Research Square

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Isolation, Characterization, and Probiotic Potential of Bacillus subtilis Strain WW12 from Wild Wolf (Canis lupus) Feces with Protective Effects Against Murine Colitis

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Abstract

Wild wolves (Canis lupus), as the closest wild relatives of domestic dogs, harbor a gut microbiota uniquely adapted to their natural environment, representing a valuable reservoir of host-adapted probiotics. In this study, we isolated *Bacillus subtilis* strains from wild wolf feces and systematically evaluated their probiotic properties, including stress tolerance, antimicrobial activity, auto-aggregation, surface hydrophobicity, antibiotic susceptibility, and genomic safety, followed by in vivo assessment using a dextran sulfate sodium (DSS)-induced colitis mouse model. Among the isolates, strain WW12 exhibited the most promising probiotic characteristics. It demonstrated high survival rates in simulated gastric (94.3%) and simulated intestinal fluids (85.7%), strong tolerance to high temperatures (65.2% survival at 70°C), and excellent auto-aggregation (78.71%) and co-aggregation with pathogenic bacteria. WW12 showed broad-spectrum antimicrobial activity against *Escherichia coli, Staphylococcus aureus* and *Salmonella Typhimurium*, with inhibition zones of 18.3 mm, 19.7 mm and 19.3 mm, respectively. The strain was sensitive to all tested antibiotics, and genomic analysis confirmed the absence of transferable antibiotic resistance genes (ARGs); the only 10 intrinsic ARGs detected were located on the chromosome, indicating a favorable safety profile. In the DSS-induced colitis model, oral administration of WW12 significantly ameliorated disease severity, as evidenced by reduced disease activity index (DAI), restored colon length, improved histopathological scores, and modulation of inflammatory cytokines (decreased IL-1β, IL-6; increased IL-10). Metagenomic analysis revealed that WW12 treatment reshaped the gut microbiota by increasing beneficial bacteria (*Bacteroides fragilis etc.*) and reducing pathogenic taxa (*Clostridioides difficile etc.*). Although not detected in canine feces after oral administration—suggesting possible intestinal mucosal colonization—WW12's efficacy in the murine model strongly supports its probiotic potential. Transcriptomic analysis further showed that WW12 downregulated the Cytokine-cytokine receptor interaction pathway and the PI3K-Akt signaling pathway. Collectively, these findings establish *Bacillus subtilis* WW12 as a safe, host-adapted probiotic candidate derived from wild wolves, with significant protective effects against intestinal inflammation, offering a novel natural intervention for inflammatory bowel disease in companion animals.

1 Introduction

Inflammatory bowel disease (IBD) in domestic dogs represents a chronic and debilitating gastrointestinal disorder characterized by persistent diarrhea, vomiting, weight loss, and abdominal discomfort, frequently accompanied by immune dysregulation and profound disruption of gut microbial homeostasis[1]. Despite the widespread recognition of canine IBD in veterinary practice, its true prevalence has remained challenging to ascertain due to the heterogeneous nature of clinical presentations and the lack of standardized diagnostic criteria. Recent large-scale epidemiological investigations, however, have provided important new insights into the magnitude of this clinical problem. A cross-sectional survey encompassing 43,517 dogs enrolled in the Dog Aging Project revealed that 30% of companion dogs experience at least one owner-reported gastrointestinal disorder during their lifetime, with inflammatory gastrointestinal disease documented in approximately 1% of the

population[2]. Notably, primary enteropathies account for the overwhelming majority of chronic diarrhea cases in dogs, with inflammatory etiologies constituting 71% of diagnoses—underscoring the significant clinical burden imposed by intestinal inflammation in canine patients[3]. Conventional therapeutic approaches for canine IBD, including glucocorticoids, immunosuppressive agents, and specialized dietary management[4], frequently yield suboptimal outcomes and are associated with a spectrum of adverse effects ranging from iatrogenic hyperadrenocorticism and gastrointestinal ulceration to heightened susceptibility to opportunistic infections[5]. This therapeutic impasse highlights an urgent and compelling need for safer, more effective, and host-compatible alternatives to address canine intestinal inflammatory disorders.

Probiotics—defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"—have emerged as a promising intervention for modulating gut microbiota composition, reinforcing intestinal barrier integrity, and attenuating mucosal inflammation[6]. Among the diverse array of probiotic candidates, *Bacillus subtilis* has garnered particular scientific and commercial attention due to its unique and highly advantageous biological attributes. Foremost among these is its capacity to form metabolically dormant endospores, which confer remarkable resistance to harsh environmental insults[7], including exposure to gastric acidity, bile salts, thermal extremes, and mechanical forces encountered during food processing, storage, and gastrointestinal transit[8]. Recent in vitro and in silico analyses of the commercially available spore-forming probiotic *Bacillus subtilis* DE111® have revealed the genomic underpinnings of its robust stress tolerance, including the presence of multi-subunit ATPases, arginine deiminase (ADI) pathway genes (argBDR), molecular chaperones (GroES/GroEL and DnaK/DnaJ), and extracellular polymeric substance (EPS) biosynthesis genes (pgsBCA) that collectively enable survival and colonization within the challenging gastrointestinal milieu[9]. In canine populations specifically, *B. subtilis* supplementation has been demonstrated to support digestive health and promote gut microbiome homeostasis, with clinical studies confirming the safety of daily administration in healthy Beagle dogs[10]. Furthermore, mounting experimental evidence has established that *B. subtilis* can ameliorate intestinal inflammation through the coordinated downregulation of the TLR4/NF-κB/NLRP3 inflammasome signaling axis while concurrently enhancing the expression of tight junction proteins—including zonula occludens-1, occludin, and claudin-1—and bolstering mucosal antioxidant capacity[11]. In the context of colitis models, *B. subtilis* isolates have been shown to exert protective effects against dextran sulfate sodium (DSS)-induced intestinal inflammation by restoring tight junction protein expression and reducing pro-inflammatory cytokine production[12]. Nevertheless, due to the fact that commercially available *Bacillus subtilis* strains are primarily developed for human use, they may exhibit poor adaptability within the canine gastrointestinal tract, which limits their clinical application and therapeutic reliability in canines[13–15].

The paradigm of host-adapted probiotics—that is, probiotic strains isolated directly from the native microbiota of target species or their phylogenetically proximate relatives—offers a compelling and biologically rational alternative to commercially available allochthonous strains. These autochthonous

strains are theoretically better equipped to colonize the gastrointestinal tract of the intended host, resist niche-specific environmental stressors, and exert precise and potent regulatory effects on host physiology compared with probiotics derived from evolutionarily distant or unrelated sources[16].

Domestic dogs (Canis lupus familiaris) share a deep and well-documented evolutionary lineage with wild gray wolves (Canis lupus), with extensive genetic and microbial evidence demonstrating that, despite millennia of domestication and profound dietary divergence, the core architecture of the canine gut microbiome retains significant phylogenetic conservation[17,18]. Free-ranging wolves inhabit pristine natural ecosystems largely undisturbed by human activity, with minimal exposure to antibiotics, representing a largely untapped and exceptionally promising source of novel probiotic strains characterized by extremely low carriage of transferable antibiotic resistance genes (ARGs), robust and diverse metabolic capabilities, and intrinsic host compatibility[19]. Indeed, pioneering investigations have successfully isolated spore-forming *Paenibacillus spp.* from the gastrointestinal tract of wild gray wolves, with comprehensive whole-genome sequencing confirming the absence of pernicious virulence determinants and the presence of antimicrobial-encoding genetic loci and carbohydrate-active enzyme systems[20,21]. Moreover, patent literature has described isolated strains of gastrointestinal bacteria from free-ranging wolves specifically for use as probiotics in canine subjects[22], underscoring the recognized potential of wolf-derived bacteria for companion animal health applications. These findings substantiate the compelling rationale that wolf-derived bacteria, including members of the order Bacillales, may constitute a fertile and underexplored source for the discovery of next-generation probiotics exquisitely tailored for canine health applications.

This study aimed to isolate and characterize wild gray wolf-derived *Bacillus subtilis* strains with enhanced probiotic potential for canine intestinal inflammation, using culture-dependent isolation and 16S rRNA identification, followed by in vitro assessments (gastrointestinal and thermal stress tolerance, surface hydrophobicity, aggregation, antimicrobial activity, antibiotic susceptibility), genomic safety evaluation (ARG analysis, COG/KEGG/CAZy annotation), and in vivo validation in a DSS-induced murine colitis model coupled with gut metagenomics and colon transcriptomics (RNA-seq, GSEA/KEGG). Our overarching goal was to obtain a high-performance, host-adapted *Bacillus subtilis* strain suitable for veterinary clinical applications, thereby providing a novel and scientifically rigorous solution for the management of canine intestinal inflammatory disorders.

2 Materials and Methods

2.1 Sample Collection

Fresh fecal samples were collected from wild wolves in a protected natural reserve using sterile collection tubes. Samples were immediately preserved on dry ice during transportation and stored at -80°C within 24 h to maintain microbial viability. All sampling procedures were non-invasive and compliant with wildlife protection regulations.

2.2 Isolation and Identification of *Bacillus subtilis* Strains

2.2.1 Enrichment and Isolation

Fecal samples (2 g) were homogenized with 10 mL of sterile phosphate-buffered saline (PBS, pH 7.2) and subjected to heat treatment at 80°C for 15 min to eliminate vegetative cells and enrich for *Bacillus* spores[23]. After cooling, the homogenate was serially diluted (10^{-1} to 10^{-6}), and 200 μL of each dilution was spread onto Luria-Bertani (LB) agar plates supplemented with 1% MR powder (as a selective carbon source). Plates were incubated at 37°C for 24–48 h, and colonies with typical *Bacillus subtilis* morphology (opaque, irregular edges, rough surface) were selected for purification by repeated streaking.

2.2.2 Morphological Identification

Purified strains were subjected to Gram staining and endospore staining, followed by observation under a light microscope (1000× magnification). Bacterial morphology was observed using scanning electron microscopy (SEM). Samples were fixed in 2.5% glutaraldehyde PBS solution at 4 ◦C for 12 h, followed by gradient dehydration with ethanol (30% to 100%), pre-freezing, freeze-drying, and gold sputtering before observation under a 5 kV accelerating voltage.

2.2.3 Molecular Identification

Genomic DNA was extracted using a bacterial genomic DNA extraction kit (TIANGEN, China). The 16S rRNA gene (primers: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-AAGGAGGTGATCCAGCC-3') were amplified by PCR[24]. PCR products were sequenced by Tsingke Biotechnology (Beijing, China), and sequences were aligned with the NCBI GenBank database using BLAST[25]. A phylogenetic tree was constructed using MEGA 11 software with the neighbor-joining method (1000 bootstrap replicates).

2.3 Stress Tolerance Assay

2.3.1 Gastrointestinal fluid tolerance

Simulated gastric fluid (pH 2.0, containing 0.35% pepsin) and intestinal fluid (pH 8.0, containing 0.1% trypsin and 1.8% bile salt) were prepared[26,27]. Bacterial suspensions (1×10^8 CFU/mL) were inoculated into the fluids (10% v/v) and incubated at 37°C for 3 h (gastric fluid) and 21 h (intestinal fluid). Viable counts were performed by plate counting, and survival rates were calculated.

2.3.2 Temperature tolerance

Bacterial suspensions were incubated at 50°C, 60°C, and 70°C for 2 h, and survival rates were determined[26].

2.4 Growth Curve of *Bacillus subtilis* strains

Overnight cultures of the three *Bacillus subtilis* strains (1 mL each) were inoculated into 100 mL of LB broth and incubated at 37 ℃. The optical density at 600 nm (OD600) was measured every 2 h until 60 h using a spectrophotometer (DeNovix, USA).

2.5 Surface Hydrophobicity and Auto-Aggregation Assays

Surface hydrophobicity was determined using the xylene method[28,29]. Bacterial suspensions (1×10^8 CFU/mL) were mixed with an equal volume of xylene, incubated at room temperature for 1 h, and the absorbance of the aqueous phase at 600 nm was measured. Hydrophobicity (%) = $[(A_0 - A_1)/A_0] \times 100\%$, where A_0 is the initial absorbance and A_1 is the absorbance after mixing.

Auto-aggregation was assessed by measuring the absorbance of bacterial suspensions (1×10^8 CFU/mL) at 600 nm at 0 h and 5 h[28,30]. Auto-aggregation (%) = $[1 - (A_5/A_0)] \times 100\%$, where A_5 is the absorbance at 5 h.

2.6 Antimicrobial Activity Assay

E. coli ATCC25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella Typhimurium* ATCC 14028 were used as indicator pathogens. Bacterial supernatants (200 μL) from 24 h LB cultures were added to Oxford cups on LB agar plates inoculated with indicator pathogens (1×10^6 CFU/mL). Plates were incubated at 37°C for 12 h, and the diameter of inhibition zones was measured[31,32].

2.7 Antibiotic Susceptibility Assay

The Kirby-Bauer disk diffusion method was used[33]. Bacterial suspensions were spread onto Mueller–Hinton agar (MHA) plates, and antibiotic discs (Gentamicin, Ciprofloxacin, Ampicillin, Cefazolin, Erythromycin, Norfloxacin, Amikacin, Sulfamethoxazole, Chloramphenicol) were placed on the plates. After 24 h incubation at 37°C, inhibition zone diameters were measured and interpreted according to CLSI standards.

2.8 Genomic Analysis

High-quality genomic DNA of the selected strain was sequenced using the Illumina NovaSeq 6000 platform. ARGs were annotated using the CARD database and ResFinder 4.1[34]. Functional genes were analyzed using COG, KEGG and CAZy databases[35].

2.9 Animal Experiment

2.9.1 Grouping and Treatment

All mice (BALB/c) were selected from 6–8 weeks male mice weighing 20 ± 2 g and free of specific pathogens. All animal experiments were approved by the Scientific Ethics and Safety Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS2025-164; Beijing, China) on October 8, 2025. All procedures conformed to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All animals were raised in a specific pathogen-free environment with a temperature

of 22 ± 3°C, a humidity of 50 ± 15%, and a 12-h light–dark cycle. All mice were given a normal diet during the experiment. All mice were divided into 5 groups (CK, DSS, DSS + WW3, DSS + WW8, and DSS + WW12), with 8 mice in each group. The specific grouping and animal experimental process are shown in Fig. 5A. During the adaptation period, all groups were given normal drinking water and feed. Starting from the second week for 21 days, the CK and the DSS groups were given normal drinking water and feed. The DSS + WW3 group was given WW3 fermentation broth every day through the gavage method (10^8 CFU/mL). The DSS + WW8 group was given WW8 fermentation broth every day through the gavage method (10^8 CFU/mL). The DSS + WW12 group was given WW12 fermentation broth every day through the gavage method (10^8 CFU/mL). To evaluate the protective effects of probiotic strains WW3, WW8, and WW12 against colitis in mice, the CK group was given normal drinking water and feed, while the other groups were given 3% DSS (Dextran Sulfate Sodium) drinking water for a week. During the modeling period, the physical condition of the mice was observed and recorded every day, including the presence of bloody stools and weight loss. The comprehensive score was used to represent the DAI[12,36].

At the end of the animal experiment, the feces of the mice were collected, stored in − 80 ℃ freezer, and blood was collected from the eyeball. The supernatant was centrifuged at 4 ℃, 3000 rpm in for 20 minutes and stored in a − 80 ℃ refrigerator. The length of the mouse colon tissue (from the anus to the end of the cecum) was measured with a ruler, photographed and recorded. Then 1 cm of colon tissue was taken, placed in a sterile centrifuge tube, fixed with 4% paraformaldehyde solution and stored at -80 ℃.

2.9.2 Clinical and Histopathological Evaluation

DAI was scored daily based on body weight change and hematochezia[36]. At the end of the experiment, colon length was measured, and colon tissues were fixed in 4% paraformaldehyde for HE staining. Histopathological scores were evaluated based on mucosal damage, inflammatory cell infiltration, and crypt destruction.

2.9.3 DNA extraction and shotgun metagenomic sequencing

Genomic DNA was extracted from fecal samples using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, United States). DNA quality was assessed by agarose gel electrophoresis and quantified using a Qubit Fluorometer. Libraries were prepared using the NEBNext DNA Library Prep Kit, and sequencing was performed on an Illumina Novaseq 6000 platform. Raw reads were filtered, host DNA was removed, and non-host reads were used for microbial taxonomy. Taxonomic profiling was performed using Kraken2[37], providing species-level resolution of gut microbial composition. Alpha diversity indices (Shannon index, Inverse Simpson index) were calculated to assess microbial richness and evenness. Group differences in microbial diversity and composition were assessed using Wilcoxon rank-sum tests for pairwise comparison for overall phylum structure variations. At the species level, the volcano plot of relative abundance of microbes was performed.

2.9.4 RNA-seq

RNA-seq was performed using intact colon tissue samples from diferent treatment groups. Total RNA was extracted using TRIzol reagent following the manufacturer's procedure. The cDNA library construction and bioinformatics analysis were used to construct cDNA libraries, which were sequenced on an Illumina NovaSeq6000 (California, USA). Gene expression levels were calculated as FPKM values. We selected genes with a fold change > 2 and a P value < 0.05. Then, we used Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to determine enrichment of upregulated and downregulated DEGs in specifc pathways.

2.10 Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was performed using SPSS 26.0 software, and differences between groups were analyzed by one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant. Graphs were generated using GraphPad Prism 8.0.

Results

3.1 Isolation and Identification of *Bacillus* Strains

The purified colonies were round, yellow, and had a rough surface (Fig. 1A). When observed under a microscope, the bacteria were blue-purple, short, rod-shaped, and blunt at both ends (Fig. 1B). Ultrastructural analysis by scanning electron microscopy (SEM) further corroborated these observations, depicting cylindrical vegetative cells with smooth cell walls and rounded ends (Fig. 1C). The cells measured approximately 0.7 μm in width and 2.5 μm in length, lacking any flagellar appendages under the imaging conditions employed (SU3500, 5.00 kV, ×10.0k magnification).

A total of 12 Gram-positive, spore-forming bacterial strains were isolated from wild wolf feces. Based on morphological, biochemical, and molecular identification, 3 strains were confirmed as *Bacillus subtilis* (designated WW3, WW8, WW12). Phylogenetic tree analysis showed that the isolates WW3 and WW8 cluster closely with strain SRCM102749, while WW12 groups with strains BEST3145 (Fig. 1D).

3.2 Probiotic Properties of Isolated Strains

3.2.1 Stress Tolerance

WW12 exhibited the highest stress tolerance among the three strains. Its survival rates in simulated gastric fluid (SGF, 3 h) and intestinal fluid (SIF, 21 h) were 94.3% and 85.7%, respectively (Fig. 2A). WW12 also showed good tolerance to high temperatures (65.2% survival at 70°C) (Fig. 2B).

3.2.2 Growth curve of strain WW12

The growth dynamics of WW3, WW8, and WW12 in LB broth are demonstrated in Fig. 3A. The strain WW12 exhibited a lag phase of approximately 20 h, during which it adapted to the new environment. This was followed by a rapid exponential growth phase from 20 to 46 h, during which vigorous metabolism and cell division occurred. The culture attained its maximum OD600 at 46 h, entering the stationary phase, likely as a consequence of nutrient depletion and the accumulation of metabolic by-products.

3.2.3 Extracellular enzyme production profile

The potential of WW3, WW8, and WW12 to produce protease and amylase was evaluated (Fig. 3B, C). The results show that WW12 has the highest amylase and protease activity among the tested strains, while WW3 has the lowest. These results suggest WW12 is a promising candidate for applications requiring high hydrolytic enzyme production.

3.2.4 Auto-aggregation and Co-aggregation with Pathogenic Bacteria

As shown in Table 1, WW12 exhibited the highest auto-aggregation rate (78.71%), which was about 3 times higher than WW3 (27.61%) and 4 times higher than WW8 (19.42%). Co-aggregation reflects the ability of probiotics to bind and neutralize pathogens. *Escherichia coli*, *Staphylococcus aureus* and *Salmonella Typhimurium* were used as pathogens. The results show that WW12 demonstrated the strongest co-aggregation capacity against *S. aureus* and *S. Typhimurium*, while WW8 was more effective against *E. coli*.

Table 1
Auto-aggregation, co-aggregation, and inhibition zone of three *bacillus subtilis* strains

Test Item	**WW3**	**WW8**	**WW12**
Auto-aggregation (%)	27.61	19.42	78.71
Co-aggregation (*Staphylococcus aureus*) (%)	32.01	19.89	38.09
Co-aggregation (*Escherichia coli*) (%)	5.96	23.88	14.05
Co-aggregation (*Salmonella Typhimurium*) (%)	18.09	24.19	44.74
Inhibition zone against *Escherichia coli* (mm)	12.9 ± 0.4	11.9 ± 0.6	18.3 ± 0.4
Inhibition zone against *Staphylococcus aureus* (mm)	19.2 ± 0.2	20.1 ± 0.2	19.7 ± 0.2
Inhibition zone against *Salmonella Typhimurium* (mm)	10.9 ± 0.6	11.8 ± 0.5	19.3 ± 0.7

3.2.5 Antimicrobial Activity

The three strains showed comparable inhibitory effects against *Staphylococcus aureus*. While WW12 showed strong antimicrobial activity against *E. coli* (inhibition zone diameter: 18.3 ± 0.4 mm) and *Salmonella Typhimurium* (19.3 ± 0.7 mm), which was significantly higher than WW3 and WW8 (Table 1).

The antimicrobial activities of WW12 against *E. coli*, *Staphylococcus aureus*, and *Salmonella Typhimurium* on plate were shown in Fig. 3D-F.

3.2.6 Antibiotic Susceptibility

WW12 was sensitive to all tested antibiotics, with no intermediate or resistant phenotypes (Table 2). Genomic analysis confirmed that WW12 lacked transferable ARGs, and only 10 detected intrinsic ARGs were located on chromosomal DNA (Fig. 4A). The COG (Clusters of Orthologous Groups) annotation classifies functional genes into categories such as carbohydrate metabolism (G), energy production (C), amino acid metabolism (E), signal transduction (T), cell wall/membrane biogenesis (M), replication and repair (L), defense mechanisms (V), and secondary metabolism (Q), among others. The distribution indicates a broad metabolic and adaptive capacity (Fig. 4B). According to the KEGG annotation, the number of metabolic enzymes are still the highest, which is according to the results of COG annotation (Fig. 4C). Based on the CAZy annotation, a total of 142 gene entries were analyzed. Some genes contain multiple CAZy domains, resulting in 156 domain assignments across five major classes: Glycoside Hydrolases (GH), Glycosyltransferases (GT), Carbohydrate-Binding Modules (CBM), Carbohydrate Esterases (CE), and Polysaccharide Lyases (PL). The diverse GH families (61) enable hydrolysis of hemicellulose, starch, inulin, trehalose, and rhamnogalacturonan. This releases fermentable sugars and produces short-chain fatty acids that benefit gut health. Multiple GT families (46) are involved in peptidoglycan biosynthesis, teichoic acid production, and exopolysaccharide (EPS) synthesis. EPS production supports biofilm formation, protecting the strain from peristalsis, bile salts, and pathogen exclusion.

Table 2
Antimicrobial susceptibility of three *bacillus subtilis* strains

Antimicrobial agents	**Zone diameter breakpoint (mm)**			**Zone diameter (mm)**		
	S≥	**I**	**R<**	**WW3**	**WW8**	**WW12**
Gentamicin	15	13 ~ 14	12	30(S)	33(S)	33(S)
Ciprofloxacin	21	16 ~ 20	15	31(S)	38(S)	41(S)
Ampicillin	15	12 ~ 14	11	17(S)	13(I)	15(S)
Cefazolin	21	18 ~ 20	17	40(S)	47(S)	23(S)
Erythromycin	23	14 ~ 22	13	32(S)	36(S)	25(S)
Norfloxacin	17	13 ~ 16	12	25(S)	35(S)	36(S)
Amikacin	17	15 ~ 16	14	30(S)	35(S)	31(S)
Sulfamethoxazole	16	11 ~ 15	10	30(S)	32(S)	40(S)
Chloramphenicol	18	13 ~ 17	12	34(S)	27(S)	21(S)

3.4 Ameliorative Effects on DSS-Induced Colitis in Mice

3.4.1 Clinical and Histopathological Improvements

In the DSS-induced colitis mouse model, the effects of WW3, WW8, and WW12 on body weight, disease activity index (DAI), colon length, histopathological changes, and inflammatory markers were evaluated. Regarding body weight changes (Fig. 5B), the DSS group showed significant weight loss, whereas the control group experienced continuous weight gain. WW12 and WW8 probiotic group alleviated weight loss. In the DAI (Fig. 5D), the DSS group exhibited significantly higher scores, while WW12 and WW8 probiotic group reduced DAI scores. In terms of colon length (Fig. 5C), the DSS group exhibited significantly shortened colons, while WW12, WW8, and WW3 groups alleviated this shortening, with the WW12 group showing the longest colon length, significantly superior to the DSS group ($p < 0.05$). In histopathological scoring (Fig. 5E-I), the DSS group exhibited the most severe pathological damage. WW12 showed markedly better tissue repair than other probiotics groups.

3.4.2 Regulation of Inflammatory Cytokines

Inflammatory marker analysis (Fig. 5K–M) revealed that the DSS group exhibited significantly elevated serum IL-1β, IL-6 and IL-10 levels. In contrast, WW3, WW8, and WW12 groups lowered IL-1β, IL-6 and IL-10 levels, with the WW12 group significantly lowering IL-1β ($p < 0.01$) and IL-10 ($p < 0.05$) levels and demonstrating superior results compared to other probiotic groups.

3.4.3 Gut microbiota analysis

In the metagenomic analysis of fecal samples, microbial diversity and community composition were compared among Control group, DSS group, WW3 group, WW8 group and WW12 group. The Shannon diversity index revealed higher microbial diversity in the WW12 group compared to the DSS group (Fig. 5I). Since all previous results indicated that WW12 was the best-performing probiotic strain, subsequent gut metagenomic analysis was mainly conducted on the three groups: CK, DSS, and WW12. Microbial abundance analysis (Fig. 6A) revealed distinct microbial compositions at the species level in CK and WW12 group compared to the DSS group. Specifically, the CK and WW12 group exhibited a higher relative abundance of probiotics, for example, *Bacteroides fragilis, Bacteroides caccae, Alistipes sp. Marseille-P5997, Pasteurella multocida, Bacteroides ovatus, Bacteroides thetaiotaomicron, Alistipes finegoldiiAlistipes shahii*

Lachnoclostridium sp. YL32, Acutalibacter muris, Alcanivorax sp. N3-2A, Muribaculum intestinale, Bacteroides caecimuris and *Lactobacillus rhamnosus*. And the CK and WW12 group exhibited a lower relative abundance of pathogen, for example, *Clostridioides difficile* and *Saccharomyces cerevisiae* (Fig. 6A). Linear Discriminant Analysis Effect Size (LEfSe) was employed to elucidate the effect sizes associated with various treatments and to identify characteristic taxa. At the species level, the top five species with significant differences between the CK and DSS groups were *Saccharomyces cerevisiae*, *Clostridioides difficile*, *Muribaculum intestinale*, Bacteroides caecimuris, and Alcanivorax sp. N3-2A.

Among them, *Saccharomyces cerevisiae* and *Clostridioides difficile* were discriminative for the DSS group, whereas *Muribaculum intestinale*, *Bacteroides caecimuris*, and *Alcanivorax sp.* N3-2A were discriminative for the CK group. The top five species with significant differences between the WW12 and DSS groups were *Saccharomyces cerevisiae*, *Clostridioides difficile*, *Bacteroides caecimuris*, *Muribaculum intestinale*, and *Lachnoclostridium sp.* YL32. Among them, *Saccharomyces cerevisiae* and Clostridioides difficile were discriminative for the DSS group, whereas *Muribaculum intestinale*, *Bacteroides caecimuris*, and *Alcanivorax sp.* N3-2A were discriminative for the WW12 group. The results indicated that, compared with the CK and WW12 groups, *Saccharomyces cerevisiae* and *Clostridioides difficile* were discriminative for the DSS group, while *Bacteroides caecimuris* and *Muribaculum intestinale* were discriminative for both the CK and WW12 groups, suggesting that WW12 may alleviate the DSS-induced colitis model by increasing the abundance of beneficial microorganisms. However, The LEfSe analysis between the WW12 and CK groups showed that *Lachnoclostridium sp.* YL32, *Acutalibacter muris*, and *Clostridiales bacterium* CCNA10 were discriminative for the WW12 group, while *Muribaculum intestinale* and Bacteroides fragilis were discriminative for the CK group. These results indicated that WW12 could partially increase the abundance of probiotics to alleviate the DSS-induced colitis model. However, compared with the CK group, the levels of *Muribaculum intestinale* and *Bacteroides fragilis* did not return to their natural intestinal abundances. This suggests that *Muribaculum intestinale* and *Bacteroides fragilis* may play important roles in maintaining intestinal health, and that WW12 may alleviate DSS-induced colitis by partially increasing the abundance of these two bacterial species. (Fig. 6B-G). Nevertheless, Bacillus subtilis was not detected in the feces of dogs fed WW12, which may indicate that WW12 has colonized the intestinal mucosal surface and possesses good colonization ability.

3.4.4 Modulation of Signaling Pathways

To elucidate the preventive mechanism of the probiotic strain WW12 against DSS-induced colitis, colon tissues were used for RNA-Seq transcriptome analysis, and differentially expressed genes (DEGs) were screened using the DESeq2 method with fold change ≥ 2 criteria and FDR < 0.05. Principal coordinate analysis (PCoA) was used to elucidate the metabolic differences among the groups. As shown in Fig. 7A, the WW12 group and the DSS group were not clearly separated, and the WW12 group was farther away from the CK group, suggesting that the mechanism by which WW12 alleviates the DSS-induced colitis model may be unique. Differential expression genes were identified and quantified using volcano plots comparing the WW12 group and the DSS group (Fig. 7B). Compared with the DSS group, the WW12 group showed 157 upregulated genes and 420 downregulated genes, indicating that WW12 may alleviate the colitis model through negative regulation. Pathway analysis of the differentially expressed genes between the WW12 group and the DSS group was performed using KEGG topology, and the results are presented in a bubble diagram (Fig. 7C). The two most significantly altered pathways were the Cytokine-cytokine receptor interaction pathway and the PI3K-Akt signaling pathway, both with p-values less than 0.01.

To further elucidate the relationship between gut microbiota and host intestinal tissue gene expression changes, Spearman correlation analysis was performed between the characteristic taxa shown in Fig. 7D

and the differentially expressed genes. As shown in Fig. 7D, *Bacillus gobiensis* was negatively correlated with most of the differentially expressed genes, while bacteria of the genus Mycolicibacterium were positively correlated with most of the differentially expressed genes, which are known to have negative effects on immunomodulation and anti-inflammatory responses. The correlation analysis indicates that an increase in the abundance of Bacillus in the gut, together with a decrease in Mycolicibacterium, positively contributes to immunomodulation and anti-inflammatory responses. This suggests that the WW12-induced increase in Bacillus abundance and decrease in Mycolicibacterium abundance may be associated with the alleviation of the intestinal inflammation model.

Discussion

The domestication of dogs from gray wolves (Canis lupus) to modern domestic dogs (Canis lupus familiaris) has imposed profound changes in diet, lifestyle, and living environment, leading to significant alterations in the gut microbiome composition and function of domestic canines [38]. These changes have been associated with an increased susceptibility to gastrointestinal disorders, including inflammatory bowel disease (IBD) and chronic enteritis. Given that wild wolves possess a unique gut microbiota adapted to their natural diet and environment [39], and their feces represent a pristine source of host-adapted probiotics [40], we reasoned that isolating probiotic candidates from wild wolf feces could offer a scientifically sound, innovative, and potentially safer strategy for developing next-generation probiotics for pet dogs. Based on this rationale, we successfully isolated and identified three *Bacillus subtilis* strains from wild wolf feces, among which WW12 exhibited excellent probiotic properties. This finding supports the feasibility of using wild wolf-derived probiotics to prevent enteritis in domestic dogs.

Stress tolerance is a critical property of probiotics, as they need to survive the harsh conditions of the GI tract [41]. WW12 showed high survival rates in simulated gastric and intestinal fluids, as well as tolerance to high temperatures and osmotic stress, which is consistent with the spore-forming ability of *Bacillus subtilis* [13]. Surface hydrophobicity and auto-aggregation are important for probiotic adhesion to intestinal epithelial cells and colonization [42]. WW12's strong hydrophobicity and auto-aggregation capacity suggest its potential for effective colonization in the host's GI tract.

Antimicrobial activity is another important probiotic property, as it helps inhibit the growth of pathogenic bacteria [43]. WW12's broad-spectrum antimicrobial activity against *E. coli* and *S. enteritidis* may be attributed to its secretion of bacteriocins, antibiotics, or other bioactive metabolites [44].

The safety of probiotics is crucial for their application, and WW12's sensitivity to all tested antibiotics and lack of transferable ARGs confirm its safety [45]. Genomic analysis confirmed that WW12 lacked transferable ARGs, and only 10 detected intrinsic ARGs were located on chromosomal DNA. Because ARGs located on chromosomes are typically non-mobile and pose a negligible risk of horizontal transfer to other intestinal bacteria, the presence of intrinsic chromosomal ARGs does not compromise probiotic

safety, whereas plasmid-borne or mobile genetic element-associated ARGs would raise serious clinical and public health concerns by potentially spreading resistance to pathogens within the gut microbiome. Notably, other *Bacillus subtilis* strains have been reported to carry similar numbers of intrinsic ARGs, such as the multidrug-resistant *B. subtilis M3* isolated from activated sludge, which also harbors ten types of ARGs on its genome. However, unlike WW12, such environmental strains often contain numerous mobile genetic elements and heavy metal resistance genes that significantly increase the risk of ARG dissemination, underscoring a key safety distinction[46]. Furthermore, the absence of transferable ARGs, combined with the low number of intrinsic chromosomal ARGs, aligns with internationally recognized probiotic safety standards, including the FAO/WHO guidelines that recommend sensitivity to clinically relevant antibiotics and the absence of transferable resistance determinants. Thus, compared to many other reported probiotic strains that may harbor transferable ARGs or elevated numbers of intrinsic ARGs, WW12 exhibits a superior biosafety profile, rendering it an ideal candidate for preventing enteritis in domestic dogs with minimal risk of contributing to the spread of antimicrobial resistance.

This study has some limitations. First, the animal experiment was conducted in mice, and further studies in dogs are needed to confirm the therapeutic effects of WW12 on canine IBD. Second, the mechanism of action of WW12 needs to be further explored at the molecular level. Third, the long-term safety and stability of WW12 in practical applications require further evaluation.

Conclusion

In summary, this study successfully isolated and characterized a novel *Bacillus subtilis* strain, WW12, from the feces of wild gray wolves. WW12 demonstrated exceptional probiotic attributes, including robust gastrointestinal and thermal stress tolerance, strong adhesion potential, broad-spectrum antimicrobial activity, and a favorable safety profile with no transferable antibiotic resistance genes. Its genomic repertoire supports versatile metabolic and adaptive capabilities, reinforcing its suitability for gastrointestinal applications. In a DSS-induced murine colitis model, WW12 effectively alleviated clinical and histopathological signs of intestinal inflammation, modulated gut microbial composition by enriching beneficial taxa and suppressing pathobionts, and regulated key inflammatory signaling pathways. Notably, the beneficial effects were associated with increased abundance of *Bacillus* and decreased *Mycolicibacterium*, positively contributing to immunomodulation. Although not detected in canine feces after oral administration—suggesting possible intestinal mucosal colonization—WW12's efficacy in the murine model strongly supports its probiotic potential. Collectively, our findings provide a scientific foundation for the development of WW12 as a next-generation, host-adapted probiotic for the prevention and adjunctive therapy of canine IBD. Future studies in dogs are warranted to confirm its translational value and long-term safety.

Declarations

Author Contribution

Songda Li and Shiyi Yang contributed equally to this work. Songda Li: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – Original Draft, Visualization. Shiyi Yang: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – Original Draft, Visualization. Hengchuan Zhang: Investigation, Data Curation, Formal analysis, Visualization. Chuyuan Xu: Investigation, Data Curation, Formal analysis. An Hu: Resources, Investigation (sample collection). Jie Liu: Data Curation, Formal analysis (genomic and bioinformatics analysis). Guilian Yang: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – Review & Editing. Ming Li: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – Review & Editing. All authors have read and approved the final version of the manuscript, agreed to its submission to Probiotics and Antimicrobial Proteins, and accept accountability for all aspects of the work.

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Figures

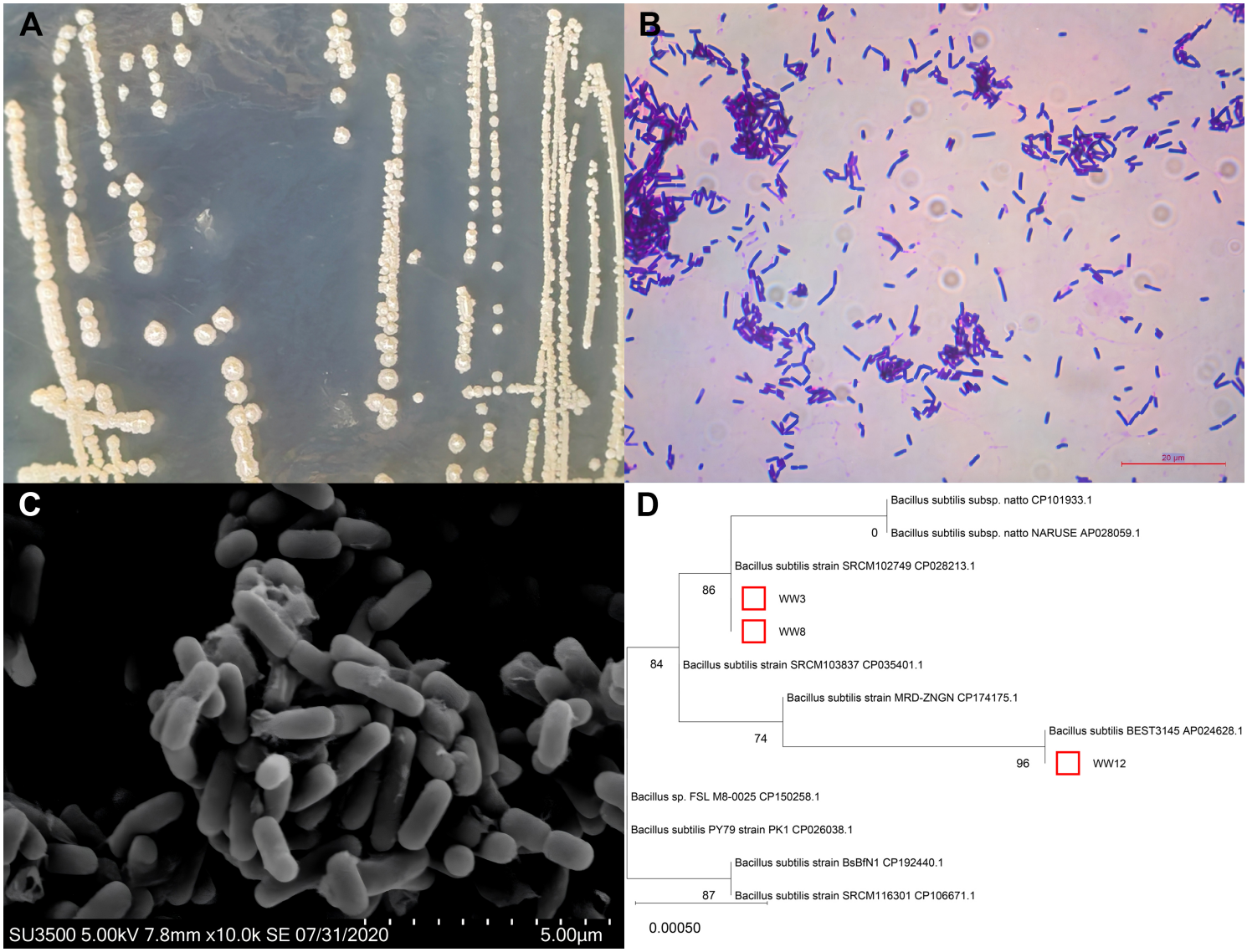


Figure 1

Isolation and identification of *Bacillus subtilis*Strain WW12. (A) Colonies of WW12 on LB agar. (B) Cellular morphology observed by Gram staining. (C) Scanning electron microscopy (SEM) morphology of WW12 (×10,000). (D) Neighbor-joining phylogenetic tree based on 16 S rRNA gene sequences. Bootstrap values > 50% are shown. Scale bar: 0.01 nucleotide substitutions per site

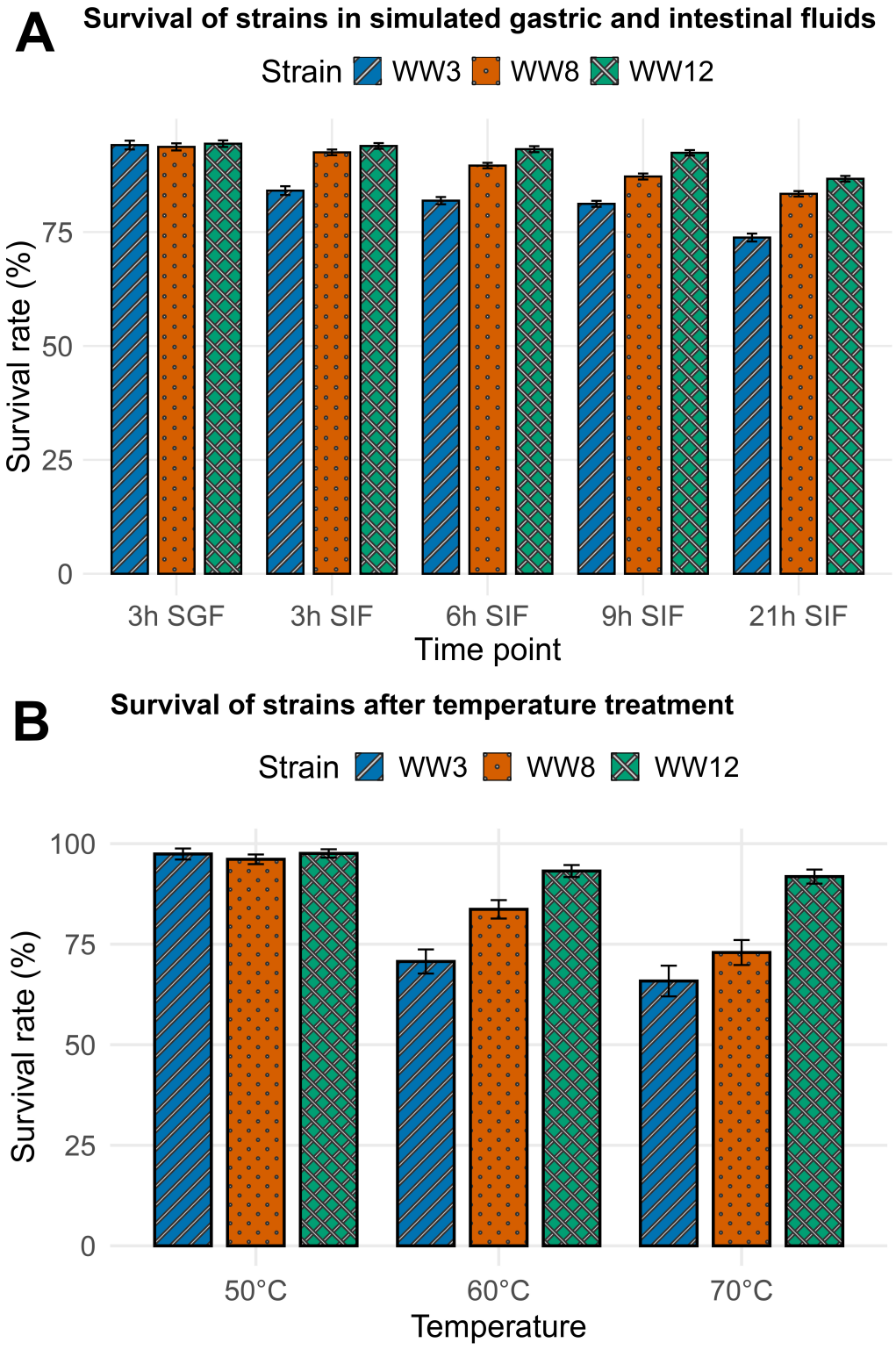


Figure 2

Stress tolerance assay of three *Bacillus subtilis*strains. (A) Survival rate of three strains of *Bacillus subtilis* in simulated gastric fuid and simulated intestinal fuid. (B) Survival rate of three strains of *Bacillus subtilis* in high temperature

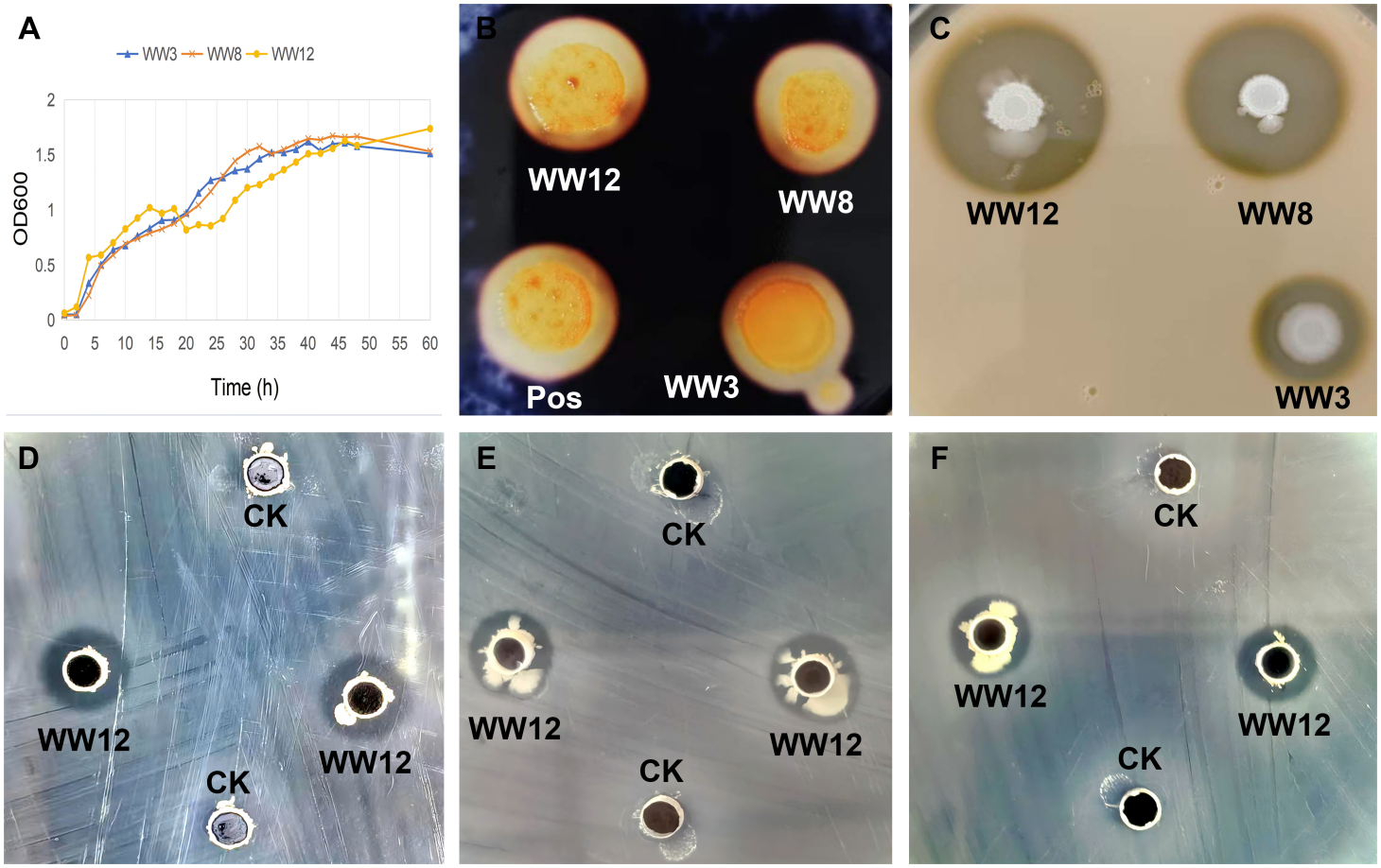


Figure 3

Probiotic properties of three Antibacterial activity of *Bacillus subtilis* strains. (A) Growth Curve of *Bacillus subtilis* strains. (B) Amylase. (C) Protease. (D) Inhibitory activity against *Escherichia coli* (E) Inhibitory activity against *Staphylococcus aureus*. (F) Inhibitory activity against Inhibition zone against *Salmonella Typhimurium*

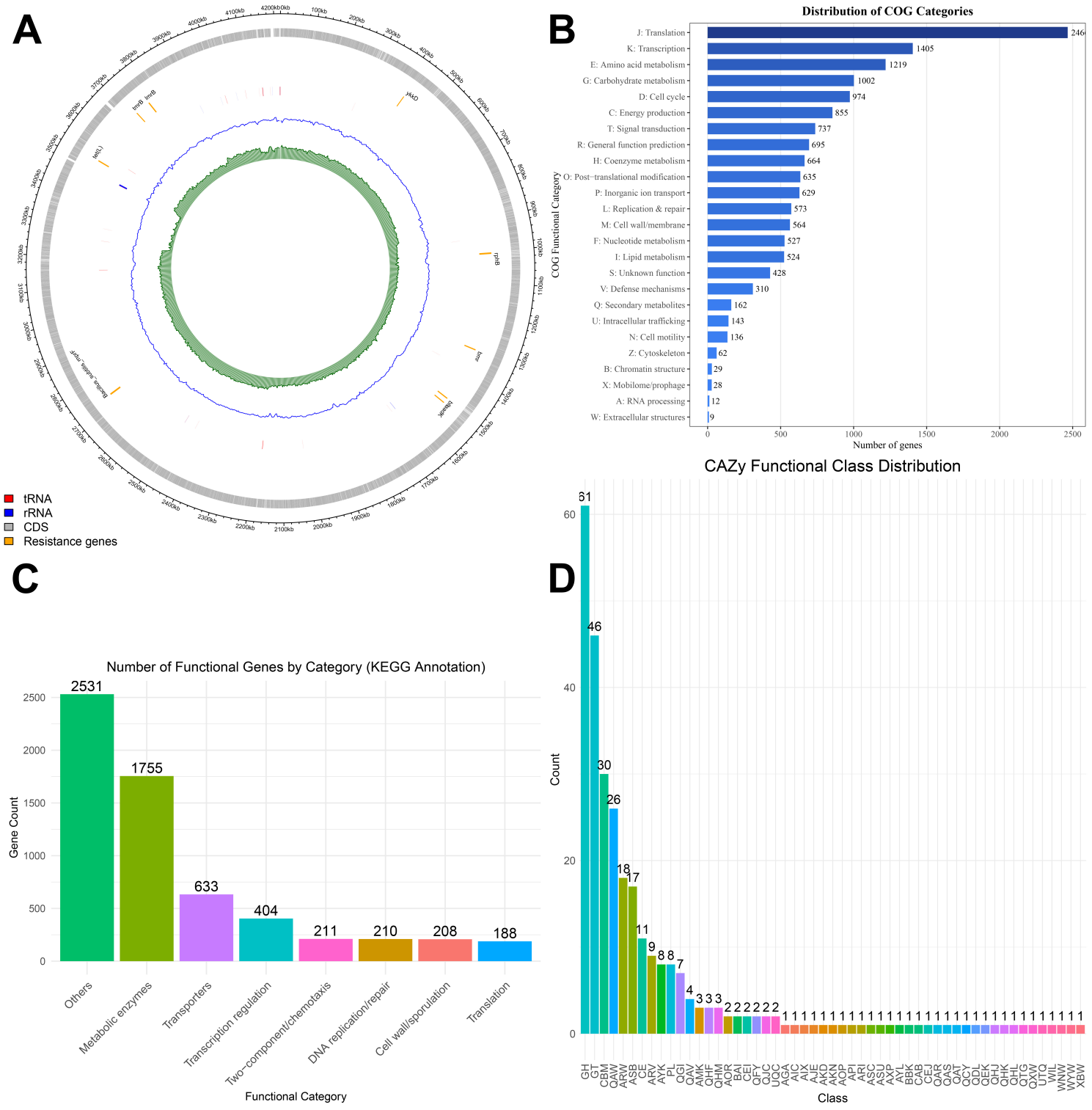


Figure 4

Genomic analysis of *Bacillus subtilis*WW12. (A) Nuclear genome circle diagram of *Bacillus subtilis* WW12. (B) Functional classification using the Clusters of Orthologous Groups (COG) database, (C) KEGG database, and (D) CAZy database

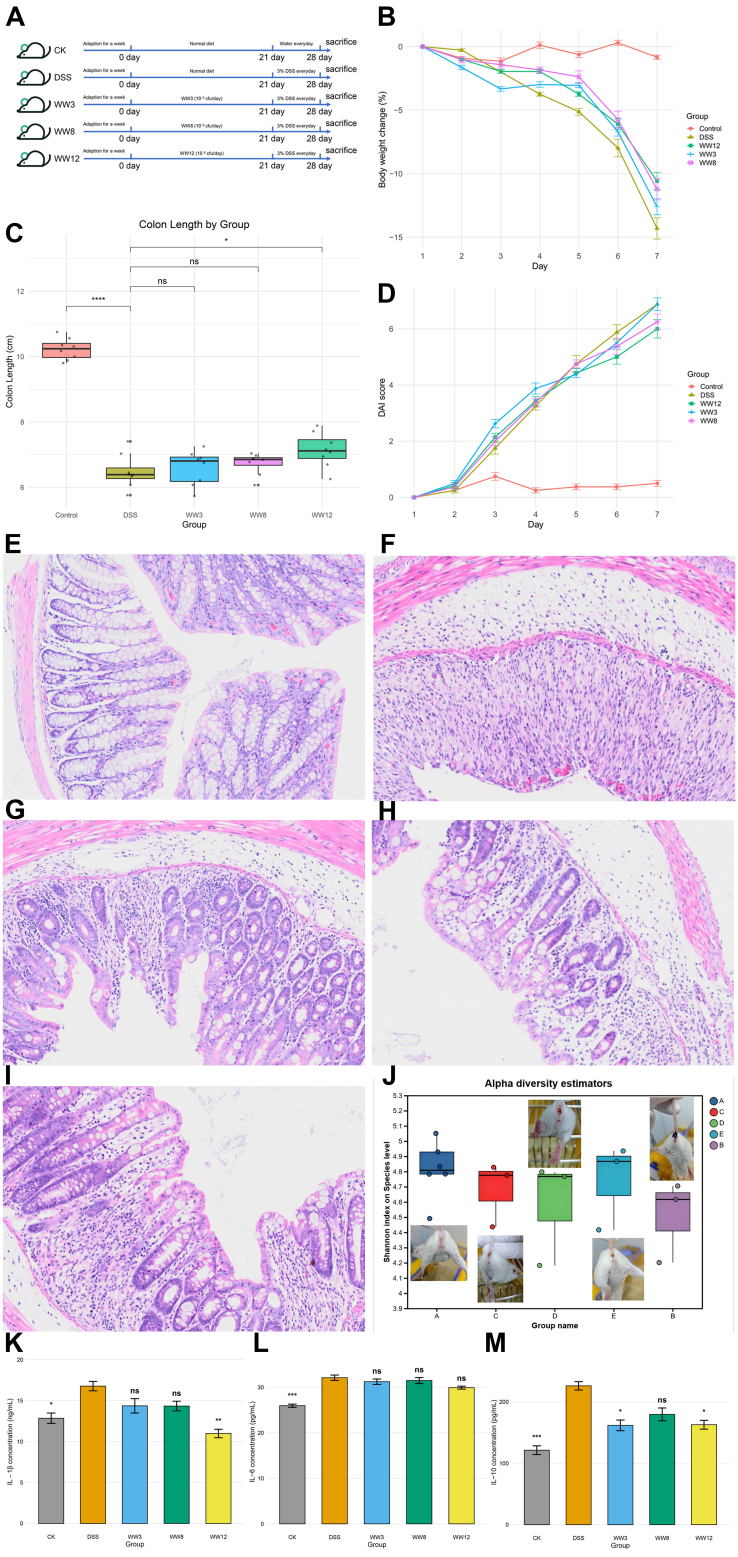


Figure 5

Anti-inflammatory properties of *bacillus subtilis* fermentation broth in the DSS model of colitis in BABAL/c mice. (A) Design of the study. (B) Loss of body weight was monitored all along the study. (C) Colon length among different groups. (D) DAI. (E) Representative colonic morphology of CK, (F) DSS, (G) WW3, (H) WW8, and (I) WW12. (J) Alpha diversity. (K) IL-1β concentration in colon. (L) IL-6 concentration in colon. (M) IL-10 concentration in colon.

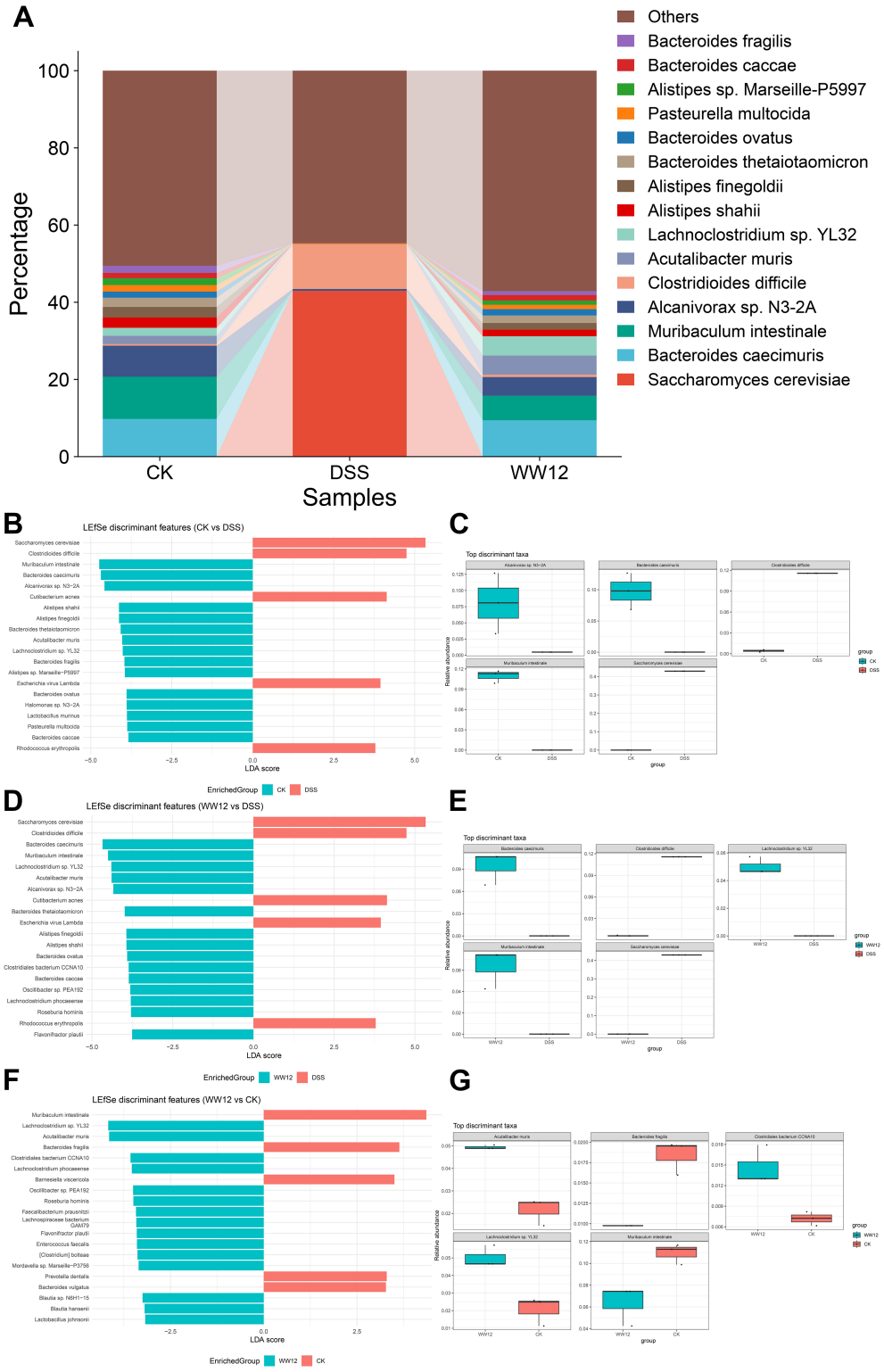


Figure 6

Characteristic taxa analysis for individual treatment groups. (A) Species-level composition analysis (B) LefSe analysis to assess the discriminative taxa between CK and DSS groups. (C) The difference bacteria analysis between CK and DSS groups. (D) LefSe analysis to assess the discriminative taxa between WW12 and DSS groups. (E) The difference bacteria analysis between WW12 and DSS groups.

(F) LefSe analysis to assess the discriminative taxa between WW12 and CK groups. (G) The difference bacteria analysis between WW12 and CK groups.

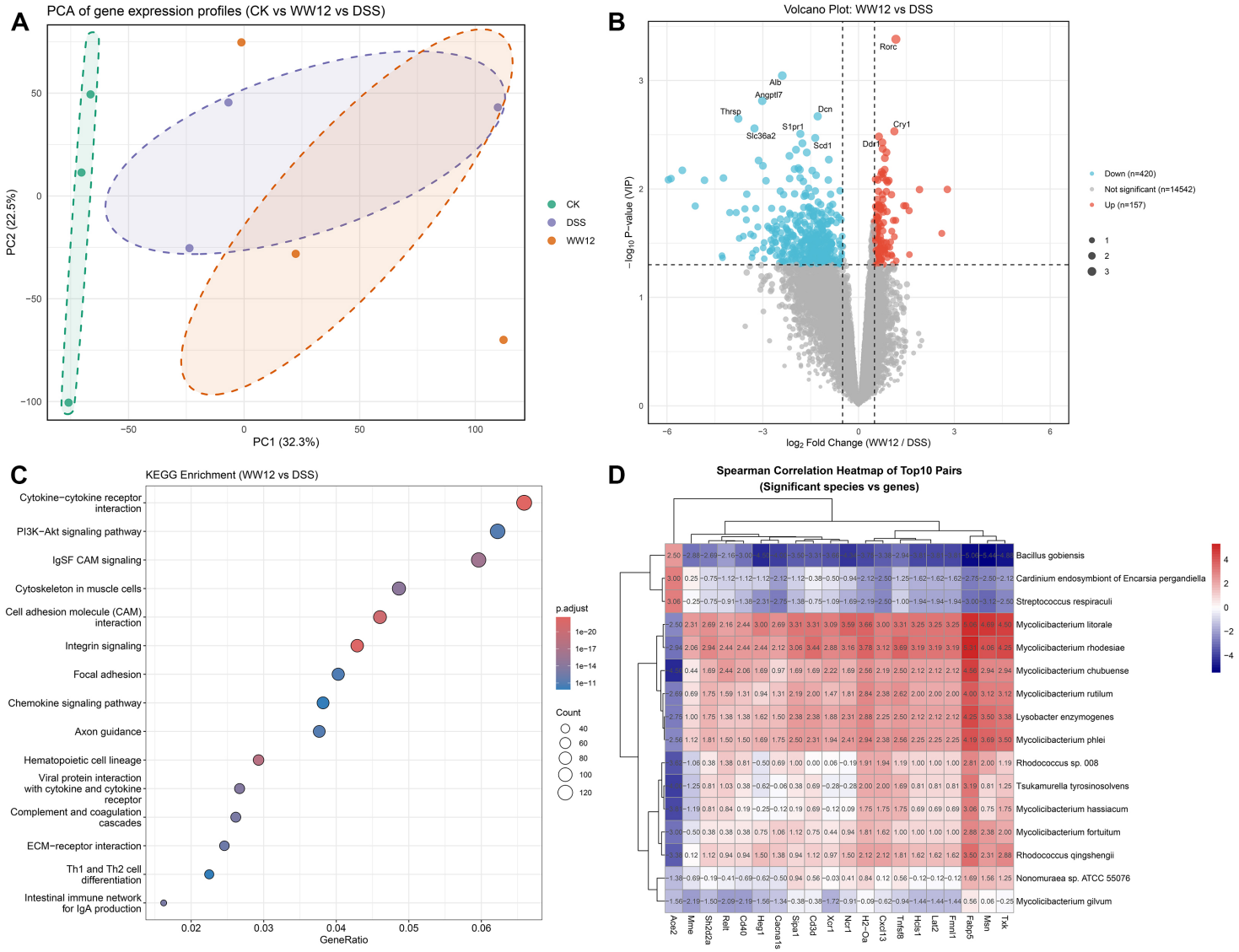


Figure 7

Transcriptome analysis of different treatment groups. (A) PCA analysis. (B) Volcano plots illustrating pairwise comparisons of relative genes levels between WW12 and DSS. group. (C) Topology analysis using KEGG pathways terms. (D) Integrative analysis of host gene expression and gut microbiota.