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Probiotics isolated from traditional fermented products of the Tibetan Plateau as a substitute for antibiotic feed additives in lamb breeding

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ABSTRACT. In recent years, the overuse of antibiotics in livestock feed has raised significant public health concerns. As a result, antibiotic-free breeding has become a key focus of sustainable agricultural development. Efforts to replace antibiotics in feed have largely focused on plant-derived compounds, extracts, and probiotics or their metabolites. In this study, four probiotic strains: *Lactobacillus delbrueckii* XH-9, *Lacticaseibacillus plantarum* GM-6, *Lactiplantibacillus rhamnosus* GM-7, and *Bacillus subtilis* N-1 were isolated from traditional fermented products of the Tibetan Plateau, and demonstrated inhibitory effects against *Escherichia coli* and *Salmonella*. A total of 45 healthy male Hu sheep (aged 30 days, with similar initial body weight) were randomly assigned to three groups: control, antibiotic, and probiotics. After 35 days of feeding, the probiotic group showed significantly reduced levels of high-density lipoprotein cholesterol (HDL-C; $P < 0.001$), total cholesterol (T-CH; $P < 0.0001$), total bilirubin (T-BiL; $P < 0.001$), creatinine (CR; $P < 0.001$), interleukin-1β (IL-1β; $P < 0.05$), and malonaldehyde (MDA; $P < 0.001$), alongside increased levels of total protein (TP; $P < 0.05$) and tumornecrosis factor-α (TNF-α; $P < 0.0001$), as well as enhanced activities of superoxide dismutase (SOD; $P < 0.05$), and catalase (CAT; $P < 0.05$). Analysis of gut microbiota composition demonstrated that antibiotic treatment significantly altered microbial community structure and promoted the growth of opportunistic pathogens. Conversely, probiotics markedly increased the relative abundance of beneficial bacteria, including *Lactobacillus* ($P < 0.05$) and *Bifidobacterium* ($P < 0.0001$). These findings collectively demonstrate that the probiotic strains effectively reduced oxidative stress, enhanced immune responses, and supported healthy gut microbiota development in growing lambs, indicating their potential as effective probiotic feed additives.

Introduction

Probiotics are live microorganisms capable of modulating host gut microbiota balance (Ding et al., 2021; Duarte and Kim, 2022), with demonstrated benefits for animal health and production performance. In healthy animals, the gut microbiota is predominantly composed of anaerobic bacteria (Aruwa et al., 2021), including *Lactobacillus*, *Bifidobacterium,* and digestive *Bacillus* species (Gomes and Malcata, 1999; Soares et al., 2019; Jha et al., 2020), accounting for more than 99% of the total

gut flora (Zhou et al., 2009). These beneficial microorganisms function through multiple mechanisms: they consume intestinal oxygen during growth and proliferation (Zolkiewicz et al., 2020), secrete bioactive compounds that lower intestinal pH (Dimidi et al., 2017), and degrade anti-nutritional factors in feed (Babot et al., 2021). These activities help establish a biological barrier that effectively prevents the colonisation and infection of opportunistic pathogens (Kunyeit et al., 2019; Gunaratnam et al., 2021).

After birth, lambs rely primarily on breast milk, which is considered the safest, and most optimal source of nutrition. However, during the weaning period, lambs are susceptible to digestive diseases, as the immature digestive system must adapt to solid feed while facing multiple stressors, including poorly digestible roughage, environmental changes, and pathogen exposure (Zhang et al., 2021; Martella et al., 2015; Hanlon et al., 2018). Consequently, approximately 90% of antibiotics used globally each year are administered for the prevention and treatment of animal diseases (Mateos et al., 1997; Gosling et al., 2018).

However, while antibiotics effectively inhibit pathogenic bacteria, they simultaneously disrupt the delicate balance of gut microbiota (Dong et al., 2019). In addition, residual antibiotics in animal waste can disperse through the entire microbial ecosystem, contributing to the emergence of drug-resistant superbugs (Liu et al., 2021a). Recognising these consequences, many nations have begun addressing antibiotic overuse, with developed countries increasingly adopting antibiotic-free farming practices as the new standard for sustainable livestock production (Golden and Mishra, 2020; Iannetti et al., 2021). Recent literature has indicated several promising antibiotic alternatives, including probiotics and their metabolites (Wenk, 2000), plant-derived compounds such as natural herbs and extracts (Kumar et al., 2014; Reddy et al., 2020), essential minerals like copper sulphate, and zinc oxide (López-Gálvez et al., 2021), certain clays (Nadziakiewicza et al., 2019), and marine-derived algae (Subhadra, 2011).

The extreme environmental conditions of the Tibetan Plateau, characterised by aridity, intense ultraviolet radiation, low oxygen levels, high atmospheric pressure, and cold temperatures, have contributed to the evolution of unique microbial communities in traditional fermented foods. Probiotics isolated from this region exhibit distinctive adaptive traits, including heavy metal resistance (Feng et al., 2022), pathogen inhibition, antioxidant capacity (Feng et al., 2020a; Wu et al., 2021), and high cellulase production (Yang et al., 2014). In this study, various traditional fermented products such as yogurt, pickles, sourdough, and silage were collected from the margins of the Tibetan Plateau. Probiotic strains were isolated and applied as feed additives for lambs, and their effects on lamb growth performance were evaluated.

Material and methods

Sample collection and strain isolation

Samples of yogurt, pickle, sourdough, and silage were collected from the Tibetan Plateau region. Each sample (1 g or 1 ml) was diluted with sterile phosphate buffered saline (PBS pH 7.4) to 3 concentrations: 1×10^{-5}, 1×10^{-6}, and 1×10^{-7}. Subsequently, 100 µl of each dilution was spread evenly onto De Man, Rogosa and Sharpe (MRS) agar plates (Solarbio, Beijing, China), and cultured at 37 °C for 48 h in anaerobic chamber (Gene Science E500, America). Single colonies were isolated using an inoculation loop, streaked on fresh MRS agar plate, and cultured under anaerobic condition for another 48 h at 37 °C. Pure isolates were subsequently inoculated into100 ml of MRS liquid medium, and cultured under anaerobic conditions for 24 h at 37 °C. Cultures were preserved in 20% glycerol solution (Rhawn, Shanghai, China) and stored at −80 °C. Prior to experimental use, frozen stocks were revived by culturing in MRS broth at 37 °C for 24 h.

Probiotics with antibacterial activity were screened using the filter paper diffusion method. Fresh cultures of indicator bacteria (100 µl) were spread evenly on MRS agar plates. Sterile filter paper discs were gently placed on medium using sterile tweezers, and 10 µl of each probiotic culture was applied to the discs. Plates were then incubated anaerobically at 37 °C for 48 h. Strains that inhibited the growth of indicator bacteria were selected and identified as *Lactobacillus delbrueckii* XH-9, *Lacticaseibacillus plantarum* GM-6, *Lactiplantibacillus rhamnosus* GM-7, and *Bacillus subtilis* N-1. All assays were performed in triplicate.

The selected probiotic strains were individually inoculated into MRS liquid medium (1% v/v) and cultured at 37 °C. The optical density of each fermentation liquid was measured spectrophotometrically at 600 nm (OD_{600}) every 2 h to plot the growth curve.

Strains identification

Total bacterial DNA was extracted using the E.Z.N.A.® bacterial genomic DNA extraction kit (Omega, Norcross, GA, USA) following the manufacturer's instructions. The 16SrRNA gene was

amplified by PCR using 10 ng of total DNA as a template. PCR products were sequenced by Shenzhen Huada Biological (Shenzhen, China). Sequencing data were submitted to NCBI GenBank and compared with reference sequences using BLAST. A phylogenetic tree was constructed using the neighbour-joining method in MEGA 6.0.

Acid and bile salt tolerance

The acid tolerance of strains XH-9, GM-6, GM-7, and N-1 was evaluated by inoculating 1% (v/v) cultures into MRS broth adjusted to pH 2.0, 3.0, 4.0, 5.0, and 6.0, and incubating anaerobically at 37 °C for 24 h. Growth was measured by OD600 using a Puxi General TU-1950 spectrophotometer (Beijing, China). For bile salt tolerance assessment, the strains were similarly cultured in MRS broth containing 0.1, 0.2, 0.3, or 0.4% bile salts (Macklin, Shanghai, China) under identical conditions, with growth quantified by OD600 measurements.

Pepsin and trypsin tolerance

The probiotic strains XH-9, GM-6, GM-7, and N-1 were first cultured in MRS broth (1% inoculum) at 37 °C for 14 h under anaerobic conditions. Then, 5% of the cultured MRS broth was transferred into 10 ml of the following solutions: MRS (pH 3.0), pepsin solution (1.0 g/100 ml, pH 8.0; Macklin, Shanghai, China), and trypsin solution (1.0 g/100 ml; Macklin, Shanghai, China). All test cultures were incubated anaerobically at 37 °C, with OD600 measurements recorded hourly to generate comparative growth curves.

Probiotic feed additive preparation

Strains XH-9, GM-6, GM-7, and N-1 preserved in glycerol stocks were individually inoculated into MRS liquid medium cultured anaerobically at 37 °C for 48 h. To collect the cells, the bacterial suspensions were centrifuged at 8 000 rpm for 5 min (Xiangyi, Changsha, China) and washed twice with sterile PBS using vortex mixing at 3 000 rpm for 1 min (Kylin-Bell Vortex Mixer QL-866, Jiangsu, China). After final centrifugation (8 000 rpm, 5 min), the cell pellets were resuspended in sterile skim milk solution (BD Difco TM Skim Milk, America), and then lyophilised under vacuum (< 10Pa) at −45~65 °C for 24–72 h (Xinbexi Biobase-BK-FD10S, Jinan, China). The viable cell count of each freeze-dried probiotic preparation was determined by plate counting.

Animal experiments

The animal trial was conducted on a farm in northwest China. All procedures involving animals were approved by the Animal Ethics Committee of Lanzhou University of Technology (Approval No: 2024-015). The lamb housing facility featured circular feeding troughs for starter feed provision and temperature-regulated water tanks maintained at 20–30 °C. The pens were constructed with mesh polypropylene flooring for optimal drainage and slip resistance. The roof was constructed from coloured steel, and the housing was surrounded by polyethylene material that could be automatically rolled up during the day for ventilation. Environmental conditions were carefully maintained, with daytime temperatures not exceeding 30 °C, nighttime temperatures remaining above 8 °C, and relative humidity consistently below 70%. Animals had *ad libitum* access to both feed and fresh water throughout the study, with all husbandry practices conforming to standard animal welfare protocols.

The study employed a randomised controlled design using 45 healthy male Hu lambs (30 days old, initial body weight 7.81 ± 0.60 kg) assigned to three treatment groups (control, antibiotic, and probiotics). All lambs were vaccinated with a combined inactivated vaccine for sheep: rapid epidemic, sudden attack, lamb dysentery, and enterotoxaemia (Zhengye, Jilin, China). The experimental setup featured five replicate pens per treatment group, with three lambs housed together in each pen (15 lambs per treatment). Pens were physically separated to prevent cross-group contamination. For sampling purposes, one randomly selected lamb from each pen (n = 5 per treatment) served as the experimental unit, ensuring independent measurements and eliminating potential interference between individuals. This design maintained biological replicates while controlling for environmental variables through standardised housing conditions.

Control lambs received standard starter feed *ad libitum*. Antibiotic group lambs were fed starter feed supplemented with 0.5% chlortetracycline (50 mg/kg feed) (Calhoun and Shelton, 1973). Probiotic group lambs were fed a starter feed supplemented with probiotics (15 g per day, providing each lamb with over 1×10^8 CFU daily) (Reuben et al., 2022). The starter feed was formulated according to the China Meat Sheep Feeding Standard (NY/T 816-2021; Table 1). In addition, each lamb was fed milk replacer at 2% of its body weight. The milk replacer was mixed with warm water (50–70 °C), stirred thoroughly, and administered via bottles 3–5 times per day. The expected daily weight gain for lambs was 200–250 g. The experiment lasted 35 days.

Table 1. Composition and nutrient levels of lamb starter feed (air-dry basis)

Item	Content, %	Item	Content, %
Diet composition		Premix[1]/%	1.00
Soybean	32.77	Nutritive Index	
Alfalfa	23.36	Digestible energy (MJ/kg)	5.29
Maize	22.49	Crude protein (%)	23.20
Rapeseed	7.33	Crude fat (%)	8.14
Cottonseed	7.00	Neutral detergent fibre (%)	17.15
Wheat bran	4.03	Acid detergent fibre (%)	5.45
$CaHPO_4$	1.00	Crude ash (%)	5.06
NaCl	0.52	Ca (%)	0.92
Mountain flour	0.50	P (%)	0.58

[1] provided per kg of starter feed: g: iron 1.10, copper 0.73, manganese 0.31, zinc 0.26, iodine 0.01, selenium 0.02, cobalt 0.22; IU: vitamin A 76 190, vitamin D 3 429, vitamin E 170; mg: vitamin B_1 23.32, vitamin B_2 28.00, vitamin B_6 22.63, vitamin B_{12} 137.13, niacin 181.01

Blood analysis

On the final day of the 35-day experiment, fasting blood samples (10 ml) were collected from the jugular vein of lambs in each treatment group using vacuum tubes (AOSAITE, Shandong, China) at 9:00. Complete blood counts were immediately analysed using a ProCyte Dx® Hematology Analyzer (IDEXX Laboratories, Westbrook, MA, USA). For serum preparation, blood samples were allowed to clot at room temperature for 30 min before centrifugation at 3 000 rpm for 10 min. The separated serum was aliquoted and stored at −80 °C until analysis. Serum biochemistry parameters, including HDL-C, LDL-C, T-CH, TP, T-BiL, ALT, AST, CR, UA, and Urea-N were quantified using a Mindray BS-420 biochemical analyser (Shenzhen, China). Oxidative stress markers (MDA, GSH-Px, SOD, CAT) and inflammatory cytokines (TNF-α, IL-6, IL-1β) were measured using commercial ELISA kits (Meilian, Shanghai, China) according to the protocols provided. Five serum samples from each group were selected for independent analysis.

DNA extraction and processing for sequencing

Fresh faecal samples were collected and immediately preserved in sterile polyethylene tubes on dry ice. Total bacterial genomic DNA was extracted using the Tiangen DNA extraction kit (Beijing, China) following the manufacturer's instructions. The extracted DNA in TE buffer was kept on dry ice and transported to Biomarker Technologies (Beijing, China) for high-throughput sequencing using the Illumina NovaSeq platform.

Data statistical analysis and chart drawing

Statistical analysis was conducted using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA) employing one-way analysis of variance (ANOVA), followed by Tukey's multiple range test at a significance level of $P < 0.05$. All experimental data are presented as arithmetic means ± standard error of the mean (SEM). Data visualisation was performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Microbial community analyses included construction of Venn diagrams using the interactive web-based platform available at http://www.bioinformatics.com.cn

Results

The tested probiotic strains XH-9, GM-6, GM-7, and N-1 exerted significant inhibitory effects against *E. coli* and *Salmonella*, with inhibition zone diameters exceeding 10 mm and 6 mm, respectively (Figure 1A). Gene sequencing results for these strains were deposited in GenBank (Accession numbers: UNPK3M9S013, UNRB55BY016, UNR6R6H9016, and UNRRYH2E013, respectively). Phylogenetic analysis showed that XH-9 was most closely related to *Lacticaseibacillus plantarum*, GM-6 to *Lactiplantibacillus rhamnosus*, GM-7 to *Lactobacillus delbrueckii*, and N-1 to *Bacillus subtilis* (Figure 1B). All strains showed a 20-h lag phase before entering logarithmic growth at 22 h. GM-7 displayed reduced growth at 38 h, while XH-9, GM-6, and N-1 retained stable growth for 4 h after 40 h before entering decline phase at 48 h.

Acid and bile salt significantly inhibited the growth of the probiotic strains (Figures 2A,B). However, when XH-9, GM-6, GM-7, and N-1 were cultured in MRS medium at pH 3.0, and with 0.3% bile salt for 24 h, the OD600 values remained above 0.5, indicating good survivability (Figures 2A,B). These strains also showed strong enzymatic tolerance, with growth curves remaining unaffected in MRS medium containing 1% pepsin or trypsin (Figures 2C,D). The results showed unimpaired proliferation capacity under these digestive conditions.

Feed supplementation with probiotics or antibiotics did not affect lamb's average daily feed intake (Table 2). Compared with CK, the probiotic group exhibited an increased average daily gain, while the antibiotic group showed a decreased gain. Feed conversion ratios followed the same trend.

After 35 days, white blood cell (WBC) levels significantly increased in the antibiotic group

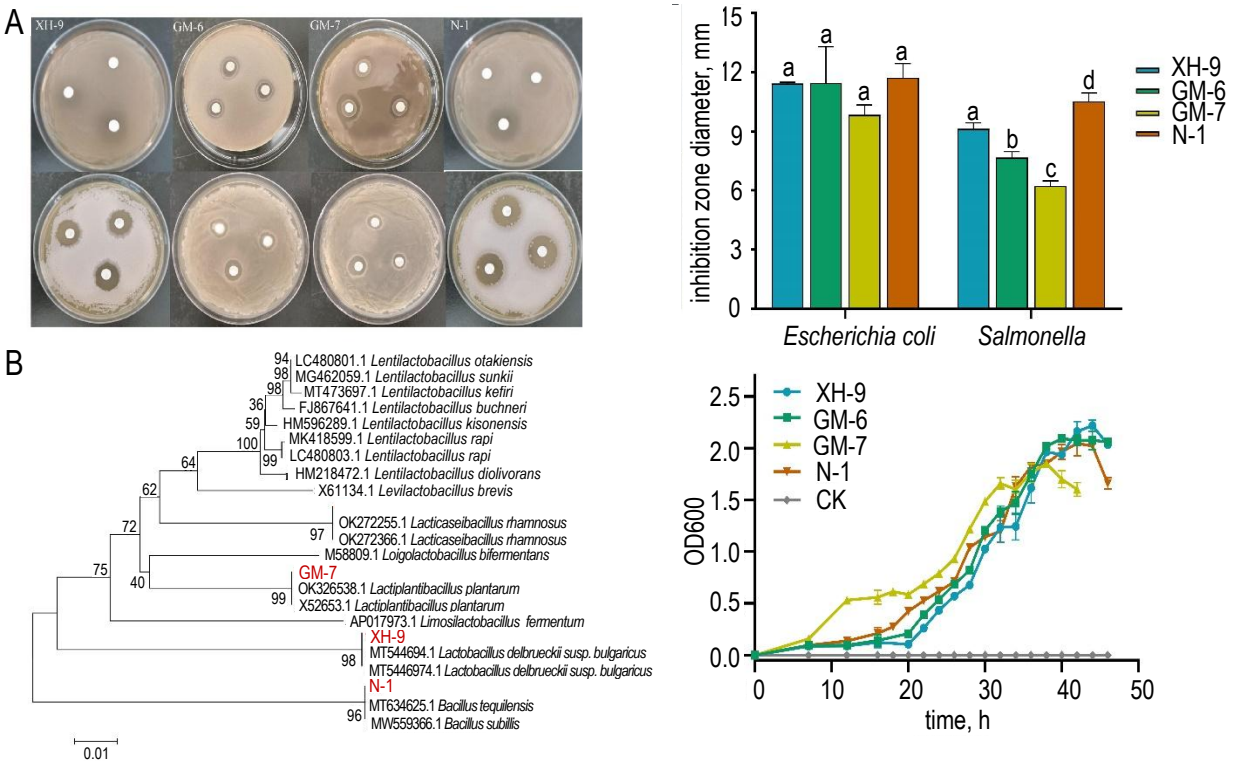

Figure 1. Screening and identification of probiotic strains. (A) Inhibition zones of different probiotics against *Salmonella* and *Escherichia coli*. (B) Phylogenetic tree of strains *Lactobacillus plantarum* XH-9, *Lactobacillus rhamnosus* GM-6, *Lactobacillus delbrueckii* GM-7, and *Bacillus subtilis* N-1 constructed using the neighbour-joining. CK – control group. Growth curves of the four strains cultured in MRS. Data are presented as means ± SD, n = 3

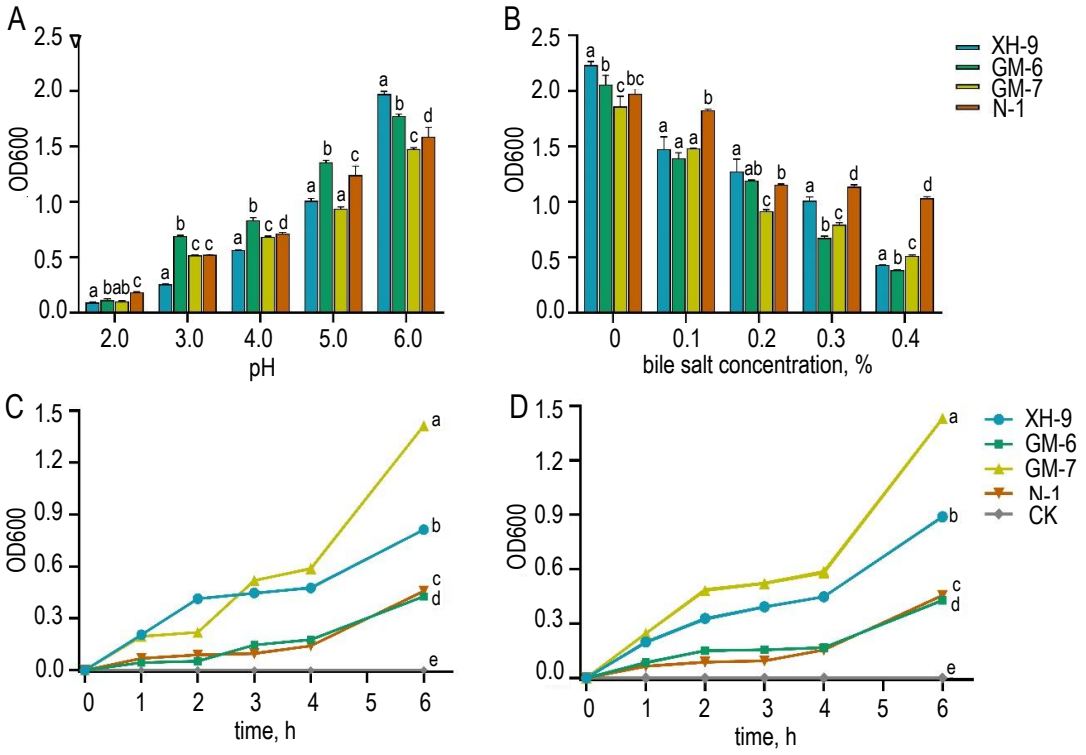

Figure 2. Growth of probiotic strains under different conditions. (A) Acid resistance assessed by growth in pH-adjusted MRS medium (24 h). (B) Bile salt tolerance tested in MRS medium supplemented with 0–0.3% bile salts (24 h). (C) Pepsin resistance in gastric fluid simulation (pepsin-containing medium). (D) Trypsin resistance in intestinal fluid simulation (trypsin-containing medium). Control: MRS broth without additives. Data are presented as means ± SD, n = 3. CK – control group, XH-9 – *Lactobacillus plantarum*, GM-6 – *Lactobacillus rhamnosus*, GM-7 – *Lactobacillus delbrueckii*, N-1 – *Bacillus subtilis*

Table 2. Growth performance of lambs

Item	Control	Antibiotic	Probiotics	SEM	*P*-values
Initial weight, kg	7.82	7.76	7.86	0.15	0.970
Final weight, kg	16.90	16.28	17.42	0.22	0.098
Average daily weight gain, kg/day	0.26	0.24	0.27	0.01	0.085
Average daily milk replacer intake, kg/day	0.232	0.239	0.231	0.002	0.209
Average daily starter food intake, kg/day	1.61	1.58	1.61	0.02	0.852
Average feed consumption/ average weight gain	7.10	7.47	6.74	0.19	0.356

SEM – standard error of the mean, n = 5 lambs per group

compared to CK ($P < 0.05$), while showing a decreasing trend in the probiotic group (Figure 3A). Red blood cell (RBC) levels remained unchanged across groups ($P > 0.05$). Further analysis of immune cells showed decreased lymphocyte levels in the antibiotic group but significantly increased counts in the probiotic group versus CK (Figure 3B; $P < 0.05$). In parallel, the proportion of eosinophils increased in the antibiotic group but declined in the probiotic group. Other immune cell types showed no significant differences between groups.

Renal function markers showed distinct responses (Figur 4D). Both treatment groups exhibited significantly decreased CR levels compared to the control group ($P < 0.001$). Moreover, antibiotics significantly increased UA levels ($P < 0.05$), while Urea-N concentration was not significantly affected in any of the groups ($P > 0.05$). Antioxidant analysis (Figure 4E) revealed that probiotics significantly decreased MDA levels ($P < 0.01$) and increased SOD and CAT activities ($P < 0.05$). No significant changes occurred in the antibiotic group ($P > 0.05$).

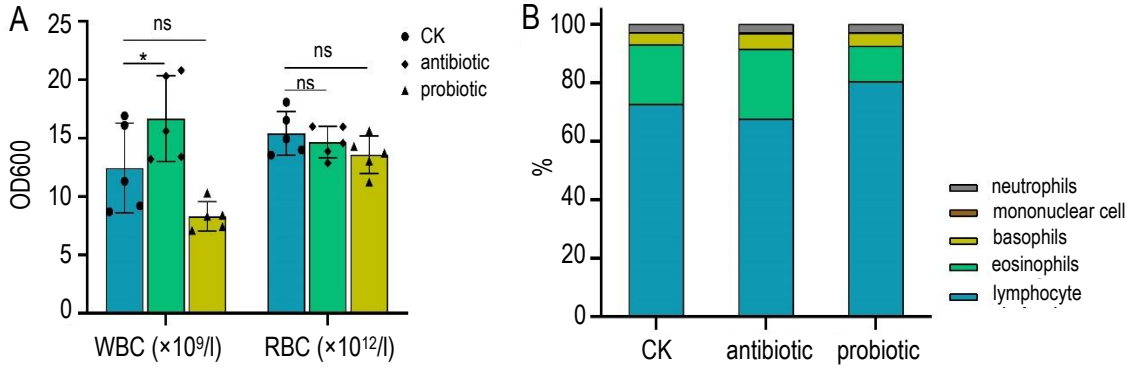

Figure 3. Blood cell parameters in lambs supplemented daily with probiotics. (A) Levels of white blood cells (WBC) and red blood cells (RBC) in different treatment groups. (B) Percentage of immune cells in different treatment groups. Bars represent mean ± SD (n=5 units per group). * $P < 0.05$. CK – control group

After 35 days of treatment, serum analysis revealed significant changes in lipid profiles (Figure 4A). Compared to control, the probiotic group showed markedly reduced HDL-C and T-CH levels ($P < 0.0001$), while higher HDL-C was observed in the antibiotic group ($P < 0.05$). LDL-C levels did not show any significant variation between the groups ($P > 0.05$). Protein and bilirubin metabolism were also affected (Figure 4B). Serum TP significantly decreased in the antibiotic group but increased in the probiotic group ($P < 0.05$) relative to CK. Total bilirubin (T-BiL) levels were significantly reduced in both treatment groups compared to the control ($P < 0.001$). Liver enzyme activity showed differential responses (Figure 4C). ALT and AST levels significantly declined in the antibiotic group ($P < 0.01$ and $P < 0.05$, respectively), while the probiotic group showed no significant changes compared to CK.

GSH-Px activity remained unaffected by either treatment ($P > 0.05$). Immune marker analysis (Figure 4F) demonstrated that the probiotic group had significantly increased TNF-α levels ($P < 0.0001$) and significantly reduced IL-1β concentrations ($P < 0.05$). Meanwhile, the antibiotic group showed no significant changes in these cytokines ($P > 0.05$). Moreover, IL-6 levels remained stable across all groups ($P > 0.05$).

The gut microbiota structure of lambs was significantly altered by both probiotic and antibiotic treatments, as demonstrated by 16S rRNA sequencing analysis. Following quality control, 1 155 424 effective reads (average: 77 028; min: 74 762; max: 79 026) from 12 samples were retained for downstream analysis. Alpha diversity indices (ACE and Chao1) indicated that both antibiotics (both $P < 0.0001$) and probiotics ($P < 0.05$ and $P < 0.001$,

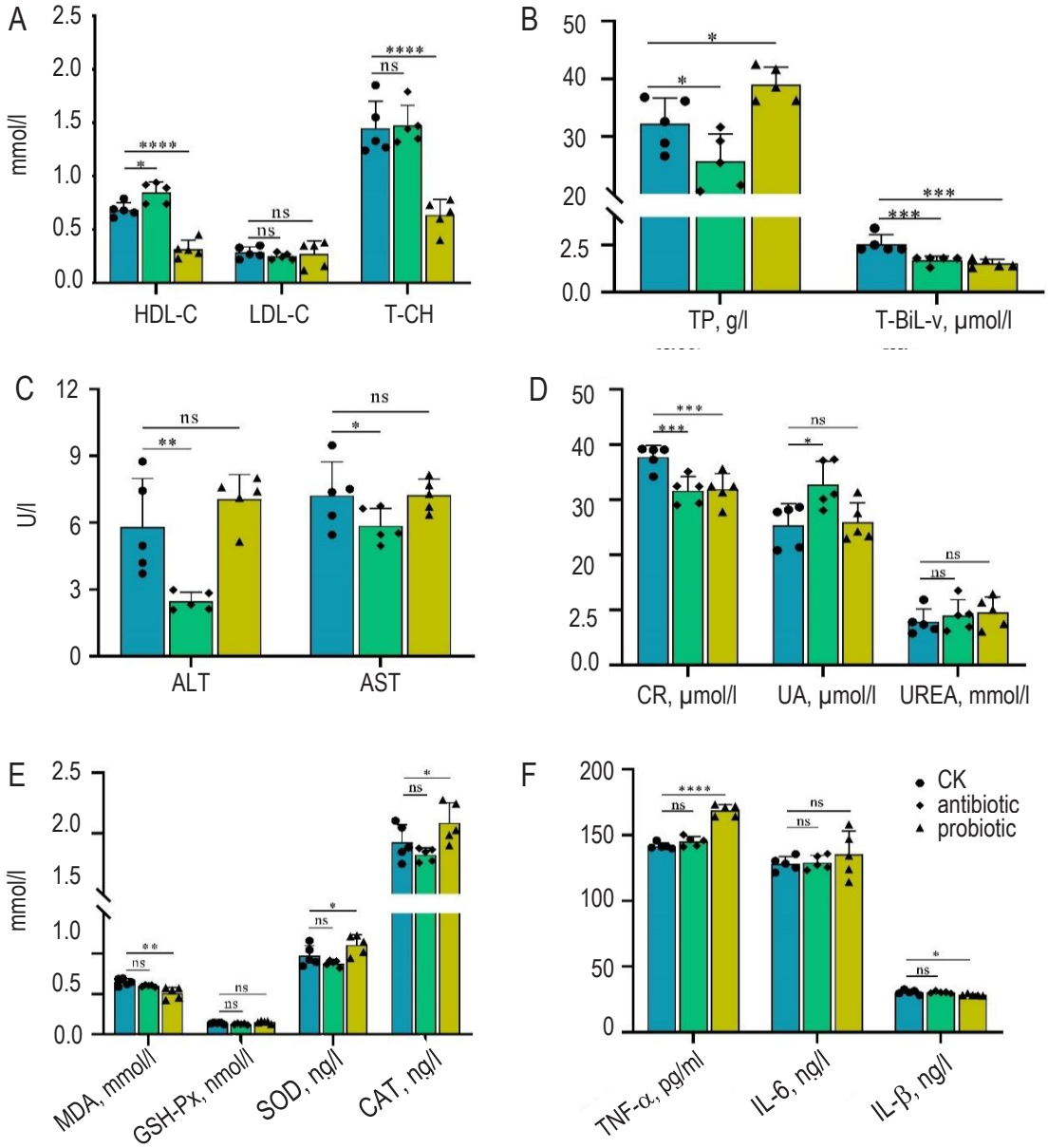

Figure 4. Serum biochemical indicators in lambs supplemented daily with probiotics. (A) Levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (T-CH). (B) Levels of total protein (TP), total bilirubin (T-BiL). (C) Levels of alanine transaminase (ALT) and aspartate transaminase (AST). (D) Levels of creatinine (CR), urea nitrogen (Urea-N), and uric acid (UA). (E) Levels of malonaldehyde (MDA), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT). (F) Levels of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β).

Bars represent mean ± SD (n = 5 units per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

respectively) significantly reduced total bacterial diversity compared to CK (Figure 5A).

Beta diversity analysis through PCoA demonstrated clear clustering of samples within treatment groups, indicating distinct microbial community structures between groups. The tight clustering of replicates within each group confirmed high intragroup similarity in microbial composition (Figure 5B).

Microbial composition analysis at the phylum level revealed that antibiotics significantly increased the proportion of *Bacteroides*, while decreasing *Firmicutes*. The probiotic group showed the opposite pattern, along with increased percentage of *Actinobacteria* compared to the CK group (Figure 5C). At the genus level, the relative abundance of *Rummeliibacillus, Lactobacillus* and *Bifidobacterium* in the intestinal tract was significantly higher in the probiotic group than in the other two groups. Both treatments reduced overall species diversity compared to CK (Figure 5E). Biomarker analysis identified *Rikenelincene*, *Ruminococcaceae* and *Prevotellaccae*

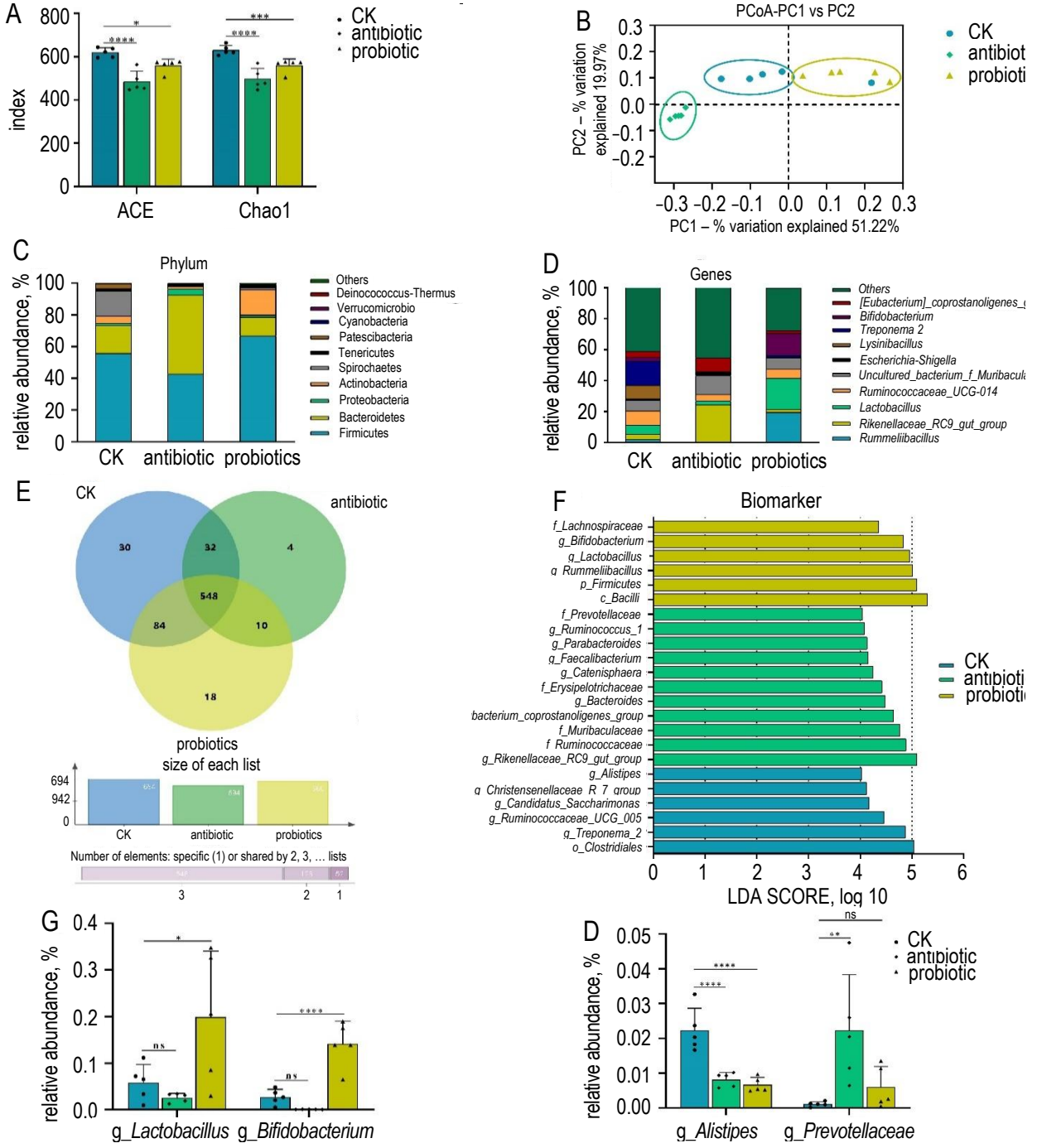

Figure 5. Gut microbiota changes in lambs supplemented daily with probiotics. (A) Alpha diversity index analysis. (B) Principal coordinates analysis (PCoA) of overall microbial community structure based on the unweighted Unifrac distances. (C) Relative abundance at the phylum level in different groups. (D) Relative abundance of genes in different groups. (E) Venn diagram analysis of OTU overlap between groups. (F) Significant microbial biomarkers identified by linear discriminant analysis effect size (LEfSe) (log10 LDA score >4). (G) Relative abundance of *Lactobacillus* and *Bifidobacterium* in different groups. (H) Relative abundance of *Alistipes* and *Prevotellaceae* in different groups. CK – control group.

Bars represent mean ± SD (n=5 units per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

as dominant in the antibiotic group, while probiotic group biomarkers matched the genus-level findings (Figure 5F). Quantitative analysis confirmed significant increases in *Lactobacillus* ($P < 0.05$) and *Bifidobacterium* ($P < 0.0001$) populations after probiotic treatments (Figure 5G). Additionally, the abundance of *Alistipes* decreased significantly in both treatment groups ($P < 0.0001$), while *Prevotellaceae* increased in the antibiotic group ($P < 0.01$; Figure 5H).

Discussion

Currently, various microbial feed additives are used in livestock production, including *Bacillus*, *Lactobacillus*, and yeast. These probiotics produce high amounts of glucose oxidase that converts glucose into H_2O_2 (Tang et al., 2016), a compound with broad-spectrum antibacterial properties. The extreme environment of the Qinghai-Tibetan Plateau contributes to the preservation of high-quality probiotics in local yogurt and feed, with studies reporting strong antagonistic effects of these isolates against pathogenic bacteria (El-Hack et al., 2018). Consistent with these findings, the four probiotics isolated in this study demonstrated significant inhibitory activity against *E. coli* and *Salmonella*. Research indicates that compound probiotics are more effective than single-strain preparations in promoting animal growth (Arsène et al., 2021). Therefore, a probiotic mixture was incorporated into the daily rations of lambs in this study.

According to previous studies, the gastric pH of young ruminants typically ranges from 5 to 6, but remains around 3 during the first two months after birth (Guilloteau et al., 2009). In addition, bile salts present in the duodenum of livestock and poultry play an important role in inhibiting exogenous bacteria, with their concentration in the digestive tract generally ranging from 0.03 to 0.30% (Maisonnier et al., 2003). In the present study, acid resistance test showed that strains GM-6, GM-7, XH-9, and N-1 were severely affected at pH 2, with a marked decline in viable cells. However, the number of viable bacteria at pH 3 was considerably higher. No significant differences in growth were observed between the probiotic strains cultured with 0.3% bile salt concentration and CK (0%), suggesting that GM-6, GM-7, XH-9, N-1 could tolerate the high osmotic pressure environment created by bile salts in the digestive tract.

Another major challenge for the survival and proliferation of lactic acid bacteria in the gastrointestinal tract of animals is their ability to resist the effects of digestive enzymes such as pepsin and trypsin (Feng et al., 2020b). Previous studies have demonstrated that various probiotic strains, including *Bacillus* and *Lactobacillus* species, can tolerate both acidic conditions and bile salts while maintaining viability in simulated gastrointestinal fluids (Li et al., 2018). In this study, experiments simulating artificial gastroenteric fluid demonstrated that the isolated probiotic strains were able to survive and proliferate. Based on these results, it can be inferred that the four strains possess sufficient resilience to survive gastric digestion. This gastric stability suggests their potential to successfully colonise the intestinal tract and exert beneficial probiotic effects in lambs.

Feed efficiency is one of the most important economic parameters for livestock operations. Previous research by He (2020) demonstrated that probiotic supplementation significantly increased daily weight gain and feed conversion ratio in growing Hu sheep. Similar benefits have been observed in calves, where probiotics improved performance and stress resilience during critical developmental stages (Kelsey and Colpoys, 2018). During weaning, lambs are particularly vulnerable to diarrhoea and growth retardation due to the transition from milk to solid feed. Studies have demonstrated that probiotic microorganisms, including lactic acid bacteria and *Saccharomyces cerevisiae*, enhance fibrolytic and proteolytic activity in the ruminant digestive tract. This improved digestive efficiency contributes to better growth performance and helps reduce stress-related responses during weaning (Arowolo and He, 2018; Bąkowski and Kiczorowska, 2021). In the current trial, although probiotics had no statistically significant effect on the feed conversion ratio in lambs, a downward trend was observed. This may be attributed to the relatively short 30-day duration of the trial, which may have been insufficient to observe measurable effects on lamb growth.

Probiotics have been reported to enhance immune function by stimulating lymphocyte proliferation in the intestinal epithelium and improving both cellular and humoral immunity (Kemgang et al., 2014). Specific studies have demonstrated that *Bifidobacteria* can stimulate the intestinal mucosa and associated lymphoid tissues (Hidalgo-Cantabrana et al., 2014), thereby activating systemic immune responses. This increases resistance against pathogens like *Salmonella typhi* and *E. coli* through coordinated action of the lymphatic and circulatory systems (Shehata et al., 2021). In this experiment, antibiotic administration led to an increase in WBC counts, while probiotics specifically increased lymphocyte proportions. The absence of significant changes in other blood parameters and inflammatory markers may be attributed to the limited antibiotic exposure, short duration of probiotic treatment, and the application of effective hygiene and disease prevention measures during the trial. Prophylactic administration of probiotics enhances immune function while reducing disease incidence in ovine production systems. This practice reduces reliance

on therapeutic antimicrobials, thereby aligning with the animal welfare principle of minimising therapeutic intervention, and concurrently mitigating the risk of antimicrobial resistance development (Sachdeva et al., 2025).

Serum biochemical parameters reflect nutrient metabolism and organ function in animals. Previous studies in rats have shown that probiotics can reduce blood triglyceride and cholesterol levels, contributing to weight loss (Sergeev et al., 2020) and prevention of type 2 diabetes (Zeng et al., 2019). Similarly, in this study, probiotics effectively reduced cholesterol accumulation in the blood of lambs. Serum markers such as T-BiL, ALT, and AST are commonly used to assess liver inflammation, while CR, UA, Urea-N indicate renal inflammatory responses. The results indicate that dietary supplementation with probiotics does not impose additional burden on the liver and kidney of lambs (El-Katcha et al., 2016). However, other studies have reported that dietary *Lactobacillus* and *Bacillus* supplementation may reduce albumin and Urea-N levels, increase globulin, ALT, and AST concentrations, enhance immune capacity, and promote amino acid metabolism (Devyatkin et al., 2021). The present findings, showing no adverse effects on liver or kidney function following probiotic supplementation, are consistent with earlier research.

Studies have shown that SOD can specifically catalyse the conversion of superoxide anion to H2O2, which is subsequently broken down into H2O and O2, thereby protecting tissues from oxidative stress (Ighodaro and Akinloye, 2018). GSH-Px directly neutralises superoxide anion and H2O2, stabilises thio-containing enzymes, and helps maintain the structural and functional integrity of cell membranes (Hassan et al., 2020). MDA serves as a marker for lipid peroxidation and oxidative tissue damage (Blanco et al., 2014; Zamboti et al., 2023). Probiotic supplementation has been demonstrated to alleviate stress-induced physiological responses and improve behavioural adaptation in transported lambs. For example, Saccharomyces boulardii effectively reduces systemic cortisol concentrations while ameliorating stress-associated behaviours, such as excessive chirping and huddling (Reddy et al., 2011; Liu et al., 2021b). The current findings revealed that probiotic administration significantly lowered serum MDA concentrations while increasing SOD and CAT activity, indicating enhanced antioxidant capacity and reduced oxidative stress. Notably, probiotic supplementation also elevated serum TNF-α levels, suggesting a potential role in immune system activation as a protective response against adverse environmental stimuli (Gil and Rueda, 2002; Catalioto et al., 2011).

The gut microbiota of animals function in a state of dynamic equilibrium; however, excessive exogenous challenges can disrupt this microbial balance. Under normal conditions, the gastrointestinal tract is predominantly colonised by anaerobic bacteria, while aerobic bacteria are often pathogenic (Freese and Schink, 2011). Prolonged antibiotic use, as a form of antimicrobial intervention, can promote the proliferation of resistant pathogenic strains within the intestine (Mao et al., 2015; Li et al., 2021). In this study, antibiotic administration significantly increased the abundance of *Prevotellaccae*, *Ruminococcaceae*, and *Rikenellaceae* in lamb intestines, which was consistent with previous studies.

Probiotics may regulate pathogenic bacteria in the gut through multiple mechanisms. First, the growth and proliferation of probiotic strains such as *Bacillus subtilis* in the animal gastrointestinal tract consume oxygen, thereby creating a strictly anaerobic environment that effectively inhibits the growth of aerobic pathogens (Browne et al., 2017). It has been reported that dietary supplementation with *Bacillus licheniformis* can increase the abundance of *Bifidobacterium* and *Lactobacillus* in the intestinal tract of pigs (Gaggìa et al., 2010), while significantly reducing the presence of opportunistic pathogens, especially *E. coli* (Dowarah et al., 2017). These findings align closely with the results of this study, supporting the role of probiotics in maintaining microbial balance and inhibiting harmful bacteria.

Second, probiotics colonise and adhere to the intestinal mucosal surface, thereby increasing host resistance and effectively reducing pathogenic damage to the gastrointestinal tract(Shu et al., 2001; Devyatkin et al., 2021). Finally, intestinal probiotics release high amounts of bacteriocins, bacteriocin-like substances, H_2O_2, and certain organic acids (Kailasapathy and Chin, 2000), which directly suppress or eliminate competing bacteria while synergising with the host's innate immune defences (Yaacob et al., 2022). The current study found that dietary probiotic supplementation increased the proportion of *Lactobacillus* and *Bifidobacterium* in the lamb intestinal tract, indicating that probiotic colonisation promoted the development and maturation of the gut microbiota, stimulated the secretion of antimicrobial factors, and reinforced the gut's physiological barrier.

While the inclusion of antibiotics in feed can promote lamb growth and reduce the incidence of infectious diseases, prolonged antibiotic

administration has been shown to impair disease resistance and feed nutrient digestion/absorption in young animals (Mingmongkolchai and Panbangred, 2018; Ban and Guan, 2021). In this study, antibiotic treatment increased the relative abundance of *Prevotellaceae* while decreasing *Alistipes* levels, reducing disease occurrence but disrupting intestinal microbiota structure. This disturbance of the gut ecosystem not only compromised intestinal barrier function but also had lasting negative impacts on long-term growth and development of lambs.

Conclusions

This study successfully isolated and characterised four novel probiotic strains: *Lactobacillus plantarum* XH-9, *Lactobacillus rhamnosus* GM-6, *Lactobacillus delbrueckii* GM-7, and *Bacillus subtilis* N-1 from Tibetan Plateau products. *In vitro* assays confirmed their potent antimicrobial activity against pathogenic bacteria. Dietary supplementation with these probiotics significantly enhanced lamb growth performance and feed efficiency while selectively promoting intestinal *Lactobacillus* populations. However, the 35-day experimental period represents a limitation of the current study. Future research should incorporate longer feeding trials to comprehensively evaluate the effects of probiotics on growth performance, serum antioxidant capacity, immune response, and gut microbiota of lambs at different growth stages.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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