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Effects of dietary *Bacillus velezensis* Y01 supplementation on growth performance, immune function, and cecal microbiota of 1 to 42 days Langya chickens

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Abstract

Bacillus velezensis (*B. velezensis*) has gained increasing recognition as a probiotic for improving animal growth performance and intestinal health. However, whether *B. velezensis* affects growth performance, immune function, and cecal microbiota in Chinese local breeds of chickens remains unclear. In this study, a total of 180 one-day-old healthy male Langya chicks were randomly divided into 3 treatment groups with 5 replicates per group and 12 chicks each replicate. Langya chicks were fed with a corn-soybean-based diet as the control group (CON), and 2 other groups were fed the same basal diet supplemented with 50 mg/kg aureomycin or 2.0×10^9 CFU/kg *B. velezensis* Y01 as the antibiotic-treated group (ANT) and the *B. velezensis*-treated group (BVT), respectively, for 42 days. Dietary supplementation with *B. velezensis* Y01 resulted in a 7.01% increase in the final body weight, a 7.27% increase in weight gain (WG), and a 7.24% increase in average daily gain (ADG) in Langya chickens at 42 days of age in the BVT group when compared to the CON group ($p<0.05$). Meantime, the final body weight was increased by 5.88%, WG by 6.18%, and ADG by 6.19% in the BVT group when compared to the ANT group ($p<0.05$). The BVT group with *B. velezensis* Y01 resulted in a 26.80% decrease in lactate dehydrogenase (LDH), a 52.92% decrease in uric acid (UA), a 20.70% decrease in cholesterol (CH), and a 40.84% decrease in urea when compared to the CON group ($p<0.05$). Furthermore, the serum immunoglobulin G (IgG) levels were increased by 36.09%, IgM by 56.08%, and interleukin-2 (IL-2) by 32.83% in the BVT group in comparison to the CON group ($p<0.05$), but IL-1, IL-6, and tumor necrosis factor-α (TNF-α) levels had no changes ($p>0.05$). Notably, *B. velezensis* Y01 enriched the abundance of Firmicutes and Bacteroidetes, resulting in the production of certain beneficial metabolites that play pivotal roles in reducing the abundance of harmful bacteria. This, in turn, decreases the virulence genes and antibiotic resistance genes in the cecal microbial communities, ultimately enhancing immunity and metabolism in Langya chickens. Collectively, these results are encouraging and suggesting that *B. velezensis* Y01 may be an effective

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alternative of antibiotic growth promoters for improving chickens' growth performance and intestinal health in poultry production.

Keywords *Bacillus velezensis*, Growth performance, Immune function, Cecal microbiota, Langya chickens

Introduction

Poultry production is an essential source of income for people living in developing countries and helps many nations' economies flourish [1, 2]. Poultry products such as meat and eggs are one of the major sources of animal protein necessary to meet the global protein demand for humans [3, 4]. As the demand for poultry meat and eggs increases, there is a need to improve production [5]. In the past, dietary antibiotics were used as growth promoters for poultry due to their ability to prevent and control diseases, increase growth performance, improve the feed conversion rate, strengthen immune function, and maintain the balance of intestinal microbiota [4, 6, 7]. However, the overuse and misuse of in-feed antibiotics in the poultry industry has increased the safety risks associated with bacterial resistance and multi-drug resistance, the drug residue in the final products, and environmental pollution. Many countries including China have thus implemented bans on the usage of antibiotics in poultry feed [4, 6, 8]. Therefore, it is an advanced research hotspot for developing and applying green, safe, and efficient antibiotic alternatives to promote poultry production. Nowadays, many scholars believe that one of the optimal alternatives to antibiotics is probiotics [9].

Probiotics are defined as living microorganisms conferring beneficial effects on the host health upon consumption in enough amounts as part of food and considered as the most promising alternatives to in-feed antibiotics in poultry production [6, 9–12]. This is due to that dietary probiotics can effectively stimulate beneficial microbiota reproduction, reduce and prevent pathogen colonization, improve the morphology of intestinal epithelial cell, enhance the host immune function without toxic side effect or drug residues, increase a variety of digestive enzymes production, and promote the host nutrient digestion and absorption on the feed [4, 6, 7, 13, 14]. The most widely used probiotics in poultry production belong to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Pediococcus*, *Clostridium*, *Saccharomyces*, and *Bacillus* [6, 11, 14–16]. Among probiotic species, *Bacillus velezensis* (*B. velezensis*) belonging to the genus *Bacillus* stands out in poultry production due to its multifaceted functional advantages. These advantages include secreting digestive enzymes to facilitate nutrient digestion and absorption, producing secondary metabolites to inhibit the growth of pathogenic microorganisms, and forming spores to withstand the high temperatures and dry conditions during the feed manufacturing processes as well as to endure the low pH and bile salt conditions within the host's gastrointestinal tract [4, 12, 16–18]. Therefore, *B. velezensis* has potential as a promising alternative to in-feed antibiotics in poultry production for improving the growth performance and modulating the intestinal microbial composition to facilitate gastrointestinal health. The beneficial functions of *B. velezensis* as a alternative to in-feed antibiotics have been proved in poultry production. For example, *B. velezensis* isolated from the manure of piglets enhances the production performance, egg quality and the plasma biochemical index in Hy-Line Brown laying hens [19]. Dietary supplementation with *B. velezensis* ZBG17 obtained from the soil samples significantly improves feed utilization efficiency and humoral immune response in Cobb broiler chickens [20]. Similarly, dietary supplementation with *B. velezensis* screened from the intestinal tracts of healthy animals as a feed additive promotes the growth performance and enhances intestinal digestion and absorption function in Arbor Acres broiler chickens by enhancing intestinal morphological structure, increasing digestive enzyme activities, and altering intestinal microbial structure [16, 17]. Dietary supplementation with *B. velezensis* BV-KNF-209 being a commercial product significantly improves the growth performance of Cobb-500 broiler chickens by enhancing immunity, increasing digestive enzyme activities, and raising the levels of intestinal short-chain fatty acids and lactic acid [4]. Dietary supplementation with *Bacillus velezensis* fermented soybean hulls increases the growth of Arbor Acres broiler chickens and promotes intestinal health by improving intestinal antioxidant capacity, suppressing pro-inflammatory gene expression, and modulating microbiota composition [21]. The benefits are achieved through the increased bioactive components in fermented soybean hulls, with a diet containing 10% fermented soybean hulls demonstrating the most pronounced effects [21]. However, the above scientific reports describing the majority of *B. velezensis* strains that promote growth performance in foreign commercial breeds of chickens originate from other animals rather than the native host, and the effects of dietary *B. velezensis* supplementation on growth performance, immune function, and cecal microbiota in Langya chickens remain unclear.

Langya chickens are Chinese fine local breeds of chickens reared widely in Shandong province due to its important economic benefits, fresh and tender in meat texture, disease resistance, stronger adaptability, higher feed utilization efficiency, small body size, and high egg production [22]. Although the emergence of *16 S rRNA*

sequencing and metagenomic sequencing has deepened insights into intestinal microbial composition and their association with an certain infective disease [16, 23, 24], there is still a knowledge gap in regard to the effectiveness of *B. velezensis* in shaping intestinal microbial metabolic potential, immune function, and the intestinal disease incidence of Langya chickens. Accordingly, a more comprehensive understanding of how the microbial taxonomic profiling regulated by *B. velezensis* affects the functional attributes of intestinal microbiota will deepen insights into intestinal microbial communities and metabolic potential as well as their role in poultry metabolism and health. Therefore, this study aims to investigate the effects and the associated mechanisms of *B. velezensis* Y01 on growth performance, immune function, and cecal microbiota and their potential metabolic functions, as well as to define the associations between cecal microbiota and intestinal health in Langya chickens.

Materials and methods

Ethics statement

All the experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Linyi University (Approval Number: LYU20240117, Linyi, Shandong, China) and the procedures adhered to the ARRIVE guidelines (https://arriveguidelines.org). After the experiments, all chickens used in this study were euthanatized by intravenous injection of pentobarbital sodium (100 mg/kg) into the wing vein. Subsequently, the loss of consciousness was rapid, followed by cessation of respiration and heartbeat, and then exsanguination, confirming euthanasia. All procedures were carried out in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals as mandated by the State Council of the People's Republic of China.

Bacterial strains and growth conditions

B. velezensis Y01 used in this experiment was isolated from the cecum of healthy and free-ranging broiler chickens, and preserved at -80℃ in the Institute of Microbe and Host Health, Linyi University. The cryopreserved *B. velezensis* Y01 was streaked onto Luria-Bertani (LB) agar plates and cultured at 37℃ overnight. To get the fermentation broth of *B. velezensis* Y01, a colony was inoculated into LB broth and incubated at 37℃ overnight with shaking (180 rpm). Bacterial cells of *B. velezensis* Y01 were prepared per day by centrifugation at 5000×g for 10 min at 4℃ and used in the following animal experiments.

Experimental design and basal diets

A total of 180 one-day-old male Langya chicks (with an average body weight of 30.66 ± 1.32 g) were purchased from Rizhao Langya Chicken Corporation LTD. They were divided into 3 treatment groups with 5 replicates per group and 12 chicks each replicate (12 chicks/cage) in a completely randomized design. The chicks in group 1 as the control group (CON) were fed with a corn-soybean-based diet; those in the other 2 groups were fed with the same basal diet, including group 2 as the antibiotic-treated group (ANT) supplemented with 50 mg/kg aureomycin, and group 3 as the *B. velezensis*-treated group (BVT) supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01. All chicks were raised in wire-floored cages (130 cm × 60 cm × 70 cm) and were able to freely obtain feed and water for the 42-day duration of the trial.

Experimental diet and feeding management

According to the Nutrient Requirement of Poultry (NRC, 1994) and China's Standard of Chick Diet (NY/T33-2004), the feeding program of Langya chickens was divided into 2 phases, including the starter phase (1–21 d) and the grower phase (22–42 d). The composition and nutrients levels of the basal diets were listed in Table 1. A lighting program of 16 h of light and 9 h of dark was used throughout the experimental period. In the first week of the experiment, the temperature of the hen-house was controlled between 33℃ and 35℃, and then stepwise decreased to 24℃ by 2℃ per week until the end of the experiment, and the relative humidity was maintained at 55–60% through natural ventilation during the whole experiment. The hen-house was cleaned and disinfected daily, and the excreta was cleared daily. These Langya chickens were subjected a routine vaccination program, and their behavior were observed daily, the morbidity and mortality were recorded daily.

Growth performance

Langya chickens of each replicate were weighed at 1 and 42 days of age. Feed intake was recorded by measuring daily feed residue and record the number of living Langya chickens to calculate weight gain (WG), average daily gain (ADG), average daily feed intake (ADFI) and feed to gain ratio (F/G) of each replicate for the different phases. Mortality was calculated and showed as cumulative mortality per group by 42 days of age

WG = Final body weight - Initial body weight

ADG = WG/(Days of test × Number of Langya chickens);

ADFI = Feed intake/(Days of test × Number of Langya chickens);

F/G = Feed intake/WG

Hematological and serum biochemical indexes analysis

At 42 days of age, one Langya chicken close to the average body weight from each replicate was randomly selected for the blood samples after 12 h fasting. The blood samples (5 mL) were collected from the wing

Table 1 Ingredient composition and nutrient levels of the basal diets (%, air-dry basis)

Ingredients (%)	Starter phase (1–21 d)	Grower phase (22–42 d)	Nutrition levels[1]	Starter phase (1–21 d)	Grower phase (22–42 d)
Corn	55.30	60.2	Metabolizable energy (MJ/kg)	12.98	13.23
Soybean meal	34.00	30	Crude protein	21.06	20.51
Cottonseed meal	4.00	3.20	Calcium	1.08	0.97
Soybean oil	3.00	3.00	Total phosphorus	0.69	0.61
Stone powder	1.00	1.20	Methionine	0.51	0.48
$CaHPO_4$	1.40	1.30	Lysine	1.15	1.09
NaCl	0.30	0.3	Threonine	0.72	0.65
Choline chloride	0.18	0.11			
Methionine	0.12	0.09			
Lysine	0.20	0.10			
Premix[2]	0.50	0.50			
Total	100.00	100.00			

[1]Metabolizable energy was calculated values; while the others were measured values

[2]Premix provided per kg of diet: VA, 10 000 IU; VD_3, 6 000 IU; VE, 160 IU; VK_3, 10 mg; VB_1, 3.2 mg; VB_2, 9.5 mg; VB_6, 8.75 mg; VB_{12}, 0.02 mg; pantothenic acid, 15 mg; folic acid, 1.25 mg; Fe, 100 mg; Cu, 10 mg; Zn, 100 mg; Mn, 120 mg; I, 0.5 mg; Se, 0.3 mg; antioxidant, 0.25 mg

veins in the anticoagulant tube with heparin sodium (10 mL). Hematological parameters of the heparin sodium-containing anticoagulant blood were measured using an auto-hematology analyzer (Mindray, BC-2800Vet, Shanghai, China), including red blood cell count (RBC), hemoglobin concentration (HGB), mean corpuscular volume (MCV), lymphocytes (LYM), white blood cell count (WBC), neutrophile granulocytes (NEU), monocytes (MONO), and platelets (PLT). Similarly, the blood samples (0.5 mL) were collected from the wing vein in the anticoagulant tube with heparin lithium (1.5 mL). 100 μL of the heparin lithium-containing anticoagulant blood were added into the veterinary biochemical reagent tray (Seamaty) to detect serum biochemical parameters such as albumin, total protein (TP), globulin, A/G ratio, bilirubin (BIL), alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bile acid (BA), amylase (AMY), lipase (LPS), creatine kinase (CK), lactate dehydrogenase (LDH), urea, uric acid (UA), glucose (GLU), cholesterol (CH), triglyceride (TG), and phosphorus using an automatic blood biochemical analyzer (Seamaty, SMT-120VP, Chengdu, Sichuan, China).

Enzyme linked immunosorbent assay (ELISA)

The above Langya chickens selected for the blood samples were used to collect the non-anticoagulated blood from the wing veins (10 mL/chicken) [4]. The non-anticoagulated blood samples were kept at room temperature for 2 h and then centrifuged at 2000 rpm for 20 min at 4℃ to obtain serum samples used for evaluating in terms of immunoglobulins and immune cytokines. The concentrations of immunoglobulins such as IgA, IgG, IgE, and IgM, as well as the levels of immune cytokines such as interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) were measured according to the manufacturer's instructions of the ELISA detection kits (Cloud-Clone, Wuhan, China).

Metagenomic sequencing

After blood collection, the above Langya chickens were anesthetized by intravenous injection of pentobarbital sodium (30 mg/kg) into the wing vein and killed by severing the jugular vein. These chickens were dissected and collected immediately the contents samples from the cecum for metagenomic DNA extraction. Metagenomic DNA was extracted from the above cecal contents samples using The QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol, and the quality of the extracted DNA was checked using 1% agarose gel electrophoresis. The purity and yield of DNA were quantified using NanoDrop2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA) and Qubit 2.0 fluorometer (Thermo Scientific). The library preparation and the paired-end sequencing were carried out by Tsingke Biotechnology Co., Ltd. (Beijing, China) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Biological informatics processing

The raw data were split and quality-filtered by removing low-quality sequence reads, adapter reads and host reads to obtain clean reads. All clean reads were spliced and assembled using the MEGAHIT software to obtain and screen Contigs with the length of over 500 bp, which were utilized to predict the open read frames (ORFs) using the Prodigal software. Subsequently, the redundancy sequences were removed from ORFs using the CD-HIT software to obtain the non-redundant initial gene catalogue. These gene catalogue were clustered into operational taxonomic units (OTUs) of 95% and the representative sequences were compared and annotated

using the non-redundant protein database (NR, ftp://ftp.ncbi.nih.gov/blast/db/) to obtain all species annotating information at levels of phylum, class, order, family, genus, and species. The top 30 phyla, orders, families, and genera in relative abundance values defined as the dominant phyla, orders, families, and genera were analyzed at the phylum, order, family, and genus levels using the R software. Alpha diversity was exhibited by shannon index, simpson, and invsimpson to confirm species diversity and uniformity of cecal content samples. Beta diversity was visualized by principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) to assess the clustering of cecal microbial samples. Linear discriminant analysis with effect size (LEfSe) was used to analyze species composition in the cecal microbial communities, for which the linear discriminant analysis (LDA) score was limited above 3.0. In addition, the functional genes were annotated using kyoto encyclopedia of genes and genomes (KEGG, http://www.genome.jp/kegg/) and carbohydrate-active enzymes database (CAZy, http://www.cazy.org/). The antibiotic resistance genes and virulence genes were annotated using the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/) and virulence factors of pathogenic bacteria (VFDB, http://www.mgc.ac.cn/VFs/main.htm), respectively.

Statistical analysis

All experimental data were tested for normality and analyzed using SPSS statistical software (version 19.0, IBM, Armonk, NY, USA) by one-factor analysis of variance (ANOVA). Multiple comparisons were conducted with the application of least significant difference (LSD) method. The test results were presented as means ± standard deviation (SD). All tests were two-sided with $p < 0.05$ and $p < 0.01$ as considered a statistically significant and highly significant.

Results

B. velezensis Y01 improves the growth performance of Langya chickens

Dietary supplementation with *B. velezensis* Y01 resulted a 7.01% increase in the final body weight, a 7.27% increase in WG, and a 7.24% increase in ADG of Langya chickens at 42 days of age in the BVT group when compared to the CON group ($p < 0.05$, Table 2). Furthermore, the final body weight was increased by 5.88%, WG by 6.18%, and ADG by 6.19% in the BVT group when compared to the ANT group ($p < 0.05$, Table 2). However, no significant differences were found in the final body weight, WG, and ADG between the CON group and the ANT group. The F/G ratio and ADFI had no significant differences within these three groups at 42 day ($p > 0.05$, Table 2).

B. velezensis Y01 affects the blood parameters of Langya chickens

To investigate the effects of *B. velezensis* Y01 on the blood parameters of Langya chickens, the hematological and serum biochemical indexes of Langya chickens were evaluated. The hematology results were shown in Table 3. The hematological indexes of Langya chickens at 42 days of age, including RBC, HGB, MCV, LYM, WBC, NEU, MONO, and PLT levels, had no significant differences within these three groups ($p > 0.05$).

The serum biochemistry results were shown in Table 4. Compared to the CON group, the ANT group with aureomycin resulted in a 26.10% decrease in LDH, and the BVT group with *B. velezensis* Y01 resulted in a 26.80% decrease in LDH, a 52.92% decrease in UA, a 20.70% decrease in CH, and a 40.84% decrease in urea in Langya chickens at 42 days of age ($p < 0.05$). In addition, the BVT group with *B. velezensis* Y01 resulted in a 31.72% decrease in urea in Langya chickens at 42 days of age in comparison to the ANT group ($p < 0.05$). While no significant differences were found in other serum biochemical characteristics of Langya chickens at 42 days of age within these three groups, including ALB, GLB, TP,

Table 2 Effects of dietary *B. velezensis* Y01 supplementation on the growth performance of 1 to 42 d Langya chickens

Items	CON[1]	ANT[2]	BVT[3]	*p*-value
Initial body weight (g)	30.21 ± 0.78	30.72 ± 1.89	31.06 ± 1.30	0.697
Final body weight (g)	500.12 ± 15.72[b]	505.45 ± 4.24[b]	535.16 ± 6.87[a]	0.002
WG (g)	469.92 ± 16.42[b]	474.74 ± 4.18[b]	504.10 ± 7.18[a]	0.003
ADG (g/d)	11.19 ± 0.39[b]	11.30 ± 0.10[b]	12.00 ± 0.17[a]	0.003
ADFI (g/d)	26.82 ± 1.21	27.57 ± 2.17	27.36 ± 0.94	0.781
F/G	2.40 ± 0.09	2.44 ± 0.20	2.28 ± 0.08	0.282

Abbreviations: WG, weight gain; ADG, average daily gain; ADFI, average daily feed intake; F:G, feed-to-gain ratio

[1]CON: the control group was fed with a corn-soybean-based diet

[2]ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin

[3]BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01

[a, b] In the same row, values with the same or no letter superscripts mean no significant difference ($p > 0.05$), while values with different letter superscripts mean significant difference ($p < 0.05$)

Table 3 Effects of dietary *B. velezensis* Y01 supplementation on the hematological indexes of 1 to 42 d Langya chickens

Items	CON[1]	ANT[2]	BVT[3]	*p*-value
RBC ($\times10^{12}$/L)	2.33 ± 0.28	2.38±0.27	2.31 ± 0.13	0.902
HGB (g/dL)	256.25 ± 17.76	243.75±28.08	276.25 ± 18.46	0.163
MCV (fL)	127.63 ± 2.27	130.15±2.89	131.63 ± 3.34	0.192
WBC ($\times10^{9}$/L)	104.23 ± 7.77	93.90 ± 12.47	121.58 ± 29.50	0.171
NEU ($\times10^{9}$/L)	90.53 ± 6.21	82.55±10.30	101.38 ± 16.89	0.187
LYM ($\times10^{9}$/L)	10.98 ± 1.37	9.08±2.08	16.38 ± 10.58	0.283
MONO ($\times10^{9}$/L)	2.73 ± 0.26	2.28±0.39	3.83 ± 2.91	0.270
PLT ($\times10^{9}$/L)	8.25±3.30	10.50 ± 6.95	10.26 ± 2.06	0.757

Abbreviations: RBC, red blood cell count; HGB, hemoglobin concentration; MCV, mean corpuscular volume; LYM, lymphocytes; WBC, white blood cell count; NEU, neutrophile granulocytes; MONO, monocytes; PLT, platelets

[1]CON: the control group was fed with a corn-soybean-based diet

[2]ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin

[3]BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^{9} CFU/kg *B. velezensis* Y01

Table 4 Effects of dietary *B. velezensis* Y01 supplementation on the serum biochemical indexes of 1 to 42 d Langya chickens

Items	CON[1]	ANT[2]	BVT[3]	*p*-value
ALB (g/L)	18.13±0.52	19.28±1.05	17.45±1.59	0.126
TP (g/L)	39.45±3.89	42.23±1.72	38.78±4.66	0.402
GLB (g/L)	21.38±3.59	22.95±0.97	21.38±3.69	0.706
A/G ratio	0.87±0.13	0.84±0.04	0.83±0.12	0.902
BIL (μmol/L)	6.95±1.55	8.27±2.97	5.63±0.99	0.238
GT (U/L)	19.25±9.5	24.50±4.20	24.67±3.06	0.633
AST (U/L)	275.25±7.93	263.50±32.17	241.50±39.75	0.315
ALT (U/L)	12.00±4.69	11.75±1.71	14.25±7.09	0.746
ALP (U/L)	823.00±331.01	900.75±260.60	891.75±93.12	0.712
BA (μmol/L)	10.67±5.98	4.74±2.55	8.23±4.93	0.254
AMY (U/L)	268.50±109.34	257.75±59.49	220.25±29.88	0.640
LPS (U/L)	13.25±2.06	14.25±1.26	13.25±0.96	0.573
LDH (U/L)	837.00±115.58[a]	618.50±46.87[b]	612.67±186.43[b]	0.054
CK (U/L)	1506±565.75	1398.50±199.07	1,535.25±1,068.91	0.960
UA (μmol/L)	259.88±84.77[a]	168.37±79.90[ab]	122.35±70.82[b]	0.091
Urea (mmol/L)	2.62±0.57[a]	2.27±0.31[a]	1.55±0.36[b]	0.019
GLU (mmol/L)	11.29±0.59	11.85±1.03	12.44±1.11	0.270
CH (mmol/L)	3.43±0.55[a]	3.38±0.33[ab]	2.72±0.38[b]	0.083
TG (mmol/L)	0.56±0.22	0.66±0.15	0.53±0.05	0.447
Calcium (mmol/L)	2.44±0.09	2.57±0.12	2.45±0.07	0.176
Phosphorus (mmol/L)	2.17±0.08	2.25±0.14	2.20±0.16	0.712

Abbreviations: ALB, Albumin; GLB, Globulin; A/G ratio, albumin/globulin ratio; TP, total protein; BIL, bilirubin; GT, glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate amino transaminase; ALP, alkaline phosphatase; BA, bile acid; AMY, amylase; LPS, lipase; CK, creatine kinase; LDH, lactate dehydrogenase; UA, uric acid; GLU, glucose; CH, cholesterol; TG, triglyceride

[1]CON: the control group was fed with a corn-soybean-based diet

[2]ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin

[3]BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^{9} CFU/kg *B. velezensis* Y01

[a, b] In the same row, values with the same or no letter superscripts mean no significant difference ($p > 0.05$), while values with different letter superscripts mean significant difference ($p < 0.05$)

A/G ratio, BIL, GT, ALT, AST, ALP, AMY, CK, GLU, calcium and phosphorus levels ($P > 0.05$).

B. velezensis Y01 increases the immune function of Langya chickens

To investigate the effects of *B. velezensis* Y01 on the immune performance of Langya chickens, the immunoglobulins and immune cytokines in the serum of Langya chickens were evaluated. The serum immunoglobulin results were shown in Table 5. Compared to the CON group, the ANT group with aureomycin resulted in a 19.15% decrease in the serum IgE levels ($p < 0.05$), and the BVT group with *B. velezensis* Y01 resulted in a 36.09% increase in the serum IgG levels and a 56.08% increase in the serum IgM levels at 42 day ($p < 0.05$). In addition, the BVT group with *B. velezensis* Y01 showed significantly

Table 5 Effects of dietary *B. velezensis* Y01 supplementation on the immune performance of 1 to 42 d Langya chickens

Items	CON[1]	ANT[2]	BVT[3]	*p*-value
IL−1 (pg/mL)	748.54 ± 115.12	826.15 ± 24.19	867.81 ± 118.78	0.279
IL−2 (pg/mL)	416.51 ± 97.26[b]	389.47 ± 37.99[b]	553.27 ± 74.73[a]	0.026
IL−6 (pg/mL)	51.01 ± 11.48	63.45 ± 8.01	56.12 ± 4.18	0.167
TNF-α (pg/mL)	122.49 ± 9.18	123.60 ± 11.09	130.41 ± 7.21	0.459
IgA (μg/mL)	2104.90 ± 253.31	1935.89 ± 185.91	2207.50 ± 263.94	0.309
IgE (μg/mL)	22.24 ± 2.05[a]	17.98 ± 1.95[b]	21.83 ± 1.32[a]	0.016
IgG (g/L)	39.26 ± 5.42[b]	40.56 ± 4.58[b]	53.43 ± 6.11[a]	0.009
IgM (μg/mL)	3409.27 ± 267.34[b]	4220.21 ± 257.89[b]	5321.25 ± 329.15[a]	0.004

Abbreviations: IL, interleukin; TNF, tumor necrosis factor; Ig, immunoglobulin

[1]CON: the control group was fed with a corn-soybean-based diet

[2]ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin

[3]BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01

[a, b] In the same row, values with the same or no letter superscripts mean no significant difference ($p > 0.05$), while values with different letter superscripts mean significant difference ($p < 0.05$)

higher levels of serum IgE (higher by 17.64%, $p < 0.05$), IgG (higher by 24.13%, $p < 0.05$), and IgM (higher by 20.69%, $p < 0.05$) at 42 day than the ANT group with aureomycin. However, no significant differences were observed in the serum IgA levels within the three groups at 42 day ($P > 0.05$).

The serum immune cytokines results were shown in Table 5. Compared to the CON and ANT group, *B. velezensis* Y01 in the BVT group resulted in a 24.71% increase and a 29.61% increase in the serum IL−2 levels at 42 day, respectively ($p < 0.05$). However, aureomycin had no significant effects on the serum IL−2 levels at 42 day as compared to the CON group ($P > 0.05$). Moreover, no significant differences were found in the serum IL-1, IL-6, and TNF-α levels within the three groups at 42 day ($P > 0.05$).

B. velezensis Y01 alters the cecal microbial composition of Langya chickens

To investigate the effects of *B. velezensis* Y01 on the cecal microbial composition in Langya chickens, we conducted metagenomic sequencing of the cecal content. The relative abundance of cecal microbiota in Langya chickens was presented at the phylum, order, family, and genus levels (Fig. 1). At the bacterial phylum level, Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, and Euryarchaeota were the top five most abundant bacterial phyla in the cecal microbiota within the three groups (Fig. 1A). Bacteroidales, Eubacteriales, unclassified Firmicutes, Campylobacterales, and Lactobacillales were the top five most abundant bacterial orders in the cecal microbiota within the three groups (Fig. 1B), and at the bacterial family level, the top five dominant bacterial families were Bacteroidaceae, Oscillospiraceae, Clostridiaceae, Lachnospiraceae, and Rikenellaceae (Fig. 1C). *Bacteroides* was the most abundant genus in the cecal microbiota within the three groups, followed by *Clostridum*, *Alistipes*, *Phocaeicola*, and *Lachnoclostridium* (Fig. 1D).

There were no differences in the alpha diversity analysis based on shannon index, simpson index, and invsimpson index among the CON, ANT and BVT groups (Fig. 2A), while the three groups were clearly separated in the beta diversity analysis based on PCA (Fig. 2B). In addition, although the CON and ANT groups were much more similar to each other than they were to the BVT group based on NMDS analysis, the ANT and BVT groups as well as the CON and BVT groups were clearly separated (Fig. 2C). Finally, the LEfSe analysis (LDA > 3) was performed to investigate which microbes in the cecal microbiota was mostly impacted by *B. velezensis* Y01. As shown in Fig. 2D, the LEfSe results revealed that *Ligilactobacillus*, *Bacteroidales*, unclassified *Bacteroidales*, unclassified *Lentisphaerae*, *Gemmiger*, *Mediterranea*, *Methanobrevibacter*, and *Lentisphaerae* were significantly enriched in the BVT group, while *Tyzzerella*, *Akkermansia*, *Methanocorpusculum*, *Bilophila*, *Cloacibacillus*, *Chlamydia*, and *Budvicia* were more abundant in the CON group, as well as *Bacteroides*, *Megamonas*, *Oscillibacter*, and *Anaeromassilibacillus* were more abundant in the ANT group.

B. velezensis Y01 alters the metabolic potential of cecal microbial communities in Langya chickens

To investigate the effects of *B. velezensis* Y01 on the metabolic potential of cecal microbial communities, the KEGG and CAZy databases were adopted to predict the gene functional profiling of cecal microbiota. As shown in Fig. 3A, the third-level KEGG pathway analysis exhibited that a total of 23 KEGG pathways were enriched in the BVT group by comparison with the observation for the CON and ANT groups, including amino acid biosynthesis (phenylalanine, tyrosine, tryptophan, lysine), amino acid metabolism (glycine, serine, threonine, glyoxylate, dicarboxylate), organic acid metabolism (pyruvate, butanoate, fatty acid), carbohydrate metabolism (glycolysis, citrate cycle, amino sugar, nucleotide sugar, fructose, mannose, galactose), vitamin metabolism (nicotinate,

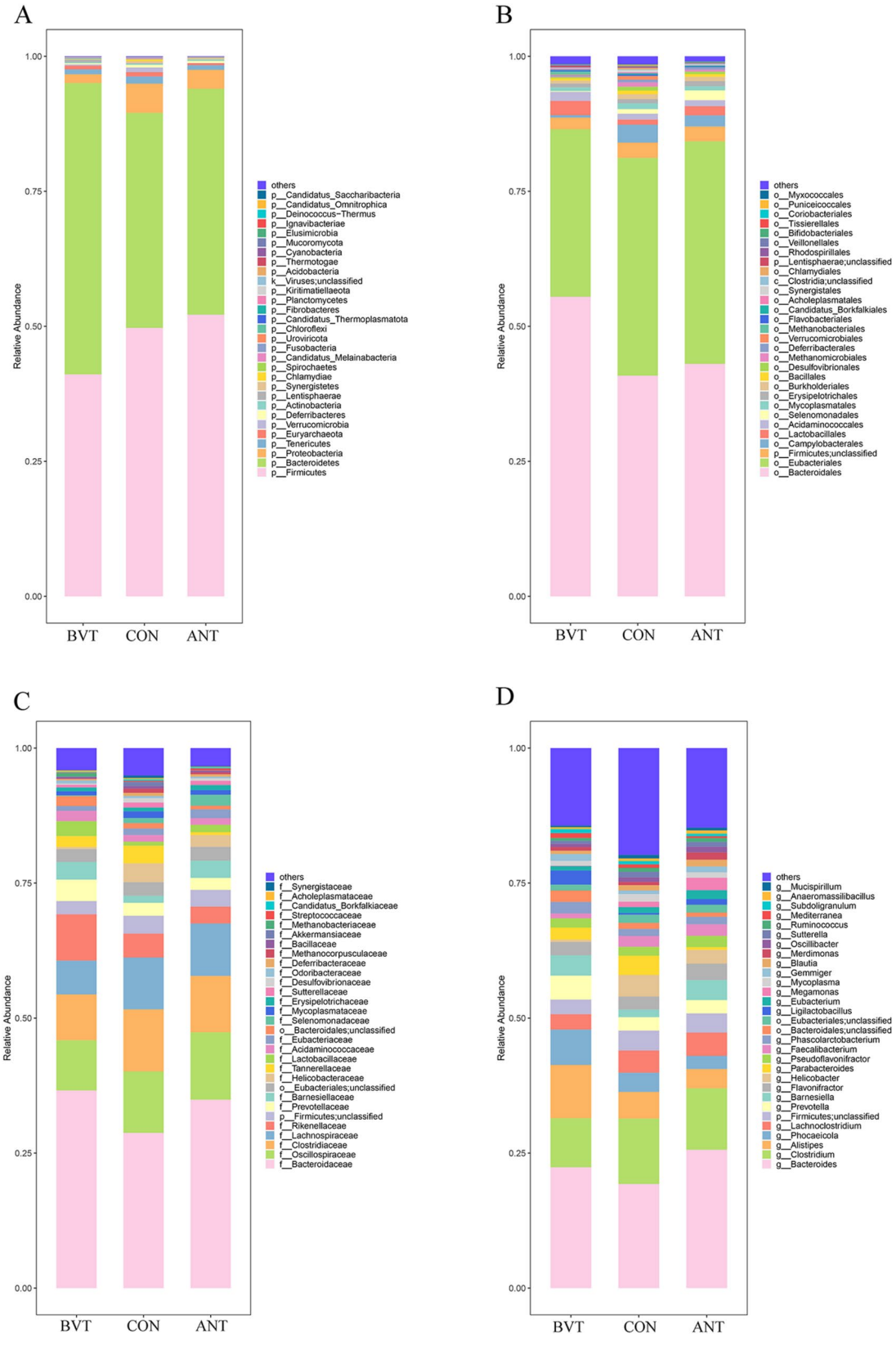

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Fig. 1 Distribution of the cecal microbiota of 1 to 42 d Langya chickens at the phylum, order, family, and genus levels. (**A**) Relative abundance of predominant bacteria at the phylum level; (**B**) Relative abundance of predominant bacteria at the order level; (**C**) Relative abundance of predominant bacteria at the family level; and (**D**) Relative abundance of predominant bacteria at the genus level. CON: the control group was fed with a corn-soybean-based diet; ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin; and BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01

nicotinamide, thiamine), vitamin biosynthesis (pantothenate, folate), and nitrogen base metabolism (pyrimidine, purine). Meanwhile, a total of 11 KEGG pathways were enriched in the ANT group by comparison with the observation for the CON and BVT groups, including carbohydrate metabolism (starch, sucrose, pentose), amino acid metabolism (cysteine, methionine, alanine, aspartate, glutamate), O-antigen nucleotide sugar biosynthesis, quorum sensing, oxidative phosphorylation, and ABC transporters. While the top 18 more abundant KEGG pathways in the CON group than the observation for the ANT and BVT groups were glycerophospholipid, methane and propanoate metabolism, DNA replication, aminoacyl-tRNA biosynthesis, ribosome, protein export, quorum sensing, oxidative phosphorylation, peptidoglycan biosynthesis, bacterial secretion system, two-component system, as well as mismatch, base excision, and nucleotide excision repair.

The gene functional annotation analysis using the CAZy database showed that a total of 25 carbohydrate-active enzymes were enriched in the BVT group, including 19 GHs (glycoside hydrolases, GH33, GH68, GH30, GH18, GH63, GH31, GH57, GH65, GH38, GH29, GH130, GH20, GH26, GH66, GH3, GH95, GH5, GH8, GH43), 5 GTs (glycosyl transferases, GT30, GT26, GT19, GT3, GT2), and 1 CE (carbohydrate esterase, CE1), as well as 20 carbohydrate-active enzymes in the ANT group, including 10 GHs (GH101, GH121, GH32, GH4, GH88, GH23, GH13, GH77, GH105, GH51), 5 GTs (GT4, GT35, GT39, GT1, GT5), 2 PLs (polysaccharide lyases, PL8, PL11), 2 CBMs (carbohydrate-binding modules, CBM48, CBM50), and 1 CE (CE10); while only 5 carbohydrate-active enzymes in the CON group, including 4 GTs (GT28, GT51, GT9, GT66) and 1 CBM (CBM6) (Table 6; Fig. 3B). In addition, the differential carbohydrate-active enzymes that analyzed using Kruskal-Wallis analysis showed that the abundance of 10 carbohydrate-active enzymes was significantly increased in the BVT group in comparison to the CON and ANT groups. These enzymes include GT19, GH95, GH31, GH3, GT25, GT8, GT30, GT21, GH66, and GH30 (Fig. 4A).

B. velezensis Y01 decreases the pathogenicity and antibiotic resistance of cecal microbiota in Langya chickens

To investigate the effects of *B. velezensis* Y01 on the pathogenicity and antibiotic resistance of cecal microbiota, the VFDB and CARD databases were adopted to predict the virulence genes and antibiotic resistance genes of cecal microbial communities. As shown in Fig. 3C, the virulence genes annotation analysis using the VFDB database showed that only 1 virulence gene such as *wbtL* was enriched in the BVT group, 39 virulence gene were enriched in the CON group, including *fliI*, *GBS_RS06585*, *flaG*, *flgL*, *cheA*, *Cj0883c*, *cheV*, *flgC*, *flhB*, *kpsD*, *flgS*, *pseC*, *hldD*, *kpsT*, *ptmA*, *flgE2*, *flgE*, *pseB*, *flhA*, *fliD*, *hddA*, *hldE*, *flhF*, *flgB*, *pseI*, *pflA*, *flgI*, *fliS*, *fliF*, *flgG2*, *cps4L*, *rfbC*, *fliR*, *cheY*, *ciaB*, *flgK*, *Cj1419c*, *gmhA2*, and *motA*, as well as 11 virulence gene were enriched in the ANT group, including *ugd*, *fliN*, *cps4J*, *groEL*, *hasC*, *gmhA*, *wbtE*, *ciaC*, *tufA*, *kpsS*, and *flgK*. These results indicated that dietary *B. velezensis* Y01 supplementation considerably reduced the virulence genes of cecal microbial communities, thereby decreasing the pathogenicity of cecal microbiota in Langya chickens.

The antibiotic resistance genes annotation analysis using the CARD database revealed that 13 antibiotic resistance genes were enriched in the BVT group, including *aac6-I*, *ant9*-I, *aph2-II*, *aph3-VII*, *CfxA2*, *ermF*, *ermT*, *lnuA*, *lnuB*, *macA*, *mefA*, *tet40*, and class A beta-lactamase coding genes, 22 antibiotic resistance genes were enriched in the CON group, including *aadA*, *OXA-61*, *TEM-1*, *catD*, *cat*, *vatB*, *cmeA*, *cmeB*, *emrB*, *qacB*, *qacEdelta1*, *sul1*, *tet44*, *tetA*, *tetL*, *tetO*, *tetX*, *vanR*, *vanS*, *vanU*, bifunctional aminoglycoside N-acetyltransferase and aminoglycoside phosphotransferase coding genes, and tetracycline resistance protein coding genes, as well as 25 antibiotic resistance genes were enriched in the ANT group, including *aadE*, *aph2-IV*, *aph2-Ie*, *aph3-I*, *aph3-III*, *aph6-I*, *bacA*, *TEM-1*, *cat*, *fosB*, *ermB*, *ermX*, *ermG*, *vatB*, *emrA*, *sul2*, *tet32*, *tetM*, *tetQ*, *tetW*, metallo-beta-lactamase coding genes, chloramphenicol exporter coding genes, class A beta-lactamase coding genes, tetracycline resistance protein coding genes, and *16 S* rRNA methylase coding genes (Fig. 3D). As shown in Fig. 4B, the differential antibiotic resistance genes that analyzed using Kruskal-Wallis analysis manifested that the abundance of 5 antibiotic resistance genes was significantly reduced in the BVT group when compared to the CON group, including *aph3-III*, *tetX*, *tet44*, *qacEdelta1*, and *ermB*. Meantime, compared to the ANT group, the abundance of 7 differential antibiotic resistance genes was significantly reduced in the BVT group. These genes include *ermG*, *aph3-III*, *tet44*, *qacEdelta1*, *ermB*, *fosB*, and *16 S* rRNA methylase coding genes (Fig. 4B). These results illustrated that dietary *B. velezensis* Y01 supplementation obviously reduced the antibiotic resistance

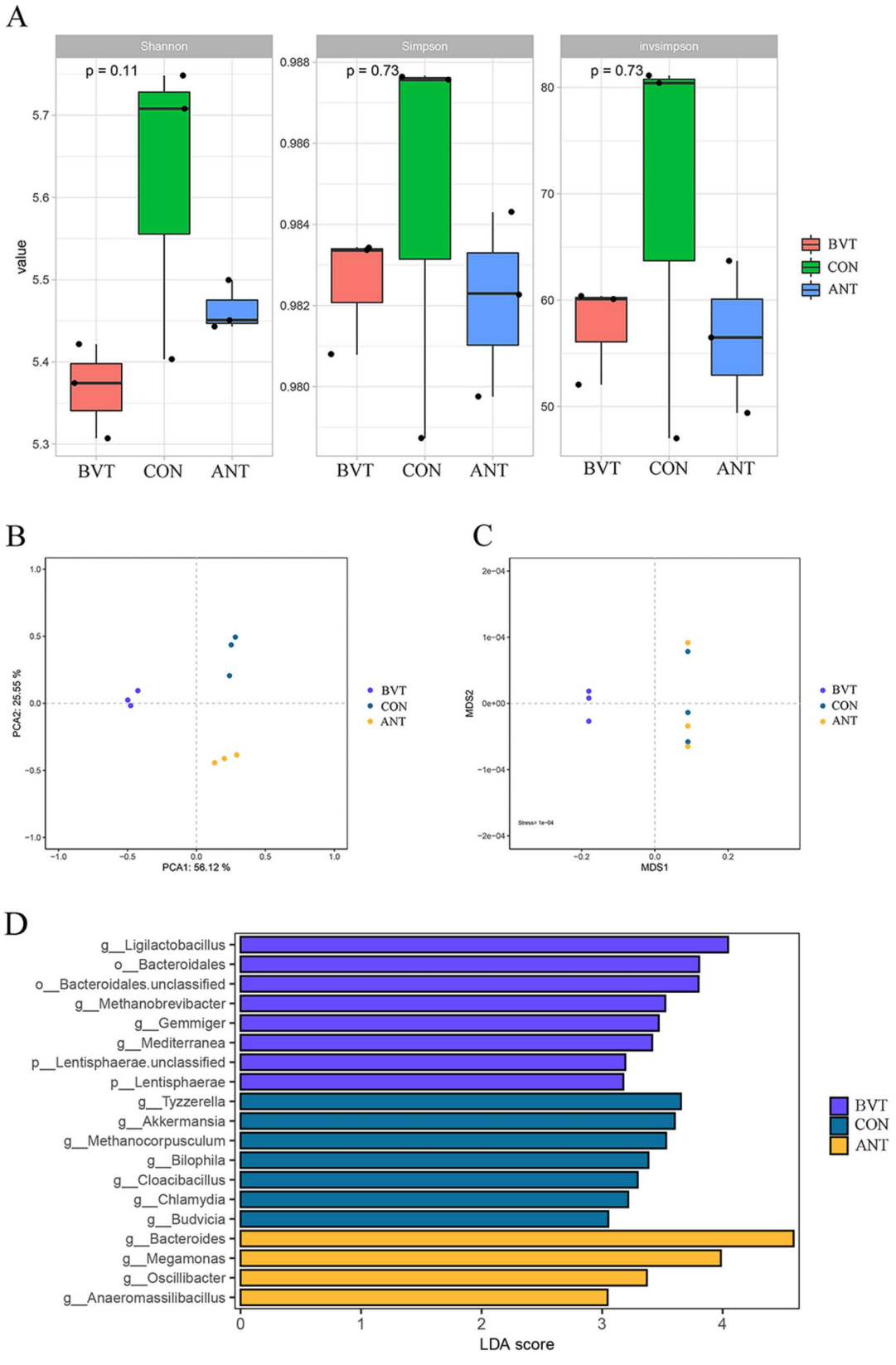

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Fig. 2 *B. velezensis* Y01 alters the cecal microbial composition of 1 to 42 d Langya chickens. (**A**) Shannon index, simpson index, and invsimpson index in alpha diversity analysis of the cecal microbial communities; (**B**) Principal component analysis (PCA) of the cecal microbial communities; (**C**) Non-metric multidimensional scaling (NMDS) analysis of the cecal microbial communities; and (**D**) Linear discriminant analysis with effect size (LEfSe) analysis of the cecal microbial communities via histogram based on the linear discriminant analysis (LDA) score. CON: the control group was fed with a corn-soybean-based diet; ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin; and BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0 × 109 CFU/kg *B. velezensis* Y01

genes of cecal microbial communities, thereby decreasing the antibiotic resistance of cecal microbiota in Langya chickens.

Discussion

In recent years, several studies have confirmed that *B. velezensis* could improve the growth performance of foreign commercial breeds of chickens by increasing nutrient digestibility and immunity capacity and improving intestinal health [4, 16, 17, 20]. However, whether *B. velezensis* affects the growth performance of Chinese local breeds of chickens has not been reported. In the present study, we observed that dietary supplementation with *B. velezensis* Y01 could improve ADG and WG of 42 d Langya chickens fed with a corn-soybean-based diet, but had no obvious effects on decreasing ADFI and F/G, which differed from the above mentioned studies [4, 16, 17, 20]. The possible reasons could be attributable to a plethora of factors, including the different strains, viability, and concentrations of *B. velezensis*, chicken species, and environmental or stress conditions.

Regarding the serum biochemistry indexes, previous studies had confirmed that continuous feeding of *Lactobacillus rhamnosus* MTCC-5897 significantly reduces the activity of serum LDH, which is indicative of normal liver and kidney functions as it is in normal range for mice [25]; and dietary supplementation of 0.2% *B. velezensis* obviously decreases the content of CH in blood plasma, which is helpful for improving the quality of eggs [19]. This is because high levels of blood plasma CH in animals increase the risk of atherosclerosis, and the intake of animal meat and egg products with low CH contents is beneficial to human health [19, 26]. The results from the present study showed that *B. velezensis* Y01 significantly decreased the activity of serum LDH and the serum CH levels in 42 d Langya chickens, which are consistent with previous findings by Bhat and Ye's [19, 25]. Moreover, *B. velezensis* Y01 also significantly decreased the contents of serum UA and Urea in 42 d Langya chickens. Serum UA is a sensitive indicator of serious kidney injury, wherein increasing values reflect UA excretion disorder from injured renal cells; and serum Urea reflects to a certain extent the glomerular filtration function, wherein increasing values indicate that the glomerular filtration function has dropped down to half of the normal value. In our study, *B. velezensis* Y01 did not lead to a significant difference in serum ALB, GLB, TP, BIL, GT, ALT, AST, ALP, AMY, CK, and GLU levels of Langya chickens, which was consistent with Liu's, but differed to Tsai's in serum GLU levels [4, 27]. The above results demonstrated that dietary *B. velezensis* Y01 supplementation could maintain the normal protein and energy metabolism of Langya chickens. However, the different results with Tsai et al. in serum GLU levels might be because dietary supplementation with *B. velezensis* Y01 can produce more GLU by hydrolyzing cellulose, glucan, and arabinoxylan, but it also improves GLU metabolism and accelerates the utilization of GLU in Langya chickens, thereby keeping normal serum GLU levels.

Immune cells and cytokines play considerable roles in the immune modulatory and defense system of an animal [4, 15, 28]. However, immune cells like LYM, NEU, and MONO in the present study were not obviously altered by dietary supplementation with *B. velezensis* Y01. Cytokines include the pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, which can exacerbate inflammation, as well as the anti-inflammatory cytokines like IL-2, IL-4 and IL-10, which can alleviate inflammation and enhance the function of cytotoxic T cells and B cells [29, 30]. In our study, *B. velezensis* Y01 in the BVT group significantly increased the serum IL-2 levels, while not altered the serum TNF-α levels in comparison with the CON and ANT groups, indicating that *B. velezensis* Y01 could enhance the immune function of Langya chickens.

Serum immunoglobulins do not only reflect the humoral immune status, but also play important roles in fighting against various infections in animals [4, 31]. There are 5 kinds of immunoglobulins, in which IgA, IgE, IgG, and IgM mainly represent the immune response of an individual. Several studies had confirmed that dietary probiotics could considerably increase serum immunoglobulin secretion in chickens, thereby enhancing the efficiency of the immune system and boosting body immunity [32, 33]. For example, *Bacillus subtilis* obviously increased the serum IgA and IgG levels at 28 d and the serum IgA levels at 42 d in broiler chickens [33]; and *B. velezensis* KNF-209 significantly improved the serum IgA and IgM levels in broiler chickens [4]. Similar results were got in the present study, *B. velezensis* Y01 significantly increased the serum IgG and IgM levels in Langya chickens as compare to the CON and ANT groups. The increased immunity may be because the biomolecules produced by *B. velezensis* and intestinal microbiota could stimulate the gut-associated immunity, including

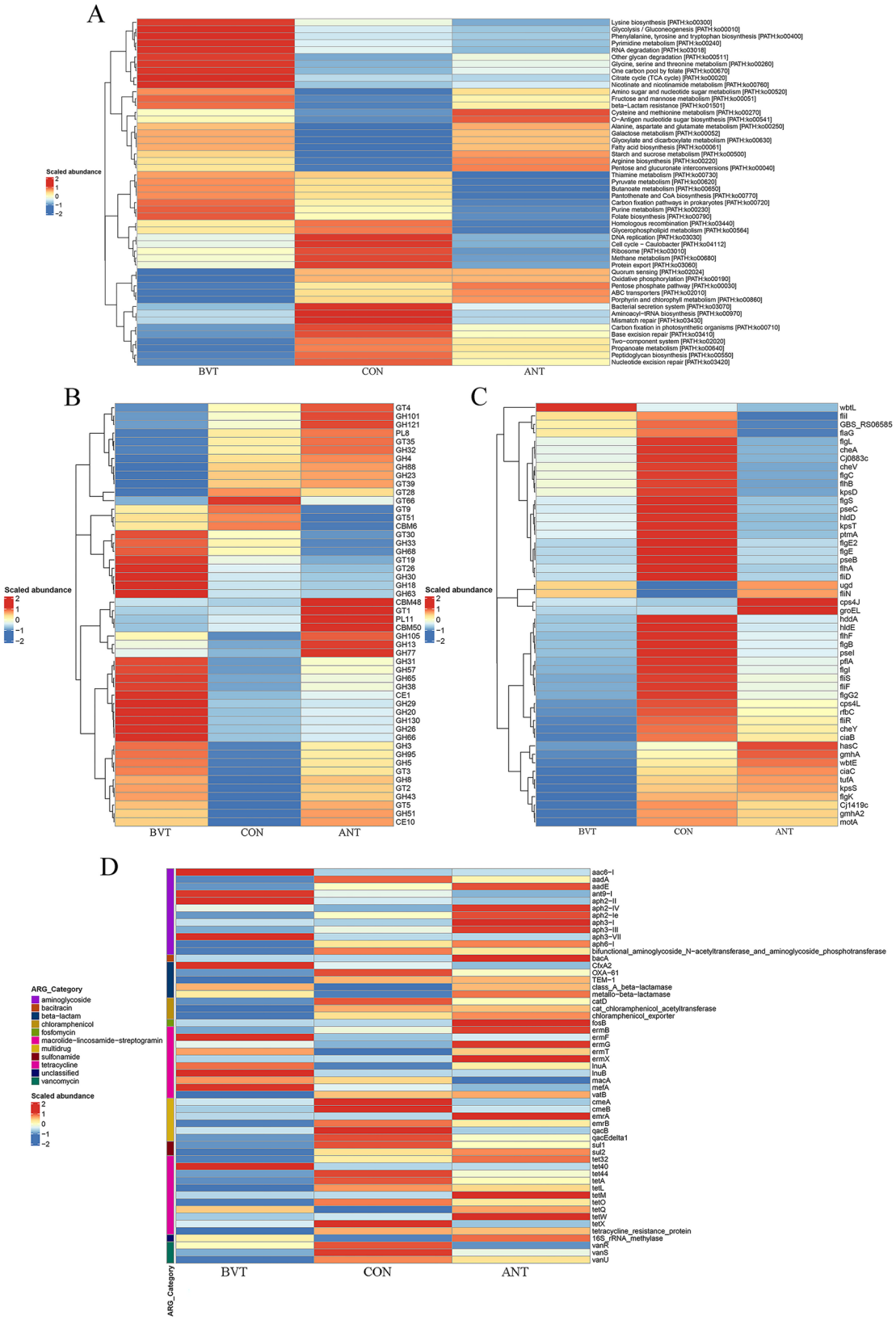

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Fig. 3 The functional genes annotation analysis of the cecal microbial communities of 1 to 42 d Langya chickens. (**A**) The third-level KEGG pathway analysis in predicted metabolic pathway of cecal microbiota; (**B**) The carbohydrate-active enzymes annotation analysis using the CAZy database in predicted metabolic function of cecal microbiota; (**C**) The virulence genes annotation analysis using the VFDB database in predicted the pathogenicity of cecal microbiota; and (**D**) The antibiotic resistance genes annotation analysis using the CARD database in predicted the antibiotic resistance of cecal microbiota. CON: the control group was fed with a corn-soybean-based diet; ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin; and BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01

proteinaceous molecules and microbial metabolites, thereby improving the growth performance of Langya chickens [32].

Cecum is a main site of fermentation and microbial colonization, which influences animal health and production [7]. Cecal microbiota is a highly complex ecosystem, which plays a crucial role in the animal's physiological function, nutrient digestion and absorption, energy homeostasis, and immune response, thus enhancing animal growth and maintaining intestinal health [23, 34]. Some studies have demonstrated that *B. velezensis* can maintain the balance of the animal's intestinal microbiota by enhancing the advantage of intestinal beneficial bacteria and reducing the colonization of intestinal harmful bacteria [7, 9, 16]. In our study, Firmicutes and Bacteroidetes were found to be the top two dominant phyla in cecal microbiota of Langya chickens. Both phyla could ferment nondigestible polysaccharides into short-chain fatty acids, including acetic acid, propionic acid, and butyric acid, which contribute to nutrient absorption and energy transformation for maintaining animal intestinal integrity [35, 36]. Moreover, the LEfSe results revealed that *B. velezensis* Y01 significantly changed the cecal microbial composition of Langya chickens by enriching the abundance of *Ligilactobacillus* and *Bacteroidales*. *Ligilactobacillus* belongs to a member of the family Lactobacillaceae, which contributes to maintaining the balance of intestinal microbiota, as well as improving the antioxidant ability and inhibiting the growth of pathogenic bacteria in the intestine [9, 37]. *Bacteroidales* are important groups of intestinal microorganisms that play a key role in immunity and metabolism [23, 38, 39]. These findings confirmed that dietary supplementation with *B. velezensis* Y01 considerably altered the cecal microbial composition, which may have a positive impact on the cecal microbial functions in Langya chickens.

In the current study, the KEGG classification analysis of cecal microbial communities showed that pathways of metabolites, including that of carbohydrate, amino acids, organic acids, and vitamins, were significantly enhanced by dietary supplementation with *B. velezensis* Y01. The results stayed in line with that of previous studies that intestinal microbial communities transmitted metabolic benefits to animals through the production of amino acids, organic acids, and vitamins, thus determining their health [40, 41]. Furthermore, analyses of predicted functions of cecal microbial communities indicated that *B. velezensis* Y01 enriched total of 25 carbohydrate-active enzymes, including 19 GHs, 5 GTs, and 1 CE, which have an crucial role in hydrolyzing amylose, cellulose, glucan, fucose, mannose, and xylose, thus enhancing nutrient digestion and absorption function of Langya chickens. Collectively, these findings illustrated that *B. velezensis* Y01 significantly altered the metabolic potential of the cecal microbial communities, which is helpful for improving nutrient digestion and absorption function in Langya chickens, thereby promoting their growth.

Previous studies have verified that *B. velezensis* has bactericidal effect on pathogen by producing secondary metabolites, including surfactin, butirosin, bacilysins, fengycin, and bacillibactins, among others, thereby reducing their colonization in the host [16, 42]. The pathogenesis of pathogen is complex and involves various bacterial virulence factors coded by a variety of virulence genes [43, 44]. In our study, the virulence genes annotation analysis showed that only 1 virulence gene such as *wbtL* was enriched by dietary supplementation with *B. velezensis* Y01. It is reported that the intestinal microbiota is a key reservoir for antibiotic resistance genes [38, 45]. The spread of antibiotic resistance genes is a major public health crisis, with the ongoing spread of antibiotic resistance genes leading to decreased efficacy of antibiotic treatments [38, 46]. In this study, *B. velezensis* Y01 considerably decreases antibiotic resistance genes being enriched in the BVT group (11 antibiotic resistance genes) in comparison with both the CON group (22 antibiotic resistance genes) and the ANT group (25 antibiotic resistance genes). These results indicated that *B. velezensis* Y01 reduced the virulence genes and antibiotic resistance genes of cecal microbial communities, thus decreasing the pathogenicity and antibiotic resistance of cecal microbiota in Langya chickens. This may be because *B. velezensis* Y01 produces secondary metabolites that inhibit the growth of harmful bacteria and increases the production of immunoglobulins and anti-inflammatory cytokines that target pathogens, particularly antibiotic-resistant pathogens, thereby diminishing the overall load of virulence genes and resistance genes in cecal microbiota. These findings imply that dietary supplementation with *B. velezensis* Y01 reduces pathogen colonization of cecal microbiota, thereby decreasing the intestinal disease incidence in Langya chickens, however, the potential effects are not indicative of long-term sustainability, as they depend on several factors, including

Table 6 The functional description of CAZy of cecal microbial community in Langya chickens

CAZy	Description	CAZy	Description
GT30	alpha-3-deoxy-D-manno-octulosonic-acid transferase	GH32	beta-(2,1)-fructosidase/ 1-exohydrolase
GH33	2-keto-3-deoxynononic acid hydrolase	GH101	endo-alpha-N-acetyl galactosaminidase
GH68	levansucrase	GH121	beta-L-arabinobiosidase
GH63	glucosylglycerate hydrolase	GH4	maltose–6-phosphate glucosidase
GT26	beta-1,4- glucosyltransferase	GT35	alpha-L-arabinofuranosidase
GH30	endo-beta-1,4-xylanase	GT4	sucrose synthase
GH18	lysozyme	PL8	hyaluronate lyase
GT19	lipid-A-disaccharide synthase	GH88	d–4,5-unsaturated beta-glucuronyl hydrolase
GH31	alpha-glucosidase	GH23	lysozyme type G
GH57	alpha-amylase	GT39	alpha-mannosyltransferase
GH65	alpha-trehalase	CBM48	GH13-binding modules
GH38	alpha-mannosidase	GT1	UDP-glucuronosyltransferase
CE1	acetyl xylan esterase	PL11	rhamnogalacturonan endolyase
GT28	beta-N-acetyl glucosaminyltransferase	CBM50	GH18, GH19, GH23, GH24, GH25, GH73-binding modules
GH130	beta-1,4-mannosylglucose phosphorylase	GH105	unsaturated rhamnogalacturonyl hydrolase
GH20	beta-hexosaminidase	GH13	maltogenic amylase
GH26	beta-mannanase	GH77	amylomaltase
GH66	dextranase	GT5	murein polymerase
GH3	beta-1,4-xylosidase	GH51	endoglucanase
GH95	alpha-L-fucosidase	CE10	arylesterase
GH8	cellulase	GH29	alpha-L-fucosidase
GT3	glycogen synthase	GT66	glycotransferase
GH5	endo-beta-1,4-glucanase/ cellulase	GT9	lipopolysaccharide N-acetylglucosaminyltransferase
GT2	cellulose synthase	GT51	murein polymerase
GH43	alpha-L-arabinofuranosidase	CBM6	cellulose-binding modules

Abbreviations: CAZy, carbohydrate-active enzymes; GT, glycosyl transferases; CE, carbohydrate esterases; GH, glycoside hydrolases; PL, polysaccharide lyases; CBM, carbohydrate-binding modules

continued probiotic administration, the evolutionary pressure from antibiotic use, and the dynamics of cecal microbiota.

Taken together, *B. velezensis* Y01 improved the growth, promoted immune function, positively modulated the cecal microbial composition, and decreased the relative abundance of harmful bacteria in the cecum of Langya chickens.

Conclusions

The present study demonstrated that dietary supplementation with *B. velezensis* Y01 resulted in a 7.01% increase in the final body weight, a 7.27% increase in weight gain (WG), and a 7.24% increase in average daily gain (ADG) in the BVT group when compared to the CON group ($p < 0.05$). Meantime, the final body weight was increased by 5.88%, WG by 6.18%, and ADG by 6.19% in the BVT group when compared to the ANT group ($p < 0.05$). The serum immunoglobulin G (IgG) levels were increased by 36.09%, IgM by 56.08%, and interleukin-2 (IL-2) by 32.83% in the BVT group in comparison to the CON group ($p < 0.05$). Compared to the ANT group, the BVT group with *B. velezensis* Y01 resulted in a 17.64% increase in the serum IgE levels, a 24.13% increase in the serum IgG levels, and a 20.69% increase in the serum IgM levels($p < 0.05$). The mechanism may be related to alterations of cecal microbial composition, increases in carbohydrate-active enzymes, and maintenance the balance of

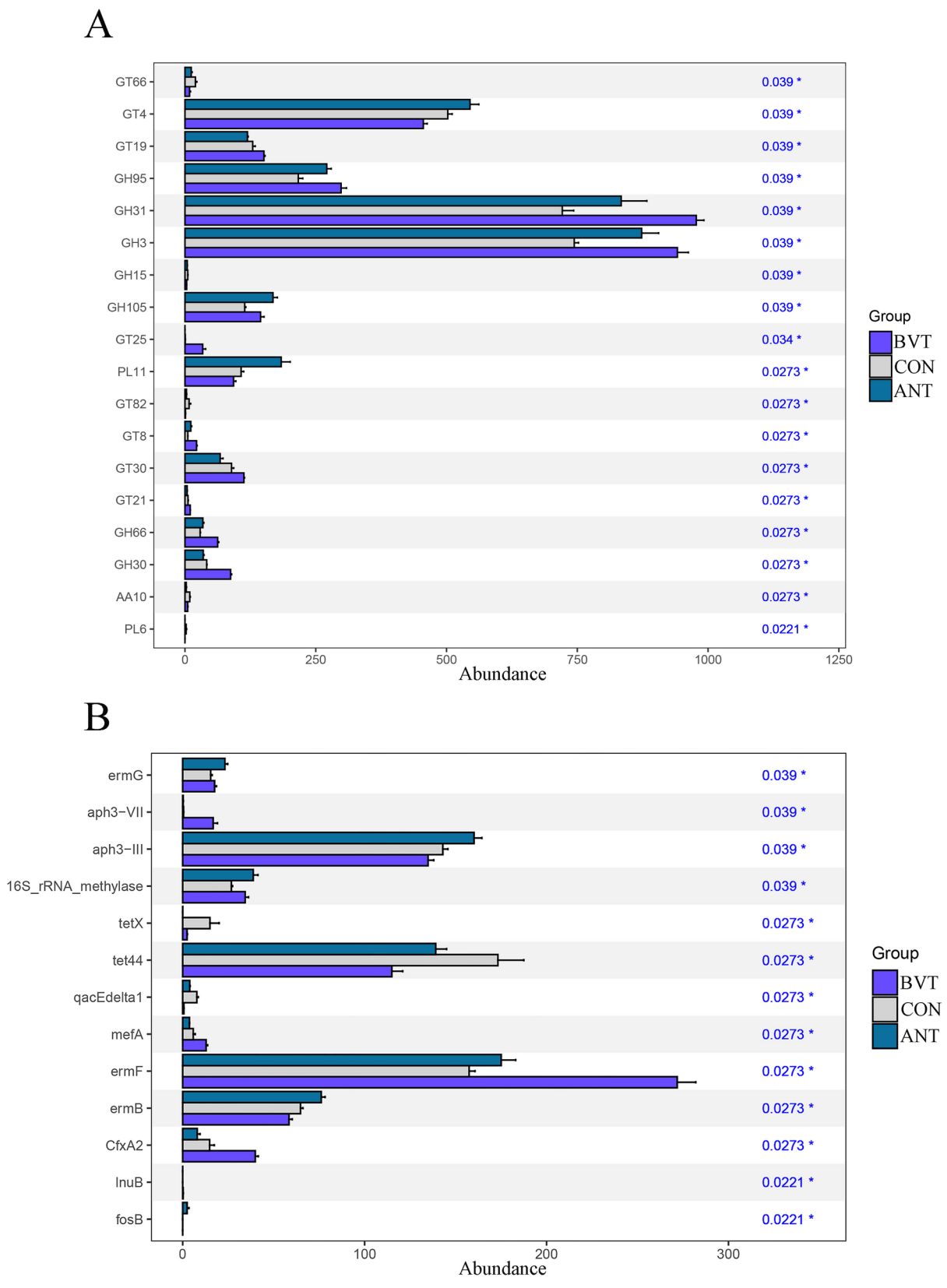

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Fig. 4 The differential carbohydrate-active enzymes and antibiotic resistance genes of 1 to 42 d Langya chickens using Kruskal-Wallis analysis. (**A**) The differential carbohydrate-active enzymes using Kruskal-Wallis analysis; and (**B**) The differential antibiotic resistance genes using Kruskal-Wallis analysis. CON: the control group was fed with a corn-soybean-based diet; ANT: the antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin; and BVT: the *B. velezensis*-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg *B. velezensis* Y01

cecal microbiota by increasing the colonization of beneficial bacteria and reducing the colonization of harmful bacteria in the cecum.

Abbreviations

B. velezensis	Bacillus velezensis
CON	The control group was fed with a corn-soybean-based diet
ANT	The antibiotic-treated group was fed with the corn-soybean-based diet supplemented with 50 mg/kg aureomycin
BVT	The B. velezensis-treated group was fed with the corn-soybean-based diet supplemented with 2.0×10^9 CFU/kg B. velezensis Y01
WG	Weight gains
ADG	Average daily gains
ADFI	Average daily feed intake
F G	Feed-to-gain ratio
RBC	Red blood cell count
HGB	Hemoglobin concentration
LYM	Lymphocytes
WBC	White blood cell count
NEU	Neutrophile granulocytes
ALT	Alanine aminotransferase
AST	Aspartate amino transaminase
ALP	Alkaline phosphatase
LDH	Lactate dehydrogenase
UA	Uric acid
CH	Cholesterol
Ig	Immunoglobulin
IL	Interleukin
TNF	Tumor necrosis factor
CAZy	Carbohydrate-active enzymes
GT	Glycosyl transferases
CE	Carbohydrate esterases
GH	Glycoside hydrolases
PL	Polysaccharide lyases
CBM	Carbohydrate-binding modules
PCA	Principal component analysis
NMDS	Non-metric multidimensional scaling
LDA	Linear discriminant analysis
KEGG	Kyoto encyclopedia of genes and genomes
CAZy	Carbohydrate-active enzymes database
CARD	Comprehensive antibiotic resistance database
VFDB	Virulence factors of pathogenic bacteria

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Author contributions
LY: investigation, methodology, experiments, writing original draft, writing review and editing, data curation and funding acquisition. ZB, JX and HF: methodology and experiments. LZ, SZ and YZ: formal analysis, methodology. XZ: conceptualization, supervision and funding acquisition. All authors read and approved the final manuscript.

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Data availability
The sequencing data for this study have been deposited in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). This data are available in the NCBI database with accession number PRJNA1226743 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1226743).

Declarations

Ethics approval and consent to participate
This study was carried out in compliance with the ARRIVE guidelines. We had obtained informed consent from Rizhao Langya Chicken Corporation LTD. to use the animals in this study. Animal experiments were conducted under animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Linyi University (Protocol Approval Number: LYU20240117). All animal work was carried out following accordance within the guidelines of the Laboratory Animal Research Center of Linyi University.

Consent for publication
Not applicable.

Conflict of interest
The authors declare no competing interests.

Clinical trial number
Not applicable.

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