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Original Research Article

Bacillus pumilus 315 improves intestinal microbiota and barrier function to alleviate diarrhea of neonatal goats

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ABSTRACT

Diarrhea is the leading cause of mortality in postnatal goat kids, seriously impacting breeding efficiency. This study aimed to explore the effects of *Bacillus pumilus 315* (*B. pumilus*) on goat kids' diarrhea and its regulatory mechanism. Thirty-six 1-day-old goat kids were assigned into four treatments, the control (CON) group and low-, medium- and high-dose groups supplemented with *B. pumilus* at 1×10^8 (BP1), 5×10^8 (BP5), and 1×10^9 CFU/d (BP10). Each group consisted of 9 replicates with 1 goat kid per replicate. The results showed that the incidence of diarrhea and fecal scores decreased significantly ($P < 0.05$). A dose of 5×10^8 CFU/d *B. pumilus* reduced pro-inflammatory factors (including tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β], interleukin-6 [IL-6], $P < 0.05$), increased the expression levels of anti-inflammatory factors (including transforming growth factor-β [TGF-β], peroxisome proliferate-activated receptor-gamma [PPAR-γ], interleukin-10 [IL-10], $P < 0.05$), immune indicators (including immunoglobulin G [IgG], immunoglobulin A [IgA], immunoglobulin M [IgM], secretory immunoglobulin A [sIgA], $P < 0.05$) and antioxidant indicators (including total antoxidative capacity [T-AOC], superoxide dismutase [SOD], glutathione peroxidase [GSH-Px], catalase [CAT], $P < 0.05$) in both jejunum and colon, and ultimately improved the barrier function of the jejunum and colon mucosa. The enhanced gut immunity and barrier function were associated with increased abundance of *Enterococcus* and *Lactobacillus* ($P < 0.05$) and decreased abundance of *Campylobacter* and *Escherichia-Shigella* ($P < 0.05$). In conclusion, dietary addition of *B. pumilus* may improve gut health by modulating the composition and function of the flora, ultimately alleviating diarrhea in goat kids.
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1. Introduction

After birth, lambs and goat kids face various challenges, including changes in nutrition, environment, and exposure to different microorganisms. During this period, the immune and digestive organs of lambs and goat kids are underdeveloped and lack adaptability, leading to a susceptibility to intestinal dysfunction and diarrhea. A study found that diarrhea prevalence in lambs was 34.21% and mortality was 15.69% (Mariano et al., 2018). Especially, the incidence of diarrhea is more than 60% within the initial 15 days of life, seriously impacting the gut health and growth performance of lambs (Fortuoso et al., 2019). The gut is an extremely complex ecosystem in which the dynamic balance among diet, microbiome and host tissue directly affects the development and physiological health of lambs (Pantazi et al., 2023). Accumulating evidence indicates that diarrhea is accompanied by corresponding changes in gut microbes, immunity and metabolites in lambs and goat kids (Wang et al., 2023). The association between the microflora and host gut has sparked interest in

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using probiotics for disease prevention and treatment in animals. Previous studies have demonstrated the effectiveness of probiotics in combating diarrhea, highlighting the recognized importance of probiotics as feed additives in animal production (Fan et al., 2021; Jiang et al., 2023). *Bacillus,* one of the most common probiotics, has been widely used in humans and animals (Cutting, 2011). It tends to survive in adverse conditions and germinate in the gut of animals due to its spore form (Hong et al., 2005). The consumption of probiotics, including *Bacillus* strains, for gut health benefits and overall well-being is on the rise in animals (Singh et al., 2022). *Bacillus subtilis* ameliorates ETEC-induced piglet diarrhea through its probiotic effects (He et al., 2020). *Bacillus licheniformis* alleviates diarrhea in weaned piglets by improving immune levels and antioxidant capacity (Yu et al., 2022). However, there is limited research on the potential of *Bacillus pumilus* (*B. pumilus*) in alleviating diarrhea in young ruminants. *B. pumilus* is a facultative anaerobic gram-positive bacterium. It participates in biological oxygen deprivation and maintains the anaerobic environment of the gastrointestinal tract (Lei et al., 2024). Studies suggested that *B. pumilus* may promote the expressions of genes related to the immune system (Zhang et al., 2023), regulate intestinal microflora (Zhang et al., 2020), and possess potential antibacterial abilities (Zeng et al., 2022).

Due to the underdeveloped intestinal tract of young ruminants and the susceptibility of their intestinal flora to change, they are particularly vulnerable to the influence of probiotics (Du et al., 2023). Thus, it was hypothesized that *B. pumilus* alleviates diarrhea in goat kids by regulating the intestinal barrier. The aim of this study was to elucidate the regulatory effects of *B. pumilus* on diarrhea and intestinal inflammation of goat kids by assessing its effect on the physical, chemical, immune, and microbial barrier functions of intestines, providing a basis for the further development and application of *B. pumilus* in ruminants.

2. Materials and methods

2.1. Animal ethics statement

The experiment was conducted on a commercial farm located in Hohhot, Inner Mongolia. The experimental procedures were approved by the Animal Ethics Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences (protocol number: IFR-CAAS-20220822).

2.2. Animals and experimental design

Thirty-six 1-day-old male Saanen goat kids (2.89 ± 0.31 kg) were randomly divided into 4 groups with 9 replicates per group and 1 goat kid per replicate. The control group (CON) was dosed daily with 10 mL sterile solution, while the low-, medium- and high-dose groups were dosed with *B. pumilus* at 1×10^8 (BP1), 5×10^8 (BP5), and 1×10^9 CFU/d (BP10). The *B. pumilus* for each treatment was dissolved into a 10-mL milk replacer solution and fed to the goat kids. The experiment lasted for 14 days. Six replicates in each group were randomly selected for slaughter and sampling on the 15th day.

The *B. pumilus 315* used in this experiment was cultured by our laboratory. The sequence access number in National Center for Biotechnology Information (NCBI) is KC790382. The viable bacteria number in the culture medium was 1×10^8 CFU/mL. The bacterial solution was stored in a refrigerator at 4 °C for the feeding experiment. *B. pumilus* solution was mixed with milk replacer and fed only once in the morning. The nutritional level of milk replacer is shown in Table 1. The milk replacer solution was kept at 40 °C during feeding. At the same time, each goat kid was guaranteed free access to a milk replacer. The daily health care of goat kids was carried out according to the daily management procedure of the farm. Within 14 days of the experiment period, colostrum was fed in the first 3 days, followed by milk replacer. All goat kids were fed thrice at 08:00, 14:00 and 20:00. The dry matter of the milk replacer was determined by drying the feed at 135 °C for 2 h (method 930.15; AOAC, 1990). The milk replacer samples were burned at 550 °C for 4 h to determine the ash content (method 942.05; AOAC, 1990). The nitrogen in feed ingredients was detected by the Kjeldahl method, and crude protein (CP) was calculated using nitrogen × 6.25 (method 991.20; AOAC, 1990). The crude fiber (CF) was determined according to the methods described by previous studies (Van Soest et al., 1991). The ether extract (EE) was detected by the Soxhlet extractor method (method 920.39; AOAC, 1990). The mineral composition of milk replacer was determined according to the methods described by the AOAC (method 985.01; AOAC,1990). The amino acid of milk replacer was determined according to the methods described by the AOAC (method 988.15; AOAC, 1990). The lactose was detected according to the methods described by the AOAC (method 972.16; AOAC, 1990).

Table 1
Nutrient levels of milk replacer (%, DM basis)[1].

Item	Contents
DM	95.90
ME, MJ/kg	12.20
CP	24.04
EE	16.10
Lactose	35.08
Ash	6.00
CF	0.70
Ca	0.84
P	0.62
Lys	2.20
Met	0.80
Thr	0.90
NaCl	1.00
Vitamin A, IU	20,000
Vitamin D, IU	6500
Vitamin E, IU	750

DM = dry matter; ME = metabolizable energy; CP = crude protein; EE = ether extract; Ash = crude ash; CF = crude fiber.
[1] Nutrient levels were analyzed values except ME which was calculated by the equation of ME = 4.411 + 0.324CP (Zhao et al., 2016).

Goat kids were weighed on the morning of the first and fifth day of the experiment, and milk replacer intake was recorded daily to characterize the growth performance. The average daily gain (ADG) and feed to gain ratio (F/G) were calculated by the following formula:

ADG = (final body weight − initial body weight)/experimental days;

F/G = feed intake/gain.

Feces were scored at 17:00 daily using a 1 to 4 scale described previously (Wang et al., 2019). Diarrhea rate and fecal score were calculated by using the following formula:

Diarrhea rate (%) =(number of kids with diarrhea × days of diarrhea)/(total number of goat kids × experimental days) × 100;

Fecal score = total fecal score/(trial days × number of goat kids).

2.3. Samples collection

A 2-cm intestinal ring was taken from the middle part of the jejunum and colon after the contents were rinsed gently with normal saline. After that, the intestinal ring was placed in a 10-mL centrifuge tube filled with 10% formalin for subsequent observation

of tissue morphology. The intestinal mucosa was scraped with a slide and collected into 2-mL cryopreservation tubes and stored in a −80 °C refrigerator for the determination of antioxidation, immunity, tight junction proteins and inflammatory factors. Jejunum and colon contents were collected into 2-mL cryopreservation tubes and stored at −80 °C for 16S high-throughput sequencing.

2.4. Intestinal mucosal biochemical parameters

The contents of mucin 2 (MUC2), claudin-1, claudin-4, occludin, zonula occludens (ZO) -1, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), peroxisome proliferator-activated receptor-γ (PPAR-γ), interleukin-10 (IL-10) and secretory immunoglobulin A (sIgA) in gut mucosa were determined by ELISA (Beijing Jinhaikeyu Biotechnology Development Co., Ltd., Beijing, China), and tested by ST-360 enzyme marker (Kehua Bioengineering Co., Ltd., Shanghai, China). The contents of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), malonaldehyde (MDA), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) in the gut mucosa were determined by the biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the samples were tested by L-3180 semi-automatic biochemical analyzer (Kehua Bioengineering Co., Ltd., Shanghai, China).

2.5. Hematoxylin-eosin staining for gut

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 produced by McAudi Industrial Group Co., Ltd. was used for image acquisition of sections. Each section of tissue was first observed under the low-power microscope. Measurement of villus height, crypt depth, mucosal thickness and muscle layer thickness of the visualized bowel using the Image-pro express image analysis and processing system. According to the pictures collected above, the number of goblet cells in each picture was counted by objective multiple (10×).

2.6. Intestinal 16S rRNA sequencing

The DNA of gut content is extracted using a DNA kit according to the manufacturer's guidelines. Then the V3–V4 region of 16S rDNA was amplified with specific primers (338F: 5′-ACTCCTACGGGAGGCAGCAG-3'; 806 R: 5′-GGACTACHVGGGTWTCTAAT-3′) with barcode. The PCR products were tested for the size of the amplified band using 1% agarose gel electrophoresis and purified with the Encourt AMPure XP nucleic acid purification kit. Through PCR amplification and enrichment of the library template, single-stranded DNA fragments were generated, and the MiSeq library was finally constructed for computer sequencing.

The Fastq data, obtained by MiSeq sequencing, were processed for quality control. After removing the barcode and primer and splicing, raw-tags were obtained, and high-quality sequences clean-tags were obtained after further removing chimeras and short sequences from row-tags. Then Quantitative Insights Into Microbial Ecology 2 (Qiime2) software was used to conduct statistical analysis of bioinformatics on amplicon sequence variants (ASVs) at 100% similar level and the Venn diagram was made with R language. Afterwards, the abundance and diversity of flora were analyzed based on ASV, and the flora structure was analyzed at the taxonomic level of the phylum and genus. Linear discriminant analysis effect size (LEfSe) analysis was performed to find bacteria genera with significant differences in abundance between groups. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) was employed to predict their functional profiles against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The correlation analysis was assessed using the Spearman algorithm and the correlation heatmap was drawn by R software ggplot2 package.

2.7. Statistical analysis

The χ2 procedure was used to compare the incidence of diarrhea between groups. The other experimental data were analyzed using the general linear model (GLM) procedure of SPSS 26.0 statistical software. The statistical model was as follows:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij},$$

where Y_{ij} is the dependent variable representing the jth observation in the ith group, μ is the overall mean, T_i is the fixed effect of the ith group, and ε_{ij} is the random error term. Duncan's method was used for multiple comparisons when the difference was significant. Polynomial contrasts were conducted to determine the linear and quadratic effects of increasing *B. pumilus* supplemental levels. Visualizations of alpha-diversity, beta diversity and LEfSe analysis were performed using R (v4.3.2). 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. $P < 0.05$ means there is a statistically significant difference. $0.05 \leq P < 0.10$ means there is a tendency to change.

3. Results

3.1. B. pumilus decreased diarrhea incidence

During the trial period, the BP1, BP5 and BP10 groups showed a decrease in the diarrhea rate ($P < 0.001$) and fecal scores ($P = 0.005$) compared with the CON group (Table 2). However, there was no significant difference among the BP1, BP5 and BP10 groups. The addition of *B. pumilus* at all levels led to a decrease in diarrhea rate at 1 to 7 days ($P = 0.047$). However, only the BP5 and BP10 groups showed a decrease in the diarrhea rate compared to the CON group at 8 to 14 days ($P = 0.001$). During 1 to 7 days, the fecal scores in the BP1 and BP5 groups were lower than that in the CON group ($P = 0.027$). During 8 to 14 days, the BP1, BP5 and BP10 groups had lower fecal scores ($P = 0.035$) compared to the CON group without significant differences among them. As shown in Table 3, no differences were observed in the initial body weight of goat kids

Table 2
Effects of *B. pumilus* on diarrhea rate and fecal score of goat kids.

Item	Group[1]				χ2	*P*-value
	CON	BP1	BP5	BP10		
Diarrhea rate, %						
1–14 d	53.17^{a}	24.58^{b}	29.03^{b}	31.20^{b}	26.755	<0.001
1–7 d	49.21^{a}	26.23^{b}	31.75^{b}	33.33^{b}	7.996	0.047
8–14 d	57.14^{a}	34.62^{ab}	26.23^{b}	29.03^{b}	15.821	0.001
Fecal score					SEM	*P*-value
1–14 d	1.93^{a}	1.41^{b}	1.42^{b}	1.49^{b}	0.153	0.005
1–7 d	1.83^{a}	1.26^{b}	1.44^{b}	1.51^{ab}	0.177	0.027
8–14 d	2.03^{a}	1.57^{b}	1.38^{b}	1.48^{b}	0.225	0.035

^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^8. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^8. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^9. CFU/d.

Table 3
Effects of *B. pumilus* on growth performance of goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatment	Linear	Quadratic
IBW, kg	2.83	2.83	3.00	2.88	0.312	0.932	0.726	0.776
FBW, kg	4.38	4.36	4.66	4.52	0.481	0.918	0.646	0.853
ADG, g/d	111.07	109.56	118.57	116.79	19.190	0.958	0.670	0.992
MRI, g/d	130.25	124.14	135.74	122.45	12.042	0.679	0.759	0.676
F/G	1.19	1.17	1.29	1.19	0.173	0.897	0.853	0.753

IBW = initial body weight; FBW = final body weight; ADG = average daily gain; MRI = milk replacer intake; F/G = feed to gain ratio.
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^8. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^8. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^9. CFU/d.

between groups ($P = 0.932$), meeting the requirements of the experiment design. Feeding *B. pumilus* had no significant effect on final body weight (FBW, $P = 0.918$), ADG ($P = 0.958$), milk replacer intake (MRI, $P = 0.679$) and F/G of goat kids ($P = 0.897$).

3.2. B. pumilus increased immunity of gut mucosa

In jejunum, with the dietary *B. pumilus* supplemental level increasing, the levels of TNF-α, IL-1β, and IL-6 exhibited a quadratic decline ($P = 0.006$, $P = 0.006$, $P = 0.008$, respectively; Table 4), whereas the levels of TGF-β, PPAR-γ, and IL-10 exhibited a quadratic increase ($P = 0.006$, $P = 0.005$, $P = 0.005$, respectively). Compared with the CON group, the contents of TNF-α, IL-1β and IL-6 decreased significantly ($P = 0.023$, $P = 0.020$, $P = 0.029$; respectively), whereas the contents of TGF-β, PPAR-γ and IL-10 increased in the BP1 and BP5 groups ($P = 0.026$, $P = 0.021$, $P = 0.023$; respectively). No significant differences in the contents of TNF-α, IL-1β, IL-6, TGF-β, PPAR-γ and IL-10 were observed between the BP10 and CON group. In the colon, with the dietary *B. pumilus* supplemental level increasing, the contents of TNF-α, IL-1β, IL-6, TGF-β, PPAR-γ and IL-10 showed a similar quadratic response ($P = 0.013$, $P = 0.009$, $P = 0.013$, $P = 0.015$, $P = 0.013$, $P = 0.015$, respectively) to that in jejunum. The contents of TNF-α, IL-1β and IL-6 significantly decreased compared with CON ($P = 0.015$, $P = 0.010$, $P = 0.016$; respectively), whereas the TGF-β, PPAR-γ and IL-10 increased in BP1, BP5 and BP10 groups ($P = 0.014$, $P = 0.014$, $P = 0.018$, respectively).

In the jejunum, with the dietary *B. pumilus* supplemental level increasing, the contents of IgG, IgA, IgM, and sIgA exhibited a quadratic increase ($P = 0.007$, $P = 0.004$, $P = 0.002$, $P = 0.008$; respectively). The BP1 and BP5 groups showed an increase in the levels of IgG, IgA, IgM, and sIgA compared with the CON group ($P = 0.026$, $P = 0.014$, $P = 0.010$, $P = 0.032$, respectively; Table 5). However, no significant differences were observed between the BP10 group and the other groups. In the colon, quadratic change ($P = 0.014$, $P = 0.014$, $P = 0.032$, $P = 0.014$; respectively) for IgG, IgA, IgM and sIgA were observed with the dietary *B. pumilus* supplemental level increasing. The contents of IgG, IgA, IgM and sIgA ($P = 0.019$, $P = 0.016$, $P = 0.024$, $P = 0.018$, respectively; Table 5) in BP1, BP5, and BP10 groups were higher than that in CON group.

3.3. B. pumilus increased antioxidation of gut mucosa

As shown in Table 6, with the dietary *B. pumilus* supplemental level increasing, the levels of T-AOC, SOD, GSH-Px, and CAT in jejunal mucosa showed a quadratic increase ($P = 0.003$, $P = 0.004$, $P = 0.005$, $P = 0.004$, respectively), whereas the content of MDA showed a quadratic decline ($P = 0.004$). Compared with the CON group, the levels of T-AOC, SOD, GSH-Px and CAT increased ($P = 0.016$, $P = 0.017$, $P = 0.017$, $P = 0.015$, respectively), while the content of MDA decreased in jejunal mucosa of the BP1 and BP5 groups ($P = 0.017$). No significant differences in the levels of T-AOC, SOD, GSH-Px, CAT and MDA were observed between the BP10 and the other groups. With the dietary *B. pumilus* supplemental level increasing, a quadratic pattern was observed in the colon for T-AOC, SOD, GSH-Px, CAT and MDA ($P = 0.020$, $P = 0.015$, $P = 0.015$, $P = 0.011$, $P = 0.014$, respectively), similar to the trend observed in the jejunum. The levels of T-AOC, SOD, GSH-Px and CAT in colonic mucosa were higher ($P = 0.028$, $P = 0.028$, $P = 0.018$, $P = 0.014$, respectively), while the content of MDA was lower ($P = 0.018$) compared with CON group.

Table 4
Effects of *B. pumilus* on intestinal inflammatory of goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatment	Linear	Quadratic
Jejunum								
TNF-α, pg/mg	35.62^{a}	32.09^{b}	32.44^{b}	33.88^{ab}	0.475	0.023	0.193	0.006
IL-1β, pg/ng	15.98^{a}	13.62^{b}	13.75^{b}	14.75^{ab}	0.320	0.020	0.157	0.006
IL-6, pg/mg	35.53^{a}	31.52^{b}	31.70^{b}	33.50^{ab}	0.567	0.029	0.191	0.008
TGF-β, ng/mg	6.44^{b}	7.30^{a}	7.23^{a}	6.86^{ab}	0.119	0.026	0.208	0.006
PPAR-γ, nmol/g	28.60^{b}	33.39^{a}	33.08^{a}	30.93^{ab}	0.653	0.021	0.191	0.005
IL-10, pg/mg	36.81^{b}	41.60^{a}	41.57^{a}	38.21^{ab}	0.674	0.023	0.177	0.005
Colon								
TNF-α, pg/mg	36.65^{a}	33.14^{b}	32.87^{b}	33.81^{b}	0.493	0.015	0.026	0.013
IL-1β, pg/ng	16.71^{a}	14.17^{b}	13.93^{b}	14.71^{b}	0.350	0.010	0.023	0.009
IL-6, pg/mg	36.91^{a}	32.71^{b}	32.29^{b}	33.52^{b}	0.601	0.016	0.028	0.013
TGF-β, ng/mg	6.17^{b}	7.06^{a}	7.18^{a}	6.93^{a}	0.129	0.014	0.021	0.015
PPAR-γ, nmol/g	27.15^{b}	32.14^{a}	32.50^{a}	31.20^{a}	0.699	0.014	0.025	0.013
IL-10, pg/mg	35.46^{b}	40.50^{a}	40.90^{a}	39.48^{a}	0.721	0.018	0.032	0.015

TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; IL-6 = interleukin-6; TGF-β = transforming growth factor-β; PPAR-γ = peroxisome proliferate-activated receptor-gamma; IL-10 = interleukin-10.
[a,b] Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^8. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^8. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^9. CFU/d.

Table 5
Effects of *B. pumilus* on intestinal immune indexes of goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatment	Linear	Quadratic
Jejunum								
IgG, g/L	6.23^{b}	7.31^{a}	7.25^{a}	6.80^{ab}	0.360	0.026	0.168	0.007
IgA, g/L	0.39^{b}	0.47^{a}	0.47^{a}	0.43^{ab}	0.026	0.014	0.144	0.004
IgM, g/L	0.79^{b}	0.88^{a}	0.87^{a}	0.83^{ab}	0.026	0.010	0.151	0.002
sIgA, ng/mL	5.74^{b}	6.41^{a}	6.40^{a}	6.08^{ab}	0.237	0.032	0.200	0.008
Colon								
IgG, g/L	5.95^{b}	7.02^{a}	7.16^{a}	6.80^{a}	0.379	0.019	0.035	0.014
IgA, g/L	0.37^{b}	0.45^{a}	0.46^{a}	0.44^{a}	0.028	0.016	0.028	0.014
IgM, g/L	0.77^{b}	0.85^{a}	0.86^{a}	0.84^{a}	0.029	0.024	0.031	0.032
sIgA, ng/mL	5.52^{b}	6.23^{a}	6.30^{a}	6.08^{a}	0.242	0.018	0.035	0.014

IgG = immunoglobulin G; IgA = immunoglobulin A; IgM = immunoglobulin M; sIgA = secretory immunoglobulin A.
^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^{8}. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^{8}. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^{9}. CFU/d.

Table 6
Effects of *B. pumilus* on the antioxidant activity of intestine in goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatment	Linear	Quadratic
Jejunum								
T-AOC, U/mg	6.92^{b}	7.77^{a}	7.71^{a}	7.29^{ab}	0.269	0.016	0.233	0.003
SOD, U/mg	67.79^{b}	78.19^{a}	77.55^{a}	72.94^{ab}	3.295	0.017	0.170	0.004
GSH-Px, U/mg	590.35^{b}	674.40^{a}	670.01^{a}	635.30^{ab}	26.468	0.017	0.135	0.005
CAT, U/mg	4.92^{b}	5.76^{a}	5.72^{a}	5.34^{ab}	0.265	0.015	0.156	0.004
MDA, nmol/mg	2.38^{a}	1.83^{b}	1.86^{b}	2.12^{ab}	0.175	0.017	0.190	0.004
Colon								
T-AOC, U/mg	6.74^{b}	7.53^{a}	7.61^{a}	7.36^{a}	0.287	0.028	0.045	0.020
SOD, U/mg	65.17^{b}	75.29^{a}	76.48^{a}	73.38^{a}	3.494	0.018	0.030	0.015
GSH-Px, U/mg	573.17^{b}	653.99^{a}	664.07^{a}	638.86^{a}	28.086	0.018	0.030	0.015
CAT, U/mg	4.72^{b}	5.55^{a}	5.63^{a}	5.37^{a}	0.274	0.014	0.029	0.011
MDA, nmol/mg	2.53^{a}	1.99^{b}	1.92^{b}	2.10^{b}	0.187	0.018	0.033	0.014

T-AOC = total antoxidative capacity; SOD = superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde.
^{a,b} Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^{8}. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^{8}. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^{9}. CFU/d.

3.4. *B. pumilus* improved intestinal morphological development

The villus height, villus height/crypt depth (VH/CD), mucosal thickness, muscle layer thickness and goblet cell number in jejunum showed a quadratic increase ($P < 0.001$, $P < 0.001$, $P = 0.015$, $P < 0.001$, $P = 0.019$, respectively) with the increase of *B. pumilus* level (Table 7 and Fig. 1). Compared with the CON group, the BP5 and BP10 groups showed significant increases in villus height and VH/CD ($P < 0.001$, $P < 0.001$, respectively), with the BP5 group exhibiting higher values than the BP10 group ($P < 0.001$). No significant impact on crypt depth was observed among the four groups. The mucosal thickness of the BP5 group was significantly higher than the other groups ($P < 0.001$). The muscle layer thickness was increased in the BP1, BP5, and BP10 groups compared with the CON group ($P < 0.001$). Additionally, the BP1 group showed a tendency towards an increase in the number of goblet cells, as opposed to the CON and BP10 groups ($P = 0.076$). No significant differences were observed in the villus height, VH/CD, mucosal thickness and goblet cells of the colon between the treatment groups.

3.5. *B. pumilus* enhanced intestinal tight junction function

Next, the parameters of tight junction proteins were measured to determine gut mucosal permeability affected by diarrhea. In jejunum, the BP1 and BP5 groups showed significant increases in the contents of claudin-1, claudin-4, MUC2, ZO-1, and occludin compared with CON group ($P = 0.019$, $P = 0.016$, $P = 0.014$, $P = 0.016$, $P = 0.017$, respectively; Table 8). However, there were no significant differences in the protein contents of claudin-1, claudin-4, MUC2, ZO-1, and occludin between the BP10 group and the other groups. In the colon, the contents of claudin-1, claudin-4, MUC2, ZO-1, and occludin significantly increased with the supplementation of *B. pumilus* ($P = 0.020$, $P = 0.022$, $P = 0.017$, $P = 0.022$, $P = 0.017$, respectively; Table 8).

3.6. *B. pumilus* inhibited diarrhea related microorganisms

By high-throughput sequencing of the V3–V4 region of bacterial 16S rRNA in jejunum contents of 24 goat kids, 1,566,351 high-quality sequences were obtained after quality control. According to the Venn diagram (Fig. S1A), the number of ASVs owned by the CON, BP1, BP5 and BP10 groups were 467, 324, 389 and 451, respectively, and the total number of ASVs shared among the four groups was 116. The *B. pumilus* supplementation had no significant effect on bacterial alpha diversity in the jejunum contents of goat kids (Fig. S1B). Anosim analysis showed the differences among the four groups ($R = 0.546$, $P = 0.001$) (Fig. S1C). Partial least squares discriminant analysis (PLS-DA) visualization analysis and non-metric multidimensional scaling (NMDS) analysis further demonstrated the CON group was separated from the BP1, BP5 and BP10 groups, indicating that significant differences between the CON group and the BP1, BP5, BP10 groups (Figs. S1D and S1E).

In the 24 samples of jejunum contents, a total of 31 phyla were detected. Bacteroidata, Proteobacteria, Firmicutes and Verrucomicrobiota were the dominant phyla in all samples with a relative abundance exceeding 1%. These four phyla accounted for over 95%

Table 7
Effects of *B. pumilus* on intestinal morphology of goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatmeat	Linear	Quadratic
Jejunum								
Villus height, μm	687.87^{c}	722.88^{bc}	936.39^{a}	746.49^{b}	17.904	<0.001	<0.001	<0.001
Crypt depth, μm	285.72	273.57	273.59	266.17	8.010	0.112	0.202	0.667
VH/CD	2.51^{c}	2.66^{bc}	3.37^{a}	2.81^{b}	0.122	<0.001	<0.001	<0.001
Mucosal thickness, μm	1062.82^{b}	1048.07^{b}	1158.25^{a}	1056.48^{b}	24.917	<0.001	0.250	0.015
Muscle layer thickness, μm	143.36^{b}	186.84^{a}	187.83^{a}	190.09^{a}	5.084	<0.001	<0.001	<0.001
Goblet cell, cell/mm^2	44.0	54.7	49.0	42.5	4.94	0.076	0.518	0.019
Colon								
Crypt depth, μm	545.46	528.95	539.81	531.26	18.682	0.782	0.643	0.844
Mucosal thickness, μm	619.97	616.21	626.09	612.82	19.386	0.927	0.836	0.771
Muscle layer thickness, μm	332.49	316.68	358.48	344.32	13.973	0.692	0.775	0.823
Goblet cell, cell/mm^2	444.5	424.6	432.9	433.6	22.43	0.350	0.313	0.203

VH/CD = villus height/crypt depth.
[a-c] Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^8. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^8. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^9. CFU/d.

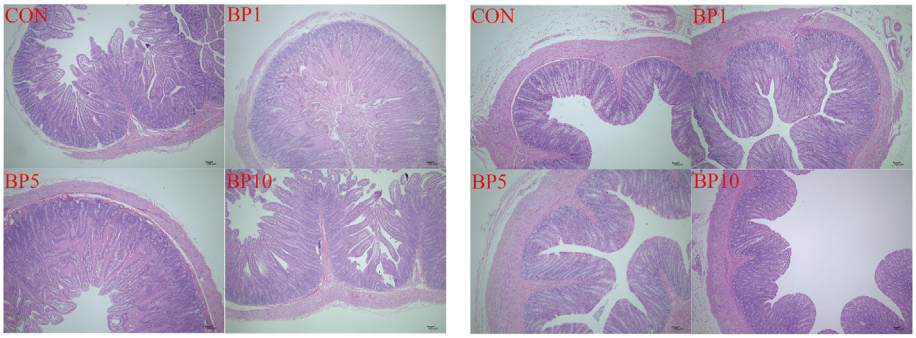

Fig. 1. Effects of *B. pumilus* on gut morphology. CON = control, BP1 = dosed daily with *B. pumilus* at 1×10^8 CFU/d, BP5 = dosed daily with *B. pumilus* at 5×10^8 CFU/d, BP10 = dosed daily with *B. pumilus* at 1×10^9 CFU/d. (magnification 200×, scale bar = 100 μm).

Table 8
Effects of *B. pumilus* on intestinal barrier function-related protein (ng/mg) in goat kids.

Item	Group[1]				SEM	*P*-value		
	CON	BP1	BP5	BP10		Treatment	Linear	Quadratic
Jejunum								
Claudin-1	1.81^{b}	2.27^{a}	2.26^{a}	2.04^{ab}	0.121	0.019	0.181	0.005
Claudin-4	1.87^{b}	2.36^{a}	2.34^{a}	2.11^{ab}	0.151	0.016	0.173	0.004
MUC2	2.34^{b}	2.73^{a}	2.70^{a}	2.54^{ab}	0.156	0.014	0.143	0.004
Occludin	2.91^{b}	3.36^{a}	3.35^{a}	3.13^{ab}	0.145	0.017	0.174	0.004
ZO-1	2.00^{b}	2.38^{a}	2.35^{a}	2.18^{ab}	0.119	0.016	0.194	0.004
Colon								
Claudin-1	1.68^{b}	2.15^{a}	2.20^{a}	2.06^{a}	0.131	0.020	0.031	0.017
Claudin-4	1.76^{b}	2.23^{a}	2.28^{a}	2.13^{a}	0.163	0.022	0.039	0.016
MUC2	2.24^{b}	2.62^{a}	2.67^{a}	2.55^{a}	0.166	0.017	0.027	0.015
Occludin	2.80^{b}	3.26^{a}	3.30^{a}	3.17^{a}	0.158	0.017	0.030	0.015
ZO-1	1.91^{b}	2.27^{a}	2.32^{a}	2.19^{a}	0.128	0.022	0.041	0.014

MUC2 = mucin 2; ZO-1 = zonula occludens-1.
[a,b] Values in the same row with no common letter superscripts mean significant difference ($P < 0.05$). ($n = 6$ per group).
[1] CON = control; BP1 = dosed daily with *B. pumilus* at 1×10^8. CFU/d; BP5 = dosed daily with *B. pumilus* at 5×10^8. CFU/d; BP10 = dosed daily with *B. pumilus* at 1×10^9. CFU/d.

of the total abundance (Fig. 2A). At the genus level, 296 genera were identified in jejunum samples from all treatments and the top 20 genera were further analyzed (Fig. 2B). The results of LEfSe analysis showed that *Lactobacillus* was significantly enriched in group BP1, while *Leuconostoc* were enriched in BP5 group (Fig. 2C). The relative abundance of *Enterococcus*, *Bacillus*, *Gilliamella* and *Bombella* in BP10 group were higher than other groups (Fig. 2C). *Streptococcus*, *Escherichia-Shigella*, *Caldanaerobius*, *Campylobacter*, *Anoxybacillus*, *Acinetobacter*, *Synechococcus CC9902*, *Candidatus_Actinomarina*, *HIMB11*, *SAR86_clade*, *Tyzzerella*, and *Clostridium_sensu_stricto_1* were enriched in CON group (Fig. 2C). Through comprehensively considering the relative abundance of each group and the role of the bacteria genus, some of the most distinctive differential bacteria genus related to diarrhea were selected for display in Fig. 2D. Dietary addition of *B. pumilus* significantly reduced the abundance of *Escherichia-Shigella* and *Campylobacter* while increasing the abundance of *Enterococcus* (Fig. 2D). The relative abundance of *Lactobacillus* in the BP1 and BP5 were significant increased

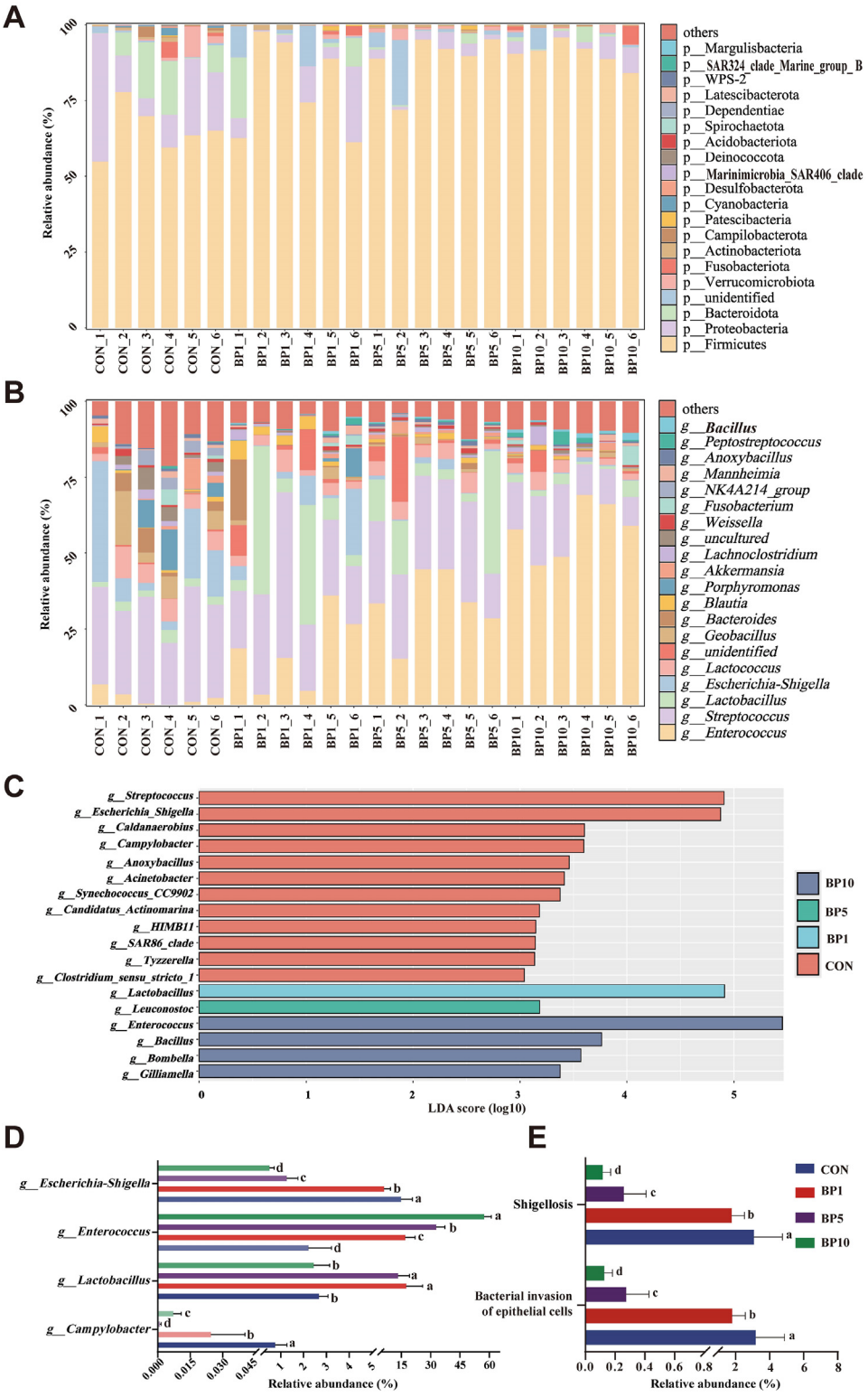

Fig. 2. Effects of *B. pumilus* on jejunal microbial composition and comparison of predicted KEGG functions. (A) Relative abundance at the bacterial phylum level in jejunum. (B) Relative abundance at the bacterial genera level in jejunum. (C) Identification of signature bacteria in the jejunum of four groups of goat kids by LEfSe analysis. (D) The relative abundance of key differential bacteria genus. (E) Predicted KEGG differential functions in functional classification level 3 ($n = 6$ per group). CON = control, BP1 = dosed daily with *B. pumilus* at 1×10^8 CFU/d, BP5 = dosed daily with *B. pumilus* at 5×10^8 CFU/d, BP10 = dosed daily with *B. pumilus* at 1×10^9 CFU/d. $^{a-d}$ Values with unlike letters were significantly different ($P < 0.05$). LEfSe = linear discriminant analysis effect size; KEGG = Kyoto encyclopedia of genes and genomes.

(Fig. 2D). The bacterial invasion of epithelial cells and Shigellosis pathways decreased with the increasing addition of *B. pumilus*, which suggests a corrective effect on immunity (Fig. 2E). Correlation analysis based on spearman correlation coefficient showed that the abundance of *Campylobacter* were positively correlated with IL-1β, while negatively correlated with IgA, IgM, PPAR-γ, CAT, T-AOC, MUC2, and ZO-1 (Fig. 3). The results also showed that the abundance of *Escherichia-Shigella* and *Lactobacillus* were positively correlated with IL-1β, IL-6 and MDA, while negatively correlated with IgA, IgG, IgM, sIgA, TGF-β, PPAR-γ, IL-10, SOD, GSH-Px, CAT, T-AOC, claudin-1, claudin-4, occludin, MUC2 and ZO-1 (Fig. 3). In addition, the abundance of *Lactobacillus* were positively correlated with TNF-α (Fig. 3).

After the high-throughput sequencing of bacterial 16S rRNA in colonic contents, 1,990,446 high-quality sequences were obtained after quality control. As shown in Fig. S2A, the number of ASVs owned by the CON, BP1, BP5 and BP10 groups were 174, 193, 198 and 172, and the total number of ASVs shared among the four groups was 87. There was no difference in bacterial alpha diversity in colonic contents (Fig. S2B). The anosim analysis showed no significant difference among the four groups (Fig. S2C). The results of LEfSe analysis showed that the *Blautia* was enriched in the CON group, the *Dorea* was enriched in the BP1 group and the *Eggerthella* was enriched in the BP5 group (Fig. S3).

4. Discussion

Diarrhea is a prevalent disease in goat kids and poses a threat to their early growth and development, as well as their future performance and intestinal health. Consequently, the prevention of goat kid diarrhea has become a major concern. In this study, *B. pumilus* 315 was administered to goat kids after birth, resulting in a significant reduction in fecal score and diarrhea rate within 15 days of age. The main internal causes of diarrhea were found to be excessive intestinal inflammatory reaction and barrier dysfunction. Further investigation revealed that the administration of *B. pumilus* positively influenced the immune barrier and microbial barrier of the jejunum, thereby improving the intestinal health of goat kids.

Diarrhea is often accompanied by intestinal inflammation (Liu et al., 2023). There is growing evidence that the colonization of *Bacillus* in the gut helps create an antibacterial environment and reduces the production of pro-inflammatory cytokines (Mazanko et al., 2022). The current study found that supplementation with *B. pumilus* reduced the levels of TNF-α, IL-1β, and IL-6, and increased the levels of TGF-β, PPAR-γ and IL-10. One significant characteristic of intestinal inflammation is the infiltration of activated neutrophils (Zhang et al., 2019). The results in this experiment demonstrated that the addition of *B. pumilus* reduced inflammatory cell infiltration and mitigated intestinal pathological changes, which aligned with the changes observed in intestinal inflammatory cytokines. These results indicated that *B. pumilus* maintains the intestinal health of goat kids by regulating the secretion of cytokines to reduce inflammation. The intestinal mucosa serves as a crucial interface for animals to interact with the internal and external environment, and it also acts as the main portal for pathogens to invade the host. To resist the invasion of pathogens, lymphoid tissues and immunocompetent cells in mucosal tissues together constitute a complete immune response network (Mayer, 2000). Previous studies have shown that immunoglobulins including IgA, IgG, IgM, sIgA play a crucial role in mucosal defense and immune reaction (Azam et al., 2023; Gong and Ruprecht, 2020; Perše and Večerić-Haler, 2019). In this study, it was found that the addition of *B. pumilus* heightened the contents of IgG, IgA and sIgA in the jejunal and colonic mucosa, indicating that *B. pumilus* had a positive effect on maintaining intestinal mucosal barrier function of goat kids. Consistent with our study, Yang et al. found that *B. pumilus SE* increased the expression of immune genes (Yang et al., 2014). In brief, the addition of *B. pumilus* to the diet alleviates diarrhea by reducing inflammation and improving immunity.

When the body stress causes diarrhea, the balance between prooxidation and antioxidation system is disrupted (Li et al., 2023). Lei et al. demonstrated that gavage of *Bacillus subtilis* improves antioxidant capacity in mice (Lei et al., 2024). Another study found that *B. pumilus* alleviates intestinal oxidative stress by modulating the nuclear factor-erythroid 2 related factor 2 (Nrf2)/kelch like epichlorohydrin associated protein 1 (Keap1) signaling pathway and reducing the production of reactive oxygen species (Wang et al., 2022). The findings of our study aligned with previous research on probiotic *Bacillus*, providing extra evidence that the supplementation of *B. pumilus* has a positive impact on antioxidant indicators (Li et al., 2019; Sun et al., 2022). Briefly, incorporating

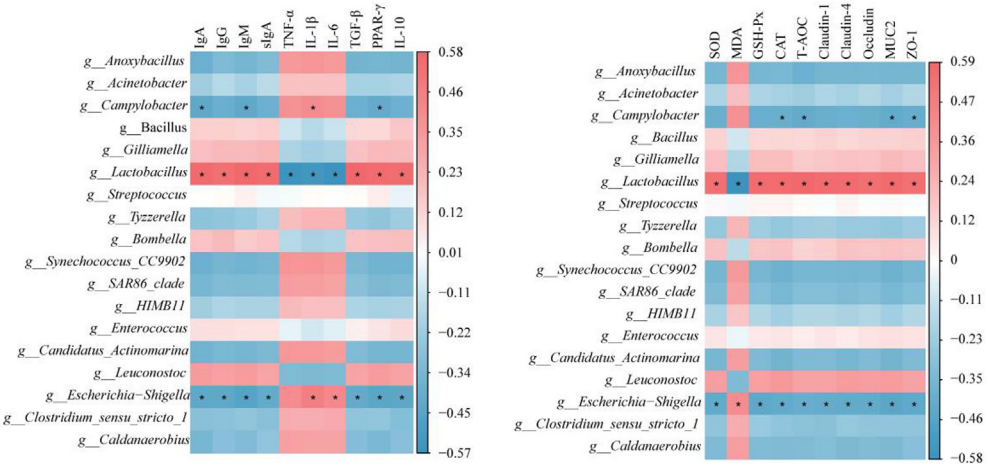

Fig. 3. The results of correlation analysis based on spearman correlation coefficient between 18 differential jejunal microbiota at the genus level and mucosal and barrier functional factors ($n = 6$ per group). IgG = immunoglobulin G; IgA = immunoglobulin A; IgM = immunoglobulin M; sIgA = secretory immunoglobulin A; TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β; IL-6 = interleukin-6; TGF-β = transforming growth factor-β; PPAR-γ = peroxisome proliferate-activated receptor gamma; IL-10 = interleukin-10; T-AOC = total antioxidative capacity; SOD = superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; MUC2 = mucin 2; ZO-1 = zonula occludens-1. *$P < 0.05$.

B. pumilus into diets helps mitigate intestinal oxidative stress associated with diarrhea.

Diarrhea is highly associated with the injury of the intestinal structure and mucosal barrier (Xiong et al., 2019). The intestinal mucosal barrier consists of a monolayer of columnar epithelial cells and tight junctions between the epithelium, which prevent harmful and pathogenic substances from entering. Tight junctions maintain the integrity of the intestinal barrier and regulate intestinal permeability, which are mainly composed of several proteins, including transmembrane proteins (such as claudins and occludins) and helper proteins (ZOs) (Qin et al., 2020). A previous study suggested that a low dose of *B. pumilus* (3×10^8 CFU/kg of feed) significantly increased the gene expression of occludin and *ZO-1* in the ileum of 14 d old broiler chickens (Bilal et al., 2021). Similarly, our study also found that the dietary addition of *B. pumilus* increased the contents of tight junction protein in the jejunum of goat kids. Interestingly, no significant changes were found between the BP10 and CON groups, which suggests that a high dose of *B. pumilus* might be ineffective as reported by Bilal et al. (2021). The competitive survival environment between strains for colonization may be the underlying mechanism (Han et al., 2021). Moreover, studies have shown that insufficient secretion or structural changes of MUC2 in the inner mucous may lead to spontaneous inflammatory reactions, resulting in a decrease of intestinal epithelial barrier function and cell proliferation (Lu et al., 2011). In this experiment, the increase of the MUC2 and tight junction protein contents suggested that *B. pumilus* improved intestinal health by strengthening the physical barrier of the jejunum. Previous studies have shown that VH/CD is directly related to diarrhea (Li et al., 2019; Pan et al., 2017), which is in accordance with our results. Intestinal peristalsis is achieved by contraction and relaxation of the muscle layer. A thinner muscle layer will reduce the peristalsis rate, causing the feed to stay in the small intestine longer and increasing the absorption time of nutrients (Liu et al., 2018). Secondly, the muscle layer thickness is directly proportional to the peristalsis rate, which theoretically should increase the digestibility of feed and improve the growth performance to a certain extent. However, in this experiment, the feed intake did not increase, which may be attributed to the young age of goat kids and the short feeding time, which still needs to be further explored. Concisely, dietary supplementation of *B. pumilus* alleviates goat kid diarrhea by altering the physiological state of the intestinal barrier.

The occurrence of diarrhea is strongly linked to gut microbiota (Li et al., 2021). Gut microbiota manipulation is crucial for the prevention and treatment of diarrhea, and methods such as dietary probiotics or fecal bacteria transplantation are widely introduced for this purpose (Li et al., 2021). Gut colonization of probiotics and dominant bacteria (such as *Lactobacillus* and *Bifidobacterium*) is enhanced by *Bacillus pumilus fsznc-09*, concurrently suppressing the proliferation of gut pathogens (such as *Staphylococcus*) (Zhang et al., 2021). In this study, *Escherichia-Shigella*, *Campylobacter*, *Enterococcus* and *Lactobacillus* were screened by LEfSe analysis as key differential microorganisms closely associated with diarrhea. It has been reported that an increased content of representatives of the *Escherichia-Shigella* genus contributes to the development of diarrhea in piglets (Gryaznova et al., 2022). In this study, the fact that *Escherichia-Shigella* abundance was reduced suggested that *B. pumilus* may have a potential role in alleviating diarrhea (Fan et al., 2021). The gastrointestinal tract of ruminants have also been frequently colonized with *Campylobacter*, which may also be one of the major causes of diarrhea (Fan et al., 2021). Oral administration of *Bacillus subtilis PS-216* spores reduces *Campylobacter* colonization in the jejunum of broiler chicken (Simunovic et al., 2022). Consistent with the above study, dietary supplementation with *B. pumilus* significantly reduced the abundance of *Campylobacter* in jejunum. The *Enterococcus* genus is believed to be commensals of the intestinal tract (Hanchi et al., 2018). However, the abundance of *Enterococcus* in the intestines of piglets with diarrhea was higher than in healthy piglets (Gryaznova et al., 2022), which is contrary to the results of this study. One possible reason is that the feeding of *B. pumilus* led to a substantial rise in the abundance of beneficial microorganisms in the *Enterococcus* genus. Numerous literature reports that strains of the genus *Lactobacillus* are able to alleviate enterotoxigenic *Escherichia coli* -induced diarrhea by modulating inflammatory factors and altering the bacterial flora (Yue et al., 2020), which corroborates the results of the present trial. Furthermore, intestinal pathogenic bacteria drive host disease by adherence and invasion to the intestinal epithelium (Poole et al., 2018). Functional prediction analysis indicated that *B. pumilus* reduced the likelihood of bacterial invasion of epithelium and shigellosis. Especially, the ability of *Shigella* to induce proinflammatory factor production in intestinal epithelial cells greatly contributes to the intense inflammatory reaction of the disease (Phalipon and Sansonetti, 2003). Interestingly, the correlation analysis in this study found that *Campylobacter* was negatively correlated with the immune capacity and barrier function of the host gut. Therefore, *B. pumilus* may reduce inflammation and enhance the intestinal barrier by improving intestinal flora, ultimately relieving diarrhea.

5. Conclusion

Our results revealed that the optimal dose (5×10^8 CFU/d) of *B. pumilus* supplementation effectively resisted diarrhea in goat kids, and a higher dose might be ineffective. By feeding *B. pumilus*, the low abundance of *Escherichia-Shigella* and *Campylobacter* revealed a low risk of pathogen invasion. A healthier flora was formed in the intestinal tract of the goat kids, which further improves intestinal immunity, antioxidant capacity, and barrier function, resulting in the reduction in diarrhea and healthy development of the gut mucosa. These findings offer a theoretical basis for the development of novel bacterial preparations to mitigate diarrhea in goat kids.

Credit Author Statement

Dingkun Fan: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Shuai Jiao:** Writing – original draft, Methodology, Investigation, Data curation. **Yuze Fu:** Investigation, Data curation. **Jixian Zhang:** Validation, Software, Data curation. **Yimin Zhuang:** Methodology, Data curation. **Juan Huang:** Methodology, Data curation. **Yanliang Bi:** Validation, Methodology. **Jianxin Zhang:** Writing – review & editing, Validation. **Naifeng Zhang:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.10.008.

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