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Bacillus velezensis as a probiotic to enhance gut health and disease resistance in American bullfrogs (*Aquarana catesbeiana*)

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Abstract: Intensive aquaculture of American bullfrogs (*Aquarana catesbeiana*) is increasingly challenged by poor water quality, disease outbreaks, and the overuse of antibiotics, underscoring the need for effective and sustainable health management strategies. This study evaluated the effects of dietary supplementation with *Bacillus velezensis* YSJ-24 on growth performance, antioxidant capacity, intestinal health, gut microbiota, and disease resistance in American bullfrogs. During a 28-day feeding trial, bullfrogs were fed a basal control diet or diets supplemented with *B. velezensis* YSJ-24 at 1×10^7, 1×10^8 or 1×10^9 CFU/g, designated BV1, BV2, and BV3, respectively. Among the supplemented groups, BV2 showed the most pronounced beneficial effects, with an 8.6% increase in weight gain rate (WGR), an 8.0% increase in specific growth rate (SGR), and a 20.0% decrease in feed conversion ratio (FCR) compared with the NC (negative control) group. Dietary supplementation with YSJ-24 significantly enhanced intestinal antioxidant capacity, as indicated by increased superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase activities (CAT), together with reduced malondialdehyde (MDA) levels ($P < 0.05$). Intestinal morphology was also improved, as evidenced by increased villus height and goblet cell density. Gut microbiota analysis revealed no significant changes in alpha diversity, although moderate compositional shifts were observed, including higher relative abundances of *Prevotella* and *Bacteroides* in the BV2 group. Following challenge with *Streptococcus agalactiae*, the survival rate of bullfrogs in the BV2 group reached 70.0%, whereas 0% survival was observed in the NC group. Overall, these findings indicate that dietary *B. velezensis* YSJ-24, particularly at 1×10^8 CFU/g, improves growth performance, intestinal health, antioxidant status, and resistance to bacterial infection in American bullfrogs. This strain may serve as a promising functional feed additive for health management in intensive bullfrog aquaculture.

Keyword: Bacillus velezensis; American bullfrog; probiotic; dietary supplementation; intestinal health; disease resistance

1. Introduction

The American bullfrog (*Aquarana catesbeiana*) has become an economically important aquaculture species globally, particularly in China, where bullfrog meat is highly valued for its nutritional profile and taste [1, 2]. As production has expanded, bullfrog farming has shifted from extensive systems to intensive, high-density models. This intensification has increased production capacity but also introduced management challenges, particularly deteriorating water quality characterized by elevated ammonia and nitrite levels and fluctuations in dissolved oxygen and pH [3, 4]. These environmental stressors impose chronic physiological stress and weaken disease resistance, increasing the risk of disease outbreaks in intensive production systems [5].

Disease outbreaks have become a major constraint limiting bullfrog aquaculture sustainability [6, 7]. Under high-density conditions, stress-induced immunosuppression facilitates pathogen proliferation, leading to reduced growth performance and elevated mortality [8]. The intestine, as the primary site of nutrient absorption and microbial colonization, plays a pivotal role in disease resistance; disruption of intestinal homeostasis compromises epithelial integrity and immune responses [5, 9]. Traditionally, chemotherapeutic agents and antibiotics have been used to control disease, but their long-term use has significant drawbacks. Frequent antibiotic application leads to the emergence of antibiotic-resistant bacteria and increases drug residue risks, raising food safety and environmental concerns [10, 11]. Moreover, antibiotics primarily target pathogens without addressing underlying causes such as impaired intestinal function [5]. These limitations have stimulated interest in non-antibiotic health management strategies aimed at preventing disease and improving host health [12].

Probiotics have emerged as a promising approach for improving health and disease resistance in aquaculture species [13, 14]. As functional feed additives, probiotics

enhance growth performance, strengthen immune responses, and stabilize intestinal microbial communities [15]. Compared with antibiotics, probiotics offer a more sustainable strategy by promoting host-microbe balance rather than directly eliminating pathogens [16]. Host-associated probiotic strains are particularly advantageous due to their enhanced survival, colonization capacity, and functional compatibility with the target species [13].

Among the various probiotic candidates, *Bacillus velezensis* has attracted increasing attention in aquaculture research [17, 18]. This species is characterized by strong stress tolerance, high spore-forming capacity, and the ability to produce a wide range of bioactive compounds. Previous studies in fish and other aquatic animals have demonstrated that *B. velezensis* can modulate intestinal microbial composition, enhance gut barrier integrity, improve nutrient utilization, and stimulate innate immune responses [12, 17]. In addition, its antagonistic activity against aquaculture-associated pathogens suggests considerable potential for reducing disease incidence without inducing antimicrobial resistance [18, 19].

Despite growing interest in probiotics for aquaculture, studies on probiotic application in bullfrogs remain limited [16]. Most existing studies have focused on general growth performance using commonly applied strains such as various lactic acid bacteria, with limited attention to spore-forming *Bacillus* species or host-adapted strains. The combined effects of probiotics on intestinal morphology, microbial community structure, and disease resistance under high-density farming conditions have not been thoroughly investigated in bullfrogs. In particular, the effects of *B. velezensis* on growth performance, intestinal health, and disease resistance in bullfrogs remain unclear [17, 20, 21]. Few studies have simultaneously examined multiple health-related endpoints, including gut structural integrity, microbial homeostasis, and resistance to pathogenic challenges, within a single experimental framework. Therefore, this study was conducted to investigate the effects of dietary supplementation with *B. velezensis* YSJ-24 on growth performance, intestinal morphology, microbial

composition, and disease resistance in American bullfrogs under intensive culture conditions. The present study provides an integrated evaluation by linking growth performance, gut health, and microbial balance, offering practical insights for improving health management and reducing disease risk in intensive bullfrog aquaculture systems.

2. Materials and methods

2.1 Animal ethics statement

All experimental procedures involving live animals were approved by the Animal Care and Use Committee of the Zhongkai University of Agriculture and Engineering (approval no. ZK202522202).

2.2 Bacillus velezensis preparation

Bacillus velezensis YSJ-24 was isolated from the intestinal tract of healthy bullfrogs and identified by 16S rRNA gene sequencing. The strain has been deposited in the Guangdong Microbial Culture Collection Center (GDMCC) under accession number GDMCC1.6706 and is preserved at the Zhongkai Innovative Institute of Animal Health Breeding (Guangzhou, China). For probiotic preparation, a single colony was inoculated into BHI broth, incubated at 37 °C with shaking at 180 rpm for 24 h, and harvested by centrifugation (4,000 × g, 10 min, 4 °C). Bacterial cells were washed twice with sterile PBS and resuspended in distilled water to a concentration of 1×10^9 CFU/mL.

2.3 Experimental bullfrogs and diet design

Healthy bullfrogs (initial weight 13.9 ± 1.7 g) were obtained from a commercial farm in Huizhou, China, and acclimated for two weeks. After fasting for 24 h, 420

bullfrogs were randomly assigned to four groups. The NC group was fed the basal diet, while the BV1, BV2, and BV3 groups were fed the basal diet supplemented with *B. velezensis* YSJ-24 at 1×10^7, 1×10^8, and 1×10^9 CFU/g diet, respectively. The basal diet was supplied by Guangzhou Fishtech Co., Ltd. Guangzhou, China, and its composition is shown in **Table 1**. A vitamin premix was added at 20 g/kg to supply both fat- and water-soluble vitamins, whereas a mineral premix was added at 5 g/kg to provide Se, Cu, Fe, Zn, Mn, I, and Co as food-grade salts. Choline chloride (50%), monocalcium phosphate, and microcrystalline cellulose were added according to the formulation shown in **Table 1**. Experimental diets were prepared daily by spraying *B. velezensis* YSJ-24 suspension onto the basal feed and were stored at 4 °C for no more than 24 h prior to feeding [22]. Each treatment had three cage replicates (floating cages, 0.5 m × 0.5 m × 0.5 m), with 35 bullfrogs per cage. Growth performance and survival were calculated per cage (n = 3 cages per treatment). Bullfrogs were fed twice daily at 08:00 and 17:00 to apparent satiety. Mortality and feed consumption were recorded daily. Water quality was maintained by daily water exchange. During the experiment, water temperature was maintained at 27.0–30.0 °C, pH at 7.2–7.8, dissolved oxygen at 3.0–5.0 mg/L, ammonia nitrogen at 0.05–0.15 mg/L, and nitrite at 0–0.02 mg/L. The experimental design of this study is illustrated in **Fig. 1**. Hereafter, the control and supplemented diets were designated as NC, BV1, BV2, and BV3, corresponding to 0, 1×10^7, 1×10^8, and 1×10^9 CFU/g of *B. velezensis* YSJ-24, respectively.

Table 1

Formulation and nutrient composition of the basal diet for bullfrogs (g/kg).

Ingredients (g/kg)		Nutrient levels (%)	
Fish meal	80	Crude protein	38.21
Soybean meal	300	Moisture	7.2
Chicken meal	60	Crude lipid	8.88
Pork meal	140	Ash	10.33
Defatted cottonseed protein	20		
Wheat flour	250		
Fish oil	15		
Soybean oil	20		
Dried blood	40		
Vitamin premixa	20		
Choline chloride (50 %)	3		
Monocalcium phosphate	15		
Microcrystalline cellulose	32		
Methionine premix	5		

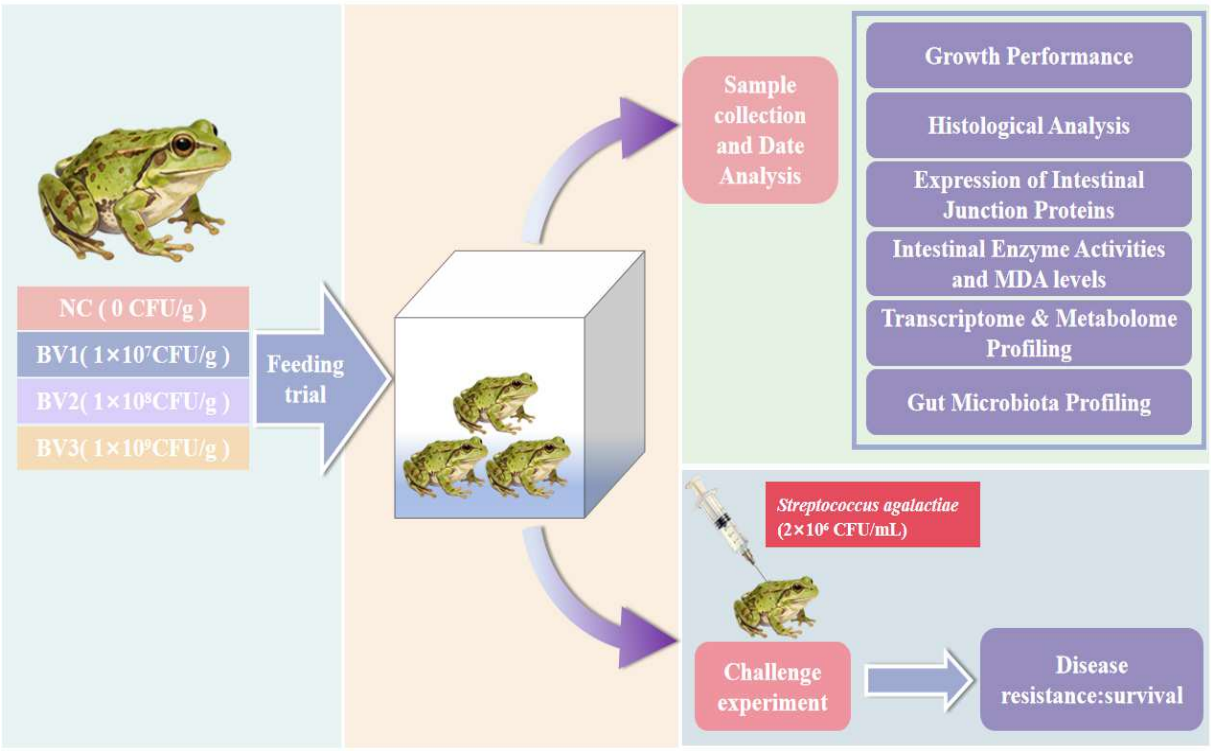


Fig. 1. Experimental design.

2.4 Sample collection and preparation

At the end of the feeding experiment, bullfrogs were fasted for 24 h before being individually weighed and deeply anesthetized with eugenol. They were then euthanized by spinal cord transection prior to tissue collection for subsequent analyses. Intestinal tissues were dissected on ice. For each treatment, three biological replicates were collected, one from each cage, with each replicate consisting of a pooled sample from three bullfrogs (nine bullfrogs per treatment in total for molecular and histological analyses). Samples were frozen in liquid nitrogen and stored at -80 °C. For histomorphology, a 1-cm anterior intestinal segment was fixed in 4% paraformaldehyde; the remaining tissues were stored at -80 °C for microbiota, transcriptomic, metabolomic, antioxidant, and gene expression analyses.

2.5 Growth performance, feed utilization, and survival rate

At the end of the feeding trial, all bullfrogs in each aquarium were counted and weighed for the calculation of weight gain rate (WGR), specific growth rate (SGR) and survival [23].

Growth indices were calculated as follows:

Weight gain rate (WGR, %) = [(final body weight − initial body weight) / initial body weight] × 100.

Specific growth rate (SGR, % day^{-1}) = [ln (final body weight) − ln (initial body weight)] × 100 / experimental days.

Feed conversion ratio (FCR) = feed consumed (g, dry weight) / weight gain (g).

Survival rate (SR, %) = (final number of bullfrogs / initial number of bullfrogs) × 100 .

2.6 Determination of intestinal enzyme activities and MDA levels

The intestinal antioxidant capacity was assessed by determining the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), along with the content of malondialdehyde (MDA). All parameters were measured using commercial assay kits purchased from Younpin Biotechnology (Shanghai, China) following the manufacturer's instructions. Absorbance values were recorded at 420 nm for SOD, 510 nm for CAT, 412 nm for GSH-Px, and 532 and 600 nm for MDA.

2.7 Intestinal tight junction-related gene expression

At the end of the feeding trial, intestinal samples were collected for gene expression analysis. Total RNA was extracted from the intestine using a HiPure Universal RNA Mini Kit (R4130-01, Magen, China) according to the manufacturer's instructions. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was verified by agarose gel electrophoresis. First-strand cDNA was synthesized using TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (AU341, TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The mRNA expression levels of tight junction-related genes, including *zo-1*, *zo-2*, *zo-3*, *occludin*, and *claudin-7*, were determined by quantitative real-time PCR (qPCR). The primer sequences used in this study are listed in **Table 2**. The qPCR reaction system and amplification conditions were performed according to previously reported procedures [24]. *β-actin* was used as the internal reference gene due to its stable expression under the experimental conditions. Relative gene expression levels were normalized to *β-actin* and calculated using the 2^-ΔΔCt method.

Table 2

Primer sequences used for quantitative real-time PCR (qRT-PCR) analysis in this study.

Gene	Sequence (5'-3')	GenBank accession no
β-actin	F: TGACTGAGAGGGGTTACAGC R: CTGCCCATCAGGCAACTCAT	[25]
zonula occludens-1 *(zo-1)*	F: CACCTGTGAAGAATACAAATGGCG R: CACACCAGCAACAAAAATACCCAC	KV926431.1
zonula occludens-2 *(zo-2)*	F: TGAGGATGGACATTTTGACCGTAG R: CATAGCCTCGGTCTGGACTGGAT	KV933782.1
zonula occludens-3 *(zo-3)*	F: AACCTCAGCAATCTTCAGGACCA R: ACCTTCTCGGAGCACCACTTTCT	KV923924.1
occludin	F: GCTCTCCACCTGGCATCATCA R: GCAAAACCCATTCCCATCTGAG	KV928934.1
claudin-7	F: GCTCTCCATTGTTCTCGGGGT R: CCCAGCAGAAAGATGAAGCCTC	KZ001064.1

2.8 Histological examination

The samples were sent to Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) for fixation and processing. Processed using conventional histological procedures, embedded in paraffin, sectioned at 3–5 μm, and stained with Periodic Acid-Schiff (PAS). The stained sections were examined under a light microscope (Nikon ECLIPSE Ci, Japan).

For each biological replicate, three sections were randomly selected for analysis. Villus height was measured in three well-oriented villi per section, yielding nine measurements per replicate. Goblet cells were counted in the entire microscopic field of each section. All measurements were performed using ImageJ software (National Institutes of Health, USA), and mean values were calculated for each biological replicate prior to statistical analysis.

2.9 Transcriptomic and metabolomic analyses

Intestinal tissues from the NC and BV (BV2) group were collected for transcriptomic analysis, with three biological replicates per group. Each biological replicate consisted of pooled intestinal samples from three individual bullfrogs to reduce individual variation. This sample size and pooling strategy are commonly considered sufficient for differential gene expression analysis in RNA-seq studies. Total RNA was extracted, and its concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). mRNA was enriched using oligo (dT)-conjugated magnetic beads and subsequently fragmented into short fragments. First-strand cDNA was synthesized by reverse transcription, followed by second-strand cDNA synthesis. After end repair, adapter ligation, and PCR amplification, the cDNA libraries were constructed and sequenced on an Illumina platform. The sequencing data were processed and analyzed using the Majorbio Cloud Platform (www.majorbio.com). The raw RNA-seq reads generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA1456330.

For metabolomic analysis, intestinal samples from the NC and BV (BV2) group were collected with three biological replicates per group. Each biological replicate consisted of pooled intestinal samples from three individual bullfrogs to minimize individual variation. The BV2 group was selected based on preliminary evaluations of growth performance and intestinal health. Samples were rapidly collected and stored at −80°C prior to analysis. Metabolites were extracted by adding 400 μL of extraction solution (methanol:water = 4:1, v/v) containing 0.02 mg/mL L-2-chlorophenylalanine as an internal standard to 50 mg of sample, followed by grinding under low-temperature conditions. The mixture was ultrasonically extracted at 5°C (40 kHz) for 30 min, incubated at −20°C, and then centrifuged for 30 min. The supernatant was collected for further analysis. Metabolomic profiling was conducted using an ultra-high-performance liquid chromatography system coupled with a Triple TOF mass

spectrometer (UHPLC-Triple TOF, AB SCIEX, USA). After chromatographic separation, metabolites were ionized and detected based on their mass-to-charge ratio (m/z). The obtained mass spectrometry data were used for qualitative identification and quantitative analysis of metabolites. DEGs were screened with $|\log_2 \text{fold change}| \geq 1$ and FDR < 0.05. DAMs were screened with VIP > 1.0 and P < 0.05.

2.10 Gut microbiota analysis

Based on the overall responses in growth performance, antioxidant capacity, intestinal morphology, and tight junction-related gene expression, the BV (BV2) group was selected for gut microbiota analysis together with the NC group. Genomic DNA was extracted from intestinal samples using a commercial DNA extraction kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The quality of extracted DNA was evaluated by 2% agarose gel electrophoresis, and DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

The extracted DNA was used as the template for PCR amplification of the V3–V4 hypervariable region of the bacterial 16S rRNA gene. The amplified products were subsequently sequenced on an Illumina NextSeq 2000 platform.

Following sequencing, paired-end (PE) reads were demultiplexed according to sample-specific barcodes. The raw reads were subjected to quality control and filtering based on sequencing quality parameters, and paired reads were merged according to their overlapping regions to obtain high-quality optimized sequences. The processed sequences were further denoised using DADA2 algorithms to generate amplicon sequence variants (ASVs), including representative sequences and abundance profiles. Based on the ASV representative sequences and abundance information, subsequent analyses, including taxonomic classification, microbial community diversity analysis,

differential abundance analysis, correlation analysis, phylogenetic analysis, and functional prediction, were performed. All microbiome data processing and statistical analyses were performed using the Majorbio Cloud Platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., China). The raw 16S rRNA gene sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA1453779.

2.11 Challenge experiment

A challenge test was conducted to evaluate the protective effects of dietary *B. velezensis* YSJ-24 against *Streptococcus agalactiae* infection. After the 28-day feeding trial, 20 bullfrogs per treatment group (NC, BV1, BV2, BV3) were intramuscularly injected in the hind leg with 0.1 mL of *S. agalactiae* suspension (2×10^6 CFU/mL). Bullfrogs were maintained on their respective diets for 14 days post-infection. Clinical signs and mortality were recorded daily, and cumulative survival rates were calculated. Surviving bullfrogs were euthanized at the end of the observation period.

2.12 Statistical analysis

Data on growth performance and survival are presented as mean ± standard deviation (SD) of three cage replicates (n = 3, 35 bullfrogs per cage). For intestinal enzyme activities, gene expression, histomorphology, transcriptomic, metabolomic, and microbiota analyses, data are presented as mean ± SD of three biological replicates (n = 3), with each biological replicate representing a pooled sample from three bullfrogs, one pooled sample per cage. Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test when significant differences were detected ($P < 0.05$). Survival after *S. agalactiae* challenge was analyzed using Kaplan–Meier survival curves. Differences among groups were compared using the

log-rank (Mantel–Cox) test in GraphPad Prism 9.0.0. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Growth performance, feed utilization, and survival

After the 28-day feeding trial, the effects of dietary *B. velezensis* YSJ-24 supplementation on growth performance parameters and survival are summarized in **Table 3**. Initial body weight did not differ significantly among experimental groups ($P > 0.05$). Compared with the NC group, dietary supplementation with *B. velezensis* YSJ-24 improved growth performance. Among supplemented groups, the BV2 group showed the best performance: final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) were 4.1%, 8.6%, and 8.0% higher than the NC group, respectively ($P < 0.05$). The BV2 group exhibited a significantly lower feed conversion ratio (FCR) ($P < 0.05$), indicating improved feed utilization efficiency. No significant differences in survival rate (SR) were observed ($P > 0.05$).

Table 3

Effects of dietary *B. velezensis* YSJ-24 supplementation levels on growth performance and survival of bullfrogs.

Parameters	NC	BV1	BV2	BV3
IBW (g)	15.6±0.1	15.5±0.1	15.58±0.1	15.5±0.1
FBW (g)	31.8±0.4^{b}	32.6±0.9^{ab}	33.1±0.7^{a}	32.3±0.5^{ab}
WGR (%)	103.8±2.1^{b}	110.2±4.5^{a}	112.7±4.2^{a}	108.1±3.7^{ab}
SGR (%/day)	2.5±0.1^{b}	2.7±0.1^{a}	2.7±0.2^{a}	2.6±0.1^{ab}
FCR	1.0±0.1^{a}	0.9±0.1^{ab}	0.8±0.1^{b}	0.9±0.1^{ab}
SR (%)	95.8±1.2	94.0±1.7	95.1±1.4	96.0±1.0

Data are presented as mean ± standard deviation (SD) of three independent biological replicates (n = 3). Each treatment consisted of three biological replicates, with three bullfrogs per replicate. Differences among treatments were analyzed using one-way ANOVA. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; SR, survival rate.

The NC, BV1, BV2, and BV3 diets contained 0, 1×10^7, 1×10^8, and 1×10^9 CFU/g of *B. velezensis* YSJ-24, respectively.

3.2 Intestinal mucosal morphology and goblet cell density

Histological analysis revealed significant differences in intestinal morphology among the experimental groups (**Fig. 2A**–**D**). Villus height was significantly lower in the NC group than in the BV1, BV2, and BV3 groups ($P < 0.05$) (**Fig. 2E**), with no significant differences observed among the supplemented groups. Goblet cell density also differed significantly among treatments (**Fig. 2F**). The BV2 group exhibited the highest goblet cell density, which was significantly higher than that in the NC, BV1, and BV3 groups ($P < 0.05$).

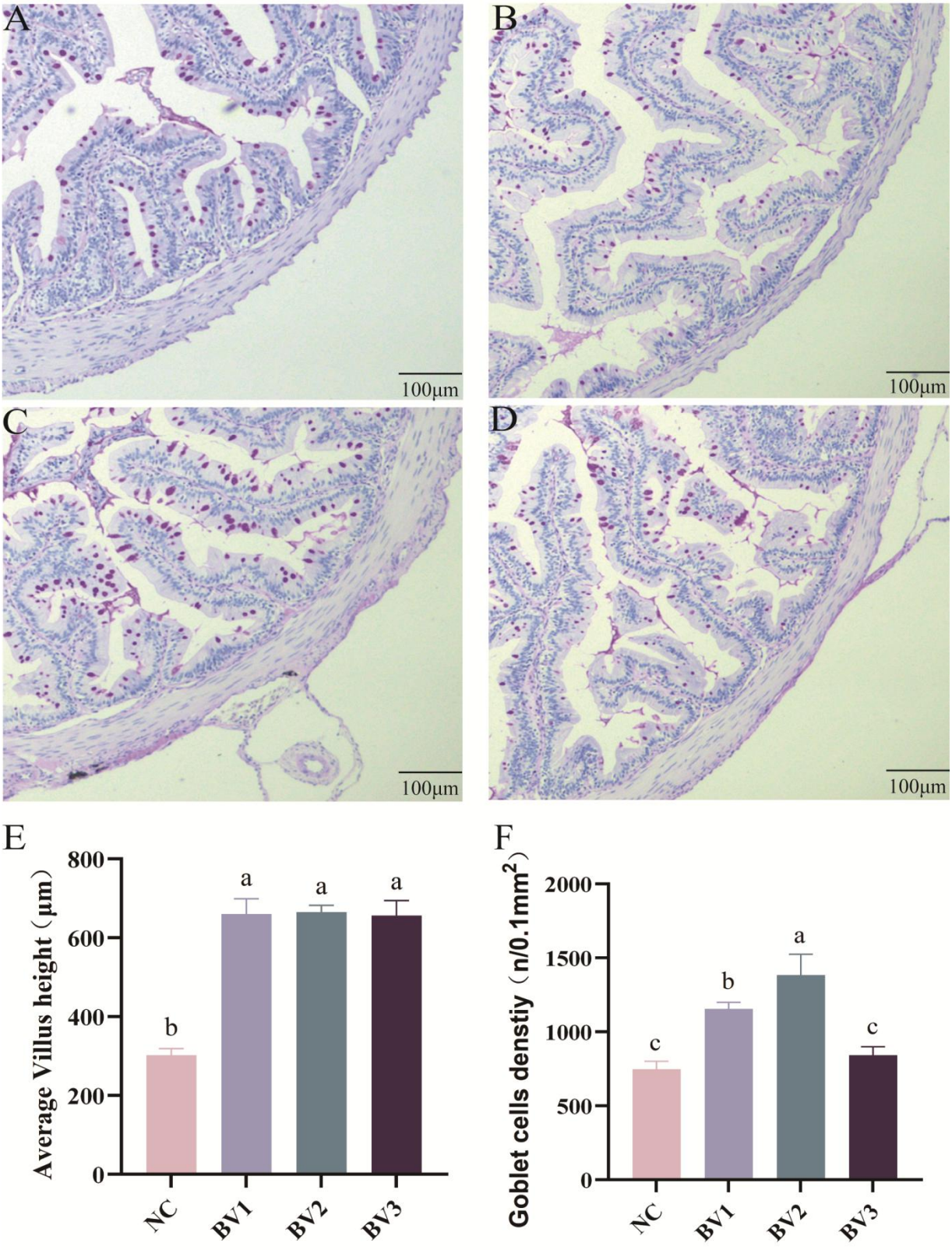


Fig. 2. Periodic acid–Schiff (PAS) staining of intestinal sections. Representative histological images of the NC (A), BV1 (B), BV2 (C), and BV3 (D) groups. Scale bar = 100 μm. (E, F) Quantification of villus height and goblet cell density.

Data are presented as mean ± standard deviation (SD) of three independent biological replicates (n = 3). Each biological replicate consisted of a pooled sample from three bullfrogs, with one replicate sampled per cage. Differences among treatments were analyzed using one-way ANOVA. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

The NC, BV1, BV2, and BV3 diets contained 0, 1×10^7, 1×10^8, and 1×10^9 CFU/g of *B. velezensis* YSJ-24, respectively.

3.3 Intestinal tight junction-related gene expression

Dietary supplementation with *B. velezensis* YSJ-24 affected the mRNA expression levels of intestinal tight junction-related genes, with marked differences observed among the experimental groups (**Fig. 3**). Compared with the NC group, the BV2 group showed significantly increased relative mRNA expression levels of *zo-1*, *zo-2*, *zo-3*, *occludin*, and *claudin-7* ($P < 0.05$). In contrast, the expression of these genes was significantly reduced in the BV3 group ($P < 0.05$). The BV1 group showed intermediate effects, with no significant differences compared to the NC group for most genes.

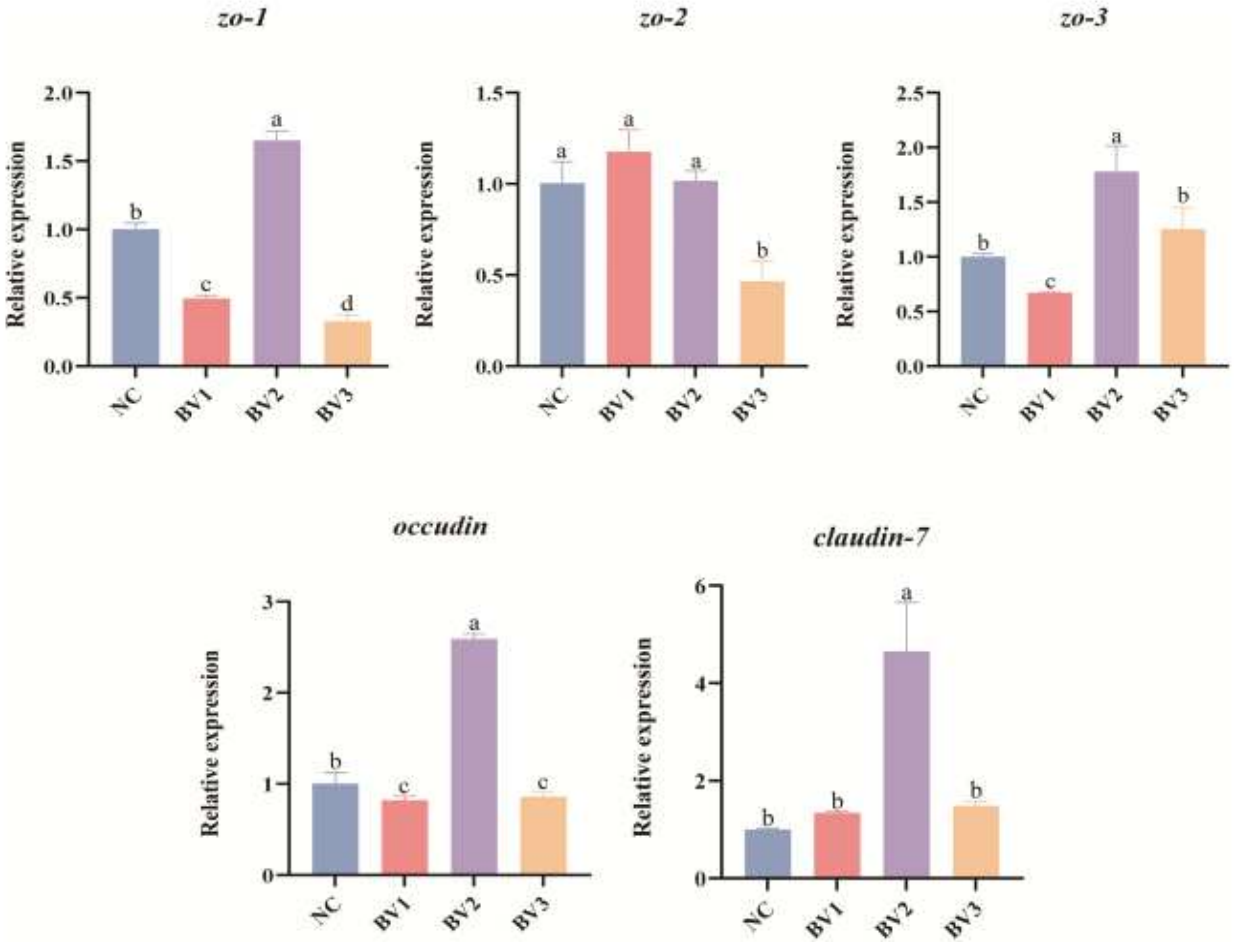


Fig. 3. Effects of dietary *B. velezensis* YSJ-24 at different levels on the relative expression of tight junction–related genes in the intestine of bullfrog. *zo-1*, zonula occludens-1; *zo*-2, zonula occludens-2; *zo-3*, zonula occludens-3; *occludin*; and *claudin-7*.
Each biological replicate consisted of a pooled sample from three bullfrogs, with one replicate sampled per cage. Differences among treatments were analyzed using one-way ANOVA. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

3.4 Intestinal enzyme activities and MDA levels

The BV2 group showed the highest intestinal SOD, CAT, and GSH-Px activities and the lowest MDA level among all groups ($P < 0.05$; **Fig. 4**). Compared with the NC group, antioxidant enzyme activities were increased and MDA levels were decreased in the BV2 group, whereas antioxidant enzyme activities were significantly decreased and MDA levels were increased in the BV3 group ($P < 0.05$). The BV1 group showed

intermediate responses. Overall, BV2 produced the most pronounced improvement in intestinal antioxidant status.

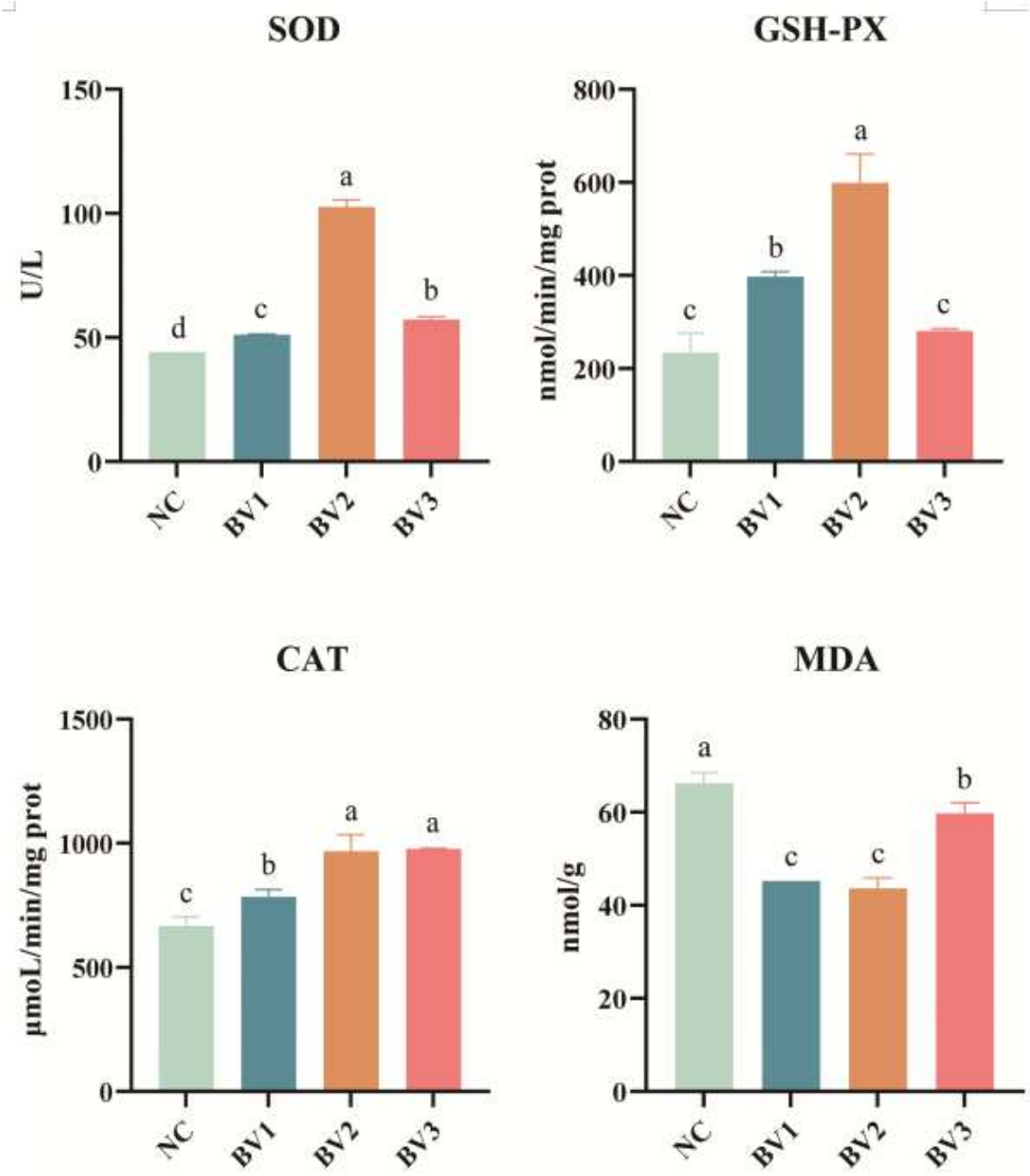


Fig. 4. Effects of dietary *B. velezensis* YSJ-24 on antioxidant enzyme activities and lipid peroxidation in the gut of bullfrog. (A–D) SOD, CAT, GSH-Px activity, and MDA content. Each biological replicate consisted of a pooled sample from three bullfrogs, with one replicate sampled per cage. Differences among treatments were analyzed using one-way ANOVA. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

3.5 Integrated transcriptomic and metabolomic analyses reveal metabolic and immune remodeling

Transcriptomic analysis revealed 63 upregulated and 64 downregulated differentially expressed genes (DEGs) ($|\log_2 FC| \geq 1$, FDR < 0.05) in the BV group compared with the NC group. Integrated KEGG pathway enrichment analysis of DEGs and differentially accumulated metabolites (DAMs) between the BV and NC groups revealed coordinated alterations in metabolic and immune-related pathways (**Fig. 5**). DAMs were mainly enriched in pathways associated with metabolism and nutrient utilization, including metabolic pathways, glycerophospholipid metabolism, bile secretion, primary bile acid biosynthesis, carbon metabolism, starch and sucrose metabolism, pentose phosphate pathway, and biosynthesis of amino acids and nucleotide sugars. In contrast, DEGs were primarily enriched in pathways related to immune response, cellular defense, and stress adaptation, including neutrophil extracellular trap formation, phagosome, ferroptosis, ABC transporters, and drug metabolism–other enzymes. Notably, several pathways, including ABC transporters, drug metabolism–other enzymes, and bile secretion, were simultaneously enriched in both transcriptomic and metabolomic datasets, suggesting coordinated regulation of immune responses, molecular transport, and metabolic homeostasis during BV exposure. Based on these enriched metabolic pathways, key DAMs potentially involved in BV-induced metabolic and immune regulation were further identified.

Further analysis of metabolites mapped to Metabolic pathways identified 24 differential metabolites, including 14 upregulated and 10 downregulated metabolites ($P < 0.05$; **Table 4**). Among them, six upregulated metabolites with potential immune relevance were highlighted: taurocholic acid, phosphatidylethanolamine, phosphatidylserine, protoporphyrin, trihydroxy bile acid, and acarbose. Taurocholic acid and trihydroxy bile acid were associated with bile acid signaling and intestinal homeostasis, whereas phosphatidylethanolamine and phosphatidylserine were related to membrane integrity, lipid metabolism, and cellular signaling. Protoporphyrin was

linked to heme biosynthesis and cellular metabolic activity, while acarbose was associated with carbohydrate metabolism.

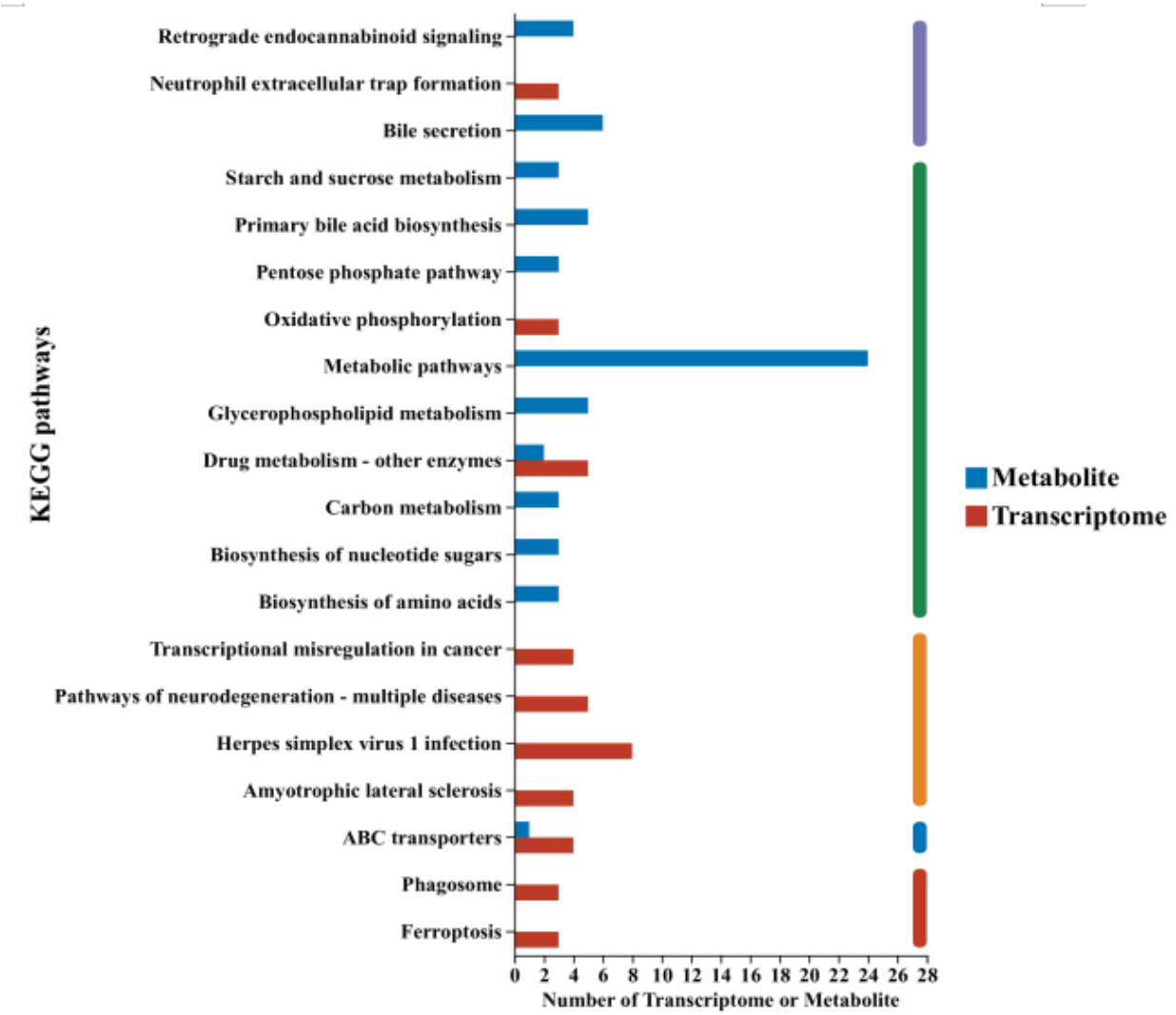


Fig. 5. KEGG pathway enrichment analysis of differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs). DEGs: $|\log_2 FC| \geq 1$, FDR < 0.05; DAMs: VIP > 1.0, P < 0.05.

Table 4

Differential metabolites mapped to metabolic pathways.

KEGG Compound ID	Metabolite	UP	Down
C00689	Trehalose 6-Phosphate	↑	
C01236	6-Phosphonoglucono-D-Lactone	↑	
C00535	Testosterone	↑	
C05122	Taurocholic Acid	↑	
C00695	Cholic Acid	↑	
C08814	Brassinolide	↑	
C00350	Pe(20:2(11Z,14Z)/20:4(5Z,8Z,11Z,14Z))	↑	
C02191	Protoporphyrin	↑	
C02737	Ps(22:2(13Z,16Z)/18:0)	↑	
C04500	N-Acetyl-D-Glucosaminyldiphosphodolichol	↑	
C00182	Glycogen	↑	
C00350	Pe(36:6)	↑	
C01301	3A,7A,12A-Trihydroxy-5B-Cholestan-26-Al	↑	
C06802	Acarbose	↑	
C00157	Pc(16:1(9Z)/18:0)		↓
C18218	16-Hydroxyhexadecanoic Acid		↓
C02741	N1-(5-Phospho-D-Ribosyl)-Amp		↓
C02737	Ps(18:2(9Z,12Z)/20:1(11Z))		↓
C00147	Adenine		↓
C00089	Sucrose		↓
C00249	Palmitic Acid		↓
C02165	Leukotriene B4		↓
C05382	Sedoheptulose 7-Phosphate		↓
C00281	D-Sedoheptulose 7-Phosphate		↓

Metabolites showing significant upregulation (↑) or downregulation (↓) are listed with their corresponding metabolite IDs. Arrows indicate relative changes in abundance compared with the NC group. Multiple lipid molecular species may be mapped to the same KEGG Compound ID due to their shared head-group structures.

3.6 Gut microbiota diversity following B. velezensis YSJ-24 Treatment

The intestinal microbiota of bullfrogs in the BV group was compared with that of bullfrogs in the NC group to evaluate the effects of dietary supplementation with *B. velezensis* YSJ-24 on gut microbial diversity and community composition. The Good's coverage index of all samples was ≥ 0.99, indicating sufficient sequencing depth to capture the majority of bacterial taxa present. The α-diversity indices, including ACE,

Chao1, Sobs, Shannon, and Simpson, did not differ significantly between the NC and BV groups ($P > 0.05$).

The taxonomic composition of the gut microbiota was analyzed at the phylum, family, and genus levels. At the phylum level, the microbiota was dominated by *Firmicutes*, *Bacteroidota*, and *Proteobacteria* (**Fig. 6A**). Compared with the NC group, the BV group exhibited a lower relative abundance of *Firmicutes* and higher abundances of *Bacteroidota* and *Proteobacteria*, although these differences were not statistically significant ($P > 0.05$). At the family level, *Streptococcaceae*, *Prevotellaceae*, *Enterobacteriaceae*, and *Lachnospiraceae* were the predominant families in both groups (**Fig. 6B**). At the genus level, *Lactococcus* was predominant in the NC group, whereas its relative abundance decreased in the BV group (**Fig. 6C**). In contrast, the relative abundances of *Prevotella* and *Bacteroides* were higher in the BV group, although no significant differences were observed ($P > 0.05$).

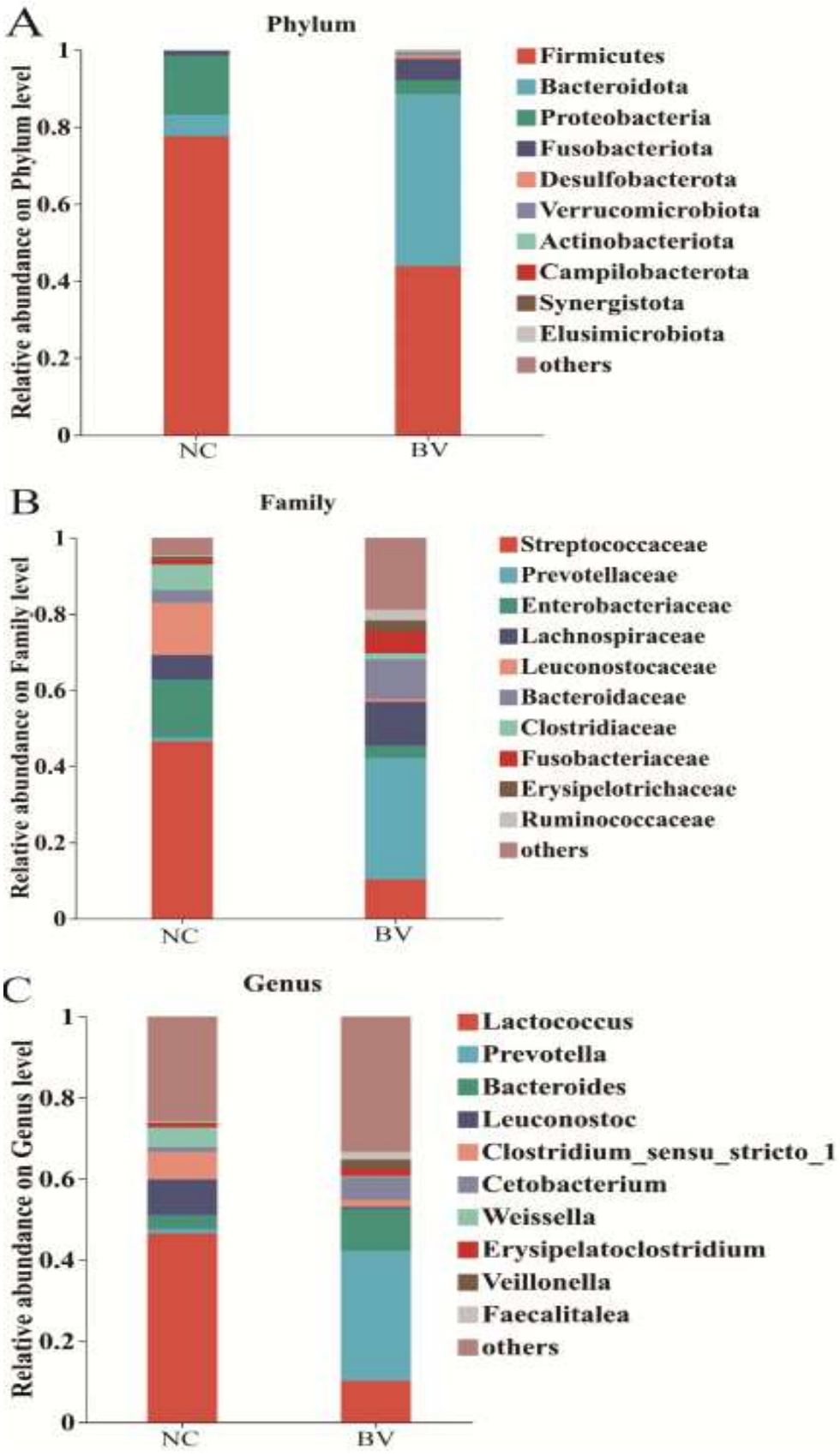


Fig. 6. Taxonomic composition of the intestinal microbiota in bullfrogs from the NC and BV2 groups at the phylum (A), family (B), and genus (C) levels. Taxa with low relative abundance were grouped as "Others".

3.7 Effects of B. velezensis YSJ-24 on the disease resistance of bullfrogs

To evaluate the protective efficacy of dietary *B. velezensis* YSJ-24 against *S. agalactiae* challenge, the cumulative survival rates of bullfrogs were monitored for 14 days post-challenge (**Fig. 7**). The NC group exhibited rapid mortality following infection, with survival progressively declining to 0% by the end of the observation period. In contrast, all *B. velezensis* YSJ-24-supplemented groups exhibited higher survival than the NC group, although the degree of protection differed among treatments. The BV1 group showed a moderate protective effect, with a final survival rate of 20%. The BV3 group exhibited an intermediate effect, with a final survival rate of 40%. Notably, the BV2 group exhibited the highest survival rate (70%) among all groups, with no additional mortality observed from day 6 onward.

All dead bullfrogs displayed clinical signs consistent with *S. agalactiae* infection, including lethargy, reduced feeding, body darkening, and hemorrhagic lesions. In addition, *S. agalactiae* was successfully reisolated from diseased bullfrogs and identified, confirming that mortality resulted from the experimental infection. These results indicate that dietary supplementation with *B. velezensis* YSJ-24 enhanced the resistance of bullfrogs to *S. agalactiae* challenge, with the BV2 group exhibiting the highest protective efficacy.

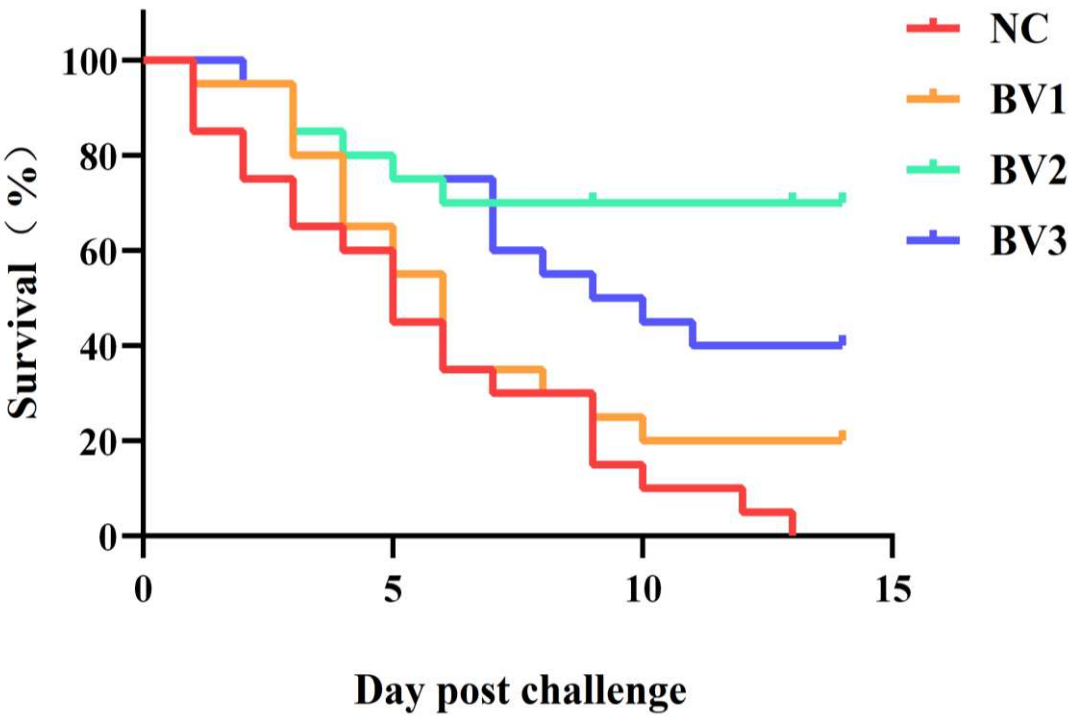


Fig. 7. Effects of dietary *B. velezensis* YSJ-24 supplementation on the resistance of bullfrogs to *Streptococcus agalactiae* challenge. Survival of bullfrogs monitored for 14 days after challenge with *S. agalactiae*.

4. Discussion

This study evaluated the effects of dietary supplementation with *Bacillus velezensis* YSJ-24 on growth performance, intestinal health, and disease resistance in American bullfrogs (*Aquarana catesbeiana*) under intensive culture conditions. The results demonstrated that *B. velezensis* YSJ-24 significantly improved growth performance, enhanced intestinal structural integrity, and increased resistance to *Streptococcus agalactiae* infection. These findings are generally consistent with previous studies reporting that *Bacillus* spp. can enhance growth performance, gut health, and disease resistance in aquatic animals [12, 26]. Taken together, the present results indicate that *B. velezensis* YSJ-24 can contribute to improved health status and production performance in bullfrogs under intensive culture conditions.

Dietary supplementation with *B. velezensis* YSJ-24 significantly improved growth performance, as indicated by increased WGR and SGR and reduced FCR, with the BV2 group exhibiting the most pronounced effects. These results are consistent with previous studies demonstrating that *Bacillus* spp. can enhance growth performance in aquatic animals [12, 27]. The enhanced growth observed in this study may be closely associated with improvements in intestinal structure and function. Histological analysis showed that *B. velezensis* YSJ-24 supplementation significantly increased villus height and goblet cell density, indicating enhanced intestinal absorptive capacity and mucosal barrier function. These findings are in agreement with previous studies reporting that probiotics can improve intestinal morphology and mucosal status in aquatic animals [28, 29]. In addition, the increased goblet cell density suggests a strengthened mucosal barrier, which may contribute to maintaining intestinal homeostasis. Together with the reduced FCR observed in the BV2 group, these structural and functional improvements likely facilitated more efficient nutrient utilization, thereby contributing to the observed enhancement in growth performance.

Consistent with the observed improvements in intestinal morphology, dietary supplementation with *B. velezensis* YSJ-24 significantly enhanced intestinal barrier function by upregulating the expression of tight junction-related genes, including *zo-1*, *zo-2*, *zo-3*, *occludin*, and *claudin-7*, with the most pronounced effects observed in the BV2 group. These results indicate an enhancement of intestinal barrier integrity, as tight junction proteins are key regulators of paracellular transport. Similar regulatory effects of Bacillus supplementation on intestinal integrity have also been reported in previous studies, where enhanced tight junction protein expression was associated with improved epithelial barrier function [30, 31]. In the present study, the elevated transcription levels of genes involved in tight junction formation suggest that Bacillus treatment may strengthen intestinal epithelial stability and promote the preservation of gut homeostasis.

In addition to barrier-related effects, dietary supplementation with *B. velezensis* YSJ-24 also enhanced antioxidant capacity, particularly in the BV2 group. The significant increases in SOD, CAT, and GSH-Px activities, together with reduced MDA levels, indicated alleviation of oxidative stress. Oxidative stress is a common challenge in intensive aquaculture and is closely associated with intestinal dysfunction and tissue damage [32]. The observed antioxidant response is consistent with previous studies reporting that probiotics can enhance antioxidant defense systems and reduce oxidative damage in aquatic animals [12, 33]. Improved antioxidant status may help protect intestinal tissues and maintain normal physiological function, thereby contributing to the overall improvement in gut health observed in this study.

Notably, the BV3 group showed lower expression of tight junction-related genes and reduced antioxidant enzyme activities compared with the NC group. This decline at the highest dose may reflect excessive metabolic burden or immune overstimulation induced by high bacterial loads, rather than beneficial modulation. As gut microbiota was analyzed only in the BV2 and NC groups, any effect of the BV3 dose on microbial homeostasis remains undetermined in the present study.

Another important aspect of this study is the integration of transcriptomic and metabolomic analyses to provide insights into functional responses associated with *B. velezensis* YSJ-24 supplementation. KEGG pathway enrichment analysis revealed that differentially expressed genes and metabolites were mainly associated with immune-related and metabolic pathways, including neutrophil extracellular trap formation and phagosome. Among these, phagosome formation and neutrophil extracellular trap (NET) formation are key components of innate immune defense, playing important roles in pathogen recognition and elimination. The enrichment of these pathways suggests that dietary supplementation with *B. velezensis* YSJ-24 influences immune-related processes and host defense capacity. Metabolomic analysis identified several upregulated metabolites, including taurocholic acid, phosphatidylethanolamine, phosphatidylserine, protoporphyrin, trihydroxy bile acid, and acarbose. Taurocholic

acid and trihydroxy bile acid are bile acid-related metabolites associated with lipid digestion and intestinal homeostasis. Phosphatidylethanolamine and phosphatidylserine are major membrane phospholipids involved in maintaining membrane integrity and cellular signaling. Protoporphyrin participates in heme metabolism and cellular metabolic processes, whereas acarbose is related to carbohydrate metabolism [34, 35]. The concurrent enrichment of immune- and metabolism-related pathways in both datasets suggests a coordinated host response at transcriptional and metabolic levels. These findings suggest that *B. velezensis* YSJ-24 supplementation may play a role in regulating host physiological status, particularly with respect to immune activity and intestinal function.

In the present study, dietary supplementation with *B. velezensis* YSJ-24 did not result in significant changes in the α-diversity indices of the gut microbiota of bullfrogs, as reflected by comparable ACE, Chao1, Sobs, Shannon, and Simpson values between the BV and NC groups. This observation is consistent with previous studies indicating that probiotic supplementation does not necessarily alter overall microbial richness or diversity, but may instead influence specific microbial taxa or functional characteristics [36, 37].

Despite the stability in diversity indices, shifts in the relative abundance of dominant taxa were observed. At the phylum level, the decrease in *Firmicutes* accompanied by a tendency toward increased *Bacteroidota* in the BV group may reflect changes in nutrient metabolism within the gut ecosystem. Members of *Bacteroidota* are known for their capacity to degrade complex polysaccharides and produce short-chain fatty acids, which contribute to intestinal health and energy harvesting [38, 39]. Although these differences were not statistically significant, they may indicate subtle restructuring of the microbial community.

At finer taxonomic levels, the numerically higher relative abundances of *Prevotella* and *Bacteroides* may suggest a tendency toward altered carbohydrate-utilizing bacterial communities, although these changes were not statistically

significant [40, 41]. In contrast, the reduction in *Lactococcus* abundance may reflect altered microbial interactions within the gut environment. In addition, the more even distribution of dominant taxa observed at the family level in the BV group may indicate improved microbial stability, which is often associated with increased resilience to environmental or pathogenic disturbances.

Importantly, these microbiota findings should be interpreted together with the improvements observed in growth performance, antioxidant capacity, intestinal barrier function, and disease resistance in the BV2 group. The absence of marked taxonomic differences suggests that the beneficial effects of *B. velezensis* YSJ-24 were not necessarily linked to large-scale restructuring of the gut microbiota, but may instead reflect subtle compositional shifts and functionally relevant microbial interactions. This interpretation is consistent with previous studies indicating that functional modulation, rather than compositional changes alone, plays an important role in host-microbiota interactions [42].

Overall, the present results suggest that *B. velezensis* YSJ-24 supplementation contributed to maintaining microbial stability while supporting a gut environment favorable for intestinal homeostasis and host health. Such stability, rather than drastic microbial restructuring, may be particularly beneficial for enhancing resilience to pathogenic infection under intensive culture conditions [43].

The challenge test with *Streptococcus agalactiae* further supported the beneficial effects of *B. velezensis* YSJ-24 on the disease resistance of bullfrogs. The BV2 group exhibited the highest survival rate following infection, indicating an enhanced capacity to withstand bacterial challenge. This improvement in disease resistance is likely associated with the combined effects of enhanced intestinal barrier function, increased antioxidant capacity, and improved physiological status observed in this study. A well-maintained intestinal environment and reduced oxidative stress are closely linked to enhanced host defense against pathogenic invasion. From an applied perspective, these findings suggest that dietary supplementation with *B. velezensis* YSJ-24 represents a

promising strategy for improving disease resistance in intensive aquaculture systems. This is particularly relevant in the context of increasing concerns over antibiotic use and resistance, where probiotics are considered a viable alternative for sustainable disease management [44, 45]. Collectively, the present findings support the application of *B. velezensis* YSJ-24 as a practical strategy for enhancing health and disease resistance in intensive bullfrog aquaculture systems.

5. Conclusion

Under intensive bullfrog aquaculture conditions, dietary supplementation with *Bacillus velezensis* YSJ-24 significantly improved growth performance, intestinal health, antioxidant capacity, and disease resistance in American bullfrogs (*Aquarana catesbeiana*). Among the tested dietary treatments, supplementation with *B. velezensis* YSJ-24 at 1×10^8 CFU/g showed the most pronounced beneficial effects, as evidenced by a 70% survival rate following *Streptococcus agalactiae* challenge. By integrating analyses of growth performance, gut health, and host defense responses, this study highlights the probiotic potential of *B. velezensis* YSJ-24 in a relatively understudied aquaculture species. Importantly, the observed maintenance of gut microbial stability and enhanced host resilience suggest that *B. velezensis* YSJ-24 contributes to sustaining microbial ecological balance. Collectively, these findings support the potential application of *B. velezensis* YSJ-24 as a functional feed additive for improving health and disease resistance in bullfrogs. As a component of integrated health management strategies, its use may help reduce antibiotic dependence and contribute to more sustainable aquaculture practices.

Author Contributions

Wensen Tan: conducted all experiments, worked with the data, and wrote the manuscript. Yueming Hu: participated in most of the experiments, assisted with data processing, and contributed to manuscript revision. Mengzhen Jiang: participated in most of the experiments and assisted with data processing. Jingyang Sun: participated

in most of the experiments, assisted with data processing, and contributed to manuscript revision. Kesong Chen, Han Lin, Hua Li, Zhixiang Bai, Yanwen Wu, and Weike Xiao: participated in most of the experiments. Jie Ma: contributed to formal analysis, writing, review, and editing. Liu Chun: guided and supervised the development of the experiments and the writing of the manuscript, and repeatedly revised the manuscript to prepare the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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