Dietary administration of *Bacillus subtilis* KC1 improves growth performance, immune response, heat stress tolerance, and disease resistance of broiler chickens

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ABSTRACT The purpose of the present study was to evaluate the probiotic properties of *Bacillus subtilis* KC1 as a feed additive in the poultry feed. Effects of the *Bacillus subtilis* supplementation on growth performance, heat-stress tolerance, resistance to *Mycoplasma gallisepticum* (**MG**) and *Salmonella* Pullorum challenge of broilers were determined. The protective effects of the *Bacillus subtilis* on liver function and immune response of broilers challenged with Aflatoxin B1 (**AFB1**) were also scrutinized. The results showed that the *Bacillus subtilis* supplementation could improve growth performance, increased body weight, relative weight of the immune organ and dressing percentage, and decrease feed conversion ratio. In addition, the *Bacillus subtilis* supplementation alleviated adverse effects caused by heat stress, MG, and *Salmonella* Pullorum challenge. Furthermore, the *Bacillus subtilis* supplementation resulted in improved liver function and enhanced immune response of broilers challenged with AFB1. In conclusion, these results suggested a tremendous potential of *Bacillus subtilis* KC1 as a feed additive in the poultry feed.

Key words: *Bacillus subtilis*, *Salmonella* Pullorum, *Mycoplasma gallisepticum*, heat stress, Aflatoxin B1

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INTRODUCTION

Dietary administration of antibiotics was first employed and confirmed to promote the growth of chickens in 1946 (Moore et al., 1946). Since then, the subtherapeutic antibiotics have been routinely used as growth promoters in poultry production for decades (Wang et al., 2020a). However, the continuous long-time exposure of commensal microbiota and pathogens to subtherapeutic antibiotics in chickens were relevant to rapid spread for antibiotic-resistant strains (Robinson et al., 2018). The increased emergence of antibiotic-resistant pathogens not only complicated the treatment for bacterial infections of chickens, but also caused a huge threat to public health (Huang et al., 2017; Robinson et al., 2018; Wang et al., 2020a). China and European Union have banned growth-promoting antibiotics used as feed additives in 2020 and 2006, respectively (Guo et al., 2020), which contributed to control the spread of antibiotic-resistant strains (Wang et al., 2020c). Besides, it has also brought great challenges to the poultry production, such as decreased growth performance and increased bacterial infection in chickens (Khalique et al., 2020; Wang et al., 2021c). Therefore, the development of safe and reliable alternatives to growth-promoting antibiotics has become a necessary goal.

Probiotics are defined as a culture of live microorganisms that can enter the digestive tract alive and confer benefits to the host when applied to human and animals (Wang et al., 2020a). Dietary supplementation of probiotics is a strategy that can enhance nutrient absorption, improve gut microbiota composition, and innate immunity, thus contribute to improved growth performance, stress tolerance, and bacterial infection resistance in chickens (Guo et al., 2020; Wang et al., 2020a, 2021b). Compared to other candidates, *Bacillus subtilis* was considered as a reliable probiotic, which has great potential as an antibiotic substitute for feed additives, due to the growth promotion and disease prevention, resistance to environmental change, and long-term storage at ambient temperature (Guo et al., 2017; Wang et al., 2021c). *Bacillus subtilis* not only regulated some nutrients

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production, such as amino acids, nucleotides, and fatty acids to improve growth performance (Park et al., 2020), but also increased the abundance of beneficial *Lactobacillus* and *Bifidobacterium* in gut by consuming the free oxygen (Latorre et al., 2015; Yang et al., 2016). Thus, isolation and characterization of suitable *Bacillus subtilis* is a potential direction for the development of alternatives to antibiotic growth promoters.

Heat stress is one of the most common environmental stressors for poultry industry, and has negative influences on animal physiology, health, and productivity (Wang et al., 2020a). *Mycoplasma gallisepticum* (**MG**) is one of the most widespread avian pathogens in chicken farms all over the world and responsible for decreased production performance and immunosuppression of chickens (Wang et al., 2021a). *Salmonella* is a food-borne disease-causing zoonotic pathogen, which not only can cause huge economic loss in poultry industry, but also a threat human health through contamination and consumption of partially cooked chicken meat (Lan et al., 2020). Aflatoxin-B1 (**AFB1**) is a common contaminant in chicken feed, which can cause liver damage and immunosuppression (Li et al., 2021; Zhu et al., 2017). Recently, a strain of *Bacillus subtilis* KC1 was isolated, and the present study was to further evaluate whether feeding *Bacillus subtilis* can improve production performance, promote heat stress tolerance, enhance *Salmonella* and MG infection resistance, and alleviate abnormal liver function and immunosuppression caused by AFB1.

MATERIALS AND METHODS

Chickens

All animal experiments were approved by Shanxi Agricultural University Animal Care and Use Committee (Shanxi, China) in accordance with Laboratory Animal-Guideline for ethical review of animal welfare (GB/T35892-2018, National Standards of the People's Republic of China). A total of 760 one-day-old commercial Arbor Acres (**AA**) broilers were purchased from a local commercial hatchery (JinNong, Taigu, Shanxi, China). Room temperature was set to 35 ± 1°C for the first week and gradually decreased to 22 ± 2°C. Water was provided ad libitum and fed control diet (Table 1). All broilers were checked negative to MG, *Salmonella* Pullorum, and other major pathogens.

Bacillus subtilis *and Culture Condition*

The *Bacillus subtilis* KC1 (Genbank no. OL721931) was used in the present study, which was isolated from the feces of healthy chickens. A single colony of the *Bacillus subtilis* was cultured in modified liquid medium (glucose 2.5 g, yeast extract 2.5 g, peptone 2.5 g, beef paste 2.5 g, NaCl 2.5 g in 500 mL of sterilized water with pH = 7.2; Guo et al., 2017), then the *Bacillus subtilis* was added in the control diet to a final concentration of 10^7, 10^8, and 10^9 CFU/kg feed.

Table 1. List of ingredients in the control diet.

Ingredients	0−42 d	Calculated nutrient levels	0−42 d
Corn	59.188	Metabolizable energy (Mcal/kg)	3.15
Soybean meal	31.900	Crude protein (g/kg)	200.00
Soybean oil	5.150	Total lysine (g/kg)	10.50
$CaHPO_4$	1.698	Total methionine (g/kg)	5.00
Limestone flour	1.095	Total methionine + cysteine (g/kg)	7.60
NaCl	0.350	Available phosphorus (g/kg)	4.00
DL-Methionine	0.248	Calcium (g/kg)	9.00
L-Lysine HCl	0.034		
Vitamin premix[a]	0.020		
Mineral premix[b]	0.200		
Choline chloride	0.100		

[a]Provided the following (per kg of complete diet): vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin E, 15 IU; vitamin K, 2.65 mg; vitamin B1, 2 mg; vitamin B12, 0.02 mg; biotin, 0.35 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; nicotinic acid, 50 mg.

[b]Provided the following (per kg of complete diet): Cu (as copper sulfate), 8 mg; Zn (as zinc sulfate), 75 mg; Fe (as ferrous sulfate), 80 mg; Mn (as manganese sulfate), 80 mg; I (as potassium iodide), 0.35 mg; Se, (as sodium selenite) 0.15mg.

Trial 1: Bacillus subtilis *Isolation and Probiotic Potency Assay*

Bacillus subtilis *Isolation* *Bacillus subtilis* was isolated from the feces of healthy chickens. Five grams feces were suspended to 50 mL sterile phosphate buffer solution (**PBS**) and mixed fully, then the mixture was incubated at 60°C for 3 h. After incubation, the mixture was diluted and spread on modified agar solid medium (glucose 2.5 g, yeast extract 2.5 g, peptone 2.5 g, beef paste 2.5 g, NaCl 2.5 g, and agar powder 10 g in 500 mL of sterilized water with pH = 7.2) at 37°C for 36 to 72 h according to a previous study (Guo et al., 2017). Suspected colony was collected and grown in liquid medium, bacterial DNA was extracted using TIANamp bacterial DNA Kit (Tiangen, Beijing, China) according to the manufacturer's guidelines. PCR amplification using 16S rRNA common primers, primer sequence as 27F: 5'-AGAGTTTGATCCTGGCTCA G-3'; 1492R: 5'-GGTTTACCTTGTTACGACT T-3' (Guo et al., 2017). The PCR product was sequenced at Beijing Genomics Institute (Beijing, China), and the nucleotide sequence was blasted at the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/B last.cgi).

Acid and Bile Tolerance Assay Evaluation of acid and bile tolerance of *Bacillus subtilis* were carried as previously described (Wang et al., 2020a). Briefly, 10^7 CFU *Bacillus subtilis* were grown in normal agar medium and agar medium which were adjusted to pH = 1.0, 2.0, 3.0, or contained 0.3% bile salt, respectively. After incubation at 37°C for 3 h, the CFU/mL of *Bacillus subtilis* was assessed to determine acid/bile tolerance.

Antimicrobial Activity Assay The antimicrobial activity of *Bacillus subtilis* was performed as described previously (Wang et al., 2020a). Briefly, 10^6 CFU target strain was grown in liquid medium (glucose 2.5 g, yeast extract 2.5 g, peptone 2.5 g, beef paste 2.5 g, NaCl 2.5 g in 500 mL of sterilized water with pH = 7.2), followed by embedding with sterilized Oxford cups into the agar

plate. Hundred μL of 10^7 CFU *Bacillus subtilis* was added to each Oxford cup and the diameter of the inhibition zone was calculated after 24 h incubation at 37°C. Target strains including *Escherichia coli* (CVCC1553), *Campylobacter jejuni* (CVCC3883), *Salmonella* Pullorum (CVCC1789), *Salmonella* Typhimurium (CVCC2220), and *Clostridium perfringens* (CVCC1125) were purchased from China Veterinary Culture Collection Center. It is worthy to mention that the above bacteria are common pathogenic bacteria of chickens.

AFB1 Degradation Ability Assay The assessment of AFB1 degradation ability of *Bacillus subtilis* was performed as described previously (Wang et al., 2019a). Briefly, 5×10^6 CFU *Bacillus subtilis* were grown in agar liquid medium and incubated with 5 μg AFB1 at 37°C for 0, 12, 24, 36, 48, and 72 h, respectively. The content of AFB1 was detected by a high-performance liquid chromatography (**HPLC**) method (Wang et al., 2019). Briefly, AFB1 was extracted by chloroform at least 5 times and condensed in methanol, then filtered by 0.22 μm membrane at least 3 times and determined by HPLC (Waters, Shanghai, China) equipped with a C18 column (5 μm, 4.6 × 150 mm, Waters). The mobile phase was composed of water and acetonitrile (40:60, v/v).

Trial 2: Effects of Bacillus subtilis *Supplementation on the Growth Performance, Relative Weight of Immune Organ, and Dressing Percentage of Broilers*

Animal Experiments Design Two hundred 1-day-old broilers were randomly allotted to 4 experimental groups in 5 replicate groups and 10 broilers per replicate group. Broilers in control group were fed control diet (Table 1) during experiment period; broilers in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^7, 10^8, and 10^9 CFU/kg feed during experiment period, respectively. Samples including immune organ tissues (spleen, bursa of Fabricius, and thymus), breast muscle tissues, and thigh muscle tissues were collected at 6 wk of age for further analysis.

Growth Performance, Relative Weight of Immune Organ, and Dressing Percentage Evaluation All broilers were euthanized by cervical dislocation at 6 wk of age. Final body weight of chickens was recorded individually. Total gain weight of chickens and feed consumption were recorded, and the feed conversion ratio (**FCR**) was calculated as follows: FCR = total feed consumption (g)/total gain weight (g). Immune organ including spleen, bursal, and thymus tissues were collected and weighed individually, the relative weight of immune organ (immune organ weight/body weight, mg/g) was calculated and recorded (Wang et al., 2020a). Heads, feet, and organs of chickens were removed manually and weighed to determine the eviscerated yield percentage (**EYP**); breast and thigh muscles of chickens were removed manually and weighed to calculate the breast muscle percentage (**BMP**) and thigh muscle percentage (**TMP**) as described earlier (Chen et al., 2018).

Trial 3: Effects of Bacillus subtilis *Supplementation on the Cardiac Response of Broilers to Acute Heat Stress*

Animal Experiments Design Eighty 1-day-old broilers were randomly divided into 8 experimental groups (n = 10). Broilers in control group were fed control diet (Table 1) during experiment period; broilers in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period. Twenty eight-days-old broilers in indicated groups were moved to preheated air chamber (Suzhou Fengshi Laboratory Animal Equipment Co. Ltd, China) at 38 ± 1°C for 0, 1, 3, and 10 h, respectively (Xu et al., 2019; Wang et al., 2020a). Then, samples including heart tissues and serum were collected for further analysis.

Myocardial Enzymes and Oxidative Stress Indicators Examination Blood samples were collected and centrifuged at 1,500 rpm for 5 min, the supernatants were used to test creatine kinase (**CK**, A032-1-1), myocardial CK (**CKMB**, H197), lactic dehydrogenase (**LDH**, A020-2-2) activities (Jiancheng Institute of Bioengineering, Nanjing, China) by using commercial kits. Heart tissues were collected and homogenized with 9 volumes of PBS buffers, and centrifuged at 1,200 rpm for 10 min. The supernatants were used to detect malondialdehyde (**MDA**, A003-1-2) content, catalase (**CAT**, A007-1-1) activity, superoxide dismutase (**SOD**, A001-3-2) activity (Jiancheng Institute of Bioengineering) by using commercial kits.

Detection of Heat Shock Proteins Expression Levels The quantitative real-time PCR (**qRT-PCR**) was conducted as described previously (Wang et al., 2020a). Briefly, total RNA was extracted using RNAiso Plus (Takara, Beijing, China) according to the manufacturer's guidelines. Two hundred ng total RNA were reverse transcribed using the HiScript cDNA Synthesis Kit (Vazyme, Nanjing, China). The heat shock proteins (**Hsps**) mRNA expression levels were examined by ChamQ Universal qPCR Master Mix (Vazyme) on a Roche 480 real-time PCR system thermocycler (Roche, Shanghai, China). Each sample was analyzed 3 times and the mRNA expression of the target genes were analyzed by $2^{-\triangle\triangle Ct}$ method (Bustin et al., 2009), following normalization with β-actin gene. The used primers are shown in Table 2.

Trial 4: Effects of Bacillus subtilis *Supplementation on the* Mycoplasma gallisepticum *Infection Resistance of Broilers*

Animal Experiments Design Two hundred 1-day-old broilers were randomly assigned into 4 experimental groups in 5 replicates (10 chickens/replicate). Broilers in the control group were fed control diet (Table 1) during

Table 2. Primers used in qRT-PCR.

Gene	Primer sequence (5'-3')	References
β-actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	(Wang et al., 2020a)
CRYAB	F: TCATGGGAAACACGAGGAGC R: ACACAGCAAACTTTCGTGGC	(Wang et al., 2020a)
Hsp 27	F: ACACGAGGAGAAACAGGATGAG R: ACTGGATGGCTGGCTTGG	(Wang et al., 2020a)
Hsp70	F: TGTGTCCATCCTTACCATTGAG R: GCTTGTGCTTACCCTTGAACTC	(Wang et al., 2020a)
AvBD3	F:ATGCGGATCGTGTACCTGCTC R:CAGAATTCAGGGCATCAACCTC	(Wang et al., 2021b)
AvBD9	F:GCAAAGGCTATTCCACAGCAG R:AGCATTTCAGCTTCCCACCAC	(Wang et al., 2021b)
AvBD10	F:TGGGGCACGCAGTCCACAAC R:ATCAGCTCCTCAAGGCAGTG	(Wang et al., 2021b)
Claudin1	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	(Wang et al., 2020a)
Occludin	F: TCGTGCTGTGCATCGCCATC R: CGCTGGTTCACCCCTCCGTA	(Wang et al., 2020a)
ZO-1	F: GCGCCTCCCTATGAGGAGCA R: CAAATCGGGGTTGTGCCGGA	(Wang et al., 2020a)

experiment period; broilers in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period; broilers in the *Mycoplasma gallisepticum* (**MG**) group were fed control diet (Table 1) during experiment period and challenged with 1×10^9 color change unit (**CCU**) mL^{-1} MG in air sac as reported previously at 1 wk of age (Wang et al., 2021a); broilers in the 10^8 *Bacillus subtilis* + MG group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period and challenged with 1×10^9 CCU mL^{-1} MG in air sac as reported previously at 1 wk of age (Wang et al., 2021a). Samples including lung tissues, breast muscle tissues, and thigh muscle tissues were collected at 6 wk of age for further analysis.

MG Detection The *mgc2* gene copy of MG was measured by quantitative PCR (**qPCR**) using a standardized PCR amplicon to establish a standard curve according to a previous study (Wang et al., 2020b). DNA was extracted from lung using TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (Takara, Beijing, China) according to the manufacturer's guidelines. The qPCR was performed on LightCycler 480 instrument, and the reaction mixture were 10 μL qPCR Mix (Vazyme), 10 μM of each forward and reverse primer (Table 2), 1 μL template DNA and 8 μL ddH2O.

Growth Performance and Dressing Percentage Evaluation Growth performance and dressing percentage evaluation were performed as same as described in *Trial 2*.

***Detection of Host Defense Peptides* Expression Levels** The **qRT-PCR** was conducted as same as described in *Trial 3*. The used primers for AvBD3, AvBD9, and AvBD10 are shown in Table 2.

Proinflammatory Cytokines TNF-α and IL-1β Detection Lung tissues were collected and homogenized with 9 volumes of PBS buffers, and centrifuged at 1,200 rpm for 10 min. The supernatants were used to detect TNF-α (H052-1) and IL-1β (H002) levels (Jiancheng Institute of Bioengineering) by using commercial kits.

Trial 5: Effects of Bacillus subtilis *Supplementation on the* Salmonella *Pullorum Infection Resistance of Broilers*

Animal Experiments Design Two hundred 1-day-old broilers were randomly divided into 4 experimental groups in 5 replicates (10 chickens/replicate). Broilers in the control group were fed control diet (Table 1) during experiment period; broilers in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period; broilers in the *Salmonella* Pullorum group were fed control diet (Table 1) during experiment period and challenged orally with 1×10^7 CFU *Salmonella* Pullorum at 2 wk of age; broilers in the 10^8 *Bacillus subtilis* + *Salmonella* Pullorum group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period and challenged orally with 1×10^7 CFU *Salmonella* Pullorum at 2 wk of age. The broilers that survived 7-d post-*Salmonella* Pullorum challenge were counted (the moribund chickens euthanized by cervical dislocation and recorded as mortality). Samples including cecal tissues and cecal contents were collected 7-d post-*Salmonella* Pullorum challenge.

Detection of Tight Junctions Related Gene Expression Levels The **qRT-PCR** was conducted as same as described in *Trial 3*. The used primers for Claudin-1, Occludin, and ZO-1 are shown in Table 2.

Fecal Shorter chain Fatty Acids Detection The fecal shorter chain fatty acids (**SCFAs**, mainly acetate, propionate, and butyrate) concentrations were detected as previously described with some modifications (Wang et al., 2020a). Cecal contents were incubated with 890 μL sodium chloride solution and 110 μL of 2 mM hydrochloric acid sodium chloride solution, and centrifuged at 12,000 rpm for 12 min. The levels of acetate, propionate, and butyrate in the cecal samples were detected using a gas chromatography (**GC**) system (7890B, Agilent, Beijing, China). The initial oven temperature was set at 85°C for 30 s, then increased 4°C per min for 12 min and held for 3 min, then increased the temperature by rising 15°C per min for 6 min and held for 2 min.

Proinflammatory Cytokines TNF-α and IL-1β Detection Cecal tissues were collected and homogenized with 9 volumes of PBS buffers, and centrifuged at 1,200 rpm for 10 min. The supernatants were used to detect TNF-α (H052-1) and IL-1β (H002) levels (Jiancheng Institute of Bioengineering) by using commercial kits.

Trial 6: Effects of Bacillus Subtilis *Supplementation on the Liver Function and Immune Response of Broilers Challenged With Aflatoxin B1*

Animal Experiments Design 1 Forty 1-day-old broilers were randomly divided into 4 experimental groups (n = 10). Broilers in the control group were fed control diet (Table 1) during experiment period; broilers

in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period; broilers in the AFB1 group were fed 1 mg/kg AFB1-contaminated control diet during experiment period; broilers in the 10^8 *Bacillus subtilis* + AFB1 group were fed 1 mg/kg AFB1-contaminated control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period. Liver tissues were collected at 6 wk of age for further analysis.

Animal Experiments Design 2 Forty 1-day-old broilers were randomly divided into 4 experimental groups (n = 10). Broilers in the control group were fed control diet (Table 1) during experiment period; broilers in *Bacillus subtilis* group were fed control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period; broilers in the AFB1 group were fed 1 mg/kg AFB1-contaminated control diet during experiment period; broilers in the 10^8 *Bacillus subtilis* + AFB1 group were fed 1 mg/kg AFB1-contaminated control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed during experiment period. All broilers were vaccinated with an attenuated IBDV vaccine (Strain B87, Zhejiang EBVAC Bioengineering, Hangzhou, China) at d 14. Serum was collected 14-d postimmunization.

Liver Function and Oxidative Stress Indicators Examination Blood samples were collected and centrifuged at 1,500 rpm for 5 min, the supernatants were used to test alanine aminotransferase (**ALT**, C009-3-1), aspartate aminotransferase (**AST**, C010-2-1) activities (Jiancheng Institute of Bioengineering) by using commercial kits. Liver tissues were collected and homogenized with 9 volumes of PBS buffers, and centrifuged at 1,200 rpm for 10 min. The supernatants were used to detect malondialdehyde (**MDA**, A003-1-2) content, catalase (**CAT**, A007-1-1) activity, superoxide dismutase (**SOD**, A001-3-2) activity (Jiancheng Institute of Bioengineering) by using commercial kits.

Serum Specific Antibody Detection Serum-specific IBDV antibody was detected as previously described by a commercial detection kit (IDEXX R Laboratory, Inc., Westbrook, ME) according to the manufacturer's guidelines (Wang et al., 2020a). The relative level of IBDV antibody was detected by calculating the S/P value as follows: [(mean value of sample hole)-(mean value of negative control hole)]/[(mean value of positive control hole)-(mean value of negative control hole). Endpoint was detected as follows: Log 10 titer = 1.09 (Log 10 S/P) + 3.36. The value was marked as positive when S/P ratio is >0.2 and negative when S/P ratio is ≤0.2.

Statistical Analysis

The data are represented as mean ± SD. Statistical analyses were carried out using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). Statistical significance was calculated by one-way or two-way ANOVA with Tukey tests for multiple-group comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

Probiotic Potential Evaluation of Bacillus subtilis

In the present study, the isolated *Bacillus subtilis* exhibited good performance to resist acid and 0.3% bile salt (Figure 1A). Furthermore, the *Bacillus subtilis* also showed good antimicrobial activities to 5 common pathogenic bacteria including *Escherichia coli, Campylobacter jejuni, Salmonella* Pullorum, *Salmonella* Typhimurium, and *Clostridium perfringens* (Figure 1B). In addition, the *Bacillus subtilis* also showed excellent performance to degrade AFB1 (Figure 1C). These results indicated that the isolated *Bacillus subtilis* has good potential as a feed additive.

Effects of Bacillus Subtilis *Supplementation on Growth Performance, Relative Weight of Immune Organ, and Dressing Percentage*

Broilers fed with the diet of 10^8 and 10^9 CFU/kg *Bacillus subtilis* showed better growth performance compared to the control group, which is characterized by the significant increase in body weight, EYP, BMP, TMP, and relative weight of thymus gland, and reduced FCR at 6 wk of age (Figures 2A−2H, all $P < 0.01$). Broilers fed with the diet of 10^7, 10^8, and 10^9 CFU/kg *Bacillus subtilis* showed markedly increased relative weight of bursal of Fabricius and spleen (Figures 2D and 2E, all $P < 0.01$).

Effects of Bacillus Subtilis *Supplementation on the Response of Broilers to Acute Heat Stress*

Broilers in control group showed significantly increased serum LDH, CK, and CKMB levels, especially at 3 h and 10 h post heat stress exposure, which indicated that heat stress caused severe heart damage (Figures 3A−3C, all $P < 0.01$). Compared to control group, the serum LDH, CK, and CKMB levels were significantly decreased in *Bacillus subtilis* supplementation group post heat stress (Figures 3A−3C, all $P < 0.01$). The control group showed significantly increased MDA levels, especially at 3-h post heat stress (Figure 3D, all $P < 0.01$), the activities of SOD and CAT were markedly elevated 1-h post heat stress and reduced significantly from 1 h to 10 h (Figures 3E−3F, all $P < 0.01$). Compared to control group, chickens fed with *Bacillus subtilis* exhibited increased SOD and CAT activities and showed reduced MDA levels during heat stress (Figures 3D−3F, all $P < 0.01$). The control group also showed that the mRNA expression levels of CRYAB, Hsp27, and Hsp70 increased significantly ($P < 0.01$) during heat stress (Figures 3G−3I, all $P < 0.01$). Compared to the control group, the mRNA expression levels of CRYAB, Hsp27 and Hsp70 were further markedly increased in *Bacillus subtilis* supplementation group during heat stress (Figures 3G−3I, all $P < 0.01$).

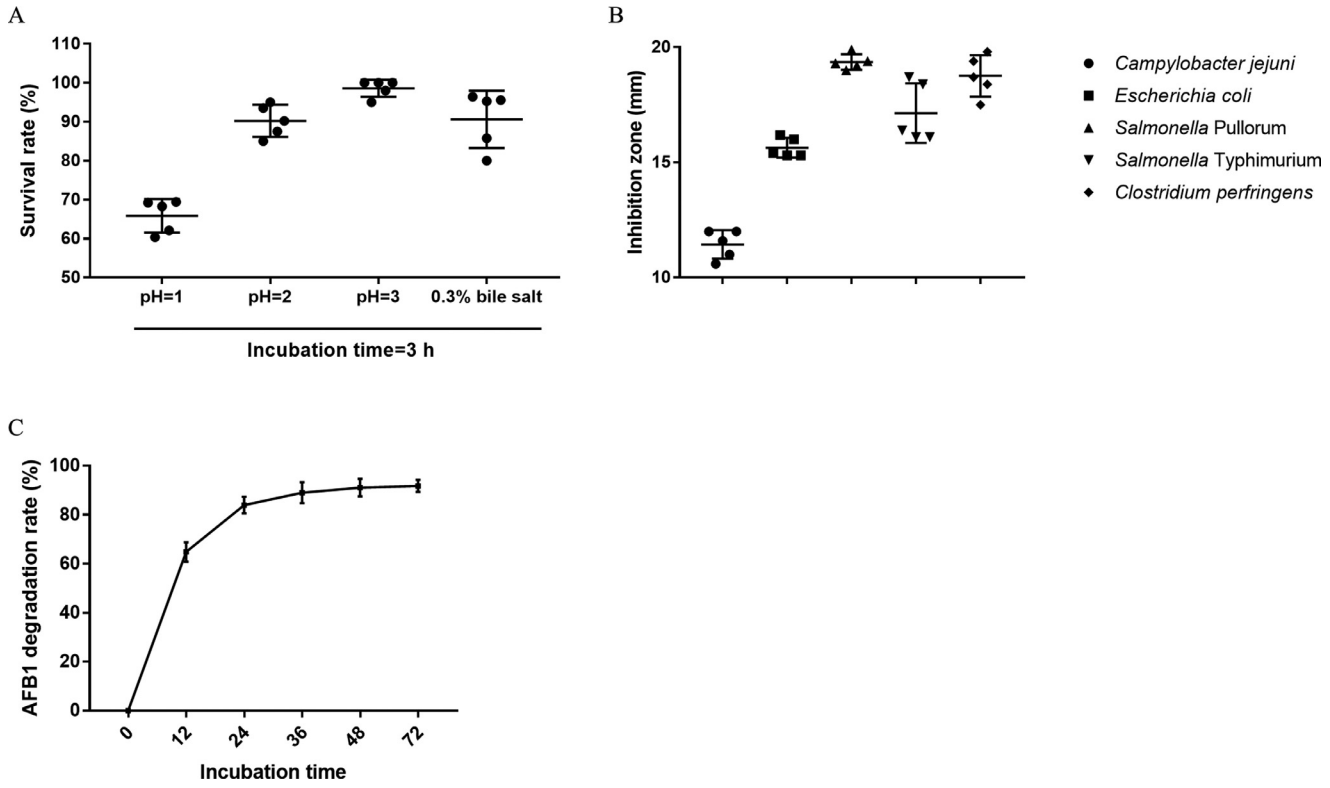

Figure 1. Assessment of probiotic properties of *Bacillus subtilis*. (A) The survival rate of isolated *Bacillus subtilis*. The agar medium adjusted to pH = 1.0, 2.0, or 3.0 to evaluate acid tolerance, and contained 0.3% bile salt to evaluate bile tolerance for 3 h. Bars represent means ± SD of five independent experiments. (B) Antibacterial activity of *Bacillus subtilis* against five pathogenic bacteria (*Escherichia coli* O78, *Campylobacter jejuni*, *Salmonella* Pullorum, *Salmonella* Typhimurium, and *Clostridium perfringens*. Bars represent means ± SD of five independent experiments. (C) Effect of *Bacillus subtilis* different incubation time on AFB1 degradation rate. The experiments were performed in the presence of 5 μg AFB1 at 37° C, pH = 7.0. Bars represent means ± SD of three independent experiments.

Effects of Bacillus Subtilis *Supplementation on the Response of Broilers to MG Challenge*

Compared to MG group, the MG colonization in lung were markedly reduced in the *Bacillus subtilis* + MG group (Figure 4A, $P < 0.01$). Compared to control group, MG infection induced poor growth performance, while the *Bacillus subtilis* + MG group showed improved growth performance which is characterized by significantly increased body weight, EYP, BMP, TMP, and decreased FCR at 6 wk of age (Figures 4B−4F, all $P < 0.01$). Furthermore, compared to the MG group, the *Bacillus subtilis* + MG group exhibited significantly increased host defense peptides AvBD3, AvBD9, and AvBD10 mRNA expression (Figures 4G−4I, all $P < 0.01$). In addition, compared to MG group, the *Bacillus subtilis* + MG group showed significantly reduced proinflammatory cytokines TNF-α and IL-1β levels (Figures 4J and 4K, all $P < 0.01$).

Effects of Bacillus subtilis *on the Response of Broilers to* Salmonella *Pullorum Challenge*

Broilers fed with *Bacillus subtilis* showed lower mortality than the control diet chickens post *Salmonell*a Pullorum challenge (Figure 5A, $P < 0.01$). Compared to the control group, *Salmonell*a Pullorum infection significantly reduced the mRNA expression levels of cecal Claudin-1, Occludin, and ZO-1 (Figures 5B−5D, $P < 0.01$), and decreased the levels of acetic acid, propionic acid, and butyric acid (Figures 5E−5G, $P < 0.01$), which were reversed by *Bacillus subtilis* supplementation (Figures 5B−5G, $P < 0.01$). In addition, compared to *Salmonell*a Pullorum group, the *Bacillus subtilis* + *Salmonella* Pullorum group showed significantly reduced proinflammatory cytokines TNF-α and IL-1β levels (Figures 5H and 5I, $P < 0.01$).

Effects of Bacillus subtilis *Supplementation on Liver Function and Immune Response of Broilers Challenged With AFB1*

Compared to the control group, broilers fed with AFB1-contaminated diet showed significantly increased serum ALT and AST levels (Figures 6A and 6B, all $P < 0.01$). Compared to the AFB1 group, broilers fed with AFB1-contaminated control diet supplemented with *Bacillus subtilis* of 10^8 CFU/kg feed markedly reduced serum ALT and AST levels (Figures 6A and 6B, all $P < 0.01$). Besides, broilers fed with AFB1-contaminated diet showed significantly increased liver MDA levels and decreased CAT and SOD activities (Figures 6C−6E, all $P < 0.01$), while broilers fed with *Bacillus subtilis* showed higher SOD and CAT activities and lower MDA levels (Figures 6C−6E, all $P < 0.01$). Infectious bursal disease virus (**IBDV**) is one of most common and important

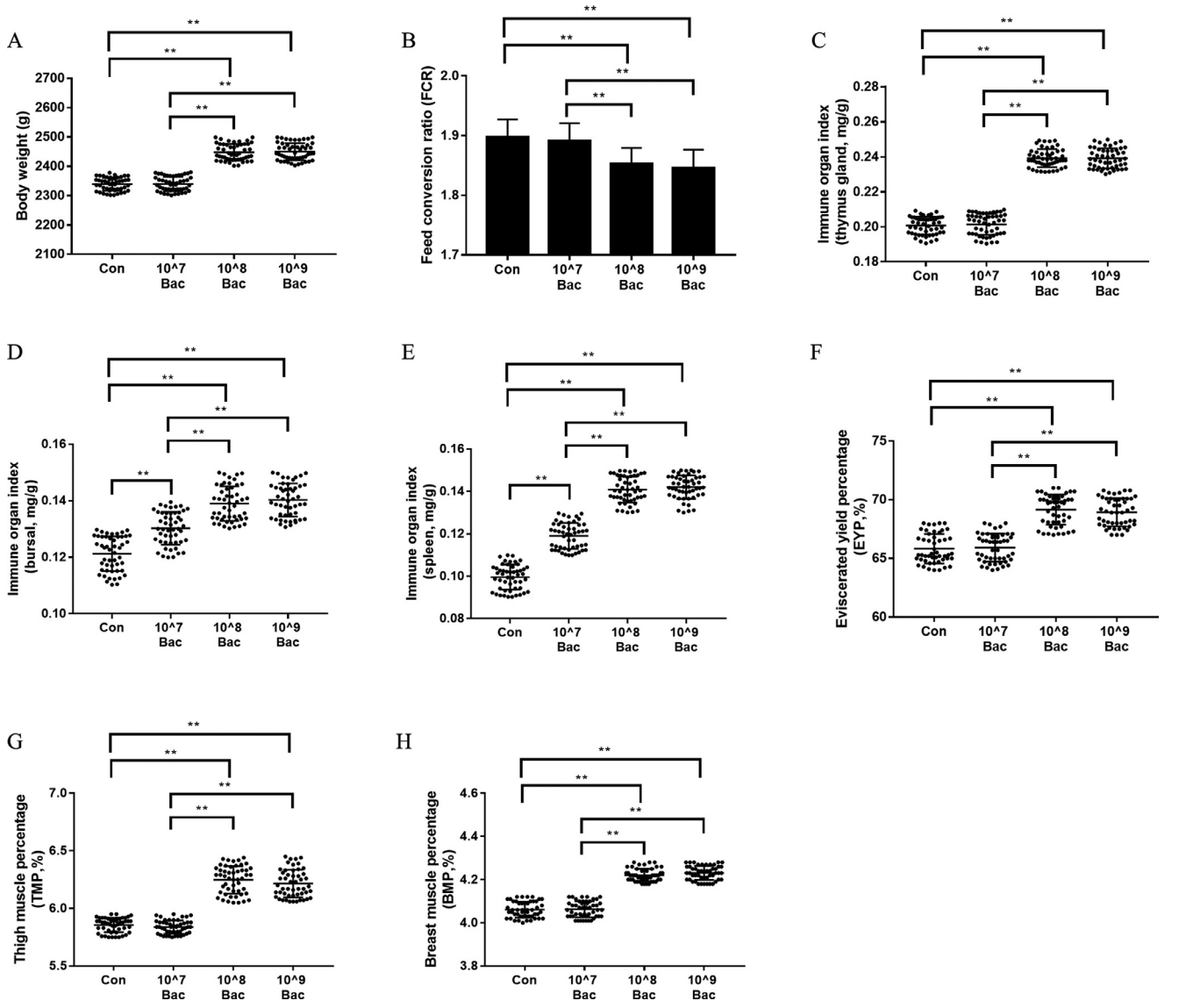

Figure 2. Effects of dietary *Bacillus subtilis* supplementation on the growth performance of broilers. (A) Effects of dietary supplementation with *Bacillus subtilis* on body weight at 6 wk of age (n = 50). (B) Effects of dietary supplementation with *Bacillus subtilis* on feed conversion ratio (FCR) at 6 wk of age (n = 50). (C−E) Effects of dietary supplementation with *Bacillus subtilis* on immune organs indices at 6 wk of age (n = 50). (F) Effects of dietary supplementation with *Bacillus subtilis* on eviscerated yield percentage (**EYP**) at 6 wk of age (n = 50). (G) Effects of dietary supplementation with *Bacillus subtilis* on thigh muscle percentage (TMP) at 6 wk of age (n = 50). (H) Effects of dietary supplementation with *Bacillus subtilis* on breast muscle percentage (BMP) at 6 wk of age (n = 50). Data were analyzed by one-way ANOVA with Tukey tests. ** indicates $P < 0.01$. Abbreviations: Bac, *Bacillus subtilis* group; Con, control group.

pathogens in chicken farms. Little information is available on whether AFB1 exposure can reduce antibody titer of IBDV after IBDV vaccine vaccination. The results showed that broilers fed with AFB1-contaminated diet showed significantly reduced serum specific antibody levels of IBDV and this could be significantly alleviated by *Bacillus subtilis* supplementation (Figure 6F, $P < 0.01$).

DISCUSSION

Probiotics were used as feed additives and reported to be mutually beneficial to the host as well as bacteria (Wang et al., 2020a). In the present study, the isolated *Bacillus subtilis* exhibited good performance to resist acid and bile salt. Furthermore, a good probiotic strain should have the ability to inhibit pathogenic microorganisms (Guo et al., 2017; Wang et al., 2020a), the isolated *Bacillus subtilis* used in the current study showed good antimicrobial activities against 5 common pathogenic bacteria including *Escherichia coli, Campylobacter jejuni, Salmonella* Pullorum, *Salmonella* Typhimurium, and *Clostridium perfringens*. Based on the above results, the effects of *Bacillus subtilis* on growth performance of broilers were further evaluated and the results confirmed that dietary administration of *Bacillus subtilis* caused higher body weight and dressing percentage of broilers, which is perhaps via control gut pathogenic bacterial proliferation and thus reducing the nutrient consumption required for maintaining immunological activity (Wang et al., 2020a). Besides, production performance improvement of broilers may be related to nutrients and extracellular digestive enzymes secreted by *Bacillus*

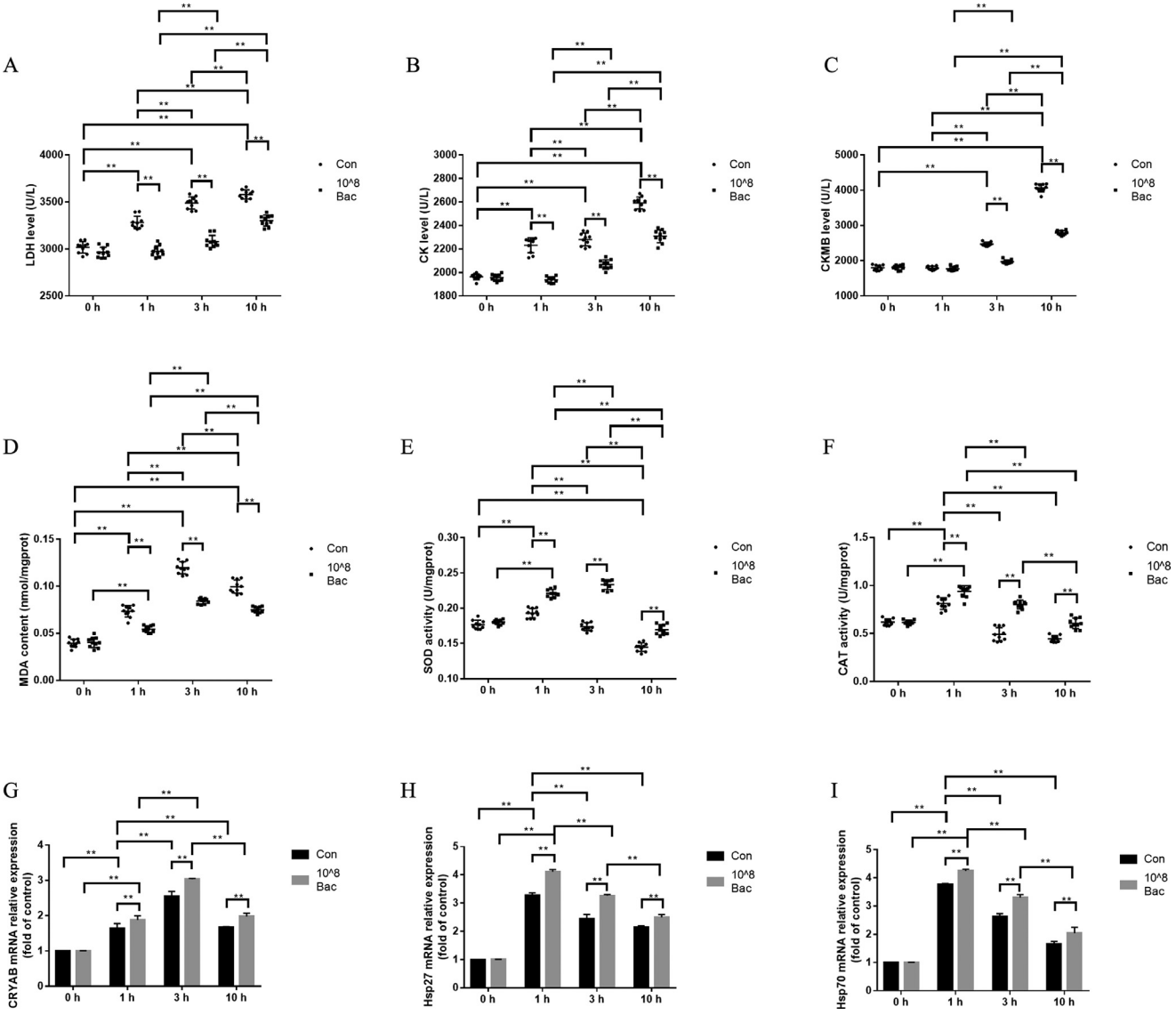

Figure 3. Effects of dietary *Bacillus subtilis* supplementation on the cardiac response of broilers to acute heat stress. (A−C) Serum cardiac damage-related enzyme activities, which were detected 0, 1, 3, 10 h post-acute heat stress (n = 10). (D−F) Oxidative stress-related indices of heart tissues, which were detected 0, 1, 3, 10 h post-acute heat stress (n = 10). (G−I) CRYAB, Hsp27, and Hsp70 mRNA expression levels of heart tissues in each group, which were detected 0, 1, 3, 10 h post-acute heat stress (n = 5). Each point represents a single bird and bars represent mean ± SD. Two-way ANOVA for repeated measurements, followed by Tukey tests. ** indicated $P < 0.01$. Abbreviations: Bac, *Bacillus subtilis* group; Con, control group.

subtilis (Sahu et al., 2008; Guo et al., 2017). In addition, the relative weight of thymus, bursa of Fabricius and spleen of the *Bacillus subtilis* supplementation groups were higher than the control group. These results indicated that *Bacillus subtilis* supplementation enhanced immunity of chickens. Because the relative weight of thymus, bursa of Fabricius and spleen are important markers of the immune status of chickens (Chen et al., 2020). Generally, immune cell growth, development, and division could induce higher animal immune organ weight, as greater absolute and relative weight indicated stronger immune function of chickens (Heckert et al., 2002; Chen et al., 2020).

Heat stress is a one of main limiting factors in poultry production because heat stress could induce abnormal metabolism and oxidative stress damage of important organs, thus resulting in poor growth performance and increased disease rate (Ahmed-Farid et al., 2021). Broilers are particularly susceptible to heat stress (Wang et al., 2020a), therefore, in addition to regulating room temperature, it is necessary to add protective additives such as anti-oxidative substances in feed to help broilers resist heat stress. Previous studies have assessed the antioxidative capacity of serval *Bacillus subtilis* and confirmed that the *Bacillus subtilis* exhibited excellent antioxidative ability (Bai et al., 2016; Cramer et al., 2018). In the current study, *Bacillus subtilis* supplementation significantly relieved the heart injury and enhanced antioxidative ability during acute heat stress. Heat shock proteins (**HSPs**) are a kind of protective proteins that stimulate cytoprotection and induce heat stress tolerance in the cells of various organs during heat stress (Wang et al., 2021a). The HSPs also could maintain the integrality of various organs by repairing damaged or misfolded proteins and promoting cell survival (Siddiqu et al., 2020; Wang et al., 2021a). In the present

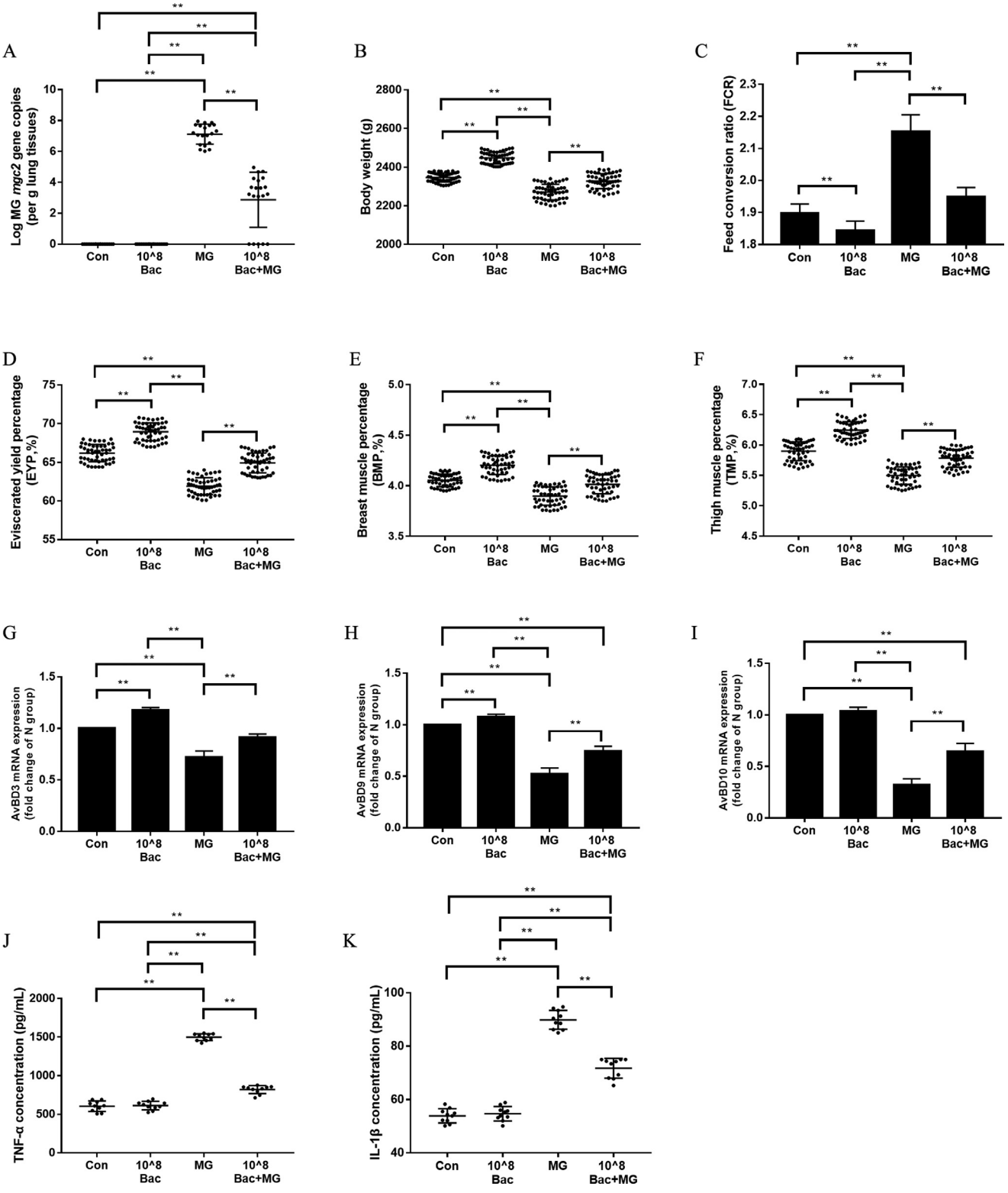

Figure 4. Effects of dietary *Bacillus subtilis* supplementation on MG infection resistance of broilers. (A) MG colonization in lung at 6 wk of age (n = 20). (B) Effects of dietary supplementation with *Bacillus subtilis* on body weight at 6 wk of age (n = 50). (C) Effects of dietary supplementation with *Bacillus subtilis* on feed conversion ratio (FCR) at 6 wk of age (n = 50). (D) Effects of dietary supplementation with *Bacillus subtilis* on eviscerated yield percentage (EYP) at 6 wk of age (n = 50). (E) Effects of dietary supplementation with *Bacillus subtilis* on thigh muscle percentage (TMP) at 6 wk of age (n = 50). (F) Effects of dietary supplementation with *Bacillus subtilis* on breast muscle percentage (BMP) at 6 wk of age (n = 50). (G–I) Effects of dietary supplementation with *Bacillus subtilis* on host defense peptides AvBD3, AvBD9 and AvBD10 mRNA expression levels of broilers challenged with MG (n = 5). (J–K) Proinflammatory cytokines of lung tissues (n = 10). Each point represents a single bird and bars represent mean ± SD. Two-way ANOVA for repeated measurements, followed by Tukey tests. ** indicated $P < 0.01$. Abbreviations: Bac, *Bacillus subtilis* group; Con, control group.

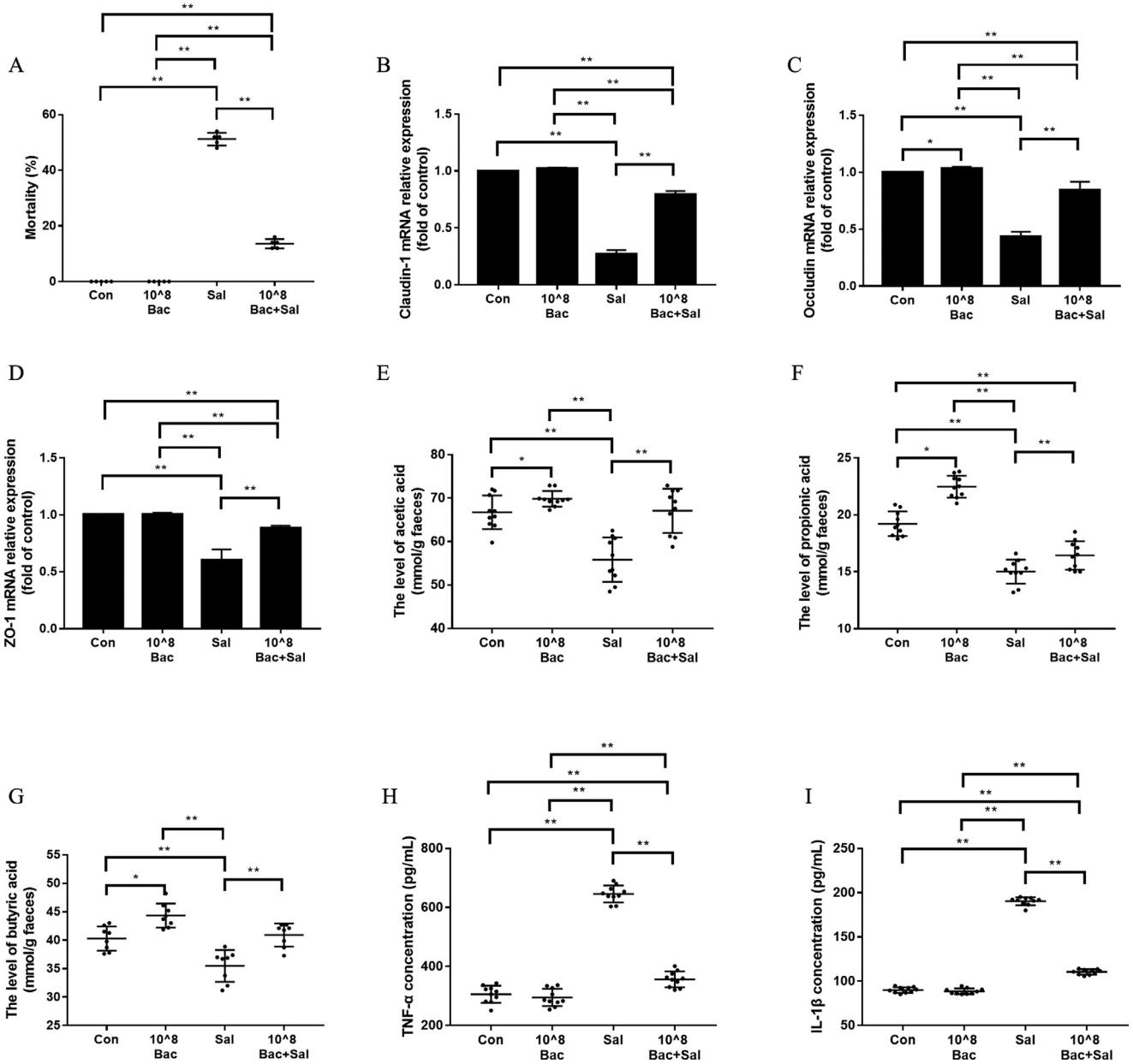

Figure 5. Effects of dietary *Bacillus subtilis* supplementation on the response of broilers to *Salmonella* Pullorum challenge. (A) Mortality of the broilers following *Salmonella* Pullorum challenge (n = 5). Each point represents the result from an independent experiment and bars represent mean ± SD. (B–D) Tight junction related gene mRNA expression of cecal tissues, which were measured 7 d post *Salmonella* Pullorum challenge (n = 5). (E–G) Effects of *Bacillus subtilis* on shorter-chain fatty acids (SCFAs) levels of cecal contents, which were measured 7 d post *Salmonella* Pullorum challenge (n = 10). (H–I) Proinflammatory cytokines of cecal tissues (n = 10). **indicates $P < 0.01$. Each point represents a single bird and bars represent mean ± SD. Two-way ANOVA for repeated measurements, followed by Tukey tests. ** indicated $P < 0.05$; ** indicated $P < 0.01$. Abbreviations: Bac, *Bacillus subtilis* group; Con, control group; Sal, *Salmonella* Pullorum group.

study, heat stress caused significant elevation in HSPs mRNA expression levels of broilers' heart, and *Bacillus subtilis* supplementation further increased the HSPs mRNA expression levels, which indicated that *Bacillus subtilis* supplementation may alleviate the adverse effects of acute heat stress by enhancing HSPs expression.

Salmonella Pullorum is one of the most isolated pathogenic microorganisms in poultry farms characterized by high morbidity and mortality in chickens, resulting in huge economic losses to poultry farmers (Li et al., 2019). Besides good breeding management and subtherapeutic antibiotics, suitable probiotics feed additives prevention is also a good measure to prevent *Salmonella* Pullorum infection in chickens (Chen et al., 2020). For example, *Bacillus subtilis* DSM17299 supplementation showed significant reduction of *Salmonella* colonization in cecum compared with control chickens (Knap et al., 2011). In the present study, the *Bacillus subtilis* showed good protective effects against *Salmonella* Pullorum challenge by decreased death rate and inflammatory injury and increased tight junctions gene expression levels of chickens. On the one hand, *Bacillus subtilis* could directly inhibit the growth of *Salmonella* (Figure 1B), and on the other hand, *Bacillus subtilis* may increase SCFAs (mainly acetate, propionate and butyrate) contents by improving intestinal microbiota composition (Neijat et al., 2019). SCFAs have been proved to

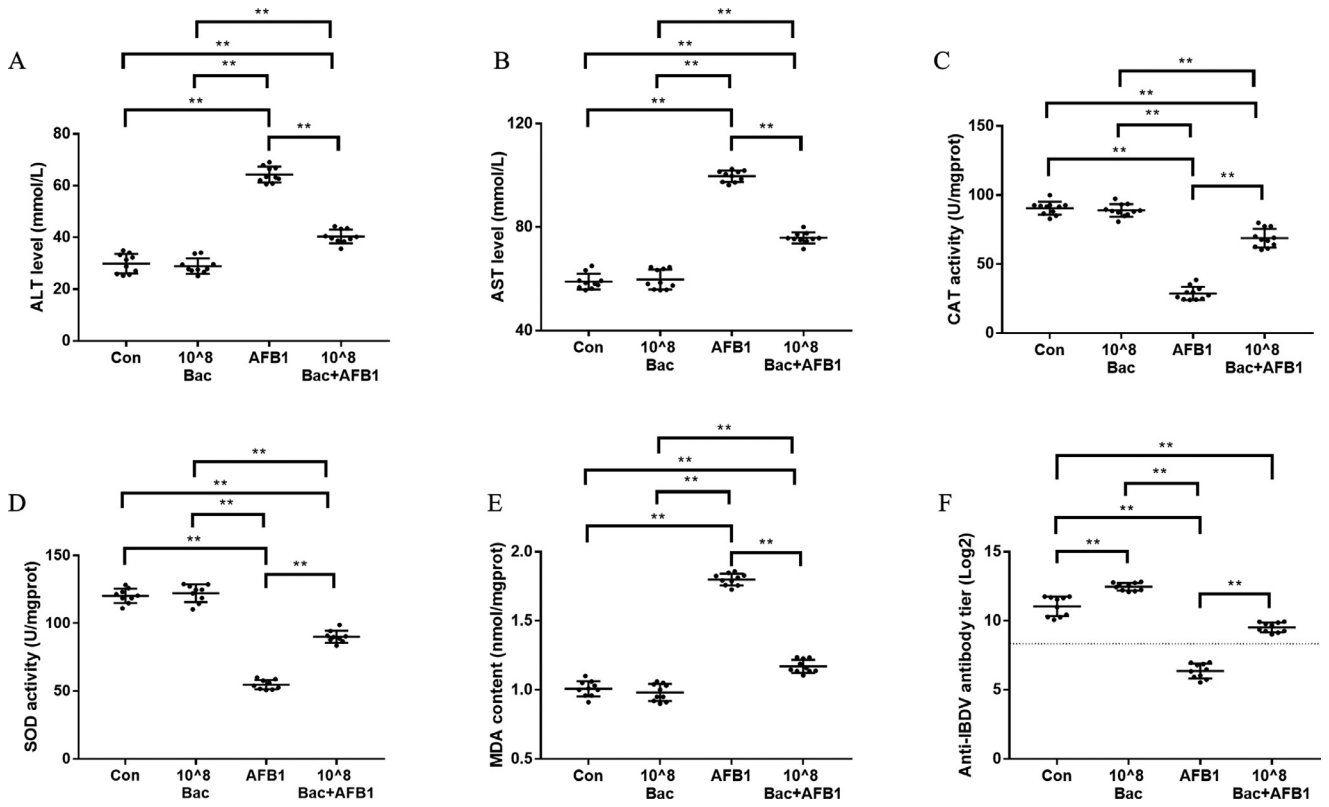

Figure 6. Effects of dietary *Bacillus subtilis* supplementation on liver function and immune response of broilers challenged with AFB1. (A) Serum ALT activity (n = 10). (B) Serum AST activity (n = 10). (C) Liver CAT activity (n = 10). (D) Liver SOD activity (n = 10). (E) Liver MDA concentration (n = 10). (F) Serum anti-IBDV specific antibody titer in chickens, which were measured 14-d postimmunization (n = 10). Log2 titers below 8.63 (which corresponds to S/P ratio <0.2) are considered negative and above 8.63 (S/P ratio >0.2) are considered positive. Two-way ANOVA for repeated measurements, followed by Tukey tests. ** indicates $P < 0.01$. Abbreviations: Bac, *Bacillus subtilis* group; Con, control group.

enhance host nonspecific immunity and inhibit *Salmonella* directly (Tsugawa et al., 2020).

MG is one of the most economically significant pathogens of broilers characterized by decreased production performance such as reduced weight gain and decreased feed conversion efficiency. In addition, MG infection could induce immunosuppression of chickens, once coinfection by MG and *Escherichia coli* or other pathogens could cause more serious economic loss to the poultry farmers (Wang et al., 2021b). Our recent studies have indicated that gut microbiota disorder was associated with MG infection and confirmed a "gut-lung axis" mechanism that oral administration of *Lactobacillus* or baicalin to chickens reduced the MG colonization and lung injury by improving gut microbiota and metabolic profiling (Wang et al., 2021a,b). In this study, oral administration of the isolated *Bacillus subtilis* effectively reduced MG colonization and enhanced host defense peptide gene expression in lung, however, the exact "gut-lung axis" mechanism of *Bacillus subtilis* against MG infection is still illusive.

Liver is the most vulnerable organ to the toxic and carcinogenic action of AFB1 which were characterized by increased MDA content and suppressed CAT and SOD enzymes activities (Li et al., 2021), and AFB1 also can cause oxidative stress and apoptosis in thymus and bursa of fabricius, thus resulting in immunosuppression (Peng et al., 2017). The use of probiotics as feed additives and to degrade AFB1 in poultry industry has increasingly gained focus (Ma et al., 2012). In the present study, the *Bacillus subtilis* supplementation group showed good protective effects against AFB1 challenge by improved liver function and enhanced serum specific IBDV antibody production. Importantly, *Bacillus subtilis* could directly degrade AFB1 (Figure 1C). While, *Bacillus subtilis* may decrease AFB1 residues by through positively regulating intestinal beneficial bacterial abundances (Chang et al., 2020).

In the present study, multiple beneficial effects of *Bacillus subtilis* were studied in chickens, including growth performance, heat stress tolerance, *Mycoplasma gallisepticum* and *Salmonella* Pullorum infection resistance, enhanced immune response and improved liver function after AFB1 challenge. These findings supported the idea that *Bacillus subtilis* as an effective feed additive in the poultry production.

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DISCLOSURES

All authors declared that there are no potential conflicts of interests.

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