



Original Research Article

Sheep-derived butyrate-producing *Clostridium beijerinckii* R8 alleviates diarrhea by shaping the gut microbiota of goat kids

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ABSTRACT

Newborn goat kids exposed to environmental stress are susceptible to diarrhea due to immature intestinal functions and undeveloped gut microbiota. Butyrate-producing bacteria as next generation probiotics benefit the maintenance of intestinal health, but the mode of regulation is still unclear. Herein, a novel butyrate-producing strain was isolated from sheep rumen and identified as *Clostridium beijerinckii* (C. *beijerinckii*) R8, thereafter goat kids were treated with C. *beijerinckii* R8 to elucidate its regulatory mechanisms on diarrhea. Thirty-six goat kids were assigned to four groups: control (CON), low dose (LCB; supplementation with 5×10^7 CFU/mL of C. *beijerinckii* R8, each with 10 mL/d), middle dose (MCB; supplementation with 5×10^8 CFU/mL of C. *beijerinckii* R8, each with 10 mL/d), high dose (HCB; supplementation with 5×10^9 CFU/mL of C. *beijerinckii* R8, each with 10 mL/d). The experiment lasted for 15 d, and 6 goat kids were randomly selected from each group for slaughter on the last day of the trial. The results showed that this isolate reduced the diarrhea rate ($P < 0.001$) and fecal scores ($P < 0.001$). In the gut, its supplementation inhibited inflammation, increased antioxidant capacity, and regulated intestinal flora. Meanwhile, C. *beijerinckii* R8 strengthened the intestinal barrier and altered the jejunum morphology. This evidence suggests that C. *beijerinckii* R8 may alleviate diarrhea in goat kids by regulating microbiota, which is directly related to intestinal barrier and immune capacity.

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1. Introduction

Newborn goat kids, especially within the two weeks after birth, face a high risk of mortality due to diarrhea due to significant changes in nutrition intake and microbial environment (Wang et al., 2023). Diarrhea is a major focus and difficulty in production management for modern intensive farms, which seriously affects production efficiency and profits. Adding probiotics to the diet to maintain gut health and promote rumen development has been

proven to be a feasible nutritional approach (Liu et al., 2019; Watanabe et al., 2022). One such example of probiotics is butyrate-producing bacteria, which interact with the host through their own properties and metabolites to improve the health of the gut. The presence of cross-feeding between butyrate-producing bacteria and intestinal flora not only aids in creating a favorable gut environment by generating volatile fatty acids efficiently (Schwab et al., 2017), but also exhibits antagonistic competition against pathogenic bacteria via boosting the competitive advantage of symbiotic bacteria (Chen et al., 2020), ultimately maintaining intestinal health through the interaction between intestinal flora and host immunity (Miao et al., 2016). Moreover, butyrate-producing bacteria avoid some drawbacks of directly feeding butyrate such as difficulty with storage due to its high volatility and poor palatability due to its rancid smell. Therefore, the dietary supplementation of butyrate-producing bacteria has become a breakthrough in current research.

Butyrate-producing bacteria, as a kind of functional bacteria and a candidate for the next generation of probiotics, exert a crucial influence on the gastrointestinal microbial community, which in turn consequentially impacts farm animals by improving

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production performance, mitigating intestinal inflammation, enhancing the body's immunity and antioxidant capacity, as well as presenting vast application prospects and significant development potential (Bui and de Vos, 2021). Research has found that a significantly lower abundance of butyrate-producing bacteria, including *Roseburia* and *Prausnitzii*, in the gut of inflammatory bowel disease patients suggests that butyrate-producing bacteria or butyrate is an important factor in disease resistance (Machiels et al., 2014). Furthermore, the notable efficacy of *Clostridium butyricum* in mitigating inflammatory bowel disease symptoms in mice and reducing mortality rates among those afflicted with this condition has been reported (Wang et al., 2018). The studies mentioned above indicate that butyrate-producing bacteria exhibit beneficially impacts intestinal health and reduces diarrhea rate. Butyrate-producing bacteria including *Clostridium butyricum*, *Faecalibacterium prausnitzii*, *Butyrivibrio fibrisolvens* and other strains in the *Roseburia* genus have been isolated and cultured. However, the few strains producing butyrate are still the key points that need to be addressed at present.

Considering the probiotic effects of butyrate-producing bacteria on microbial community and the butyrate-mediated interaction between intestinal flora and host immunity, it is valuable to study the regulatory effects of butyrate bacteria supplementation on the intestinal immune barrier and microflora in regard to diarrhea. To the best of our knowledge, the application of butyrate-producing bacteria to alleviate diarrhea and improve intestinal health in ruminants is still rare. Our hypothesis was that butyrate-producing *Clostridium beijerinckii* (*C. beijerinckii*) R8 alleviates diarrhea by shaping the gut microbiota of goat kids. Therefore, the objective of this study was to screen a strain exhibiting elevated production of butyrate and investigate the impact of dietary addition of the strain on diarrhea, serum biochemical indices, intestinal mucosal barrier and microflora of goat kids.

2. Materials and methods

2.1. Ethics statement

The animal trial was conducted following the ARRIVE (Animal Research Reporting of in Vivo Experiments) guidelines at Shenda Ranch of Shengjian Biotechnology Co., Ltd., Hohhot, Inner Mongolia. The protocols for feeding and slaughtering in this study were approved by the Animal Ethics Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (approval number: IFR-CAAS-20220822).

2.2. Isolation and identification of butyrate-producing bacteria from sheep rumen

The isolation procedure of butyrate-producing bacteria was as follows: 1) 10 g of rumen contents collected from sheep rumen were added into 90 mL distilled water and placed in 80 °C water baths for 10 min to eliminate non-spore bacteria; 2) the mixture was transferred to reinforced clostridium medium (RCM) for anaerobic culture at 37 °C and 21% CO₂ concentration for 48 h; 3) the mixture after culture was subjected to a water bath at a temperature of 80 °C for 10 min and transferred to trypticase sulfite neomycin agar medium (TSN) for anaerobic selective enrichment culture at 37 °C for 48 h; 4) according to the gradient dilution medium of 10⁻³, 10⁻⁵ and 10⁻⁷, the mixture after enrichment was coated with TSN medium plate at 37 °C and cultured anaerobically with 21% CO₂ for 48 h; 5) the screened strains were purified and cultured for more than 3 generations to obtain the screened strains. The obtained strains were sequenced and identified by RuiBio BioTech Co., Ltd., China. Isolates were stored at -80 °C in 50%

glycerol storage solution. The obtained strains were prepared into fermentation broth for acid production detection (1% inoculation, 37 °C, 21% CO₂ anaerobic culture for 48 h, and the inoculation concentration was 10¹² CFU/mL).

The RCM formula was as follows: yeast extract 3 g, beef extract 10 g, tryptone 15 g, glucose 5 g, soluble starch 1 g, NaCl 5 g, sodium acetate anhydrous 3 g, cysteine hydrochloride 0.5 g, agar 20 g, with distilled water added to 1000 mL. The TSN formula was as follows: yeast extract 10 g, tryptone 15 g, Na₂SO₃ 1 g, ferric citrate 0.5 g, neomycin sulfate 0.05 g, polymyxin B 0.02 g, agar 20 g, with distilled water added to 1000 mL.

Selection of strains ($n = 8$) characterized as high butyrate-yielding for DNA extraction. Initial taxonomic characterization of the ruminal butyrate-producing bacteria isolated in this study was performed through sequencing of the 16S rRNA gene using the universal primers P0 (5'-AGAGTTTGATCMTGG-3') and PC3 (5'-ACGGGCGGTGTGTRC-3'). The following conditions were used for each amplification cycle (total of 30 cycles): 95 °C for 5 min, 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, followed by a final step at 72 °C for 10 min. The reactions had approximately: 1 μL of genomic DNA, 0.5 μL of each primer (20 μmol/L), 2 μL of dNTP mixture (10 mmol/L), 2 μL of 10 × PCR buffer, 0.2 μL of Taq polymerase (5 U μL) for a reaction mixture of 50 μL. The PCR product was confirmed on a 1.5% agarose gel.

The 16S rRNA sequence of strain R8 was queried against the NCBI 16S ribosomal RNA sequences database using BLAST (Benson et al., 2015). Clostridium sequences with ≥98.65% identity were retrieved from the NCBI database for comparative analyses. A phylogenetic tree of the 16S rRNA gene was constructed in MEGA 7.0 with 1000 replicates, utilizing the Neighbor-Joining Algorithm (Kumar et al., 2016). *Streptococcus intermedius* C270 was selected as the outgroup to root the phylogenetic tree.

2.3. Animals, diets, and experimental design

Thirty-six male Saanen goat kids of similar body weight (2.98 ± 0.51 kg) at 1 day of age were chosen and randomized to four groups ($n = 9$ per group): CON (control group; supplementation with sterile solution); LCB (low-dose group; supplementation with 5 × 10⁷ CFU/mL of *C. beijerinckii* R8, each with 10 mL/d); MCB (middle-dose group; supplementation with 5 × 10⁸ CFU/mL of *C. beijerinckii* R8, each with 10 mL/d); HCB (high-dose group; supplementation with 5 × 10⁹ CFU/mL of *C. beijerinckii* R8, each with 10 mL/d). The *C. beijerinckii* R8 used in this experiment was the strain with the strongest butyrate-producing ability identified by the above experiment in this study. Since diarrhea is common in goat kids under two weeks of age, the experiment lasted for 14 d (Sun et al., 2022). Six replicates of each group were sacrificed on the 15th day after weighing. Goat kids and milk replacer were weighed for calculation of average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F). Meanwhile, the diarrhea severity of goat kids in each group was recorded. Feces were evaluated on a scale of 1 to 4 as previously described (Wang et al., 2019). An animal was considered to have diarrhea if its fecal score was ≥2. Diarrhea rate (%) = (number of kids with diarrhea × days of diarrhea)/(total number of goat kids × experimental days) × 100.

During the experiment, colostrum was fed for the first 3 d, followed by milk replacer purchased from a commercial company; the chemical composition of which was analyzed (Table 1). All goat kids were fed thrice daily at 08:00, 14:00, and 20:00. The milk replacer solution containing *C. beijerinckii* R8 was administered once daily at 08:00. The milk replacer solution was kept at 42 °C during feeding. The dry matter was determined by drying the feed at 135 °C for 2 h (method 930.15; AOAC, 1990). Determination of crude protein in feed ingredients was by the Kjeldahl method (method 991.20;

Table 1
Ingredients and chemical composition of the commercial milk-replacer.

Item	Amount
Ingredients, g/kg	
Skim milk powder	600
Milk whey powder	160
Lactose	100
Animal fat	90
Premix ¹	50
Nutrient levels², % DM basis	
Total	1000
DM	95.90
CP	24.04
EE	16.10
Ash	6.00
CF	0.70
Ca	0.84
P	0.62
ME, MJ/kg	12.20

DM = dry matter; CP = crude protein; EE = ether extract; ash = crude ash; CF = crude fiber; ME = metabolizable energy.

¹ Provided the following per kilogram premix: vitamin A, 80,000 IU; vitamin D₃, 4250 IU; vitamin E, 100 IU; iron, 200 mg; cobalt, 1 mg; copper, 25 mg; manganese, 125 mg; zinc, 150 mg; selenium, 1 mg.

² Nutrient contents were analyzed values except ME which was calculated by the equation of ME = 4.411 + 0.324CP (Zhao et al., 2016).

AOAC, 1990). The ether extract was determined by the Soxhlet extractor method (method 920.39; AOAC, 1990). The ash was determined by drying at 550 °C for 4 h after cauterization (method 942.05; AOAC, 1990). The crude fiber was determined using an ANKOM 220 fibre analyser (ANKOM Technology, USA) (method 962.09; AOAC, 1990). The minerals composition of the milk replacer was determined according to the methods described by the AOAC (method 985.01; AOAC, 1990).

2.4. Sample collection and analysis

Serum samples ($n = 24$) were obtained by jugular vein blood collection prior to slaughtering. Then, six goat kids in each group were selected for slaughter. The collected jejunal and colonic tissue samples were stored at room temperature in a centrifuge tube containing 10% formalin for subsequent observation of tissue morphology. The collected intestinal mucosa and contents were placed in a 2-mL frozen storage tube at -80 °C for subsequent analysis.

The contents of serum alanine transaminase (ALT), aspartate transaminase (AST), diamine oxidase (DAO), and D-lactic acid were analyzed based on the procedure described by Wang et al., (2020). Samples of jejunal and colonic mucosa were preserved at a temperature of -80 °C. Intestinal mucosa (1 g) was pulverized and added into 9 mL normal saline. The corresponding colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for superoxide dismutase (SOD), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), peroxisome proliferator-activated receptor- γ (PPAR- γ), claudin-1, claudin-4, occludin, MUC2, ZO-1, IgA, IgG, IgM and sIgA in the jejunal and colonic mucosa were evaluated using Multiskan FC (Thermo Scientific, USA) (Zhang et al., 2022).

The tissue was fixed and dehydrated with the automatic dehydrator, embedded, and sliced with paraffin, stained with

hematoxylin and eosin, and finally sealed with neutral gum. The BA210 digital three-mode micro camera system (McAudi Industrial Group Co. Ltd., China) was used for image acquisition of sections. The collected images were imported to Motic Images Advanced 3.2 for data analysis (Wu et al., 2023).

Genomic DNA was extracted from jejunum and colon content using DNA Kits. The V3–V4 region of 16S rRNA was amplified with specific primers with barcode: 338F: 5'-ACTCCTACGGGAGGCAG-CAG-3' and 806R: 5'-GGACTACHVGGGTATCTAAT-3'. The PCR products after amplification were purified using the Encourt AMPure XP kit. PCR amplification and enrichment of the library template were used to generate single-stranded DNA fragments and finally the MiSeq library was constructed for DNA sequencing. The raw data was divided into different samples based on the barcode sequence, which was then filtered and spliced using Pear software (v0.9.11) (Zhang et al., 2014). Sequences with a quality score of 20 or lower were excluded. Splicing was carried out with a minimum overlap setting of 10 bp and a P -value setting of 0.0001. VSEARCH (v2.7.1) software was employed to eliminate sequences shorter than 230 bp and to remove chimeric sequences using the UCHIME method (Rognes et al., 2016; Edgar et al., 2011). The clean tags were denoised using the DADA2 plug-in in Qiime2 software to generate amplicon sequence variants (ASV) (Bolyen et al., 2019). An analysis based on the ASV determined the relative abundance and diversity of the flora by using total sum normalization. Additionally, the flora structure was analyzed by examining the classification at both the phylum and genus levels. The study utilized the Kruskal–Wallis test to identify species showing significant abundance differences between groups, the Wilcoxon rank-sum test to compare differences within groups, and linear discriminant analysis to evaluate the impact of these distinct species. To gain insights into the changes in jejunum and colon microbiota, PICRUST2 was employed to predict their functional profiles against the KEGG database (Douglas et al., 2020). The correlation analysis was assessed using the Spearman algorithm after the Kolmogorov–Smirnov test and the correlation heatmap was drawn by R software ggplot2 package.

2.5. Statistical analysis

The data for growth performance, diarrhea severity, serum, jejunum, and colon biochemical parameters were analyzed by one-way analysis of variance with IBM SPSS Statistics (version 26.0, IBM Corp, Armonk, New York, USA). The χ^2 procedure and Duncan method were used to compare the incidence of diarrhea between groups, and the dose effect of *C. beijerinckii* R8 in the diet was analyzed by linear and quadratic regression. Statistical significance was defined as $P < 0.05$. Visualizations were performed using GraphPad Prism 8 (GraphPad Software, Dotmatics, Boston, Massachusetts, USA).

3. Results

3.1. Isolation and identification of butyrate-producing bacteria

A total of 203 colonies were isolated from the contents of sheep rumen, which were grown on agar plate media (Fig. 1A). Eighty-one isolates showed butyrate production activity (Fig. 1A). The butyrate production of the top 10% isolates is shown (>1.5 g/L) in Fig. 1A and B. The R8 colony with the highest yield of butyrate was selected and individual morphology and colony characteristics were observed after further purification. Colony characteristics and morphology of thalli on RCM medium are shown in Fig. 1C. The surface of the R8 colony is sticky, and the edge of the colony forms a white or milky circle of protuberances, irregularly circular in shape. The thalli were

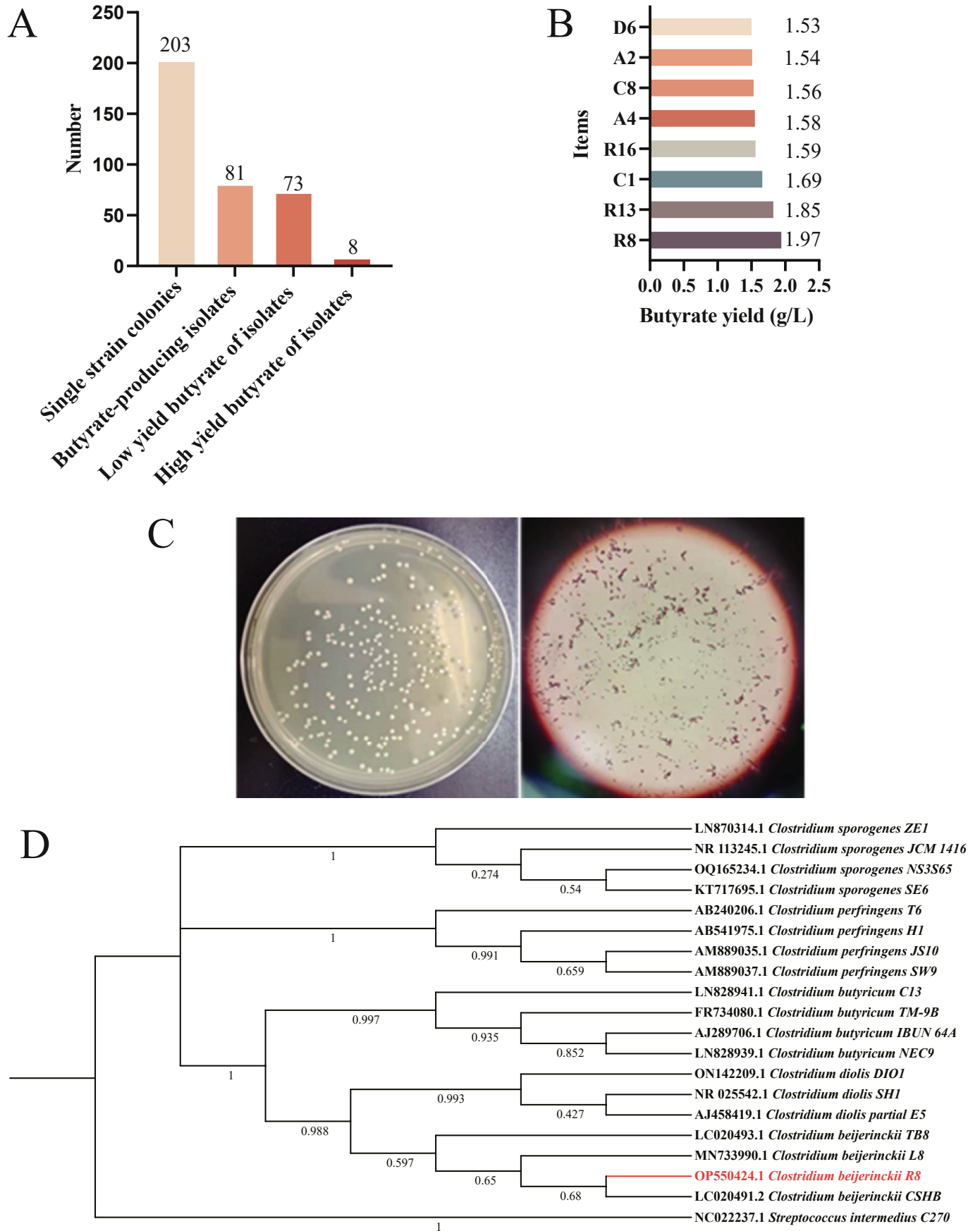


Fig. 1. Classification and phylogeny of isolated butyrate bacteria. (A) Number of isolates and strains during enrichment and isolation. (B) Butyrate yield of isolates. (C) Colony characteristics (left) and cell morphology (right) of isolates. (D) Phylogenetic tree derived from 16S rRNA gene sequence of *C. beijerinckii* R8.

Table 2
Effects of feeding *C. beijerinckii* R8 on diarrhea rate and fecal score of goat kids.

Item	Groups ¹				χ^2	P-value
	CON	LCB	MCB	HCB		
Diarrhea rate, %						
1–14 d	53.17 ^a	24.60 ^b	17.32 ^b	23.81 ^b	46.232	<0.001
1–7 d	49.21 ^a	25.40 ^b	15.78 ^b	24.19 ^b	20.862	<0.001
8–14 d	57.14 ^a	31.75 ^b	20.63 ^b	23.81 ^b	23.286	<0.001
Fecal score						
					SEM	
1–14 d	1.93 ^a	1.42 ^b	1.19 ^b	1.36 ^b	0.241	<0.001
1–7 d	1.83 ^a	1.40 ^b	1.19 ^b	1.37 ^b	0.144	0.005
8–14 d	2.03 ^a	1.49 ^b	1.19 ^b	1.35 ^b	0.161	<0.001

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

straight, rod-shaped, solitary, gram-positive and spore-forming (Fig. 1C). The 16S rRNA sequence of R8 was deposited in GenBank with the accession code OP550424.1. This sequence was aligned with a subset of 18 publicly accessible *Clostridium* spp. The alignment showed that the strain isolated from the rumen formed a single lineage with *C. beijerinckii* R8 group (Fig. 1D). Also, by conducting BLASTn searches on the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, visited on 5 June 2023), it was discovered that *C. beijerinckii* L8, *C. beijerinckii* TB8, and *C. beijerinckii* CSHB

(accession numbers: MN733990.1, LC020493.1, and LC020491.2, respectively) exhibited nucleotide identities ranging from 99.48% to 99.78%. The query coverage was found to be at least 100% with a minimum alignment score of 2431.

3.2. Diarrhea and growth of goat kids

Throughout the trial period or at each trial stage, the fecal score and diarrhea rate of the LCB, MCB and HCB groups were significantly reduced compared with the CON group ($P < 0.001$), but no statistical distinctions were observed among the LCB, MCB, and HCB groups (Table 2). As the supplemental dose of *C. beijerinckii* R8 increased, the fecal scores of the goat kids showed a significant decrease from 1 to 14 d ($P < 0.001$) and 8 to 14 d ($P < 0.001$). However, there were no differences in ADG and ADFI among the four groups (Table 3). The G:F of LCB, MCB, and HCB was lower than that of the CON group ($P = 0.026$).

3.3. Intestinal mucosal inflammatory markers and antioxidant index

Inflammatory factors of the intestinal mucosa are shown in Table 4. In the jejunum, the LCB, MCB, and HCB groups exhibited significantly lower levels of IL-1 β ($P = 0.006$), IL-6 ($P = 0.015$), and TNF- α ($P = 0.005$) compared with the CON group. Conversely, the LCB, MCB, and HCB groups demonstrated higher levels of TGF- β ($P = 0.023$), PPAR- γ ($P = 0.018$), and IL-10 ($P = 0.012$) than CON group. No differences were observed between the LCB, MCB, and

Table 3
Effect of *C. beijerinckii* R8 on growth performance of goat kids.

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
IBW, kg	2.91	2.85	3.05	2.76	0.784	0.641	0.662	0.618
FBW, kg	4.47	5.03	4.85	4.54	0.114	0.259	0.368	0.089
ADG, g/d	111.11	139.29	128.73	126.58	4.266	0.131	0.084	0.181
ADFI, g/d	146.24	150.91	161.35	144.64	3.972	0.457	0.439	0.298
G:F	1.40 ^a	1.15 ^b	1.25 ^b	1.14 ^b	0.331	0.026	0.029	0.051

IBW = initial body weight; FBW = final body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = feed to gain ratio; SEM = standard error of the mean.

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

Table 4
Effects of feeding *C. beijerinckii* R8 on intestinal inflammation in goat kids.

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
Jejunal mucosa								
TNF- α , pg/mg	36.17 ^a	33.24 ^b	32.57 ^b	32.29 ^b	0.483	0.005	0.002	0.039
IL-1 β , pg/mg	16.35 ^a	14.33 ^b	13.66 ^b	13.81 ^b	0.340	0.006	0.002	0.049
IL-6, pg/mg	36.11 ^a	32.83 ^b	31.90 ^b	32.59 ^b	0.531	0.015	0.007	0.033
TGF- β , ng/mg	6.30 ^b	7.10 ^a	7.15 ^a	7.05 ^a	0.115	0.023	0.017	0.032
PPAR- γ , mmol/g	27.83 ^b	32.00 ^a	32.40 ^a	32.17 ^a	0.617	0.018	0.009	0.044
IL-10, pg/mg	36.08 ^b	40.23 ^a	40.57 ^a	41.31 ^a	39.603	0.012	0.004	0.120
Colonic mucosa								
TNF- α , pg/mg	36.64 ^a	34.94 ^{ab}	33.98 ^b	32.87 ^b	0.465	0.016	0.002	0.700
IL-1 β , pg/mg	16.62 ^a	15.45 ^{ab}	14.91 ^b	14.13 ^b	0.312	0.021	0.003	0.722
IL-6, pg/mg	36.49 ^a	35.29 ^{ab}	34.42 ^b	32.32 ^b	0.542	0.022	0.003	0.598
TGF- β , ng/mg	6.17 ^b	6.60 ^{ab}	6.84 ^a	7.15 ^a	0.119	0.014	0.002	0.765
PPAR- γ , mmol/g	27.16 ^b	29.57 ^{ab}	30.92 ^a	32.55 ^a	0.641	0.010	0.001	0.708
IL-10, pg/mg	35.12 ^b	38.51 ^{ab}	39.02 ^a	40.74 ^a	0.644	0.010	0.001	0.420

TNF- α = tumor necrosis factor- α ; IL-1 β = interleukin-1 β ; IL-6 = interleukin-6; TGF- β = transforming growth factor- β ; PPAR- γ = peroxisome proliferator-activated receptor γ ; IL-10 = interleukin-10.

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

Table 5
Effects of feeding *C. beijerinckii* R8 on the intestinal antioxidant index in goat kids.

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
Jejunal mucosa								
T-AOC, U/mg	6.63 ^b	7.47 ^a	7.60 ^a	7.52 ^a	0.115	0.009	0.004	0.021
SOD, U/mg	64.40 ^b	75.27 ^a	76.04 ^a	75.52 ^a	1.414	0.008	0.004	0.020
GSH-Px, U/mg	563.04 ^b	654.76 ^a	658.10 ^a	656.42 ^a	11.535	0.006	0.003	0.018
CAT, U/mg	4.65 ^b	5.55 ^a	5.57 ^a	5.51 ^a	0.114	0.010	0.006	0.017
MDA, mmol/mg	2.56 ^a	1.99 ^b	1.95 ^b	1.98 ^b	0.075	0.011	0.005	0.025
Colonic mucosa								
T-AOC, U/mg	6.74 ^b	7.11 ^{ab}	7.18 ^{ab}	7.59 ^a	0.105	0.044	0.003	0.897
SOD, U/mg	65.17 ^b	70.03 ^{ab}	71.04 ^{ab}	76.08 ^a	1.321	0.036	0.002	0.966
GSH-Px, U/mg	573.17 ^b	609.39 ^{ab}	613.93 ^{ab}	656.60 ^a	10.307	0.047	0.003	0.852
CAT, U/mg	4.72 ^b	5.13 ^{ab}	5.18 ^{ab}	5.62 ^a	0.108	0.049	0.002	0.953
MDA, mmol/mg	2.53 ^a	2.27 ^{ab}	2.21 ^{ab}	1.94 ^b	0.071	0.032	0.002	0.938

T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; MDA = malondialdehyde.

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

HCB groups. In the colon, the MCB and HCB groups exhibited significantly lower levels of TNF- α ($P = 0.016$), IL-1 β ($P = 0.021$), and IL-6 ($P = 0.022$) compared with the CON group, while the MCB and HCB groups displayed higher levels of TGF- β ($P = 0.014$), PPAR- γ ($P = 0.010$), and IL-10 ($P = 0.010$). No significant differences were observed between the LCB and CON groups, as well as between the LCB and MCB and HCB groups.

In the jejunal mucosa, the concentration of SOD ($P = 0.008$), T-AOC ($P = 0.009$), GSH-Px ($P = 0.006$) and CAT ($P = 0.010$) in LCB, MCB and HCB exhibited a significant increase compared with the CON group (Table 5). Meanwhile, the content of MDA was lower ($P = 0.011$) in LCB, MCB and HCB compared with the CON group. Moreover, in the colonic mucosa, the content of SOD ($P = 0.036$), T-AOC ($P = 0.044$), GSH-Px ($P = 0.047$), and CAT ($P = 0.049$) in the HCB group was significantly higher than those in the CON group, while no significant difference was observed when compared with the other two groups. Dietary addition of *C. beijerinckii* R8 significantly decreased ($P = 0.032$) the content of MDA in the colonic mucosa.

3.4. Intestinal mucosal barrier functions

Jejunal villous height did not differ between the four groups (Table 6). The supplementation of *C. beijerinckii* R8 reduce the crypt depth ($P = 0.004$) and mucosal thickness ($P < 0.001$) of the jejunum compared with the CON group. The supplementation of *C. beijerinckii* R8 enhanced jejunal villous height to crypt depth ratio (VH/CD) ($P = 0.004$) compared with the CON group. The supplementation of *C. beijerinckii* R8 increased ($P < 0.05$) jejunal muscular

thickness ($P < 0.001$) to varying degrees. The different doses of *C. beijerinckii* R8 did not induce any significant alterations in colonic crypt depth, mucosal thickness, or muscle thickness.

In comparison to the CON group, the addition of *C. beijerinckii* R8 resulted in an increase in the levels of IgA ($P = 0.020$), IgG ($P = 0.018$), and IgM ($P = 0.032$) of jejunal mucosa (Table 7). In the colon, the levels of mucosal IgA ($P = 0.017$), IgG ($P = 0.014$), and IgM ($P = 0.016$) gradually increased with the supplementation of *C. beijerinckii* R8. The levels of sIgA in jejunal and colonic mucosa were similar among the four groups.

The content of intestinal mucosal protein is shown in Table 8. Feeding *C. beijerinckii* R8 increased the contents of claudin-1, claudin-4, MUC2, occludin and ZO-1 in the jejunal ($P = 0.021$, $P = 0.023$, $P = 0.014$, $P = 0.017$, $P = 0.021$, respectively) and colonic ($P = 0.015$, $P = 0.020$, $P = 0.016$, $P = 0.017$, $P = 0.021$, respectively) mucosa of the LCB, MCB, and HCB groups.

In terms of the serum biochemical parameters (Table S1), the level of ALT ($P < 0.001$), AST ($P = 0.006$), DAO ($P = 0.019$), and D-lactic acid ($P < 0.001$) in the LCB, MCB, and HCB groups was found to be lower compared with the CON group.

3.5. Intestinal microbiota

The microflora of 24 jejunal content samples from goat kids was characterized by 16S rRNA sequencing. The 16S rRNA gene sequencing of the samples yielded 1,499,772 quality sequences, with an average of 62,490 reads per sample. The examination identified a total of 2937 ASVs. As shown in Fig. S1, the Chao1, Observed species, PD-whole tree,

Table 6
Effects of feeding *C. beijerinckii* R8 on intestinal morphology in goat kids.

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
Jejunum								
Villous height, μm	716.20	654.15	671.88	709.62	12.957	0.281	0.987	0.056
Crypt depth, μm	280.96 ^a	236.28 ^b	251.33 ^b	240.42 ^b	5.023	0.004	0.014	0.087
VH/CD	2.40 ^b	2.80 ^a	3.06 ^a	2.94 ^a	0.066	0.004	0.001	0.043
Mucosal thickness, μm	1156.30 ^a	961.19 ^b	1025.38 ^b	1006.10 ^b	16.109	<0.001	0.006	0.005
Muscular thickness, μm	138.89 ^c	204.24 ^a	181.32 ^b	193.82 ^{ab}	3.177	<0.001	<0.001	<0.001
Colon, μm								
Crypt depth	547.61	559.26	560.81	578.57	7.723	0.623	0.203	0.895
Mucosal thickness	620.02	653.58	654.19	636.94	7.725	0.348	0.455	0.102
Muscular thickness	332.35	339.15	324.29	352.22	7.035	0.549	0.480	0.455

VH/CD = villous height to crypt depth ratio.

^{a-c} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

Table 7
Effects of feeding *C. beijerinckii* R8 on intestinal immunity function-related proteins in goat kids.

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
Jejunal mucosa								
IgA, g/L	0.38 ^b	0.45 ^a	0.46 ^a	0.45 ^a	0.012	0.020	0.011	0.042
IgG, g/L	6.05 ^b	7.04 ^a	7.12 ^a	7.16 ^a	0.144	0.018	0.009	0.043
IgM, g/L	0.78 ^b	0.86 ^a	0.86 ^a	0.85 ^a	0.111	0.032	0.018	0.054
slgA, µg/mL	5.78	6.17	6.24	6.23	0.082	0.168	0.057	0.212
Colonic mucosa								
IgA, g/L	0.37 ^b	0.41 ^{ab}	0.43 ^a	0.46 ^a	0.011	0.017	0.002	0.822
IgG, g/L	5.95 ^b	6.48 ^{ab}	6.74 ^a	7.13 ^a	0.143	0.014	0.002	0.754
IgM, g/L	0.77 ^b	0.81 ^{ab}	0.83 ^a	0.86 ^a	0.010	0.016	0.002	0.756
slgA, µg/mL	5.52	5.84	6.07	6.13	0.091	0.053	0.009	0.437

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; slgA = secretory immunoglobulin A.

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

Table 8
Effects of feeding *C. beijerinckii* R8 on intestinal barrier function-related proteins in goat kids (ng/mg).

Item	Groups ¹				SEM	P-value		
	CON	LCB	MCB	HCB		Treatment	Linear	Quadratic
Jejunal mucosa								
Claudin-1	1.74 ^b	2.16 ^a	2.19 ^a	2.15 ^a	0.061	0.021	0.013	0.037
Claudin-4	1.80 ^b	2.22 ^a	2.26 ^a	2.24 ^a	0.064	0.023	0.011	0.054
MUC2	2.28 ^b	2.63 ^a	2.66 ^a	2.63 ^a	0.050	0.014	0.008	0.035
Occludin	2.85 ^b	3.27 ^a	3.30 ^a	3.24 ^a	0.060	0.017	0.013	0.026
ZO-1	1.94 ^b	2.26 ^a	2.30 ^a	2.28 ^a	0.048	0.021	0.010	0.048
Colonic mucosa								
Claudin-1	1.68 ^b	1.91 ^{ab}	2.02 ^a	2.19 ^a	0.061	0.015	0.002	0.780
Claudin-4	1.76 ^b	1.98 ^{ab}	2.10 ^a	2.67 ^a	0.063	0.020	0.003	0.813
MUC2	2.24 ^b	2.43 ^{ab}	2.53 ^a	2.66 ^a	0.051	0.016	0.002	0.756
Occludin	2.80 ^b	3.01 ^{ab}	3.12 ^a	3.29 ^a	0.060	0.017	0.002	0.822
ZO-1	1.91 ^b	2.07 ^{ab}	2.18 ^a	2.30 ^a	0.049	0.021	0.003	0.814

MUC2 = mucoprotein 2; ZO-1 = zonula occludens-1.

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$).

¹ CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

and Shannon indexes of bacterial richness and diversity were no different among the four groups. PCoA and NMDS analysis showed an inability to distinguish among the four groups of samples (Figs. S2A and B). Overall, 27 phyla were commonly found in the jejunum samples from all treatments. Of them, Firmicutes, Proteobacteria, Bacteroidota and Verrucomicrobiota were the major phyla in jejunal content (relative level >1%) and exceeded more than 94% of the total abundance (Fig. 2A). There was a significant decrease in the relative abundance of Proteobacteria and Bacteroidota, while the relative abundance of Firmicutes showed a significant increase upon supplementation with *C. beijerinckii* R8. From jejunal contents, 367 genera were detected, and the top 20 genera were considered and accounted for approximately 84% of the relative abundance of all genera (Fig. 2B). Notably, the enrichment of *Escherichia-Shigella* and *Escherichia coli* in the jejunum of the CON group was significantly reduced by feeding *C. beijerinckii* R8 (Fig. 2C). *Lactobacillus acidophilus* was enriched in the LCB group, while *Eubacterium* and *Corynebacterium* were enriched in the MCB group and *Lactobacillus*, *Enterococcus* and *Conservatibacter* were enriched in the HCB group (Fig. 2C). PICRUSt2 was used to predict functional abundance of jejunal bacteria (Fig. S3). At the first functional level, the cellular processes of jejunal bacteria pathway were decreased ($P < 0.001$) with *C. beijerinckii* R8 supplementation (Fig. 2D). At the second functional level, the infectious disease: bacterial, drug resistance: antimicrobial and immune system pathways were decreased ($P < 0.001$) with *C. beijerinckii* R8 supplementation (Fig. 2E). At the third functional level, bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection and shigellosis pathways were decreased with the with *C. beijerinckii* R8 supplementation (Fig. 2F).

A total of 2,035,970 sequences were obtained from the colon contents of 24 goat kids, with an average of 84,832 reads per sample. The examination identified a total of 1742 ASVs. As shown in Fig. S1, the Chao1, Observed species, PD-whole tree, and Shannon indexes of bacterial richness and diversity were no different among the four groups. PCoA and NMDS analysis showed an inability to distinguish among the four groups of samples (Figs. S2C and D). From all treatments, 7 phyla were present in colon contents. The total abundance of Bacteroidota, Proteobacteria, and Firmicutes (relative level >1%) exceeded 98 percent of the total abundance of the colon for CON, LCB, MCB, and HCB (Fig. 2G). In the colon, 95 genera were detected, 48 genera were common from all treatments, and the top 20 genera accounted for more than 95% of total abundance (Fig. 2H). The abundance of *Escherichia-Shigella* in the colon accounted for 1.17%, 0.52%, 0.54% and 0.42% of the sequences for the CON, LCB, MCB, and HCB groups, respectively, which were significantly reduced ($P < 0.001$) by feeding *C. beijerinckii* R8 (Fig. 2H). The distribution of functional gene abundance was no different in colonic bacteria (Fig. S4).

3.6. Correlation analysis between intestinal bacteria and intestinal health

The association between microbial composition and intestinal barrier index was analyzed based on Pearson's rank correlation coefficient. In the jejunum, *Escherichia-Shigella* was negatively correlated with SOD, T-AOC, GSH-Px, CAT, IL-10, TGF- β , and PPAR- γ (Fig. 3A). The *Escherichia-Shigella* was positively correlated with

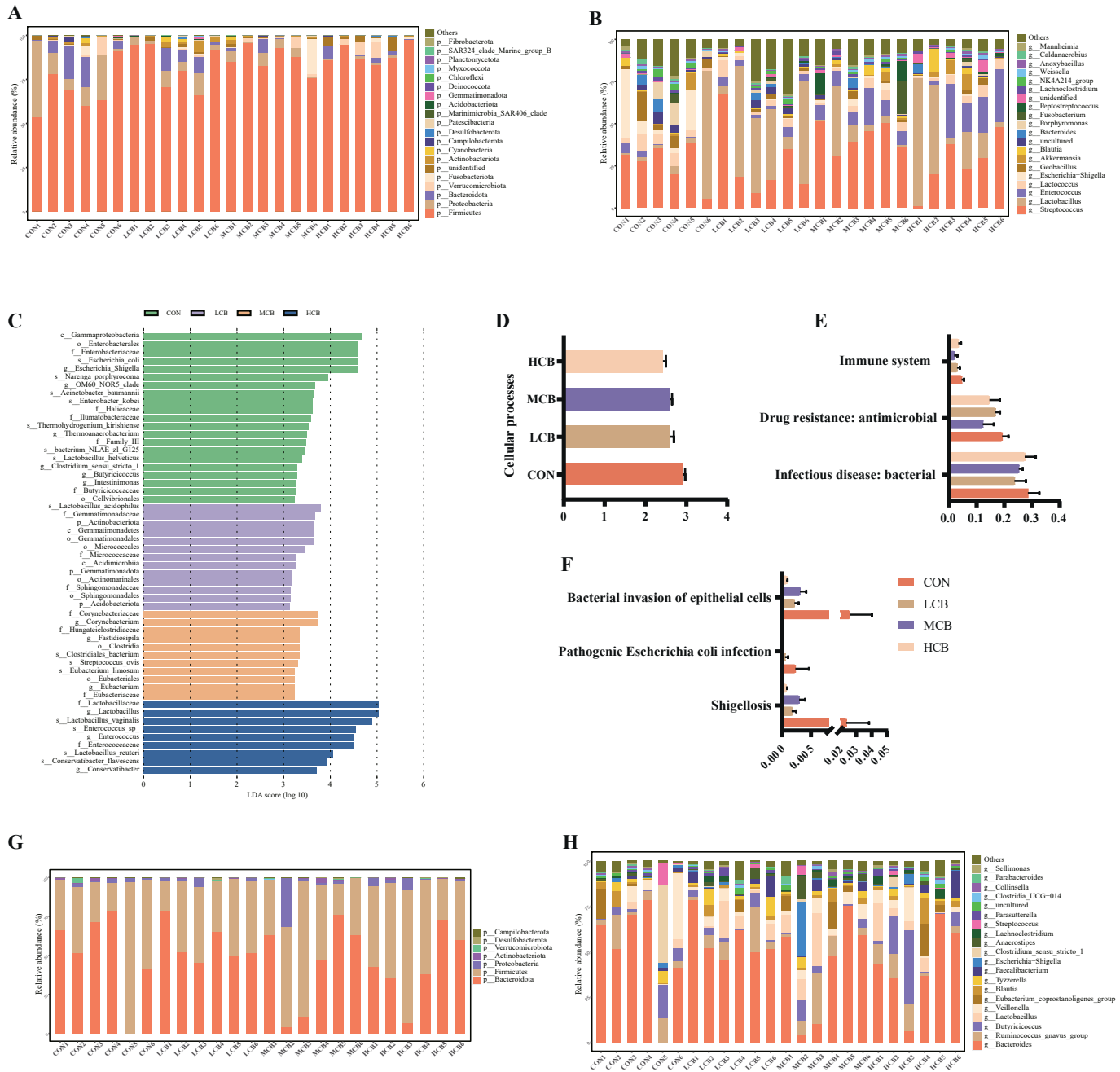


Fig. 2. Effects of *C. beijerinckii* R8 on intestinal microbial composition and comparison of predicted KEGG functions. (A) Relative abundance at the bacterial phylum level in jejunum. (B) Relative abundance at the bacterial genera level in jejunum. (C) Identification of crucial bacteria in the jejunum of four groups of goat kids by LEfSe analysis. (D to F) Predicted KEGG differential functions in functional classification levels 1, 2 and 3, respectively. (G) Relative abundance at the bacterial phylum level in colon. (H) Relative abundance at the bacterial genera level in colon. CON, supplemented with sterile solution; LCB, supplemented with 5×10^7 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; MCB, supplemented with 5×10^8 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d; HCB, supplemented with 5×10^9 CFU/mL of *C. beijerinckii* R8 and each with 10 mL/d.

MDA, TNF- α , IL-1, and IL-6 (Fig. 3A). In addition, *Escherichia-Shigella* was negatively correlated with IgA, IgG, IgM, claudin-1, claudin-4, occludin, MUC2, and ZO-1 (Fig. 3B). *NK4A214* and *Porphyromonas* were negatively correlated with sIgA (Fig. 3B). In the colon, *Faecalibacterium* was positively correlated with SOD, T-AOC, GSH-Px, CAT, IL-10, PPAR- γ , TGF- β , IgA, IgG, IgM, sIgA, claudin-1, claudin-4, occludin, MUC2, and ZO-1 (Fig. 3C–D). Meanwhile, *Faecalibacterium* was negatively correlated with MDA, TNF- α , IL-1, and IL-6 (Fig. 3C). The association analysis using intestinal composition and functional prediction revealed that *Escherichia-Shigella* and *Bacteroides* were significantly correlated with three pathways at the first functional level, six pathways at the second

functional level, and four pathways of the top 20 pathways at the third functional level (Fig. S3). The immune system was the main pathway involved in the regulation of intestinal inflammation, which was significantly correlated with the relative abundance of *Escherichia-Shigella*. In the colon, *Escherichia-Shigella* was significantly correlated with one pathway at the first functional level, four pathways at the second functional level, and two pathways of the top 20 pathways at the third functional level (Fig. S4). The infectious disease: bacterial pathway was the main pathway involved in the regulation of intestinal inflammation, which was significant correlated with the relative abundance of *Escherichia-Shigella*.

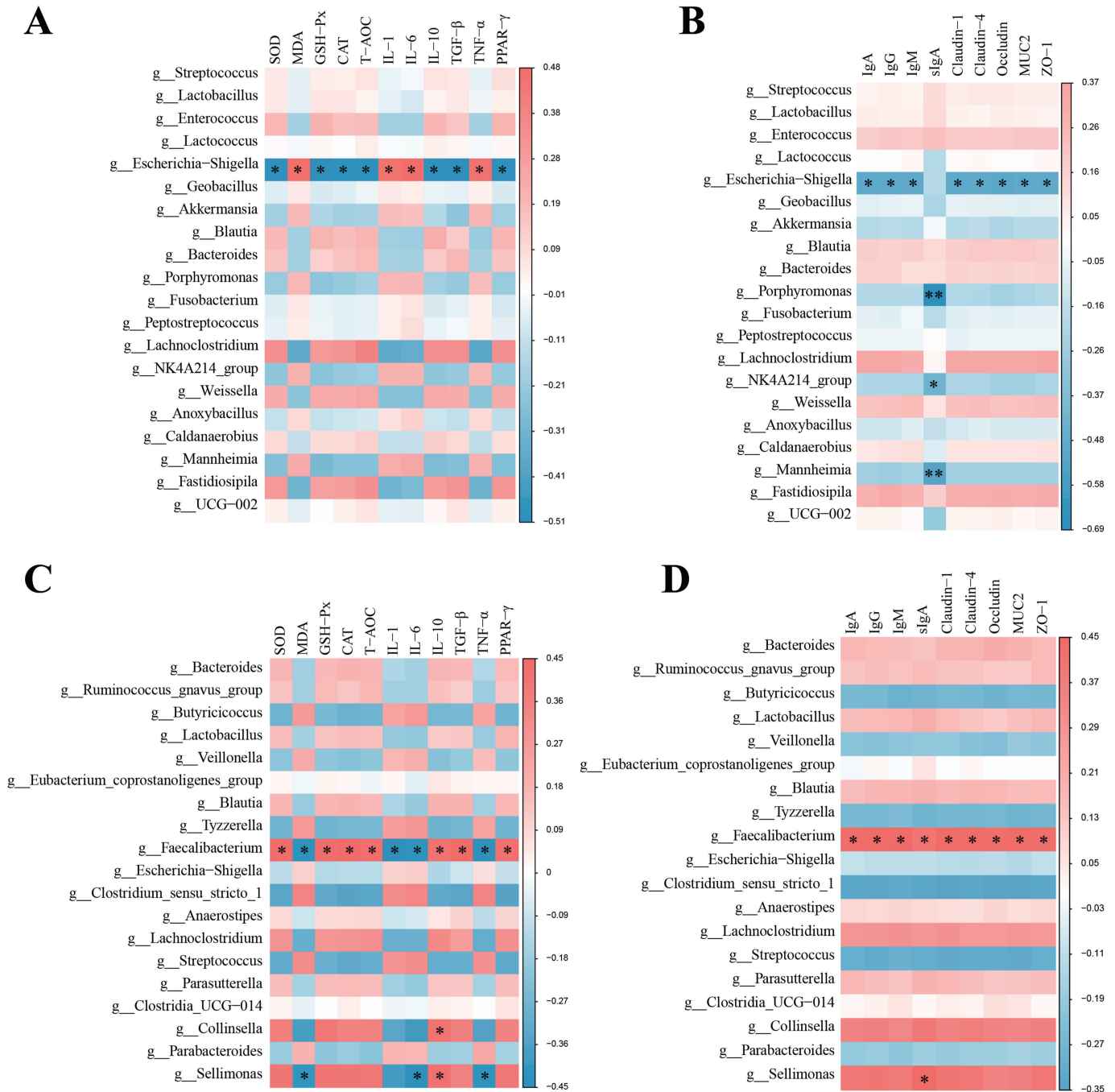


Fig. 3. Correlation analysis based on Spearman correlation coefficient. (A) The results of association analysis between antioxidant markers and inflammatory factors and jejunal microbiota at genus level via the Pearson correlation coefficient of rank correlation. (B) The results of association analysis between immunity index and barrier function and jejunal microbiota at genus level via the Pearson correlation coefficient of rank correlation. (C) The results of association analysis between antioxidant markers and inflammatory factors and colonic microbiota at genus level via the Pearson correlation coefficient of rank correlation. (D) The results of association analysis between immunity index and barrier function and colonic microbiota at genus level via the Pearson correlation coefficient of rank correlation. * $P < 0.05$; ** $P < 0.01$. T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; MDA = malondialdehyde; TNF- α = tumor necrosis factor- α ; IL-1 β = interleukin-1 β ; IL-6 = interleukin-6; TGF- β = transforming growth factor- β ; PPAR- γ = peroxisome proliferator-activated receptor- γ ; IL-10 = interleukin-10; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; sIgA = secretory immunoglobulin A; MUC2 = mucoprotein 2; ZO-1 = zonula occludens-1.

4. Discussion

Eighty-one strains of butyrate-producing bacteria were isolated with the integrated method, of which only eight were defined as high butyrogenic strains. The strain with the highest yield of butyrate was selected and defined as *C. beijerinckii* R8. It should be noted that the *C. beijerinckii* R8 was isolated from sheep rumen.

Adding *C. beijerinckii* R8 to the diet to regulate the intestinal microflora or even physiological health of goat kids is an application of rumen microbial transplantation. Generally, dietary butyrate-producing bacteria supplementation improves the growth performance (Li et al., 2021), modulates the muscle development (Dou et al., 2023) and reduces the diarrhea rate (Chen et al., 2018) of animals, but the experimental results are varied. In

the present study, distinct intestinal mucosal barrier and microbiota responses to supplementing the diet with *C. beijerinckii* R8 were observed. Dietary *C. beijerinckii* R8 significantly reduced the fecal score and diarrhea rate in goat kids during the trial period.

Changes in the structure of intestinal flora is an important marker of diarrhea (Liu et al., 2023). The invasion of host tissues by harmful bacteria and opportunistic pathogens leads to diseases and inflammation. The balance between intestinal microbiota and host immunity is the key to resisting pathogen invasion and preventing inflammation. Disruption of the balance between the host and gut microbes not only has the potential to result in inflammatory diseases (Abenavoli et al., 2022), but may also contribute to autoimmune diseases (Garrett et al., 2010). Previous studies indicated that ingestion of *Clostridium butyricum* stimulated the expansion of beneficial bacteria and inhibited the growth of unwanted bacteria, which had a positive impact on the intestinal ecosystem (Cao et al., 2019; Zhang et al., 2017). The LEfSe analysis in this study revealed a significant association between intestinal inflammation and the differential microorganisms *Escherichia-Shigella* and *Lactobacillus*, which provided additional evidence to support the above conclusion (Hou et al., 2015; Sun et al., 2021). Therefore, the results indicated that the lower *Escherichia-Shigella* in the *C. beijerinckii* R8 diet group was beneficial and decreased the diarrhea rate of goat kids. *Faecalibacterium* dominates the intestinal flora and serves as a potential biomarker to identify intestinal diseases, especially intestinal inflammation and irritable bowel syndrome (Rabiei et al., 2019). *Faecalibacterium* participates in polysaccharide metabolism to produce a variety of metabolites, which can alleviate intestinal inflammation (Miquel et al., 2015), promote immune response, and maintain intestinal homeostasis (Sokol et al., 2008). In this study, our data indicated that *Faecalibacteria* in the colon was significantly associated with immune indexes and tight junction protein. Therefore, the improvement of colon immunity may be related to the genus *Faecalibacteria*. The differences in functional pathways were mainly associated with *Escherichia-Shigella*, which further corroborated that *C. beijerinckii* R8 altered gut microbial function by reducing the abundance of *Escherichia-Shigella*. In brief, the addition of *C. beijerinckii* R8 appeared to maintain gut health by shaping the microbiota.

Diarrhea occurs alongside intestinal inflammation and a disruption of the intestinal microbiota (Dahiya and Nigam, 2023; Liu et al., 2023). The presence of dysbiosis, which involves an impaired intestinal barrier, results in an expedited interaction between the host's immune cells and microbiota (Potrykus et al., 2021). According to scientific studies, altering the microbiota in different disease conditions may decrease inflammation (Huang et al., 2023; Taylor et al., 2022; Xu et al., 2021). Probiotics provide beneficial impacts on the host by regulating the microbiota to suppress inflammation and enhance the integrity of the intestines (So et al., 2023; Zhao et al., 2020). Consistent with the results of previous studies, dietary supplementation with *C. beijerinckii* R8 decreased the IL1 β and TNF α level, while increasing the content of TGF- β and affecting the expression of IL-10 (Fu et al., 2021; Wang et al., 2016; Zhao et al., 2020). In addition, our study suggested that feeding *C. beijerinckii* R8 up-regulates PPAR- γ content and down-regulates IL-6 content, which may be associated with relieving intestinal inflammation and reducing the rate of diarrhea. Generally, serum levels of ALT and AST are important indicators of systemic inflammation. When inflammation occurs, the level of ALT and AST will increase rapidly. The results of this experiment suggested that *C. beijerinckii* R8 decreases the content of ALT and AST and protects intestinal barrier functions. Immunoglobulin IgA, IgG and IgM are the main antibodies that reflect immune status, whose content indicate the immune level of intestinal mucosa to a certain extent. Feeding microbes directly has the potential to maintain

animal health via enhancing immune capacity (Zhang et al., 2023, 2017). Consistent with this, ingestion of *C. beijerinckii* R8 increased the levels of IgA, IgG, IgM in the jejunum and colon in our study, which indicated better immunity and faster immune response. In summary, the evidence indicated that *C. beijerinckii* R8 could regulate the content of intestinal mucosal inflammatory factors and reduce intestinal inflammatory response, thereby enhancing the intestinal immunity and promoting the intestinal health of goat kids.

The permeability of the intestinal epithelium relies on the tight junction proteins, which consist mainly of occludin, zonula occludens (ZO) and claudins (Ghosh et al., 2021). The increased permeability of the gut allows inflammatory mediators to interact with the host, ultimately leading to inflammation (Ghosh et al., 2021). A study found that the ingestion of *Clostridium butyricum* in weaned piglets promoted the RNA expression of tight junction proteins in the intestines (Zong et al., 2019). Similarly, the levels of jejunal and colonic tight junction proteins were significantly increased by adding *C. beijerinckii* R8. Moreover, the increased MUC2 content provided a mucus lining for the intestine, which complemented the intestinal physical barrier to inhibit direct microbial-host communication (Potrykus et al., 2021). An important manifestation of intestinal barrier damage is the release of DAO and D-lactic acid into the serum. As important indicators of intestinal permeability, DAO (Xiao et al., 2019) and D-lactic acid (Zhao et al., 2011) are blocked and unable to enter the peripheral blood supply when intestinal permeability is reduced. According to our study, the supplementation of *C. beijerinckii* R8 in milk replacer resulted in a notable decrease in both D-lactic acid and DAO activity levels of newborn goat kids, which suggested that *C. beijerinckii* R8 exerts positive effects in reducing intestinal permeability. In our current work, the significantly increased tight junction proteins in the intestines signified lower intestinal permeability which could facilitate remission of inflammation (Ghosh et al., 2021). Intestinal histomorphology has been extensively utilized in the evaluation of intestinal development and function (Chen et al., 2018). Diarrhea may be attributed to villous atrophy and crypt hypertrophy, which reduce nutrient digestion and absorption (Huang et al., 2015; O'Loughlin et al., 1991). The crucial mechanism behind this phenomenon relates to the direct association between increased VH/CD and enhanced epithelial turnover (Ichikawa et al., 1999). Previous studies indicated that probiotics as feed additives continually increased the VH/CD of piglets (Pan et al., 2017) and ameliorated diarrhea significantly (Wang et al., 2019). Similar to the above studies, dietary supplementation with *C. beijerinckii* R8 increased jejunal VH/CD and improved diarrhea. Thus, the addition of *C. beijerinckii* R8 could accordingly alter the intestinal physiological state, thereby affecting diarrhea rate and fecal scores.

Accumulated data from animal experiments suggest that oxidative stress results in impairment to the intestinal mucosal layer and dysbiosis, and ultimately stimulates the immune response (Li et al., 2023). Recently, a study suggested that dietary addition of *Clostridium butyricum* improves the antioxidant capacity of goats under immune stress (Zhang et al., 2023). The mechanism underlying this phenomenon is related to the fact that H₂ produced by the fermentation of *Clostridium butyrate* rapidly spreads into tissues and cells, selectively reacting with potent oxidizing agents including hydroxyl radicals and nitrite anions in cells and regulating gene expression to reduce oxidative stress (Slezak et al., 2016). In this study, *C. beijerinckii* R8 significantly improved the antioxidant capacity of goat kids. Another study has shown that sodium butyrate improved intestinal epithelial barrier damage induced by oxidative stress through the AMPK-mitophagy pathway (Li et al., 2022), which may partly explain this result. A previous study found that IPEC-J2 cells produced MDA in response

to ETEC stimulation, and the concentration of ETEC directly correlates with the increase with MDA content, aligning with the results obtained from this experiment (Dou et al., 2021). The findings from this experiment align with the previous research, indicating that *C. beijerinckii* R8 exerts positive antioxidant effects.

5. Conclusion

In this study, a novel strain of *C. beijerinckii* R8 was isolated and utilized as a feed additive to investigate its effects on microflora composition, intestinal barrier, and diarrhea in goat kids. By incorporating *C. beijerinckii* R8 into the diet, a more balanced and beneficial microflora was established in the gut, which was achieved by inhibiting the growth of *Escherichia-Shigella* and stimulating the reproduction of *Lactobacillus*. The altered microflora enhanced intestinal immune response and antioxidant capacity through microbiome-host crosstalk. Meanwhile, *C. beijerinckii* R8 improved intestinal health by reducing intestinal permeability. These changes ultimately led to a reduction in diarrhea rates in goat kids. Overall, this study contributes to our knowledge of the vital roles played by gut microbiota in both immunity and diarrhea, thereby providing support for the utilization of *C. beijerinckii* R8 in production systems of goat kids.

Availability of data and materials

The datasets used and analyzed during the current study are available from the NCBI.

Sequence Read Archive (SRA), accession number PRJNA1088740 (Supplementary file 2).

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Author contributions

Naifeng Zhang: Conceptualization, experimental design, draft proofing, and funding acquisition resources. **Dingkun Fan:** Investigation, formal analysis, writing—original draft. **Yuze Fu:** animal trials, sample collection, data curation. **Jixian Zhang:** analysis and data collection. **Yanliang Bi and Tao Ma:** data analysis and proofing. **Qiyu Diao:** Writing - review & editing. All authors have read and approved the final manuscript.

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Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2024.06.004>.

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