Host-derived bacillus spp. as probiotic additives for improved growth performance in broilers

Bei Wang,* Qiong Wu,‡ Shengzu Yu,* Qi Lu,* Xuan Lv,* Miao Zhang,* Ying Kan,* Xiqiang Wang,* Yingqi Zhu,* Guijun Wang,*,† and Qing Wang*,†,[1]

*College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China; †Anhui Province Key Laboratory of Veterinary Pathobiology and Disease Control, Hefei 230036, China; and ‡Animal Husbandry and Veterinary Service Centre of Jiujiang, Wuhu 241012, China

ABSTRACT In recent years, the utilization of antibiotics in animal feed has been restricted, probiotics have been increasingly used to replace antibiotics in maintaining animal health. The aim of this study was to screen and evaluate probiotics with excellent probiotic potential from the gut of healthy goslings for clinical application. Thirteen strains of *Bacillus* (named AH-G201 to AH-G2013), including 2 strains of *Bacillus subtilis (B. subtilis)*, 6 strains of *Bacillus licheniformis (B. licheniformis)* and 5 strains of *Bacillus amyloliquefaciens (B. amyloliquefaciens)*, were isolated and identified. Then, acid and bile salts tolerance tests were performed to screen probiotics strains that could survive under different environments. The effects of screened probiotics on the growth of pathogenic *Escherichia coli* (***E. coli***) and *Salmonella* were assessed. Furthermore, we performed the drug resistance tests and safety tests in animals. The results showed that *B. Subtilis* AH-G201, *B. licheniformis* AH-G202 and AH-G204 exhibited higher gastrointestinal resistance under in vitro conditions, and showed a moderate level of resistance to the tested antibiotics. Importantly, AH-G201 and AH-G202 showed 24 to 60% inhibition rate against pathogenic *E. coli* and *Salmonella.* Moreover, the safety analysis of AH-G201 and AH-G202 suggested that the 2 probiotics strains have no adverse effects on body weight gain and feed intake in the broilers, and in addition, they have significantly improved growth performance. Finally, we analyzed effects of *B. Subtilis* AH-G201and *B. licheniformis* AH-G202 on growth performance, immune organ index and the feces microbes of broilers. The results showed that broilers fed with high doses (5×10^9 CFU/mL, for single strain) of a mixture of AH-G201 and AH-G202 exhibited good growth performance, and exhibited the greatest gain in spleen weight and the highest lactic acid bacteria counts. These findings indicate that the combined addition of *B. Subtilis* AH-G201 and *B. licheniformis* AH-G202 has the potential to replace antibiotics and to improve the growth performance of broilers.

Key words: *Bacillus*, probiotics, strain screening, growth performance, feed additives

2023 Poultry Science 102:102240
https://doi.org/10.1016/j.psj.2022.102240

INTRODUCTION

Bacillus is a spore-forming genus of bacterium that is found in the intestines of animals and confers resistance against harmful microorganisms (Holzapfel et al., 2001). In the breeding industry, antibiotics are widely used to prevent infection by pathogenic microorganisms and effectively improve the growth performance of animals (Bunyan et al., 1977; Li et al., 2018). However, the widespread use of antibiotics is associated with serious consequences, including increased breeding costs, drug residues in animal products, and drug resistance (Barton, 2000; Shivaramaiah et al., 2011). As a result, the European Union (**EU**) has phased out the use of antibiotics as feed additives, and these restrictions are being gradually implemented at a global scale too (Bogaard et al., 2000; Sorum and Sunde, 2001). Therefore, the use of probiotic feed additives in the animal husbandry sector have receiving increasing attention as a cost-effective alternative to combatting diseases in animals and improving breeding performance (Reuter, 2001; Zhu and Joergert, 2003). The characteristics and disease resistance of probiotics have being widely investigated, and they are gradually being implemented in production practices (FAO, 2006). In China, 35 microbial species have received approval for use as feed additives according to the Feed Additives Catalogue (2013). These species include spore-producing

Received April 26, 2022.
Accepted August 19, 2022.

[1]Corresponding author: qingwang@ahau.edu.cn

bacteria of the *Bacillus* genus. *Bacillus* species have high stability and are resistant to temperature changes, acid, and salt (Deniz et al., 2011). Moreover, some essential nutrients, such as digestive enzymes and vitamins that support animal growth could be produced by *Bacillus* species (Li et al., 2019; Mun et al., 2021; Zhang et al., 2021a). In addition, *Bacillus* species could inhibit the reproduction of harmful aerobic bacteria by consuming intestinal oxygen and, thereby, promoting the growth of beneficial anaerobic bacteria (Sanders et al.,2003; Gao et al., 2017). Several studies have shown that infection-resistant *Bacillus subtilis* promoted growth to some extent, increased the production of β-defensins in birds, and increases resistance to *Escherichia coli* and anti-Newcastle disease virus in broilers (Dong et al., 2020).

Although ample studies have been conducted involving *B. subtilis* or *B. licheniformis* in broilers, the effects of different *Bacillus* strains on animals were significantly different. Therefore, the objective of the present study was to screen and evaluate the effects of continuous dietary supplementation with *B. subtilis* and *B. licheniformis*, on growth performance, immune organ index and gut microflora in broilers. Our findings will be beneficial for broilers with some novel ideas for antibiotic substitutes.

MATERIALS AND METHODS

Experimental Animals

The experiments were conducted on 40 one-day-old and 12 seven-day-old broilers purchased from the Hefei poultry breeding farm. During the feeding process, broilers were given free access to feed to ensure that they consumed sufficient feed and water. All the animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals set by Anhui Agricultural University. Ethical approval was obtained from the Animal Care and Use Committee of Anhui Agricultural University.

Bacillus Isolation and Identification

Bacillus strains were isolated from the fresh intestinal contents of healthy Zhedong goslings from a farm in Anhui Province. The intestinal content collected from each gosling was diluted in 0.9% NaCl solution. Next, the suspension was heated at 80°C for 20 min, and diluted from 10^{-1} to 10^{-4} using 0.9% NaCl solution by the 10-fold dilution method. An aliquot of 100 μL of each dilution was plated on nutrient agar, and incubated at 37°C for 24 h to isolate individual colonies. Individual colonies of *Bacillus* were selected based on their morphological features and purified by 4 zone streak plate cultivation on nutrient agar. After 24 h, strains were selected for further characterization and Gram staining.

PCR Amplification and DNA Sequencing of 16S rRNA

Total genomic DNA was extracted from *Bacillus* species cultured overnight in Luria-Bertani (**LB**) medium. The genomic DNA was used as the template for PCR, and partial 16S rRNA sequencing of the PCR-amplified 1,540-bp fragment was performed using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1527R (5′-AAAGGAGGTGATCCAGCC-3′) (Hoa et al., 2001; Khaneja et al., 2010). The primers were synthesized by General Biol Co. Ltd., Anhui, China. PCR was performed in a 20 μL reaction system consisting of 10 μL of 2 × Taq PCR Master Mix (TaKaRa Bio Inc., Dalian, China), 2 μL of DNA template, 0.5 μL of a primer, and 7 μL of sterile deionized H_2O. The reaction conditions were as follows: initial denaturation at 95°C for 3 min; 32 cycles of denaturation at 94°C for 15 s, renaturation at 55°C for 15 s, and extension at 72°C for 90 s; and final extension at 72°C for 5 min. The purity of the PCR products was determined with 1% agarose gel electrophoresis. The sequencing of the 16S rRNA gene was performed by General Biol Co. Ltd., Anhui, China. The sequences obtained were compared to sequences deposited in the GenBank nonredundant nucleotide database by BLAST analysis.

Biochemical Characterization of Bacillus Strains

Characterization of *Bacillus* strains using microbiological and biochemical methods was performed according to Bergey's Manual of Systematic Bacteriology. The bacterial biochemistry kits were purchased from Qingdao Haibo Biotechnology Co., Ltd, China. Tests for measuring glucose levels, citrate utilization, nitrate reduction, and liquefaction of gelatin, as well as the Voges-Proskauer test, were performed.

Tolerance to Acid and Bile Salts

Before tests to measure tolerance to acid and bile salts were conducted, the purified bacteria were cultured on 2% LB medium that was rotated at a speed of 180 r/min at 37°C for 18 h. Then, LB media at different pH levels (1, 2, 3, and 4) were prepared with 5% hydrochloric acid, and the isolated *Bacillus* strains were inoculated in these media to test their salt tolerance. The media were rotated for 6 h at 180 r/min at 37°C, and the OD_{600} of the suspension was measured.

For measuring bile tolerance, the LB media were prepared with bile salts (Hopebiol Co., Ltd, Qingdao, China) at concentrations of 1, 2, and 3 g/L and a 10% concentration of *Bacillus* and rotated at 180 r/min for 6 h at 37°C. After culture, the OD_{600} of the suspension was measured.

Antibiotic Susceptibility

To evaluate antibiotic resistance and sensitivity, antibiograms for the isolated strains were obtained using the radial diffusion method, according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 1997). The susceptibility of the

Bacillus isolates to gentamicin, erythromycin, clindamycin, penicillin, carbenicillin, cefepime, azithromycin, doxycycline, florfenicol, and norfloxacin was determined by culturing in nutrient agar plates at 37°C for 24 h. The zone of inhibition around each drug susceptibility paper (Hang Zhou Microbial Reagent Co., Ltd, Hangzhou, China) was measured using vernier calipers.

In Vitro Inhibition of Pathogens

Salmonella and *E. coli* strains isolated from chickens were selected as indicator strains. They were activated in a 37°C liquid LB medium and cultured at 180 r/min for 24 h. When the viable count of bacteria was 1×10^8 CFU/mL, 100 μL of indicator bacteria was mixed with the isolated bacillus strains (AH-G201, AH-G202, and AH-G204) in 5 mL of liquid LB medium under the same culture conditions. After 24 h of co-culture, the bacterial solution was gradient diluted, coated with a triangular stick on a Mac plate, and cultured at 37°C for 24 h. After culture, the number of *E. coli* and *Salmonella* colonies was counted and the bactericidal rate was calculated by the following formula: Bactericidal rate(%) = (N1-N2)/ N2 × 100%, where N1 is the number of viable bacteria of *E. coil / Salmonella* culture on mac plate for 24 h, N2 is the numberof viable bacteria of *E. coil / Salmonella* on mac plate after 24 h co-culture with AH-G201/AH-G202/ AH-G204, respectively.

In Vivo Safety Evaluation

Twelve 7-day-old broilers chickens were used for in vivo safety evaluation. All the chickens were housed at 37°C with free access to feed and water. After 3 d of acclimatization, the chickens were randomly divided into 4 groups of 3 animals each: 1) basal diet and gavage with 0.2 mL of 0.9% sterile saline (control group), 2) basal diet and gavage with mixture of AH-G201 (1mL, 5×10^{10} CFU/mL) and AH-G202 (1 mL, 5×10^{10} CFU/mL), 3) basal diet and gavage with AH-G201 (1 mL, 5×10^{10} CFU/mL), and basal diet and gavage with AH-G202 (1 mL, 5×10^{10} CFU/mL). Behavior, food and water intake, and fecal properties were observed daily. Body weight and food intake were measured before and after the gavage treatment. After 14 d of daily gavaging, the chickens were euthanized by exsanguination and immediately autopsied. The liver, kidney, spleen, muscular stomach, glandular stomach, and intestinal tract were collected and examined for pathological changes.

Growth Performance

A total of 40 broiler chickens were randomly divided into 4 groups of 10 each. The basal diets were formulated according to the nutrient requirements of AMINOChick 2.0 and the Chinese Feeding Standard of Chicken, and their ingredient composition and nutrient levels are shown in Table 1. The control group was fed a basal diet only. The high-dose treatment groups were fed basal diets and received gavage with mixture strains of AH-G201 (1 mL, 5.0×10^9 CFU/mL) and AH-G202 (1 mL, 5.0×10^9 CFU/mL); the medium-dose treatment groups were fed basal diets and received gavage with mixture of AH-G201 (1 mL, 2.5×10^9 CFU/mL) and AH-G202 (1 mL, 2.5×10^9 CFU/mL); the low-dose treatment groups were fed basal diets and received gavage with mixture of AH-G201 (1 mL, 1.0×10^9 CFU/mL) and AH-G202 (1 mL, 1.0×10^9 CFU/mL). After 42 d of continuous feeding, the animals in each group were weighed to calculate their average weight. Chickens that were weak or sick were eliminated before the trial. During the trial, the mortality was recorded daily, and the feed consumption was adjusted to body weight. The body weight and mortality were recorded every week, and the average daily gain (**ADG**) and Feed: Gain ratio (**F/G**) were calculated based on body weight.

Immune Organ Indices

At the end of the trial period (42 d), the broilers of each group were weighed. Then, the birds were exsanguinated and scalded for collection of tissue from the thymus gland, spleen, and bursa of fabricius. The tissues were cleaned and weighed to calculate the immune organ index (**IOI**). The formula for calculating IOI was as follows: %IOI = 100 × (immune organ weight [g]/body weight).

Microflora Count

The fecal samples were collected on d 21 and 42 of the experiment. Three healthy broilers with similar body weight were randomly selected from each group, and 0.1 g of fresh fecal samples were collected with sterile cotton swabs. Sterile normal saline was diluted from 10^{-1} to 10^{-8} by the 10-fold dilution method. Then, 100 μL of the 10^{-5} dilution was coated on a nutrient agar plate, and 100 μL of the 10^{-8} dilution was pipetted onto Man, Rogosa, and Sharpe (**MRS**) medium. Three

Table 1. Composition of ingredients and nutrients in the feed provided to the experimental animals.

Items	Content
Corn	43.63%
Bran	7%
Fine rice bran	11%
Soybean meal	24.5%
Wheat	8.0%
NaCl	0.37%
Methionine	0.15%
$CaHPO_4$	1.9%
Limestone powder	0.45%
Vitamin A	1,500 IU
Vitamin D_3	200 IU
Vitamin K3	0.5 mg
Vitamin E	10 IU
Pantothenic acid	10 mg
Iron	80 mg
Zinc	40 mg
Selenium	0.15 mg

parallel controls for each sample were cultured at 37°C for 24 h. Colony count was performed with the plate counting method, and the number of viable aerobic bacteria and lactic acid bacteria (**LAB**) per gram of fecal sample was calculated.

Statistical Analysis

The data were presented as mean ± standard deviation (**SD**) and were analyzed using one-way ANOVA followed by Tukey's multiple comparison using SPSS 23.0 software (SPSS Inc.). Differences between groups were considered significant at P values of <0.05. Graphs were generated using GraphPad Prism 9 software.

RESULTS

Identification of Isolated Strains

Thirteen strains of *Bacillus* were isolated from the intestinal tract of 10 healthy geese and named serially from AH-G201 to AH-G2013. The common morphological characteristics of the isolated strains were coarse-looking opaque colonies that were stained white and had folds on agar plates (Figure 1A). Gram staining revealed positively stained rod-shaped bacilli, ovoid spores with blunt ends on both sides (Figure 1A).

Among the 13 identified strains, AH-G201 and AH-G203 were cultured on nutrient agar plates for 12 h. The colonies were irregular with an obvious ring-shaped bulge in the center, and the edges were not smooth. Microscopic observations for AH-G202, AH-G204, AH-G205, AH-G206, AH-G207, and AH-G208 were consistent with those for AH-G201, and the colony was stained white and had obvious folds after culture on nutritional agar. Microscopic examination of the AH-G209−AH-G2013 strains revealed short *bacilli* with an oval medium-sized spore, white staining, folding, irregular colonies, a rough and opaque surface, and a horizontal protrusion in the middle of the nutrient agar plates.

According to the Handbook of Systematic Identification of Common Bacteria, the biochemical data shown in Table 2 indicate that the AH-G201−G2013 strains belong to *Bacillus*.

The strains were assessed by 16S rRNA sequencing and BLAST analysis for identification of species. Analysis of the phylogenetic relationship based on the 16S rRNA sequences presented in Figure 1B revealed that the AH-G201 and AH-G203 strains are closely related to *B. subtilis*. Further, AH-G202, AH-G204, AH-G205, AH-G206, AH-G207, and AH-G208 were closely related to *B. licheniformis*, and AH-G209, AH-G2010, AH-G2011, AH-G2012, and AH-G2013 were closely related to *B. amylolyticus*.

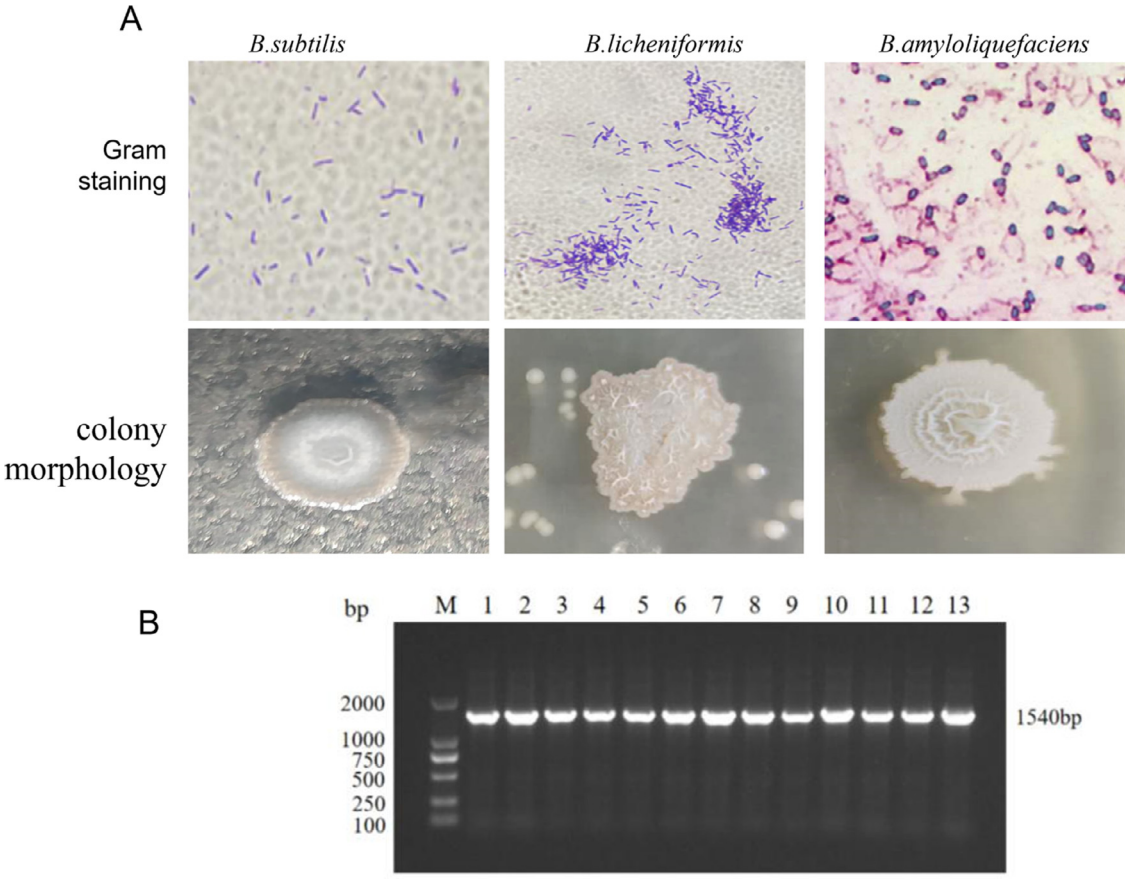

Figure 1. Isolation and identification of *Bacillus* strains. (A) Depict the Gram staining results of the isolated strains and the morphological features. AH-G201 and AH-G203 depict the observations for *B. subtilis*. AH-G202 and AH-G204−AH-G208 depict the observations for *B. licheniformis*. AH-G209 and AH-G2013 depict the observations for *B. amyloliquefaciens*. (B) PCR findings: 1−13 present the 16S rRNA results for AH-G201−AH-G2013.

Table 2. Results of the biochemical characterization of *Bacillus.*

Items/strains[1]	AH-G201	AH-G202	AH-G203	AH-G204	AH-G205	AH-G206	AH-G207	AH-G208	AH-G209	AH-G2010	AH-G2011	AH-G2012	AH-G2013
Glucose	+	+	+	+	-	+	+	+	-	-	+	+	+
Citrate utilization	+	+	+	+	-	+	-	-	-	-	-	-	+
Nitrate reduction	+	+	-	+	+	+	+	+	+	-	+	+	+
V.P test	+	+	+	+	+	+	+	+	+	+	+	+	-
Liquefaction of gelatin	+	-	+	-	+	+	-	+	+	-	-	+	+

+means the result was positive; - means the result was negative.
[1]Abbreviations: V.P test, Voges-Proskauer reaction.

Screening of the Strains

The 13 isolated strains were analyzed for biological characteristics, including acid and bile salt tolerance, antibiotic susceptibility, antimicrobial activity, and in vivo safety.

After 6 h of culture at pH 1.0−4.0, the 13 isolated strains exhibited higher survival under low pH conditions (Figure 2). In particular, the OD_{600} values of AH-G201, AH-G202, AH-G204, AH-G2007, AH-G2010, and AH-G2013 at pH 4.0 were >0.2. Interestingly, the OD_{600} values of AH-G201 at pH 1.0 was >0.2.

The results for tolerance to bile salts are shown in Figure 3. AH-G201, AH-G202, AH-G204, and AH-G2013 were able to survive in the presence of 1 g/L and 2 g/L bile salts, but only the AH-G201 strain was able to survive in the presence of 3 g/L bile salts (OD_{600} >0.2).

The data in Table 3 show that all the isolated strains exhibited resistance to penicillin, weak resistance to florfenicol and norfloxacin. Further, the AH-G202 strain

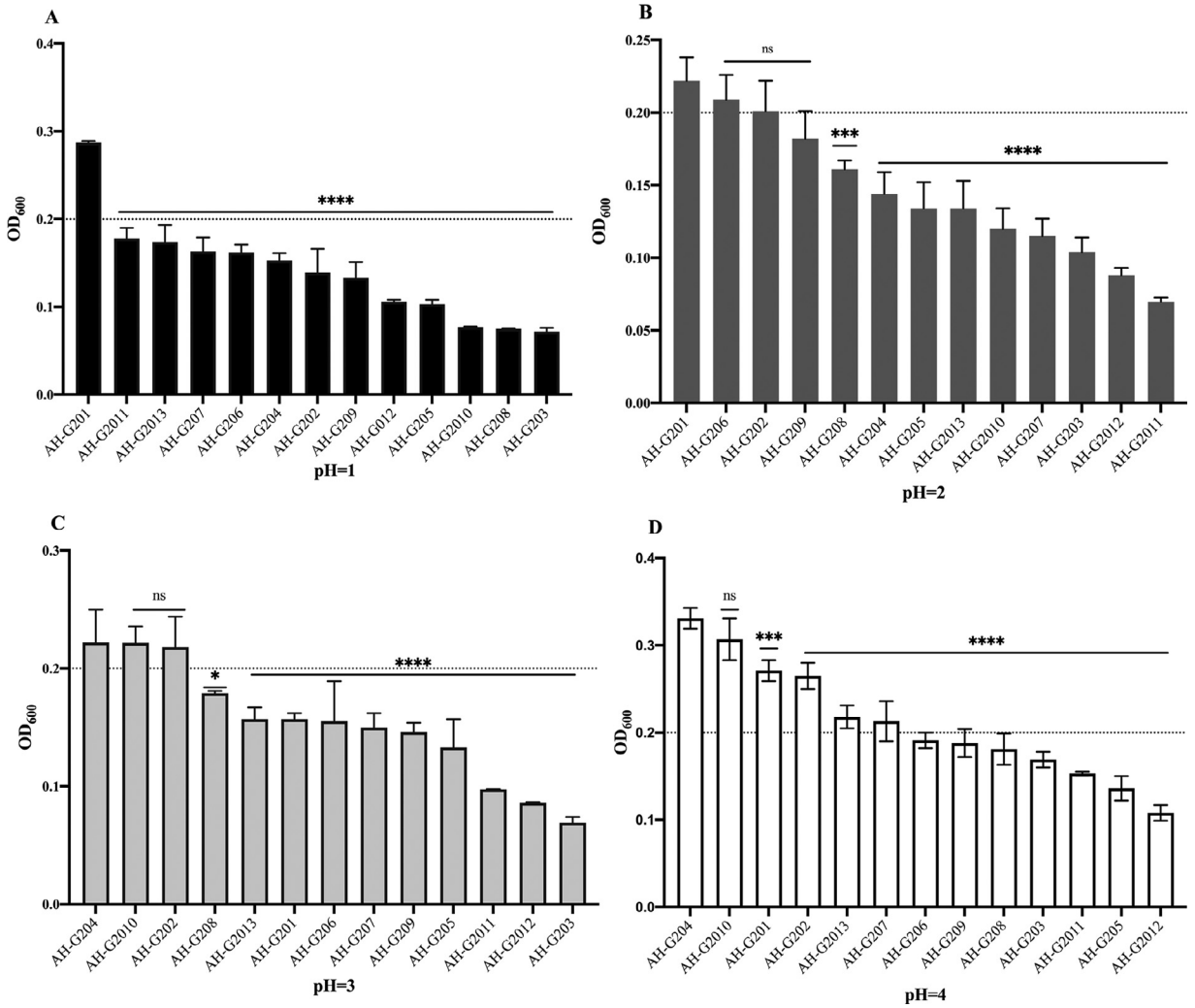

Figure 2. Tolerance of *Bacillus* strains to different pH levels. After 6 h incubation in pH = 1 (A), pH = 2 (B), pH = 3 (C), or pH = 4 (D), the OD_{600} of the isolated strains were calculated and ranked. Values displayed are the mean ± SD. The highest OD_{600} of isolated strain was selected as a reference in the same concentration, the remaining 12 strains were compared to the reference with significant differences shown as: $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. The dotted line ($OD_{600} = 0.2$) represent the positive critical line.

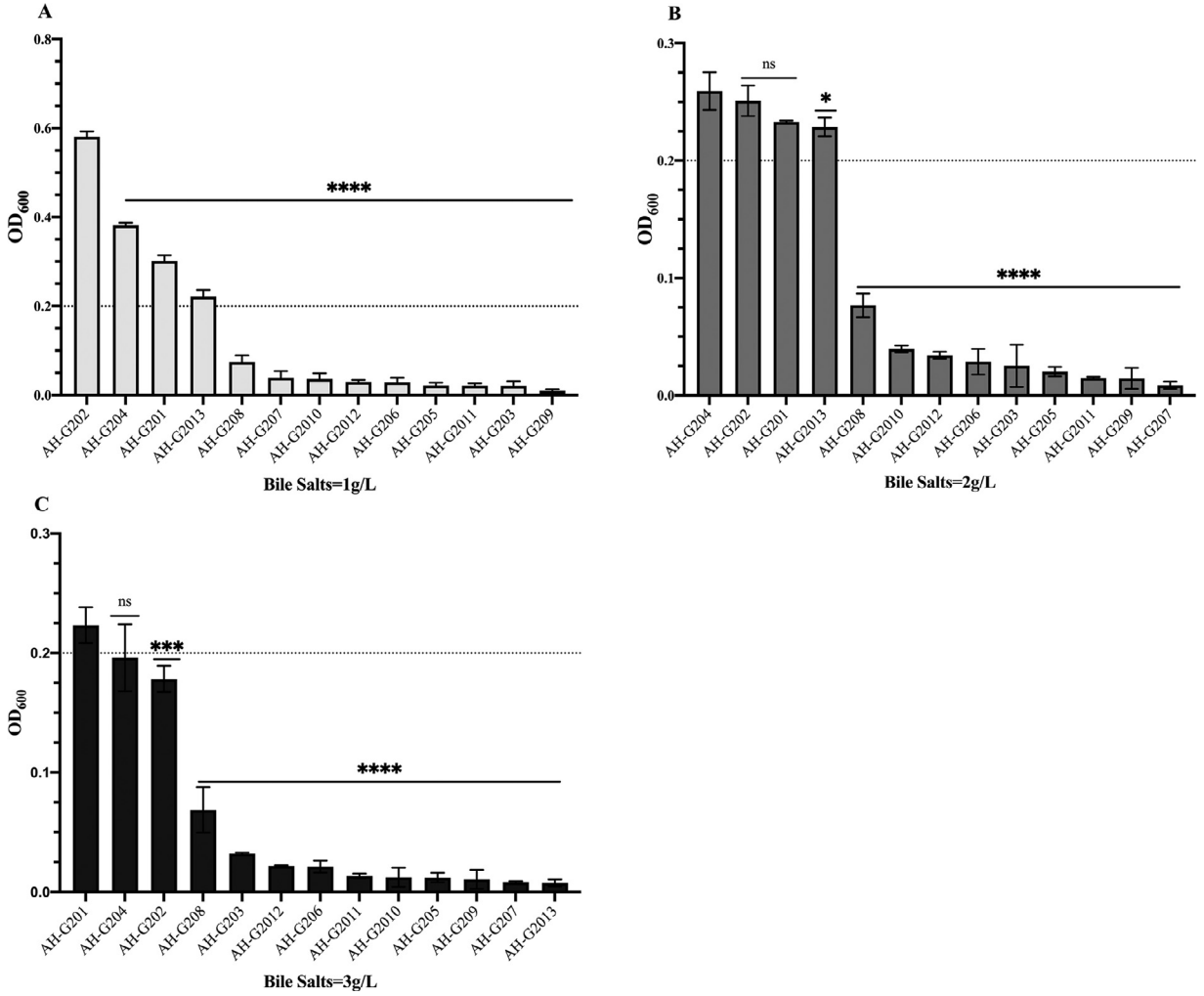

Figure 3. Tolerance of *Bacillus* strains to different concentrations bile salts. After 6 h incubation in 1g/L of bile salts (A), 2 g/L of bile salts (B), or 3 g/L of bile salts (C), the OD_{600} of the isolated strains were calculated and ranked. Values displayed are the mean ± SD. The highest OD_{600} of isolated strain was selected as a reference in the same concentration, the remaining 12 strains were compared to the reference with significant differences shown as: $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. The dotted line ($OD_{600} = 0.2$) represent the positive critical line.

exhibited strongest resistance to 5 drugs, including clindamycin (DA), penicillin (G), azithromycin (AZM), erythromycin (E), carbenicillin (AM). AH-G203, AH-G2010, AH-G2011 and AH-G2013 exhibited resistance only to penicillin. Overall, the isolates exhibited different levels of sensitivity to different antibiotics. AH-G201, AH-G202, AH-G204, AH-G205, and AH-G207 showed resistance to 3 to 5 drugs.

To sum up, the findings indicate that the AH-G201, AH-G202, and AH-G204 strains exhibited some tolerance to changes in intestinal and gastric pH and bile salt. Besides, 3 isolated strains showed a moderate level

Table 3. Results of the drug resistance test of *Bacillus* strains.

Antimicrobial agents[1]	Inhibition zone diameters/mm			Strains												
	R	I	S	AH-G201	AH-G202	AH-G203	AH-G204	AH-G205	AH-G206	AH-G207	AH-G208	AH-G209	AH-G210	AH-G211	AH-G212	AH-G213
GM	≤12	12∼15	≥15	S	S	S	S	I	I	S	I	R	S	S	S	S
DA	≤14	14∼20	≥21	R	R	I	I	R	I	I	I	I	I	I	I	I
G	≤28		≥29	R	R	R	R	R	R	R	R	R	R	R	R	R
AM	≤13	14∼16	≥17	R	R	S	S	I	I	R	I	I	I	I	R	I
FEP	≤14	15∼17	≥18	R	I	S	S	R	R	R	R	S	S	I	S	S
AZM	≤13	14∼17	≥18	I	R	I	R	I	S	I	I	I	I	S	I	I
E	≤13	14∼22	≥23	I	R	I	R	R	I	I	I	I	I	I	I	I
Doxycycline	≤12	13∼15	≥16	S	S	S	S	S	S	R	S	S	S	S	S	S
FFC	≤12	13∼17	≥18	S	S	S	S	S	S	S	S	S	S	S	S	S
NOR	≤12	13∼16	≥17	S	S	S	S	S	S	S	S	S	S	S	S	S

[1]Abbreviations: AM, carbenicillin; AZM, azithromycin; DA, clindamycin; E, erythrocin; G, penicillin; GM, Gentamicin; FEP, cefepime; S, Susceptible; R, resistance; I, intermediate.

Table 4. In vitro results of the pathogen inhibition test of *Bacillus*.

	Positive control group (10^8 CFU/mL)		*E. coli* group (10^8 CFU/mL)		*Salmonella* group (10^8 CFU/mL)	
Strain	*E. Coli*[1]	*Salmonella*	CFU	Bactericidal rate (%)	CFU	Bactericidal rate (%)
AH-G204	30	50	32	-6.7	68	-36
AH-G201			16	46.44	20	60
AH-G202			13	56.67	38	24

[1]Abbreviations: *E. coli, Escherichia coli.*

Table 5. Results of safety tests in *Bacillus* strains.

Items/diets	Basal diet (control group)	Basal diet + AH-G201 + AH-G202	Basal diet + AH-G201	Basal diet + AH-G202
Average body weight before feeding (g)	50.2	65.0	59.7	63.6
Average body weight after 2 weeks (g)	223.9[b]	263.5[a]	251.3[a]	254.9[a]
Average daily weight gain	12.4	14.2	13.7	13.7

Values were displayed as the mean ± SD, and significance was analyzed by Student's *t* test.
[a,b]Different superscript letters in the same row indicate significant difference ($P < 0.05$). Same superscript letters or no letters in the same row indicate no significant difference ($P > 0.05$).

Table 6. Effect of *Bacillus* supplementation at different doses on the growth performance of broilers.

Items	High-dose group (The dose of single strain 5.0×10^9 CFU/mL)	Medium-dose group (The dose of single strain 2.5×10^9 CFU/mL)	Low-dose group (The dose of single strain 1.0×10^9 CFU/mL)	Control group (no supplementation)
Body weight (g)	801.56 ± 9.62^{A}	800.97 ± 67.53^{A}	769.6 ± 50.6^{AB}	713.64 ± 5.83^{B}
ADG (g)	19.34 ± 1.23^{A}	18.93 ± 0.74^{A}	18.35 ± 0.73^{AB}	17.3 ± 0.55^{B}
ADFI (g)	34.98	34.98	34.32	34.32
F:G ratio	1.82 ± 0.12^{A}	1.85 ± 0.07^{A}	1.87 ± 0.07^{AB}	1.98 ± 0.06^{B}

Values were displayed as the mean ± SD, and significance was analyzed by Student's *t* test.
[A,B]Different superscript letters in the same row indicate significant difference ($P < 0.01$). Same capital letters or no letters in the same row indicate no significant difference ($P > 0.05$).

of resistance to the tested antibiotics. Therefore, the AH-G201, AH-G202, and AH-G204 strains were selected for the next studies.

The data in Table 4 show that AH-G201 exhibited a 46.44% inhibition rate against pathogenic *E. coli* and a 60% inhibition rate against pathogenic *Salmonella* under in vitro conditions. Further, AH-G202 exhibited a 56.67% inhibition rate against pathogenic *E. coli* and a 24% inhibition rate against pathogenic *Salmonella*. The inhibition rate of AH-G204 against both pathogenic bacteria was less than 1%. The results indicated that the AH-G201 and AH-G202 strains had an inhibitory effect on the experimental pathogens, but the AH-G204 strain did not.

The results of in vivo safety analysis of AH-G201 and AH-G202 are shown in Table 5. The results indicated that the 2 strains were safe for use as food additives in poultry. The behavior, food and water intake, and stool characteristics (gray brown and cylindrical) of the experimental animals were normal, and autopsy examination did not reveal any pathological changes either. After feeding for 15 d, the average body weight of the broilers in the experimental group was significantly higher than that in the control group ($P < 0.05$).

Growth Performance

As shown in Table 6, the broilers fed with mixed *Bacillus* strains in high-dose group and medium-dose group had significantly higher body weight, and ADG than the control group. In addition, the F:G ratio in the high-dose group and medium-dose group were significantly lower than in the control group($P < 0.01$). However, there was no difference in these parameters between the high-dose group and the medium-dose group.

Immune Organ Indexes

The immune organ indexes calculated for each group are shown in Figure 4. After 42 d of the trial, there was no significant difference in the organ indexes for the bursa of fabricius and thymus among the treatment groups. Significant differences were found in the spleen index between the high-dose group and control groups. Moreover, the high-dose group showed the highest spleen weight gain, but there was no significant difference in spleen weight gain between the middle-dose group and low-dose group. However, these 2 groups showed no difference increase in spleen weight gain in comparison to the control group ($P < 0.05$).

Microflora Count

The counts of feces aerobic bacteria and lactic acid bacteria (LAB) are shown in Figure 5. At 21 d after the start

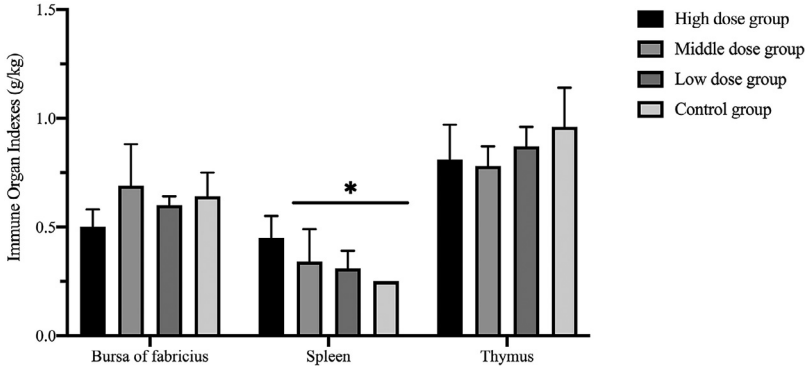

Figure 4. Effects of *Bacillus* supplementation on the immune organ indexes of broilers. The indexes of the bursa of fabricius, spleen, and thymus for broilers were calculated after feeding for 42 days. Values displayed are the mean ± SD. $^{*}P < 0.05$ indicates differences among the four groups.

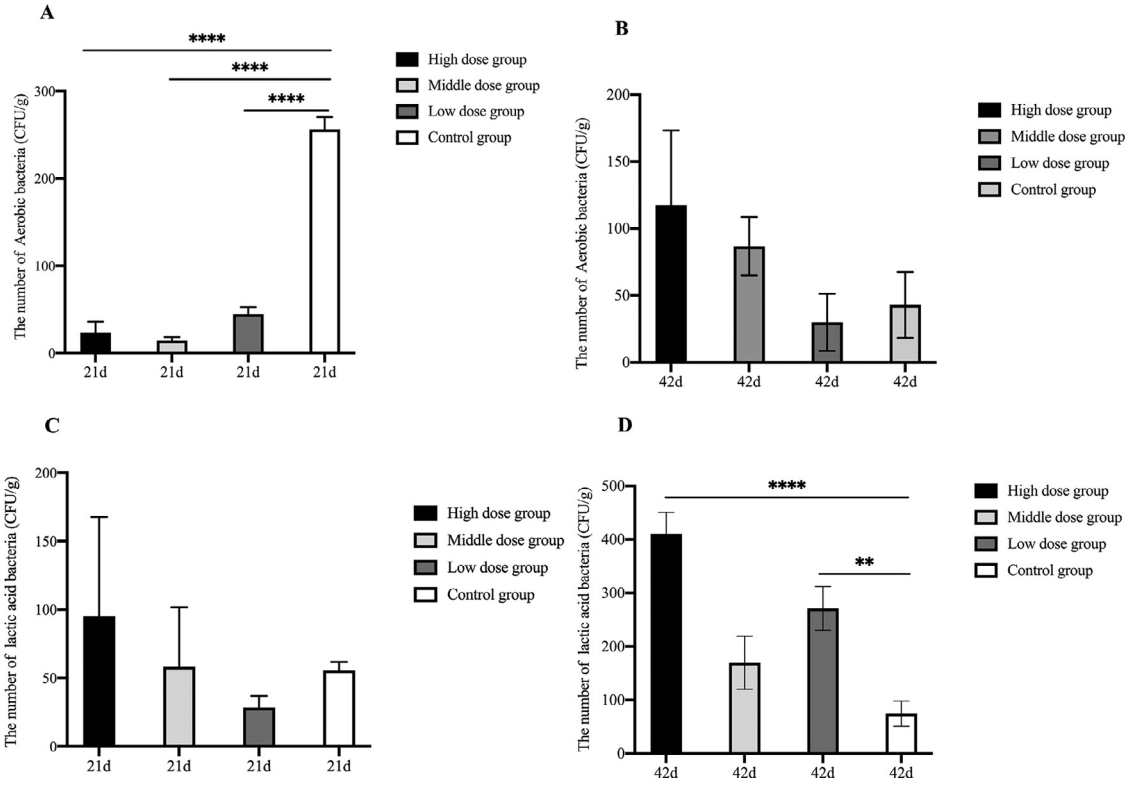

Figure 5. Effect of *Bacillus* supplementation on the aerobic bacteria and *Lactobacillus* counts in the intestinal tract of broilers. Aerobic bacteria (A and B) and *Lactobacillus* (C and D) counts in the cecal gut of broiler chickens after feeding for 21 days and 42 days. Values are displayed as the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences among the four groups.

of the trial, the feces aerobic bacteria counts were significantly lower in the treatment groups than in the control group. However, there was no significant difference in the LAB count between any of the groups. After feeding for 42 d, the LAB counts were significantly higher in the treatment groups than in the control group.

DISICUSION

In this study, we isolated and screened 13 strains of *Bacillus* from the intestinal tracts of healthy goslings and identified 2 strains (AH-G201 and AH-G202) that could germinate in the gut and the stomach, and exhibited resistance to penicillin and sensitivity to gentamicin and doxycycline. The addition of the *B. subtilis* strain AH-G201 and the *B. licheniformis* strain AH-G202 to the broilers was found significantly promote the growth performance compared to the addition of a single bacterial strain. Our findings indicated that the AH-G201 and AH-G202 strains may be useful as probiotic supplements instead of antibiotics, and may be beneficial for improving the growth of broilers and regulating intestinal flora.

Bacillus strains are commonly uses as probiotic (Hoa et al., 2001;Khaneja et al., 2010; Liu et al., 2020). In this study, 13 strains of *Bacillus* were isolated from the intestinal tract of healthy goslings, and were found to be suitable for use as probiotics in broilers. In addition, as the intestinal tract is the preferred environment

for probiotics, the strains isolated from the intestinal tract can be easily implanted in poultry and exert the maximum probiotic effect.

In order to examine the potential benefits of probiotics in the gastrointestinal tract, they need to be screened for their biological characteristics. Exposure to the gastrointestinal tract was the main stress that could reduce the viability of most ingested probiotics, due to the low pH (1.5−3.5) value of gastric juices and the antimicrobial activity of bile salts in intestinal fluid (Casula and Cutting, 2002; Li et al., 2019). Hence, probiotics need to be able to survive in the gastrointestinal conditions in order to perform their physiological functions (Huang et al., 2014). Our study has shown that the AH-G201 and AH-G202 strains could survive to some extent under simulated gastric juice (pH 1−4) and bile acid conditions. These results indicated that the AH-G201 and AH-G202 strains could probably survive and perform their physiological functions under in vivo gastrointestinal conditions. Moreover, a large number of studies have shown that Bacillus strains can inhibit a variety of pathogenic bacteria (Shu and Gill, 2001; Guo et al., 2017; Jayaraman et al., 2017; Markowiak and Śliżewska, 2017). Of the target strains tested in our research, inhibitory activity was detected against *E. coli* and *Salmonella*. It is also important for probiotics to be safe for the host and have no transferable or acquired antimicrobial resistance (Qiao et al., 2002). In our study, AH-G201 and AH-G202 were found to be resistant to penicillin and clindamycin, and sensitive to doxycycline, florfenicol, and norfloxacin. These findings indicated that our target strains have some bactericidal ability that could potentially protect against pathogenic bacteria and have a low risk of conferring resistance to antibiotics. Therefore, these 2 strains exhibit properties that make them suitable as probiotic supplements in broilers.

Poultry farming is essentially an economic activity (Zhang et al., 2021a), and growth performance characteristics are important indicators of the economic benefits of broiler production (Yang et al., 2016). As a spore-producing probiotic, B. subtilis and *B. licheniformis* can both promote growth (Deniz et al.,2011; Gao et al.,2017; Lin et al., 2017; Li et al., 2019; Dong et al., 2020; Mohammad et al., 2021; Mun et al., 2021; Zhang et al., 2021a,b; Mun et al., 2021). Our results show that adding B. subtilis AH-G201 along with B. licheniformis AH-G202 significantly promoted growth performance in the broilers, as the broilers that were fed both AH-G201 and AH-G202 showed a remarkable gain in body weight, average daily weight gain and reduce the F:G ratio especially in high-dose and middle-dose treatment group. These findings indicated that the addition of mixed strains of probiotics to the diet of broilers may have commercial benefits.

The development state of immune organs (the thymus, spleen, and bursa of fabricius) directly impacts immune function of broilers, and immune organ index have used to evaluate the development of immune organ (Sikandar et al., 2017; Fitri et al., 2022). Our results showed that feeding with both *B. subtilis* and *B. licheniformis* could promoted weight gain of the spleen in the broilers after 42 d of supplementation, which indicated that *B. subtilis* and *B. licheniformis* could promote the development of spleen. Similar to our findings, previous research has reported that *B. subtilis* could modulate immune function in broilers (Guo et al., 2016). However, the effects of mixture probiotics on immune function of broilers in our study still need to be further explored.

The gut is a hypoxic microenvironment whereas some resident microbes are aerobic to maintain the balance of the flora (Chen et al., 2020). According to previous reports, *Bacillus,* a kind of aerobic bacterium, could grow and consume oxygen to inhibit the growth of harmful aerobic bacteria (*Escherichia coli and Salmonella)*, whereas promote the dominant bacteria (lactic acid bacteria [LAB] and *Bifidobacterium*) increased in the cecum (Sanders et al., 2003; Gao et al., 2017; Chen et al., 2020; Zhang et al., 2021b), similar with our results of 21 d feeding. In addition, we think there may be 2 reasons. Firstly, combined with the results of previous pathogen inhibition studies in vitro, AH-G201 and AH-G202 had an inhibitory effect on *E. coli* and *Salmonella*. We speculate that AH-G201 and AH-G202 can inhibit aerobic pathogens and promote the growth of LAB after 21 d feeding. Secondly, the colonization and growth rate of the screened *Bacillus* may be different due to the age, breed, and regional differences of selected chickens. Therefore, we speculate that the counts of mixture *Bacillus* strains colonization in the intestinal tract did not reach the logarithmic growth stage after 21 d of feeding, and there was no significant difference in the promoting effect of LAB. Previous studies have reported that the supplementation with *B. licheniformis* to broilers' diet could significant increase the colonization of both *B. licheniformis* and *B. subtilis* in gut with the age (Mohammad et al., 2021). In our study, after 42 d of feeding, the broilers were in the growing period, and the number of *B. licheniformis* and *B. subtilis* were at the logarithmic growth phase and stationary phase. Therefore, after 42 d of feeding, the number of *Bacillus* as the aerobic bacteria in treatment groups was higher than that in control. In addition, increasing the number of *Bacillus* increase the oxygen consuming to inhibit the growth of harmful aerobic bacteria and the numbers of LAB increased. Hence, our results showed that the number of aerobic bacteria in high-dose and medium-dose group was higher than that in other groups, but had no difference between these group after 42 d of feeding.

In conclusion, *B. subtilis* AH-G201 and *B. licheniformis* AH-G202 exhibite characteristics that support their use as probiotics in broilers. The results of our animal experiments demonstrate that compound probiotics can significantly promote the growth performance of broiler chickens. Therefore, *B. subtilis* AH-G201 and *B. licheniformis* AH-G202 could potentially be used instead of antibiotics in broilers.

ACKNOWLEDGMENTS

This work was supported by the Special Project of Extension of Agricultural Science and Technology in Anhui Agricultural University (grant number: 2021kjtg001), Shennong Scholar Project of Anhui Agricultural University (grant number: rc392101), Natural Science Foundation of Anhui Agricultural University (grant number: k2039006).

DISCLOSURES

The authors declare no competing interests.

REFERENCES

Barton, MD. 2000. Antibiotic use in animal feed and its impact on human healt. Nutr. Res. Rev. 13:279–299.

Bogaard, A. E. J. M.van den, N. London, and E. E. Stobberingh. 2000. Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. J. Antimicrob. Chemother. 45:663–671.

Bunyan, J., Jeffries L. Jeffries, Jessica R. Sayers, A. L. Gulliver, and K. Coleman. 1977. Antimicrobial substances and chick growth promotion: the growth-promoting activities of antimicrobial substances, including fifty-two used either in therapy or as dietary additives. Poult. Sci. 18:283–294.

Casula, G., and S. M. Cutting. 2002. Bacillus probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68:2344–2352.

Chen, Y., L. Zhang, G. Hong, C. Huang, W. Qian, T. Bai, J. Song, Y. Song, and X. Hou. 2020. Probiotic mixtures with aerobic constituent promoted the recovery of multi-barriers in DSS-induced chronic colitis. Life Sci. 240:117089.

Deniz, G., A. Orman, F. Cetinkaya, H. Gencoglu, Y. Meral, and I. I. Turkmen. 2011. Effects of probiotic (Bacillus subtilis DSM 17299) supplementation on the caecal microflora and performance in broiler chickens. Rev. Med. Vet. 162:538–545.

Dong, Y., R. Li, Y. Liu, L. Ma, J. Zha, X. Qiao, T. Chai, and B. Wu. 2020. Benefit of dietary supplementation with bacillus subtilis BYS2 on growth performance, immune response, and disease resistance of broilers. Probiotics Antimicrob. Proteins. 12:1385–1397.

Fitri, D., S. Sugiharto, Y. Turrini, W. Endang, I. W. Hanny, and A. Tugay. 2022. Growth performance, blood variables, intestinal bacterial content, and morphological measurements of broilers supplemented with Lactobacillus casei-fermented mixture of red rice and aromatic ginger. Vet. World. 15:818–826.

Food Agriculture Organization/World Health Organization [FAO/WHO]. 2006. Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO Working Group. FAO, Rome, Italy.

Gao, Z., H. Wu, L. Shi, X. Zhang, R. Sheng, F. Yin, and R. Gooneratne. 2017. Study of Bacillus subtilis on growth performance, nutrition metabolism and intestinal microflora of 1 to 42 d broiler chickens. Anim. Nutr. 3:109–113.

Guo, M., G. Hao, B. Wang, N. Li, R. Li, L. Wei, and T. Chai. 2016. Dietary administration of bacillus subtilis enhances growth performance, immune response and disease eesistance in cherry valley ducks. Front. Microbiol. 7:1975.

Guo, M., F. Wu, G. Hao, Q. Qi, R. Li, N. Li, L. Wei, and T. Chai. 2017. Bacillus subtilisImproves immunity and disease resistance in rabbits. Front. Immunol. 8:354.

Hoa, TT., L. H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P. H. Van, and SM. Cutting. 2001. Fate and dissemination of Bacillus subtilis spores in a murine model. Appl. Environ. Microbiol. 67:3819–3823.

Holzapfel, WH., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. 73:365S–373S.

Huang, H. Y., H. Y. Hsieh, V. An. King, L. L. Chi, and J. H. Tsen. 2014. To pre-challenge lactic acid bacteria with simulated gastrointestinal conditions is a suitable approach to studying potential probiotic properties. J. Microbiol. Methods. 107:138–146.

Jayaraman, S., P. P. Das, P. C. Saini, B. Roy, and P. N. Chatterjee. 2017. Use of Bacillus Subtilis PB6 as a potential antibiotic growth promoter replacement in improving performance of broiler birds. Poult. Sci 96:2614–2622.

Khaneja, R., L. Perez-Fons, S. Fakhry, L. Baccigalupi, S. Steiger, E. To, G. Sandmann, T. C. Dong, E. Ricca, P. D. Fraser, and S. M. Cutting. 2010. Carotenoids found in Bacillus. J. Appl. Microbiol. 108:1889–1902.

Li, Ao., Y. Wang, Z. X. Li, H. Qamar, K. Mehmood, L. Zhang, J. Liu, H. Zhang, and J. Li. 2019. Probiotics isolated from yaks improves the growth performance, antioxidant activity, and cytokines related to immunity and inflammation in mice. Microb. Cell Fact. 18:112.

Li, N., Q. Wang, Y. Wang, An. Sun, Y. Lin, Y. Jin, and X. Li. 2018. Oral probiotics ameliorate the behavioral deficits induced by chronic mild stress in mice via the gut microbiota-inflammation axis. Front. Behav. Neurosci. 12:266.

Lin, Y., S. Xu, D. Zeng, X. Ni, M. Zhou, Y. Zeng, H. Wang, Y. Zhou, H. Shu, K. Pan, and G. Li. 2017. Disruption in the cecal microbiota of chickens challenged with Clostridium perfringens and other factors was alleviated by Bacillus licheniformis supplementation. PLoS One 12:1–18.

Liu, Y., T. Yan, X. Li, Y. Duan, X. Yang, and X. Yang. 2020. Effects of Bacillus subtilis and antibiotic growth promoters on the growth performance, intestinal function and gut microbiota of pullets from 0 to 6 weeks. Animal 28:1–10.

Markowiak, P., and K. Śliżewska. 2017. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients 9:1021.

Mohammad, A., M. Akteruzzaman, T. A. Ferdous, S. S. Islam, B. C. Das, M. P. Siddique, and S M. L. Kabir. 2021. Dietary supplementation of Bacillus-based probiotics on the growth performance, gut morphology, intestinal microbiota and immune response in low biosecurity broiler chickens. Vet. Anim. Sci. 14:100216.

Mun, D., H. H. Kyoung, M. Kong, S. Ryu, K. B. Jang, J. Baek, K. Park, M. Song, and Y. Kim. 2021. Effects of Bacillus-based probiotics on growth performance, nutrient digestibility, and intestinal health of weaned pigs. J. Anim. Sci. Technol. 63:1314–1327.

Qiao, H., L. C. Duffy, E. Griffiths, D. Dryja, A. Leavens, J. Rossman, G. Rich, M. Riepenhoff-Talty, and M. Locniskar. 2002. Immune responses in rhesus rotavirus-challenged Balb/c mice treated with bifidobacteria and prebiotic supplements. Pediatr. Res. 51:750–755.

Reuter, G. 2001. Probiotics—possibilities and limitations of their application in food, animal feed, and in pharmaceutical preparations for men and animals. Berl. Munch. Tierarztl. Wochenschr. 114:410–419.

Sanders, M. E., L. Morelli, and T. Tompkins. 2003. Sporeformers as human probiotics: Bacillus, Sporolactobacillus, and Brevibacillus. Compr. Rev. Food Sci. Food Saf. 2:101–110.

Shivaramaiah, S., N. R. Pumford, M. J. Morgan, R. E. Wolfenden, A. D. Wolfenden, A. Torres-Rodriguez, B. M. Hargis, and G. Tellez. 2011. Evaluation of Bacillus species as potential candidates for direct-fed microbials in commercial poultry. Poult. Sci. 90:1574–1580.

Shu, Q., and H. S. Gill. 2001. A dietary probiotic (Bifidobacterium lactis HN019) reduces the severity of Escherichia coli O157:H7 infection in mice. Med. Microbiol. Immunol. 189:147–152.

Sikandar, A., H. Zaneb, M. Younus, S. Masood, A. Aslam, M. Shah, and H. Rehman. 2017. Growth performance, immune status and organ morphometry in broilers fed Bacillus subtilis-supplemented diet. S. Afr. J. Anim. Sci. 47:378–388.

Sorum, H., and M. Sunde. 2001. Resistance to antibiotics in the normal flora of animals. Vet. Res. 32:227–241.

Yang, J., K. Qian, W. Zhang, Y. Xu, and Y. Wu. 2016. Effects of chromium-enriched bacillus subtilis KT260179 supplementation on chicken growth performance, plasma lipid parameters, tissue chromium levels, cecal bacterial composition and breast meat quality. Lipids Health Dis. 15:188.

Zhang, N., L. Wang, and Y. Wei. 2021a. Effects of Bacillus pumilus on growth performance, immunological indicators and gut microbiota of mice. J. Anim. Physiol. Anim. Nutr. (Berl) 105:797–805.

Zhang, S., G. Zhong, D. Shao, Q. Wang, Yan. Hu, T. Wu, C. Ji, and S. Shi. 2021b. Dietary supplementation with Bacillus subtilis promotes growth performance of broilers by altering the dominant microbial community. Poult. Sci. 100:100935.

Zhu, X., and R. D. Joergert. 2003. Composition of microbiota in content and mucus from ceca of broiler chickens as measured by fluorescent in situ hybridization with group-specific, 16S rRNA-targeted oligonucleotide probes. Poult. Sci. 82:1241–1249.