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RESEARCH

Characterization and Assessment of Sheep-Origin Probiotic *Bacillus licheniformis* B63 Strain for Potential Use in Intestinal Health and Disease

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Abstract

Bacterial diarrhea causes serious losses for the sheep industry. Antibiotic resistance acquired by diarrheal bacteria is still a hurdle in the care of animal health. Thus, it is urgent to develop effective alternatives to antibiotics for controlling bacterial diarrhea. We initially isolated *Bacillus* spp. from Xinjiang fine wool sheep fecal and determined their properties of hemolysis and tolerance to acid and bile salts to identify potential candidates. Subsequently, we studied the position of a candidate in phylogenetic trees by 16S rRNA sequences and its susceptibility to antibiotics, ability to inhibit diarrheal bacteria, and toxicity, as well as its effects on animal health. Fourteen *Bacillus* spp. strains were isolated from sheep fecal. We identified the non-hemolysis B63 strain, which exhibited a high tolerance to acid and bile salts. Phylogenetic analysis indicated that the B63 strain is a new strain of *Bacillus licheniformis*. The *B. licheniformis* B63 strain was prompt to form spores, susceptible to commonly used antibiotics, and able to inhibit diarrhea-associated bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*. Animal studies determined that *B. licheniformis* B63 at 4×10^8 CFU/mL was non-toxic to mice and SD rats. Supplement with *B. licheniformis* B63 promoted the body weight gain of mice, reduced the inflammatory interleukin 6, and increased the jejunum villus height of SD rats. The newly isolated, non-hemolysis, spore-forming *B. licheniformis* B63 strain should be considered an optimal strain for the development of an effective probiotic supplement to control diarrheal diseases and promote the health of sheep and other animals.

Keywords Sheep · *Bacillus licheniformis* B63 · Probiotic · Bacterial diarrhea

Introduction

Bacteria-associated diarrhea in lamb results in malnutrition, body weight loss, dehydration, and even animal death [1]. Xinjiang is one of the five traditional grazing areas in China, and the sheep industry is one of the major husbandry industries. Bacterial diarrhea may induce 20% mortality in lambs with significant economic losses that seriously endanger the development of the sheep industry in Xinjiang [2].

The long-term and routine uses of antibiotics in the treatment of bacterial diarrhea in lamb not only alter the intestinal microbiota but also result in bacterial resistance to antibiotics and failure of the treatment [2]. Thus, it is urgent to advance therapeutic strategies for controlling bacterial diarrhea and emergent antibiotic resistance. Probiotics, using live microorganisms, have been proposed to be alternatives for antibiotics [3], by improving intestinal microbiota, regulating the host immune system, strengthening the intestinal epithelial barrier, and reducing the pathogen population [4, 5].

The microorganisms *Bacillus*, *Lactobacillus*, and *Saccharomyces*, isolated from the gastrointestinal tract and fermented dairy products, have been used as probiotics to control intestinal diseases [6–8]. *Bacillus* has been recognized as a promising candidate for developing probiotics

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due to its ability to colonize, reproduce, and form stable endosporogenesis to survive in abiotic conditions for long-term production and storage [9]. In addition, *Bacillus* produces a variety of functional metabolites, including antibiotics, bioinsecticides, enzymes, and lipopeptides, that also increase its biological and commercial value in the development of effective probiotics [10].

Bacillus licheniformis species have been generally regarded as safe (GRAS) by the United States Food and Drug Administration (US FDA) [11]. Diets supplemented with *Bacillus licheniformis* have been shown to increase body weight gain, villus height and digestive enzyme activities, and animal growth [12]. Dietary supplementation of commercially available probiotics containing *Bacillus licheniformis* in sheep helps animal growth, improves antioxidant capacity and immune performance, and increases the abundance of beneficial bacteria in the intestine [13]. The commercial probiotic mixture B-2999D, containing *Bacillus licheniformis*, has been shown to increase the body weight of 2-month-old sheep, enhance immunity, improve the intestinal microbiota, and maintain normal metabolic processes [14]. It has also been shown that the use of microbes isolated from the host gut optimizes the effectiveness of probiotics [15]. However, what role *Bacillus licheniformis* may play in promoting animal health and the intestinal microbiota remains to be addressed.

In this study, we initially isolated a novel strain B63 of *Bacillus licheniformis* from the fecal of healthy fine wool sheep in Xinjiang and determined its biochemical properties, 16S rRNA sequencing, and associated phylogenetic tree. Subsequently, we studied the safety and efficacy of B63 in the control of diarrhea and determined the adequacy of B63 in developing an optimized probiotic supplement for sheep.

Materials and Methods

Isolation and Morphological Properties of *Bacillus*

Fresh fecal samples were collected from 6-month-old Xinjiang fine wool sheep purchased from an intensive sheep farm at the Animal Central Hospital of Xinjiang Agricultural University. After mixing 3 fecal samples, 2.5 g of the mixture was suspended in 20 mL of sterilized saline (0.85% NaCl) and heated at 80 °C for 20 min. After centrifugation (4 °C, 3000 rpm, 3 min), 200 μL of suspension was mixed with 800 μL of LB broth (Hopebio, China), followed by incubation at 37 °C for 24 h. Serial dilutions were prepared and plated onto LB agar plates (Hopebio, China), followed by incubation at 37 °C for 24 h. Single colonies were isolated and cultured that was repeated for 2–5 times to develop pure colonies. After Gram staining, the morphology of bacteria isolated from individual colonies was evaluated microscopically [16].

Hemolysis Test

Bacteria were diluted, plated on the blood (5% sterile defiber sheep blood) agar plates (Beijing Solarbio Science & Technology, China), and incubated at 37 °C for 24 h [17]. The hemolysis was evaluated and classified based on the lysis of red blood cells in the medium around the colonies: the green zones around colonies (α-hemolysis), clear zones around colonies (β-hemolysis), and no zone around colonies (γ-hemolysis). The strain was considered safe if γ-hemolysis was detected [18].

Acid and Bile Salt Tolerance

To determine acidic tolerance, bacterial suspension was mixed with LB broth prepared at pH 2, 3, 4, 5, and 7, as well as 6.68 (control group), followed by incubation at 37 °C for 3 h. LB broth was acidified with 1 mol/L hydrochloric acid (HCl). To determine bile salt tolerance, bacterial suspension was mixed with bile salts at 0.1%, 0.2%, and 0.3% ox-bile (*w*/*v*) (Solarbio, China), followed by incubation at 37 °C for 3 h. Growth was monitored by determining the OD_{600nm} in a spectrophotometer (Synergy HTX BioTek, USA) [19].

Biochemical and Molecular Biological Identification of *Bacillus*

Biochemical characterization was performed using *Bacillus* biochemical identification strips (Hopebio, China). First, inoculate the bacteria in different biochemical tubes, including Voges-Proskauer (VP), citrate, propionate, D-xyloss, L-arabinose, D-mannitol, gelatin liquefaction, nitrate reduction, starch hydrolysis, and growth tests under 7% NaCl and a pH value of 5.7. Then, it was incubated at 37 °C for 48 ~ 96 h. After the incubation, VP reagent, nitrate reduction reagent, and Lugo's iodine reagent were added to the corresponding biochemical tube, and the change in color or state of the biochemical tube was observed, and the results were judged according to the instructions.

Molecular biological identification was performed using 16S rRNA sequence analysis. High-quality genomic DNA was extracted from bacterial isolates using the EasyPure Bacteria Genomic DNA Kit (TransGen Biotech, China). Then, the 16S rRNA gene was partially amplified by the polymerase chain reaction (PCR) using universal bacterial primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Sangon Biotech, China) [20]. PCR reactions contained 5 μL of template DNA, 0.5 μL of 10 pmol of each primer (forward and reverse), 10 μL 250 units of Taq polymerase (Cwbiotech,

China), and 4 μL of ddH_2O (Cwbiotech, China). The PCR reaction (95 °C for 5 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 90 s, final extension at 72 °C for 10 min, then cooled to 4 °C) was performed in a QIAamplifier 96 PCR machine (QIAGEN, China). DNA sequencing was carried out by Sangon Biotech (Shanghai, China). A sequence similarity search was conducted using GenBank BLAST. The phylogenetic tree was determined using MEGA7 software with bootstrap analysis using 1000 replications to assess the relative stability of branches [20].

Growth Rate

Prior to the experiment, the bacteria were added to LB broth at 1% *v*/*v* (volume of solute/volume of solution). The B63 strain solution was incubated by shaking (180 rpm) at 37 °C [18]. The absorbance at 600 nm was measured every 2 h with a spectrophotometer for 24 h.

Antibiotic Susceptibility and Antibacterial Test

Bacterial susceptibility to antibiotics was determined using the Kirby-Bauer disk diffusion method [21]. Briefly, bacteria were grown in LB broth at 37 °C for 10 h, followed by spreading on LB agar plates placed with antibiotic disks of clindamycin, vancomycin, gentamicin, kanamycin, streptomycin, chloramphenicol, erythromycin, ampicillin, oxacillin, penicillin G, norfloxacin, ciprofloxacin, tetracycline, cefotaxime, and cefoperazone at concentrations that meet the antibiotic standard content of microbiological drug sensitive paper (Hangzhou Binhe Microorganism Reagent, China). All LB plates were incubated at 37 °C for 24 h, and then the diameter of the inhibition zone was measured.

An antibacterial test of bacteria was investigated against three indicator bacteria by the agar-well diffusion method. The indicator strain was grown in LB broth medium, overlaid on LB agar plates, and allowed to stand at room temperature for 30 min. The bacteria were transferred in an Oxford cup on overlaid agar, incubated at 37 °C, and subsequently examined for inhibition zones at 24 h. The experiments were run in triplicate, and the mean value of the inhibition zone was determined [22] as indicator microorganisms: *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*.

Animal

Kunming mice (male and female, 24.85 ± 1.92 g) and Sprague–Dawley (SD) rats (male, 301.55 ± 21.15 g) were used for the acute and subacute toxicity evaluation, respectively. The animals were purchased from the Experimental Animal Center of Xinjiang Medical University (SCXK2018-0002). They had free access to clean tap water and commercial rat chow ad libitum during the study period.

Acute Toxicity

A total of 36 mice were randomly allocated into three groups of 12 mice each (6 females and 6 males). After overnight fasting, the extract of bacteria (4×10^8 CFU/mL, B63 group) was administered at single doses of 0.1 mL/10 g by intraperitoneal injection. The control group received the same volume of distilled water. The blank group was not treated. The animals were observed for mortality and clinical symptoms of toxicity during the first 30 min after dosing and daily thereafter for 7 days. The symptoms of toxicity to observe include movement, fur, dietary consumption, and defecation. The body weight of mice was recorded from day 1 to day 7. On the 7th day, the animals were sacrificed, and the organ coefficient was calculated (organ coefficient = organ weight/body weight × 100%). At the end of the experiments, the major organs (liver and spleen) of both animal groups were quickly fixed in 4% paraformaldehyde. Following fixation, the tissues were subjected to routine processing including dehydration, embedding in paraffin, preparing 5 μm sections, and staining with hematoxylin and eosin (H&E). The histological observation was performed using a Pannoramic MIDI Digital Slide Scanner (Hungary) equipped with a slice scanner [23].

Subacute Toxicity

To detect any subacute toxicity of *B. licheniformis* B63 in animals, SD rats were orally administered with bacterial mixtures for 4 weeks. Forty SD rats were randomly divided into five groups ($n = 8$) as follows: (1) the black group which received standard rat chow for 4 weeks, (2) the control group which received the black group diet and added 1 mL/100 g of PBS, (3) the low-dose group which received the black group diet and added 1 mL/100 g of *B. licheniformis* B63 (4×10^6 CFU/mL, B63-L group), (4) the medium-dose group which received the black group diet and added 1 mL/100 g of *B. licheniformis* B63 (4×10^7 CFU/mL, B63-M group), and (5) the high-dose group which received the black group diet and added 1 mL/100 g of *B. licheniformis* B63 (4×10^8 CFU/mL, B63-H group). Toxicity signs, mortality, and body weight gain were observed daily throughout the experiment.

At the end of the test, the SD rats were fasted overnight. We measured body weight and collected organs for organ coefficient calculation, and organ pathological sections were made. Blood was obtained by the orbital venous plexus method and collected in EDTA sample tubes and additive-free tubes. The whole blood samples collected in EDTA tubes were processed immediately for hematological analysis with an automated hematology analyzer (Beijing Baolingman Sunshine Technology Co., Ltd., BM830). The parameters evaluated were white blood cell (WBC), monocyte (MON), red blood cell (RBC), hematocrit (HCT),

granulocyte (GRA), lymphocyte percentage (LYM), hemoglobin (HGB), platelet (PLT), and platelet hematocrit (PCT) [23]. The blood samples collected in additive-free tubes were centrifuged (4 °C, 3000 rpm, 15 min), and the obtained serums were analyzed using ELISA kits (SenBeiJia Biological Technology Co., Ltd., Nanjing, China). Parameters measured included immunoglobulins (IgA, IgG, and IgM) and interleukins (IL-2, IL-6, and IL-10). Then, the major organs (liver and spleen), jejunum, and colon of both animal groups were collected and quickly fixed in 4% paraformaldehyde; the procedure was the same as the acute toxicity test.

Statistical Analysis

GraphPad Prism 8 and one-way analysis of variance (ANOVA) were used to determine statistical significance. A P value < 0.05 was considered significant.

Results

Isolation of probiotic *Bacillus spp.*

Based on the bacterial shape, a total of 14 distinguishable colonies were determined and selected for purification (Table 1). Gram-staining revealed all these isolates with a short rod shape and a spore structure as a typical morphology of *Bacillus* spp. (Fig. 1). The hemolysis test determined that the B41 and B63 strains showed γ-hemolysis (Fig. 2), and the other strains showed β-hemolysis, as listed in Table 1. The results indicated that B41 and B63 strains were not hemolytic and suitable for further studies of probiotic strains.

Table 1 Hemolysis test results of 14 single colonies

Name	α-Hemolysis	β-Hemolysis	γ-Hemolysis
B12 strain	−	+	−
B14 strain	−	+	−
B23 strain	−	+	−
B32 strain	−	+	−
B33 strain	−	+	−
B41 strain	−	−	+
B42 strain	−	+	−
B51 strain	−	+	−
B52 strain	−	+	−
B62 strain	−	+	−
B63 strain	−	−	+
B71 strain	−	+	−
B81 strain	−	+	−
B82 strain	−	+	−

"+" denotes positive reaction and "−" denotes negative reaction

Acid and Bile Salt Tolerance

To determine whether B41 and B63 strains may survive under gastrointestinal conditions, we studied the tolerance of B41 and B63 to various pH values at 2, 3, 4, 5, and 7, as well as the tolerance to bile salts at 0.1%, 0.2%, and 0.3%. As shown in Fig. 3, the B41 and B63 strains showed low tolerance to pH 2, 3, and 4 in contrast to their high tolerance to pH 5 and 7; however, B63 appeared to have higher tolerance than B41 to pH 4 to 7. In addition, the B63 strain, but not B41, exhibited a wide tolerance to bile salts. The result further indicated the candidacy of the B63 strain as a probiotic strain.

Biochemical Identification and Phylogenetic of B63 Strain

To understand the B63 strain, we used biochemical identification strips and 16S rRNA sequencing to determine its biochemical properties and phylogenetic position. As listed in Table 2, the VP, citrate, propionate, gelatin liquefaction, nitrate reduction, and starch hydrolysis tests were positive. The D-xylose, L-arabinose, D-mannitol, 7% sodium chloride, and pH 5.7 growth tests were negative. The phylogenetic analysis of 16S rRNA gene sequences determined that the B63 strain was closely related to *Bacillus licheniformis* NR074923.1 with an identity of 99.85%. Searching the GenBank database, bacterial strains showing ≥ 98% similarity of 16S rRNA gene sequences with the B63 strain were selected to construct a phylogenetic tree using MEGA7 software to determine the genetic distance of each strain, as schemed in Fig. 4. Accordingly, the B63 was a *Bacillus licheniformis*, named as *Bacillus licheniformis* B63, deposited as CGMCC 26746 in the China General Microbiological Culture Collection Center.

Growth Rate

To investigate the growth characteristics of *B. licheniformis* B63, we measured the absorbance of the culture medium to determine the growth curves of the *B. licheniformis* B63 in LB broth. As shown in Fig. 5, the *B. licheniformis* B63 started to enter the logarithmic phase at 2 h and reached the stable phase at 10 h, which was extended to 24 h. Thus, the log phase data indicated the *B. licheniformis* B63 proliferation between 2 and 10 h.

Antibiotic Susceptibility

The antibiotic-susceptible property is a prerequisite for a safe probiotic strain. We used the Kirby-Bauer method to determine the susceptibility of the B63 strain to 15 antibiotics. As shown in Table 3, the *B. licheniformis* B63 was fully

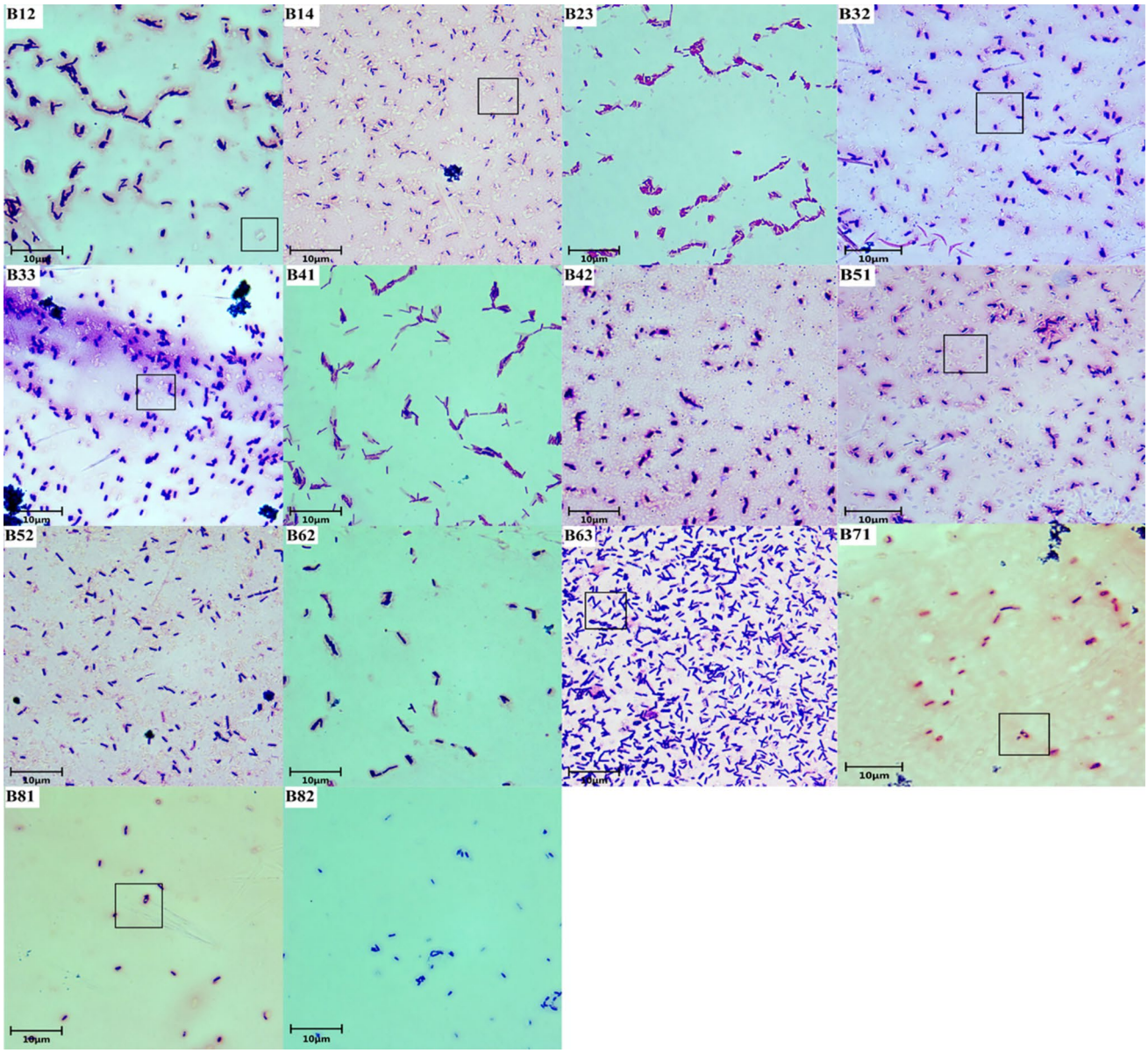

Fig. 1 Gram staining results of *Bacillus* spp. Gram-positive bacteria were purple, Gram-negative bacteria were red, and the spore structure was transparent in the bacteria or free in the bacteria. "□" represents spore structure

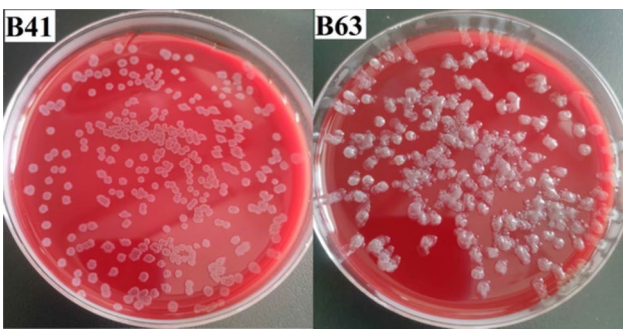

Fig. 2 γ-Hemolysis results of B41 and B63 strains. γ-Hemolysis is a phenomenon that does not destroy the structure of red blood cells and does not form a hemolysis ring

sensitive to vancomycin, gentamicin, kanamycin, streptomycin, chloramphenicol, erythromycin, norfloxacin, ciprofloxacin, and tetracycline; however, it was resistant to clindamycin and penicillin G. Thus, in general, *B. licheniformis* B63 was suitable to be a probiotic strain.

Antibacterial Test

To investigate the ability of *B. licheniformis* B63 to inhibit diarrhea-associated pathogenic bacteria, we used the Oxford cup antibacterial test and detected that the B63 strain was inhibitory to pathogenic bacteria, including *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus*

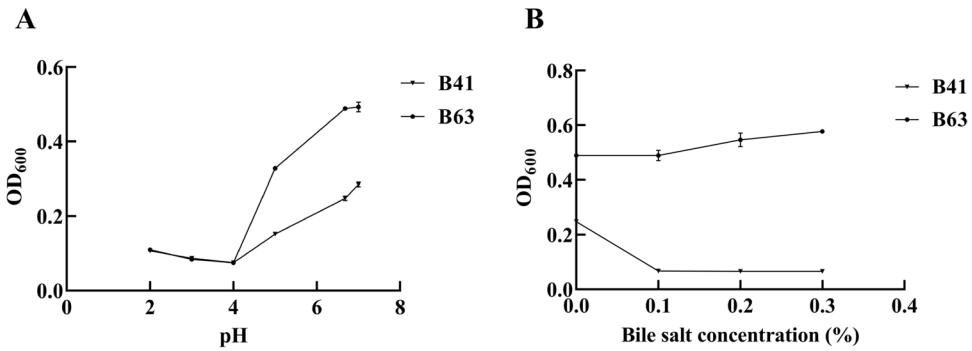

Fig. 3 The survival of strains under different acid concentrations (**A**). The survival of strains under different bile salt concentrations (**B**). The data were expressed as the mean ± SD. Bar was not shown in the figure because SD < 0.01

aureus (Table 4). The result clearly indicated that *B. licheniformis* B63 was able to inhibit three diarrhea-associated pathogenic bacteria.

Table 2 Biochemical identification of isolated B63 strain

Item	B63 strain
VP	+
Citrate	+
Propionate	+
D-Xylose	−
L-Arabinose	−
D-Mannitol	−
Gelatin liquefaction	+
7% sodium chloride	−
pH 5.7	−
Nitrate reduction	+
Starch hydrolysis	+

"+" denotes positive reaction and "−" denotes negative reaction. Refer to Bergey's Bacterial Identification Manual (Eighth Edition) for the determination of results

Acute Toxicity Animal Study

To study the acute toxicity of *B. licheniformis* B63, we injected (I.P) 4×10^8 CFU/mL of *B. licheniformis* B63 into mice. Seven days after the injection, mice continued to gain weight even higher than the control group and showed no detectable adverse or lethal effect (Fig. 6A). Autopsy did not detect any pathologic effects on the kidney, heart, liver, lungs, and spleen, as well as no change detectable in liver and spleen organ coefficients (Fig. 6B, C). Histological examination did not reveal any pathological changes in the spleen and liver (Fig. 7). Accordingly, these results indicated that the *B. licheniformis* B63 at 4×10^8 CFU/mL was non-toxic to mice and possibly beneficial to animal health.

Subacute Toxicity Animal Study

No mortality or toxicity was detectable during the 28-day period. We also did not detect any significant changes in body weight gained between animal groups (Fig. 8). After autopsy, pathological changes were not detectable in the internal organs, and the coefficients in the thymus, spleen, and liver were similar between groups (Table 5). Table 6

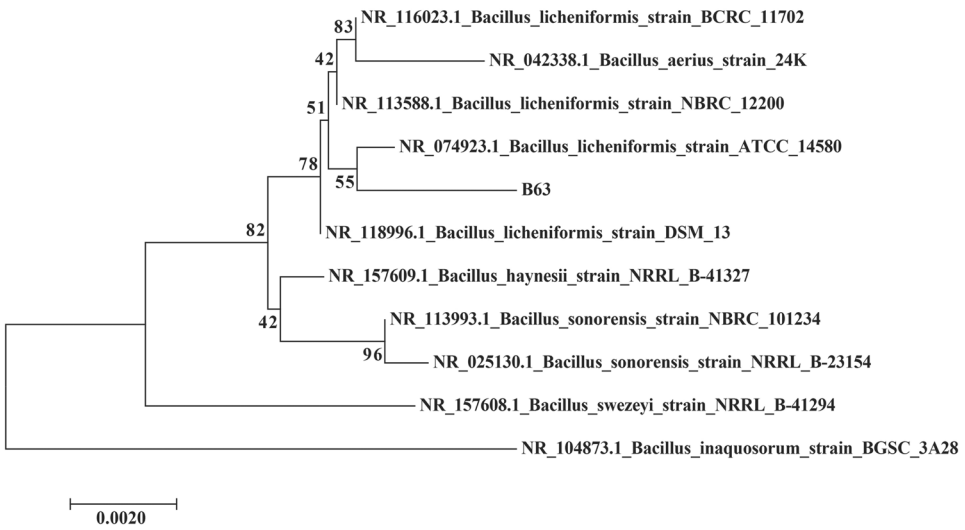

Fig. 4 Phylogenetic tree based on the 16S rRNA gene sequence of B63 strains

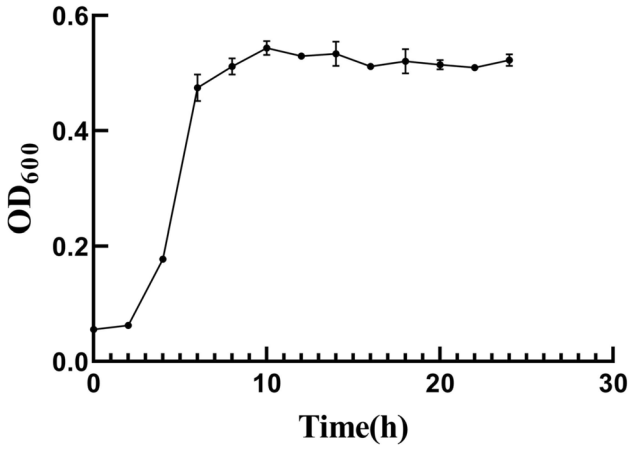

Fig. 5 The *B. licheniformis* B63 strains' growth time curve. The data were expressed as the mean ± SD. Bar was not shown in the figure because SD < 0.01

summarizes the levels of hematological parameters in the blank, control, and *B. licheniformis* B63 groups. In addition, there were no detectable changes at the level of WBC, MON, RBC, HCT, GRA, LYM, HGB, PLT, and PCT between each group. Histopathological evaluation of the spleen and liver did not reveal any lesions (Fig. 9). Thus, oral administration of rats with *B. licheniformis* B63 up to 4×10^8 CFU/mL was safe without producing any adverse effects.

Table 4 Antibacterial test of *B. licheniformis* B63

Pathogen name	Diameter of antibacterial ring (mm)
Escherichia coli	19.19 ± 0.31
Salmonella typhi	19.12 ± 0.56
Staphylococcus aureus	17.40 ± 3.71

The data were expressed as the mean ± SD. No bacteriostatic effect (diameter < 10 mm), moderate bacteriostatic effect (10 mm < diameter < 15 mm), and highly bacteriostatic (diameter > 15 mm)

Intestinal Immunity Promotion

To address whether *B. licheniformis* B63 was able to induce intestinal immunity in SD rats, we used the technique of ELISA to determine the content of serum immunoglobulins (IgA, IgG, and IgM) and interleukins (IL-2, IL-6, and IL-10). As shown in Fig. 10, the IL-6 level was significantly lower in the B63 group than in the control group ($P < 0.05$). Pathological evaluation did not reveal any intestinal ulceration, inflammatory cell exudation, intestinal mucosal degeneration, or necrosis (Fig. 11A–C). Histological examination of villus height and crypt depth of the jejunum revealed that the villus height, but not the crypt depth, was significantly higher in the B63-L and B63-M groups than the blank group, and the villus height was significantly higher in the B63-L group than the B63-M group (Figs. 11A

Table 3 Antibiotic susceptibility test of *B. licheniformis* B63

Types of antibiotics	Antibiotics name	Drug concentration	Diameter of inhibition zone (mm)	Susceptibility
Lincomycins	Clindamycin	2 μg/pc	11.22 ± 2.09	R
Peptides	Vancomycin	30 μg/pc	23.21 ± 3.12	S
Aminoglycosides	Gentamicin	10 ± 2.5 μg/pc	21.12 ± 2.15	S
	Kanamycin	30 μg/pc	22.84 ± 2.18	S
	Streptomycin	10 μg/pc	15.96 ± 1.36	S
Chloramphenicol	Chloramphenicol	30 μg/pc	22.93 ± 3.86	S
Macrolides	Erythromycin	15 μg/pc	31.54 ± 0.33	S
β-Lactams	Ampicillin	10 μg/pc	16.15 ± 0.91	I
	Oxacillin	1 μg/pc	13.66 ± 5.57	I
	Penicillin G	10 U/pc	14.86 ± 1.35	R
Quinolones	Norfloxacin	10 μg/pc	35.36 ± 2.35	S
	Ciprofloxacin	5 μg/pc	35.51 ± 2.35	S
Tetracyclines	Tetracycline	30 μg/pc	34.12 ± 1.86	S
Cephalosporins	Cefotaxime	30 μg/pc	16.61 ± 2.42	I
	Cefoperazone	75 μg/pc	17.80 ± 0.99	I

The results were judged with reference to "Standards for Antibacterial Drug Susceptibility Test by Disk Method" WS/T 125–1999. μg/pc represents a piece of paper on the unit dose of antibiotics and "pc" represents a piece of antibiotic paper

S sensitive, *I* intermediate, *R* resistant

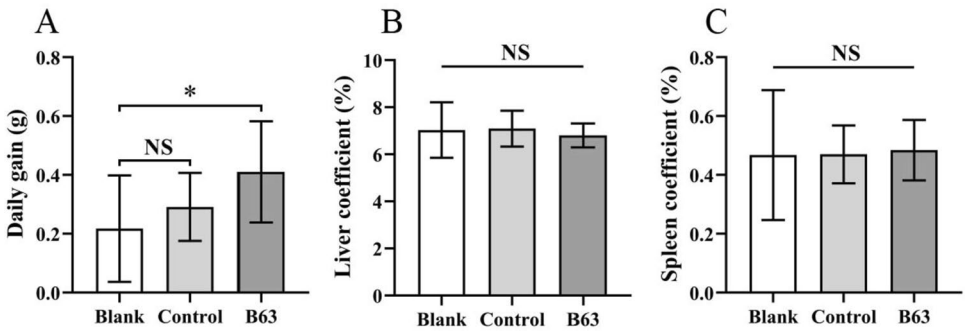

Fig. 6 The results of acute toxicity study. **A** Daily gain of mice. **B** Spleen coefficient of mice. **C** Liver coefficient of mice. The data were expressed as the mean ± SD (*$P < 0.05$)

and 12A, B). The V/C values were significantly higher in the B63-L and B63-M groups than in the blank group (Fig. 12C). *B. licheniformis* B63 had no significant effect on colon crypt depth (Figs. 11C and 12D). Thus, the results indicated that *B. licheniformis* B63 was able to promote intestinal immunity.

Discussion

In this communication, we demonstrated that we isolated a novel non-hemolytic B63 strain of *B. licheniformis* from the intestine of sheep. Our in vitro studies determined that *B. licheniformis* B63 was inhibitory to pathogenic bacteria, susceptible to antibiotics, and able to colonize the intestinal tract. Our in vivo studies revealed the ability of *B. licheniformis* B63 to enhance the intestinal immunity of animals. According to probiotic criteria [24–28] and considering the ability of *B. licheniformis* B63 to not only inhibit diarrheal pathogens but also promote intestinal immunity, the *B. licheniformis* B63 may serve as an optimal probiotic strain to control diarrheal diseases and promote the health of sheep.

The ability of *B. licheniformis* B63 to inhibit diarrhea-associated *Escherichia coli*, *Salmonella typhi*, and

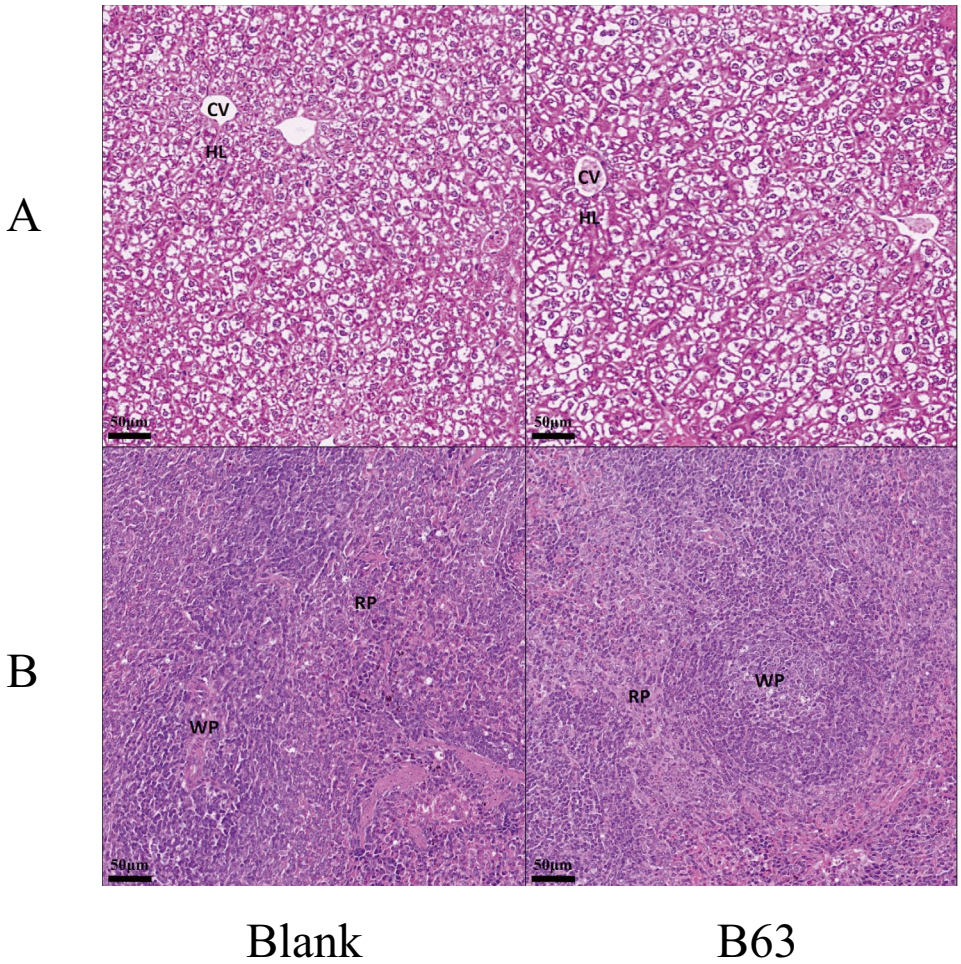

Fig. 7 Representative photomicrographs of liver and spleen from Kunming mice treated daily for 7 days with standard chow diet (blank group) or intraperitoneal injection B63 strains (4×10^8 CFU/mL). **A** In the liver micrograph, the centrilobular vein (CV) and hepatic lobule (HL) are visible in all images. **B** In the spleen micrograph, the white pulp (WP) and red pulp (RP) are visible in all images. H&E staining was used. Magnification: × 20 for the liver and × 10 for the spleen

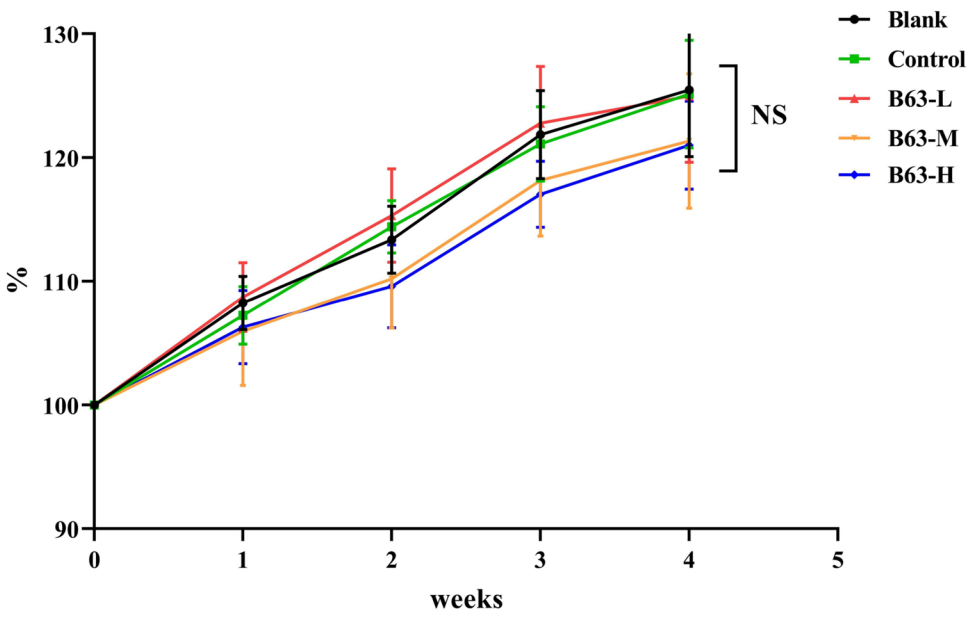

Fig. 8 The line diagram of weight changes in SD rats during subacute toxicity test. The data were expressed as the mean ± SD

Staphylococcus aureus is consistent with a *B. licheniformis* strain isolated from yak [29]. *Escherichia coli* is the most common diarrhea-associated pathogen in animals [30], seriously harming animal health. Thus, the application of *B. licheniformis* B63 to the control of diarrhea-associated pathogens was conceivable to help maintain animal growth. However, whether the *B. licheniformis* B63 strain is inhibitory to intestinal pathogenic bacteria in sheep and other animals remains to be studied.

Our phylogenetic analysis indicated that the newly isolated *B. licheniformis* B63 strain was closely related to, but distinguishable from, other reported *B. licheniformis* strains isolated from various resources [31–33]. The prompt ability of *Bacillus spp.* to produce spores against environmental stress was considered an optimal criterion for the development and storage of probiotic strains [34]. The *B. licheniformis* B63 was highly susceptible to 15 commonly used antibiotics, in contrast to many other identified antibiotic-resistant *B. licheniformis* strains [35]. The World Health Organization has recently indicated that the acquisition of cephalosporin resistance has become a common concern in the control of bacterial pathogens [29]. The B63 strain was

Table 5 Organ coefficient of subacute toxicity test

Item	Blank	Control	B63-L	B63-M	B63-H
Thymus coefficient (%)	0.09 ± 0.02	0.10 ± 0.03	0.10 ± 0.02	0.08 ± 0.03	0.11 ± 0.03
Spleen coefficient (%)	0.22 ± 0.03	0.22 ± 0.03	0.19 ± 0.02	0.18 ± 0.02	0.20 ± 0.04
Liver coefficient (%)	2.99 ± 0.23	2.81 ± 0.14	2.84 ± 0.83	2.78 ± 0.46	3.09 ± 0.55

The data were expressed as the mean ± SD

Table 6 Hematological analysis of subacute toxicity test

Item	Blank	Control	B63-L	B63-M	B63-H
RBC (10^{12}/L)	6.21 ± 1.12	5.40 ± 2.00	5.04 ± 1.62	4.87 ± 1.19	5.34 ± 1.66
MCV (fL)	60.23 ± 1.25	59.91 ± 2.77	63.66 ± 2.12	65.43 ± 2.99	62.76 ± 2.52
HCT (%)	36.93 ± 6.98	32.26 ± 11.68	32.15 ± 10.74	31.85 ± 8.48	33.41 ± 10.62
PCT (%)	0.32 ± 0.28	0.45 ± 0.36	0.24 ± 0.12	0.23 ± 0.24	0.30 ± 0.20
WBC (10^9/L)	7.39 ± 3.45	8.49 ± 4.26	5.90 ± 4.24	6.26 ± 3.65	7.11 ± 2.68
LYM (10^9/L)	4.49 ± 2.51	5.63 ± 3.25	3.22 ± 1.89	3.88 ± 2.33	4.44 ± 1.58
MON (10^9/L)	1.56 ± 0.56	1.24 ± 0.56	1.48 ± 1.55	1.09 ± 0.76	1.32 ± 0.76
GRA (10^9/L)	1.33 ± 0.60	1.63 ± 0.84	1.21 ± 0.88	1.25 ± 0.88	1.35 ± 0.62
HGB (g/L)	125.76 ± 23.38	111.26 ± 39.63	102.53 ± 31.56	98.98 ± 25.33	112.96 ± 28.28

The data were expressed as the mean ± SD

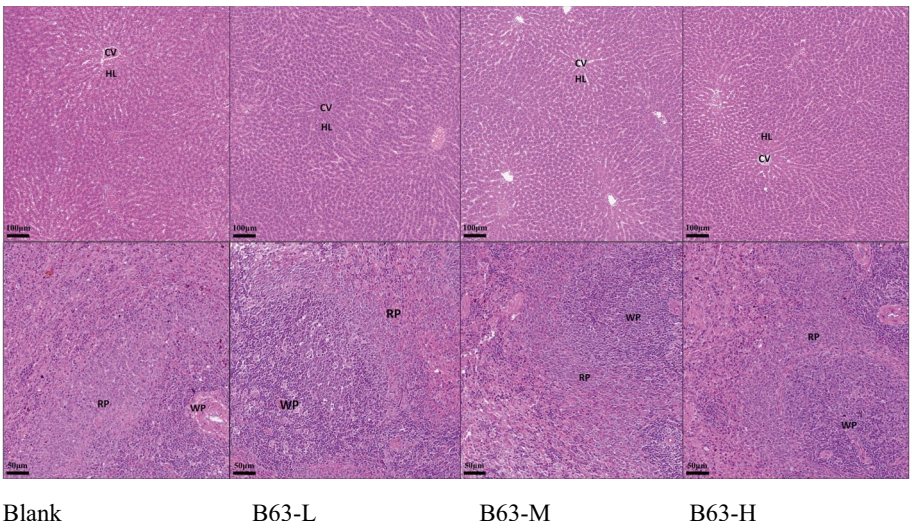

Fig. 9 Representative photomicrographs of spleen and liver from SD rats treated daily for 4 weeks with standard chow diet (blank group) or PBS mixture of *B. licheniformis* B63 strains at a low dose (4×10^6 CFU, B63-L), a medium dose (4×10^7 CFU, B63-M), and a high dose (4×10^8 CFU, B63-H). **A** In the liver micrograph, the centrilobular vein (CV) and hepatic lobule (HL) are visible in all images. **B** In the spleen micrograph, the white pulp (WP) and red pulp (RP) are visible in all images. H&E staining was used. Magnification: × 10 for the liver and × 20 for the spleen

somewhat resistant to cephalosporin. Whether the B63 strain was able to help control cephalosporin-resistant pathogens remains to be studied.

Our animal studies revealed the ability of *B. licheniformis* B63 to not only accelerate body weight gain in mice but also promote intestinal immunity in rats, consisting with the results of other studies of *B. licheniformis* or other probiotics [36–39]. Supplementation of animals with *B. licheniformis* B63 resulted in reducing the inflammatory cytokine IL-6 in the serum and improving intestinal structure. IL-6 elevation is associated with tissue damage and infection as an indicator of pathological effects in inflammatory diseases [40] [41] [42]. The structure of the intestinal mucosa is associated with gut health [39]. Villi are critical components of the digestive tract, and their geometry is an indicator of the absorptive capacity of the small intestine [43]. Thus, the ability of *B. licheniformis* B63 to reduce anti-inflammatory effects and enhance intestinal health, conceivably contributing to increased nutrient absorption and animal body weight gain. Although the efficacy of probiotics may be animal species-specific [44], our animal models revealed that the sheep *B. licheniformis* B63 strain was capable of providing

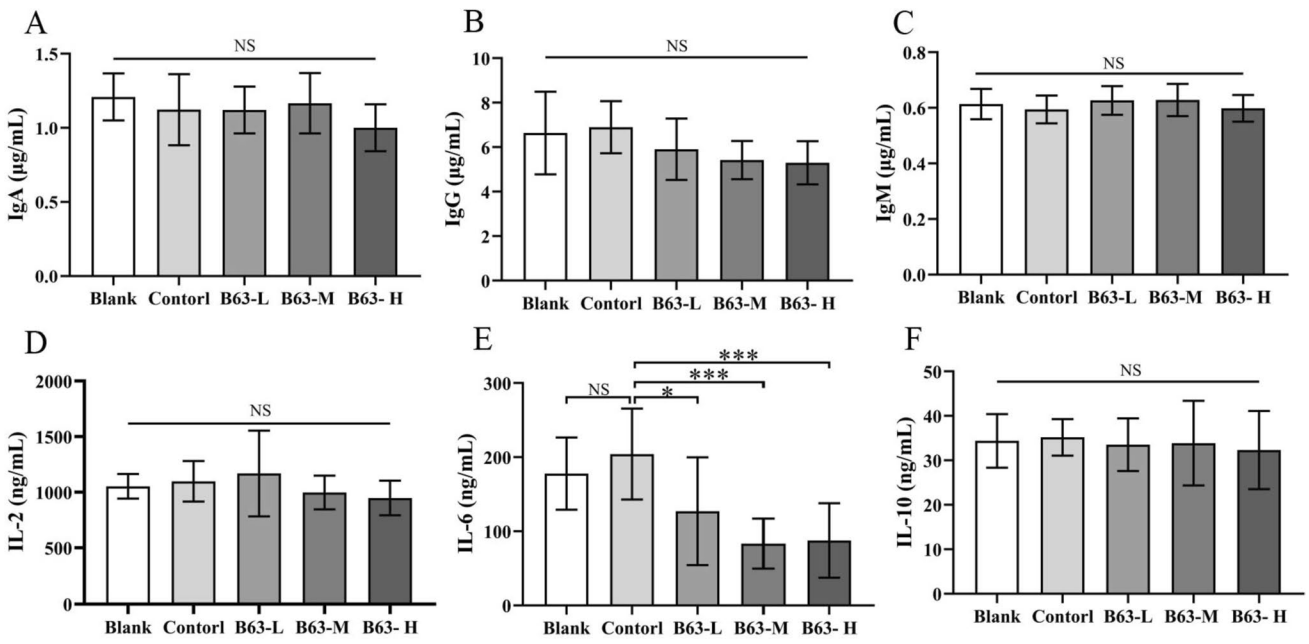

Fig. 10 Results of serum immunoglobulin and cytokine concentrations in SD rats. The data were expressed as the mean ± SD (*$P<0.05$, ***$P<0.001$)

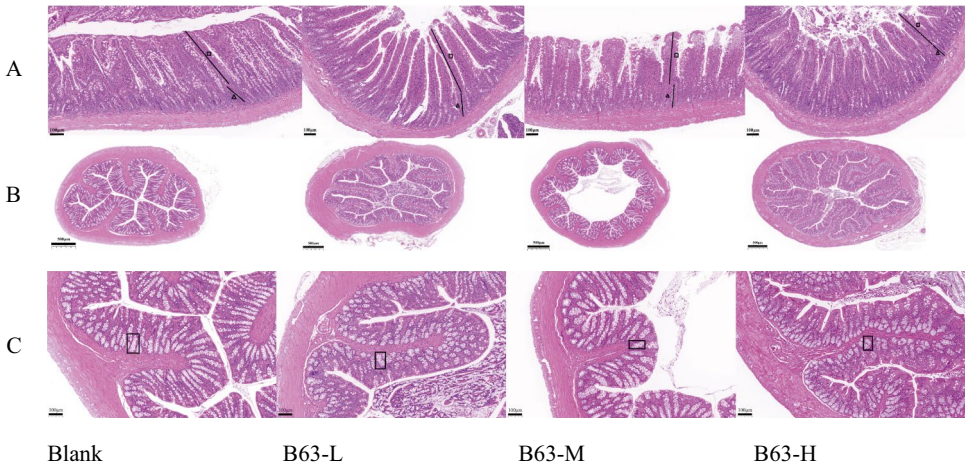

Fig. 11 H&E stained of jejunum and colon. Histopathology showed that the intestinal mucosa was structurally intact, with neatly arranged epithelial cells and no degeneration or necrosis of intestinal tissues. **A** H&E results of jejunum in each group under × 10, "□" represents the villus height measurement site and "△" represents the crypt depth measurement site. **B** H&E results of the colon in each group under × 20. **C** H&E results of the colon in each group under × 10, in the "□" was the site for measuring the depth of the crypt

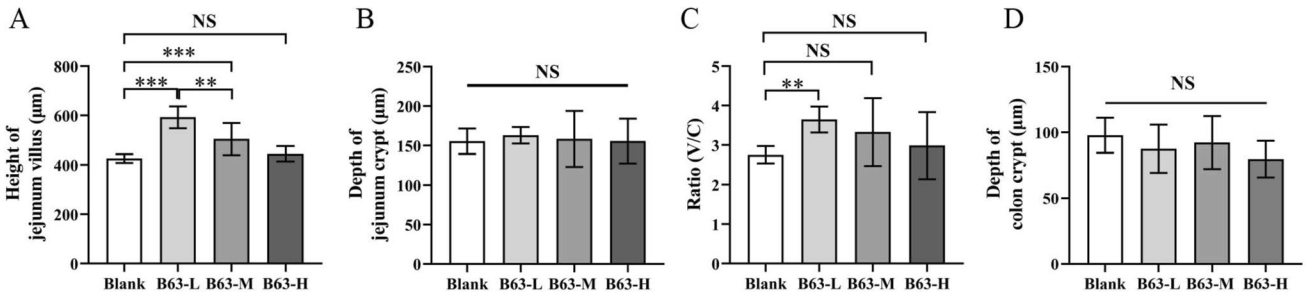

Fig. 12 Effects of probiotics on morphometry of jejunum and colon in mice. **A** The length of jejunum villus. **B** The depth of the jejunum crypt. **C** Ratio of jejunal villus height to crypt depth. **D** The depth of the colon crypt. The data were expressed as the mean ± SD (*$P < 0.05$; ***$P < 0.001$)

somewhat benefits to mice and rats. However, whether the *B. licheniformis* B63 strain may be beyond a species-specific strain in food animals remains to be clarified.

Conclusion

The newly isolated, non-hemolytic, spore-forming *B. licheniformis* B63 strain was able to inhibit diarrhea-associated bacteria. Supplementation of the B63 strain enhanced the intestinal structure and immunity of animals, as well as improved animal growth. Accordingly, the *B. licheniformis* B63 strain should be considered an optimal probiotic strain for the development of an effective probiotic strain to control diarrheal diseases and promote the health of sheep and other animals.

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Author Contributions Conceptualization, M.H., Y.L. and M.Y.; writing—original draft preparation, M.H., Y.L. and M.Y.; writing—review and editing, N.L., Y.S.,G.Y. and J.W.; formal analysis, F.Y., Q.P., S.J., R.S. and X.W.. All authors reviewed the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article. The probiotics strains (Bacillus licheniformis B63) have been deposited to NCBI GenBank (GenBank Accession SUB13897788 Bacillus OR665461).

Declarations

Ethical Approval The animal study was authorized by the Ethics Committee of Xinjiang Agricultural University (no. 2017013).

Conflict of Interest The authors declare no competing interests.

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