039/b904958d

Liu, P., Meagher, R. J., Light, Y. K., Yilmaz, S., Chakraborty, R., Arkin, A. P., et al. (2011). Microfluidic fluorescence in *situ* hybridization and flow cytometry (μFlowFISH). *Lab. chip* 11, 2673–2679. doi: 10.1039/c1lc20151d

Liu, S., Moon, C. D., Zheng, N., Huws, S., Zhao, S., and Wang, J. (2022). Opportunities and challenges of using metagenomic data to bring uncultured microbes into cultivation. *Microbiome* 10, 76. doi: 10.1186/s40168-022-01272-5

Lloréns-Rico, V., Simcock, J. A., Huys, G. R. B., and Raes, J. (2022). Single-cell approaches in human microbiome research. *Cell* 185, 2725–2738. doi: 10.1016/j.cell.2022.06.040

Luo, X., Chen, J. Y., Ataei, M., and Lee, A. (2022). Microfluidic compartmentalization platforms for single cell analysis. *Biosensors* 12, 58. doi: 10.3390/bios12020058

Maake, T. W., Aiyegoro, O. A., and Adeleke, M. A. (2021). Effects of Lactobacillus rhamnosus and Enterococcus faecalis Supplementation as Direct-Fed Microbials on Rumen Microbiota of Boer and Speckled Goat Breeds. *Vet. Sci.* 8, 103. doi: 10.3390/vetsci8060103

Madhu, B., Miller, B. M., and Levy, M. (2023). Single-cell analysis and spatial resolution of the gut microbiome. *Front. Cell. Infect. Microbiol.* 13. doi: 10.3389/fcimb.2023.1271092

Marsh, T. L. (1999). Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2, 323–327. doi: 10.1016/S1369-5274(99)80056-3

McGenity, T., van der Meer, J. R., and de Lorenzo, V. (2010). *Handbook of hydrocarbon and lipid microbiology*. Ed. K. N. Timmis (Berlin: Springer), 4716.

Megur, A., Daliri, E. B., Balnionytė, T., Stankevičiūtė, J., Lastauskienė, E., and Burokas, A. (2023). *In vitro* screening and characterization of lactic acid bacteria from Lithuanian fermented food with potential probiotic properties. *Front. Microbiol.* 14, 1213370. doi: 10.3389/fmicb.2023.1213370

Mende, D. R., Aylward, F. O., Eppley, J. M., Nielsen, T. N., and DeLong, E. F. (2016). Improved environmental genomes *via* integration of metagenomic and single-cell assemblies. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00143

Meruvu, H., and Harsa, S. T. (2023). Lactic acid bacteria: isolation-characterization approaches and industrial applications. *Crit. Rev. Food Sci. Nutr.* 63, 8337–8356. doi: 10.1080/10408398.2022.2054936

Min, B. R., Pinchak, W. E., Hume, M. E., and Anderson, R. C. (2021). Effects of condensed tannins supplementation on animal performance, phylogenetic microbial changes, and *in vitro* methane emissions in steers grazing winter wheat. *Animals: an Open Access J. MDPI* 11, 2391. doi: 10.3390/ani11082391

Mizrahi, I., Wallace, R. J., and Moraïs, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nat. Rev. Microbiol.* 19, 553–566. doi: 10.1038/s41579-021-00543-6

Mordant, A., and Kleiner, M. (2021). Evaluation of sample preservation and storage methods for metaproteomics analysis of intestinal microbiomes. *Microbiol. Spectr.* 9, e0187721. doi: 10.1128/Spectrum.01877-21

Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A., et al. (2010). Use of ichip for high-throughput in *situ* cultivation of "uncultivable" microbial species. *Appl. Environ. Microbiol.* 76, 2445–2450. doi: 10.1128/AEM.01754-09

Nobu, M. K., Narihiro, T., Rinke, C., Kamagata, Y., Tringe, S. G., Woyke, T., et al. (2015). Microbial dark matter ecogenomics reveals complex synergistic networks in a methanogenic bioreactor. *ISME J.* 9, 1710–1722. doi: 10.1038/ismej.2014.256

Noel, S. J., Olijhoek, D. W., Mclean, F., Løvendahl, P., Lund, P., and Højberg, O. (2019). Rumen and fecal microbial community structure of holstein and Jersey dairy cows as affected by breed, diet, and residual feed intake. *Animals: an Open Access J. MDPI* 9, 498. doi: 10.3390/ani9080498

Park, J., Kerner, A., Burns, M. A., and Lin, X. N. (2011). Microdroplet-enabled highly parallel co-cultivation of microbial communities. *PloS one* 6 (2), e17019. doi: 10.1371/journal.pone.0017019

Peng, N., Peng, X. Q., and Le, M. (2020). The application of microbiome culturomics in veterinary medicine. *Acta Veterinaria Zootechnica Sin.* 51, 2942–2953. doi: 10.11843/j.issn.0366-6964.2020.12.004

Pepoyan, A. Z., Tsaturyan, V. V., Badalyan, M., Weeks, R., Kamiya, S., and Chikindas, M. L. (2020). Blood protein polymorphisms and the gut bacteria: impact of probiotic Lactobacillus acidophilus Narine on Salmonella carriage in sheep. *Beneficial Microbes* 11, 183–189. doi: 10.3920/BM2019.0138

Pittaluga, A. M., Yang, F., Gaffney, J. R., Embree, M., and Relling, A. E. (2023). Effect of supplementation with ruminal probiotics on growth performance, carcass characteristics, plasma metabolites, methane emissions, and the associated rumen microbiome changes in beef cattle. *J. Anim. Sci.* 101, skac308. doi: 10.1093/jas/skac308

Poothong, S., Tanasupawat, S., Chanpongsang, S., Kingkaew, E., and Nuengjamnong, C. (2024). Anaerobic flora, Selenomonas ruminis sp. nov., and the bacteriocinogenic Ligilactobacillus salivarius strain MP3 from crossbred-lactating goats. *Sci. Rep.* 14, 4838. doi: 10.1038/s41598-024-54686-6

Prakash, V., Madhavan, A., Veedu, A. P., Babu, P., Jothish, A., Nair, S. S., et al. (2023). Harnessing the probiotic properties and immunomodulatory effects of fermented food-derived Limosilactobacillus fermentum strains: implications for environmental enteropathy. *Front. Nutr.* 10, 1200926. doi: 10.3389/fnut.2023.1200926

Prakash, O., Pandey, P. K., Kulkarni, G. J., Mahale, K. N., and Shouche, Y. S. (2014). Technicalities and glitches of terminal restriction fragment length polymorphism (T-RFLP). *Indian J. Microbiol.* 54, 255–261. doi: 10.1007/s12088-014-0461-0

Qi, P., Lv, J., Yan, X., Bai, L., and Zhang, L. (2023). Microfluidics: insights into intestinal microorganisms. *Microorganisms* 11, 1134. doi: 10.3390/microorganisms11051134

Ratan, Z. A., Zaman, S. B., Mehta, V., Haidere, M. F., Runa, N. J., and Akter, N. (2017). Application of fluorescence *in situ* hybridization (FISH) technique for the detection of genetic aberration in medical science. *Cureus* 9, e1325. doi: 10.7759/cureus.1325

Reid, G. (2006). Safe and efficacious probiotics: what are they? *Trends Microbiol.* 14 (8), 348–352. doi: 10.1016/j.tim.2006.06.006

Ren, A., Zhang, B., and Zhou, C. S. (2016). Research progress in separation and ldentification of ruminal microorganisms in ruminants. *J. Domest. Anim. Ecol.* 37, 85–89. doi: 10.3969/j.issn.1673-1182.2016.06.016

Rodrigues, C. F., Azevedo, N. F., and Miranda, J. M. (2021). Integration of FISH and microfluidics. *Methods Mol. Biol.* 2246, 249–261. doi: 10.1007/978-1-0716-1115-9_16

Schofield, B. J., Lachner, N., Le, O. T., McNeill, D. M., Dart, P., Ouwerkerk, D., et al. (2018). Beneficial changes in rumen bacterial community profile in sheep and dairy calves as a result of feeding the probiotic Bacillus amyloliquefaciens H57. *J. Appl. Microbiol.* 124, 855–866. doi: 10.1111/jam.13688

Schütte, U. M., Abdo, Z., Bent, S. J., Shyu, C., Williams, C. J., Pierson, J. D., et al. (2008). Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Appl. Microbiol. Biotechnol.* 80, 365–380. doi: 10.1007/s00253-008-1565-4

Sreepathi, N., Kumari, V. B. C., Huligere, S. S., Al-Odayni, A. B., Lasehinde, V., Jayanthi, M. K., et al. (2023). Screening for potential novel probiotic Levilactobacillus brevis RAMULAB52 with antihyperglycemic property from fermented Carica papaya L. *Front. Microbiol.* 14, 1168102. doi: 10.3389/fmicb.2023.1168102

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., and Watson, M. (2019). Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nat. Biotechnol.* 37 (8), 953–961. doi: 10.1038/s41587-019-0202-3

Sun, J. F., and Lin, Y. T. (2009). Isolation, identification and quantitative analysis of ruminal microorganisms. *China Dairy Cattle* 04), 18–23. doi: 10.3969/j.issn.1004-4264.2009.04.008

Tan, J. Y., Wang, S., Dick, G. J., Young, V. B., Sherman, D. H., Burns, M. A., et al. (2020). Co-cultivation of microbial sub-communities in microfluidic droplets facilitates high-resolution genomic dissection of microbial 'dark matter'. *Integr. biology: quantitative Biosci. nano to macro* 12, 263–274. doi: 10.1093/intbio/zyaa021

Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997). A genomic perspective on protein families. *Science* 278 (11), 631–637. doi: 10.1126/science.278.5338.631

Tidjani Alou, M., Naud, S., Khelaifia, S., Bonnet, M., Lagier, J. C., and Raoult, D. (2020). State of the art in the culture of the human microbiota: new interests and strategies. *Clin. Microbiol. Rev.* 34, e00129–e00119. doi: 10.1128/CMR.00129-19

Tyagi, A. K., Kumar, S., Choudhury, P. K., Tyagi, B., and Tyagi, N. (2020). Conjugated linoleic acid producing potential of lactobacilli isolated from goat (AXB) rumen fluid samples. *Asian-Australasian J. Anim. Sci.* 33 (8), 1233–1241. doi: 10.5713/ajas.19.0080

Valm, A. M., Mark Welch, J. L., Rieken, C. W., Hasegawa, Y., Sogin, M. L., Oldenbourg, R., et al. (2011). Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4152–4157. doi: 10.1073/pnas.1101134108

Varada, V. V., Tyagi, A. K., Banakar, P. S., Das, A., Tyagi, N., Mallapa, R. H., et al. (2022). Autochthonous Limosilactobacillus reuteri BFE7 and Ligilactobacillus salivarius BF17 probiotics consortium supplementation improves performance, immunity, and selected gut health indices in Murrah buffalo calves. *Vet. Res. Commun.* 46, 757–767. doi: 10.1007/s11259-022-09896-6

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PloS One* 8, e57923. doi: 10.1371/journal.pone.0057923

Wan, X., Yang, Q., Wang, X., Bai, Y., and Liu, Z. (2023). Isolation and cultivation of human gut microorganisms: A review. *Microorganisms* 11 (4), 1080. doi: 10.3390/microorganisms11041080

Wang, R., Cai, W. T., Wang, X. L., Gao, J., and Huang, M. (2019a). Progress in Chinese medicine-probiotics compound microecological preparations for livestock and poultry. *Chin. J. Biotechnol.* 35, 972–987. doi: 10.13345/j.cjb.180443

Wang, Y., Gong, L., Wu, Y. P., Cui, Z. W., Wang, Y. Q., Huang, Y., et al. (2019b). Oral administration of Lactobacillus rhamnosus GG to newborn piglets augments gut barrier function in pre-weaning piglets. *J. Zhejiang Univ. Sci. B* 20, 180–192. doi: 10.1631/jzus.B1800022

Wang, Y., Li, Z., Jin, W., and Mao, S. (2022). Isolation and characterization of ruminal yeast strain with probiotic potential and its effects on growth performance, nutrients digestibility, rumen fermentation and microbiota of Hu sheep. *J. fungi* 8, 1260. doi: 10.3390/jof8121260

Wang, S. J., Li, X., Liu, L. Y., Dan, Y. L., Xiao, Z. T., Wang, Q. Y., et al. (2021). Screening and identification of probiotics from yak dung and milk in Tibet. *China Brewing* 40, 43–48. doi: 10.11882/j.issn.0254-5071.2021.07.009

Waters, J. C. (2009). Accuracy and precision in quantitative fluorescence microscopy. *J. Cell Biol.* 185 (7), 1135–1148. doi: 10.1083/jcb.200903097

Watterson, W. J., Tanyeri, M., Watson, A. R., Cham, C. M., Shan, Y., Chang, E. B., et al. (2020). Droplet-based high-throughput cultivation for accurate screening of antibiotic resistant gut microbes. *eLife* 9, e56998. doi: 10.7554/eLife.56998.sa2

Wen, L., Li, G., Huang, T., Geng, W., Pei, H., Yang, J., et al. (2022). Single-cell technologies: From research to application. *Innovation* 3, 100342. doi: 10.1016/j.xinn.2022.100342

Wu, H. H., Wang, M. G., Xu, H. W., Wang, D. M., and Yang, D. (2018). Improvement of experimental methods for the biological and biochemical identification of microorganisms. *Experiment Teach. Apparatus* 35 (05), 34–40.

Xie, H. W., Weng, B., Li, X. Y., Chen, M., Guo, M. N., Zheng, L. L., et al. (2021). Research on the application of microecological preparations. *Guangdong Chem. Industry* 48, 17–19. doi: 10.3969/j.issn.1007-1865.2021.24.008

Xiong, R. Y., Yang, Q. N., Shang, Y., and Wang, L. M. (2020). Screening and function of probiotics and their application in animal feed. *Feed Res.* 43, 147–150. doi: 10.13557/j.cnki.issn1002-2813.2020.03.038

Xu, B., Hu, B., Wang, J., Lan, Y., Zhu, Y., Dai, X., et al. (2018a). Virgibacillus indicus sp. nov. and Virgibacillus profundi sp. nov, two moderately halophilic bacteria isolated from marine sediment by using microfluidic streak plates. *Int. J. Syst. Evol. Microbiol.* 68, 2015–2023. doi: 10.1099/ijsem.0.002782

Xu, Y., and Zhao, F. (2018b). Single-cell metagenomics: challenges and applications. *Protein Cell* 9, 501–510. doi: 10.1007/s13238-018-0544-5

Yadav, K. K., Nimonkar, Y., Poddar, B. J., Kovale, L., Sagar, I., Shouche, Y., et al. (2022). Two-dimensional cell separation: a high-throughput approach to enhance the culturability of bacterial cells from environmental samples. *Microbiol. Spectr.* 10, e0000722. doi: 10.1128/spectrum.00007-22

Yang, S., Luo, J., Chen, Y., Wu, R., Liu, H., Zhou, Z., et al. (2022). A buffalo rumen-derived probiotic (SN-6) could effectively increase simmental growth performance by regulating fecal microbiota and metabolism. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.935884

Yang, S., Ma, S., Chen, J., Mao, H., He, Y., Xi, D., et al. (2010). Bacterial diversity in the rumen of Gayals (Bos frontalis), Swamp buffaloes (Bubalus bubalis) and Holstein cow as revealed by cloned 16S rRNA gene sequences. *Mol. Biol. Rep.* 37, 2063–2073. doi: 10.1007/s11033-009-9664-6

Yin, J., Chen, X., Li, X., Kang, G., Wang, P., Song, Y., et al. (2022). A droplet-based microfluidic approach to isolating functional bacteria from gut microbiota. *Front. Cell. Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.920986

Young, K. D. (2007). Bacterial morphology: why have different shapes? *Curr. Opin. Microbiol.* 10, 596–600. doi: 10.1016/j.mib.2007.09.009

Yu, Y., Wen, H., Li, S., Cao, H., Li, X., Ma, Z., et al. (2022). Emerging microfluidic technologies for microbiome research. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.906979

Yu, M. F., Zhao, X. M., Cai, H., Yi, J. M., and Hua, G. H. (2020). Dihydropyridine enhances the antioxidant capacities of lactating dairy cows under heat stress condition. *Animals: an Open Access J. MDPI* 10, 1812. doi: 10.3390/ani10101812

Yu, X., Zhao, F. Y., and Zhao, Z. H. (2023). Research progress in culturomics of beneficial bacteria in the intestines. *Food Sci.* 44 (23), 365–371. doi: 10.7506/spkx1002-6630-20221210-103

Yuan, C. L., Yu, Z. Y., Lin, Y. T., and Wang, L. H. (2015). Effects of probiotics on the calves and ts affecting factors. *China Dairy Cattle* 08, 9–12. doi: 10.3969/j.issn.1004-4264.2015.08.002

Zhang, X., Ke, W., Ding, Z., Xu, D., Wang, M., Chen, M., et al. (2022). Microbial mechanisms of using feruloyl esterase-producing Lactobacillus plantarum A1 and grape pomace to improve fermentation quality and mitigate ruminal methane emission of ensiled alfalfa for cleaner animal production. *J. Environ. Manage.* 308, 114637. doi: 10.1016/j.jenvman.2022.114637

Zhang, J., Liu, Y. X., Guo, X., Qin, Y., Garrido-Oter, R., Schulze-Lefert, P., et al. (2021). High-throughput cultivation and identification of bacteria from the plant root microbiota. *Nat. Protoc.* 16, 988–1012. doi: 10.1038/s41596-020-00444-7

Zhang, D., Yang, Y., Qin, Q., Xu, J., Wang, B., Chen, J., et al. (2019). MALDI-TOF characterization of protein expression mutation during morphological changes of bacteria under the impact of antibiotics. *Anal. Chem.* 91, 2352–2359. doi: 10.1021/acs.analchem.8b05080

Zhao, Z., Fan, B., Chen, D., Men, D., Wang, J., and Chen, J. (2016). Development of droplet microfluidics enabling high-throughput single-cell analysis. *Molecules* 21, 881. doi: 10.3390/molecules21070881

Zheng, W., Zhao, S., Yin, Y., Zhang, H., Needham, D. M., Evans, E. D., et al. (2022). High-throughput, single-microbe genomics with strain resolution, applied to a human gut microbiome. *Science* 376, eabm1483. doi: 10.1126/science.abm1483

Zhong, H., Zheng, N., Wang, J., and Zhao, S. (2023). Isolation and pan-genome analysis of Enterobacter hormaechei Z129, a ureolytic bacterium, from the rumen of dairy cow. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1169973

Zhou, W., Le, J., Chen, Y., Cai, Y., Hong, Z., and Chai, Y. (2019b). Recent advances in microfluidic devices for bacteria and fungus research. *TrAC Trends Anal. Chem.* 112, 175–195. doi: 10.1016/j.trac.2018.12.024

Zhou, N., Sun, Y. T., Chen, D. W., Du, W., Yang, H., and Liu, S. J. (2019a). Harnessing microfluidic streak plate technique to investigate the gut microbiome of Reticulitermes chinensis. *Microbiol. Open* 8, e00654. doi: 10.1002/mbo3.654

Zhou, R., Yang, G., Zhang, Y., and Wang, Y. (2023). Spatial transcriptomics in development and disease. *Mol. Biomed.* 4, 32. doi: 10.1186/s43556-023-00144-0

Zhu, Z., Kristensen, L., Difford, G. F., Poulsen, M., Noel, S. J., Abu Al-Soud, W., et al. (2018). Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. *J. dairy Sci.* 101, 9847–9862. doi: 10.3168/jds.2017-14366

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Comparative analysis of intestinal microbiota composition between free-ranged captive yak populations in Nimu County

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The intestinal microbiota assumes a pivotal role in modulating host metabolism, immune responses, overall health, and additional physiological dimensions. The structural and functional characteristics of the intestinal microbiota may cause alterations within the host's body to a certain extent. The composition of the gut microbiota is associated with environmental factors, dietary habits, and other pertinent conditions. The investigation into the gut microbiota of yaks remained relatively underexplored. An examination of yak gut microbiota holds promise in elucidating the complex relationship between microbial communities and the adaptive responses of the host to its environment. In this study, yak were selected from two distinct environmental conditions: those raised in sheds (NS, n=6) and grazed in Nimu County (NF, n=6). Fecal samples were collected from the yaks and subsequently processed for analysis through 16S rDNA and ITS sequencing methodologies. The results revealed that different feeding styles result in significant differences in the Alpha diversity of fungi in the gut of yaks, while the gut microbiota of captive yaks was relatively conserved. In addition, significant differences appeared in the abundance of microorganisms in different taxa, phylum *Verrucomicrobiota* was significantly enriched in group NF while *Firmicutes* was higher in group NS. At the genus level, *Akkermansia*, *Paenibacillus*, *Roseburia*, *Dorea*, *UCG_012*, *Anaerovorax* and *Marvinbryantia* were enriched in group NF while *Desemzia*, *Olsenella*, *Kocuria*, *Ornithinimicrobium* and *Parvibacter* were higher in group NS ($P<0.05$ or $P<0.01$). There was a significant difference in the function of gut microbiota between the two groups. The observed variations are likely influenced by differences in feeding methods and environmental conditions both inside and outside the pen. The findings of this investigation offer prospective insights into enhancing the yak breeding and expansion of the yak industry.

KEYWORDS

captive yaks, free-range yaks, feeding style, gut microbiota, Nimu County, sequencing, Tibet autonomous region

Introduction

The intestinal microecology constitutes a dynamic ecosystem characterized by continuous fluctuations. Ruminant gastrointestinal tracts harbor vast populations of microorganisms, primarily comprising bacteria ((>98%), fungi (>0.1%), viruses, and protozoa (Larabi et al., 2020). In recent years, gut microbiota has been recognized as a signaling hub that integrates environmental inputs, genetic factors, and immune system signals to influence host metabolism, immunity, and infection responses (Thaiss et al., 2016). The composition of the gut microbiota is constantly shaped by multiple factors, such as dietary habits, seasons, lifestyle, antibiotics, or diseases (Rinninella et al., 2019). It is already established that the gut microbiota exerts a significant influence on animal digestive processes, with dietary composition standing out as a primary determinant in shaping the composition and function of the gut microbiota (Fan et al., 2023). The changes in gut microbiota also reflect the health status of the host, and the imbalance of gut microbiota has been associoated with various diseases (Wu et al., 2019).

Till now, research on the gut microbiota of ruminants were mainly focused on the type of animal, growth stages, and production performance. The composition and function of gut microbiota were explored through high-throughout sequencing analysis of their intestinal contents from different segments. Within diverse ruminant species, *Firmicutes* and *Bacteroidetes* emerge as predominant phyla, a pattern consistently observed across ruminants including sheep, yaks, and elk (Zeng et al., 2017; Xu et al., 2021; Lin et al., 2023). Furthermore, microbial communities within different segments of the intestine exhibit specificity, with the diversity and richness of microbial populations typically observed higher in the large intestine than that in the small intestine.

The yak, a distinctive cattle breed primarily inhabiting high-altitude regions (above 3000 meters), epitomizes a species evolved by natural selection, exhibiting remarkable adaptation to the cold, low-pressure, and hypoxic conditions prevalent in such environments. Its unique attributes render it an invaluable resource for the inhabitants of high-altitude areas (Li et al., 2020). It is generally believed that domesticating yaks originated in Xizang, China. Yaks are an important source of milk and meat products and play an important role in local economic development (Kong et al., 2024). In recent years, due to the promotion of sustainable and healthy development of ecological animal husbandry, the management of yak breeding has gradually shifted from year-round grazing to warm season grazing and cold season breeding in farms. However, many herders still adopt traditional grazing and breeding models. Exploring the impact of yak feeding patterns on the physical condition and economic value of yaks is crucial for the development of high-altitude areas. Previous studies have shown that cold and hypoxic environments may lead to changes in the gut microbiota composition (Ramos-Romero et al., 2020; Wang et al., 2020). Compared to the animals in plain areas, the high-altitude hypoxic environment in the Qinghai Tibet Plateau may shape distinct gut microbiomes in yaks (Wen et al., 2022). Therefore, this study explores the impact of different feeding models on the richness and diversity of yak gut microbiota, providing recommendations for improved feeding plans for yaks.

In this study, we utilized high-throughput sequencing (HTS) to investigate the effects of different feeding strategies on the gut microbiota structure and function of yaks in Nimu County. HTS is a molecular biology technique with high sequencing flux, fast detection speed, and low error rate, widely used in the field of microbiology research, and has been widely used in the exploration of gut microorganisms (Lee et al., 2020; Lu et al., 2023). The application of HTS in determining genomic sequences has been demonstrated for the first time through genome sequencing of *Acinetobacter baumannii*. Using this technology, we have explored the diversity, richness, structure, and functions of gut microbiota in yaks under different feeding conditions, to offer recommendations for the promotion of the healthy and sustainable development of the yak breeding industry.

Materials and methods

Sample collection

Fresh fecal samples were collected from 6 free-range yaks (group NF, n=6) and 6 captive yaks (group NS, n=6), belonging to herdsman and standardized yak farm, respectively, from Nimu County, Lhasa City, Xizang Autonomous Region, China (an average altitude above 3800 meters with an average annual temperature of 7°C). The fecal material was immediately collected after excretion using a sterile swab to avoid contamination and only the fresh feces were collected for subsequent testing. Fecal samples were snap-frozen using liquid nitrogen in sterile cryotubes and were stored at -80°C for subsequent testing.

DNA extraction, PCR amplification, and library construction

Before DNA extraction, samples were vortexed for homogenization. OMEGA Soil DNA Kit (D5625–01) (Omega Bio-Tek, Norcross, GA, USA) was used to extract the genomic DNA (gDNA) from feces, quality and quantity of DNA was assessed and was stored at −20°C. According to the previous reports, the selected hypervariable regions of bacterial 16S rRNA genes (V3–V4) and fungal ITS genes were amplified by PCR using specific primers with Barcode and high-fidelity DNA polymerase (Lundberg et al., 2013; Wen et al., 2022; Lu et al., 2023). PCR products were subjected to 2% agarose gel electrophoresis, and DNA fragments were excised and purified by using Quant-iT Pico Green dsDNA Assay Kits.

Based on the preliminary quantitative results from electrophoresis, the recovered products of PCR amplification were detected and quantified using the Microplate reader (BioTek, FLx800) fluorescence quantitative system. The resulting proportions were combined according to the sequencing requirements of each sample. Library construction was performed using the TruSeq Nano DNA LT Library Prep Kit from Illumina.

The quality of the prepared library was assessed using Agilent Bioanalyzer 2100 and Promega QuantiFluor. Once the library passed the quality control, it was subjected to sequencing.

Bioinformatics and statistical analysis

The raw reads obtained in FASTQ format were pair-ended and preprocessed using cutadapt software to identify and remove adapters. Following trimming, the qualified paired-end reads from the previous step were subjected to quality filtering, noise reduction, splicing, chimerism removal, and other quality control analyses. These procedures were conducted according to the default parameters of QIIME 2 using DADA2 (Callahan et al., 2016; Bolyen et al., 2019), to obtain consensus sequences and ASV abundance tables. The consensus sequences were annotated and blasted against Silva database Version 138 using classify-sklearn with the default parameters. Based on taxonomic information, statistical analysis of community structure was conducted at various classification levels. Alpha and Beta diversity analysis was conducted to compare and analyze microbial community structure within and between communities, respectively (Lozupone et al., 2007; Mokany et al., 2011). LEFSe (Linear discriminant analysis effect size) and STAMP difference analysis were used to evaluate the significance level of species abundance difference and obtain the bacterial and fungal species information with significant differences between the two groups. PICRUSt2 (Phylogenetic Investigation of Communities by Recommendation of Unobserved States) was employed to predict the metabolic function of the flora based on marker gene (16S/ITS) sequence (Douglas et al., 2020).

The Wilcoxon test and ANOSIM were used to evaluate significant differences between different groups. P values < 0.05 were declared as statistically significant.

Results

Sequence analysis

Twelve fecal samples were collected from free-range yaks (NF, n=6) and captive yaks (NS, n=6). Amplicon sequences of 16S and ITS were conducted to explore the differences in gut microbiota between yaks under captive feeding and grazing conditions. A total of over 1,711,000 raw data reads were obtained, with 848,297 for NF and 863,496 for NS in the bacterial analysis, and 853,474 for NF and 833,245 for NS in the fungal analysis (Supplementary Tables 1, 2). After filtering, 1,640,000 sequences were retained for bacteria (NF=813,069, NS=827,157) and 1,623,000 for fungi (NF=810,811, NS=812,794). In this study, both sequencing results exhibited high-quality scores (Q20>95%, Q30>89%), suggesting high accuracy in base detection and meeting the requirements for further analysis. Additionally, rarefaction and Shannon curves showed a gradual flattening with the increasing number of sequencing data, indicating reasonable sequencing depth and a sufficiently large amount of data (Figures 1A, B, D, E). The sequences generated from the V3/4 and ITS regions were clustered into 8949 bacterial ASVs and 1541

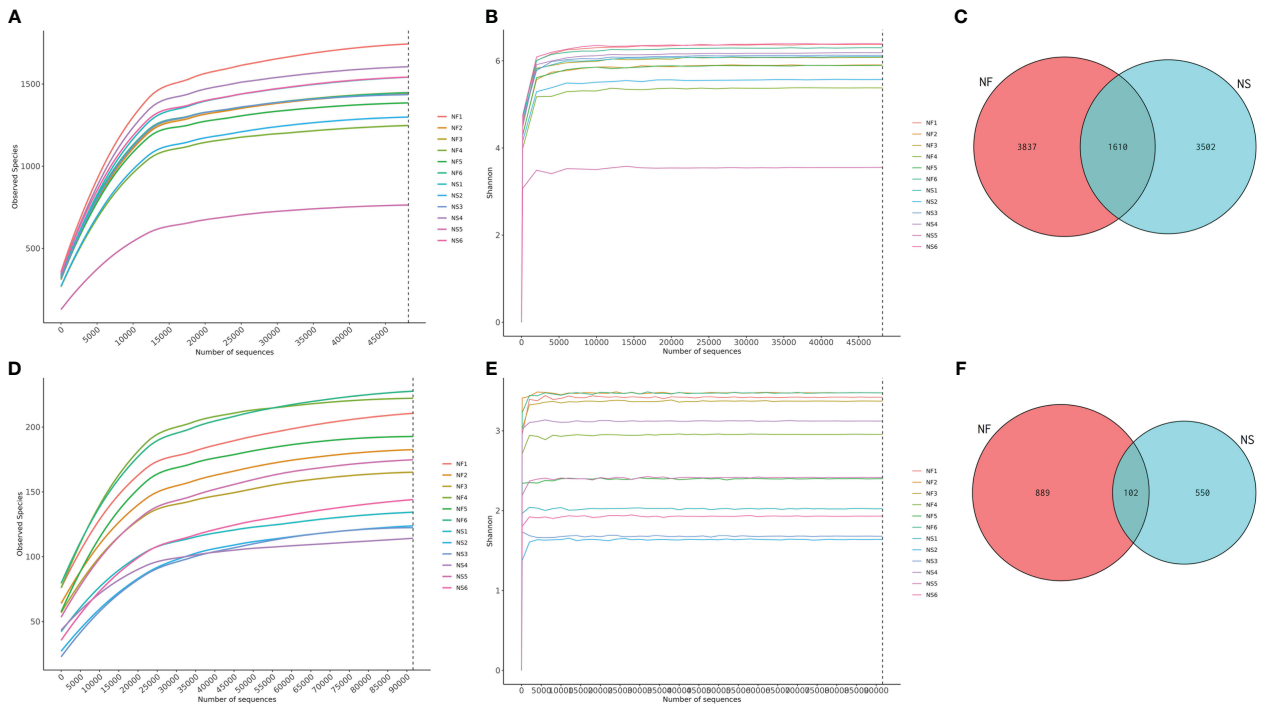

FIGURE 1
Sequencing data feasibility analysis and OTUs distribution. **(A)** Rarefaction curve of bacterial sequences, **(B)** Shannon curve of bacterial sequences, **(C)** Gut bacterial ASVs distribution in different groups, **(D)** Rarefaction curve of fungal sequences, **(E)** Shannon curve of fungal sequences, **(F)** Gut fungal ASVs distribution in different groups.

fungal ASVs. Based on the clustering analysis results of ASVs, we analyzed common and unique ASVs between the two groups. Group NF yielded 3,837 bacterial ASVs, while group NS yielded 3,502 bacterial ASVs, with 1,610 ASVs shared between the two groups (Figure 1C). Additionally, 889 fungal ASVs were identified in group NF, and 550 fungal ASVs were identified in group NS, with 102 ASVs in common (Figure 1F).

The effect of feeding styles on the diversity of yak Intestinal Flora

Alpha diversity and beta diversity of the yak gut microbial fraction were calculated to explore the effects of different feeding styles on the gut microbiota of yaks. The Chao1 estimator and ACE estimator were used to describe the richness of a community, while the Shannon index and Simpson index were used to show the diversity of species in the samples. There was no significant difference between the two groups in bacterial alpha diversity (Table 1). However, four fungal alpha diversity indices were significantly higher in group NF than in group NS ($P<0.05$) (Table 2), indicating that the richness and evenness of gut fungal community in grazing yaks were higher than those in captive yaks (Figure 2). Using the statistical algorithm Bray Curts for beta diversity analysis, the graph provides an intuitive observation of the distance between group samples. Samples with high similarity in community structure tend to cluster together, while samples with significant differences in community structure tend to be far apart. In the bacterial principal component analysis (PCA) plot (Figure 3A), dots representing the two groups are not distinctly

TABLE 1 The indexes of alpha diversity of gut bacteria.

sample	observed_species	ACE	Chao1	Shannon	Simpson
NF1	1744	1810.216342	1813.068	9.2011	0.9944
NF2	1441	1489.527862	1496.598	8.5193	0.9826
NF3	1449	1501.954038	1511.16	8.768	0.9914
NF4	1248	1286.138338	1292.0323	7.762	0.9746
NF5	1386	1423.854067	1421.3465	8.5043	0.9876
NF6	1448	1489.130506	1483.9211	9.09	0.9955
NS1	1542	1594.533257	1586.7194	8.7926	0.9909
NS2	1301	1352.702004	1363.16	8.0396	0.981
NS3	1438	1478.330731	1479.4455	8.8396	0.9923
NS4	1607	1652.719655	1651.52	8.9251	0.991
NS5	765	802.3223353	809.0143	5.1313	0.8759
NS6	1543	1598.782962	1595.0238	9.222	0.9956

TABLE 2 The indexes of alpha diversity of gut fungi.

sample	observed_species	ACE	Chao1	Shannon	Simpson
NF1	211	234.6421	234.8824	4.9356	0.9344
NF2	183	194.9086	190.7727	5.0141	0.9497
NF3	166	179.0274	176.0588	4.8642	0.9462
NF4	223	228.6601	236.125	4.2639	0.8891
NF5	193	198.4924	197.4	3.4616	0.839
NF6	228	240.1577	240.65	5.0139	0.946
NS1	134	143.6319	140.5625	2.9206	0.6597
NS2	124	132.2439	131.5	2.3643	0.6645
NS3	123	135.0197	131.5	2.425	0.6749
NS4	114	122.9013	136.75	4.5044	0.9351
NS5	176	192.3004	189.1429	3.4846	0.8078
NS6	144	161.6346	156.0476	2.786	0.7583

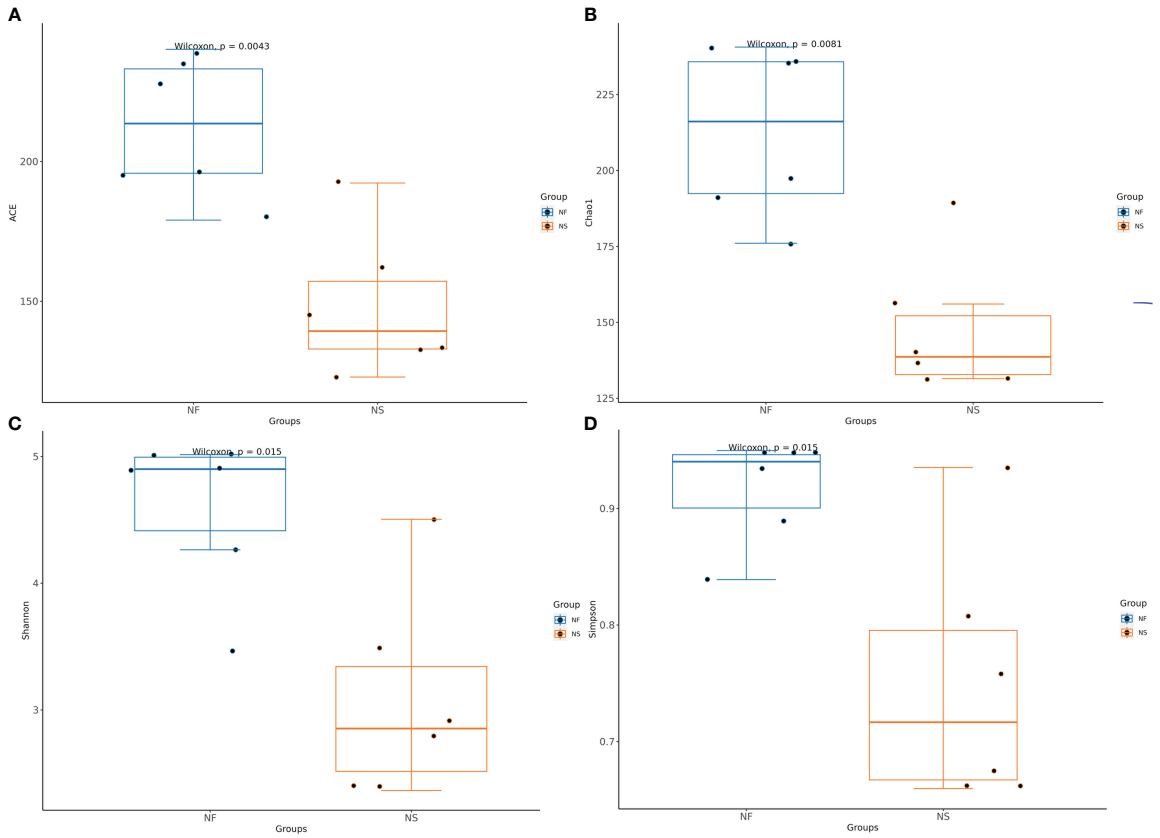

FIGURE 2
Comparative analysis of the Alpha diversity in gut fungi between house feeding and captive yaks. **(A)** ACE index, **(B)** Chao1 index, **(C)** Shannon index, **(D)** Simpson index.

dispersed, suggesting a less clear separation. In contrast, the fungal PCA plot (Figure 3D) exhibits a clear distinction between dots belonging to the two groups. PCoA analysis (Figures 3B, E) demonstrates that the distance between sample dots within the group is relatively close, while the distance between groups is relatively far in both bacterial and fungal analyses. This indicates noticeable independent clustering. The NMDS plot (Figures 3C, F) further supports these findings (ANOSIM, bacterial: p = 0.018, fungal: p=0.02).

These results suggest that different feeding styles have led to variations in the species composition structure of the yak gut microbiota community. The PCA, PCoA, and NMDS analyses collectively highlight the distinct microbial community patterns between yaks subjected to different feeding conditions.

The effect of feeding regime on the community composition of intestinal bacteria

Based on the results of species annotation, choosing the top 10 taxa with the highest abundance ranking at levels of phylum and genus for each sample, a column accumulation chart of relative species abundance was generated to visualize the species with higher relative abundance and their proportion at different taxa.

At the phylum level, *Firmicutes* was the chief phyla in the two groups (NF: 63.52%, NS:71.65%) while it was more abundant in group NS. The phylum with the second highest abundance was *Bacteroidota* (NF: 21.62%, NS:18.91%), followed by *Actinobacteriota* (NF: 9.74%, NS:6.69%) (Figure 4A). The *Firmicutes*/*Bacteroidetes* ratio is often associated with body health (Magne et al., 2020), in this study, the *Firmicutes*/*Bacteroidetes* ratio was relatively higher in group NS. At the genus level, the primary genus in group NF was *UCG-005*, while the primary genera in group NS was an uncultured genus belonging to the family *Planococcaceae*, which was enricher in group NS (NF: 0.0837%%, NS:14.32%). In addition, the abundance of *Christensenellaceae_R-7_group* was higher in group NF while the abundance of *Romboutsia* was higher in group NS (Figure 4B).

The effect of feeding regimes on the community composition of intestinal fungi

At the phylum level, *Ascomycota* has the top abundance in both groups (NF: 75.25%, NS:32.01%) in classified phylum, followed by *Basidiomycota* (NF: 0.37%, NS:0.18%) and *Firmicutes* (NF: 0.0233%, NS:0.0326%), together with uncultured phylum, they accounted for over 99.95% of the total number (Figure 4C). It is worth mentioning that a large number of unclassified fungi was detected in the intestines of domesticated yaks, which was

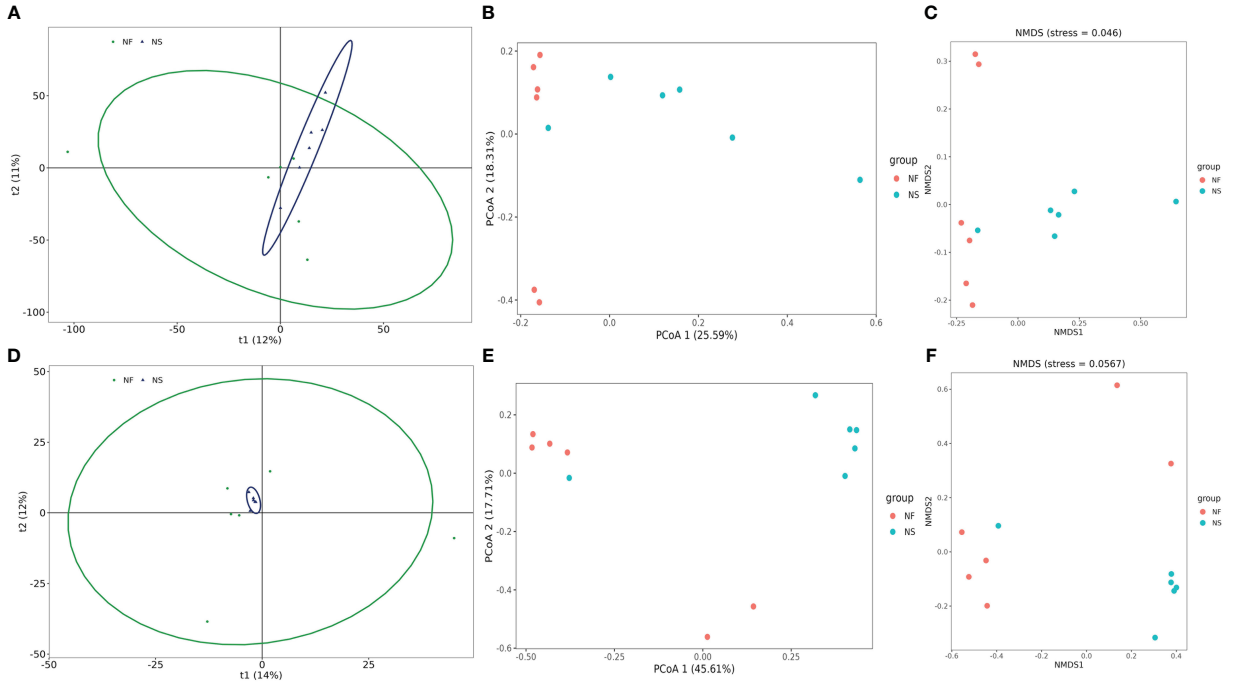

FIGURE 3
Comparative analysis of the gut bacterial and fungal beta diversities between house feeding and captive yaks. **(A, D)** gut bacterial and fungal PCA plots, **(B, E)** gut bacterial and fungal PCoA plots, **(C, F)** gut bacterial and fungal NMDS plots.

significantly higher than that of grazing yaks. An unidentified family belonging to *Pleosporales* was more abundant in group NF (6.69%) than in group NS (0.53%). Genus *Scleromitrula* was much richer in group NS (11.13%) than group NF (7.05%) while *Myrothecium* was richer in group NF (3.94%) than in group NS (1.74%) (Figure 4D).

Significant impact of different feeding regimes on intestinal microbiota

LEfSe analysis performs linear discriminant analysis (LDA) on samples according to different grouping conditions based on their taxonomic composition, identifying communities or species that have a significant impact on sample partitioning (Segata et al., 2011). In this study, LEfSe analysis was performed based on the abundance of species from two groups, species with LDA values > 2 were established biomarkers with statistical differences between groups.

A total of 39 biomarkers were detected in intestinal microbiota (Figures 5A, B). Phylum *Verrucomicrobiota* was significantly enriched in group NF, in this phylum, genus *Akkermansia* was significantly richer in NF, too. The abundance of Firmicutes was higher in group NS. In the class Bacilli belonging to Firmicutes, genus *Paenibacillus* was significantly richer in group NF while *Desemzia* was much more in group NS. In order Bacillales, genus *Solibacillus* was richer in group NF. In order *Oscillospirales* belonging to class *Clostridia*, *Oscillibacter*, *Candidatus_Soleaferrea* and *Incertae_Sedis* were enriched in group NF while *Clostridium: methylpentosum*_group was enriched in group NS. Besides, the abundance of genera *Roseburia*, *Dorea*, *UCG_012*, *Anaerovorax* and *Marvinbryantia* were higher in group NF. In addition, *Olsenella*, *Kocuria*, *Ornithinimicrobium* and *Parvibacter* were more abundant in group NS while *Alloprevotella*, *Coriobacteriaceae_UCG_002* and *Mailhella* were richer in group NF ($P<0.05$ or $P<0.01$). In intestinal fungi, the genus *Acremonium* belonging to *Iae_ncertSedis* in phylum *Ascomycota* was significantly richer in group NF ($P<0.01$) (Figures 5C, D).

Metabolic alterations in gut microbiota to adapt to different feeding methods

PICRUSt was utilized to compare species composition information obtained from high-throughput sequencing data with information in the database, infer functional gene composition in samples, and analyze functional differences between various samples or groups (Ijoma et al., 2021). KEGG (Kyoto Encyclopedia of Genes and Genomes, primary database for studying metabolic pathways, integrating genomic information, chemical information, system information, and disease and health information (Kanehisa et al., 2017). COG (Clusters of Orthologous Groups) is a database for annotation of homologous proteins which can predict protein function well. PCA analysis revealed the similarity or difference in microbial community functions between the two groups on an overall level, results revealed that the bacterial functions were similar within the group, but more dispersed between groups while the dots of fungal functions were

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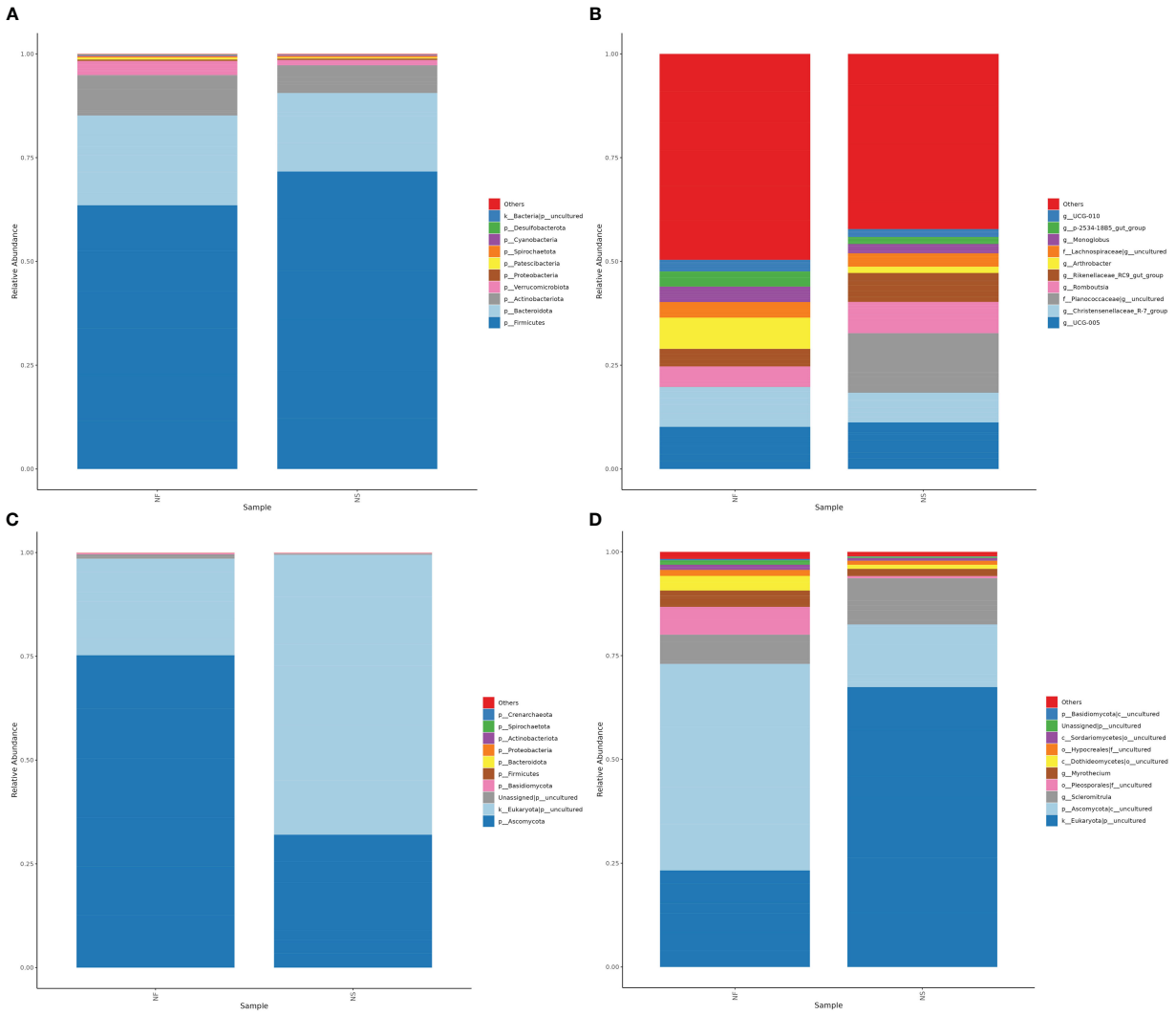

FIGURE 4
The relative abundance of bacterial **(A, B)** and fungal **(C, D)** taxa at the levels of Phylum and Genus.

relatively scattered overall (Figures 6A, B). Results obtained from LDA combined with LEfSe analysis showed 13 significant differences in KEGG pathways between the two groups. Bacterial KEGG pathways including Biosynthesis of other secondary metabolites, Endocrine system, and nervous system were higher in group NF while Signal transduction was richer in group NS ($P<0.05$) (Figure 6C). Fungal KEGG pathways such as Signal transduction, Metabolism of other amino acids, and Cancer-specific types were distinctively richer in group NF while the "replication and repair," nervous system was observed higher in group NS ($P<0.05$) (Figure 6E). The significant differences between the two groups in metabolic pathways of KEGG l3 are shown in Figure 7. 16 significant differences were recorded in COG function prediction between group NS and NF. Energy production and conversion, Nucleotide transport, and metabolism were found in higher tendency in group NF while signal transduction mechanisms were in group NS($P<0.05$) in intestinal bacteria (Figure 6D). Energy production and conversion, Inorganic ion transport and metabolism, RNA processing and modification were detected richer in group NF while Replication_recombination and repair, Defense mechanisms were found higher in group NS ($P<0.05$ or $P<0.01$) in intestinal fungi (Figure 6F).

Discussion

Yak (*Bos grunniens*) is one of the main domesticated cattle breeds in China, having dominant livestock species in the pastoral areas of the Qinghai Tibet Plateau since ancient times, renowned for its adaptability to high-altitude cold environments (>3000m above sea level). Domestic yaks are thought to have originated from Xizang, China, currently, two feeding modes viz. free range and artificial breeding are being practiced for domestic yaks. In recent years, many scholars have expressed their assertive view that factors such as diet and feeding environment shape the gut microbiota (Zmora et al., 2019). In yaks, there are also reports suggesting that different feeding modes/regimes have changed the

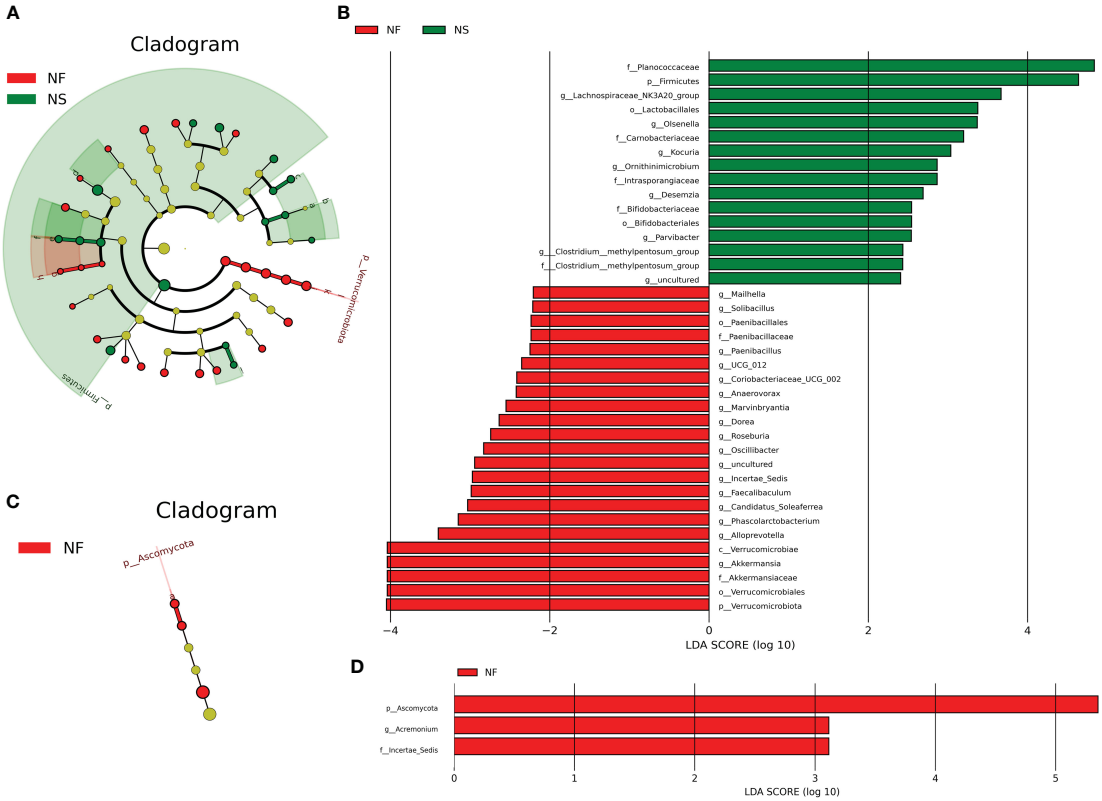

FIGURE 5
LEfSe analysis of intestinal bacterial **(A, B)** and fungal community **(C, D)** between house feeding and captive yaks at the levels of Phylum, Class, Order, Family, Genus, and Species.

structure and diversity of yak gut microbiota (Wen et al., 2022; Zhu et al., 2023).

In the current study, 16S and ITS rDNA sequencing analyses were employed to explore the differences in gut microbiota structure and function of yaks in Nimu County, Lhasa City under different feeding approaches. Fresh fecal samples of yaks belonging to the same age groups, yak farms, and free-ranged yaks were randomly collected for analysis. The results exhibited significant variations in the diversity, structure, and function of gut microbiota among two groups of yaks. Though no significant differences were observed from the results of bacterial alpha diversity of the two groups, the richness and evenness of gut fungal community in grazing yaks were found significantly higher than those in captive yaks. In addition, PCA plots showed that the aggregation phenomenon of points in the NS group can be observed, implying that the composition of gut microbiota in yaks fed in captivity was closely associated, especially with the fungal community. Captive yaks generally consume prepared feed (including alfalfa hay, oat hay, corn straw, and artificially prepared mixed feed), while the food for grazing yaks is undefined. Presumably, the regularity of diet and the stability of food ingredients are related to the relatively conservative gut microbiota of captive yaks, the complexity of environmental conditions may promote the increase of gut fungal richness in grazed yaks, the structural and functional changes of gut microbiota serve to enable yaks to adapt to the changing environment.

At the phylum level, Firmicutes and Bacteroidetes are widely recognized as the most abundant phyla in the gut microbiota of ruminants, including yaks (de Menezes et al., 2011; Nie et al., 2017; Zhang et al., 2020b). Results of the current study were consistent with this viewpoint of authors supported the already established data. They play a crucial role in the microbial ecology of ruminant intestines, for Firmicutes can effectively decompose lignin and cellulose, while Bacteroidetes can decompose nonfibrous feed material (Dowd et al., 2008; Shanks et al., 2011). Zhu et al. compared the intestinal microbiota of grazing and house-feeding yaks in Linzhou and found the grazing yaks had decreased level of Firmicutes($P<0.05$) microbiota and relatively increased levels of Bacteroidetes, and a decreased ratio of Firmicutes/Bacteroidetes, which was consistent with results of the current study (Zhu et al., 2022). Artificially prepared food has relatively high protein, polysaccharide, and fat content, therefore house feeding yaks can steadily digest well-grounded, nutritious food. On the contrary, grazing yaks lack balanced feed, especially during the cold seasons around the year. Previous studies have shown a positive correlation between the relatively massive increase of Firmicutes and a highly nutritious diet. Bacteroides can promote decompose polysaccharides and proteins, improve the utilization rate of assimilated nutrients, and lead to improved digestion and absorption of nutrients (Zhang et al.,

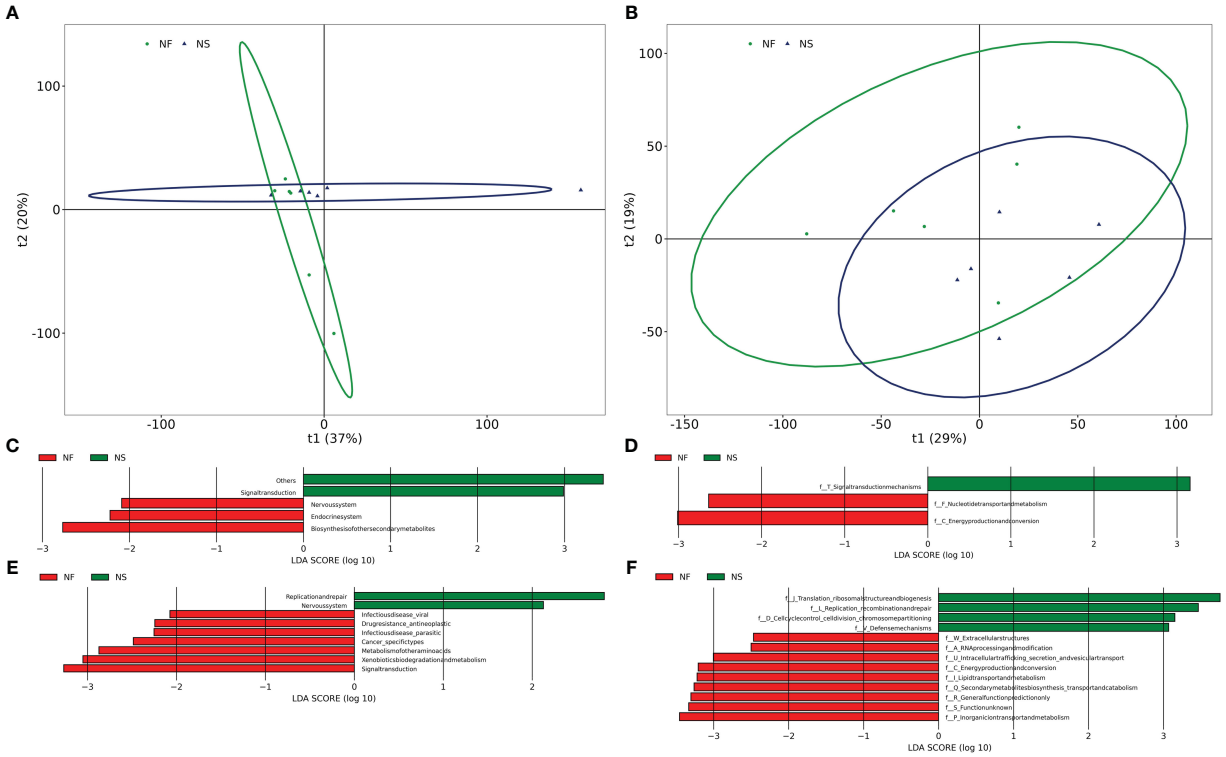

FIGURE 6
Comparative analysis of the function of gut bacterial **(A, C, E)** and fungal **(B, D, F)** between house feeding and captive yaks.

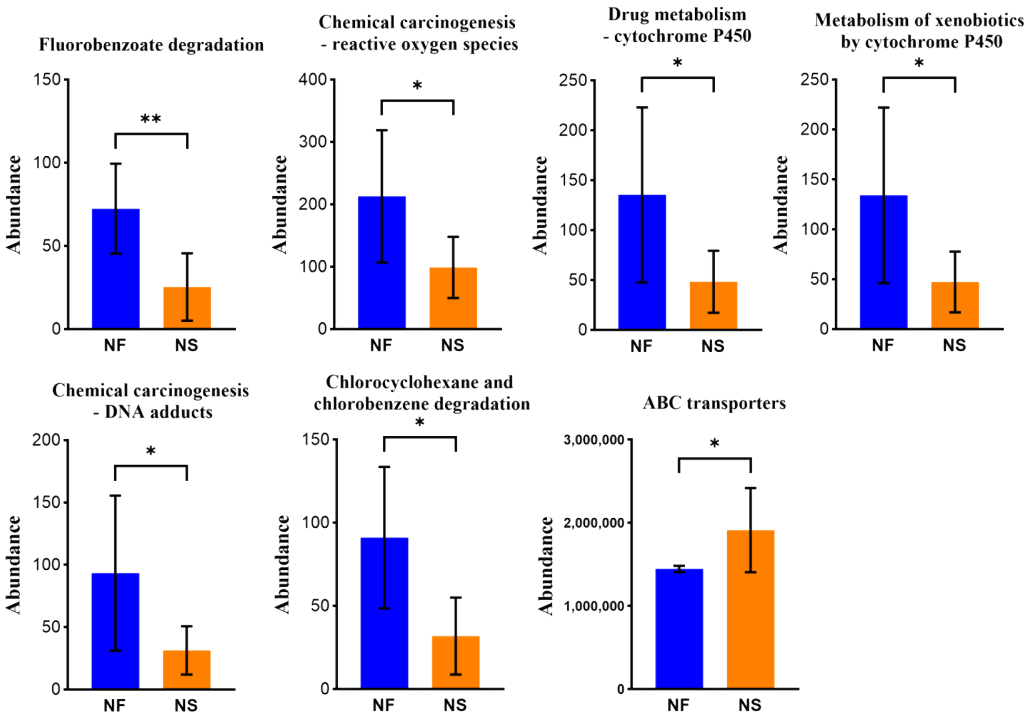

FIGURE 7
Iconic different pathways between two groups in metabolic pathways of KEGG l3. *P<0.05, **P<0.01.

2020b). Therefore, it can be speculated that the enrichment of Firmicutes in the NF group was for better digestion of high-nutrient foods, while the elevation of Bacteroides in the NS group was for better utilization of the scarce food and adaptation to the environment with limited resources.

We further analyzed the differences in cultured bacteria between the two groups and found that 37 attributes showed significant changes. *Lachnospiracea*e sp. was related to the adaptation of high-grain diets (Chen et al., 2011), and higher abundance of Lachnospiraceae_NK3A20_group was found inbeef cattle fed with heated water in winter (He et al., 2023). Furthermore, Lachnospiraceae_NK3A20_group was significantly positively correlated with the rumen propionate and isovalerate (He et al., 2023). In addition, the same genus was associated to the digestion and absorption of proteins in the intestine, as well as the metabolism of glycerophospholipids, and the increased concentration of short-chain fatty acids (SCFAs) such as propionic acid (Liu et al., 2022; Ma et al., 2023). Compared to grazing yaks, house-feeding yaks existed in a warmer environment, with a higher protein and grain content in their diet. Thus increase of *Lachnospiraceae_NK3A20_group* was also a response of the gut microbiota to the feeding environment. Members of *Clostridium:methylpentosum_group* were also linked to the production of SCFAs (Himelbloom and Canale-Parola, 1989). Short chain fatty acids are the main products of intestinal microbes breaking down indigestible carbohydrates, and also generally considered as vital sources of energy to Propionate esters, which have the characteristics of reducing cholesterol, reducing fat storage, anti-cancer, and anti-inflammatory effects. Butyrate is crucial for maintaining the integrity of the intestinal wall and is also beneficial for the nervous system. Acetate helps to maintain intestinal pH to slightly acidic making it suitable for the colonization of beneficial microorganisms, and can also prevent opportunistic pathogenic bacteria from entering and adhering to intestinal epithelium (Lee et al., 2020; Moniri and Farah, 2021). Several genera belonging to *Lachnospiraceae* were found significantly increased in group NF, its members are the main source of short-chain fatty acids production, however, the different taxa of *Lachnospiraceae* were also related to different intestinal and extra-intestinal diseases. *Dorea* sp. can degrade polysaccharides, and oligosaccharides, directly related to glucose metabolism, and metabolizes to produce acetate (Palmnäs-Bédard et al., 2022). *Marvinbryantia* sp. and *Roseburia* sp. produce butyrate (Chen et al., 2021). In addition, genera belonging to other families such as *Faecalibaculum* and *Oscillibacter* increased in group NF, they are also producers of SCFAs. Among them, *Oscillibacter* was found in higher conc. Among the intestines of free-range yaks in Qinghai Province compared to captive feeding yaks (Wen et al., 2022), it was found in agreement with the results of the current studies. SCFAs produced by microorganisms can help resist the invasion of external pathogenic bacteria and alleviate intestinal diseases. Disconcertingly, several genera (*Paenibacillaceae*, *Verrucomicrobiota*, *Akkermansia*, *Ruminococcaceae*, *Marvinbryantia*, etc) which significantly increased in the NF group have been reported to be positively associated with metabolic diseases such as diabetes and obesity, including some SCFAs producers (Palmas et al., 2021; Rondanelli et al., 2021; Deledda et al., 2022). The abundance of Lactobacillus and *Olsenella* relatively decreased in group NF, while *Coriobacteriaceae_* UCG_ 002 relative increased, similar tendencies were also found in the gut microbiota of animals with intestinal diseases such as colitis and inflammatory bowel disease (Zhang et al., 2020a; Zhang et al., 2023). It can be speculated that although the body has made adaptations, the complex environment in pastoral areas still has certain adverse effects on the health status of grazing yaks.

Although research on fungal microbiota is still in its early stages, fungi do have a significant impact on the homeostasis of gut microbiota, they coexist with bacterial microorganisms and greatly expand the gut microbiota that interacts with the intestinal immune system. Mainly, fungal microorganisms are responsible for decomposing lignocellulose in the intestine and achieving this function through the secretion of cell wall degrading enzymes and physical permeation (Wang et al., 2021). The significant enhancement of the phylum Ascomycota and genus Acremonium contributed to the unique gut fungal signature found in the NF group in this study ($P<0.01$). The enrichment of Ascomycota was previously found in yaks infected by *Cryptosporidium parvum* (Lu et al., 2023), grazed yaks are easily exposed to parasitic pathogens in the wild, and the significant increase of Ascomycota may suggest the potential presence of infection in yaks. However, metagenomic analysis was needed to further confirmation.

Differences in multiple metabolic pathways between two groups were found through microbial function prediction. Biosynthesis of other secondary metabolites and catabolism were detected richer in the grazing group. Microbial metabolites have effects on animal gut health and span cardiovascular and nervous systems as well (Thomas et al., 2022). The metabolic pathways related to the utilization of exogenous energy in grazing microorganisms are also prominent, in addition to significantly enriched nucleotide, lipid, and inorganic transport and metabolism. It is said that ruminants can utilize diet or endogenous nonprotein nitrogen (NPN) to furnish protein requirements. The aggregation of these functions by microorganisms also reflects their adaptation to food-deficient environments through efficient feed utilization. In addition, many disease-related metabolic pathways are also enhanced in the grazing group, such as cancer-specific types, infectious disease viral/parasitic, and drug resistance antineoplastic, we believe that living in pastoral areas increases the probability of yaks being exposed to pathogens and parasites. In group NS, the significant enriched defense mechanism and replication and repair were detected, implying that the microbial community of captive yaks has a certain degree of defense and self-repair ability, and a certain degree of conservatism and stability.

In current research, we found subtle significant differences in the gut microbiota of yaks under different feeding conditions, with different feed ingredients shaping the proportion and structure of microorganisms involved in nutrient utilization while dissimilar external environments also contribute to differences in microbial function and metabolism. The ecological environment and dietary structure are important driving factors for intestinal microbial succession, and the gut microbiota will change appropriately under various external stimuli to adapt to the habitat environment, further analysis of the connections between these variations and environmental factors is required.

Conclusion

This study explored the composition and function of the intestinal microbiota in grazed yaks and house-feeding yaks and described the dynamic changes of the gut microbial community. The structure and diversity of the intestinal microflora of yaks were changed and such variation was more pronounced in fungal communities. Besides, we also found that variation in the abundance or function of certain microbe may be related to an adaptation to the external environment, including distinct alternations in some dominant phyla (Firmicutes and Ascomycota). Figuring out the effect of these factors on the intestinal microbial community can help us to understand the interaction mechanism between microbes and the host and the environment, provide a reference for better rearing of yaks, and a novel perspective for the development of prebiotics and microecological agents.

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 below: https://www.ncbi.nlm.nih.gov/, PRJNA1098018.

Ethics statement

This study was guided and approved by the ethics committee of Nanjing Agricultural University (NJAU.No20220305025). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ: Investigation, Writing – original draft. SL: Investigation, Writing – original draft. YC: Formal analysis, Methodology, Writing – original draft. HW: Data curation, Resources, Writing – original draft. KL: Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. WB: Conceptualization, Funding acquisition, Writing – original draft.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1420389/full#supplementary-material

SUPPLEMENTARY TABLE 1
The data of 16S rRNA high-throughput sequencing.

SUPPLEMENTARY TABLE 2
The data of ITS genes high-throughput sequencing.

References

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Chen, Y., Penner, G. B., Li, M., Oba, M., and Guan, L. L. (2011). Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Appl. Environ. Microbiol.* 77, 5770–5781. doi: 10.1128/AEM.00375-11

Chen, Z., Radjabzadeh, D., Chen, L., Kurilshikov, A., Kavousi, M., Ahmadizar, F., et al. (2021). Association of insulin resistance and type 2 diabetes with gut microbial diversity: A microbiome-wide analysis from population studies. *JAMA Netw. Open* 4, e2118811. doi: 10.1001/jamanetworkopen.2021.18811

Deledda, A., Palmas, V., Heidrich, V., Fosci, M., Lombardo, M., Cambarau, G., et al. (2022). Dynamics of gut microbiota and clinical variables after ketogenic and mediterranean diets in drug-naïve patients with type 2 diabetes mellitus and obesity. *Metabolites* 12(11):1092. doi: 10.3390/metabo12111092

de Menezes, A. B., Lewis, E., O'donovan, M., O'neill, B. F., Clipson, N., and Doyle, E. M. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. *FEMS Microbiol. Ecol.* 78, 256–265. doi: 10.1111/j.1574-6941.2011.01151.x

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Dowd, S. E., Callaway, T. R., Wolcott, R. D., Sun, Y., Mckeehan, T., Hagevoort, R. G., et al. (2008). Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol.* 8, 125. doi: 10.1186/1471-2180-8-125

Fan, L., Xia, Y., Wang, Y., Han, D., Liu, Y., Li, J., et al. (2023). Gut microbiota bridges dietary nutrients and host immunity. *Sci. China Life Sci.* 66, 2466–2514. doi: 10.1007/s11427-023-2346-1

He, T., Yi, G., Wang, X., Sun, Y., Li, J., Wu, Z., et al. (2023). Effects of heated drinking water during the cold season on serum biochemistry, ruminal fermentation, bacterial community, and metabolome of beef cattle. *Metabolites* 13(9):995. doi: 10.3390/metabo13090995

Himelbloom, B. H., and Canale-Parola, E. (1989). Clostridium methylpentosum sp. nov.: a ring-shaped intestinal bacterium that ferments only methylpentoses and pentoses. *Arch. Microbiol.* 151, 287–293. doi: 10.1007/BF00406553

Ijoma, G. N., Nkuna, R., Mutungwazi, A., Rashama, C., and Matambo, T. S. (2021). Applying PICRUSt and 16S rRNA functional characterization to predicting co-digestion strategies of various animal manures for biogas production. *Sci. Rep.* 11, 19913. doi: 10.1038/s41598–021-99389–4

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* 45, D353–d361. doi: 10.1093/nar/gkw1092

Kong, Q., Chen, X., Liu, Y., Ali, F., Idrees, A., Ataya, F. S., et al. (2024). Sodium acetate and sodium butyrate attenuate diarrhea in yak calves by regulating gut microbiota and metabolites. *Heliyon* 10, e26564. doi: 10.1016/j.heliyon.2024.e26564

Larabi, A., Barnich, N., and Nguyen, H. T. T. (2020). New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy* 16, 38–51. doi: 10.1080/15548627.2019.1635384

Lee, J., D'aigle, J., Atadja, L., Quaicoe, V., Honarpisheh, P., Ganesh, B. P., et al. (2020). Gut microbiota-derived short-chain fatty acids promote poststroke recovery in aged mice. *Circ. Res.* 127, 453–465. doi: 10.1161/CIRCRESAHA.119.316448

Li, K., Li, Z., Zeng, Z., Li, A., Mehmood, K., Shahzad, M., et al. (2020). Prevalence and molecular characterization of Cryptosporidium spp. in yaks (Bos grunniens) in Naqu, China. *Microb. Pathog.* 144, 104190. doi: 10.1016/j.micpath.2020.104190

Lin, L., Lai, Z., Zhang, J., Zhu, W., and Mao, S. (2023). The gastrointestinal microbiome in dairy cattle is constrained by the deterministic driver of the region and the modified effect of diet. *Microbiome* 11, 10. doi: 10.1186/s40168-022-01453-2

Liu, Y., Liu, C., Wu, H., Meng, Q., and Zhou, Z. (2022). Small intestine microbiome and metabolome of high and low residual feed intake angus heifers. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.862151

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Lu, S., Zou, W., Chen, X., Sun, G., Cidan, Y., Almutairi, M. H., et al. (2023). Effects of Cryptosporidium parvum infection on intestinal fungal microbiota in yaks (Bos grunniens). *Microb. Pathog.* 183, 106322. doi: 10.1016/j.micpath.2023.106322

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., and Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nat. Methods* 10, 999–1002. doi: 10.1038/nmeth.2634

Ma, W., Yuan, M., Chang, S., and Wang, C. (2023). N-carbamylglutamate supplementation regulates hindgut microbiota composition and short-chain fatty acid contents in Charollais and Small Tail Han crossbred sheep. *Front. Vet. Sci.* 10. doi: 10.3389/fvets.2023.1230190

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P., et al. (2020). The firmicutes/bacteroidetes ratio: A relevant marker of gut dysbiosis in obese patients? *Nutrients* 12(5):1474. doi: 10.3390/nu12051474

Mokany, K., Harwood, T. D., Overton, J. M., Barker, G. M., and Ferrier, S. (2011). Combining α- and β-diversity models to fill gaps in our knowledge of biodiversity. *Ecol. Lett.* 14, 1043–1051. doi: 10.1111/ele.2011.14.issue-10

Moniri, N. H., and Farah, Q. (2021). Short-chain free-fatty acid G protein-coupled receptors in colon cancer. *Biochem. Pharmacol.* 186, 114483. doi: 10.1016/j.bcp.2021.114483

Nie, Y., Zhou, Z., Guan, J., Xia, B., Luo, X., Yang, Y., et al. (2017). Dynamic changes of yak (Bos grunniens) gut microbiota during growth revealed by polymerase chain reaction-denaturing gradient gel electrophoresis and metagenomics. *Asian-Australas J. Anim. Sci.* 30, 957–966. doi: 10.5713/ajas.16.0836

Palmas, V., Pisanu, S., Madau, V., Casula, E., Deledda, A., Cusano, R., et al. (2021). Gut microbiota markers associated with obesity and overweight in Italian adults. *Sci. Rep.* 11, 5532. doi: 10.1038/s41598–021-84928-w

Palmnäs-Bédard, M. S. A., Costabile, G., Vetrani, C., Åberg, S., Hjalmarsson, Y., Dicksved, J., et al. (2022). The human gut microbiota and glucose metabolism: a scoping review of key bacteria and the potential role of SCFAs. *Am. J. Clin. Nutr.* 116, 862–874. doi: 10.1093/ajcn/nqac217

Ramos-Romero, S., Santocildes, G., Piñol-Piñol, D., Rosés, C., Pagés, T., Hereu, M., et al. (2020). Implication of gut microbiota in the physiology of rats intermittently exposed to cold and hypobaric hypoxia. *PloS One* 15, e0240686. doi: 10.1371/journal.pone.0240686

Rinninella, E., Cintoni, M., Raoul, P., Lopetuso, L. R., Scaldaferri, F., Pulcini, G., et al. (2019). Food components and dietary habits: keys for a healthy gut microbiota composition. *Nutrients* 11(10):2393. doi: 10.3390/nu11102393

Rondanelli, M., Gasparri, C., Peroni, G., Faliva, M. A., Naso, M., Perna, S., et al. (2021). The potential roles of very low calorie, very low calorie ketogenic diets and very low carbohydrate diets on the gut microbiota composition. *Front. Endocrinol. (Lausanne)* 12. doi: 10.3389/fendo.2021.662591

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Shanks, O. C., Kelty, C. A., Archibeque, S., Jenkins, M., Newton, R. J., Mclellan, S. L., et al. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. *Appl Environ Microbiol.* 77 (9), 2992–3001. doi: 10.1128/AEM.02988–10

Thaiss, C. A., Zmora, N., Levy, M., and Elinav, E. (2016). The microbiome and innate immunity. *Nature* 535, 65–74. doi: 10.1038/nature18847

Thomas, J. P., Modos, D., Rushbrook, S. M., Powell, N., and Korcsmaros, T. (2022). The emerging role of bile acids in the pathogenesis of inflammatory bowel disease. *Front. Immunol.* 13. doi: 10.3389/fimmu.2022.829525

Wang, Y., Fu, Y., He, Y., Kulyar, M. F., Iqbal, M., Li, K., et al. (2021). Longitudinal characterization of the gut bacterial and fungal communities in yaks. *J. Fungi (Basel)* 7 (7):559. doi: 10.3390/jof7070559

Wang, Y., Zhou, R., Yu, Q., Feng, T., and Li, H. (2020). Gut microbiome adaptation to extreme cold winter in wild plateau pika (Ochotona curzoniae) on the Qinghai-Tibet Plateau. *FEMS Microbiol. Lett.* 367, fnaa134. doi: 10.1093/femsle/fnaa134.32840567

Wen, Y., Li, S., Wang, Z., Feng, H., Yao, X., Liu, M., et al. (2022). Intestinal microbial diversity of free-range and captive yak in Qinghai province. *Microorganisms* 10(4):754. doi: 10.3390/microorganisms10040754

Wu, Y., Wan, J., Choe, U., Pham, Q., Schoene, N. W., He, Q., et al. (2019). Interactions between food and gut microbiota: impact on human health. *Annu. Rev. Food Sci. Technol.* 10, 389–408. doi: 10.1146/annurev-food-032818-121303

Xu, C., Liu, W., Sun, B., Zhang, S., Zhang, S., Yang, Y., et al. (2021). Multi-omics analysis reveals a dependent relationship between rumen bacteria and diet of grass- and grain-fed yaks. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.642959

Zeng, Y., Zeng, D., Ni, X. Q., Zhu, H., Jian, P., Zhou, Y., et al. (2017). Microbial community compositions in the gastrointestinal tract of Chinese Mongolian sheep using Illumina MiSeq sequencing revealed high microbial diversity. *Amb Express* 7 (1):75. doi: 10.1186/s13568-017-0378-1

Zhang, Y., Gan, Y., Bao, H., and Wang, R. (2023). Perturbations of gut microbiome and metabolome of pigs infected with Mycoplasma hyorhinis. *J. Sci. Food Agric.* 103, 6219–6232. doi: 10.1002/jsfa.12690

Zhang, X. L., Xu, T. W., Wang, X. G., Geng, Y. Y., Liu, H. J., Hu, L. Y., et al. (2020b). The effect of transitioning between feeding methods on the gut microbiota dynamics of yaks on the qinghai-tibet plateau. *Anim. (Basel)* 10(9):1641. doi: 10.3390/ani10091641

Zhang, W., Zou, G., Li, B., Du, X., Sun, Z., Sun, Y., et al. (2020a). Fecal microbiota transplantation (FMT) alleviates experimental colitis in mice by gut microbiota regulation. *J. Microbiol. Biotechnol.* 30, 1132–1141. doi: 10.4014/jmb.2002.02044

Zhu, Y., Cidan, Y., Sun, G., Li, X., Shahid, M. A., Luosang, Z., et al. (2023). Comparative analysis of gut fungal composition and structure of the yaks under different feeding models. *Front. Vet. Sci.* 10. doi: 10.3389/fvets.2023.1193558

Zhu, Y., Li, X., Lousang, Z., Suolang, Z., Suolang,, Ciyang,, et al. (2022). House feeding pattern increased male yak fertility by improving gut microbiota and serum metabolites. *Front. Vet. Sci.* 9. doi: 10.3389/fvets.2022.989908

Zmora, N., Suez, J., and Elinav, E. (2019). You are what you eat: diet, health and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* 16, 35–56. doi: 10.1038/s41575-018-0061-2

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Interpretation of the effects of rumen acidosis on the gut microbiota and serum metabolites in calves based on 16S rDNA sequencing and non-target metabolomics

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Introduction: Rumen acidosis is one of the most common diseases in beef cattle. It severely affects the normal development of calves and poses a significant threat to the farming industry. However, the influence of rumen acidosis on the gut microbiota and serum metabolites of calves is currently unclear.

Objective: The aim of this study is to investigate the changes in the gut microbiota and serum metabolites in calves after rumen acidosis and analyse the correlation.

Methods: Eight calves were selected as the rumen acidosis group, and eight health calves were selected as the healthy group. The faecal gut microbiota and serum metabolites of calves were detected respectively using 16S rDNA high-throughput sequencing and non-target metabolomics. The correlation between gut microbiota and serum metabolites was analyzed by Spearman correlation analysis.

Results: Differential analysis of the diversity and composition of gut microbiota between eight male healthy (Health) and eight male rumen acidosis (Disease) calves revealed that rumen acidosis increased the abundance of the gut microbiota in calves. At the phylum level, compared to the Healthy group, the relative abundance of Proteobacteria in the Disease group significantly decreased ($P<0.05$), while the relative abundance of Desulfobacterota significantly increased in the Disease group ($P<0.05$). At the genus level, compared to the Disease group, the relative abundance of *Alloprevotella*, *Muribaculaceae*, *Succinivibrio*, *Prevotella*, *Agathobacter* and *Parabacteroides* significantly increased in the Healthy group ($P<0.05$), while the relative abundance of *Christensenellaceae_R-7* and *Monoglobus* significantly decreased in the Healthy group ($P<0.05$). Differential analysis results showed the Healthy group had 23 genera with higher abundance, while the Disease group had 47 genera with higher abundance. Serum metabolomics results

revealed the differential metabolites associated with rumen acidosis, including nicotinamide, niacin, L-glutamic acid and carnosine, were mainly enriched in the nicotinate and nicotinamide pathway and the histidine pathway.

Conclusion: The occurrence of rumen acidosis can induce changes in the gut microbiota of calves, with a significant increase of the *Christensenellaceae_R-7* genus and a significant decrease of *Prevotella* and *Succinivibrio* genera. In addition, the occurrence of rumen acidosis can also induce changes in serum metabolites including niacin, niacinamide, L-glutamine, and carnosine, which may serve as the diagnostic biomarkers of rumen acidosis of calves.

KEYWORDS

calves, rumen acidosis, gut microbiota, serum metabolites, correlation analysis

1 Introduction

Rumen acidosis is an important disease of the digestive system, and it seriously affects the health of cattle (Trevisi et al., 2018). In the clinical practice, it usually presents as the subacute symptoms with the typical signs of anorexia, depression, eyes sunken and muscle tremors. The cattle being severely affected may exhibit recumbency or opisthotonus, groan and excrete yellow-brown watery feces. From the above clinical symptoms, it can be seen that the rumen acidosis can lead to several intestinal diseases. The gut microbiota is closely related to the healthy status, nutrient metabolism, immune function, and the onset and development of disease (Niederwerder, 2018). The gut microbiota is involved in the regulation of various metabolic pathways in the host, forming interactions such as the host-microbe metabolic axis, the host-microbe signalling axis and the immune-inflammatory axis, which are closely related to the multiple organs in the body, including the gut, liver, muscle and brain (Welch et al., 2022). For example, when high-concentrate feeding is used to induce rumen acidosis, a large amount of undigested feed enters the hindgut, leading to fermentation in the hindgut and lowering the pH of the feces to around 6.42 (Gressley et al., 2011). High-throughput sequencing of the V1-V3 variable regions in bacteria revealed significant changes in the microbial community in the feces. Rumen acidosis causes large amounts of rumen fermentable substrates to enter the hindgut, producing large amounts of volatile fatty acids and lactic acid, which damage the intestinal epithelial cells. By altering the structure of the microbial community in the hindgut, diarrhea is induced, severely affecting the animal health and production (Mao et al., 2012). Rumen acidosis can activate the innate immune response in cattle, increasing the levels of serum amyloid A, lipopolysaccharide-binding protein and haptoglobin (Khafipour et al., 2009). This activation of the systemic immune response can be used as a diagnostic marker for rumen acidosis and also indicate the association between rumen acidosis and systemic health disorders (Zebeli and Metzler-Zebeli, 2012). In addition, further research has shown that rumen acidosis could also cause metabolic diseases such as ketosis and hyperlactatemia in dairy cows (Aditya et al., 2018; Tang et al., 2024). Currently, there is relatively little research on the changes in the fecal microbiota in calves with rumen acidosis. To analyze the changes in the gut microbiota structure and serum metabolites of rumen acidosis in calves, the microbial 16S rDNA V3-V4 region sequences in the feces and the serum metabolites of the healthy and rumen acidosis in calves were determined used the Illumina Miseq sequencing platform and LC-MS/MS in this study. To further elucidate the pathogenesis of rumen acidosis of calves, community structure diversity, differential metabolites screening, metabolic pathway analysis and the correlation analysis were performed.

2 Materials and methods

2.1 Main instruments and reagents

Ultra-performance liquid chromatograph (Waters 2D UPLC, Waters, USA); high-resolution mass spectrometer (Q-Exactive, Thermo Fisher Scientific, USA); chromatographic column: ACQUITY UPLC BEH C18 (1.7 μm, 2.1*100 mm, Waters, USA); low-temperature high-speed centrifuge (Centrifuge 5430, Eppendorf); vortex mixer (QL-901, Qilinbell Instrument Manufacturing Co., Ltd., China); water purification system (Milli-Q Integral, Millipore Corporation, USA); freeze vacuum concentrator (Maxi Vacbeta, GENE COMPANY); internal standards: L-leucine-d3, L-phenylalanine (13C-9.99%), L-tryptophan-d5, progesterone-2,3,4-13C3; methanol (A454-4) and acetonitrile (A996-4) were MS grade (Thermo Fisher Scientific, USA); formic acid ammonia (17843-250G, Honeywell Fluka, USA), formic acid (50144-50 mL, DIMKA, USA) and the water provided by the water purification system.

2.2 Experimental animals

The animals were provided by Zhangye Wanhe Grass Livestock Industry Science and Technology Development Co., Ltd. According to the performance and clinical signs (Oetzel, 2017), eight male calves were selected as the rumen acidosis group (Disease), and eight male healthy calves (with the similar age and weight to the rumen acidosis group) were selected as the healthy group (Health). Calves in the two groups were reared in the same feeding environment. The feed was supplied by Zhangye Wanhe Grass Livestock Industry Science and Technology Development Co., Ltd. The concentrated feed consists of corn, corn germ meal, soybean meal, distillers grains, baking soda and premix (Table 1). Written informed consent was obtained from the owners for the participation of their animals in this study.

2.3 Sample collection

2.3.1 Feces sample collection

5 g feces samples were collected from each calf in the healthy group and the rumen acidosis group. Then the samples were placed in cryovials, and then immediately freezed in liquid nitrogen for the subsequent analysis.

2.3.2 Serum sample collection

Blood samples were collected from the jugular vein of calves in the healthy and rumen acidosis groups. Then the serum was separated (centrifuged at 3500 rpm for 15 min) and stored at -80°C.

2.4 Metabolite extraction

The samples were thawed slowly at 4°C. Then, 100 μL of the sample was transferred to a 96-well plate and 300 μL of pre-cooled extraction solution (methanol:acetonitrile=2:1, v:v) along with 10 μL of internal standard were added. The mixture was made vortex for 1 min, and then centrifuged at 4°C, 4000 rpm for 20 min. After centrifugation, 300 μL of the supernatant was transferred to a freeze vacuum concentrator and dried. 150 μL complex solution (methanol: H_2O=1:1, v:v) was added for re-dissolution. The solution was made vortex for 1 min, then centrifuged at 4°and 4000 rpm for 30 min. After that, the supernatant was transferred to the sample vials. To ensure the quality control, 10 μL of the supernatant from each sample was mixed to create the QC samples. These QC samples were used to evaluate the repeatability and stability of the LC-MS/MS analysis process.

TABLE 1 The composition of the basal diet.

Composition	Content(%)
Corn	52.00
Corn germ meal	12.00
Soybean meal	23.00
Distillers grains	8.00
Baking soda	1.00
Premix	4.00

2.5 LC-MS/MS analysis

Waters 2D UPLC (Waters, USA) conjunction with the Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA) were utilized for metabolite separation and detection.

2.5.1 Chromatographic conditions

BEH C18 column (1.7 μm, 2.1*100 mm, Waters, USA) was used. In the positive ion mode, the mobile phase consisted of 0.1% formic acid in aqueous solution (A) and 0.1% formic acid in methanol (B). In the negative ion mode, the mobile phase consisted of 10 mM formic acid ammonium solution (A) and 10 mM formic acid ammonium solution in 95% methanol (B). The gradient elution conditions were as follows: 0-1 min, 2% B; 1-9 min, 2%-98% B; 9-12 min, 98% B; 12-12.1 min, 98% B-2% B; 12.1-15 min, 2% B. The flow rate was 0.35 mL/min, the column temperature was 45°C, and the injection volume was 5 μL.

2.5.2 Mass spectrometry conditions

Q-Exactive mass spectrometer was used for the primary and secondary mass spectrometry data acquisition. The mass scanning range was 70 - 1050 m/z, with a primary resolution of 70,000, AGC of $3e^6$, and the maximum injection time of 100 ms. The top 3 ions were selected for fragmentation based on precursor ion intensity, and secondary information was collected. The secondary resolution was set at 17,500, AGC was set at $1e^5$, and the maximum injection time was set at 50 ms. The fragmentation energy was set at 20, 40, and 60 eV. The ion source (ESI) parameters were set as follows: sheath gas flow rate at 40, auxiliary gas flow rate at 10, spray voltage at 3.80 KV in the positive ion mode and 3.20 KV in the negative ion mode, ion transfer tube temperature at 320°C, and auxiliary gas heater temperature at 350°C.

2.6 OTUs clustering result statistics

The software USEARCH (v7.0.1090) was used to cluster the assembled Tags into OTUs. The main process is as follows:

a. UPARSE was used to cluster at 97% similarity, obtaining representative sequences of OTUs.
b. UCHIME (v4.2.40) was used to remove chimeras from the representative sequences of OTUs. For 16S and ITS, pre-existing chimera databases were used for comparison and removal. The chimera databases used were gold database (v20110519) for 16S and UNITE (v20140703) for ITS, selected based on sequencing regions.
c. The usearch global method was used to align all Tags to the representative sequences of OTUs, obtaining the OTUs abundance table for each sample.

The DADA2 (Divisive Amplicon Denoising Algorithm) method in the software QIIME2 was used to denoise the data and obtain Amplicon Sequence Variants (ASVs), which were sequences with 100% similarity. Then, a feature table (Feature, a general term for ASV/ASV, etc.) was obtained. The main process was as follows:

a. Qiime tools were used to import filtered paired-end sequences.
b. Qiime DADA2 denoise-paired command was used to build the feature table based on the DADA2 method.
c. Qiime tools were used to export to convert the feature table into a format that can be directly viewed.

2.7 Data processing

The data were expressed as "mean ± standard deviation" and the statistical analysis was performed by GraphPad Prism 7.0. $P<0.05$ was considered as statistically significant.

3 Results

3.1 Effects of rumen acidosis in calves on the gut microbiota

3.1.1 Effects of rumen acidosis in calves on the OTUs of the gut microbiota

The healthy group (Health) had 1139 operational taxonomic units (OTUs), while the rumen acidosis group (Disease) had 1319 OTUs, with 1009 OTUs shared between the two groups (Figure 1A). The PLS-DA analysis results clearly demonstrate a separation between the two groups (Figure 1B).

3.1.2 Effects of rumen acidosis in calves on the gut microbiota diversity and richness

The analysis results of alpha diversity clearly demonstrated that there were differences in the richness of gut microbiota between the two groups. The rumen acidosis group (Disease) had significantly higher sobs, chao, and ace indices than the healthy group (Health) ($P<0.05$) (Figure 2). It strongly suggested that the rumen acidosis had a direct impact on the richness of the gut microbiota in the calves.

3.1.3 Effects of rumen acidosis on the composition of the gut microbiota in the calves

The gut microbiota of calves was primarily composed of Firmicutes, Bacteroidota, Spirochaetota, Proteobacteria, Cyanobacteria, Verrucomicrobiota, Desulfobacterota, Patescibacteria, Actinobacteriota, and Elusimicrobiota, with Firmicutes and Bacteroidota being the dominant phyla. The results indicated that the relative abundance of Proteobacteria was significantly lower in the rumen acidosis group (Disease) compared to the healthy group (Health) ($P<0.05$). Moreover, the rumen acidosis group (Disease) showed a significantly higher relative abundance of Desulfobacterota than the healthy group (Health) ($P<0.05$) (Figure 3A).

At the genus level, twenty-eight dominant bacterial genera were identified, including *Alloprevotella*, *Muribaculaceae*, *Succinivibrio*, *Lachnospiraceae_NK4A136*, *Treponema*, *Bacteroides*, *Prevotella*, *Agathobacter*, *Clostridia_UCG-014*, *Clostridium*, *Alistipes*, *Anaerosporobacter*, *Prevotellaceae_NK3B31*, *Phascolarctobacterium*, *Parabacteroides*, *Eubacterium coprostanoligenes*, *Lachnospiraceae UCG-007*, *Christensenellaceae R-7*, *Oscillibacter*, *Ruminococcus torques*, *Roseburia*, *Gastranaerophilales*, *Prevotellaceae UCG-003*, *Clostridia vadinBB60*, *Monoglobus*, *Bacteroidales RF16*, *Akkermansia*, and *Prevotellaceae UCG-004*. The results indicated that the healthy group (Health) had a significantly higher relative abundance of *Alloprevotella*, *Muribaculaceae*, *Succinivibrio*, *Prevotella*, *Agathobacter*, and *Parabacteroides* compared to the rumen acidosis group (Disease) ($P<0.05$). Furthermore, the healthy group (Health) had a significantly lower relative abundance of *Christensenellaceae_R-7* and *Monoglobus* ($P<0.05$) (Figure 3B). When setting a predefined LDA value of >2.4, the results revealed that the healthy group (Health) had 23 genera with higher abundance, whereas the rumen acidosis group (Disease) had 47 genera with higher abundance at different taxonomic levels. This is demonstrated in Figure 4.

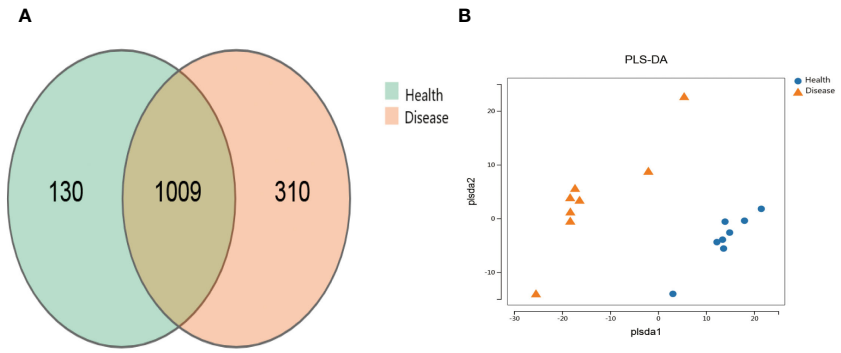

FIGURE 1
Effect on the distribution of gut microbiota OTUs of rumen acidosis in calves. Health, the healthy group; Disease, the rumen acidosis group. **(A)** represents the OTUs of the gut microbiota of the healthy group and the rumen acidosis group. **(B)** represents the PLS-DA analysis results of the healthy group and the rumen acidosis group.

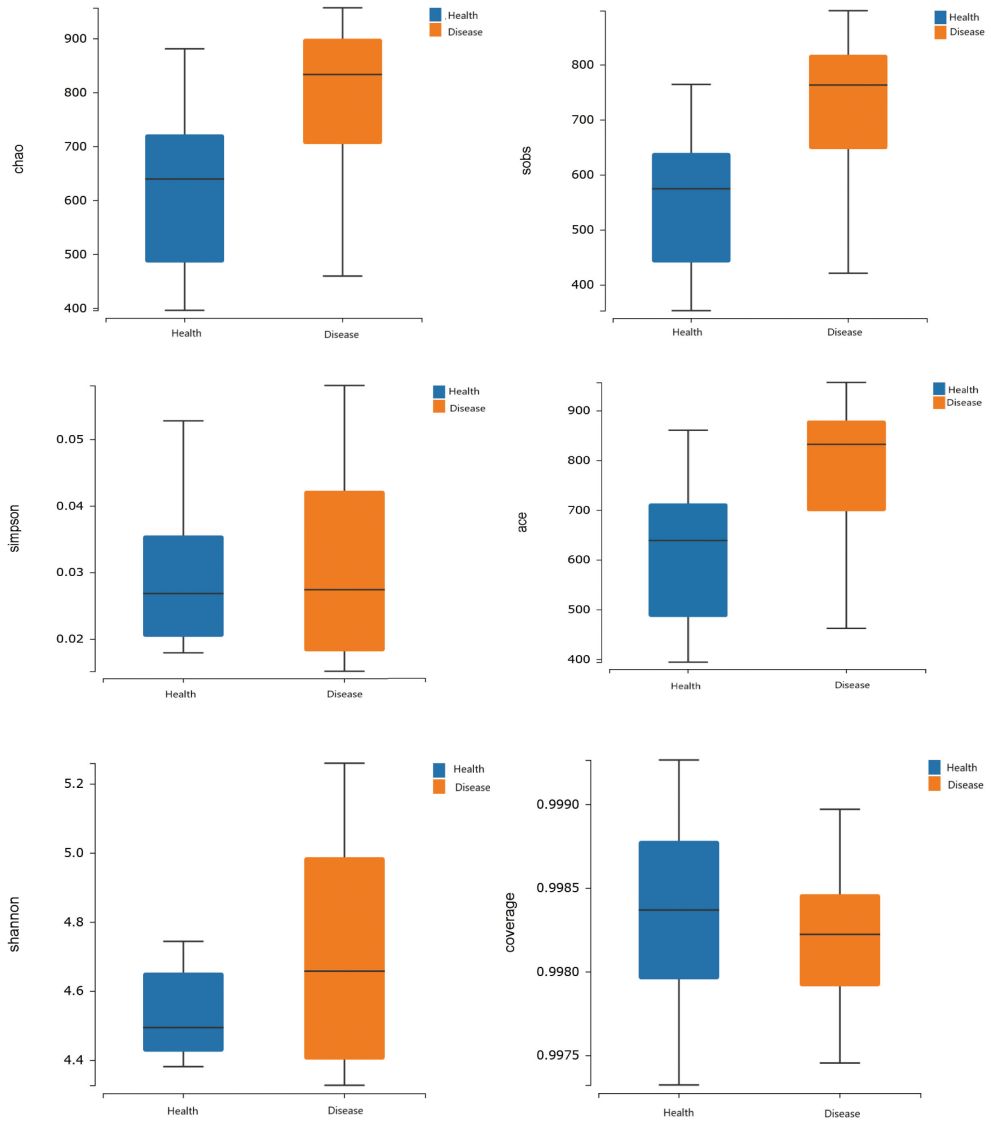

FIGURE 2
Effects of rumen acidosis on the α diversity of the gut microbiota in calves.

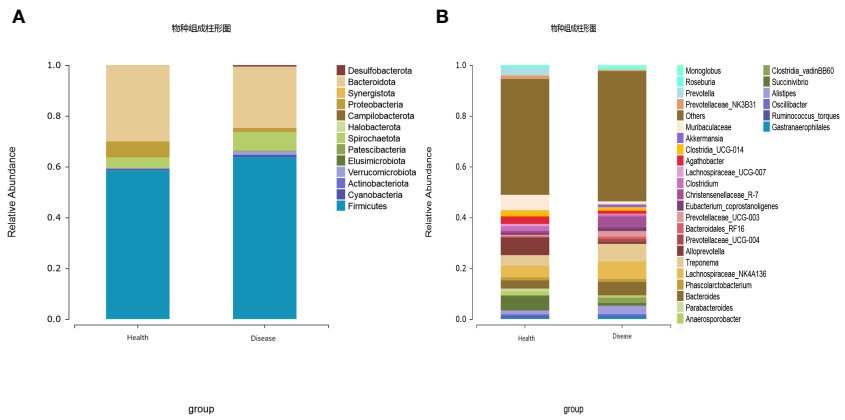

FIGURE 3
Effect of rumen acidosis on the gut microbiota of calves **(A)** represents phylum level; **(B)** represents genus level.

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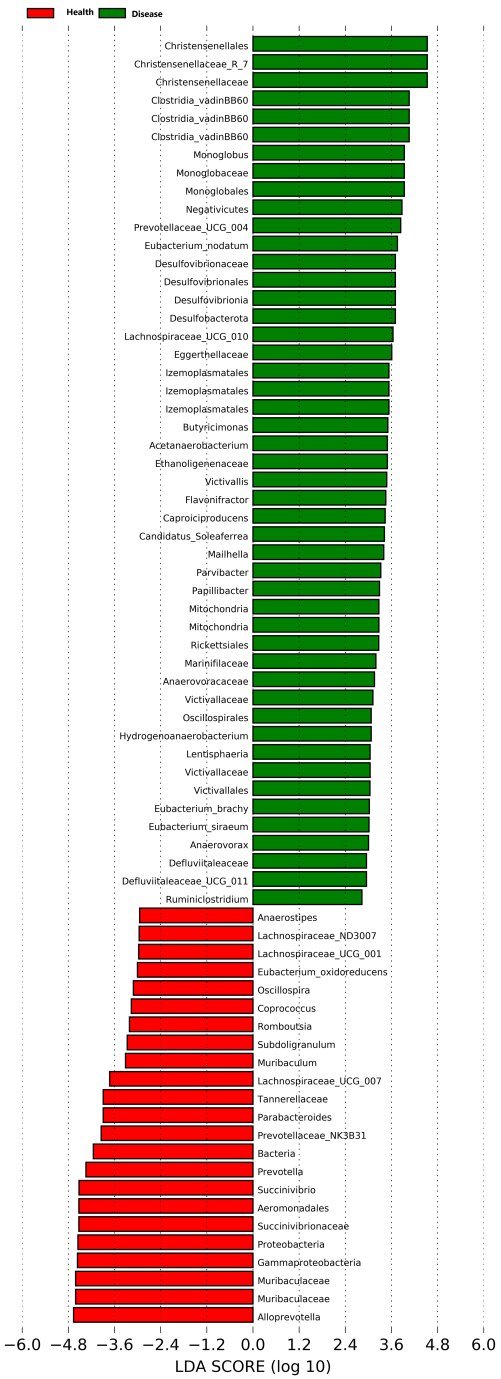

FIGURE 4
LDA analysis of rumen acidosis on the gut microbiota of calves.

3.2 Effects of rumen acidosis in calves on the serum metabolites

3.2.1 Instrument stability and total ion chromatograms in the positive and negative ion modes

The total ion chromatograms of the six QC samples were consistent and reliable. The excellent stability of the instrument in both positive and negative ion modes was demonstrated in Figures 5A, B, and the reproducibility of the metabolomics method based on LC-MS/MS was evidenced in this experiment. The base peak chromatograms (BPC) of all the QC samples overlapped well, with only minor fluctuations in retention time and peak response intensity. This confirmed that the instrument was in a good condition and the signal stability was maintained throughout the sample analysis process.

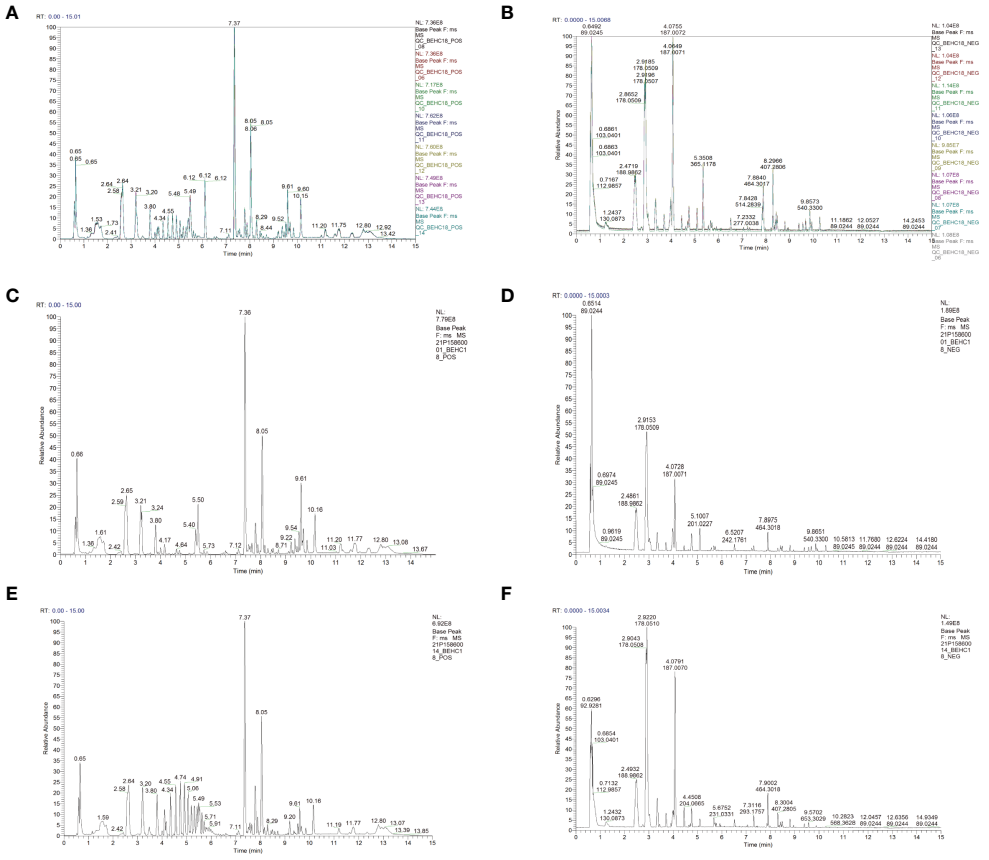

FIGURE 5
The instrumental stability and total ion current chromatogram of calves serum LC-MS/MS in the positive and negative ion modes **(A)** (positive ion); **(B)** (negative ion); **(C)** Health (positive ion); **(D)** Health (negative ion); **(E)** Disease (positive ion); **(F)** Disease (negative ion).

3.2.2 PCA analysis

PCA analysis results clearly demonstrated a distinct separation between the health and disease groups in terms of the metabolites detected in positive and negative ion modes. These findings strongly suggested that the rumen acidosis severely disrupted the normal physiological metabolism of calves, resulting in the significant changes in endogenous physiological metabolites (Figures 6A, B). In both positive and negative ion modes, the PCA score charts visually reflected the contribution of all metabolites to the intergroup differences. The outliers that were further away from the other metabolites had a greater contribution to the intergroup differences and were more likely to be the differential metabolites of rumen acidosis in calves, as shown in Figures 6C, D. The data confidently demonstrated a greater number of metabolites were detected in the negative ion mode.

3.2.3 PLS-DA analysis of serum metabolites in the positive and negative ion modes

The positive and negative ion modes were used to conduct PLS-DA analysis on the serum metabolites of the healthy and disease groups. The sample points of the two groups were distinctly separated in both ion modes, and each group's samples showed a tendency to be closer to their respective groups to varying degrees. These findings strongly suggested that rumen acidosis significantly impacted the normal metabolism of calves serum (Figures 7A, B). The PLS-DA model's effectiveness was evaluated using R^2Y and Q^2. The study demonstrated that the PLS-DA score plot performed well and had high predictive ability, as evidenced by R^2Y and Q^2 values of 0.85 and 0.81, respectively. Additionally, the permutation test indicated that the model was not overfitting, with a Q^2 intercept value less than 0 (Figures 7C, D).

3.2.4 Screening of differential metabolites

The differential metabolites are shown in Table 2 (in the Supplementary materials). A total of 139 differential metabolites were identified in the serum of calves in the positive ion mode using the PLS-DA model (VIP>1, Ratio>1.2 or Ratio<0.83, $P<0.05$), with 59 upregulated and 80 downregulated metabolites. In the negative ion mode, 39 differential metabolites were identified in the serum of calves, including 30 upregulated and 9 downregulated metabolites.

3.2.5 Metabolic pathway enrichment analysis

The metabolite pathways with $P<0.05$ were confidently selected as the most relevant to rumen acidosis in calves. In the positive ion mode, three metabolic pathways were decisively chosen, including Nicotinate and nicotinamide metabolism, Aldosterone-regulated sodium reabsorption, and Cortisol synthesis and secretion. 27 metabolic pathways were confidently identified in the negative

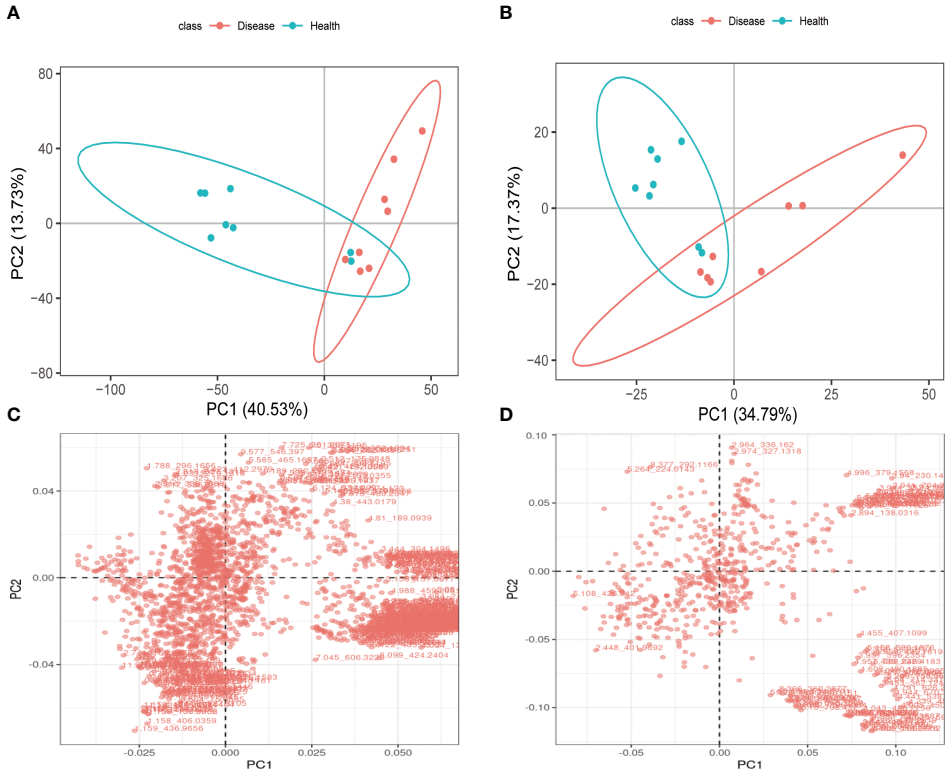

FIGURE 6
The PCA analysis and differential metabolites in each group of calves serum under LC-MS/MS positive ion **(A, C)** and negative ion **(B, D)** models.

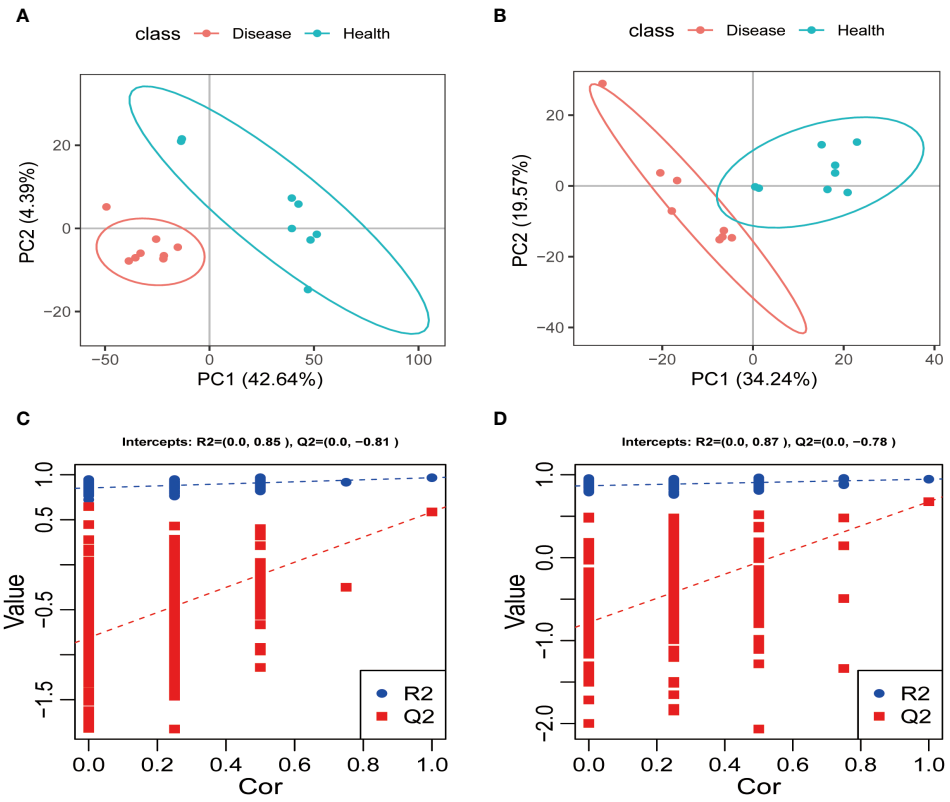

FIGURE 7
The PLS-DA analysis and scores of each group in the positive **(A, C)** and negative ion **(B, D)** models.

ion mode, including Arginine and Proline metabolism, Glyoxylate and Dicarboxylate metabolism, Neuroactive Ligand-Receptor Interaction, Aminoacyl-tRNA Biosynthesis, Protein Digestion and Absorption, Histidine metabolism, Butanoate metabolism, Glutathione metabolism, Taste Transduction, Beta-Alanine metabolism, Alanine, Aspartate and Glutamate metabolism, Ferroptosis, Arginine biosynthesis, Taurine and hypotaurine metabolism, Retrograde endocannabinoid signaling, Nitrogen metabolism, Proximal tubule bicarbonate reclamation, D-Glutamine and D-glutamate metabolism, Synaptic vesicle cycle, Gap junction, Phospholipase D signaling pathway, Long-term depression, Circadian entrainment, GABAergic synapse, Glutamatergic synapse, Long-term potentiation, and FoxO signaling pathway (Figure 8).

3.3 Correlation analysis between gut microbiota and serum metabolites

The spearman correlation analysis showed a negative correlation (r=0.89; *P<0.01*) between the relative concentration of (3β, 4β, 15α, 16β, 25s)-4, 8, 15, 16, 26-pentahydroxycholest-5-en-3-yl β-d-xylopyranoside and the *Alloprevotella* genus, and a positive correlation (r=0.90; *P<0.01*) with the *Anaerovorax* genus. The concentration of 2-aminoethyl (2r)-2-hydroxy-3-[(1z,12z)-1,12-nonadecadien-1-yloxy] propyl hydrogen phosphate was found to be negatively correlated with the presence of the *Christensenellaceae_R-7* genus (r=-0.94, *P<0.01*). Similarly, the concentration of Oligomycin a was also found to be negatively correlated with the presence of the *Christensenellaceae_R-7* genus (r=-0.92, *P<0.01*). The concentration of (3β,4β,15α,16β,25s)-4,8,15,16,26-pentahydroxycholest-5-en-3-yl β-d-xylopyranoside showed a positive correlation with the *Christensenellaceae_R-7* genus (r = 0.92, *P < 0.01*), while the concentration of Gemfibrozil exhibited a negative correlation with the same genus (r=-0.89, *P < 0.01*). The concentration of N - [(2s,3r,4e,6e)-1,3-dihydroxy-4,6-tetradecadien-2-yl] icosanamide showed a negative correlation with the presence of the *Christensenellaceae_R-7* genus (r=-0.89, *P < 0.01*).The concentration of (3β, 4β, 15α, 16β, 25s) - 4, 8, 15, 16, 26 - pentahydroxycholest-5-en-3-yl β-d-xylopyranoside showed a negative correlation with the presence of the *Coprococcus* genus (r=-0.93, *P<0.01*). Conversely, the concentration of Gemfibrozil showed a positive correlation with the presence of the *Coprococcus* genus (r=0.93, *P<0.01*). Furthermore, the concentration of Ricinelaidic acid exhibited a negative correlation with the presence of the *Izemoplasmatales* genus (r=-0.94, *P<0.01*). The negative correlation between the concentration of Oligomycin a and the *Izemoplasmatales* genus (r=-0.93, *P<0.01*), as well as the concentration of Sl3675000 and O-(hydroxy{(2r)-2-hydroxy-3-[(2-methoxyicosyl)oxy]propoxy}phosphoryl)-l-serine with the *Izemoplasmatales* genus (r=-0.90, *P<0.01*), demonstrates a clear relationship between these variables. Conversely, the concentration of Gemfibrozil and N-acetyl-l-alanine exhibited significant positive correlation with the *Lachnospiraceae_ND3007* genus (r=0.90 and 0.89, *P<0.01*) respectively. The concentration of 5,8-dihydro-6-(4-methyl-3-penten-1-yl)-1,2,3,4-tetration was strongly correlated with the presence of the *Lachnospiraceae_UCG_007* genus (r=0.90, *P<0.01*). Similarly, the concentration of (3β,4β,15α,16β,25s) - 4,8,15,16,26-pentahydroxycholest-5-en-3-yl β- d - xylopyranoside was highly correlated with the presence of the *Mailhella* genus (r=0.96, *P<0.01*). The concentration of Gemfibrozil and N-acetyl-l-alanine showed negative correlation with the *Mailhella* genus (r=-0.92

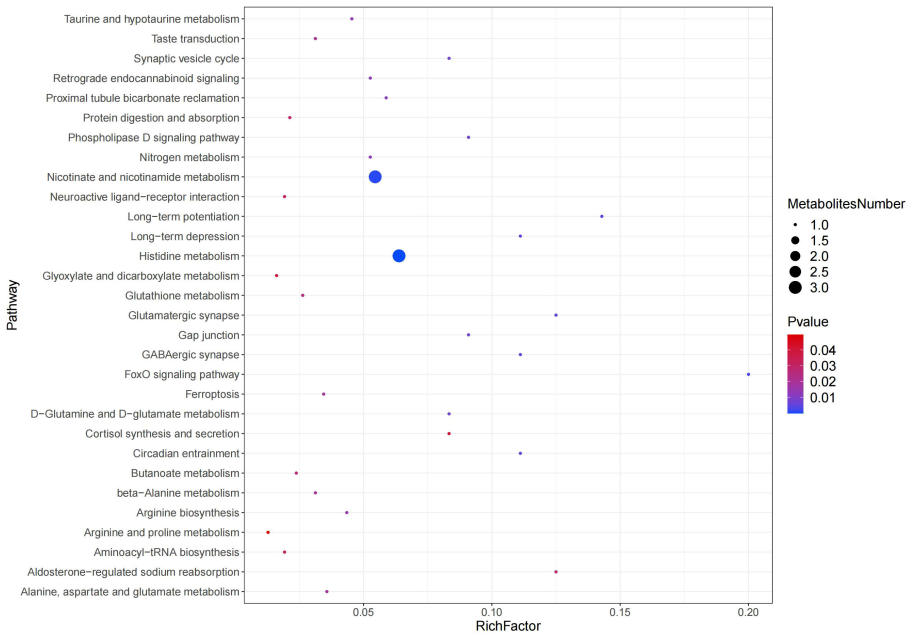

FIGURE 8
Pathway enrichment analysis results in the positive and negative ion modes.

and -0.89, *P<0.01*) respectively. Epelsiban concentration was also negatively correlated with the *Paeniclostridium* genus (r= -0.89, *P<0.01*) (Figure 9).

4 Discussion

4.1 Effect of rumen acidosis on the gut microbiota in calves

Under normal physiological conditions, the gut is maintained in a relatively stable state by the close attachment of physiologically dominant anaerobic bacteria to the intestinal mucosa, forming a gut barrier (Chen et al., 2022). However, when calves experience rumen acidosis, the fermentation pattern in the abomasum changes, thereby affecting the stability and diversity of the rumen microbiota (Faniyi et al., 2019).

Alpha diversity analysis of the gut microbiota revealed a sample coverage index of over 99%, indicating that the sample size was sufficient to accurately reflect the types and diversity of gut microbiota in calves with rumen acidosis. In addition, the diversity index (Sob index) and abundance index (Chao index and ACE index) in the rumen acidosis group were significantly higher than those in the healthy group, indicating an increased proportion of harmful bacterial genera or the proliferation of other harmful bacteria in the calves gut due to rumen acidosis, leading to increase the diversity of gut microbiota. This finding was consistent with the description of J. C. Plaizier et al (Plaizier et al., 2018). In this study, a total of 10 phyla and 28 dominant bacterial genera were detected in the rumen acidosis group. At the phylum level, the dominant phyla in both the rumen acidosis group and the healthy group were Firmicutes and Bacteroidetes. The Proteobacteria phylum was significantly lower in calves with rumen acidosis compared to the healthy group, while the Desulfobacterota phylum was significantly higher. The relative abundance of Firmicutes showed an increasing trend in the rumen acidosis group, while the relative abundance of Bacteroidetes showed a decreasing trend. This finding contradicted the results of a study conducted by K. Wen et al., indicating that rumen acidosis significantly reduced the abundance of Proteobacteria, one of its signature phyla, and greatly disrupted the diversity of normal gut microbiota (Wen et al., 2021). Furthermore, the study also demonstrated that subacute rumen acidosis increased the relative abundance of Firmicutes in calves, which was consistent with the findings of the aforementioned study (Fu et al., 2022). Another study showed that rumen acidosis increased the relative abundance of Firmicutes and decreased the relative abundance of Bacteroidetes (Nagata et al., 2018). These studies indicated that the rumen acidosis indeed altered the normal gut microbiota in calves.

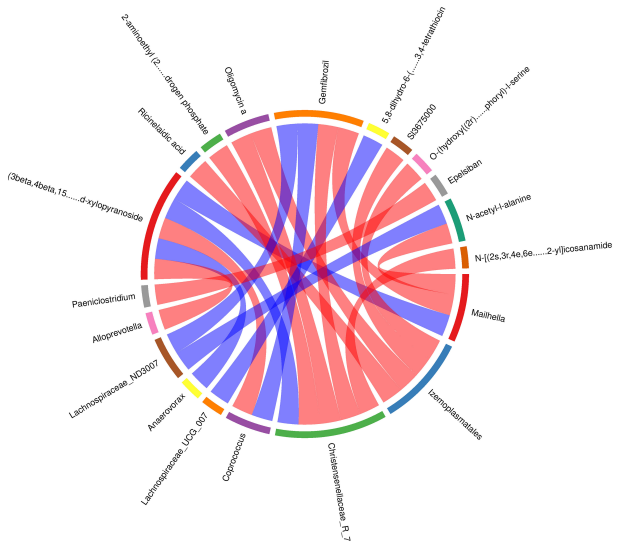

FIGURE 9
Correlation analysis of gut microbiota and metabolites in calves with rumen acidosis.

At the genus level, there were significant differences in the composition and proportion of gut microbial genera between the two groups. *Prevotella* and *Succinivibrio* were dominant genera (Li et al., 2022). In this study, the abundance and relative abundance of *Prevotella* (Ji et al., 2023), *Muribaculaceae*, *Succinivibrio* (Connors et al., 2018), *Prevotella* (Lourenco et al., 2020), *Agathobacter*, and *Parabacteroides* (Zafar and Saier, 2021) were significantly lower in the rumen acidosis group compared to the healthy group of calves, while the abundance and relative abundance of *Christensenellaceae_R-7* and *Parabacteroides* were significantly higher. *Prevotella* is a protein-degrading bacterium in the rumen and intestinal mucosa of ruminants, mainly degrading the semi-fiber components of food and promoting the degradation of non-fiber polysaccharides and pectin (Pang et al., 2022). Rumen acidosis leads to the change in the gut microbiota in calves, resulting in the decreased feed efficiency. *Succinivibrio* is a dominant genus in the Proteobacteria phylum (Tapio et al., 2017), which produces succinic acid through hydrogen utilization and plays an important role. Not only does this compete with hydrogenotrophic methanogens for substrates, but succinate is a precursor for propionate production (McCabe et al., 2015; Bailoni et al., 2021), and most propionate in ruminants is produced via the succinate pathway. Ramayo-Caldas et al. found that *Succinivibrionaceae* could improve feed efficiency, reduced methane emission, and increased the propionate concentration in Holstein cows (Ramayo-Caldas et al., 2020). *Christensenellaceae_R-7* belongs to the Firmicutes phylum (Waters and Ley, 2019). Combined with the analysis at the phylum level, it could be inferred that the relative abundance of the Firmicutes phylum in the gut microbiota of calves with rumen acidosis showed an increasing trend, further indicating that rumen acidosis in calves altered the abundance of gut microbiota. LDA analysis results of the gut microbiota in calves with rumen acidosis revealed that it could affect the normal gut microbiota community in calves, promote the rapid growth and proliferation of harmful bacterial communities and inhibit the proliferation of beneficial bacterial communities, result in an imbalance of the normal microbial flora.

4.2 Effect of rumen acidosis of calves on serum metabolites

Non-targeted metabolomics is a high-throughput data-driven approach widely used in the study of inflammation-related

metabolic markers and pathways, providing a theoretical basis for the diagnosis and appropriate treatment of inflammation-related diseases. In this study, the non-targeted metabolomics based on LC-MS/MS platform combined with multivariate data statistical analysis methods were used to analyze the serum of calves in the rumen acidosis and healthy groups. The study found that the differential metabolites associated with rumen acidosis of calves mainly included niacin, nicotinic acid, L-glutamine and carnosine. These differential metabolites were mainly enriched in the niacin and nicotinic acid metabolism pathway and the histidine metabolism pathway. Niacin is the main precursor of nicotinamide adenine dinucleotide (NAD^+) and its involvement in the metabolic pathway affects the synthesis of related metabolites. Nicotinic acid, also known as vitamin B3, is converted to niacin through transamination in the body, and niacin is the main precursor of NAD^+ (Penberthy, 2009; Zhai et al., 2009). In the synthesis of NAD^+, nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in this reaction pathway. NAMPT catalyzes the transfer of the phosphoribosyl group from 5'-phosphoribosyl-1'-pyrophosphate to nicotinamide mononucleotide (NMN) and pyrophosphate. NMN is converted to NAD^+ under the action of nicotinamide adenine dinucleotide transferase (Luk et al., 2008; Sampath et al., 2015). NAD+ acts as an activator of SIRT1 and activates SIRT1 by regulating the ratio of NAD^+/NADH. SIRT1 is a positive regulator of NF-κB, which is considered as an important transcription factor involved in the production of pro-inflammatory cytokines (Imai, 2009; Matsushita et al., 2013; Revollo et al., 2007; Zha et al., 2023). The study by Yu Ma et al. have found that regulating niacin and nicotinic acid metabolism could achieve anti-inflammatory effects (Ma et al., 2016). Zhou et al. found that disruption of niacin and nicotinamide metabolism could lead to a series of adverse reactions caused by inflammation and oxidative stress induced by hypertension (Zhou et al., 2023). This study found that the levels of niacin and nicotinamide metabolites in the serum of calves with rumen acidosis were significantly lower than those in the healthy group, leading to a decrease in the biosynthesis of NAD^+. The reduction in NAD^+ synthesis inhibits the activation of SIRT1, thereby reducing the inhibition of the NF-κB pathway, leading to the release of inflammatory factors and ultimately rumen acidosis. In conclusion, niacin and nicotinamide inhibit the occurrence of inflammation in calves by regulating niacin and nicotinamide metabolism.

Carnosine is a dipeptide molecule (β-alanyl-L-histidine) with anti-inflammatory, antioxidant, anti-glycation, and chelating properties (Prakash et al., 2021). Zheng et al. found that the presence of carnosine could increase the secretion of IL-10, GM-CSF, and TNF-α in the body while reduce the secretion of IL-8 (Zheng et al., 1996). In addition, carnosine has the antioxidant and anti-aging properties, including better maintenance of muscle strength and pH buffering properties, playing an important role in the stability and anti-fatigue (Guiotto et al., 2005). According to previous studies, the metabolism of alanine, aspartic acid, and glutamic acid is related to carnosine metabolism and is regulated by carnosine metabolism (Sookoian and Pirola, 2012). In addition, certain metabolic processes such as pyruvate metabolism, β-alanine metabolism, histidine metabolism, pantothenic acid and coenzyme A biosynthesis are also related to carnosine metabolism (Ostfeld and Hoffman, 2023). This study found that the level of carnosine in the serum of calves with rumen acidosis was significantly lower than that in the normal group, indicating that rumen acidosis affected the normal amino acid metabolism in calves, thereby affecting the synthesis of carnosine. It also suggests that inflammation inhibits the anti-inflammatory effect of carnosine. In conclusion, carnosine can be used as one of the metabolic markers to determine rumen acidosis in calves.

Glutamine is the main fuel and biological precursor of mammalian intestinal cells (Reeds and Burrin, 2001), including ruminants such as cattle (Okine et al., 1995) and sheep (Beaulieu et al., 2001), and involved in maintaining intestinal mucosal integrity (Potsic et al., 2002) and inhibiting the activation of inflammatory cytokines (Kim and Kim, 2017). L-glutamine is usually used as a functional antioxidant and energy supplement in the body. It is converted to glutamate and ammonia through deamination in the mitochondria of the small intestine, providing energy for the small intestine (Wang et al., 2022). This study found that the level of L-glutamine in the serum of calves with rumen acidosis was significantly lower than that in the calves of the healthy group, indicating that rumen acidosis affected the normal mechanism of the calf intestine. The decrease in L-glutamine leads to a decrease in the fuel and biological precursor required for the calves energy metabolism, resulting in the insufficient energy supply and ammonia conversion. Rumen acidosis disrupts the metabolism in the abomasum, leading to a decrease in the level of L-glutamine, which in turn causes an imbalance in the intestinal ecology of calves. Therefore, the level of L-glutamine in serum can also be used as one of the metabolic markers for evaluating rumen acidosis in calves.

4.3 Correlation analysis between serum metabolites and gut microbiota in calves with rumen acidosis

In the rumen acidosis group, compared to the normal calves, there was a significant increase in the abundance of the *Christensenellaceae_R-7* genus. This increase in the genus could affect the normal physiological functions of calves, leading to disrupt their glycolytic function. Calves over-ferment glucose, producing excessive amounts of acetate and butyrate, thereby interfering with normal glucose metabolism pathways. It further confirmed that the association between rumen acidosis in calves and dysbiosis of the microbial community. Additionally, (3beta, 4beta, 15alpha, 16beta, 25s)-4, 8, 15, 16, 26-pentahydroxycholest-5-en-3-yl beta-d-xylopyranoside is associated with monosaccharide synthesis. In this study, we found a positive correlation between the concentration of (3beta, 4beta, 15alpha, 16beta, 25s)-4, 8, 15, 16, 26-pentahydroxycholest-5-en-3-yl beta-d-xylopyranoside in serum metabolites and the *Christensenellaceae_R-7* genus in the gut microbiota. This suggested that an increase in this metabolite promoted the proliferation of the *Christensenellaceae_R-7* genus. Furthermore, Gemfibrozil can inhibit 1-O-β-glucuronidation (Tornio et al., 2017). The study found a negative correlation between the

concentration of Gemfibrozil and the *Christensenellaceae_R-7* genus. This indicated that the proliferation of the *Christensenellaceae_R-7* genus inhibited the production of Gemfibrozil metabolites in serum, thereby affecting the normal physiological function of this metabolite. In summary, through the correlation analysis of gut microbiota and serum metabolites in calves, rumen acidosis primarily affected the excessive fermentation of products in the body's glucose metabolism pathway, leading to metabolic disorders and loss of regulation of normal pathways, resulting in the onset of disease in calves. Therefore, it could be proven that rumen acidosis not only altered the gut microbiota in calves but also intervened with the diversity and abundance of post-intestinal microbial communities by affecting normal metabolic reactions.

5 Conclusion

The occurrence of rumen acidosis can induce changes in the gut microbiota of calves, with a significant increase of the *Christensenellaceae_R-7* genus and a significant decrease of *Prevotella* and *Succinivibrio* genera. Additionally, the occurrence of rumen acidosis can also induce changes in the serum metabolites including niacin, niacinamide, L-glutamine, and carnosine.

Data availability statement

The data of 16s DNA has been uploaded. ID PRJNA1126434 http://www.ncbi.nlm.nih.gov/bioproject/1126434 Metabolomic data is being uploaded.

Ethics statement

The animal studies were approved by Animal Ethics Committee of Lanzhou Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy of Agricultural Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FW: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing, Formal analysis, Validation, Visualization. PJ: Visualization, Writing – review & editing, Formal analysis, Methodology. HY: Methodology, Writing – review & editing, Data curation, Formal analysis, Software, Validation. XZ: Project administration, Writing – review & editing, Conceptualization, Resources, Supervision. XW: Writing – review & editing, Conceptualization, Project administration, Resources, Supervision.

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Conflict of interest

Author XZ was employed by Zhangye Wanhe Grass Livestock Industry Science and Technology Development Co., Ltd.

The remaining authors declared 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/fcimb.2024.1427763/full#supplementary-material

References

Aditya, S., Humer, E., Pourazad, P., Khiaosa-Ard, R., and Zebeli, Q. (2018). Metabolic and stress responses in dairy cows fed a concentrate-rich diet and submitted to intramammary lipopolysaccharide challenge. *Animal.* 12, 741–749. doi: 10.1017/S1751731117002191

Bailoni, L., Carraro, L., Cardin, M., and Cardazzo, B. (2021). Active rumen bacterial and protozoal communities revealed by RNA-based amplicon sequencing on dairy cows fed different diets at three physiological stages. *Microorganisms*. 9, 754. doi: 10.3390/microorganisms9040754

Beaulieu, A. D., Overton, T. R., and Drackley, J. K. (2001). Substrate oxidation by isolated ovine enterocytes is increased by phlorizin-induced glucosuria. *Can. J. Anim. Sci.* 81, 585–588. doi: 10.4141/A01-032

Chen, M., Lin, W., Li, N., Wang, Q., Zhu, S., Zeng, A., et al. (2022). Therapeutic approaches to colorectal cancer via strategies based on modulation of gut microbiota. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.945533

Connors, J., Dawe, N., and Van Limbergen, J. (2018). The role of Succinate in the regulation of intestinal inflammation. *Nutrients*. 11, 25. doi: 10.3390/nu11010025

Faniyi, T. O., Adegbeye, M. J., Elghandour, M. M. M. Y., Pilego, A. B., Salem, A. Z. M., Olaniyi, T. A., et al. (2019). Role of diverse fermentative factors towards microbial community shift in ruminants. *J. Appl. Microbiol.* 127, 2–11. doi: 10.1111/jam.14212

Fu, Y., He, Y., Xiang, K., Zhao, C., He, Z., Qiu, M., et al. (2022). The role of rumen microbiota and its metabolites in subacute ruminal acidosis (SARA)-induced inflammatory diseases of ruminants. *Microorganisms*. 10, 1495. doi: 10.3390/microorganisms10081495

Gressley, T. F., Hall, M. B., and Armentano, L. E. (2011). Ruminant Nutrition Symposium: Productivity, digestion, and health responses to hindgut acidosis in ruminants. *J. Anim. Sci.* 89, 1120–1130. doi: 10.2527/jas.2010-3460

Guiotto, A., Calderan, A., Ruzza, P., and Borin, G. (2005). Carnosine and carnosine-related antioxidants: a review. *Curr. Med. Chem.* 12, 2293–2315. doi: 10.2174/0929867054864796

Imai, S. (2009). The NAD World: a new systemic regulatory network for metabolism and aging–Sirt1, systemic NAD biosynthesis, and their importance. *Cell Biochem. Biophys.* 53, 65–74. doi: 10.1007/s12013-008-9041-4

Ji, H., Tan, D., Chen, Y., Cheng, Z., Zhao, J., and Lin, M. (2023). Effects of different manganese sources on nutrient digestibility, fecal bacterial community, and mineral excretion of weaning dairy calves. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1163468

Khafipour, E., Krause, D. O., and Plaizier, J. C. (2009). A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* 92, 1060–1070. doi: 10.3168/jds.2008-1389

Kim, M. H., and Kim, H. (2017). The roles of Glutamine in the intestine and its implication in intestinal diseases. *Int. J. Mol. Sci.* 18, 1051. doi: 10.3390/ijms18051051

Li, J., Lian, H., Zheng, A., Zhang, J., Dai, P., Niu, Y., et al. (2022). Effects of different roughages on growth performance, nutrient digestibility, ruminal fermentation, and microbial community in weaned Holstein calves. *Front. Vet. Sci.* 9. doi: 10.3389/fvets.2022.864320

Lourenco, J. M., Kieran, T. J., Seidel, D. S., Glenn, T. C., Silveira, M. F. D., Callaway, T. R., et al. (2020). Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. *PloS One* 15, e0231533. doi: 10.1371/journal.pone.0231533

Luk, T., Malam, Z., and Marshall, J. C. (2008). Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity. *J. Leukoc. Biol.* 83, 804–816. doi: 10.1189/jlb.0807581

Ma, Y., Bao, Y., Wang, S., Li, T., Chang, X., Yang, G., et al. (2016). Anti-inflammation effects and potential mechanism of Saikosaponins by regulating nicotinate and nicotinamide metabolism and Arachidonic acid metabolism. *Inflammation*. 39, 1453–1461. doi: 10.1007/s10753-016-0377-4

Mao, S., Zhang, R., Wang, D., and Zhu, W. (2012). The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC Vet. Res.* 8, 237. doi: 10.1186/1746-6148-8-237

Matsushita, T., Sasaki, H., Takayama, K., Ishida, K., Matsumoto, T., Kubo, S., et al. (2013). The overexpression of SIRT1 inhibited osteoarthritic gene expression changes induced by interleukin-1β in human chondrocytes. *J. Orthop Res.* 31, 531–537. doi: 10.1002/jor.22268

McCabe, M. S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R. A., et al. (2015). Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised Succinivibrionaceae and an increase of the methanobrevibacter Gottschalkii clade in feed restricted cattle. *PloS One* 10, e0133234. doi: 10.1371/journal.pone.0133234

Nagata, R., Kim, Y. H., Ohkubo, A., Kushibiki, S., Ichijo, T., and Sato, S. (2018). Effects of repeated subacute ruminal acidosis challenges on the adaptation of the rumen bacterial community in Holstein bulls. *J. Dairy Sci.* 101, 4424–4436. doi: 10.3168/jds.2017-13859

Niederwerder, M. C. (2018). Fecal microbiota transplantation as a tool to treat and reduce susceptibility to disease in animals. *Vet. Immunol. Immunopathol.* 206, 65–72. doi: 10.1016/j.vetimm.2018.11.002

Oetzel, G. R. (2017). Diagnosis and management of subacute ruminal acidosis in dairy herds. *Vet. Clin. North Am. Food Anim. Pract.* 33, 463–480. doi: 10.1016/j.cvfa.2017.06.004

Okine, E. K., Glimm, D. R., Thompson, J. R., and Kennelly, J. J. (1995). Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. *Metabolism*. 44, 325–331. doi: 10.1016/0026-0495(95)90162-0

Ostfeld, I., and Hoffman, J. R. (2023). The effect of β-Alanine supplementation on performance, cognitive function and resiliency in soldiers. *Nutrients*. 15, 1039. doi: 10.3390/nu15041039

Pang, K., Dai, D., Yang, Y., Wang, X., Liu, S., Huang, W., et al. (2022). Effects of high concentrate rations on ruminal fermentation and microbiota of yaks. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.957152

Penberthy, W. T. (2009). Nicotinamide adenine dinucleotide biology and disease. *Curr. Pharm. Des.* 15, 1–2. doi: 10.2174/138161209787185779

Plaizier, J. C., Danesh, Mesgaran., M., Derakhshani, H., Golder, H., Khafipour, E., Kleen, J. L., et al. (2018). Review: Enhancing gastrointestinal health in dairy cows. *Animal* 12, s399–s418. doi: 10.1017/S1751731118001921

Potsic, B., Holliday, N., Lewis, P., Samuelson, D., DeMarco, V., and Neu, J. (2002). Glutamine supplementation and deprivation: effect on artificially reared rat small intestinal morphology. *Pediatr. Res.* 52, 430–436. doi: 10.1203/00006450-200209000-00021

Prakash, M. D., Fraser, S., Boer, J. C., Plebanski, M., de Courten, B., and Apostolopoulos, V. (2021). Anti-cancer effects of Carnosine-A dipeptide molecule. *Molecules*. 26, 1644. doi: 10.3390/molecules26061644

Ramayo-Caldas, Y., Zingaretti, L., Popova, M., Estellé, J., Bernard, A., Pons, N., et al. (2020). Identification of rumen microbial biomarkers linked to methane emission in Holstein dairy cows. *J. Anim. Breed Genet.* 137, 49–59. doi: 10.1111/jbg.12427

Reeds, P. J., and Burrin, D. G. (2001). Glutamine and the bowel. *J. Nutr.* 131, 2505S–8S,discussion 2523S-4S. doi: 10.1093/jn/131.9.2505S

Revollo, J. R., Grimm, A. A., and Imai, S. (2007). The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals. *Curr. Opin. Gastroenterol.* 23, 164–170. doi: 10.1097/MOG.0b013e32801b3c8f

Sampath, D., Zabka, T. S., Misner, D. L., O'Brien, T., and Dragovich, P. S. (2015). Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) as a therapeutic strategy in cancer. *Pharmacol. Ther.* 151, 16–31. doi: 10.1016/j.pharmthera.2015.02.004

Sookoian, S., and Pirola, C. J. (2012). Alanine and aspartate aminotransferase and glutamine-cycling pathway: their roles in pathogenesis of metabolic syndrome. *World J. Gastroenterol.* 18, 3775–3781. doi: 10.3748/wjg.v18.i29.3775

Tang, R., Yang, W., Song, J., Xiang, K., Li, S., Zhao, C., et al. (2024). The rumen microbiota contributed to the development of mastitis induced by subclinical ketosis. *Microb. Pathog.* 187, 106509. doi: 10.1016/j.micpath.2023.106509

Tapio, I., Snelling, T. J., Strozzi, F., and Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8, 7. doi: 10.1186/s40104-017-0141-0

Tornio, A., Neuvonen, P. J., Niemi, M., and Backman, J. T. (2017). Role of gemfibrozil as an inhibitor of CYP2C8 and membrane transporters. *Expert Opin. Drug Metab. Toxicol.* 13, 83–95. doi: 10.1080/17425255.2016.1227791

Trevisi, E., Riva, F., Filipe, J. F. S., Massara, M., Minuti, A., Bani, P., et al. (2018). Innate immune responses to metabolic stress can be detected in rumen fluids. *Res. Vet. Sci.* 117, 65–73. doi: 10.1016/j.rvsc.2017.11.008

Wang, S., Wang, F., Kong, F., Cao, Z., Wang, W., Yang, H., et al. (2022). Effect of supplementing different levels of L-Glutamine on holstein calves during weaning. *Antioxidants*. 11, 542. doi: 10.3390/antiox11030542

Waters, J. L., and Ley, R. E. (2019). The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 17, 83. doi: 10.1186/s12915-019-0699-4

Welch, C. B., Ryman, V. E., Pringle, T. D., and Lourenco, J. M. (2022). Utilizing the gastrointestinal microbiota to modulate cattle health through the microbiome-gut-organ axes. *Microorganisms*. 10, 1391. doi: 10.3390/microorganisms10071391

Wen, K., Zhao, M. M., Liu, L., Khogali, M. K., Geng, T. Y., Wang, H. R., et al. (2021). Thiamine modulates intestinal morphological structure and microbiota under subacute ruminal acidosis induced by a high-concentrate diet in Saanen goats. *Animal*. 15, 100370. doi: 10.1016/j.animal.2021.100370

Zafar, H., and Saier, M. (2021). Gut Bacteroides species in health and disease. *Gut Microbes* 13, 1–20. doi: 10.1080/19490976.2020.1848158

Zebeli, Q., and Metzler-Zebeli, B. U. (2012). Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. *Res. Vet. Sci.* 93, 1099–1108. doi: 10.1016/j.rvsc.2012.02.004

Zha, D., Yang, Y., Huang, X., Wang, Z., Lin, H., Yang, L., et al. (2023). Nicaraven protects against endotoxemia-induced inflammation and organ injury through modulation of AMPK/Sirt1 signalling in macrophages. *Eur. J. Pharmacol.* 946, 175666. doi: 10.1016/j.ejphar.2023.175666

Zhai, R. G., Rizzi, M., and Garavaglia, S. (2009). Nicotinamide/nicotinic acid mononucleotide adenylyltransferase, new insights into an ancient enzyme. *Cell Mol. Life Sci.* 66, 2805–2818. doi: 10.1007/s00018-009-0047-x

Zheng, L. M., Ojcius, D. M., Garaud, F., Roth, C., Maxwell, E., Li, Z., et al. (1996). Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. *J. Exp. Med.* 184, 579–584. doi: 10.1084/jem.184.2.579

Zhou, Z., Chen, J., Cui, Y., Zhao, R., Wang, H., Yu, R., et al. (2023). Antihypertensive activity of different components of Veratrum alkaloids through metabonomic data analysis. *Phytomedicine*. 120, 155033. doi: 10.1016/j.phymed.2023.155033

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Investigating the regulatory effect of Shen Qi Bu Qi powder on the gastrointestinal flora and serum metabolites in calves

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Object: To investigate the effects of Shen Qi Bu Qi Powder (SQBQP) on the average daily gain, blood indexes, gastrointestinal microflora, and serum metabolites of calves.

Methods: A total of 105 calves were randomly assigned to three groups (n = 35 per group): the control group (C, fed with a basal diet for 21 days) and two treatment groups (SQBQP-L and SQBQP-H, fed with the basal diet supplemented with 15 and 30 g/kg of SQBQP), respectively for 21 days. The active components of SQBQP were identified using LC-MS/MS. Serum digestive enzymes and antioxidant indices were determined by ELISA kits and biochemical kits, respectively. Serum differential metabolites were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), while flora in rumen fluid and fecal were analyzed by 16S rDNA sequencing. Further correlation analysis of gastrointestinal flora and serum metabolites of SQBQP-H and C groups were performed with Spearman's correlation.

Results: The principal active components of SQBQP mainly includes polysaccharides, flavonoids, and organic acids. Compared to the control group (C), calves in the SQBQP-H (high dose) and SQBQP-L (low dose) groups showed a significant increase in serum amylase (AMS) levels ($P<0.001$), while lipase content significantly decreased ($P<0.05$). Additionally, the average daily gain, T-AOC, and cellulase content of calves in the SQBQP-H group significantly increased ($P<0.05$). *Proteobacteria* and *Succinivibrio* in the rumen flora of the SQBQP-H group was significantly lower than that of the C group ($P<0.05$). The relative abundance of *Proteobacteria, Actinobacteria, Candidatus_Saccharibacteria, Deinococcus_Thermus, Cyanobacteria*, and *Succinivibrio* in the SQBQP-H group was significantly increased ($P<0.05$), while the relative abundance of *Tenericutes* and *Oscillibacter* was significantly decreased ($P<0.05$). Serum metabolomics analysis revealed 20 differential metabolites, mainly enriched in amino acid biosynthesis, β-alanine metabolism, tyrosine, and tryptophan biosynthesis metabolic pathways ($P<0.05$). Correlation analysis results showed that *Butyrivibrio* in rumen flora and *Oscillibacter_valericigenes* in intestinal flora

were significantly positively correlated with average daily gain, serum biochemical indexes, and differential metabolite (-)-Epigallocatechin (R>0.58, *P*<0.05).

Conclusion: SQBQP can promote calves weight gain and enhance health by modulating gastrointestinal flora and metabolic processes in the body.

KEYWORDS

calves, average daily gain, serum indicators, gastrointestinal flora, serum metabolites, correlation analysis

1 Introduction

In light of the rapid expansion of the beef cattle breeding industry in China, the intensification degree is on the rise. The off-site fattening mode has emerged as the dominant paradigm in this context. Factors such as transportation, driving, vaccination stimuli, and heat stress negatively impact calves, leading to reduce immunity and growth performance, and increase morbidity and mortality (Deters and Hansen, 2022). To support the "Antibiotic Reduction Action" initiative by the Ministry of Agriculture and Rural Affairs of China and to promote the healthy, green, and sustainable development of the beef cattle industry, it is crucial to conduct research on the efficacy of Chinese veterinary compounds for improving calf health and growth.

Shen Qi Bu Qi Powder (SQBQP) is a self-made prescription by us, which primarily comprises natural Chinese herbal medicines, including *Radix Astragali* and *Rhizoma Atractylodis macrocephalae* and so on. The prescription has the effects of strengthening the stomach, replenishing qi, and consolidating the surface. Previous research conducted by us found that SQBQP could improve the immune index of 3-month-old calves, and could not effect on the liver and kidney function. The active ingredients of traditional Chinese medicine (TCM) regulate the immune level of animals by affecting the antioxidant level (Huo et al., 2022), digestive enzyme activity (Liu et al., 2023d; Long et al., 2020), rumen, and intestinal microbial flora (Che et al., 2022; Chen et al., 2023), thus achieving the desired health care effect. Some studies found that TCM was rich in active ingredients such as polysaccharides, flavonoids, and saponins (Zhu and Yu, 2018), which play an important role in bacteriostasis (Shi et al., 2022), anti-inflammatory (Lin et al., 2015), promoting growth (Liu et al., 2023b), and improving immunity effects (Liu et al., 2019). The medicinal and food-related TCM like *Radix Astragali* and *Rhizoma Atractylodis macrocephalae* have demonstrated extensive applications in breeding industry, including the rearing of aquatic animals, poultry, pigs, sheep, cows, and rabbits (Salazar et al., 2019; Swelum et al., 2021; Zou et al., 2022). The serum antioxidant index is a crucial indicator for assessing oxidative stress in animals. The active ingredients present in *Radix Astragali* and *Rhizoma Atractylodis macrocephalae* include polysaccharides, organic acids, and flavonoids, which have been demonstrated to effectively enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in serum. These ingredients have been shown to reduce the damage caused by oxidative free radicals to cell membranes and to maintain intestinal barrier function (Hao et al., 2020; Wang et al., 2021). Serum digestive enzyme activity is a key indicator of the animal's digestion and absorption abilities. TCM practices have been shown to enhance the activity of digestive enzymes, including pepsin, trypsin, and amylase, thus facilitating the nutrient digestion and absorption and improving the production performance (Harikrishnan et al., 2022). The rumen microbial flora plays an important regulatory role in the nutrition and metabolism of ruminants. The active ingredients in TCM have been shown to enhance feed degradation and fermentation, thereby improving the feed utilization by regulating the structure of gastrointestinal microbial flora (Kholif and Olafadehan, 2021; Harikrishnan et al., 2022; Rabee et al., 2024). These ingredients selectively promote the growth of beneficial bacteria, inhibit harmful bacteria, maintain rumen environment stability, and enhance body immunity (Liu et al., 2023b; Ma et al., 2023). Analyzing serum metabolites provides a deeper understanding of the effects of TCM on the animal metabolism and elucidates the comprehensive multi-component, multi-channel, and multi-target mechanisms of TCM action (Liu et al., 2023a).

In this study, SQBQP was used to feed calves, and its effects on the growth performance, serum antioxidant capacity, digestive enzyme activity, rumen and intestinal microflora, and serum metabolism of calves were analyzed. The aim was to systematically investigate the mechanisms of promoting calf health and weight gain of SQBQP, thereby support its effective application in the healthy breeding of calves.

2 Materials and methods

2.1 Preparation of Shen Qi Bu Qi powder

Radix Astragali, Radix Codonopsis pilosulae, Radix Saposhnikoviae divaricatae, and Rhizoma Atractylodis macrocephalae were crushed. Then they were passed through a 100-mesh sieve and mixed in proportion to create the final preparation.

2.2 LC-MS/MS analysis of SQBQP samples

The active components of SQBQP were subjected to analysis by Waters 2D UPLC (Waters, USA) in conjunction with a Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic conditions were as follows: the chromatographic column used was a Hypersil GOLD a Q column (100*2.1 mm, 1.9 µm, Thermo Fisher Scientific, USA). The mobile phases employed were 0.1% formic acid in water (liquid A) and 100% acetonitrile containing 0.1% formic acid (liquid B). The elution gradients were as follows: The mobile phases were as follows: 0-2 min, 5% B; 2-22 min, 5%-95% B; 22-27 min, 95% B; 27.1-30 min, 5% B. The flow rate was 0.3 mL/kg. The column temperature was maintained at 40°C, the flow rate was set at 0.3 mL/min, and the injection volume was fixed at 5 µL. Mass spectrometry: The primary and secondary mass spectrometry data were collected using a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA). The mass-to-charge ratio range for mass spectrometry scanning was 150-1500, with a primary resolution of 70,000, an AGC of $1e^6$, and a maximum injection time (IT) of 100 ms. The top three parent ions were selected for fragmentation, and secondary information was subsequently collected. The secondary resolution was 35,000, the AGC was $2e^5$, the maximum injection time (IT) was 50 ms, and the stepped voltage was set to 20, 40, and 60 eV. The settings for the ion source (ESI) were as follows: the sheath gas flow rate was 40, the auxiliary gas flow rate was 10, the spray voltage (kV) was 3.80 for positive ion mode, and 3.20 for negative ion mode, the capillary temperature was 320°C, and the temperature of the auxiliary gas heater was 350°C. The TCMSP database was used to compare the results of LC-MS/MS detection.

TABLE 1 Composition of basic diet (%).

Item	Contents (%)
Corn concentrate	
ADF	4.11
NDF	9.44
P	0.56
DM	88.95
CP	11.56
EF	3.21
Ash	5.8
Ca	0.46
Na	0.644
Mg	0.26
Wheat straw	
ADF	50.75
NDF	72.58
P	0.16
DM	91.15
CP	2.42
EF	1.91
Ash	9.15
Ca	0.2

ADF, acid detergent fiber; NDF, neutral detergent fiber; DM, dry matter; CP, crude protein; EE, crude fat; Ash, crude ash.

2.3 Experimental animals

A total of 105 three-month-old Simmental hybrid bull calves with a mean body weight of 122.55 ± 15.01 kg were randomly assigned to three groups, with 35 calves in each group. There was no significant difference in the body weight of the calves among the three groups. This experiment was conducted at the beef cattle farm of Wanhe Grass Livestock Industry Science and Technology Development Co., Ltd. in Zhangye City, Gansu Province, China. Calves in the control group (C) were fed with a basal diet daily (Table 1) for 21 days. Calves in the SQBQP-L group were fed with a basal diet supplemented with 15 g/kg of SQBQP for 21 days, and calves in the SQBQP-H group were fed with a basal diet supplemented with 30 g/kg of SQBQP for 21 days. The calves were fed twice daily, at 7:00-8:00 am and 2:30-3:30 pm, taking food and water freely.

2.4 Sample collection

At the end of the experiment, the blood (10 mL) was collected from the tail vein of all experimental animals before morning feeding. The serum was separated by centrifugation at 3500 rpm for 15 mins and stored at -80°C. Six calves were randomly selected from each group for ruminal fluid collection, which was filtered and stored in a refrigerator at -80°C. Additionally, 14 calves were randomly selected from each group, and approximately 5 g of fresh feces were collected and stored at -80°C.

2.5 Average daily gain and daily feed intake of calves

Initial and final weights were recorded before and after the test and the daily weight gain was calculated.

$$\text{Average daily gain of calves} = (\text{body weight of each calf after the test} - \text{body weight of each calf before the test}) / \text{feeding days}.$$

$$\text{Feed intake} = \text{total daily feed intake per group} / \text{number of calves per group}.$$

2.6 Detection of serum antioxidant and digestive enzyme indexes

Four Enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biological Co., Ltd.) were used to determine

amylase (AMS, YJ921025), cellulase (Cellulase, YJ551029), protease (Protease, YJ361025), and lipase (Lipase, YJ295405) in calf serum. In addition, serum total antioxidant capacity (T-AOC, A015-2-1), total superoxide dismutase (T-SOD, A001-1-2), malondialdehyde (MDA, A003-1), catalase (CAT, A007-1-1), and reduced glutathione (GSH, A006-2-1) activities were measured using the biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7 Detection of rumen fluid and fecal samples

A total of six rumen fluid samples and fourteen fecal samples were selected from each group for the gastrointestinal flora detection. For PCR amplification, 30 ng of qualified genomic DNA and corresponding fusion primers were used to configure the PCR reaction system and set the PCR reaction parameters. The PCR amplification products were purified using Agencourt AMPure XP magnetic beads, dissolved in the elution buffer, labeled, and the library construction was completed. The Agilent 2100 Bioanalyzer was used to determine the fragment range and concentration of the library. The qualified library was sequenced on a sequencer based on the size of the inserted fragment. After data filtering, the remaining high-quality clean data is used for post-analysis. Through the overlapping relationship between reads, reads are spliced into tags. Tags were clustered into OTUs and compared with the database and species annotation. Based on the OTU and annotation results, sample species complexity analysis and intergroup species difference analysis were performed.

2.8 Serum metabolomics analysis

A total of six serum samples were selected from each group for detecting serum metabolites. Metabolites were separated and detected by Waters 2D UPLC (Waters, USA) and Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA). The chromatographic column used was a BEH C18 chromatographic column (1.7 μm 2.1 * 100 mm, Waters, USA). In the positive ion mode, the mobile phase consisted of 0.1% aqueous formic acid solution (A) and 100% methanol (B) containing 0.1% formic acid. In the negative ion mode, the mobile phase consisted of 10 mM ammonium formate aqueous solution (A) and 95% methanol (B) containing 10 mM ammonium formate. The following gradient was used for elution: 0-1 min, 2% B solution; 1~9 min, 2%~98% B solution; 9~12 min, 98% B solution; 12~12. 1 min, 98% B solution~2% B solution 12. 1~15 min, 2% B liquid. The flow rate was 0.35 mL/min, the column temperature was 45°C and the injection volume was 5 μL. Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) was used to collect primary and secondary mass spectrometry data. The MS scan mass-to-nucleus ratio range was 70-1050, the first order resolution was 70,000, the AGC was $3e^6$ and the maximum injection time (IT) was 100 ms. According to the parent ion strength, Top3 was selected for fragmentation, and secondary information was collected. The secondary resolution was 17,500, the AGC was $1e^5$, the maximum injection time (IT) was 50 ms and the fragmentation energy (stepped once) was set to 20, 40, 60 eV. The ion source (ESI) parameters were set as follows: Sheath gas flow rate was 40, auxiliary gas flow rate was 10, spray voltage (| KV |) was 3.80 in positive ion mode, 3.20 in negative ion mode, capillary temperature was 320°C, auxiliary gas heater temperature was 350°C.

2.9 Statistical methods

Statistical analysis was performed using the GraphPad. Prism. 9.5 software. The data were presented in the form of X ± SEM, and T-test and one-way analysis of variance were used for group comparison. $P<0.05$ indicated a significant difference, and $P<0.01$ indicated an extremely significant difference.

3 Results

3.1 LC-MS/MS analysis of SQBQP sample analysis

The TCMSP database was used to compare the results of LC-MS/MS detection. The results showed that 27 active components were identified in SQBQP, including polysaccharides, amino acids, flavonoids, nucleic acids, organic acids, and alcohols. The top three components were polysaccharides, flavonoids, and organic acids (Figure 1, Table 2).

3.2 Effects of the SQBQP on the average daily gain and the daily feed intake of calves

The average daily gain (ADG) of calves in the SQBQP-H group was significantly higher than that in the C group (Figure 2A). There was no significant change in the daily feed intake of calves between groups (Figure 2B). It was concluded that SQBQP could significantly increase the average daily gain of calves ($P<0.05$).

3.3 Effects of the SQBQP on the antioxidant indexes of the serum of calves

By measuring the antioxidant indices such as GSH, CAT, and T-SOD in the calf serum, it was found that compared with the control group (C), the GSH content in the SQBQP-L group was significantly increased ($P<0.01$), and the T-AOC content in the SQBQP-H group was significantly increased ($P<0.001$). Both CAT and T-SOD showed an increasing trend, but the difference was not significant ($P>0.05$). MDA showed a decreasing trend, but the difference was not significant ($P>0.05$). These results indicated that SQBQP could improve the antioxidant capacity of calves (Figures 3A–E).

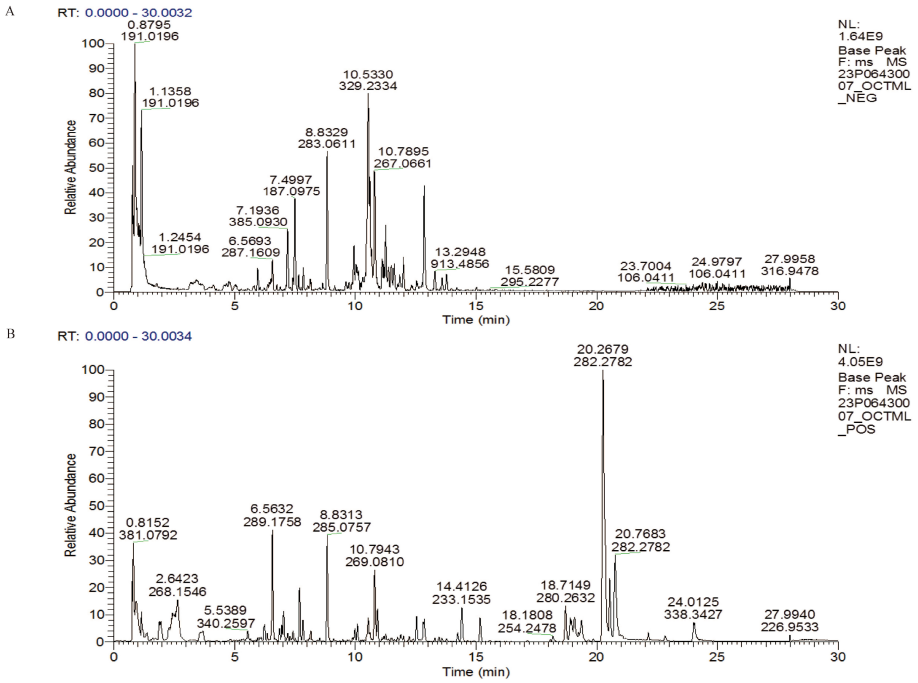

FIGURE 1
LC-MS/MS total ion chromatogram in SQBQP. **(A)** Negative ion mode diagram; **(B)** Positive ion mode diagram.

3.4 Effects of the SQBQP on serum digestive enzymes of calves

The contents of AMS, lipase, cellulase, and protease digestive enzymes in the calf serum were measured. Compared to the control group (C), the contents of cellulase ($P<0.05$) and AMS ($P<0.001$) were significantly increased in the SQBQP-H group. The content of cellulase in SQBQP-L group had an increasing trend, but the difference was not significant ($P>0.05$). A significant decrease in the lipase content ($P<0.05$) was observed in both the SQBQP-L and SQBQP-H groups. The results demonstrated that SQBQP significantly enhanced the digestive capacity of calves (Figures 4A–D).

3.5 Effects of SQBQP-H on the rumen and intestinal flora of calves

3.5.1 Effects of SQBQP-H on α and β diversity, phylum and genus level richness of rumen microbiota in calves

The total number of operational taxonomic units (OUT) in group C was 1265, while the total number of OUTs in group SQBQP-H was 1146. Principal coordinate analysis (PCoA) demonstrated a certain degree of similarity between the SQBQP-H group and the C group (Figures 5A, B). The analysis of α and β diversity accurately reflected the species and structural diversity of the rumen microbial community. Compared to the C group, there were no significant difference in the rumen microbial Chao1, ACE, Shannon, and Simpson indexes of calves in the SQBQP-H group (Figures 5C–F, $P>0.05$). Furthermore, no significant differences were observed between the two groups in the core microbiota at the phylum and genus levels (Figures 5G, H, $P>0.05$). However, it was observed that the SQBQP-H group exhibited a significant reduction in the relative abundance of *Succinivibrio* and *Butyrivibrio* at the genus level (Figure 5H, $P<0.05$). The detection of rumen microbial flora revealed that SQBQP down-regulated the relative abundance of certain microbial flora without affecting the core microbial flora in the rumen of calves. It resulted in maintaining a dynamic balance of rumen microbial flora and promoting forage digestion and absorption in the rumen.

3.5.2 Effects of SQBQP-H on α and β diversity, phylum and genus richness of intestinal flora in calves

The total number of operational taxonomic units (OUT) in group C was 1646, while the total number of OUTs in group SQBQP-H was 1688. Principal coordinate analysis (PCoA) demonstrated a certain degree of similarity between the SQBQP-H group and the C group (Figures 6A, B). The α and β diversity analysis of the two groups accurately reflected the species and structural diversity of the intestinal microbial community. Compared to the C group, there were no significant differences in the Chao1, ACE, Shannon, and Simpson indexes of rumen microorganisms in the SQBQP-H group (Figures 6C–F, $P>0.05$). However, the relative abundance of *Proteobacteria*, *Actinobacteria*, *Candidatus_Saccharibacteria*, *Deinococcus_Thermus*, and *Cyanobacteria* at the phylum level in TOP10 of the SQBQP-H group was significantly increased compared to the C group (Figure 6G, $P<0.05$). Conversely, the relative abundance of

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TABLE 2 A detailed characterization of the components included in the SQBQP.

NO.	Name	Source of compound Chinese medicine	RT/ min	Molecular Weight (da)	Delta Mass (ppm)
1	Adenine	*Radix Astragali, Radix Codonopsis pilosulae, Radix Saposhnikoviae divaricatae, Rhizoma Atractylodis macrocephalae*	0.939	135.05444	-0.401931342
2	Citric acid	*Radix Astragali, Rhizoma Atractylodis macrocephalae*	1.16	192.0268	-1.053490593
3	Galactitol	*Radix Astragali, Rhizoma Atractylodis macrocephalae*	1.047	182.07912	0.442739992
4	L-isoleucine	*Radix Astragali*	1.344	131.09465	0.129853029
5	Maleic acid	*Radix Astragali*	0.922	116.01091	-0.432249479
6	Lactose	*Radix Astragali*	1.315	388.1216	134472.9735
7	Succinic acid	*Radix Astragali*	1.24	118.02659	-0.185995698
8	Sucrose	*Radix Astragali*	0.966	342.11611	-0.291907329
9	Thymidine	*Radix Astragali*	1.786	242.09003	-0.991738137
10	Uridine	*Radix Astragali*	1.166	244.06908	-1.867001845
11	D-erythronic acid	*Radix Astragali*	1.242	136.03712	-0.355699107
12	Mucic acid	*Radix Astragali*	0.788	210.03741	-0.725651461
13	Mannitol	*Radix Astragali*	0.956	182.07899	-0.278588206
14	Ferulic acid	*Radix Saposhnikoviae divaricatae*	5.527	194.05797	0.308934059
15	D-(+)-trehalose	*Radix Saposhnikoviae divaricatae*	1.35	342.1162	-0.045956437
16	L-sorbose	*Radix Saposhnikoviae divaricatae*	0.832	180.06329	-0.54807851
17	D-(+)-glucosamine	*Radix Saposhnikoviae divaricatae*	0.935	179.07925	-0.660385374
18	Naringenin	*Radix Codonopsis pilosulae, Radix Saposhnikoviae divaricatae*	9.55	272.06819	-1.024026333
19	Daidzein	*Radix Codonopsis pilosulae, Rhizoma Atractylodis macrocephalae*	8.393	254.05778	-0.503055877
20	D-glutamic acid	*Radix Codonopsis pilosulae*	0.998	147.0531	-0.377546123
21	D-proline	*Radix Codonopsis pilosulae*	0.977	115.06327	-0.522967837
22	Eriodictyol	*Radix Codonopsis pilosulae*	8.123	288.06448	3.794530125
23	Eupatilin	*Radix Codonopsis pilosulae*	11.547	344.08954	-0.190103823
24	1 3-dicaffeoylquinic acid	*Rhizoma Atractylodis macrocephalae*	5.631	516.12629	-0.933028349
25	Isochlorogenic acid b	*Rhizoma Atractylodis macrocephalae*	6.958	516.12627	-0.983478089
26	3 5-dicaffeoylquinic acid	*Rhizoma Atractylodis macrocephalae*	7.136	516.12619	-1.138324133

Tenericutes was significantly decreased (Figure 6G, $P<0.05$). At the genus level, the relative abundance of *Succinivibrio* in the TOP10 of the SQBQP-H group was significantly increased, while that of *Oscillibacter* was significantly decreased (Figure 6H, $P<0.05$). The results demonstrated that SQBQP-H not only maintained the steady state of rumen microbial flora in calves but also reduced the relative abundance of harmful intestinal flora. It increased the growth and reproduction of beneficial flora, promoted the digestion and absorption of nutrients, and improved the energy utilization and average daily gain.

3.6 Effects of SQBQP-H on serum metabolites of calves

Partial least squares discriminant analysis (PLS-DA) revealed a clear distinction between the two groups (Figure 7A), indicating that SQBQP-H significantly altered the serum metabolite profile. The heat map demonstrated the differential metabolites aggregated between the control group (C) and the SQBQP-H group, revealing significant metabolic differences. Furthermore, the overall distribution of differential metabolites is illustrated in the volcano plot (Figure 7B).

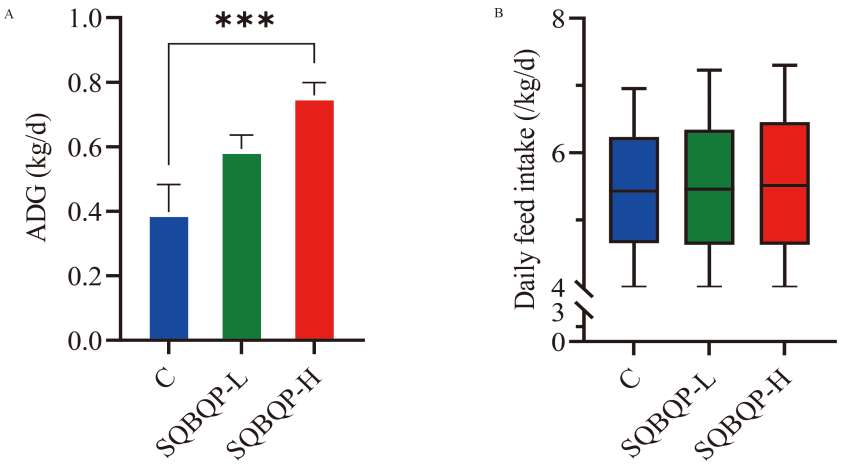

FIGURE 2
Effects of SQBQP on the average daily gain and daily feed intake of calves. **(A)** Average daily gain, **(B)** Daily feed intake. The date are expressed as SEM ± mean (n=35) and asterisks indicate significant differences (***P<0.001).

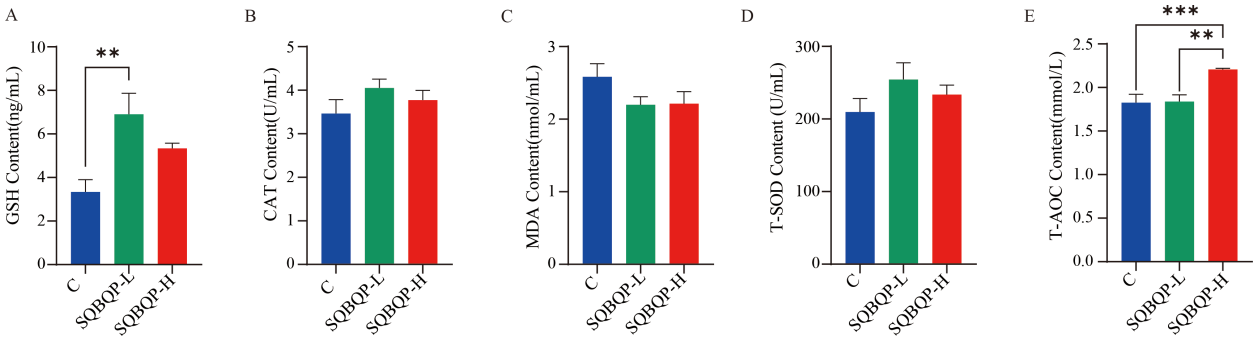

FIGURE 3
Effect of SQBQP on the serum antioxidants in calves. **(A)** GSH content, **(B)** CAT content, **(C)** MDA content, **(D)** T-SOD content, **(E)** T-AOC content. The data were expressed as SEM ± mean (n=6), and the asterisk indicated that there were significant differences according to one-way analysis of variance (**P<0.01, ***P<0.001).

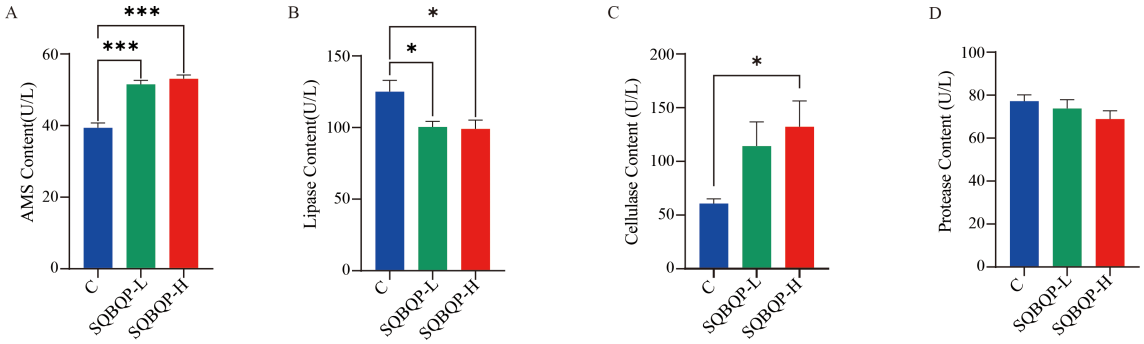

FIGURE 4
Effects of SQBQP on serum digestive enzymes in calves. **(A)** AMS content, **(B)** Lipase content, **(C)** Cellulase content, **(D)** Protease content. The data were expressed as the standard error of the mean SEM ± mean (n=6), with the asterisk indicating that there were significant differences according to one-way analysis of variance (*P<0.05, ***P<0.001).

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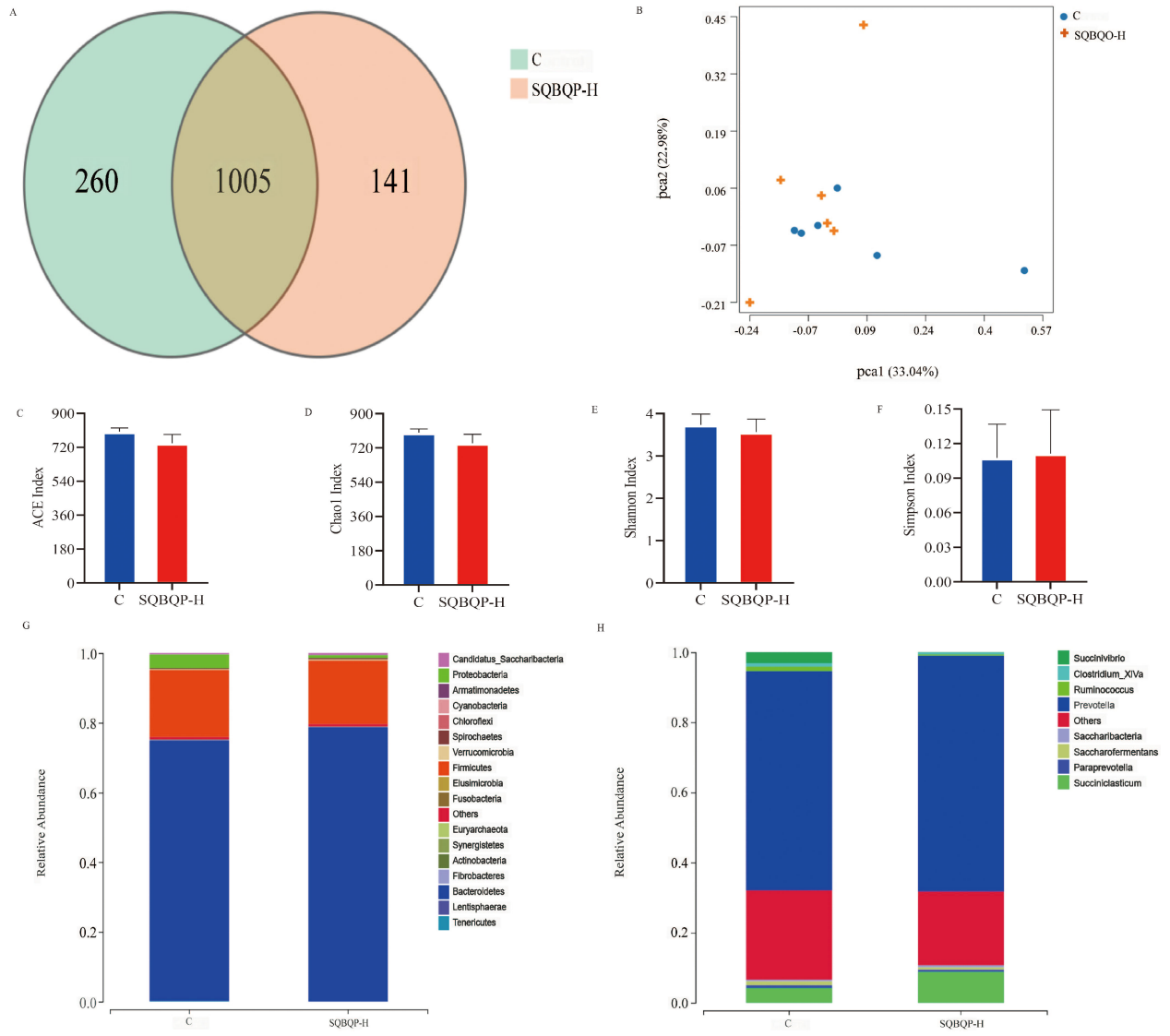

FIGURE 5
Effects of SQBQP-H on the rumen microflora composition in calves. **(A)** Venn diagram, **(B)** PCoA diagram, **(C)** Chao 1 index, **(D)** ACE index, **(E)** Shannon index, **(F)** Simpson index, **(G)** Phylum level, **(H)** Genus level (n=6).

In both positive and negative ion modes, a total of 150 metabolites exhibited significant changes. Among these, 108 metabolites were up-regulated, such as (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-picerol. Additionally, 42 metabolites were down-regulated, such as arginine and Glycine-DL-phenylalanine.

The impact of SQBQP on the metabolic pathway of calves was investigated using KEGG pathway analysis. The top20 metabolic pathways of calves in the SQBQP-H group were found to be affected, as illustrated in Figure 7C. The analysis results revealed that SQBQP could regulate the serum metabolic pathways in calves including amino acid biosynthesis, β-alanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, protein digestion and absorption, ABC transporters, and other metabolic pathways.

3.7 Correlation analysis between gastrointestinal flora and average daily gain and serum indexes

The Spearman correlation method was employed to further investigate the relationship between gastrointestinal flora and average daily gain (ADG) and serum indexes. Overall, the correlation between gastrointestinal microbiota and ADG and serum indexes exhibited changes following the SQBQP intervention in calves (Figures 8A, B). The SQBQP intervention influenced weight gain and serum antioxidant and digestive enzyme indexes by regulating the homeostasis of gastrointestinal microbiota in calves. The study found that the reduction in *Succinivibrio* in the rumen microbial community of calves following the SQBQP intervention

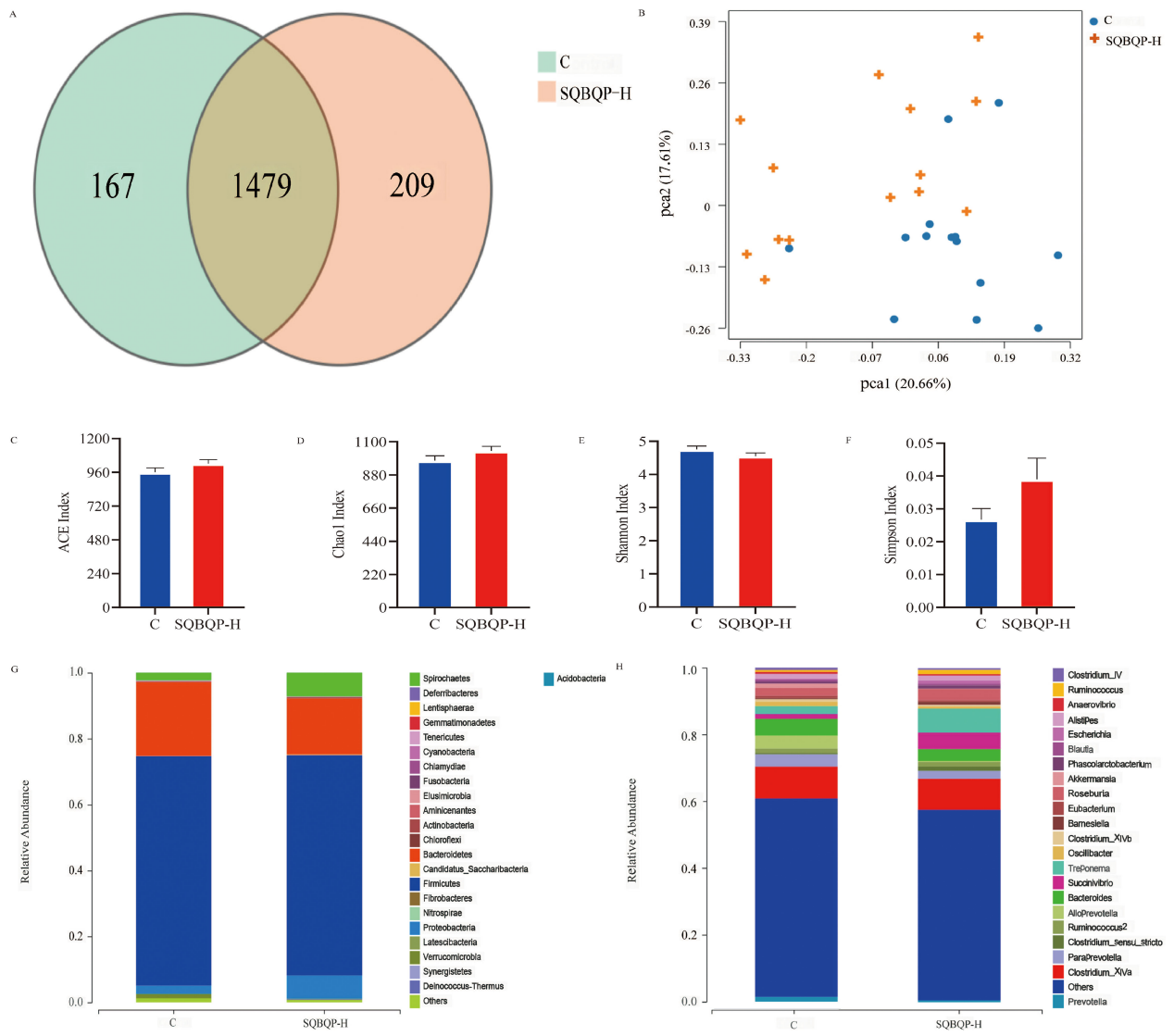

FIGURE 6
Effects of SQBQP-H on the composition of intestinal flora in calves. **(A)** Venn diagram, **(B)** PCoA diagram, **(C)** Chao 1 index, **(D)** ACE index, **(E)** Shannon index, **(F)** Simpson index, **(G)** Phylum level, **(H)** Genus level (n=14).

was negatively correlated with ADG (R<-0.6, *P*<0.05) (Figure 8A). Additionally, in SQBQP-treated calves, *Oscillibacter* was negatively correlated with CAT, T-AOC (R<-0.6, *P*<0.05), ADG, GSH, and Cellulase (R<-0.5, *P*> 0.05). Furthermore, *Oscillibacter* was found to be positively correlated with lipase (R>0.6, *P*<0.05) (Figure 8B). The results demonstrated that SQBQP could significantly enhance the average daily gain and serum indices of calves by regulating the relative abundance of *Succinivibrio* in rumen bacteria and *Oscillibacter* in intestinal bacteria. This further indicated that SQBQP could be employed as a traditional Chinese medicine compound to promote the growth and development of calves.

3.8 Correlation analysis between rumen flora and intestinal flora

The correlation analysis results between the level of rumen flora and intestinal flora in calves were illustrated in Figure 9. It was demonstrated that *Succinivibrio* in the intestinal flora exhibited a positive correlation with *Prevotella* in the rumen flora (R>0.4, *P*>0.05). This data indicated a correlation between different bacteria in the gastrointestinal tract of calves, suggesting that one bacteria can indirectly influence the growth of another bacteria in the different internal environments.

3.9 Correlation analysis between serum metabolites and average daily gain and serum indexes

The results of correlation analysis between serum metabolites, average daily gain (ADG), and serum indices showed that metabolites were correlated with weight gain and serum indices after the SQBQP intervention (Figure 10). This study found that the serum metabolite (-)-Epigallocatechin was positively

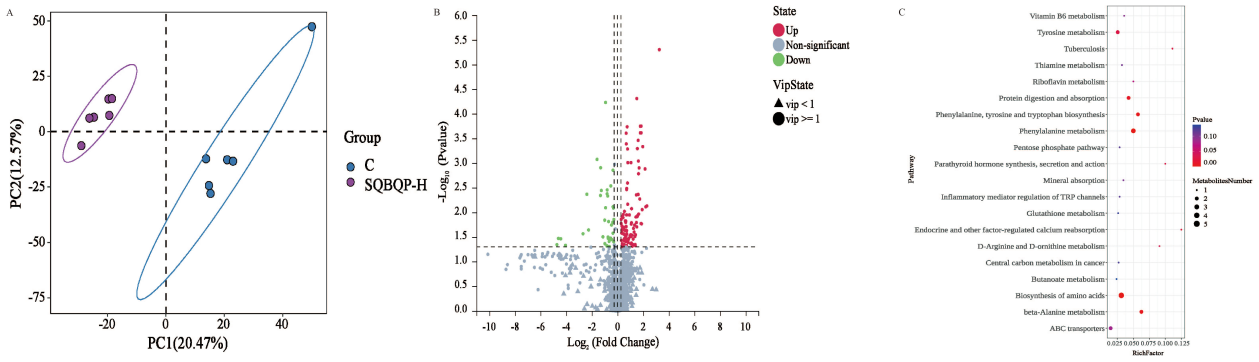

FIGURE 7
Effects of SQBQP-H on the serum metabolites in calves. **(A)** PLS-DA score plot of orthogonal partial least squares discriminant analysis of serum metabolites in the C and SQBQP-H groups, **(B)** Volcano map, each point represents a metabolite. The significantly upregulated metabolites were indicated by red dots, while the downregulated metabolites were indicated by green dots, and the metabolites with no significant difference were indicated by grey, **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG)-enriched serum differential metabolites. The color of the spot indicates the P value. The greater the degree of red, the more significant the enrichment. The size of the spots represents the number of different metabolites that are enriched (n=6).

correlated with T-AOC, AMS, cellulase, GSH, ADG, and CAT (R>0.58, P<0.05). Trans-cinnamaldehyde was found to be positively correlated with T-AOC, AMS, cellulase, GSH, T-SOD and ADG (R>0.58, P<0.05). Trans-piceatannol was positively correlated with T-AOC, cellulase, AMS, GSH, ADG, and CAT (R>0.6, P<0.05). P-hydroxyphenylacetic acid demonstrated a positive correlation with lipase, protease, and malondialdehyde (R>0.4, P<0.05) while exhibiting a negative correlation with ADG, AMS, T-AOC, and cellulase (R<-0.55, P<0.05). Gly-DL-phenylalanine was found to be negatively correlated with AMS, cellulase, T-SOD, ADG, T-AOC, and GSH (R<-0.6, P<0.05). The results indicated that SQBQP could significantly enhance ADG and serum indices of calves by regulating the content of various metabolites. Consequently, SQBQP can be employed as a traditional Chinese medicine compound to enhance the growth and development of calves.

3.10 Correlation analysis between serum metabolites and gastrointestinal flora

The correlation analysis results between differential metabolites in the calf serum and the level of rumen flora (Figure 11A). Twenty differential metabolites were strongly correlated with rumen flora (R>0.6, P<0.05). Metabolites such as (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-picerol were negatively correlated with *Butyrivibrio*. Additionally, P-hydroxyphenylacetic acid was positively correlated with *Butyrivibrio*. The correlation analysis results between serum differential metabolites and intestinal flora species levels in calves (Figure 11B). Twenty differential metabolites were strongly correlated with the intestinal flora (R>0.6, P<0.05). Metabolites such as (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-piceatannol were negatively correlated with *Oscillibacter_valericigenes* in the intestinal flora. In contrast, Glycine-DL-phenylalanine was positively

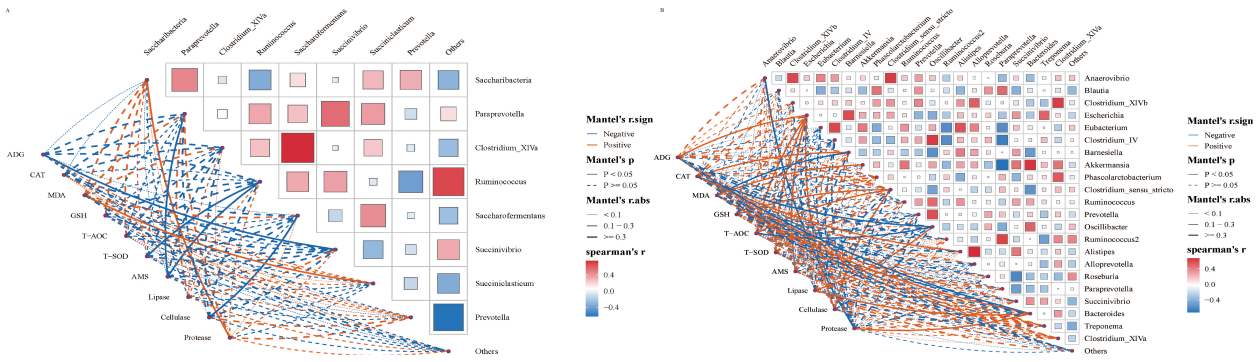

FIGURE 8
Correlation analysis between gastrointestinal flora and average daily gain and serum indexes. **(A)** Correlation between rumen flora and average daily gain and serum indicators, **(B)** Correlation between intestinal flora and average daily gain and serum indicators. The red and blue lines represent positive correlation and negative correlation, respectively. The thicker the line is, the stronger the correlation is. P<0.05 is the solid line.

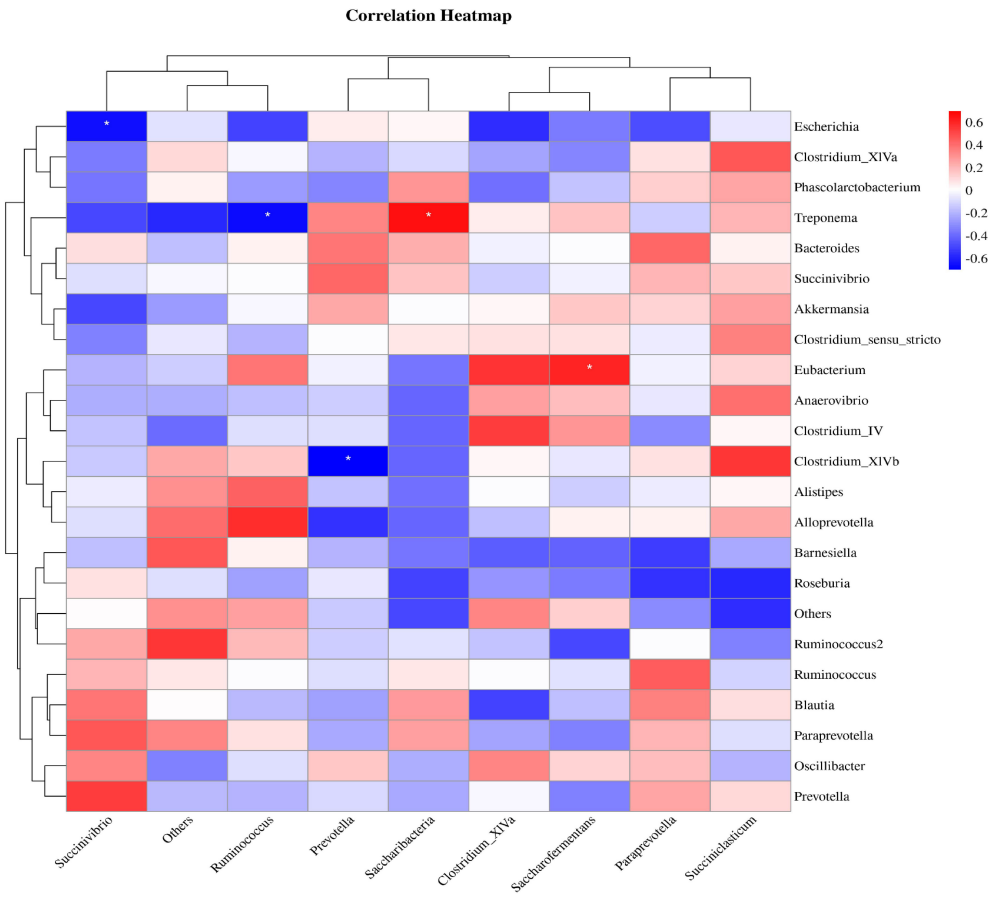

FIGURE 9
Correlation analysis between rumen flora and intestinal flora. Red indicates a positive correlation, blue indicates a negative correlation, and * represents $P<0.05$.

correlated with *Oscillibacter_valericigenes*. These results indicate that SQBQP can regulate the abundance of gastrointestinal microflora in calves and indirectly affect the expression of serum metabolites.

4 Discussion

In the cattle production, some factors, including transportation, extreme temperatures, driving, and vaccination stimuli, can induce the stress response in calves, which in turn reduces their disease resistance of calves. Therefore it is of great importance to screen TCM or compound for calf healthcare according to the requirements of the 'antibiotic reduction action' in animal husbandry production proposed by China, to ensure the health of calves and improve breeding efficiency. The advantages of TCM include the green, natural, and non-resistant properties. This study analyzed the effects of SQBQP on ADG, blood indexes, gastrointestinal microflora, and serum metabolites of 3-month-old calves based on clinical practice. LC-MS/MS was employed to identify and quantify the active components present in SQBQP. The results showed that the primary substances in SQBQP mainly includes polysaccharides, flavonoids, and organic acids. It was demonstrated that astragalus polysaccharide (Jia et al., 2019), codonopsis pilosula polysaccharide (Gao et al., 2020), atractylodes macrocephala polysaccharide (Li et al., 2022), and saposhnikovia divaricata polysaccharide (Fan et al., 2023) could enhance the antioxidant and antibacterial capabilities. Flavonoids have been shown to confer various health benefits, exhibiting antibacterial, anti-inflammatory, anti-tumor, and antioxidant effects (Tagousop et al., 2018; Calis et al., 2020; Zeng et al., 2022). Organic acids have the effects of anti-tumor, anti-inflammatory, and free radical scavenging (Wei et al., 2013; Erukainure et al., 2017). Although the components of TCM are complex and the mechanisms of action are diverse, the evidence of their curative effect is irrefutable. TCMs such as *Radix Astragali* and *Rhizoma Atractylodis macrocephalae*, are rich in polysaccharide active ingredients (Zeng et al., 2019; Chen et al., 2020). These ingredients significantly enhance the antioxidant capacity of calves and promote their growth and development, particularly in improving the average daily weight gain of calves.

The redox state is a significant indicator of the overall health status of the body. Animals, including livestock and poultry, can rely on a variety of antioxidant enzymes to regulate the redox balance. This study found that after the administration of SQBQP on calves, there was a significant increase in the serum GSH content, a increase tendency of CAT and T-SOD content, and a decrease tendency of MDA content. These findings indicate that SQBQP can enhance the

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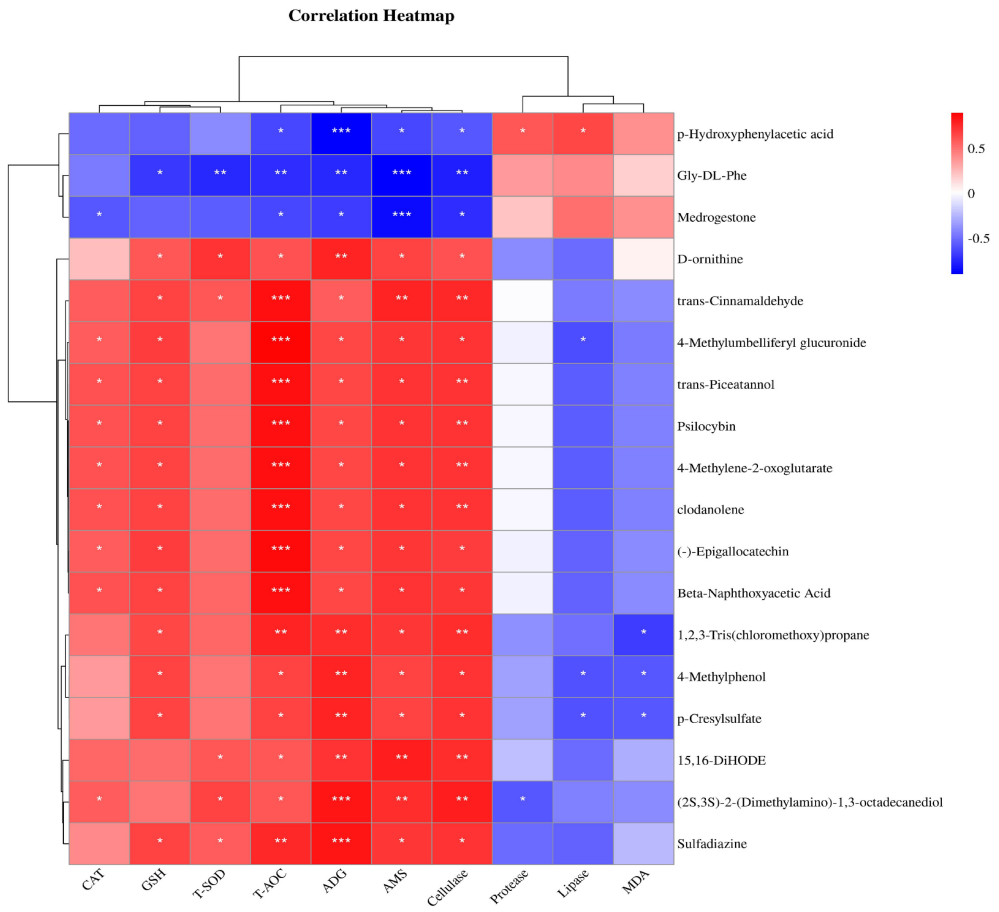

FIGURE 10
Correlation analysis between serum metabolites and average daily gain and serum indexes. Red represents positive correlation, green represents negative correlation, * represents $P<0.05$, ** represents $P<0.01$, *** represents $P<0.001$.

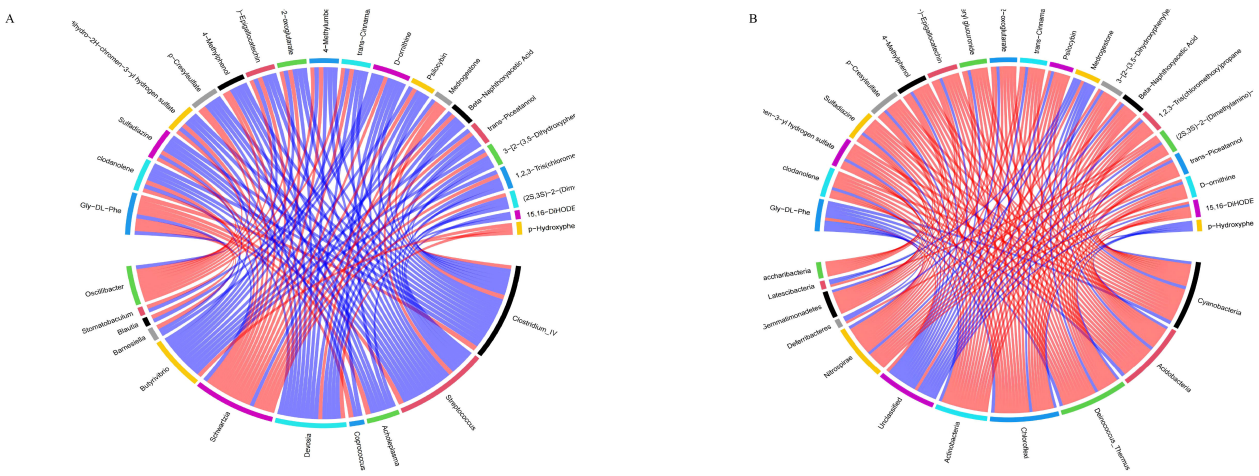

FIGURE 11
Correlation analysis between differential metabolites in serum and gastrointestinal flora. **(A)** correlation between serum differential metabolites and rumen flora, **(B)** Correlation between serum differential metabolites and intestinal flora; red and blue lines represent positive and negative correlations, respectively.

antioxidant capacity of calves. The active ingredients in TCM, such as polysaccharides and flavonoids, can activate the antioxidant system, increase the activity of antioxidant enzymes, alleviate oxidative stress and enhance the body's antioxidant capacity by increasing serum levels of CAT, GSH, T-SOD and T-AOC and decreasing levels of MDA. (Chen et al., 2019b; Zeng et al., 2019; Wang et al., 2022b). Some studies have shown that the flavonoid eupatilin has a range of pharmacological activities, including anticancer, anti-inflammatory, antioxidant, neuroprotective, anti-allergic, and cardioprotective properties (Lu et al., 2023, 2024). Other studies have shown that ferulic acid, a naturally occurring compound with antioxidant and antimicrobial properties, improves calf growth performance by enhancing the calf's antioxidant capacity (Peña-Torres et al., 2021). The experiment results demonstrated that SQBQP enhanced the antioxidant capacity of calves and effectively alleviated the oxidative stress.

Digestive enzymes are produced by various tissues and organs of animals and transported to target organs to decompose and digest specific substances. Cellulose is produced in the rumen of calves to break down cellulose and provide energy. Proteases, amylases, and lipases are present in the gastrointestinal tract, where they can decompose and digest nutrients, provide energy for daily requirements. The results of this study indicate that the feeding calves with SQBQP can significantly increase the activity of cellulase and amylase, while the activity of lipase is significantly reduced. Some previous research has shown that adding sunflower shells to the diet can significantly increase the amylase activity in bovine serum (Kondrashova et al., 2021). In this study, SQBQP significantly increased serum amylase activity in calves but decreased lipase activity. This suggests that serum determination can be used instead of material determination in target organs, warranting further research. Adding Bacillus subtilis to piglet diets has been shown to significantly increase the activity of amylase and lipase in the ileum (Deng et al., 2020). Substituting corn with wheat in the diet of beef cattle significantly enhances the activity of amylase, lipase, protease, and cellulase in the gastrointestinal tract (Liu et al., 2016). Naringenin affects lipid metabolism in serum primarily by directly inhibiting pancreatic lipase activity (Liu et al., 2022). In contrast, ferulic acid may indirectly affect serum digestive enzyme function by improving intestinal health and enhancing antioxidant capacity (Gong et al., 2019). The findings demonstrate that SQBQP can enhance the digestive and absorptive capabilities of calves, thereby promoting their growth by influencing serum levels of digestive enzymes.

The complex microbial flora in the gastrointestinal tract of ruminants plays a crucial role in maintaining homeostasis, regulating energy metabolism, and activating intestinal immunity (Dill-McFarland et al., 2019). By regulating feed composition, it can enhance feed utilization and animal growth performance (Jiang et al., 2021; Nguse et al., 2022; Chen et al., 2019a). This study demonstrated that SQBQP significantly impacted the regulation of rumen and intestinal microbial communities in calves and notably increased their ADG. The dominant phyla in all test samples were *Bacteroidetes, Firmicutes*, and *Proteobacteria*. In the SQBQP-H group, the abundance of *Firmicutes* in rumen fluid and feces decreased, while the relative abundance of *Bacteroidetes* in the rumen increased. Furthermore, the overall relative abundance of *Firmicutes* and *Bacteroidetes* was higher than that in the control group (C group). Both *Firmicutes* and *Bacteroidetes* are key players in the fermentation of fiber and the degradation of carbohydrates, producing various cellulases to hydrolyze macromolecular compounds such as cellulose and sugar (Jami et al., 2014; Xie et al., 2023). This process promotes fat deposition, milk fat production, and the digestion and absorption of nutrients (de Melo et al., 2023; Song et al., 2023). SQBQP has been shown to maintain the homeostasis of the rumen environment in calves by regulating the relative abundance of *Bacteroidetes* and *Firmicutes*. The increased relative abundance of *Proteobacteria* in the intestinal microflora of the SQBQP-H group was linked to the degradation and fermentation of biopolymers. Additionally, the study revealed that *Actinobacteria, Candidatus_Saccharibacteria, Deinococcus_Thermus, and Cyanobacteria* were markedly elevated in the intestinal tract of the SQBQP-H group, with *Actinobacteria* playing a pivotal role in the biodegradation of lignocellulose (Briggs et al., 2021). The study also found that the relative abundance of *Prevotella* in the rumen of the SQBQP-H group was higher than that in the C group, whereas its abundance in the intestine was lower. Flavonoids have been shown to enhance the decomposition and utilization of plant polysaccharides by increasing the relative abundance of *Prevotell* (Ghimire et al., 2022). This suggests that the increased abundance of *Prevotella* in the rumen of calves in the SQBQP-H group facilitates feed digestion and utilization. Conversely, the relative abundance of *Succinivibrio* in the rumen bacteria of the SQBQP-H group was significantly reduced, while its abundance in the intestinal bacteria was considerably increased. This indicates a competitive relationship between *Prevotella* and *Succinivibrio* in the rumen, where an increase in *Prevotella* inhibits *Succinivibrio* growth, a dynamic reversed in the intestine. *Succinivibrio* could ferment a diverse range of sugars, primarily producing acetic acid and succinic acid, with minor amounts of formic acid and lactic acid (Liu et al., 2023c). Correlation analysis results suggest a positive relationship between the abundance of *Prevotella* in rumen flora and *Succinivibrio* in intestinal flora, indicating similar roles in the different organ environments. Furthermore, correlation analysis results between gastrointestinal flora, ADG of calves, and serum indices revealed that SQBQP intervention reduced the relative abundance of *Succinivibrio* in the rumen and significantly increased the ADG of calves. After SQBQP intervention, the relative abundance of *Oscillibacter* in the intestine was reduced, while the serum levels of CAT and T-AOC significantly increased, and the lipase levels decreased. These findings demonstrate that SQBQP exerts a positive regulatory effect on the rumen and intestinal flora of calves. An increase in the relative abundance of beneficial flora resulted in an enhanced feed digestion and absorption, subsequently improving ADG, antioxidant capacity, and the digestive efficiency in calves.

Alterations in the serum metabolite concentrations have a direct impact on the health status of animals (Guasch-Ferré et al., 2016; Wang et al., 2020; Wu et al., 2022). In this study, three down-regulated and 17 up-regulated differential serum metabolites were identified, exhibiting strong correlations with ADG and serum indicators. Among these metabolites, (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-piceatannol were positively correlated with T-AOC, AMS, cellulase, GSH, ADG, and CAT. Conversely, hydroxyphenylacetic acid and Glycine-DL-phenylalanine were negatively correlated with these

indicators. These findings suggest that alterations in the concentration of these metabolites may significantly enhance the ADG and serum indicators of calves. Numerous prospective studies have investigated the correlation between gastrointestinal microbiota and serum metabolites in both humans and animals (Zhang et al., 2021; Wang et al., 2022a). Our correlation analysis of rumen flora and serum metabolites revealed that (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-piceatannol were negatively correlated with *Butyrivibrio*. Additionally, P-hydroxyphenylacetic acid was positively correlated with *Butyrivibrio*. Similarly, the analysis between intestinal flora and serum metabolism indicated that metabolites such as (-)-Epigallocatechin, Trans-cinnamaldehyde, and Trans-piceatannol were negatively correlated with *Oscillibacter valericigenes* in the intestinal flora, while Glycine-DL-phenylalanine was positively correlated with *Oscillibacter valericigenes*. This study found that differential metabolites showed a correlation with their corresponding gastrointestinal microbial flora in terms of significant up-regulation or down-regulation. Numerous studies have demonstrated that these metabolites can enhance animal growth performance and promote gastrointestinal health. For instance, β-naphthyloxyacetic acid is an auxin metabolite that stimulates mammalian guanylate cyclase activity. (-)-Epigallocatechin (Umme et al., 2021), Trans-cinnamaldehyde (Wang et al., 2022c), and Trans-pistol have been shown to possess anti-inflammatory and antioxidant properties, while Trans-cinnamaldehyde also exhibits antibacterial activity (Wu et al., 2020). D-ornithine, a key metabolite for energy conversion, has been observed to increase the body weight of young goats (Wang et al., 2023). SQBQP, a traditional Chinese medicine compound, has demonstrated antioxidant, anti-inflammatory, and antibacterial properties. This study found that SQBQP intervention in calves significantly up-regulate the content of these metabolites in calf serum, reduced the relative abundance of *Butyrivibrio* in rumen bacteria, and *Oscillibacter_valericigenes* in intestinal bacteria. Further studies revealed that SQBQP can up-regulate calf serum metabolites, affect various metabolic pathways, including amino acid biosynthesis, β-alanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, protein digestion and absorption, and ABC transporter pathways. Amino acid biosynthesis plays a pivotal role in protein synthesis, energy provision, and participation in the metabolic pathways. Through conjugated and independent pathways, organisms can convert inorganic substances into amino acids, regulate by feedback inhibition and gene regulation. Prenatal nutrient supplementation significantly affected β-alanine metabolism in pregnant cows (Schalch Junior et al., 2022). Moreover, yam has been found to regulate abnormal fecal flora caused by diarrhea in rats and participate in β-alanine metabolism, as well as phenylalanine, tyrosine, and tryptophan biosynthesis (Zhang et al., 2023). Protein digestion and absorption, along with ABC transporters are the crucial metabolic pathways for calf gastrointestinal health (Huang et al., 2020). Tyrosine metabolism is also vital for the normal metabolism of perinatal dairy cows (Lopreiato et al., 2023; Zhao et al., 2024). Combining weight gain, serum partial indicators, microbiome, metabolomics, and their correlation analysis, it was confirmed that SQBQP promotes gastrointestinal health and increases the average daily gain of calves by regulating the abundance of gastrointestinal flora, serum metabolites, and metabolic pathways.

5 Conclusion

The study demonstrated that SQBQP could enhance the ADG of calves, improve serum indices, regulate the relative abundance of gastrointestinal flora, and influence the serum metabolite changes in calves. Further correlation analysis of the gastrointestinal microbiome and metabolomics revealed that SQBQP could enhance the body weight and antioxidant capacity of calves by regulating the relative abundance of gastrointestinal microbial flora, including *Succinivibrio*, *Butyrivibrio*, and *Oscillibacter_valericigenes*, and the levels of metabolites such as Trans-cinnamaldehyde, (-)-Epigallocatechin, Trans-piceatannol, P-hydroxyphenylacetic acid, and Glycine-DL-phenylalanine. Moreover, the study identified specific bacterial species, namely *Butyrivibrio* and *Oscillibacter_valericigenes*, and key metabolites including Trans-cinnamaldehyde, (-)-Epigallocatechin, Trans-piceatannol, P-hydroxyphenylacetic acid, and Glycine-DL-phenylalanine, which were found to promote gastrointestinal health in calves. These results provided the preliminary evidence of the potential of SQBQP as a functional feed additive. However, further systematic research and long-term monitoring are needed to fully evaluate the potential for the widespread use.

Data availability statement

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics statement

The animal study was approved by animal ethics committee of Gansu Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HY: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. JR: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. PJ: Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. XSZ: Data curation, Investigation, Methodology, Software, Writing – review & editing. ZM: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – review & editing. CL: Data curation, Investigation, Methodology, Software, Writing – review & editing. NZ: Data curation, Investigation, Methodology, Software, Writing – review & editing. TM: Data curation, Investigation, Methodology, Software, Writing – review & editing. XPZ: Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. YH: Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing. YW: Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – review & editing.

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Conflict of interest

Author XZ was employed by Zhangye Wanhe Animal Husbandry Industry Technology Development Co.

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.

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References

Briggs, J. A., Grondin, J. M., and Brumer, H. (2021). Communal living: glycan utilization by the human gut microbiota. *Environ. Microbiol.* 23, 15–35. doi: 10.1111/1462-2920.15317

Calis, Z., Mogulkoc, R., and Baltaci, A. K. (2020). The roles of flavonols/flavonoids in neurodegeneration and neuroinflammation. *Mini Rev. Med. Chem.* 20, 1475–1488. doi: 10.2174/1389557519666190617150051

Che, Q. Y., Luo, T. T., Shi, J. H., He, Y. H., and Xu, D. L. (2022). Mechanisms by which traditional Chinese medicines influence the intestinal flora and intestinal barrier. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.863779

Chen, S. Y., Deng, F., Jia, X., Liu, H., Zhang, G. W., and Lai, S. J. (2019a). Gut microbiota profiling with differential tolerance against the reduced dietary fiber level in rabbits. *Sci. Rep.* 91, 1–9. doi: 10.1038/s41598-018-36534-6

Chen, F., Huang, G., Yang, Z., and Hou, Y. (2019b). Antioxidant activity of Momordica charantia polysaccharide and its derivatives. *Int. J. Biol. Macromol.* 138, 673–680. doi: 10.1016/j.ijbiomac.2019.07.129

Chen, G., Li, Z. Q., Liu, S. L., Tang, T., Chen, Q. H., Yan, Z. M., et al. (2023). Fermented Chinese herbal medicine promoted growth performance, intestinal health, and regulated bacterial microbiota of weaned piglets. *Anim. (Basel).* 13, 476. doi: 10.3390/ani13030476

Chen, L., Long, R., Huang, G., and Huang, H. (2020). Extraction and antioxidant activities *in vivo* of pumpkin polysaccharide. *Ind. Crops Prod.* 146, 112199. doi: 10.1016/j.indcrop.2020.112199

de Melo, H. S. A., Ítavo, L. C. V., de Castro, A. P., Ítavo, C. C. B. F., de Araújo Caldas, R., Mateus, R. G., et al. (2023). Effect of whole oilseeds in the diet on bacterial diversity in the solid fraction of the ruminal content of steers. *Trop. Anim. Health Prod.* 55, 32. doi: 10.1007/s11250-022-03442-x

Deng, B., Wu, J., Li, X., Zhang, C., Men, X., and Xu, Z. (2020). Effects of Bacillus subtilis on growth performance, serum parameters, digestive enzyme, intestinal morphology, and colonic microbiota in piglets. *AMB Express.* 10, 212. doi: 10.1186/s13568-020-01150-z

Deters, E. L., and Hansen, S. L. (2022). Long-distance transit alters the liver and skeletal muscle physiology of beef cattle. *Animal* 16, 100555. doi: 10.1016/j.animal.2022.100555

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., and Suen, G. (2019). Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85, e02141–e02118. doi: 10.1128/AEM.02141-18

Erukainure, O. L., Hafizur, R. M., Choudhary, M. I., Adhikari, A., Mesaik, A. M., Atolani, O., et al. (2017). Anti-diabetic effect of the ethyl acetate fraction of Clerodendrum volubile: protocatechuic acid suppresses phagocytic oxidative burst and modulates inflammatory cytokines. *BioMed. Pharmacother.* 86, 307–315. doi: 10.1016/j.biopha.2016.12.035

Fan, H. T., Sun, M., Li, J., Zhang, S. F., Tu, G. Z., Liu, K. C., et al. (2023). Structure characterization and immunomodulatory activity of a polysaccharide from Saposhnikoviae Radix. *Int. J. Biol. Macromol.* 233, 123502. doi: 10.1016/j.ijbiomac.2023.123502

Gao, Z. Z., Zhang, C., Jing, L. R., Feng, M., Li, R., and Yang, Y. (2020). The structural characterization and immune modulation activities comparison of Codonopsis pilosula polysaccharide (CPPS) and selenizing CPPS (sCPPS) on mice *in vitro* and vivo. *Int. J. Biol. Macromol.* 160, 814–822. doi: 10.1016/j.ijbiomac.2020.05.149

Ghimire, S., Cady, N. M., Lehman, P., Peterson, S. R., Shahi, S. K., Rashid, F., et al. (2022). Dietary isoflavones alter gut microbiota and lipopolysaccharide biosynthesis to reduce inflammation. *Gut Microbes* 14, 2127446. doi: 10.1080/19490976.2022.2127446

Gong, L. X., Wang, H. N., Wang, T. X., Liu, Y. L., Wang, J., and Sun, B. G. (2019). Feruloylated oligosaccharides modulate the gut microbiota *in vitro* via the combined actions of oligosaccharides and ferulic acid. *J. Funct. Foods.* 60, 103453. doi: 10.1016/j.jff.2019.103453

Guasch-Ferré, M., Hruby, A., Toledo, E., Clish, C. B., Martínez-González, M. A., Salas-Salvadó, J., et al. (2016). Metabolomics in prediabetes and diabetes: A systematic review and meta-analysis. *Diabetes Care* 39, 833–846. doi: 10.2337/dc15-2251

Hao, X. Y., Wang, P. J., Ren, Y. S., Liu, G. T., Zhang, J. X., Leury, B., et al. (2020). Effects of Astragalus membranaceous roots supplementation on growth performance, serum antioxidant and immune response in finishing lambs. *Asian-Australas J. Anim. Sci.* 33, 965–972. doi: 10.5713/ajas.19.0295

Harikrishnan, R., Devi, G., Doan, H. V., Tapingkae, W., Balasundaram, C., Arockiaraj, J., et al. (2022). Changes in immune gene expression, immune response, digestive enzymes -antioxidant status, and growth of catla (Catla catla) fed with Astragalus polysaccharides against Edwardsiellosis disease. *Fish Shellfish Immunol.* 121, 418–436. doi: 10.1016/j.fsi.2022.01.022

Huang, M. Z., Cui, D. A., Wu, X. H., Hui, W., Yan, Z. T., Ding, X. Z., et al. (2020). Serum metabolomics revealed the differential metabolic pathway in calves with severe clinical diarrhea symptoms. *Anim. (Basel).* 10, 769. doi: 10.3390/ani10050769

Huo, X. Q., Gu, Y., and Zhang, Y. L. (2022). The discovery of multi-target compounds with anti-inflammation activity from traditional Chinese medicine by TCM-target effects relationship spectrum. *J. Ethnopharmacol.* 293, 115289. doi: 10.1016/j.jep.2022.115289

Jami, E., White, B. A., and Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PloS One* 9, e85423. doi: 10.1371/journal.pone.0085423

Jia, N., Qiao, H. R., Zhu, W., Zhu, M. H., Meng, Q. H., Lu, Q., et al. (2019). Antioxidant, immunomodulatory, oxidative stress inhibitory, and iron supplementation effect of Astragalus membranaceus polysaccharide-iron (III) complex on iron-deficiency anemia mouse model. *Int. J. Biol. Macromol.* 132, 213–221. doi: 10.1016/j.ijbiomac.2019.03.196

Jiang, C. X., Ding, L. M., Dong, Q. M., Wang, X. J., Wei, H. Y., Hu, C. S., et al. (2021). Effects of root extracts of three traditional Chinese herbs as dietary supplements on dry matter intake, average daily gain, rumen fermentation, and ruminal microbiota in early weaned yak calves. *Anim. Feed Sci. Technol.* 278. doi: 10.1016/j.anifeedsci.2021.115002

Kholif, A. E., and Olafadehan, O. A. (2021). Essential oils and phytogenic feed additives in ruminant diet: chemistry, ruminal microbiota and fermentation, feed utilization and productive performance. *Phytochem. Rev.* 206, 1–22. doi: 10.1007/s11101-021-09739-3

Kondrashova, K., Shejjda, E., Shajahmetova, E., Lebedev, S., Miroshnikov, S., Duskaev, G., et al. (2021). PSVII-22 Changes in biochemical composition of saliva and blood of cattle after sunflower husk included in the diet. *J. Anim. Sci.* 3, 338–339. doi: 10.1093/jas/skab235.622

Li, W. Y., Zhou, X. Y., Xu, S. W., Cao, N., Li, B. X., Chen, W. B., et al. (2022). Lipopolysaccharide-induced splenic ferroptosis in goslings was alleviated by polysaccharide of atractylodes macrocephala koidz associated with proinflammatory factors. *Poult Sci.* 101, 101725. doi: 10.1016/j.psj.2022.101725

Lin, Y. C., Chang, C. W., and Wu, C. R. (2015). Anti-nociceptive, anti-inflammatory and toxicological evaluation of Fang-Ji-Huang-Qi-Tang in rodents. *BMC Complem. Altern. Med.* 15, 10. doi: 10.1186/s12906-015-0527-5

Liu, S., Han, Z., Trivett, A. L., Lin, H., Hannifin, S., Yang, D., et al. (2019). Cryptotanshinone has curative dual anti-proliferative and immunotherapeutic effects on mouse Lewis lung carcinoma. *Cancer Immunol. Immunother.* 68, 1059–1071. doi: 10.1007/s00262-019-02326-8

Liu, J., Pang, Y. Q., Yang, H. X., Huo, D. N., Liu, X. X., and Chen, X. X. (2023a). Gut microbiota and urine metabolomics based exploration of coreopsis tinctoria nutt. tea on type 2 diabetic mellitus. *Arab J. Chem.* 16, 105037. doi: 10.1016/j.arabjc.2023.105037

Liu, S., Wang, K. J., Lin, S. Z., Zhang, Z. D., Cheng, M., Hu, S. S., et al. (2023b). Comparison of the effects between tannins extracted from different natural plants on growth performance, antioxidant capacity, immunity, and intestinal flora of broiler chickens. *Antioxid. (Basel Switzerland).* 12, 441. doi: 10.3390/antiox12020441

Liu, S., Xiao, G. X., Wang, Q., Tian, J. P., Feng, X., Zhang, Q. Y., et al. (2023c). Effects of dietary Astragalus membranaceus and Codonopsis pilosula extracts on growth performance, antioxidant capacity, immune status, and intestinal health in broilers. *Front. Vet. Sci.* 10. doi: 10.3389/fvets.2023.1302801

Liu, C. W., Zeng, H. Z., Jiang, R. G., Wang, K. F., Ouyang, J., Wen, S., et al. (2023d). Effects of mulberry leaf fu tea on the intestines and intestinal flora of goto-kakizaki type 2 diabetic rats. *Foods* 12, 4006. doi: 10.3390/foods12214006

Liu, H., Zhao, H., Che, J. J., and Yao, W. J. (2022). Naringenin protects against hypertension by regulating lipid disorder and oxidative stress in a rat model. *Kidney Blood Press Res.* 47, 423–432. doi: 10.1159/000524172

Liu, Y. F., Zhao, H. B., Liu, X. M., You, W., Cheng, H. J., Wan, F. C., et al. (2016). Substitution of wheat for corn in beef cattle diets: digestibility, digestive enzyme activities, serum metabolite contents and ruminal fermentation. *Asian-Australas J. Anim. Sci.* 29, 1424–1431. doi: 10.5713/ajas.15.0866

Long, L. N., Kang, B. J., Jiang, Q., and Chen, J. S. (2020). Effects of dietary Lycium barbarum polysaccharides on growth performance, digestive enzyme activities, antioxidant status, and immunity of broiler chickens. *Poult Sci.* 99, 744–751. doi: 10.1016/j.psj.2019.10.043

Lopreiato, V., Alharthi, A. S., Liang, Y. S., Elolimy, A. A., Bucktrout, R. , Socha, M. T., et al. (2023). Influence of cobalt source, folic acid, and rumen-protected methionine on performance, metabolism, and liver tissue one-carbon metabolism biomarkers in peripartal holstein cows. *Anim. (Basel).* 13, 2107. doi: 10.3390/ani13132107

Lu, Y. L., Li, D., Huang, Y. Y., Sun, Y. Y., Zhou, H. M., Ye, F. R., et al. (2023). Pretreatment with eupatilin attenuates inflammation and coagulation in sepsis by suppressing JAK2/STAT3 signaling pathway. *J. Inflammation Res.* 16, 1027–1042. doi: 10.2147/jir.s393850

Lu, Y. Y., Min, Q. Q., Zhao, X. Y., Li, L., Zhao, G. J., and Dong, J. Z. (2024). Eupatilin attenuates doxorubicin-induced cardiotoxicity by activating the PI3K-AKT signaling pathway in mice. *Mol. Cell. Biochem.* 479, 869–880. doi: 10.1007/s11010-023-04769-1

Ma, K. Y., Bai, T. T., Hu, P. F., Zhao, M., Xiu, Z., Surilige,, et al. (2023). Sanwei sandalwood decoction improves the function of the gut microbiota in heart failure. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1236749

Nguse, M., Yang, Y., Fu, Z. L., Xu, J. C., Ma, L., and Bu, D. P. (2022). Phyllanthus emblica (Amla) fruit powder as a supplement to improve preweaning dairy calves' health: effect on antioxidant capacity, immune response, and gut bacterial diversity. *Biol. (Basel).* 11, 1753. doi: 10.3390/biology11121753

Peña-Torres, E. F., Dávila-Ramírez, J. L., Peña-Ramos, E. A., Valenzuela-Melendres, M., Pinelli-Saavedra, A., Avendaño-Reyes, L., et al. (2021). Effects of dietary ferulic acid on growth performance, carcass traits and meat quality of heifers. *J. Sci. Food Agric.* 101, 548–554. doi: 10.1002/jsfa.10666

Rabee, A. E., Mohamed M Ghandour, M., Sallam, A., Elwakeel, E. A., Mohammed, R. S., Sabra, E. A., et al. (2024). Rumen fermentation and microbiota in Shami goats fed on condensed tannins or herbal mixture. *BMC Vet. Res.* 20, 35. doi: 10.1186/s12917-024-03887-2

Salazar, L. F. L., Nero, L. A., Campos-Galvão, M. E. M., Cortinhas, C. S., Acedo, T. S., Tamassia, L. F. M., et al. (2019). Effect of selected feed additives to improve growth and health of dairy calves. *PloS One* 14, e0216066. doi: 10.1371/journal.pone.0216066

Schalch Junior, F. J., Polizel, G. H. G., Cançado, F. A. C. Q., Fernandes, A. C., Mortari, I., Pires, P. R. L., et al. (2022). Prenatal supplementation in beef cattle and its effects on plasma metabolome of dams and calves. *Metabolites* 12, 347. doi: 10.3390/metabo12040347

Shi, Q. Q., Li, X., Zhu, D. J., Jiang, J. J., and Li, X. G. (2022). Comprehensive analysis of the antibacterial and anti-hepatoma activity of metabolites from jujube fruit. *Food Biosci.* 47. doi: 10.1016/j.fbio.2021.101511

Song, C., Zhang, T. T., Xu, D. H., Zhu, M. M., Mei, S. H., Zhou, B. J., et al. (2023). Impact of feeding dried distillers' grains with solubles diet on microbiome and metabolome of ruminal and cecal contents in Guanling yellow cattle. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1171563

Swelum, A. A., Hashem, N. M., Abdelnour, S. A., Taha, A. E., Ohran, H., Khafaga, A. F., et al. (2021). Effects of phytogenic feed additives on the reproductive performance of animals. *Saudi J. Biol. Sci.* 28, 5816–5822. doi: 10.1016/j.sjbs.2021.06.045

Tagousop, C. N., Tamokou, J. D., Ekom, S. E., Ngnokam, D., and Voutquenne-Nazabadioko, L. (2018). Antimicrobial activities of flavonoid glycosides from Graptophyllum granulosa and their mechanism of antibacterial action. *BMC Complem. Altern. Med.* 18, 252. doi: 10.1186/s12906-018-2321-7

Umme, H., Kandagalla, S., Sharath, B. S., Jyothsna, K., and Manjunatha, H. (2021). Network pharmacology approach uncovering pathways involved in targeting Hsp90 through curcumin and epigallocatechin to control inflammation. *Curr. Drug Discovery Technol.* 18, 127–138. doi: 10.2174/1570163816666191210145652

Wang, M. W., Huang, H. J., Wang, L., Yang, H. S., He, S. W., Liu, F., et al. (2021). Herbal extract mixture modulates intestinal antioxidative capacity and microbiota in weaning piglets. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.706758

Wang, Y., Liu, X. J., Chen, J. B., Cao, J. P., Li, X., and Sun, C. D. (2022a). Citrus flavonoids and their antioxidant evaluation. *Crit. Rev. Food Sci. Nutr.* 62, 3833–3854. doi: 10.1080/10408398.2020.1870035

Wang, Y., Nan, X. M., Zhao, Y. G., Jiang, L. S., Wang, H., Zhang, F., et al. (2022b). Changes in the profile of fecal microbiota and metabolites as well as serum metabolites and proteome after dietary inulin supplementation in dairy cows with subclinical mastitis. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.809139

Wang, H. X., Peng, Z. H., and Sun, H. M. (2022c). Antifungal activities and mechanisms of trans-cinnamaldehyde and thymol against food-spoilage yeast Zygosaccharomyces rouxii. *J. Food Sci.* 87, 1197–1210. doi: 10.1111/1750-3841.16075

Wang, D. D., Tang, G. F., Li, Y. Y., Yu, J. J., Lei, X. J., Cao, Y. C., et al. (2023). Differences in serum metabolome profile explain individual variations in the growth performance of young goats. *J. Proteomics.* 288, 104982. doi: 10.1016/j.jprot.2023.104982

Wang, H. Y., Xu, R. Y., Zhang, H., Su, Y., and Zhu, W. Y. (2020). Swine gut microbiota and its interaction with host nutrient metabolism. *Anim. Nutr.* 6, 410–420. doi: 10.1016/j.aninu.2020.10.002

Wei, G., Guan, Y., Yin, Y., Duan, J., Zhou, D., Zhu, Y. R., et al. (2013). Anti-inflammatory effect of protocatechuic aldehyde on myocardial ischemia/reperfusion injury *in vivo* and *in vitro*. *Inflammation* 36, 592–602. doi: 10.1007/s10753-012-9581-z

Wu, Z. A., Du, Z. Z., Tian, Y. Y., Liu, M., Zhu, K. L., Zhao, Y. F., et al. (2022). Inulin accelerates weight loss in obese mice by regulating gut microbiota and serum metabolites. *Front. Nutr.* 9. doi: 10.3389/fnut.2022.980382

Wu, J. R., Zhong, W. J., Chen, Z. D., Zhu, B. Q., Jiang, Y. Y., and Wierzbicki, P. M. (2020). The protective impact of Trans-Cinnamaldehyde (TCA) against the IL-1b induced inflammation *in vitro* osteoarthritis model by regulating PI3K/AKT pathways. *Folia Histochem. Cytobiol.* 58, 264–271. doi: 10.5603/FHC.a2020.0025

Xie, K. L., Chang, S. H., Ning, J., Guo, Y. R., Zhang, C., Yan, T. T., et al. (2023). Dietary supplementation of Allium mongolicum modulates rumen-hindgut microbial community structure in Simmental calves. *Front. Microbiol.* 14. doi: 10.3389/fmicb.2023.1174740

Zeng, P., Li, J., Chen, Y. L., and Zhang, L. J. (2019). The structures and biological functions of polysaccharides from traditional Chinese herbs. *Prog. Mol. Biol. Transl. Sci.* 163, 423–444. doi: 10.1016/bs.pmbts.2019.03.003

Zeng, X., Li, J. X., Lyu, X., Chen, J., Chen, X., and Guo, S. (2022). Untargeted metabolomics reveals multiple phytometabolites in the agricultural waste materials and medicinal materials of codonopsis pilosula. *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.814011

Zhang, Q., Zhang, X., Wang, Q., and Chen, S. Q. (2023). Dioscoreae rhizoma starch improves chronic diarrhea by regulating the gut microbiotas and fecal metabolome in rats. *Food Sci. Nutr.* 11, 6271–6287. doi: 10.1002/fsn3.3567

Zhang, Q., Zhang, Y. M., Zeng, L., Chen, G. W., Zhang, L., and Liu, M. F. (2021). The role of gut microbiota and microbiota-related serum metabolites in the progression of diabetic kidney disease. *Front. Pharmacol.* 12. doi: 10.3389/fphar.2021.757508

Zhao, X. Y., Wang, Y. X., Wang, L. Y., Sun, S. Q., Li, C. Y., Zhang, X. W., et al. (2024). Differences of serum glucose and lipid metabolism and immune parameters and blood metabolomics regarding the transition cows in the antepartum and postpartum period. *Front. Vet. Sci.* 11. doi: 10.3389/fvets.2024.1347585

Zhu, D. P., and Yu, B. (2018). Synthesis of the diverse glycosides in traditional Chinese medicine. *Chin. J. Chem.* 368, 681–691. doi: 10.1002/cjoc.201800156

Zou, W. J., Deng, L. L., Wu, H. D., Liu, Z. Y., Lu, W., and He, Y. Y. (2022). Untargeted metabolomics profiling reveals beneficial changes in the milk of sows supplemented with fermented compound Chinese medicine feed additive. *Anim. (Basel).* 12, 2879. doi: 10.3390/ani12202879

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Relationship between pathogenic *E.coli* O78-induced intestinal epithelial barrier damage and Zonulin expression levels in yaks

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To explore whether the intestinal damage of yak colibacillosis resulted from the regulation of Zonulin expression by its pathogenic bacteria, the overexpression and interference plasmids of Zonulin were designed and cultured in Tranwell after cell transfection. Then qRT-PCR and Western blot were used to detect the results of cell transfection, 200 mL 1×105 CFU/mL *E.coli* O78 was added for 4 hours, transmembrane resistance was measured by transmembrane resistance meter, FD4 fluorescence concentration in the lower chamber was detected by enzyme labeling instrument, bacterial translocation was measured by CFU counting method, and epithelial mucin (MUC1, MUC2) and tight junction protein (FABP2, Occludin, ZO-1) were detected by qRT-PCR.

Results: The Zonulin gene overexpression and knockout cell lines were successfully constructed, the TEER value of the barrier of Zonulin overexpression cell lines began to decrease at 1 h after the addition of *E.coli* O78 and reached the lowest value at 4 h, and the TEER value of Zonulin interference cell lines decreased within 1-4 h after the addition of *E.coli* O78. At 4 h, the FD4 passing capacity of Zonulin overexpression cell lines was significantly higher than that of interfering cell lines, reaching twice as much as siRNA-1. The amount of bacterial translocation in overexpressed cell lines increased rapidly within 1-4 h, and the concentration of *E.coli* in the lower chamber was significantly higher than that in the siRNA-1 group at 4 h, but there was no significant change in the siRNA-1 group in the 1-4 h. There was no significant change in the mRNA level of MUC1 in Zonulin overexpression and interference cell lines after the addition of *E.coli* O78. In the overexpression group, the mRNA levels of MUC2, Occludin, and ZO-1 were significantly decreased, and the mRNA level of FABP2 was increased considerably. These results suggest

stimulate epithelial cells to secrete Zonulin protein. Many Zonulin proteins regulate the opening of tight junction structures, reduce the transmembrane resistance of the cell barrier, and improve the permeability of the cell barrier and the amount of bacterial translocation.

KEYWORDS

yak, pathogenicity *E.coli* O78, intestinal epithelial cell barrier, cell transfection, TEER, Zonulin

1 Introduction

Yak is an endemic breed of cattle on the Qinghai-Tibet Plateau, which occupies a dominant position in Xizang's animal husbandry and is an important part of livestock in this area (Liu et al., 2023). Yak colibacillosis is an infectious disease caused by *E.coli* (*Escherichia Coli, E.coli*) infection, showing typical symptoms such as high fever and diarrhea. The morbidity and mortality of the disease are very high, especially affecting calves and female yaks. Diarrhea in calves and yaks infected with pathogenic *E.coli* is considered to be one of the main diseases. Diarrhea occurs in sick calves 3-5 days after birth and 3-10 days after weaning. Severe dehydration and electrolyte imbalance caused by diarrhea are one of the important causes of calf death, with a fatality rate of more than 90% (Hong, 2015; Raz, 2013; Wang et al., 2022). In recent years, Escherichosis coliform in yak has brought great challenges to the yak breeding industry. The growth retardation and death of diseased yaks are the direct causes of economic losses of herdsmen, and diarrhea yaks can also cause the spread of pathogens in the environment, making it difficult for treatment (Rehman et al., 2017). At present, the conventional method for the treatment of colibacillosis in yaks is to use antibiotics. Long-term use of antibiotics will cause problems such as drug residues and intestinal flora imbalance ((Dong et al., 2020; Jia et al., 2018). At the same time, due to the complex physiological structure of the gastrointestinal system of ruminants, antibiotic treatment alone is not enough to solve gastrointestinal flora-related diseases. Probiotics are defined as living microbial supplements that have a beneficial effect on the host by improving the composition of intestinal microorganisms (Majidi-Mosleh et al., 2017). The most commonly used probiotics are *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus saliva*, *Bifidobacterium*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus thermophilus* and so on (Hossain et al., 2012). *Lactobacillus* (*Lactobacillus, LAB*) is a Gram-positive, lactic acid-producing thick-walled bacteria, which is the most common probiotic in the intestinal tract of mammals. The efficacy of lactic acid bacteria in improving bacterial diarrhea has been widely recognized, lactic acid bacteria are members of the normal microbiota in the intestine, which work together with other microorganisms in the intestine to defend against pathogenic bacteria, improve the intestinal flora, thereby alleviating bacterial diarrhea, and also reduce the possibility of pathogen transmission due to feces during yak breeding (Sadowska et al., 2010; Vinderola et al., 2007). Studies have shown that feeding lactic acid bacteria plays a beneficial role in increasing animal meat production and reducing bovine diarrhea (Kober et al., 2022). The detection of intestinal pathogens after feeding lactic acid bacteria showed that probiotics reduced the number of pathogenic bacteria by increasing the number of beneficial bacteria in the intestinal environment and inhibiting them (Fooks and Gibson, 2002; Liu et al., 2022). It can effectively reduce intestinal pathogens and balance animal intestinal flora. It is reported that after *LAB* feeding, the content of many kinds of beneficial bacteria in the intestinal tract of calf yak was higher had higher terpenoid and polyketone metabolism, and showed an inhibitory effect on some viruses at the same time (Kobayashi et al., 2017).

The intestinal barrier is a kind of defensive structure, that separates the aseptic tissue in the body from the microflora *in vitro*, and is the first line of defense of the intestinal tract against external pathogenic bacteria, its core mechanism is the selective permeation function (Felipe-Lopez et al., 2023; Rogers et al., 2023). This selective infiltration ensures the normal digestive function of the intestine and blocks the possibility of pathogens entering the tissues of the body. Microscopic scientists in the 19th century observed and defined the paracellular space between adjacent epithelial cells. Now this intercellular structure is called the intercellular junction complex, which consists of tight junctions (TightJunctions, TJ), adhesive junctions (Adherens Junctions) and desmosomes (Desmosomes) (Cereijido et al., 2004; Farquhar and Palade, 1963). It has been found that the main function of tight junction is to maintain the surface polarity of intestinal epithelial cells and reversibly prevent the spread of macromolecules and microorganisms inside and outside the epithelial cells (Kucharzik et al., 2001; Yu and Yang, 2009). Tight junction proteins mainly include transmembrane proteins Occludin, tight junction proteins Claudin, tight junction structural proteins ZO-1, ZO-2, ZO-3, and so on. The functions of these proteins have been gradually understood. It is the binding of these protein complexes with membrane lipids that control the junction and dispersion of tight junction structures and regulate paracellular diffusion, resulting in

changes in intestinal permeability (Mitic and Anderson, 1998). Blocking protein (Occludin) is the first intact membrane protein found in a tight junction. It affects the permeability of the intestinal epithelial cell barrier and regulates the entry of macromolecules (Mazzon et al., 2002; Mccarthy et al., 1996). Tight junction protein Claudin is a quadruple transmembrane protein that makes up the TJ chain, including four transmembrane domains (Transmembrane, TM), two extracellular loops containing conserved residues, and two short intracellular hydrophobic terminals (N-and C-terminals) (Tao et al., 2005) and contains 27 subtypes, and different subtypes determine the physiological characteristics of TJ. Studies have shown that the overexpression of Claudin-2 significantly increases the ionic conductivity of epithelial cells. When the expression of Claudin protein is abnormal, it usually leads to the reversible opening of tight junction structure, affecting the possibility of intestinal contents entering the intestine, and the close adhesion between cells to form permeable epithelium (Furuse et al., 2001). Subsequent studies showed that Claudin-2 formed a cation-selective paracellular pathway in addition to the tight junction permeability pathway (Amasheh et al., 2002; Colegio et al., 2002). These studies show that Claudins affect the function of TJ structure, regulate the charge of ion conductance, and form a selective intercellular pathway. The Zonula occludens (ZO) protein family, which includes ZO-1, ZO-2, and ZO-3, is a scaffold protein of TJ and belongs to the membrane-associated guanosine kinase-like protein (Maguk) family (Gonzalez-Mariscal et al., 2000). Many proteins contribute to the integrity of intestinal barriers and tight junctions. In the intestinal mucosa, the mucus layer plays an important role in stabilizing the host and microbial environment. Changes in the mucus layer or the interaction between abnormal microorganisms and the mucus layer have been proven to be the main causes of intestinal inflammation (Rokhsefat et al., 2016). The mucus layer secretes mucus hydrogel, which prevents the rapid transfer of bacteria on the surface of the intestinal barrier by adhering to the pathogen (Leoncini et al., 2024). Among them, the mucin family (Mucin, MUCs) is the main protein component of mucus on the mucous surface, which is mainly produced by goblet cells. MUC1 and MUC2 can adhere to pathogenic bacteria and protect intestinal epithelial mucosa (Cox et al., 2023; Noah et al., 2011). Changes in mucin production can weaken the intestinal mucus barrier and cause bacterial translocation and immune response, while mucosal inflammatory diseases such as ulcerative colitis are usually due to impaired expression of MUC2 protein (Boltin et al., 2013). Fatty acid binding protein (fatty acid-binding protein, FABP) is involved in the transport of intracellular fatty acids from the membrane to the membrane. After binding to FABP, fatty acids maintain their solubility and transport to the organelles (Huang et al., 2022). At present, at least 9 kinds of FABP protein family have been identified. FABP2, FABP1, and FABP9 are the three fatty acid-binding proteins specific to the small intestine (Hotamisligil and Bernlohr, 2015). Among them, FABP2 is the most abundant FABP in the small intestine, including membrane FABP2 and soluble FABP2, accounting for about 2% of intestinal epithelial cell proteins (Ockner and Manning, 1974). FABP2 is usually used as an indicator of intestinal functional integrity, and its release is affected by intestinal inflammation and intestinal microbiota (Dutta et al., 2019; Guerrant et al., 2016; Stevens et al., 2018).

Zonulin protein was originally considered a cholera vibrio toxin analog, which is related to the pathogenesis of diarrhea. In subsequent studies, Zonulin was identified as a precursor of haptoglobin 2 (Haptoglobin2, HP2). The release of Zonulin secreted by animal bodies is usually associated with diarrhea. The latest reports suggest that Zonulin is also associated with diseases such as diabetes and bipolar disorder (Sturgeon and Fasano, 2016; Wood et al., 2020), as the only known intestinal function regulator, the continuous study of its regulatory mechanism makes Zonulin protein has great potential for the treatment of diarrheal diseases. It has been reported that pathogens can increase the production of Zonulin in intestinal mucosa and combine with intestinal mucosal receptors through autocrine or paracrine, so as to improve intestinal permeability, promote intestinal inflammation, and increase the translocation ability of pathogenic bacteria (Sturgeon and Fasano, 2016).

In previous studies in our laboratory, we explored the effects of pathogenic *E.coli* on the intestinal epithelial barrier and intestinal functional proteins and proved that LAB has the ability to restore the destruction of the intestinal barrier caused by *E.coli* (Zhang et al., 2024a). Combined with their effects on the expression of Zonulin protein, we speculate that the damage of yak *E.coli* to the intestinal tract is due to the regulation of Zonulin expression by pathogenic bacteria. In this experiment, we will design Zonulin overexpression plasmid and siRNA, transfect cells, and culture TransWell to construct yak intestinal epithelial cell barrier of Zonulin overexpression and interference. After adding 200 μL 1×10^5 CFU/mL *E.coli O78* to the treated epithelial cell barrier for 4 hours, the transmembrane resistance of the epithelial barrier was measured by transmembrane resistance meter, the bacterial translocation was measured by CFU counting method, and the FD4 fluorescence concentration in the lower chamber was detected by enzyme labeling instrument. The expression levels of epithelial mucin (MUC1, MUC2) and tight junction protein (FABP2, Occludin, ZO-1) were detected by qRT-PCR to explore whether the damage of intestinal epithelial barrier function caused by pathogens was affected by the change of Zonulin expression level.

2 Materials and methods

2.1 Preparation of pathogenic *Escherichia Coli*

Pathogenic *Escherichia coli O78* was derived from diarrheal yaks in Linzhi City, Xizang Autonomous region, and was preserved by the Clinical Key Laboratory, School of Animal Science, Xizang College of Agriculture and Animal Husbandry. The bacterial strains were resurrected, inoculated on nutrient Agar(01-023, Aobox, Beijing, China), and cultured in a constant temperature incubator (LRH-150, Shenglan, Jiangsu, China) at 37°C for 24 hours. Then a single colony was selected and put into the nutritious broth (HB0108, Hopebio, Qingdao, China) and cultured in a constant temperature shaker incubator (BSD-TF370, Boxun, Shanghai,

China) at 37°C for 18-24 hours. Eosin-methylene blue Agar (HB0107, Hopebio, Qingdao, China) was used to detect bacteria, and colony forming unit (CFU) counting method was used to determine the required strain concentration.

2.2 Cell culture and grouping

Yak intestinal epithelial cells were isolated from 30-to 60-day-old yak embryos obtained from Nyingchi slaughterhouse. Cut the calf's small intestine into a disposable petri dish, rinse with aseptic PBS, put the small intestine into a sterile Cillin bottle, add the appropriate amount of aseptic PBS, cut it into tissue blocks around 1 mm^3 with aseptic scissors, and rinse with aseptic PBS. Yak intestinal epithelial cells were cultured in DMEM-F12 (Invitrogen, CA, USA) + 10% fetal bovine serum (PAN, Adenbach, Germany) in a 5% CO_2 and 37°C incubator.

Our laboratory has used an ABC staining kit (SA1002, Boster, Wuhan, China) to detect the binding of anti-cytokeratin 18 antibody (BB12213553, BIOSS, Beijing, China), and identified the cultured cells as intestinal epithelial cells (Zhang et al., 2024b).

About 80% of the cells in the full cell bottle were digested with 0.25% trypsin (EDTA included) (25200072, Gibco, USA), and the cells were transferred to the PC membrane Transwell chamber with a pore diameter of 0.4 μm, and the number of cells was 1×10^5/well. The culture medium was changed after 48 hours and continued to be cultured for 12 hours. The experimental groups were divided into the control group (Control group, normal cultured cells) and the model group (Model group, treated with 200 μL 1×10^5 CFU/mL *E.coli O78* for 4 h) (Zhang et al., 2024a; Zhang et al., 2024b).

2.3 Construction of intestinal epithelial cell line of yak with Zonulin overexpression

2.3.1 Plasmid vector construction

According to the bovine Zonulin gene sequence (NM_001040470.2) on NCBI and the restriction site on pcDNA3.1 (+) vector, primers for Zonulin were designed by Premier6.0 and synthesized by Chengdu Qingke Biological Technology Co., Ltd. The total RNA of bovine intestinal tissue was extracted and reverse transcribed into cDNA, and then PCR was performed with Zonulin gene-specific primers. The products were digested with NheI and BamHI, and the products were recovered by gel. After ligation and transformation, the clones were selected for PCR and double restriction endonuclease digestion. Sequencing comparison of positive clone liquid to Jingke Biology Co., Ltd.

2.3.2 Plasmid DNA extraction

A plasmid extraction kit (D6826030000E31V014, OMEGA, USA) was used for plasmid extraction. The plasmid bacteria were resuscitated and cultured in the medium containing ampicillin antibiotics to amplify the plasmid. The bacterial solution of 50-200 mL was centrifuged at 4000 × g at room temperature for 10 min, and the bacteria were collected. The medium was abandoned and the 10 mL SolutionI/RNaseA mixture was added to the precipitation. The cells were completely re-suspended by a liquid transfer gun blowing or swirling. Add 10 mL Solution II and turn the centrifuge tube up and down 8-10 times to get a clear lysate. Add N3Buffer precooled by 5 mL and turn the centrifuge tube up and down 10 times until white flocculent precipitates are formed. The pyrolysis solution is collected by a syringe filter. ETR Solution is added to the lysate by 0.1x volume of ETR Solution, and the test tube is reversed 10 times. Then the 10 min is placed in an ice bath and 5 min is bathed in water at 42°C. At 25°C, 4000 × g centrifugation for 5 min, the Solution will form a blue layer at the bottom of the test tube. Transfer the supernatant to another new 50 mL test tube, add 0.5 times the volume of anhydrous ethanol at room temperature, gently reverse the test tube 6-7 times, and place 1-2 min at room temperature. The HiBind® DNA Maxi binding column was placed in a 50 mL collection tube, 20 mL of filtrate was added to the HiBind® DNA Maxi binding column, the 3 min was centrifuged by 4000xg at room temperature, and the filtrate was discarded. Re-insert the column, add 10 mL HBC Buffer, centrifuge for 3 min at room temperature 4000 × g, and discard the filtrate. The combined column was re-inserted, 10 mL DNA Wash Buffer was added, 4000 × g 3 min was centrifuged at room temperature, and the filtrate was discarded. The highest speed centrifuge dries the binding column matrix for 10 min. Place the HiBind ®DNA Maxi binding column on a clean 50 mL centrifuge tube, add 1-3 mL Endo-Free Elution Buffer directly to the HiBind ®DNA Maxi binding column matrix (the amount added depends on the expected end product concentration), and rest the 5 min at room temperature. 4000 × g centrifugal 5 min was used to elute DNA. Discard the column and store the DNA at -20°C.

2.3.3 Cell transfection

Yak intestinal epithelial cells in the logarithmic growth phase were cultured in 6-well plates, and lipo3000 transfection reagents were used to transfect pcDNA3.1-NC and pcDNA3.1-Zonulin liposomes when the cells grew to 80% and 90%, with 3 repeats in each group. The transfection system was configured as shown in Table 1. Add 2mL of complete medium to each well. The diluted transfection reagent and diluted DNA were mixed and placed for 15 min at room temperature and then added to the corresponding well. After 48 hours, the total RNA and protein of the cells were extracted, and the expression of the recombinant plasmid was detected by qRT-PCR and WB to verify the overexpression of Zonulin.

TABLE 1 Zonulin overexpression transfection system.

	Reagent name	Gauge/hole
Dilution transfection reagent	Opti-MEM	125μL
	Lipo 3000	7.5μL
Diluted DNA	Opti-MEM	125μL
	DNA	5μg
	P3000	10μL

TABLE 2 siRNA sequence.

Gene name	Gene ID	Justice chain sense (5′-3′)	Antisense chain antisense (5′-3′)
cattle-HP-1	id:280692	GGACAUCACUCCUACUUUA(dT)(dT)	UAAAGUAGGAGUGAUGUCC(dT)(dT)
cattle-HP-2	id:280692	GACAGAAGGUACCUGUCAA(dT)(dT)	UUGACAGGUACCUUCUGUC(dT)(dT)
cattle-HP-3	id:280692	GGUUCGCUAUCAGUGCAAA(dT)(dT)	UUUGCACUGAUAGCGAACC(dT)(dT)

2.4 Zonulin interferes with the construction of intestinal epithelial cell line of yak

2.4.1 siRNA sequence design

According to the CDS region sequence of the Zonulin gene, Zonulin small interference RNA was designed using the siRNA online design website https://rnaidesigner.invitrogen.com/rnaiexpress/, and siRNA was synthesized by Qingke Biotechnology Co., Ltd. The siRNA sequence is shown in Table 2.

2.4.2 siRNA transfection

The dry siRNA powder was diluted to 20 μM with ddH_2O without RNA enzyme and stored at -20 °C after sub-packaging. Lipo3000 transfection reagent was used for cell transfection without adding P3000. The transfection system is shown in Table 3. Complete medium 2 mL was added to each well, diluted transfection reagent and diluted siRNA were mixed and 15 min was placed at room temperature to add to the corresponding well. After 48 hours, the total RNA and protein were extracted, and the expression of Zonulin was detected by qRT-PCR and WB to verify the effect of Zonulin interference.

2.5 Effects of *E. coli* on Zonulin overexpression and interference with cell barrier

2.5.1 Establishment of epithelial barrier *in vitro* of yaks

After overexpressing and interfering with the construction of the yak intestinal epithelial cell line, the monolayer epithelial barrier model of cultured cells in the TransWell chamber was established, and the transmembrane resistance of the upper and lower compartments was measured every 12 hours to determine the construction of cell barrier. For the establishment of Zonulin overexpression and interference monolayer cell barrier, *E.coli O78* with 1×10^5 CFU was cultured for 4 h, and the grouping names were pcDNA3.1-Zonulin and siRNA-1.

TABLE 3 siRNA transfection system.

	Reagent name	Gauge/hole
Dilution transfection reagent	Opti-MEM	125μL
	Lipo 3000	7.5μL
Diluted siRNA	Opti-MEM	125μL
	siRNA-1/2/3	75pmol

2.5.2 Detection of transmembrane resistance

The transmembrane resistance of the monolayer epithelial barrier was measured by a Transmembrane Resistance Meter (MERS00002, Merck, Germany). The cells successfully constructed with Zonulin overexpression and interference were inoculated into the Transwell chamber of 0.4 μm PC membrane. After 48 hours of incubation, the corresponding concentration and dose of *E.coli* O78 were added according to the experimental group in 2.2 above at 60 hours. The transmembrane resistance of intestinal epithelial cell barrier in different treatment groups was measured by a transmembrane resistance meter, and a TransWell with only culture medium was set as a blank control to measure the level of empty resistance. After the instrument was calibrated, the short electrode was inserted into the upper chamber of each group and the long electrode was inserted into the lower chamber of each group, and the readings were made on the transmembrane resistance meter. The change of transmembrane resistance during 1-4 h was recorded. The measured readings are processed and the final transmembrane resistance = (measurement group reading-blank group reading) × surface area is calculated.

2.5.3 FD4 permeability detection

The permeability of the epithelial barrier was evaluated by FD4 throughput. The methods are as follows: the cells successfully constructed with Zonulin overexpression and interference were inoculated into the Transwell chamber of 0.4 μm PC membrane, the medium was changed after 48 hours of culture, and the cells were treated with *E.coli O78* at the 60th hour. After the addition of *E.coli O78*, the upper chamber culture medium of the intestinal epithelial barrier model of different treatment groups was replaced with the medium containing 20 μg/mL FITC-Dextran (4KD), and the lower chamber culture medium was replaced by aseptic PBS. After being cultured for 4 hours in the constant temperature incubator of 37°C and 5% CO_2, the lower chamber PBS was collected and the relative fluorescence intensity across the cell barrier was measured under 485 nm excitation wavelength and 538 nm emission wavelength by fluorescence enzyme labeling instrument. The concentration of FD4 was calculated according to the standard curve.

2.5.4 Detection of bacterial translocation

The number of *E.coli O78* crossing the cell barrier into the lower chamber was calculated by the CFU counting method. The methods are as follows: the cells successfully constructed with Zonulin

overexpression and interference were inoculated into the Transwell chamber of 0.4 μm PC membrane, and the yak intestinal epithelial cell barrier model was constructed and treated with *E.coli O78* in the same way. At the 4th hour after the addition of *E.coli O78*, the lower chamber culture medium of TransWell was collected for bacterial CFU count. The lower chamber culture medium diluted by aseptic PBS was used to 10^{-8}, and 200 μL of bacterial PBS suspension with suitable dilution concentration (10^{-5}, 10^{-6}, 10^{-7}) was added to the aseptic plate, and the unsolidified eosin methylene blue Agar medium at about 40°C was added to the plate, and the liquid was evenly distributed by shaking the plate. After the culture medium was solidified and incubated in a constant temperature incubator for more than 16 hours, a single colony appeared. The morphology of the colony was observed to distinguish and record the number of *E.coli O78*, *E.coli O78* concentration = colony number × dilution times × 5. And calculate the average concentration of *E.coli O78* (n= 3).

2.6 Effects of Zonulin overexpression and interference on the expression of barrier protein mRNA

Total RNA was extracted from the treated yak intestinal epithelial cells, and the expression levels of mucin genes (MUC1, MUC2) and tight junction-related protein genes (FABP2, Occludin, ZO-1) were detected by qRT-PCR.

2.7 Data processing

The statistical analysis of the data is carried out by *GraphPadPrism9.0*. Each group of experiments was repeated more than 3 times. For the data of normal distribution, the data were expressed by mean ± standard deviation. For continuous variables, the values are the median and quartile range of data with non-normal distribution. For classified variables, take the percentage value. For the comparison of the two groups, the P value is obtained by using *one-way Student t-test* test and *Mann-Whitney* nonparametric test to determine the difference between groups with normal distribution data. For multiple group comparisons, the P-value is derived by the one-way ANOVA (continuous variable) or Chi-square test (categorical variable), and then the *Bonferroni* test is used to compare the group means. For all data, p <0.05 was considered statistically significant.

3 Results

3.1 Overexpression of Zonulin and interference with the construction of cell line

The results of overexpression of Zonulin and interference with cell line construction are shown in Figure 1. After qRT-PCR

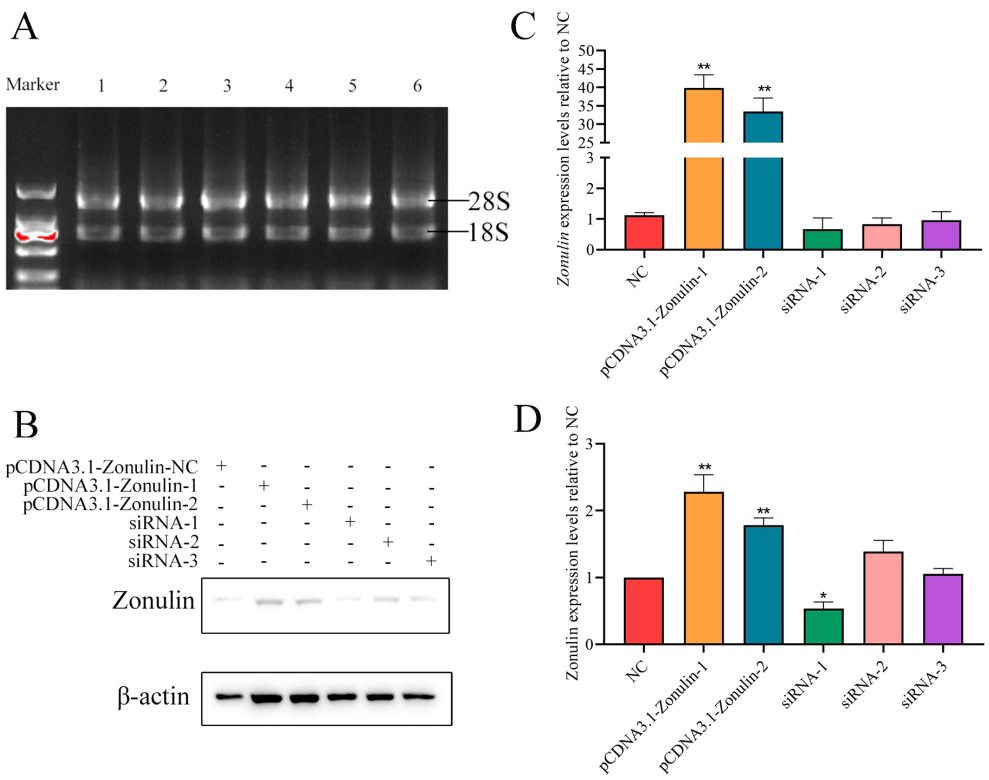

FIGURE 1
Results of Zonulin overexpression and siRNA interference efficiency detection. **(A)** RNA integrity verification. **(B)** results of WB gray value of Zonulin protein. **(C)** overexpression and interference of Zonulin mRNA relative NC expression. **(D)** relative NC expression of overexpression and interference of Zonulin protein. *p<0.05, **p<0.01.

detection, the expression level of Zonulin mRNA in overexpressed cell lines was more than 40 times that of the control group (Figure 1B). The results of interfering with the expression level of Zonulin mRNA in cell lines showed that siRNA-1 had the best effect on Zonulin knockdown. The effect of overexpression and knockdown verified by Western-blot is shown in Figures 1C, D. The overexpression and knockout cell lines of the Zonulin gene were successfully constructed, which were consistent with the expression level of mRNA, and the effect of siRNA-1 knockdown was the best.

3.2 Detection of barrier function of epithelial cells in yak

3.2.1 Detection results of transmembrane resistance

The change of transmembrane resistance of the Zonulin overexpression cell line with time was shown in Figure 2A. The transmembrane resistance of the cell barrier began to decrease at 1 h, decreased to less than 100 Ω cm^2 at 2 h, and reached the lowest value at 4 h (Figure 2B). The change of transmembrane resistance of the Zonulin interference cell line is shown in Figure 2A. The transmembrane resistance showed a downward trend in 1-4 h and decreased to the lowest value of 160 Ω cm^2 at 4 h. At 4 h, the transmembrane resistance of Zonulin overexpression was significantly higher than that of interfering cell lines ($p < 0.01$) (Figure 2B).

3.2.2 FD4 permeability test results

The amount of FD4 passed by the Zonulin overexpressing cell line was significantly higher than that of the interfering cell line ($p < 0.01$), which was twice as much as that of siRNA-1 at 4 h.

3.2.3 Detection results of bacterial translocation

The change of the number of *E.coli O78* in the lower chamber of TransWell at 1-4 h was shown in Figure 2. The bacterial translocation of overexpressed cell lines increased rapidly within 1-4 h, and the concentration of *E.coli O78* in the lower chamber reached about 8×10^5 CFU/mL at 4 h, which was higher than that in the siRNA-1 group. In siRNA-1 group, the amount of bacterial translocation was less than 2×10^5 CFU/mL in 1-4 h, and there was little change in the whole.

3.3 qRT-PCR test results

The results of the detection of the Zonulin overexpression and mRNA expression levels of mucin and tight junction-associated proteins in cell lines are shown in Figure 3. There was no significant change in the expression level of MUC1 between the two groups, and there was no significant difference in the expression level of mRNA between the two treatment groups ($p > 0.05$), which was consistent with our previous results (Figure 3B). The mRNA levels of MUC2, Occludin, and ZO-1 decreased significantly ($p < 0.05$)

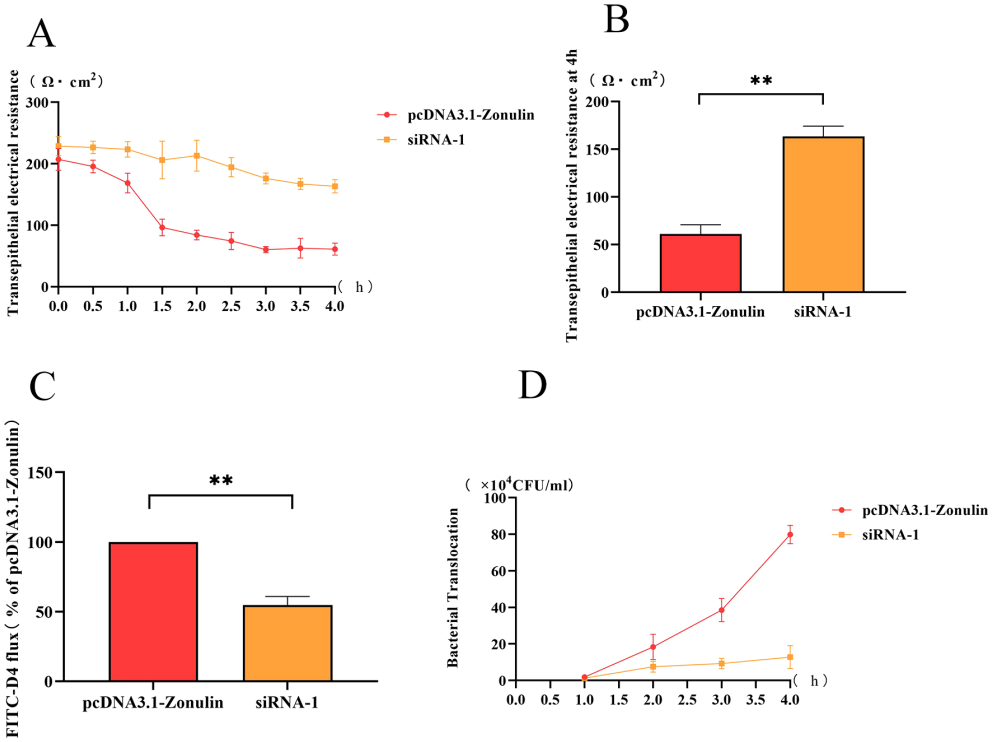

FIGURE 2
The changes of intestinal barrier function after Zonulin overexpression and interference. **(A)** *E.coli O78* affected the change of transmembrane resistance of intestinal epithelial barrier during 1-4 h. **(B)** the transmembrane resistance of intestinal epithelial barrier at 4 h. **(C)** the content of FD4 in the lower chamber at 4 h. **(D)** the amount of *E.coli* translocation changed during 1-4 h. ***p*<0.01.

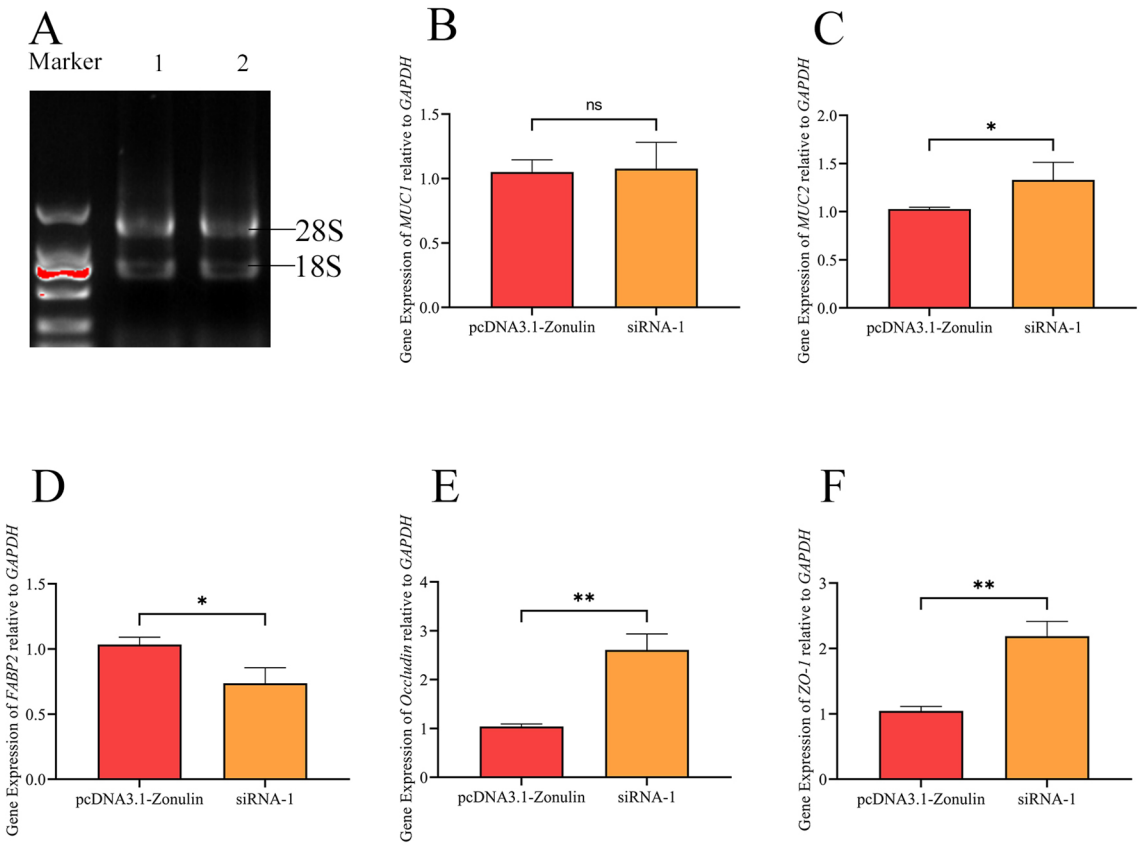

FIGURE 3
The effect of *E.coli O78* on the expression of mucin and tight junction associated protein mRNA in intestinal epithelial barrier after Zonulin overexpression and interference. **(A)** RNA integrity verification. **(B)** the relative expression of MUC1 was compared. **(C)** the relative expression of MUC2 was compared. **(D)** the relative expression of FABP2 was compared. **(E)** the relative expression of Occludin was compared. **(F)** the relative expression of ZO-1 was compared. *$p<0.05$, **$p>0.01$, $^{ns}p < 0.05$.

(Figures 3C, E, F), while the mRNA levels of FABP2 increased significantly ($p < 0.05$) (Figure 3D).

4 Discussion

The intestinal barrier has a complex multi-layer structure, which is the physical and functional barrier between the body and the intestinal contents. The destruction of the intestinal barrier is one of the main causes of inflammatory bowel disease and diarrhea, as well as the disorder of intestinal microbiota and the translocation of harmful molecules (Camara-Lemarroy et al., 2018; Martini et al., 2017; Schumann et al., 2017). A tight junction structure is the main structure for the intestinal tract to maintain selective permeability and cellular barrier. Zonulin is a tight junction regulator, and its concentration has a great impact on intestinal permeability. Bacterial stimulation induces the release of Zonulin, which is usually accompanied by significant secretion of Zonulin, which stimulates the intestinal barrier. The induction of TJ by Zonulin may be the host's defense mechanism. Defensive flushing is carried out through the secretion of intestinal fluid to avoid the colonization of bacteria in the small intestine (El et al., 2002). Zonulin acts through PAR2 and EGF receptors in intestinal epithelial cells, resulting in tight junction uncoupling, thereby increasing intestinal permeability (Fasano, 2011). Although diarrhea caused by the secretion of Zonulin flushes the intestinal tract to expel pathogens, the opening of TJ results in the disappearance of the selective permeability of the cell barrier, followed by the entry of bacteria into the body and the occurrence of intestinal inflammation and infection. Further stimulation of bacteria in turn continues to affect the release of Zonulin from intestinal epithelium, causing a vicious cycle of diarrhea.

In this experiment, we constructed the barrier of Zonulin inhibition and overexpression of yak intestinal epithelial cells to explore whether Zonulin gene expression affects the pathogenicity of *E.coli O78* and the regulation of tight junction-related genes. After Zonulin knock down, the effect of *E.coli O78* on intestinal barrier function was weakened. Compared with the overexpression group, FD4 permeability and bacterial translocation decreased significantly, and the transmembrane resistance of the intestinal barrier was maintained to some extent. It is suggested that in the case of overexpression of Zonulin, bacteria act on the intestinal mucous layer to destroy the intestinal barrier and stimulate epithelial cells to secrete Zonulin protein. A large amount of Zonulin protein regulates the opening of a tight junction

structure, reduces the transmembrane resistance of the cell barrier, and increases the permeability of the cell barrier and the possibility of bacteria penetrating through the cell barrier. The downregulation of MUC2 represents the disappearance of the intestinal mucus layer and weakens the adhesion to bacteria. Then it brings the bacterial translocation and breaks through the barrier, which is consistent with the result indicated by the amount of bacterial translocation. We found that in the Zonulin overexpression cell line, the mRNA expression levels of tight junction proteins ZO-1 and Occludin decreased, which represented the destruction of the tight junction structure of the epithelial barrier. The down-regulation of mRNA of the constituent protein ZO-1 led to the instability of the tight junction structure, and the subsequent down-regulation of Occludin indicated the destruction of tight junction function, which was consistent with the previously reported functional study of Zonulin (Camara-Lemarroy et al., 2020). FABP2 is the most abundant fatty acid-binding protein in the small intestine (Hotamisligil and Bernlohr, 2015). Our study found that the overexpression of Zonulin relatively caused the increase of the expression of FABP2, which proved that there was a positive correlation between Zonulin and FABP2, which was also directly related to the overgrowth of bacteria in the small intestine, which was consistent with the results of previous studies (Zhang et al., 2024a). In contrast to overexpression, Zonulin knockdown maintained the mRNA expression of ZO-1 and Occludin proteins at the same time, which represented the integrity of tight junction structure and normal function of tight junction protein. It has been reported that inhibition of Zonulin can protect the tight junction of the intestinal barrier from pathogen exposure and inhibit activation of astrocytes (Zhao et al., 2023), and the use of Zonulin antagonist on respiratory epithelial cells significantly maintained the abundance and distribution of tight junction protein and the stability of transmembrane resistance (Kalsi et al., 2020). Our knockdown of Zonulin also maintains the mRNA expression of key tight junction proteins and protects the normal function of the cellular barrier.

In previous experiments in our laboratory, we explored the protective effect of *LAB* on intestinal barrier damage caused by *E.coli* O78 and obtained that LAB can regulate the expression of Zonulin, control the stability of the intestinal barrier, and protect the tight junction structure destroyed by *E.coli O78* (Zhang et al., 2024a). Similarly, the knockdown cell line of Zonulin also has the ability to resist the damage of the intestinal barrier caused by *E.coli O78*, while controlling the integrity of the intestinal tight junction structure and normal function. Combined with the results of previous experiments, we speculated that *E.coli O78* contact with the cellular barrier will cause the release of Zonulin, followed by the loss of intestinal tight junction function and the increase of cellular barrier permeability. At the same time, *E.coli O78* can destroy the mucus barrier, resulting in bacterial translocation and increased barrier permeability. *Lactobacillus Lac-2* could regulate the release of Zonulin from the intestinal epithelial barrier of yaks and reduce the stimulation of *E.coli O78* to the intestinal barrier. When the secretion of Zonulin decreased, *E.coli O78* could not cause the destruction of intestinal tight junction-related functions. At the same time, *Lactobacillus Lac-2* could compete with pathogens and had antibacterial ability, which affected the activity and translocation of *E.coli O78*, and reduced the number of *E.coli O78* translocation through the barrier. It also reduced the damage of *E.coli O78* to the mucus layer, so we observed the up-regulation of MUC2 and the decrease of bacterial translocation. From the above results, we speculate that *LAB* can restore the intestinal epithelial barrier damage caused by *E.coli O78*. On the one hand, its function is to reduce the opening of tight junction structures caused by Zonulin regulation by down-regulating the expression level of Zonulin. On the other hand, the competition and inhibition of *LAB* on *E.coli O78* growth reduced the damage of the barrier mucus layer caused by *E.coli O78*. The combined effects of the two aspects resulted in the increase in transmembrane resistance, the decrease in bacterial translocation, a decrease in barrier permeability, the increase in mucin expression level, the increase in tight junction protein expression level, the decrease in Zonulin protein expression level and the decrease in FABP2 protein expression level after *LAB*. From the results, we think that Lactobacillus Lac-2 from yak can recover the damage of the intestinal barrier caused by *E.coli O78*, and it is expected to be an effective microbial intervention therapy for E.coli disease in yaks. However, the results of our study only show that yak-derived *E.coli* O78 causes the destruction of the intestinal barrier by affecting the changes of intestinal Zonulin protein levels, and the mechanism of the intestinal barrier disruption caused by the restoration of *E.coli* O78 by yak-derived Lactobacillus Lac-2 is still unclear and needs to be further explored.

In conclusion, *E.coli* O78 from yak can cause damage to the intestinal barrier by influencing changes in the level of Zonulin protein in the yak gut, including downregulation of tight junction protein expression, disturbance of intestinal microbiota, and increased intestinal permeability. This study provides theoretical support for the mechanism of probiotics in the treatment of yak colibacillosis caused by *E.coli* O78, and has a certain effect on the improvement of yak breeding industry in Tibetan areas. Future research will explore the specific mechanism by which probiotics can achieve the effect of treating yak colibacillosis.

5 Conclusions

In this experiment, we constructed a yak intestinal epithelial cell line with overexpression and interference of Zonulin and investigated the effects of *E.coli O78* on intestinal barrier transmembrane resistance, FD4 permeability, and bacterial translocation, as well as the mRNA expression levels of mucin and tight junction related genes MUC1, MUC2, FABP2, ZO-1 and Occludin under the condition of Zonulin overexpression and interference. The results showed that Zonulin knockout could resist the destruction of the intestinal epithelial barrier caused by *E.coli O78*, significantly reduce the permeability of epithelial cells, maintain the integrity of epithelial barrier, significantly increase the mRNA expression level of mucin and tight junction protein, and maintain the integrity of intestinal mucosa and tight junction structure. It is proved that the damaging effect of yak-derived *E.coli* on the intestinal tract of yak comes from its regulation of Zonulin.

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 manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

XR: Data curation, Methodology, Writing – original draft, Writing – review & editing. BS: Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. ZC: Methodology, Writing – review & editing. JZ: Methodology, Writing – review & editing. SW: Methodology, Writing – review & editing. RL: Methodology, Writing – review & editing. MS: Methodology, Writing – review & editing. HD: Methodology, Writing – review & editing. QW: Conceptualization, 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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References

Amasheh, S., Meiri, N., Gitter, A. H., Schoneberg, T., Mankertz, J., Schulzke, J. D., et al. (2002). Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J. Cell Sci.* 115, 4969–4976. doi: 10.1242/jcs.00165

Boltin, D., Perets, T. T., Vilkin, A., and Niv, Y. (2013). Mucin function in inflammatory bowel disease: an update. *J. Clin. Gastroenterol.* 47, 106–111. doi: 10.1097/MCG.0b013e3182688e73

Camara-Lemarroy, C. R., Metz, L., Meddings, J. B., Sharkey, K. A., and Wee, Y. V. (2018). The intestinal barrier in multiple sclerosis: implications for pathophysiology and therapeutics. *Brain* 141, 1900–1916. doi: 10.1093/brain/awy131

Camara-Lemarroy, C. R., Silva, C., Greenfield, J., Liu, W. Q., Metz, L. M., and Yong, V. W. (2020). Biomarkers of intestinal barrier function in multiple sclerosis are associated with disease activity. *Multiple Sclerosis J.* 26, 1340–1350. doi: 10.1177/1352458519863133

Cereijido, M., Contreras, R. G., and Shoshani, L. (2004). Cell adhesion, polarity, and epithelia in the dawn of metazoans. *Physiol. Rev.* 84, 1229–1262. doi: 10.1152/physrev.00001.2004

Colegio, O. R., Van Itallie, C. M., Mccrea, H. J., Rahner, C., and Anderson, J. M. (2002). Claudins create charge-selective channels in the paracellular pathway between epithelial cells. *Am. J. Physiology-Cell Physiol.* 283, C142–C147. doi: 10.1152/ajpcell.00038.2002

Cox, K. E., Liu, S., Lwin, T. M., Hoffman, R. M., Batra, S. K., and Bouvet, M. (2023). The mucin family of proteins: candidates as potential biomarkers for colon cancer. *Cancers* 15(5):1491. doi: 10.3390/cancers15051491

Dong, H., Liu, B., Li, A., Iqbal, M., Mehmood, K., Jamil, T., et al. (2020). Microbiome analysis reveals the attenuation effect of lactobacillus from yaks on diarrhea via modulation of gut microbiota. *Front. Cell. Infection Microbiol.* 10. doi: 10.3389/fcimb.2020.610781

Dutta, D., Methe, B., Amar, S., Morris, A., and Lim, S. H. (2019). Intestinal injury and gut permeability in sickle cell disease. *J. Trans. Med.* 17, 183. doi: 10.1186/s12967-019-1938-8

El, A. R., Panigrahi, P., Bamford, P., Berti, I., Not, T., Coppa, G. V., et al. (2002). Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology* 123, 1607–1615. doi: 10.1053/gast.2002.36578

Farquhar, M. G., and Palade, G. E. (1963). Junctional complexes in various epithelia. *J. Cell Biol.* 17, 375–412. doi: 10.1083/jcb.17.2.375

Fasano, A. (2011). Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiol. Rev.* 91, 151–175. doi: 10.1152/physrev.00003.2008

Felipe-Lopez, A., Hansmeier, N., Danzer, C., and Hensel, M. (2023). Manipulation of microvillar proteins during Salmonella enterica invasion results in brush border effacement and actin remodeling. *Front. Cell. Infection Microbiol.* 13. doi: 10.3389/fcimb.2023.1137062

Fooks, L. J., and Gibson, G. R. (2002). *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. *FEMS Microbiol. Ecol.* 39, 67–75. doi: 10.1111/j.1574-6941.2002.tb00907.x

Furuse, M., Furuse, K., Sasaki, H., and Tsukita, S. (2001). Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J. Cell Biol.* 153, 263–272. doi: 10.1083/jcb.153.2.263

Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Semin. Cell Dev. Biol.* 11, 315–324. doi: 10.1006/scdb.2000.0178

Guerrant, R. L., Leite, A. M., Pinkerton, R., Medeiros, P. H., Cavalcante, P. A., Deboer, M., et al. (2016). Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PloS One* 11, e158772. doi: 10.1371/journal.pone.0158772

Hong, S. (2015). Diagnosis and control of colibacillosis in calves and yaks. *Chin. J. Anim. Husbandry Veterinary Abstracts* 31, 136.

Hossain, M. E., Ko, S. Y., Kim, G. M., Firman, J. D., and Yang, C. J. (2012). Evaluation of probiotic strains for development of fermented Alisma canaliculatum and their effects on broiler chickens. *Poultry Sci.* 91, 3121–3131. doi: 10.3382/ps.2012-02333

Hotamisligil, G. S., and Bernlohr, D. A. (2015). Metabolic functions of FABPs–mechanisms and therapeutic implications. *Nat. Rev. Endocrinol.* 11, 592–605. doi: 10.1038/nrendo.2015.122

Huang, X., Zhou, Y., Sun, Y., and Wang, Q. (2022). Intestinal fatty acid binding protein: A rising therapeutic target in lipid metabolism. *Prog. Lipid Res.* 87:101178. doi: 10.1016/j.plipres.2022.101178

Jia, Z., Chen, A., Bao, F., He, M., Gao, S., Xu, J., et al. (2018). Effect of nisin on microbiome-brain-gut axis neurochemicals by Escherichia coli-induced diarrhea in mice. *Microbial Pathogenesis* 119, 65–71. doi: 10.1016/j.micpath.2018.04.005

Kalsi, K. K., Jackson, S., and Baines, D. (2020). Zonulin antagonist protects against tight junction disruption by Pseudomonas aeruginosa in airway epithelial cells. *FASEB J.* 34. doi: 10.1096/fasebj.2020.34.s1.05094

Kobayashi, H., Kanmani, P., Ishizuka, T., Miyazaki, A., Soma, J., Albarracin, L., et al. (2017). Development of an *in vitro* immunobiotic evaluation system against rotavirus infection in bovine intestinal epitheliocytes. *Beneficial Microbes* 8, 309–321. doi: 10.3920/BM2016.0155

Kober, A., Riaz, R. M., Mehwish, H. M., Villena, J., and Kitazawa, H. (2022). Immunomodulation potential of probiotics: A novel strategy for improving livestock health, immunity, and productivity. *Microorganisms* 10(2):388. doi: 10.3390/microorganisms10020388

Kucharzik, T., Walsh, S. V., Chen, J., Parkos, C. A., and Nusrat, A. (2001). Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *Am. J. Pathol.* 159, 2001–2009. doi: 10.1016/S0002-9440(10)63051-9

Leoncini, G., Cari, L., Ronchetti, S., Donato, F., Caruso, L., Calafa, C., et al. (2024). Mucin expression profiles in ulcerative colitis: new insights on the histological mucosal healing. *Int. J. Mol. Sci.* 25(3):1858. doi: 10.3390/ijms25031858

Liu, G., Kragh, M. L., Aabo, S., Jensen, A. N., and Olsen, J. E. (2022). Inhibition of Virulence Gene Expression in Salmonella Dublin, Escherichia coli F5 and Clostridium perfringens Associated With Neonatal Calf Diarrhea by Factors Produced by Lactic Acid Bacteria During Fermentation of Cow Milk. *Front. Microbiol.* 13 828013. doi: 10.3389/fmicb.2022.828013

Liu, X., Gao, J., Liu, S., Cheng, Y., Hao, L., Liu, S., et al. (2023). The uniqueness and superiority of energy utilization in yaks compared with cattle in the highlands: A review. *Anim. Nutr.* 12, 138–144. doi: 10.1016/j.aninu.2022.09.011

Majidi-Mosleh, A., Sadeghi, A. A., Mousavi, S. N., Chamani, M., and Zarei, A. (2017). Ileal MUC2 gene expression and microbial population, but not growth performance and immune response, are influenced by in ovo injection of probiotics in broiler chickens. *Br. Poultry Sci.* 58, 40–45. doi: 10.1080/00071668.2016.1237766

Martini, E., Krug, S. M., Siegmund, B., Neurath, M. F., and Becker, C. (2017). Mend your fences: the epithelial barrier and its relationship with mucosal immunity in inflammatory bowel disease. *Cell. Mol. Gastroenterol. Hepatol.* 4, 33–46. doi: 10.1016/j.jcmgh.2017.03.007

Mazzon, E., Sturniolo, G. C., Puzzolo, D., Frisina, N., and Fries, W. (2002). Effect of stress on the paracellular barrier in the rat ileum. *Gut* 51, 507–513. doi: 10.1136/gut.51.4.507

Mccarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S., Rogers, R. A., et al. (1996). Occludin is a functional component of the tight junction. *J. Cell Sci.* 109, 9) 2287–2298. doi: 10.1242/jcs.109.9.2287

Mitic, L. L., and Anderson, J. M. (1998). Molecular architecture of tight junctions. *Annu. Rev. Physiol.* 60, 121–142. doi: 10.1146/annurev.physiol.60.1.121

Noah, T. K., Donahue, B., and Shroyer, N. F. (2011). Intestinal development and differentiation. *Exp. Cell Res.* 317, 2702–2710. doi: 10.1016/j.yexcr.2011.09.006

Ockner, R. K., and Manning, J. A. (1974). Fatty acid-binding protein in small intestine. Identification, isolation, and evidence for its role in cellular fatty acid transport. *J. Clin. Invest.* 54, 326–338. doi: 10.1172/JCI107768

Raz, S. (2013). Research progress of colibacillosis in yaks. *Livestock Poultry Industry* 08), 8–10. doi: 10.19567/j.cnki.1008-0414.2013.08.004

Rehman, M., Iqbal, K., Shar, F., Huang, Ä淑成S., Lan, Y., Mehmood, K., et al. (2017). Antibiotic Resistance of Escherichia coli in Free-Ranging Yaks (Bos grunniens) from Tibetan Plateau, China. *Pakistan Veterinary J.* 37, 139–144.

Rogers, A. P., Mileto, S. J., and Lyras, D. (2023). Impact of enteric bacterial infections at and beyond the epithelial barrier. *Nat. Rev. Microbiol.* 21, 260–274. doi: 10.1038/s41579-022-00794-x

Rokhsefat, S., Lin, A., and Comelli, E. M. (2016). Mucin-microbiota interaction during postnatal maturation of the intestinal ecosystem: clinical implications. *Digestive Dis. Sci.* 61, 1473–1486. doi: 10.1007/s10620-016-4032-6

Sadowska, B., Walencka, E., Wieckowska-Szakiel, M., and Rozalska, B. (2010). Bacteria competing with the adhesion and biofilm formation by Staphylococcus aureus. *Folia Microbiologica* 55, 497–501. doi: 10.1007/s12223-010-0082-x

Schumann, M., Siegmund, B., Schulzke, J. D., and Fromm, M. (2017). Celiac disease: role of the epithelial barrier. *Cell. Mol. Gastroenterol. Hepatol.* 3, 150–162. doi: 10.1016/j.jcmgh.2016.12.006

Stevens, B. R., Goel, R., Seungbum, K., Richards, E. M., Holbert, R. C., Pepine, C. J., et al. (2018). Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. *Gut* 67, 1555–1557. doi: 10.1136/gutjnl-2017-314759

Sturgeon, C., and Fasano, A. (2016). Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. *Tissue Barriers* 4, e1251384. doi: 10.1080/21688370.2016.1251384

Tao, H. Y., Li, S. J., and Tan, X. J. (2005). Advances in quaternary transmembrane protein molecules. *Foreign Med. (Internal Medicine)* 10), 422–424.

Vinderola, G., Matar, C., and Perdigon, G. (2007). Milk fermented by Lactobacillus helveticus R389 and its non-bacterial fraction confer enhanced protection against Salmonella enteritidis serovar Typhimurium infection in mice. *Immunobiology* 212, 107–118. doi: 10.1016/j.imbio.2006.09.003

Wang, B. J., Si, L. J. Z., and Chen, J. C. (2022). Epidemiological characteristics and control of colibacillosis in yaks in Tibet. *Anim. Husbandry Veterinary Sci. (Electronic edition)* 22), 76–78.

Wood, H. L., Deboer, M. D., and Fasano, A. (2020). Zonulin as a potential putative biomarker of risk for shared type 1 diabetes and celiac disease autoimmunity. *Diabetes-Metabolism Res. Rev.* 36, e3309. doi: 10.1002/dmrr.3309

Yu, Q. H., and Yang, Q. (2009). Diversity of tight junctions (TJs) between gastrointestinal epithelial cells and their function in maintaining the mucosal barrier. *Cell Biol. Int.* 33, 78–82. doi: 10.1016/j.cellbi.2008.09.007

Zhang, J., Ren, X., Wang, S., Liu, R., Shi, B., Dong, H., et al. (2024a). Microbial interventions in yak colibacillosis: Lactobacillus-mediated regulation of intestinal barrier. *Front. Cell. Infection Microbiol.* 14. doi: 10.3389/fcimb.2024.1337439

Zhang, J., Shi, B., Lu, S., Wang, S., Ren, X., Liu, R., et al. (2024b). Metagenomic analysis for exploring the potential of Lactobacillus yoelii FYL1 to mitigate bacterial diarrhea and changes in the gut microbiota of juvenile yaks. *Microbial Pathogenesis* 186, 106496. doi: 10.1016/j.micpath.2023.106496

Zhao, X., Zhang, T., Zheng, Y., Zhao, Z., Ding, W., Zhang, Z., et al. (2023). Gut microbiota from short-chain chlorinated paraffin-exposed mice promotes astrocyte activation by disrupting the intestinal tight junction via zonulin upregulation. *J. Agric. Food Chem.* 71, 8192–8202. doi: 10.1021/acs.jafc.3c01058

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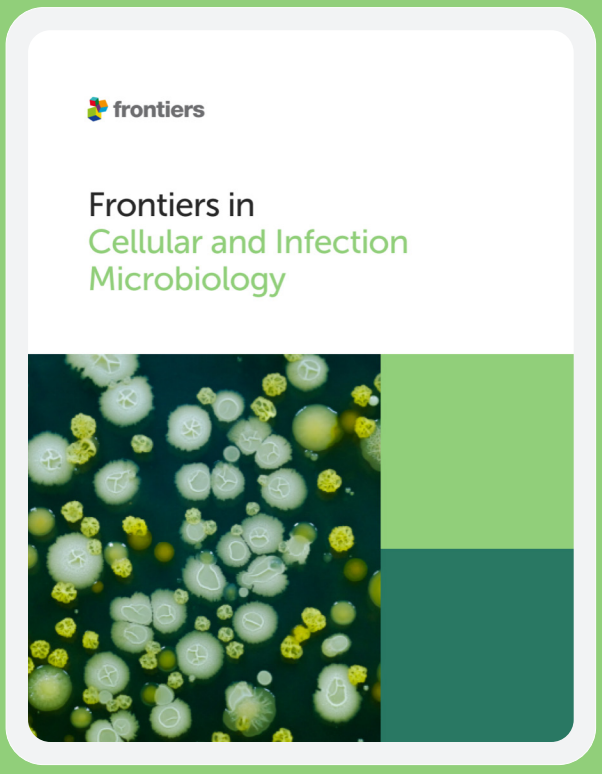

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