



Article

Lactobacillus johnsonii DY2 Isolated from Yaks Alleviated Acute *Escherichia coli* Infection via Modulating Inflammatory Responses, Antioxidant Capacity, and Gut Microbiota

Yuhui Liu ¹, Yanlei Dong ¹, Muhammad Safdar ^{2,3} , Mingming Liu ^{2,*} and Kun Li ^{1,*}

¹ College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China; 9221710603@stu.njau.edu.cn (Y.L.); dongyanlei@stu.njau.edu.cn (Y.D.)

² School of Basic Medicine, Hubei University of Arts and Science, Xiangyang 441000, China; msafdar@cuvas.edu.pk

³ Department of Breeding & Genetics, Cholistan University of Veterinary and Animal Sciences, Bahawalpur 63100, Pakistan

* Correspondence: lmm_2010@hotmail.com (M.L.); lk3005@njau.edu.cn (K.L.)

Simple Summary

This study explored a beneficial bacterium, *Lactobacillus johnsonii* DY2, isolated from the feces of yaks, to see whether it could help protect against acute *Escherichia coli* infection. The increasing problem of antibiotic resistance makes it necessary to find safe, natural alternatives to prevent and control bacterial diseases. Laboratory tests showed that DY2 can inhibit *E. coli* growth. When given to mice for three weeks before infection, DY2 reduced disease severity—preventing weight loss, lowering bacterial spread, and maintaining normal organ health. It also reduced inflammation and oxidative stress by balancing key immune molecules and boosting antioxidant enzymes. Under the microscope, DY2-treated mice showed healthier intestinal tissues compared to infected controls. Moreover, gut microbial analysis revealed that DY2 increased the diversity and abundance of beneficial bacteria, helping restore gut balance during infection. These findings suggest that *Lactobacillus johnsonii* DY2 enhances natural defense systems through anti-inflammatory, antioxidant, and microbiota-stabilizing effects. This yak-derived probiotic could be a promising and environmentally friendly tool to help protect animals and humans from infections in an era when antibiotic resistance is a growing concern.

Abstract

The escalating challenge of antimicrobial resistance has spurred interest in probiotics as alternatives for combating bacterial infections. This study aimed to isolate and characterize probiotic *Lactobacillus johnsonii* (*L. johnsonii*) from yak feces with protective efficacy against acute *Escherichia coli* (*E. coli*) infection. In vitro, DY2 supernatant inhibited the growth of *E. coli*. In vivo, mice pretreated orally with DY2 (1×10^9 CFU/mL) for 21 days before *E. coli* challenge exhibited significantly reduced weight loss ($p < 0.001$), lower bacterial translocation in the intestines ($p < 0.001$), and normalized organ indices ($p < 0.05$) compared to untreated infected controls. DY2 modulated host immune and oxidative responses by significantly lowering serum levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6; $p < 0.001$ to $p < 0.05$) and malondialdehyde (MDA; $p < 0.001$), while elevating levels of the anti-inflammatory IL-10 ($p < 0.05$) and antioxidant enzymes (SOD, GSH-Px, T-AOC; $p < 0.001$ to $p < 0.01$). Histologically, DY2 preserved intestinal mucosal integrity, with reduced villus shortening and inflammatory infiltration ($p < 0.001$ for villus length in key segments). 16S rRNA sequencing of intestinal microbiota revealed enhanced α -diversity ($p < 0.05$ to $p < 0.001$), community stability, and enrichment of beneficial genera such as



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Butyricimonas in DY2-treated mice. Conclusively, *Lactobacillus johnsonii* DY2 protects against acute *E. coli* infection via anti-inflammatory, antioxidant, gut barrier strengthening, and microbiota-modulating activities. Yak-derived lactobacilli are promising probiotics with excellent antibacterial properties.

Keywords: 16S rRNA sequencing; *Escherichia coli*; gut microbiota; *Lactobacillus johnsonii*; probiotics; Yaks

1. Introduction

The rising threat of antimicrobial resistance (AMR) has made conventional antibiotics increasingly unreliable for treating bacterial infections, driving urgent interest in non-antibiotic alternatives [1]. Among these, probiotics—particularly lactic acid bacteria such as lactobacilli—have gained considerable attention for their potential to promote host health through mechanisms including direct pathogen inhibition, immune system regulation, and reinforcement of intestinal barrier function, as well as restoration of microbial balance in the gut [2]. *Lactobacillus* species are particularly relevant for acute bacterial infections due to their ability to inhibit pathogen adhesion, produce bacteriocins, and modulate acute inflammatory responses in models of gastroenteritis and sepsis [3]. This potential is closely associated with their role in modulating the gut microbiota, which is a critical determinant of host homeostasis that provides colonization resistance against pathogens and guides immune development [4]. Additionally, a common feature of many gastrointestinal and systemic diseases is gut dysbiosis, or disruption of the gut microbial community [5]. Therefore, the strategies that maintain or restore a resilient gut microbiome may offer a promising therapeutic approach.

Despite considerable progress, key challenges persist in developing effective probiotics against acute infections. The beneficial effects of probiotics are highly strain-specific [6], driving the need for continuous discovery and rigorous characterization of novel isolates. Furthermore, although in vitro and chronic disease models are reliable, comprehensive in vivo assessments of acute infections under specific conditions—that analyze the interplay between immunomodulation, oxidative stress mitigation, and microbial community resilience—remain limited [7,8]. Recently, there has been increasing interest in exploring novel probiotics in unique animals. Species like the yak (*Bos grunniens*), which thrive in extreme high-altitude environments on the Qinghai–Tibet Plateau at altitudes above 3000 m, may host microbial symbionts with strong adaptive features [9,10]. Yaks exhibit unique adaptations such as enhanced hypoxia tolerance, efficient nutrient utilization in low-oxygen environments, and a resilient gut microbiota enriched in fiber-degrading bacteria that aid survival in cold, high-altitude conditions [11]. Microbes that have co-evolved with such hosts may possess unusual robustness and functional traits that standard laboratory or livestock-derived strains lack [12,13].

Acute *E. coli* infections remain a major global health concern, causing intestinal inflammation, epithelial barrier breakdown, and potential systemic complications [14,15]. The pathological cascade is driven by excessive pro-inflammatory cytokines, oxidative tissue damage, and disturbance of the gut microbiota [16]. Although antibiotics remain the mainstay of treatment, their use often exacerbates dysbiosis and fuels AMR, highlighting the need for prophylactic approaches that enhance the host's intrinsic defense systems [17]. Prophylactic strategies that bolster intrinsic host defenses are therefore highly desirable. Lactobacilli, for instance, can inhibit pathogens via competitive exclusion and antimicrobial metabolite production [18,19]. However, a holistic understanding of their protective role

during complex and variable infections—encompassing their capacity to dampen systemic inflammation and oxidative stress while simultaneously shaping the structure and stability of the gut microbiota—have not been fully elucidated.

L. johnsonii is a natural inhabitant of the mammalian gut and has repeatedly shown anti-inflammatory and barrier-protective properties [20,21]. *L. johnsonii* was selected due to its well-documented anti-inflammatory, barrier-protective, and antimicrobial properties against enteric pathogens, making it ideal for testing in an acute infection model [22]. We reasoned that a strain isolated from resilient animals like yaks, adapted to harsh niches, may possess enhanced functional properties [23–25].

In this study, we isolate and characterize probiotic *Lactobacillus johnsonii* (*L. johnsonii*) from yak feces with protective efficacy against acute *Escherichia coli* (*E. coli*) infection. Therefore, it may provide the integrated evidence that a yak-derived *L. johnsonii* strain confers broad-spectrum protection against acute enteric *E. coli* infection through coordinated anti-inflammatory, antioxidant, barrier-strengthening, and microbiota-modulating activities—revealing the largely untapped potential of such isolates.

2. Materials and Methods

2.1. Isolation and Identification of Isolates

Fresh fecal samples were collected from healthy adult yaks grazing in Xizang, China. They were immediately placed on dry ice and transported to the laboratory at Nanjing Agricultural University and stored at -80°C in a refrigerator for the subsequent isolation, identification and screening of *Lactobacillus* spp. [26]. The thawed samples (0.5 g) were suspended in 7 mL sterile phosphate-buffered saline (PBS; Servicebio, Wuhan, China) with purity 99.9%, vortexed briefly and then incubated in a shaker at 37°C for 3 h. The supernatant was inoculated on sterile Mann Rogosa Sharp (MRS) Agar medium from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China) and then inverted into a constant temperature incubator at 37°C for 24 h under anaerobic conditions. The milky white, round and raised colonies of suspected *Lactobacillus* in MRS Agar plates were picked out and purified three times until the single colonies in the whole plate had the same shape and size. Finally, a single isolate, designated DY2, was preserved in MRS broth from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China) supplemented with 20% glycerol from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) at -80°C .

2.2. Molecular Identification of the Isolates

Genomic DNA was extracted using a commercial bacterial DNA kit from Tiangen Biotech (Beijing, China) according to the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') [27]. PCR was performed in a 50 μL reaction volume with the following cycling conditions: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 45 s, 56°C for 30 s, and 72°C for 2 min; final extension at 72°C for 10 min. Amplicons were verified by 1% agarose gel electrophoresis, purified, and sequenced bidirectionally by Sangon Biotech (Shanghai, China). Sequences were compared against the National Center for Biotechnology Information (NCBI) nr database using Basic Local Alignment Search Tool (BLAST), and a neighbor-joining phylogenetic tree was constructed with MEGA 7.0 software using 1000 bootstrap replicates.

2.3. Growth Curves of the Isolate

DY2 was inoculated (1% *v/v*) into MRS broth from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China) and incubated at 37°C with shaking (180 rpm). Optical density

at 600 nm (OD_{600}) was measured every 2 h for 30 h using a spectrophotometer (Shimadzu UV-1900, Kyoto, Japan). Experiments were performed in triplicate.

2.4. In Vitro Antibacterial Tests

The antibacterial activity of DY2 culture supernatants against *Escherichia coli* ATCC 25922 was evaluated using the agar well diffusion assay [28]. Overnight *E. coli* cultures were grown in Luria–Bertani (LB) broth at 37 °C with shaking and adjusted to a 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). LB agar plates were evenly inoculated with the standardized bacterial suspension using a sterile swab to form a uniform lawn. Wells of 6 mm diameter were aseptically punched into the agar, and 100 μ L of cell-free supernatant from 24-h DY2 cultures (centrifuged at $10,000 \times g$ for 10 min at 4 °C and filter-sterilized through a 0.22 μ m membrane) was added. Plates were allowed to pre-diffuse at room temperature for 30 min and then incubated aerobically at 37 °C for 12 h. Antibacterial activity was determined by measuring the diameters of the clear inhibition zones around the wells with a digital caliper in two perpendicular directions, and the average diameter was recorded. All assays were performed in triplicate, and LB broth without supernatant served as a negative control.

2.5. Animal Experimental Design

Thirty outbred ICR mice (15 males, 15 females; 6–8 weeks old, 25–30 g) were obtained from the Center for Comparative Medicine, Yangzhou University (Yangzhou, China). Animals were kept under specific pathogen-free conditions with a 12-h light/dark cycle, controlled temperature (22 ± 2 °C), and ad libitum access to standard rodent chow and water. After a 7-day acclimation period, mice were randomly divided into three groups ($n = 10$ per group, balanced by sex): control (C), infection model (M), and DY2-pretreated (T). Mice in group T received daily oral gavage of DY2 suspension (1×10^9 CFU/mL in saline, 10 mL/kg body weight) for 21 consecutive days. The dose (1×10^9 CFU/mL) was based on efficacy in mouse probiotic models [29]. Groups C and M received equivalent volumes of sterile saline. On day 21, mice in groups M and T were challenged intraperitoneally with 0.1 mL of pathogenic *E. coli* (1×10^8 CFU/mL; laboratory-preserved clinical isolate) [30]. The intraperitoneal (IP) route was chosen to model severe systemic infection with bacterial translocation, mimicking advanced enteric infections where pathogens disseminate beyond the gut, leading to sepsis and indirect gut effects via inflammation [15]. Animals were monitored weekly for body weight, food and water intake, fecal consistency, and general health throughout the experimental period. Body weight was recorded using a calibrated digital balance, while food and water consumption were measured by weighing feed and water daily and calculating weekly intake per animal. Fecal consistency was assessed visually and scored using a standardized scale (e.g., 0 = normal, 1 = soft, 2 = loose, 3 = diarrhea). Any signs of illness or abnormal behavior were noted to assess overall health. All procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nanjing Agricultural University (NJAU.No20230915137), and ethical approval was obtained prior to the start of the study. Animals were housed under controlled environmental conditions (temperature, humidity, and light/dark cycle) with ad libitum access to feed and water throughout the study.

2.6. Collection of Samples

On day 22 (24 h post-challenge), mice were anesthetized with ketamine and xylazine, and euthanized by cervical dislocation. The 24-h post-challenge time point was selected to capture acute responses, as used in similar *E. coli* sepsis models where inflammation peaks early. Blood was collected via retro-orbital sinus puncture. Heart, liver, spleen, lungs, and

kidneys were excised and weighed. Segments of duodenum, jejunum, ileum, cecum, and colon, along with rectal contents, were collected aseptically for downstream analyses [31].

2.7. Analysis of Bacterial Load in Organs

Duodenum, jejunum, and ileum tissues were aseptically excised, rinsed with cold sterile phosphate-buffered saline (PBS; Servicebio, Wuhan, China) to remove luminal contents, and homogenized in sterile PBS at 1:9 (*w/v*) using a glass-Teflon homogenizer on ice for downstream analysis. Serial 10-fold dilutions were plated on LB agar from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China) and incubated aerobically at 37 °C for 24 h. Colonies were morphologically confirmed as *E. coli*-like; selective media (e.g., MacConkey) could enhance specificity in future work. Colony-forming units (CFU) per gram of tissue were calculated.

2.8. Serum Antioxidants and Cytokine Analysis

Serum was separated by centrifugation (3500 × *g*, 10 min, 4 °C) and stored at −80 °C. Levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and total antioxidant capacity (T-AOC) were quantified using commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the manufacturer's protocols [32]. Serum cytokines (TNF- α , IL-1 β , IL-6, IL-10) were measured by ELISA from ABclonal Biotechnology (Wuhan, China). All assays were performed in duplicate.

2.9. Histopathological Analysis

Duodenum, jejunum, and ileum tissues were aseptically excised, rinsed with cold sterile phosphate-buffered saline (PBS; Servicebio, Wuhan, China) to remove luminal contents, and homogenized in sterile PBS using a glass-Teflon homogenizer (Daihan Scientific, Republic of Korea) for downstream analyses. For histological examination, tissue sections were fixed in 10% neutral-buffered formalin (Sigma-Aldrich, Saint Louis, MO, USA), embedded in paraffin using a Leica EG1150H embedding station (Leica, Germany), sectioned at 5 μ m thickness with a Leica RM2235 microtome (Leica, Germany), stained with hematoxylin and eosin (H&E; Sigma-Aldrich, USA), and observed under an Olympus GX41 microscope (Olympus Co., Tokyo, Japan) equipped with an Olympus DP25 camera (Olympus Co., Japan) to assess structural integrity and cellular morphology. Measurements were taken from 10 well-oriented villi and crypts per section, as standard [33].

2.10. DNA Extraction and 16S rRNA Gene Sequencing

Total genomic DNA was extracted from rectal contents using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). DNA concentration and purity were assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was confirmed by 1% agarose gel electrophoresis. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using barcoded primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [34]. PCR reactions (25 μ L) contained 5 μ L 5 × FastPfu buffer, 0.25 μ L FastPfu polymerase (5 U/ μ L), 2 μ L dNTPs (2.5 mM), 1 μ L each primer (10 μ M), 1 μ L template DNA, and 14.75 μ L ddH₂O. Cycling conditions: 98 °C for 5 min; 25 cycles of 98 °C for 30 s, 53 °C for 30 s, 72 °C for 45 s; final extension at 72 °C for 5 min. Amplicons were purified using VAHTS DNA Clean Beads (Vazyme, Nanjing, China), quantified with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA), and pooled equimolarly. Paired-end sequencing (2 × 250 bp) was performed on an Illumina MiSeq platform with MiSeq Reagent Kit v3 at Bioyi Biotechnology Co., Ltd. (Wuhan, China).

2.11. Statistical and Bioinformatics Analysis

All the data are expressed as mean $\pm$ standard deviation (SD). Differences among groups were analyzed by one-way ANOVA followed by Tukey's post hoc test using SPSS 26.0 (IBM Corp., Milpitas, CA, USA). Sex was included as a covariate in ANOVA; no significant sex effects were found. Graphs were generated with GraphPad Prism 10.0 (GraphPad Software, Boston, MA, USA). $p < 0.05$ was considered statistically significant. The raw sequencing data were processed using QIIME2 (2022.2). Reads were quality-filtered, denoised, merged, and chimera-removed using DADA2 to generate amplicon sequence variants (ASVs). Taxonomy was assigned using the Greengenes2 database (2022.10). Alpha-diversity metrics (Chao1, Faith's phylogenetic diversity, observed features, Shannon, and Simpson indices) and rarefaction curves were calculated. Beta-diversity was assessed by Bray–Curtis dissimilarity with principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS), and significance tested by PERMANOVA. Differential taxa were identified using Linear Discriminant Analysis Effect Size (LEfSe) with LDA score > 2.0 and $p < 0.05$.

3. Results

3.1. Morphological Observation of the Isolates

The isolate, named DY2, formed characteristic milky-white, round, and raised single colonies with neat edges and smooth, moist surfaces when grown on MRS agar plates, which is typical for many *Lactobacillus* species (Figure 1a). In addition, these colonies appeared uniform after repeated streaking and purification, further supporting the purity and consistency of the isolated strain.

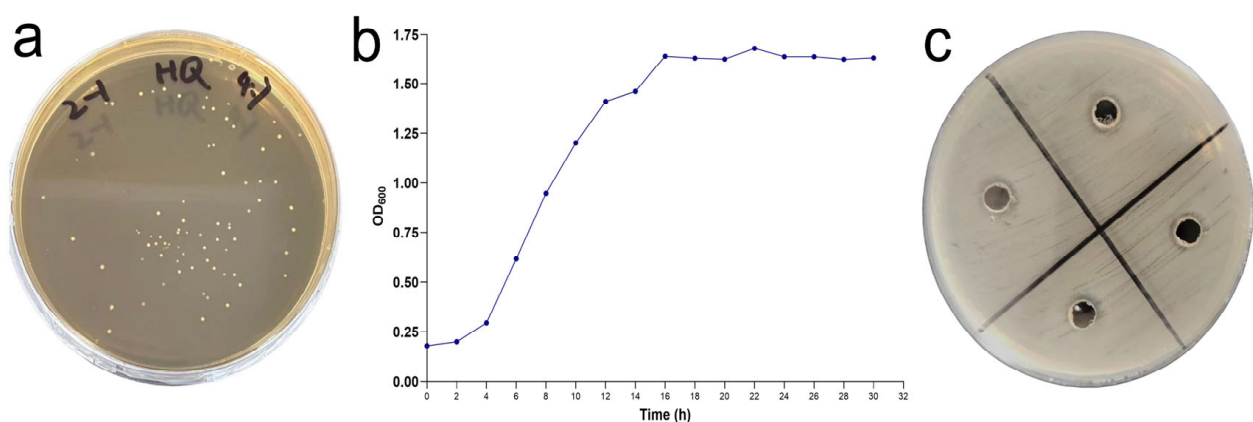


Figure 1. Phenotypic characterization and in vitro antimicrobial activity of the isolated strain. (a) Colony morphology; (b) Growth curves of the isolate over 30 h (Optical density at 600 nm); (c) Inhibitory activity of the isolate against *Escherichia coli* (*E.coli* ATCC 25922). Magnification: 200 $\times$ (histology); C = control, M = model, T = DY2-treated.

3.2. Identification by Molecular Biology

Phylogenetic analysis was performed using the neighbor-joining algorithm and p -distance model in MEGA 7.0 software, generating a robust tree that clearly grouped DY2 with reference strains (Figure 2). Moreover, 16S rRNA gene sequence analysis demonstrated that DY2 shared 99.66% homology with *Lactobacillus johnsonii*, providing strong molecular evidence for its species-level identification and distinguishing it from closely related lactobacilli.

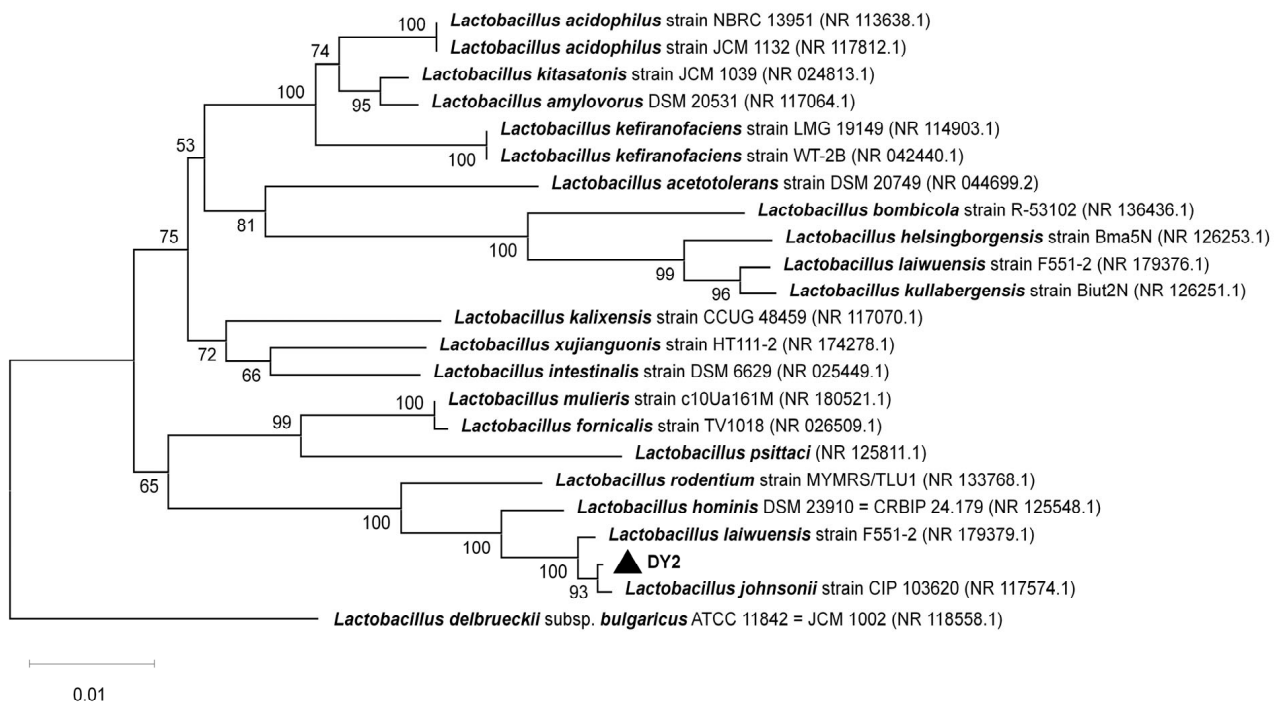


Figure 2. Phylogenetic tree of the isolate based on Neighbor-Joining distance analysis of 16S rRNA gene sequences.

3.3. Growth Curve

DY2 entered the logarithmic growth phase rapidly, within the first 2 h of incubation in MRS broth under optimal conditions. Moreover, it progressed smoothly to the stationary phase by approximately 16 h, after which optical density remained stable for the duration of monitoring (Figure 1b). This growth curve reflects efficient nutrient utilization and robust viability, which are desirable traits for potential probiotic applications.

3.4. In Vitro Antibacterial Results

The inhibitory activity of DY2 was assessed by the agar well diffusion assay, in which the antimicrobial effect was determined based on the diameter of inhibition zones formed by the cell-free supernatant against the indicator pathogen. The supernatant exhibited clear antibacterial activity against *Escherichia coli* ATCC 25922, forming well-defined inhibition zones (Figure 1c). Specifically, the average diameter of these zones across replicates was 14.11 ± 1.42 mm, indicating moderate but consistent antagonistic potential likely mediated by secreted metabolites.

3.5. Effect of DY2 on Body Weight, Bacterial Load, and Organ Index in Mice

From the onset of the experiment, mice in the control group (C) exhibited a consistent and progressive increase in body weight, accompanied by normal behavioral activities, healthy fur condition, regular food intake, and overall good mental status. Similarly, all groups displayed an upward trend in body weight during the 21-day pretreatment period, but the DY2-pretreated group (T) exhibited a noticeably higher rate of gain compared to the others. On day 21, mice in groups M and T received the intraperitoneal *E. coli* injection to induce acute infection. At this point, group M experienced clear weight loss, along with signs of distress, while group T showed almost no such decline and maintained better vitality (Figure 3a). In addition, these observations suggest that prolonged oral administration of DY2 enhanced host resistance, significantly reducing the severity of infection-related physiological impacts. Moreover, compared to group C, the bacterial loads

in the duodenum, jejunum, and ileum were dramatically higher in group M ($p < 0.001$), confirming that *E. coli* caused substantial intestinal barrier damage and intraluminal and bacterial translocation. Likewise, the organ indices of the liver, spleen, kidney, and lung were markedly increased in the M group compared with those in the C group ($p < 0.05$), reflecting systemic infection and associated organ enlargement or inflammation, whereas no differences were seen in the heart index. In addition to these changes, DY2 pretreatment in group T led to significant reductions in the liver, spleen, and kidney indices compared to group M ($p < 0.05$; Figure 3c).

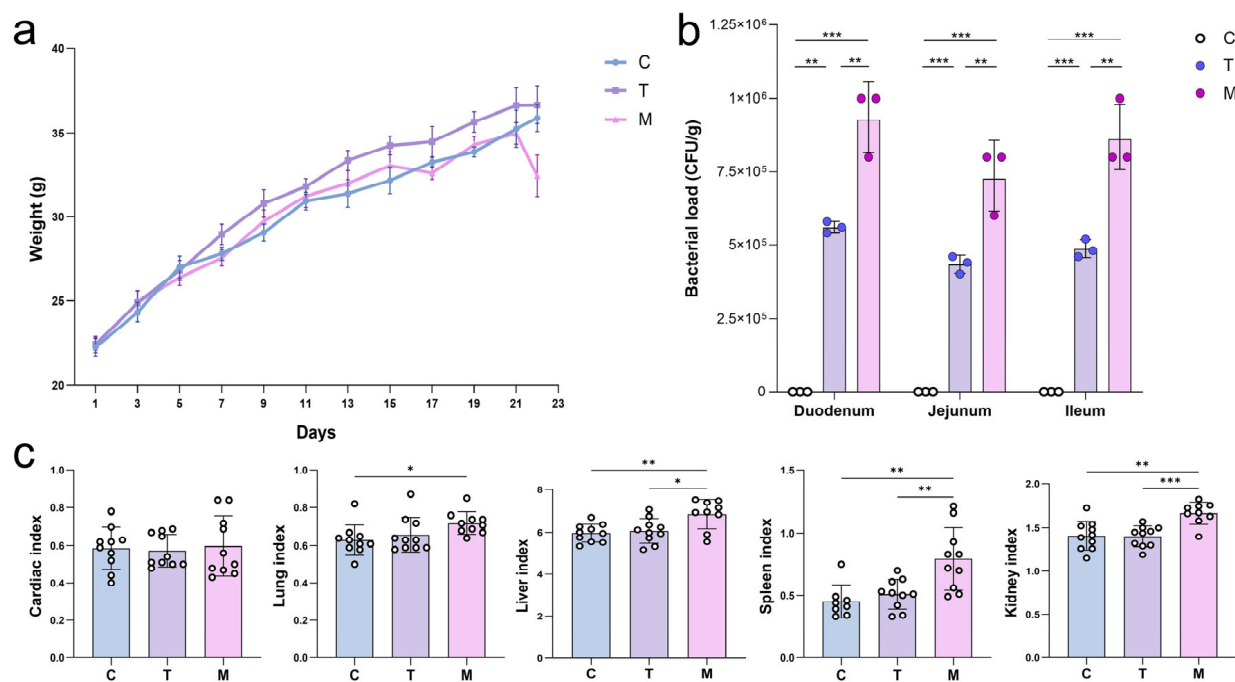


Figure 3. Effects of the isolate on body weight, organ index, and bacterial load. (a) Changes in body weight of mice; (b) Bacterial loads; (c) Organ index (Organ weight/Body weight). Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean $\pm$ SD. C, control group; M, infection model group; T, DY2-pretreated group.

3.6. Serum-Related Index Detection

To evaluate DY2's influence on systemic inflammatory and oxidative responses during acute infection, serum levels of key cytokines and antioxidant markers were quantified. The results demonstrated that *E. coli* challenge in group M induced a pronounced pro-inflammatory response, as evidenced by significantly elevated serum levels of IL-1 β , IL-6, and TNF- α compared with group C (Figure 4). In contrast, DY2 pretreatment in group T reduced these pro-inflammatory cytokines to varying but meaningful degrees. Simultaneously, the anti-inflammatory cytokine IL-10, which was reduced in group M, was significantly upregulated in group T ($p < 0.05$). In addition, these cytokine shifts were complemented by improvements in oxidative stress markers. Infection in group M led to reduced levels of T-AOC, SOD, and GSH-Px, alongside markedly elevated MDA as an indicator of lipid peroxidation. Similarly, DY2 pretreatment reversed these trends, restoring antioxidant enzyme activities and decreasing MDA (Figure 4).

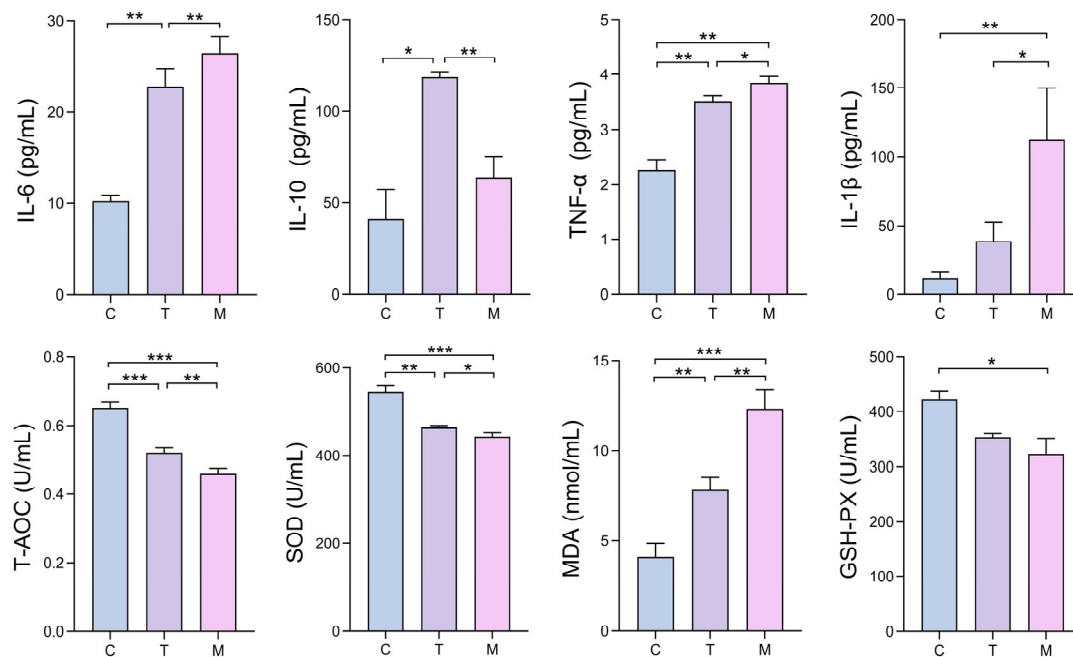


Figure 4. Measurement of inflammatory factors and oxidative stress indicators in mouse serum. Inflammatory factors include interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). Oxidative stress indicators include malondialdehyde (MDA), superoxide dismutase (SOD), glutathione pe-oxidase (GSH-Px), and total antioxidant capacity (T-AOC). Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean $\pm$ SD. C, control group; M, infection model group; T, DY2-pretreated group.

3.7. Analysis of Intestinal Pathological Sections

To assess DY2's impact on intestinal tissue integrity, paraffin sections from the duodenum, jejunum, and ileum were subjected to hematoxylin and eosin (H&E) staining for histopathological analysis (Figure 5). In group C, the intestinal mucosa appeared completely normal, with intact morphology, no evidence of inflammation, well-preserved epithelial cells (including absorptive and goblet cells), clear brush borders, tightly arranged cells without shedding. No neutrophil infiltration was observed in the lamina propria, and the muscularis mucosa remained intact (Figure 5a). In stark contrast, group M displayed severe inflammatory injury, including damaged villus structure with shortening and blunting, destroyed epithelial integrity, inflammatory cell infiltration in the lamina propria and sub-mucosa, and deepened crypts. However, the extent of damage in group T was considerably milder, with villi that were largely intact and only slightly shortened, reduced inflammatory infiltration, and overall preservation of mucosal structure (Figure 5a,b). In addition, these histological improvements demonstrate that prophylactic DY2 administration provided significant protection to the intestinal barrier during acute infection.

3.8. The Structural Composition of Intestinal Microorganisms

To investigate DY2's effects on the gut microbiota, rectal contents were subjected to 16S rRNA gene sequencing. Raw read counts ranged from over 91,600 in group C to 102,000 in group T and 101,000 in group M. After quality control, denoising, sequence merging, and chimera removal, at least 46,400, 53,400, and 53,100 high-quality sequences were obtained for groups C, T, and M, respectively (Figure 6). Using the DADA2 method for denoising, amplicon sequence variants (ASVs) were generated and clustered for species annotation. In total, 12,611 ASVs were identified across the three groups: 2488 in group C, 3398 in group T, and 2955 in group M, with 403 ASVs shared among all groups and a notably high number of unique ASVs (2563) in group T (Figure 6a). This distribution suggests

that DY2 pretreatment positively influenced microbial richness. Moreover, rarefaction curves flattened out for all samples, confirming that the sequencing depth was adequate to capture most of the existing diversity (Figure 6b). In addition, rank-abundance curves, which plot ASV abundance against rank, showed relatively flat and even distributions in group C, indicating homogeneous community composition with small differences in ASV abundance. In contrast, group M curves were steeper, reflecting lower species evenness. The curves for group T were smoother than those of group M, implying that probiotic pretreatment enhanced community resilience and stability (Figure 6c).

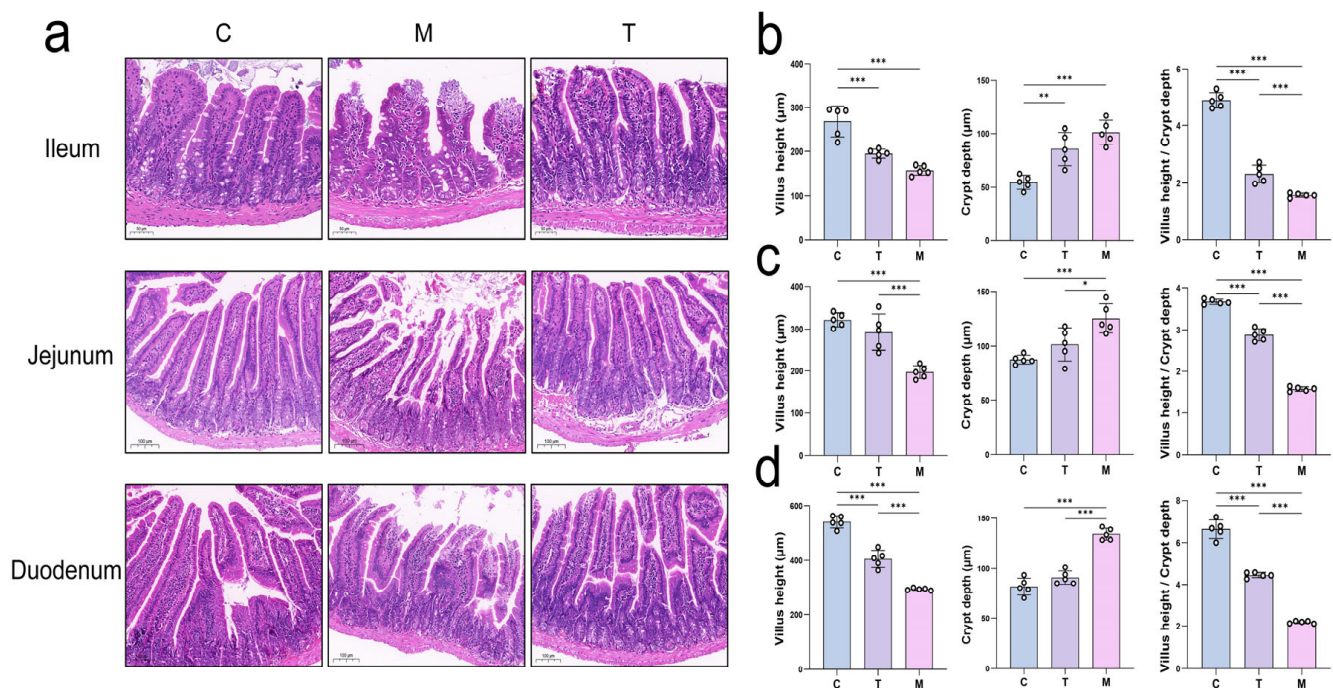


Figure 5. Analysis of intestinal pathological sections in mice. (a) HE staining sections of the ileum (20×), jejunum (10×), and duodenum (10×) of mice; (b) The length of the ileal villi and the depth of the ileal crypt; (c) The length of the jejunal villi and the depth of the jejunal crypt; (d) The length of the duodenal villi and the depth of the duodenal crypt. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SD. C, control group; M, infection model group; T, DY2-pretreated group.

Alpha-diversity analysis further compared intra-group microbial variation, revealing higher values across multiple indices (including Faith's phylogenetic diversity, Chao1, observed species richness, Shannon, and Simpson) in group T compared to group C. In contrast, group M showed relatively lower diversity (Figure 6d).

Beta-diversity, which measures inter-community differences, was visualized using principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS), showing scattered sample distributions and clear separation among groups (Figure 7a). Additionally, Linear Discriminant Analysis Effect Size (LEfSe) identified 19 significantly enriched bacterial taxa across the groups ($p < 0.05$), with differences spanning five taxonomic levels (Figure 7b).

Relative abundance bar charts highlighted shifts at multiple taxonomic ranks (Figure 8). At the phylum level, the top four were *Bacteroidota* (70.85% in C, 65.37% in T, 47.40% in M), *Firmicutes_D* (16.45% in C, 4.50% in T, 31.30% in M), *Firmicutes_A* (2.50% in C, 13.76% in T, 9.97% in M), and *Actinobacteriota* (5.78% in C, 4.99% in T, 7.25% in M). *Campylobacterota* was notably higher in T (7.57%) than in M (0.63%). At the family level, the relative abundances of *Lactobacillaceae* and *Lachnospiraceae* were elevated in the M group compared

with the C group. while *Muribaculaceae* decreased; DY2 pretreatment elevated *Bacteroidaceae*, *Lachnospiraceae*, and *Helicobacteraceae* while reducing *Lactobacillaceae* and *Turicibacteraceae*. At the genus level, infection enriched *Lactobacillaceae*, *Turicibacteraceae*, and *Limosilactobacillus* but depleted *Paramuribaculum* and *Duncaniella*; in addition, group T showed increased *Helicobacter_C_479931* and decreased *Turicibacteraceae*.

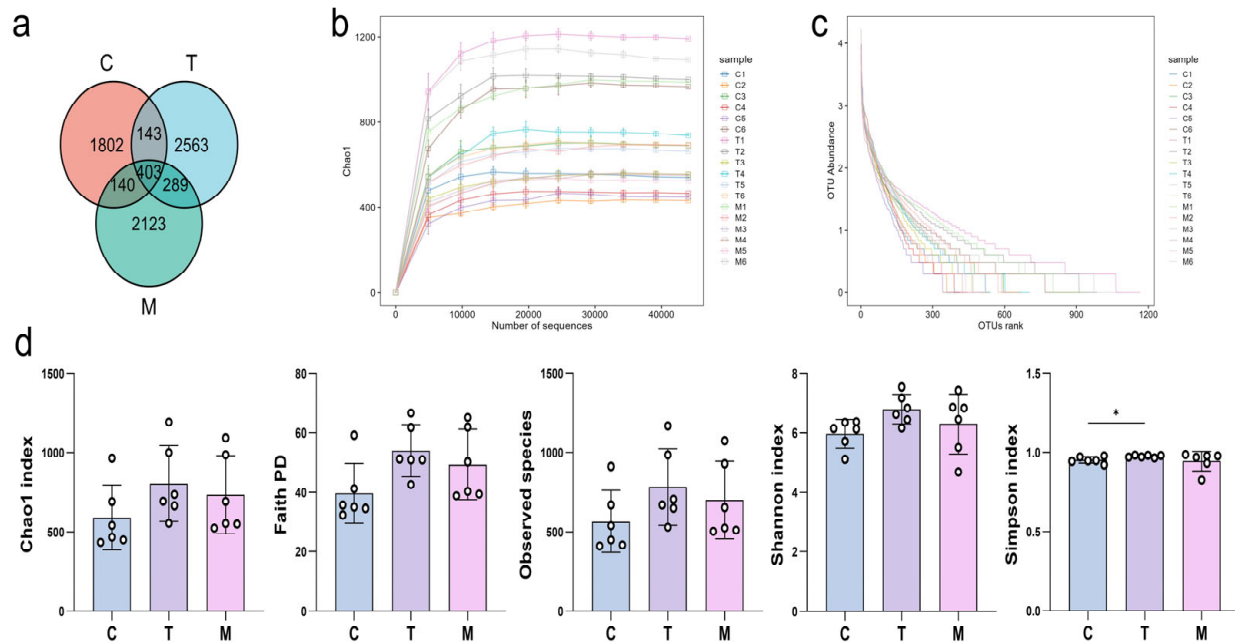


Figure 6. Sequencing results of the mouse intestine. (a) ASV composition Venn graph; (b) Rarefaction curve; (c) Rank abundance curve; (d) α -diversity analysis, including Chao1 index, Faith's phylogenetic diversity, Observed species, Shannon diversity index, Simpson dissimilarity index. Statistical significance is indicated as * $p < 0.05$. Data are presented as mean $\pm$ SD. C, control group; M, infection model group; T, DY2-pretreated group.

3.9. Intestinal Differential Microbiota

Re-analysis of sequencing data specifically at the phylum and genus levels identified a total of 4 phyla and 16 genera with statistically significant differential abundance (Figure 9). Compared to groups C and T, group M exhibited significantly higher relative abundances of several genera, including *Turicibacter*, *C19*, *Brachybacterium*, *UBA644*, *Emergencia*, *SFMI01*, and *Xylanivirga*. In contrast, abundances of *Paramuribaculum*, *Cryptobacteroides*, *Parabacteroides B 862066*, and *Bifidobacterium 388775* were decreased in group M. Notably, group T showed significant enrichment of health-associated genera such as *Evtepieia*, *Acutalibacter*, *Borkfalkia*, and particularly *Butyricimonas*. Moreover, at the phylum level, *Firmicutes_D* and *Firmicutes_B_370539* increased markedly in group M, whereas *Bacteroidota* and *Campylobacterota* remained significantly lower in M compared to both C and T. In addition to these specific shifts, the overall pattern reinforces DY2's capacity to counteract infection-driven dysbiosis and promote a more favorable microbial ecosystem.

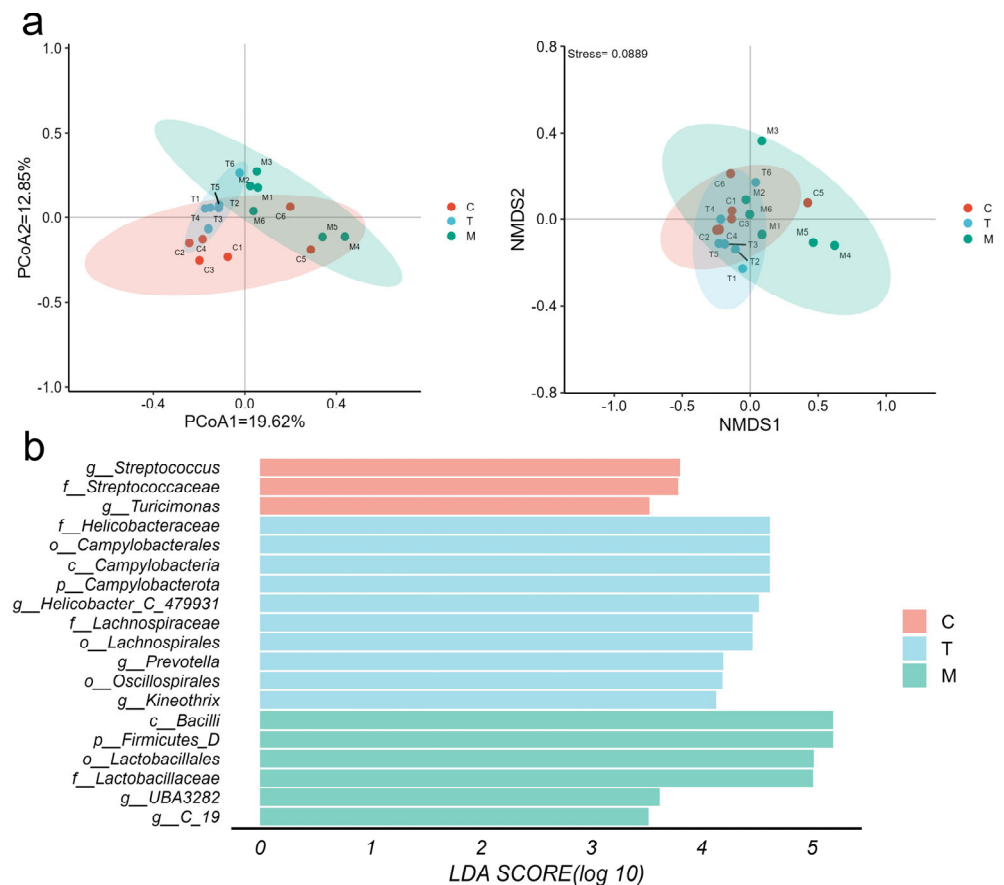


Figure 7. Microbiota structure analysis of mice in different groups. (a) β -Diversity analysis, including PcoA (Principal coordinates analysis) and NMDS (Non-metric Multidimensional Scaling); (b) LefSe (LDA Effect Size) analysis of samples between groups (Linear discriminant analysis, LDA). C, control group; M, infection model group; T, DY2-pretreated group.

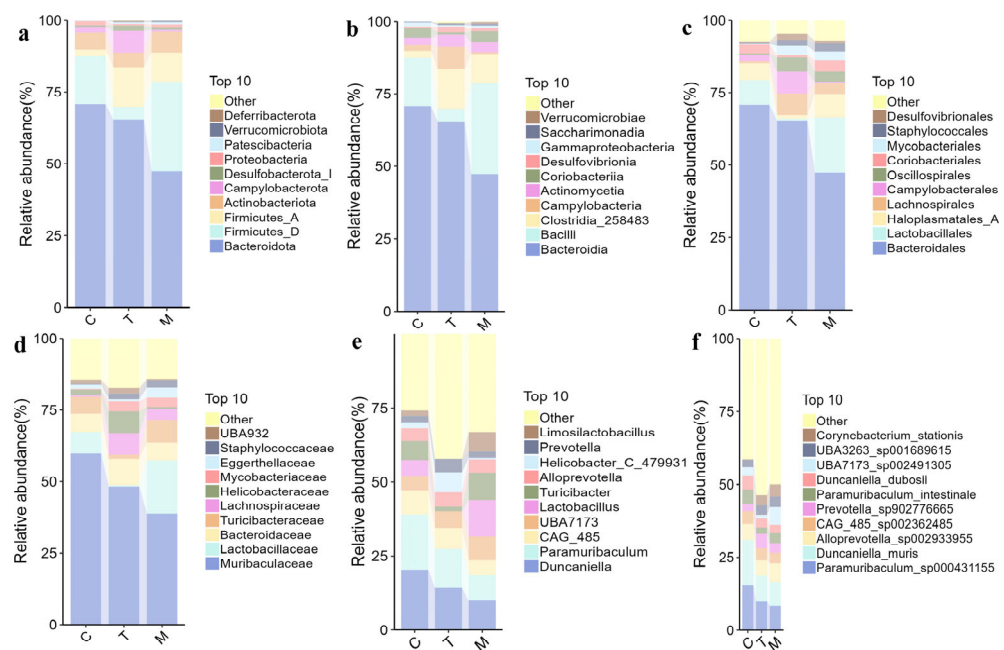


Figure 8. A diagram of the structural composition of the intestinal flora in mice at different levels. (a) Phylum, (b) class, (c) order, (d) family, (e) genus, and (f) species. C, control group; M, infection model group; T, DY2-pretreated group.

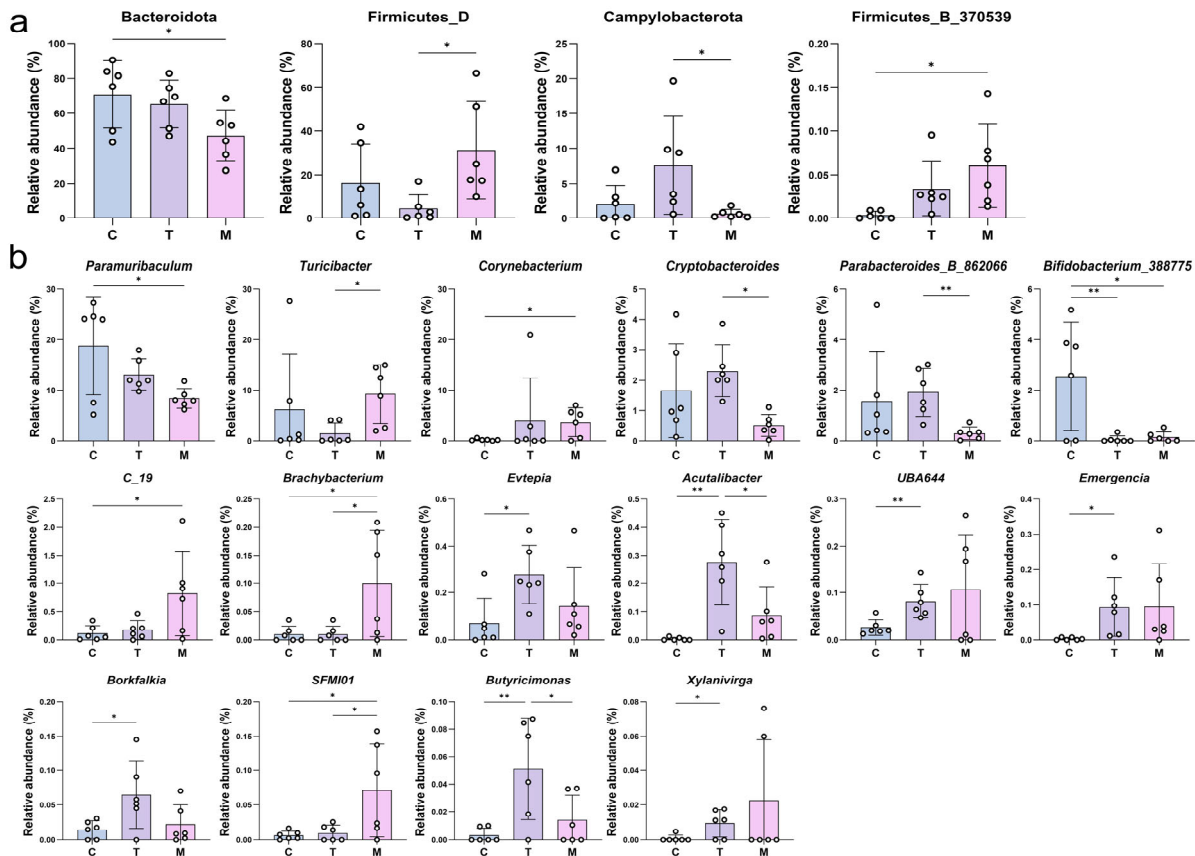


Figure 9. Analysis of differences in intestinal microbiota in mice. (a) Phylum and (b) genus. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$. Data are presented as mean $\pm$ SD. C, control group; M, infection model group; T, DY2-pretreated group.

4. Discussion

In this study, we demonstrated that the yak-derived *Lactobacillus johnsonii* DY2 confers promising protective effects against acute *E. coli* infection in this model through coordinated multi-level mechanisms. Specifically, DY2 simultaneously modulated systemic inflammation, bolstered antioxidant defenses, preserved intestinal epithelial integrity, and stabilized the gut microbial ecosystem. In addition to these individual effects, the collective findings underscore DY2's robust probiotic potential, particularly its ability to enhance host resilience against pathogenic challenges in a harsh infectious context. Moreover, the strain's origin from high-altitude yaks—animals adapted to extreme environmental stressors—may contribute to its superior functional attributes, setting it apart from many conventionally sourced lactobacilli.

Cytokines play central roles in orchestrating immune responses during infection. More than 200 cytokines have been identified to date [35], and among them, IL-6 is a versatile mediator whose serum levels typically rise sharply in response to bacterial challenge. Increasing evidence shows that IL-6 family members drive acute inflammatory conditions, including sepsis and macrophage activation syndrome [36]. Similarly, IL-1 β acts as a master regulator of inflammation by inducing transcription of numerous downstream pro-inflammatory genes, while also contributing to homeostasis in various organ systems [37]. In contrast, the anti-inflammatory cytokine IL-10 is essential for restraining excessive immune activation, bolstering innate defenses, and promoting tissue repair during infection [38]. TNF- α is a pivotal mediator of intestinal inflammatory responses and plays a well-established role in the pathogenesis of inflammatory bowel disease (IBD). Moreover, it acts synergistically to enhance the expression of downstream pro-inflammatory cytokines,

including IL-1 β and IL-6 [39]. In the present study, DY2 treatment markedly attenuated *E. coli*-induced inflammatory responses by significantly reducing the secretion of IL-1 β , IL-6, and TNF- α , while concurrently restoring the levels of the anti-inflammatory cytokine IL-10. These results align with prior reports on *L. johnsonii* strains that attenuate inflammatory signaling via TLR and MAPK pathway modulation [40,41]. These effects may involve pathways such as Nrf2 or TLR–MAPK, though direct evidence is lacking and requires further study. However, unlike some strains where anti-inflammatory effects vary by context, DY2 consistently mitigated severe systemic inflammation, suggesting greater stability possibly linked to its adaptation in the yak gastrointestinal tract. Comparatively, well-studied probiotics like *L. rhamnosus* GG and *L. plantarum* also reduce pro-inflammatory cytokines [42–45], but often with weaker effects on IL-10 upregulation during acute infection, highlighting a potentially distinguishing feature of DY2.

In addition to immune modulation, oxidative stress represents a critical component of infection pathology [46]. Total antioxidant capacity (T-AOC) is widely recognized as an integrative indicator of an organism's ability to scavenge free radicals and preserve redox homeostasis [45]. Antioxidant enzymes, notably superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), function cooperatively to eliminate excessive reactive oxygen species (ROS) and mitigate oxidative cellular damage [47]. In contrast, malondialdehyde (MDA) accumulation reflects the extent of lipid peroxidation and membrane injury [48]. Our results demonstrated that DY2 pretreatment significantly enhanced SOD, GSH-Px, and T-AOC activities, accompanied by a pronounced reduction in MDA levels following *E. coli* challenge. This profile mirrors observations with other effective *Lactobacillus* strains that enhance oxidative resistance through Nrf2 pathway activation [49,50]. Therefore, the Nrf2 signaling pathway is a pivotal regulator of antioxidant responses [51,52]. In addition, certain LAB (e.g., *L. delbrueckii*) were shown to attenuate LPS-induced intestinal oxidative damage by triggering a TLR–Btk–Nrf2 signaling cascade in intestinal mucosa [53]. This integrative response suggests a synergistic relationship between DY2's bioactive metabolites and host cellular antioxidant machinery.

Histopathological examination revealed that mice pretreated with DY2 maintained intact villus structure, reduced crypt deepening, and diminished inflammatory infiltration—outcomes consistent with prior work showing that *L. johnsonii* strains protect epithelial integrity by upregulating tight junction expression and limiting pathogen translocation [54,55]. These observations echo findings for *L. johnsonii* MG, which enhanced tight-junction-mediated barrier integrity via binding between bacterial GAPDH and host junctional adhesion molecule JAM-2 [56,57]. The experimental results showed that DY2 reduced bacterial load in duodenum, jejunum, and ileum by over 40%, indicating strengthened mucosal barriers. Moreover, in *E. coli*-infection models, *Lactobacillus* supplementation has been shown to upregulate expression of tight junction proteins (Occludin, Claudin-1, ZO-1), improve mucin production, and reduce pro-inflammatory cytokines, together leading to restored mucosal architecture and reduced pathogen translocation [58,59].

Gut microbiota disruptions are a hallmark of intestinal inflammation [60,61], and our results confirmed that acute *E. coli* infection reduced microbial α -diversity and markedly altered community composition in mice. In contrast, DY2 pretreatment preserved and even enhanced diversity, improved evenness, and enriched beneficial taxa such as *Butyricimonas* and *Borkfalkia*. Microbiota resilience is increasingly recognized as a determinant of infection outcomes, where diversity loss exacerbates inflammation and barrier collapse [62,63]. Butyrate-producing bacteria are known to maintain epithelial barrier integrity, enhance mucin production, suppress NF- κ B activation, and support Treg induction [64,65]. For instance, supplementation of butyrate-producing bacteria to dysbiotic microbiota from Crohn's disease patients restored barrier integrity in vitro [66]. Interestingly, DY2 uniquely

increased Campylobacterota abundance, a taxon rarely associated with probiotic intervention [67]. Although the biological relevance remains unclear, it may indicate a niche-filling compensatory mechanism that contributes to overall community stability. The capacity of DY2 to foster such ecological shifts under infection indicates it may act not only directly but also indirectly by reshaping host–microbe interactions toward a more health-promoting ecosystem. This observation agrees with recent findings that targeted probiotic–prebiotic interactions can shift dysbiotic communities toward functional recovery [68,69].

Despite these promising insights, several limitations must be acknowledged. This study relied on a single outbred ICR mouse model and one pathogenic *E. coli* strain (laboratory-preserved clinical isolate), which may limit the generalizability of the protective effects to other host strains, *E. coli* variants (including antibiotic-resistant strains), infection routes, or species. The intraperitoneal (IP) challenge, while reproducible for inducing severe systemic inflammation, translocation, and downstream intestinal barrier/microbiota disruption, does not fully recapitulate localized enteric infection compared to oral/intragastric administration; future studies should validate findings with oral models. Bacterial loads in tissues were quantified on non-selective LB agar with morphological confirmation only; selective media (e.g., MacConkey or EMB agar) would enhance strain specificity and confirm the pathogenic challenge isolate. The 24 h post-challenge time point captures peak acute inflammatory, oxidative, barrier, and dysbiosis responses, as supported by similar *E. coli* sepsis models [32], but does not assess longer-term recovery or adaptive changes. No strain-specific qPCR or selective CFU plating was performed to confirm DY2 viability, transient/persistent colonization, or gastrointestinal passage after oral gavage. While clear phenotypic benefits were observed, the precise molecular mechanisms—such as potential involvement of Nrf2, TLR–MAPK, or STAT3–IL-10 pathways, or production of specific antimicrobial metabolites (e.g., bacteriocins, exopolysaccharides) remained to address in future studies. Additionally, 16S rRNA sequencing provides robust compositional analysis but limited functional, strain-level, or metabolomic resolution and cannot quantify DY2 or its metabolites. Future investigations incorporating metatranscriptomics, metabolomics, gnotobiotic models, diverse pathogens, oral challenge routes, and colonization tracking would address these gaps and strengthen mechanistic and translational insights.

5. Conclusions

Our study demonstrates that the yak-derived *Lactobacillus johnsonii* DY2 protects against acute *E. coli* infection in this model through coordinated anti-inflammatory, antioxidant, gut barrier-strengthening, and microbiota-modulating activities. DY2 not only inhibits pathogen proliferation but also markedly attenuates systemic inflammatory responses, enhances host antioxidant defenses, preserves intestinal epithelial integrity, and promotes gut microbiota resilience under severe infectious stress. By restoring the balance between pro- and anti-inflammatory cytokines, reducing oxidative damage, limiting bacterial translocation, and enriching beneficial microbial taxa such as *Butyrivibrio*, DY2 effectively strengthens the host's intrinsic defense systems. Given these multifaceted benefits, *L. johnsonii* DY2 represents a promising probiotic candidate for the prevention of acute enteric infections. These findings highlight yak-derived *L. johnsonii* as promising probiotics with excellent antibacterial properties, which is particularly important in the era of escalating antimicrobial resistance.

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