Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.332

RESEARCH ARTICLE

The influence of *Bacillus amyloliquefaciens* BS32 Isolate in Enhancing Growth, Immune Response, and Regulating Intestinal Gene Expression in *Salmonella*-Challenged Broilers

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ARTICLE HISTORY (25-814)

Received: August 18, 2025
Revised: December 02, 2025
Accepted: December 09, 2025
Published online: December 20, 2025

Key words:
Bacillus
Gene expression
Growth performance
Gut microbiota
Histology
Probiotics
Salmonella

ABSTRACT

Salmonella infection remains a significant concern in the poultry production industry. It causes substantial economic losses worldwide, including decreased growth rates, increased mortality, and a heightened risk of foodborne transmission to humans. The objective of this study was to isolate and characterize *Bacillus* species from broiler feces and to evaluate the probiotic potential of *Bacillus amyloliquefaciens* BS32 (BaBS32) isolate, with particular emphasis on its capacity to improve broiler health and to prevent the adverse effects of *Salmonella* infection. Following antimicrobial testing of 40 isolates against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the BaBS32 isolate demonstrated the largest inhibition zones, measuring 30mm and 26 mm, respectively. Morphological, biochemical, and 16S rDNA sequencing analyses revealed that BS32 shared 99% similarity with *Bacillus amyloliquefaciens* DSM 25840. The BaBS32 isolate exhibited several key probiotic characteristics, including robust survival at pH 2.5 (85.2%) and in 0.3% bile salt (78.6%), strong biofilm-forming ability, phosphate solubilization, and indole-3-acetic acid production. Antibiotic susceptibility testing indicated that the BaBS32 isolate is safe, exhibiting broad sensitivity and no multidrug resistance. In *vitro* assays demonstrated that BaBS32 possesses dose-dependent antioxidant and antibacterial activities, with DPPH radical scavenging reaching 90% at 320 mg/mL and an inhibitory zone of 8.5-34 mm against pathogenic bacteria. Dietary supplementation of broilers with BaBS32 isolate at doses of 80-320 mg/kg resulted in improved growth performance, enhanced antioxidant enzyme activities, and strengthened immunity, meanwhile, decreasing liver enzyme levels and markers of oxidative stress. Additionally, BaBS32 treatment reduced the expression of inflammatory and apoptotic genes induced by *Salmonella*, notably *BAX*, and *Caspase*- 3 (up to 2.6 and 1.85- fold, respectively), and upregulated genes associated with tight junction integrity and immune response. On the other hand, microbial count decreased, where *E. coli* and *Salmonella* populations by 61%, and an enrichment of beneficial lactic acid bacteria by 55%. Histopathological examination indicated that BaBS32 isolate mitigated intestinal damage caused by *Salmonella*, restoring villus height and improving mucosal structures. *Bacillus amyloliquefaciens* BS32 isolate exhibits significant probiotic potential, capable of enhancing broiler health and resistance against *Salmonella* infection.

To Cite This Article: Aminah Allohibi, 2025. The influence of *Bacillus amyloliquefaciens* BS32 isolate in enhancing growth, immune response, and regulating intestinal gene expression in *Salmonella*-challenged broilers. Pak Vet J, 45(4): 1638-1649. http://dx.doi.org/10.29261/pakvetj/2025.332

INTRODUCTION

Salmonella infection poses a considerable global challenge in poultry production, bearing significant economic and public health repercussions (Wibisono *et al.,* 2020). The infections increase mortality rates, reduce growth efficiency, and cause substantial losses in the poultry industry (Zhou *et al.,* 2020). Notably, poultry-based products such as meat and eggs remain among the primary causes of human salmonellosis, which can lead to substantial morbidity worldwide. Contamination that may occur during manufacturing and processing poses risks to animal health and presents significant public health challenges (Ali and Alsayeqh, 2022). This highlights the importance of adopting effective control strategies throughout the poultry production chain to safeguard consumers and maintain the industry productivity.

The traditional approaches to controlling *Salmonella* in poultry involve the utilization of vaccines, strict biosecurity measures, antibiotics, and improved farm hygiene. (Ruvalcaba-Gómez *et al.,* 2022). Vaccination provides partial protection across serovars and exhibits an unpredictable duration of immunity, although it offers targeted protection. The management and enhancement of livestock growth have historically relied on antibiotics; however, their excessive use has accelerated the development of antimicrobial resistance (AMR). This phenomenon has diminished treatment efficacy and facilitated the proliferation of resistant zoonotic pathogens within the human food supply. International health authorities underscore the urgent need to mitigate AMR through the adoption of antibiotic alternatives in livestock, thereby ensuring the safety of both animal and human health (Kasimanickam *et al.*, 2021).

Under these circumstances, there is a heightened interest in probiotics as an alternative to antibiotics, which are unsustainable in poultry production. Probiotics, defined as live microorganisms that confer health benefits to the host, serve to improve gut microbial balance, enhance immune responses, and inhibit pathogenic microorganisms through competitive exclusion (Mazziotta *et al.,* 2023). The most notable species in this category are the *Bacillus* species, which are remarkable for their ability to produce strong spores resistant to gastrointestinal conditions, to colonize, and to remain active over an extended period (Todorov *et al.,* 2022). Furthermore, *Bacillus* species produce antimicrobial metabolites, promote the secretion of digestive enzymes, and regulate host immunity. These factors collectively enhance nutrient utilization and increase disease resistance. Previous studies suggest that dietary supplementation with *Bacillus* probiotics improves growth performance in broilers, evidenced by increased body weight, elevated feed intake, and a favorable feed-to-gain ratio. Notably, the administration of both *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been associated with greater villus height, an expanded mucosal surface area, and improved intestinal morphology, thereby facilitating more effective nutrient absorption. These advancements directly lead to increased output and economic gains for poultry producers. Additionally improving nutritional value, *Bacillus* probiotics are also protective against enteric pathogens, particularly, *Salmonella* (Mazkour *et al.,* 2022). Their extrusion of ecological niches into the gut, production of bacteriocins and antibacterial metabolites to suppress pathogen growth, and activation of the immune system are manifestations of competitive exclusion (Hashem, 2025). Earlier research has demonstrated a decline of over 60 % in *Salmonella* and *Escherichia coli* counts in broilers fed on *Bacillus* probiotics with significant increases in the population of beneficial lactic acid bacteria (Ringø *et al.,* 2020). These microbial modifications can be utilized to restore gut homeostasis, improve barrier integrity, and decrease pathogen colonization and shedding. At the molecular level, *Bacillus* probiotics influence the expression of genes related to maintaining epithelial barrier function and immune regulation. These regulatory mechanisms promote improved intestinal health, enhanced resistance to disease, and overall systemic well-being. Although substantial evidence supports the efficacy of *Bacillus*, there remain knowledge gaps regarding the specific molecular pathways involved during *Salmonella* infection, particularly concerning the relationship between gut microbiota modulation and host physiological outcomes. Moreover, the identification and assessment of new *Bacillus* isolates with improved probiotic properties remain necessary (Su *et al.,* 2025).

Based on this, the current study aimed to isolate and molecularly characterize a novel isolate, *Bacillus amyloliquefaciens* BS32, and then assess its probiotic potential through both in vitro and in vivo methodologies. The objectives of the study were: (1) to establish the antimicrobial, antioxidant, and gastrointestinal survival properties of BaBS32; (2) to evaluate the effects on growth performance, liver function, oxidative stress, and immune parameters of broilers when BaBS32 was utilized both under standard conditions and in the presence of Salmonella challenge; (3) to determine the impact of BaBS32 on genes associated with gut intestinal barrier function, as well as intestinal inflammation and apoptosis; and (4) to assess the effects on gut microbial populations and intestinal histomorphology to validate BaBS32 as an effective and safe probiotic alternative to antibiotics in poultry production. Consequently, this research will contribute to enhancing scientific knowledge and support the practical application of a promising Bacillus probiotic that can improve the health and productivity of broilers, while concurrently mitigating the persistent threat of *Salmonella* infection in poultry systems.

MATERIALS AND METHODS

***Bacillus* isolation, screening, and identification:** Fecal samples from broiler chickens were collected from poultry farm cages and placed in sterile containers, then transported to the microbiology laboratory within 24 hours. A 10 g sample of these feces was homogenized in 90 mL of peptone buffer to achieve a 10^{-1} dilution, which was subsequently serially diluted to 10^{-7}. All dilution samples were inoculated onto Luria-Bertani (LB) agar plates and incubated at 37°C for 24 hours. The isolates screened based on the most promising antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected for further analysis. Bacterial identification was conducted using conventional morphological, biochemical, and physiological characterization methods. Cell wall lysis was accomplished by sequentially applying mutanolysin, achromopeptidase, and lysostaphin as cell wall lytic enzymes. DNA was purified using phenol-chloroform extraction. The DNA pellets were separated by electrophoresis on a 1.5% agarose gel using Tris/borate/EDTA (TBE) buffer, and stained with ethidium bromide to visualize under UV light. Fragment sizes were estimated based on a 3000 bp molecular weight ladder. For genetic characterization, the 16S rRNA gene was amplified via PCR using primers UBC-827 (5'-AC)8G-3' and UBC-901 (5'(CA)8RY-3'). Partial sequencing of the amplified products was performed. The RNAmmer version 1.2 software was employed to reconstruct gene sequences from whole-genome shotgun data, which were then compared with those of related *Bacillus* species.

Probiotic and safety properties: Acid resistance was measured using a spectrophotometer at 650nm at hourly intervals in triplicate. The bile salt tolerance test involved inoculating 1 mL of bacterial culture into 9mL of LB broth with 0.1 M NaOH, followed by incubation at 37°C for 3 hours. OD readings at 650 nm were recorded and justified to 0.08±0.05 to standardize bacterial counts. Each acid-tolerant isolate's bile tolerance was then tested by adding 100µL of the overnight culture into 0.3% bile salts in LB broth. Viability was checked by sampling 100µL at 0, 1, 2, 3, and 4 hours, then plating on LB agar. Growth of colonies indicated a positive result, while no colonies indicated a negative.

Calculation of the survival rate was

$$Survival\ rate\ (\%) = \frac{OD\ after\ treatment}{OD\ before\ treatment}\ X\ 100$$

To perform a safety assessment, antibiotic susceptibility was evaluated by plating isolates on nutrient media at a final concentration of 10^6 CFU/g. Standard antibiotic discs, including tetracycline (30 µg), azithromycin, erythromycin, ceftriaxone, and gentamicin, were employed. The plates were incubated at 42 °C for 48 hours.

Biological activities

Antioxidant activity: BS isolate at concentrations of 10, 20, 40, 80, 160, and 320µg/mL was evaluated for DPPH radical-scavenging activity. An ethanolic DPPH solution (0.5 mL) was combined with 1 mL of each sample extract, and the mixture was allowed to darken for 30 minutes. Absorbance measurements were taken at 517nm. The IC50 values were determined as the minimum concentration of each substance required to achieve 50% scavenging of the DPPH radical. The percentage of DPPH scavenging activity was calculated using the following formula.

$$\%\ DPPH\ scavenging\ activity = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100$$

Antibacterial activity: To evaluate the antibacterial effects, *Bacillus* isolate, six concentrations (10, 20, 40, 80, 160, and 320µg/mL) were prepared. Eight-millimeter discs were moistened with each concentration for 30 minutes. These discs were subsequently tested against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Salmonella typhi*, *Escherichia coli*, and *Klebsiella pneumoniae*. The incubation was followed by measuring inhibition zones in millimeters.

Experimental design: The entire study was conducted at a private farm in Rabigh City, Rabigh Governorate, Makkah Province, Saudi Arabia. All animal experiments were performed in accordance with the ethical standards of the institutional research committee and followed the national and international guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the appropriate Institutional Animal Care and Use Committee (IACUC). All procedures were designed to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

A randomized distribution of 360 broiler chicks was divided into six groups based on initial body weight on day one. Each group comprised six replicates of ten chicks. The treatments included a control group, T1-T3 groups of non-infected broilers supplemented with either 80, 160, or 320mg/kg of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, and T5 *Salmonella*-challenged broilers and treated with BaBS32 at 320mg/kg. The supplementation doses were selected based on previous research. For the *Salmonella* challenge, 0.5mL of *Salmonella* culture (1 x 10^8 CFU/mL) was administered into the crop using a syringe fitted with a sterile gavage cannula. The birds were housed in three-level battery cages equipped with automated watering systems and had unrestricted access to feed and water. The composition of the basal diet was prepared according to previously established specifications.

Growth performance: Growth performance parameters, including live body weight (LBW), feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), performance index (PI), and growth rate (GR), were calculated in accordance with the methodologies of Saad *et al.* (2022) and (Brody and Lardy, 1946): Body weight gain

(BWG) = Final body weight (FBW) − Initial body weight (IBW)
Growth rate (GR) = $(LBW_{35} - LBW_1) / [0.5 \times (LBW_1 + LBW_{35})]$
Performance index (PI) = BWG / FCR

Biochemical parameters: Three chicks per group were anesthetized using an R550 Multioutput Laboratory Small Animal Anesthesia Machine, which allows gas flow adjustments from 0 to 2.0L/min. Blood serum was obtained by sampling the hepatic portal vein, then centrifuged at 3000 rpm for 15 minutes. The serum levels of AST, ALT, the AST/ALT ratio, and ALP were analyzed. Liver tissues were rinsed with cold 0.9% saline, weighed, and stored at -70°C. Levels of MDA and activities of SOD, GSH, and CAT were measured, along with the total antioxidant capacity (TAC). Immunoglobulins IgG, IgA, and IgM were quantified using sandwich ELISA at an absorbance of 450nm.

Gene expression: The RNA extracted from the intestinal tissue of the chicken was subsequently dissolved in diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was determined using OD 260/280 measurement. In semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), 3µg of RNA was loaded, and the samples were denatured at 70°C for five minutes. cDNA synthesis was conducted using 0.5 ng oligo (dT) primers, 2µL of 10X reverse transcription buffer, 2 µL of 10mM deoxynucleotide triphosphates (dNTPs), and 1µL of 100µM reverse transcriptase. The mixture was incubated at 42°C, then at 70°C for 10 minutes. Gene expression levels were quantified using the 2-ΔΔCT method, with actin serving as the reference gene (Table 1).

Microbial quantification of the intestine: The aseptically collected post-mortem intestinal digest was homogenized, frozen, and stored at 4°C. Total viable bacteria, *E. coli*, total yeast and molds, and *Lactobacillus* spp. counts were reported in log10 CFU/g digesta.

Intestine histology: The intestinal tissues were kept in 10% formalin (48 hours), washed with distilled water (30 minutes), and dried using graded alcohol (70 and 90 %). Clearing was performed using xylene cycles of 50% xylene (60 min), 50 % alcohol, and pure xylene (90 min). The samples were fixed in paraffin, then sliced at 4-5 µm and stained with Hematoxylin and Eosin.

Statistical analysis: The Shapiro-Wilk test was used to assess normality, and the Levene test was used to assess homogeneity of variance. A one-way ANOVA was performed because both assumptions were satisfied ($P>0.05$). Data are presented as mean ± SE. Post hoc comparisons were conducted using Fisher's least significant difference (LSD) test. The level of statistical significance was set at $P\leq0.05$.

RESULTS

Forty bacterial cultures were collected from soil samples labeled BS 1 to BS 40. These isolates were screened their antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* to identify the most promising candidates for further probiotic evaluation. Four selected isolates demonstrated greater inhibitory zones against both pathogens, BS32 exhibited the largest zones of inhibition, measuring 30.0 mm against *S. aureus* and 26 mm against *P. aeruginosa*. This considerable antibacterial activity suggested a high likelihood of probiotic effectiveness (Table 2), thereby selecting BS32 for detailed characterization. Microscopic analysis revealed that BS32 is a Gram-positive, motile, rod-shaped bacterium that exists as single cells, consistent with biochemical profiling results. When cultured on LB agar, BS32 formed flat, round colonies with irregular margins and a pale cream coloration. The isolate solubilized 62.4mg/L of $Ca_3(PO_4)_2$ within seven days, produced 8.9 ppm of indole-3-acetic acid (IAA) in the presence of tryptophan, and catalyzed the conversion of 0.7mol/h/vial of acetylene to ethylene in a nitrogen-free malate medium. Additionally, BS32 used 1-aminocyclopropane-1-carboxylic acid (ACC) as its sole carbon source and demonstrated the capacity to form biofilms on glass surfaces. These morphological and biochemical characteristics indicated a close relation to *Bacillus* species. Further characterization via MALDI- TOF and 16S rDNA sequencing identified the isolate as *Bacillus amyloliquefaciens* BS32, exhibiting 99% similarity to *Bacillus amyloliquefaciens* DSM 25840. PCR amplification produced a single 350-bp band corresponding to the 16S rRNA gene, thereby confirming the accuracy of the protocol. Phylogenetic analysis further supported its close relationship with other *Bacillus* strains, suggesting that BaBS32 represents a novel isolate of *Bacillus amyloliquefaciens* (Fig. 1).

Probiotic properties:

PH and bile salts tolerance: Table 2 indicates that isolates, especially *Bacillus amyloliquefaciens* BS32, possess the necessary probiotic properties. BaBS32 showed the highest survival rates in acidic conditions (pH 2.5; 85.2±3.1%) and in 0.3% bile salts (78.6±2.8%). These characteristics are important because effective gut colonization requires resistance to gastric acidity and bile exposure. BaBS32 exhibited higher tolerance and antimicrobial activity than the other isolates, with BS9 showing the lowest values. Overall, these findings demonstrate the strong probiotic potential of BaBS32.

Antibiotic susceptibility: Four isolates, BS32, BS15, BS10, and BS9, showed variable sensitivity to tetracycline, azithromycin, erythromycin, and gentamicin, with inhibition zones exceeding the CLSI susceptibility threshold (19mm). BS32 had the largest inhibition zones in the group. Responses to ceftriaxone were intermediate, i.e., BS32 (18±0.8mm) and BS15 (16±0.7mm), whereas BS10 (14±0.6mm) and BS9 (12±0.5mm) were considered resistant. Notably, no isolates exhibited multidrug resistance. These findings endorse the safety of the isolates, particularly BS32, for use as probiotics.

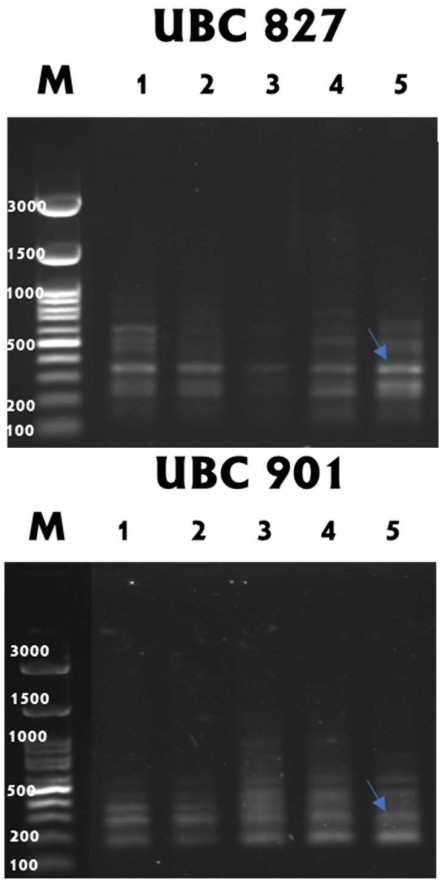

Fig. 1: 16S rRNA genes of *Bacillus* isolate, Lane 1, Ladder [(L) 100–3,000 bp]; Lane 2 to 5, Positive controls (P, *Bacillus amyloliquefaciens* DSM 25840); Lane 6, identified isolate at 350bp.

Biological activities

Antioxidant activity: The dose-dependent antioxidant value of *Bacillus amyloliquefaciens* BS32, as measured by DPPH radical-scavenging activity, is shown in Fig. 2. The efficiency of the antioxidants also increased gradually as the concentration rose; the Scavenging activity rose from 35% at 10mg/mL to almost 90% at 320mg/mL. Each increase in concentration led to a significant increase in antioxidant activity ($P<0.05$), indicating a strong, progressively increasing antioxidant effect.

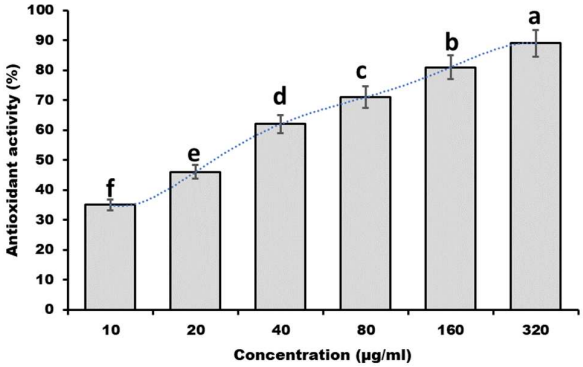

Fig. 2: Antioxidant activity of *Bacillus amyloliquefaciens* BS32 isolate against DPPH free radicals. Lowercase letters above columns indicate significant differences (P<0.05).

Table 1: Primer sequences and characteristics for target genes

Target Gene	Primer Sequence (5'→3')	Product Length (bp)	GenBank Reference
TBP	F: CGTGCAAGCTCTGTTTAGTG R: AAGCATTCCGGCAAAGCAGC	106	NM_001396193.1
OCLN	F: AGGTCTACAACAGCATCACA R: ATGCCTTCCCAAAAGGACT	157	NM_205128.1
MUC1	F: GGGAATCTGTGGCTTGTTGA R: TTCTCAGCATCTCTCCCCAA	83	XM_040680153.2
JAM2	F: TCCTGCAGCGCTGACTTCAT R: CGGACTCAATTACAAGCAGC	138	NM_001397141.1
CD4	F: ACCGACATCTGTGGAGCAGC R: TCCAAGGGAAAGCTCTTCAC	197	NM_204649.2
CD8α	F: AACAGTGACAGTGGTGGTC R: CCTGAGTAGGTGGTATGGGA	98	NM_001048080.1
IL1β	F: CTGCCTGCAGAAGAAGCCC R: TGTCAGCAAAGTCCCGCTC	164	NM_204524.2
IL6	F: AACAACCTCAACCTCCCCAA R: AGGTCTGAAAGGCGAACAGA	112	NM_204628.2
TLR4	F: GTTTGGTGCTTGGAAGCTTG R: CGAGCTGTTGCCACCCCTTA	146	NM_001030693.2
BAX	F: TGACCCTCTGACCCTAGCTT R: ATCCCAGCACTTTGAGAGGT	134	NM_001291430.2
CASP3	F: AGGTGGAGGAGCTCTCCTAC R: CCTGAGCGTGGTCCATCTTC	199	NM_204725.2
GAPDH	F: CCACATGGCATCCAAGGACT R: GAACTGAGCGGTGGTGAAGG	101	NM_204305.2

TBP: TATA-box binding protein (reference gene), OCLN: Occludin (tight junction protein), MUC1: Mucin 1 (epithelial barrier marker), JAM2: Junctional adhesion molecule 2, CD4/CD8α: Cluster of differentiation 4/8α (T-cell markers), IL1β/IL6: Interleukin-1β/Interleukin-6 (pro-inflammatory cytokines), TLR4: Toll-like receptor 4 (innate immunity), BAX: BCL2-associated X protein (apoptosis regulator), CASP3: Caspase 3 (apoptosis executor), GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (reference gene), F/R: Forward/Reverse primer, bp: Base pairs.

Table 2: Survival Rate (%) of *Bacillus amyloliquefaciens* B32 against low pH and bile salt (0.3%)

Isolate	Inhibition Zone (mm)	Survival Rate (%)	
		pH 2.5	0.3% Bile Salt
BS32	30.0 (*S. aureus*) 26.0 (*P. aeruginosa*)	85.2±3.1	78.6±2.8
BS15	27.5 (*S. aureus*) 24.3 (*P. aeruginosa*)	79.4±2.7	72.3±2.5
BS10	26.7 (*S. aureus*) 23.1 (*P. aeruginosa*)	75.8±2.9	68.9±2.3
BS9	25.3 (*S. aureus*) 22.0 (*P. aeruginosa*)	70.1±3.0	65.4±2.1

Data are presented as mean±SD

Antibacterial activity: Inhibition zones increased steadily with increasing BaBS32 suspension concentration. The maximum sensitivity was observed in *Staphylococcus aureus* (34.0±1.7mm), *Streptococcus pyogenes* (32.8±1.6mm), and *Listeria monocytogenes* (31.5±1.5mm). *Klebsiella pneumoniae* and *Escherichia coli* were relatively insensitive, with the inhibition zone of 27.5±1.2mm and 28.3±1.3mm, respectively.

Table 3: Antibiotic resistance profiles of selected *bacillus* isolates

Antibiotic (30 µg)	BS32	BS15	BS10	BS9	CLSI Interpretation
Tetracycline	22±1.0	20±0.9	18±0.8	15±0.7*	S (≥19 mm)
Azithromycin	25±1.2	23±1.1	21±1.0	19±0.9	S (≥19 mm)
Erythromycin	28±1.3	25±1.2	23±1.1	20±1.0	S (≥19 mm)
Ceftriaxone	18±0.8	16±0.7	14±0.6*	12±0.5*	I (15–18 mm), R (≤14 mm)
Gentamicin	30±1.5	28±1.4	25±1.2	22±1.1	S (≥19 mm)

(Inhibition Zone Diameter, mm±SD; n=3) R, resistant, S, sensitive, I, susceptible

Table 4: Antibacterial activity of *Bacillus amyloliquefaciens* BS32 at various concentrations against pathogenic bacteria

Pathogenic Bacteria	Inhibition Zone Diameter (mm) at Different Concentrations (µg/mL)					
	10	20	40	80	160	320
Staphylococcus aureus	12.5±0.8	18.2±1.0	24.7±1.3	30.0±1.5	32.5±1.6	34.0±1.7
Streptococcus pyogenes	11.8±0.7	17.5±0.9	23.2±1.2	28.5±1.4	31.0±1.5	32.8±1.6
Listeria monocytogenes	10.5±0.6	16.0±0.8	21.5±1.1	27.2±1.3	29.8±1.4	31.5±1.5
Salmonella typhi	9.8±0.5	14.5±0.7	19.8±1.0	25.9±1.2	28.3±1.3	30.2±1.4
Escherichia coli	8.5±0.4	13.2±0.6	18.5±0.9	24.8±1.1	27.0±1.2	28.8±1.3
Klebsiella pneumoniae	7.2±0.3	12.0±0.5	17.0±0.8	23.6±1.0	25.8±1.1	27.5±1.2

*(Inhibition Zone Diameter, mm±SD; n=3) *

In Vivo experiment

Growth performance of *Salmonella*-challenged broilers: Table 5 depicts the impact of dietary BS32 supplementation on growth performance between 10 and 35days. The BS32 treatments (T1-T3) demonstrated significantly higher final body weight (FBW), body weight gain (BWG), feed intake (FI), and performance index (PI), with the magnitude of improvements being dose-dependent (P<0.05). The greatest enhancement was observed in the T3 group (320 mg/kg). *Salmonella* challenge (T4) markedly reduced FBW, BWG, FI, and PI, while increasing the feed conversion ratio (FCR). Notably, partial mitigation of these adverse effects was achieved through BS32 supplementation at 320 mg/kg in *Salmonella*-challenged birds (T5), with most growth parameters returning to baseline levels and FCR approaching control values.

Blood biochemistry markers: Table 6 outlines parameters related to liver and kidney function, oxidative stress, and immune response. AST and ALT showed significant dose-dependent changes across dietary BS32 groups (T1-T3), indicating hepatoprotective effects. The oxidative stress marker MDA decreased, while antioxidant enzymes (SOD, GSH, and CAT) increased, especially in the highest-dose group (T3). *Salmonella* challenge in T4 led to increased levels of AST, ALT, MDA, and uric acid, along with impairments in immunity and antioxidant activity. However, supplementation with BS32 at 320mg/kg (T5) significantly reduced these changes, regulating parameters to normal. BaBS32 also significantly increased immunoglobulin levels (IgG and IgA), suggesting improved humoral immunity. There were no notable differences in thyroid hormones (T3, T4) among the groups. Overall, BaBS32 acts as an important protective agent against liver damage, oxidative stress, and immune problems, even during *Salmonella* infection.

Gene expression against *Salmonella* infection: The results concerning the impact of dietary *Bacillus amyloliquefaciens* BS32 on intestinal gene expression related to tight junction integrity and immune regulation in broilers are summarized in Table 7. Supplementation with BaBS32 (160 mg/kg T2 and 320 mg/kg T3) in non-infected birds notably elevated the expression of tight junction genes. Specifically, the levels of OCLN, MUC1, and JAM2 increased by approximately 58%, 63%, and 68%, respectively, with an additional 47% increase observed at T3. Conversely, the Salmonella challenge (T4) caused significant downregulation of barrier-associated genes, with OCLN, MUC1, and JAM2 decreasing by 8%, 12%, and 5%, respectively, relative to the control, indicating compromised mucosal integrity. Nevertheless, birds challenged (T5) and supplemented with BaBS32 at 320 mg/kg demonstrated a notable recovery in tight junction gene expression, with increases of 55%, 39%, and 36% in OCLN, MUC1, and JAM2, respectively, compared to the control. Furthermore, there was modulation of immune marker genes: BaBS32 (T3) upregulated CD4 and CD8a by 76% and 61% in healthy broilers, respectively, while Salmonella challenge resulted in significant downregulation, with reductions of 18% and 9% in CD4 and CD8a, respectively, below control levels. In vivo, the supplementation of BaBS32 (T5) in infected birds facilitated the restoration of immune gene expression, evidenced by increases of 49% in CD4 and 42% in CD8a relative to the control. Furthermore, TLR4 expression was downregulated across all BaBS32-stimulated groups, with a maximum reduction of 28% relative to the control, suggesting a potential anti-inflammatory regulatory role. All these data collectively demonstrate that BaBS32 supplementation enhances tight junction integrity and immune gene expression in healthy broilers and counteracts *Salmonella*-induced suppression, with a beneficial effect of 76%.

Fig. 3 (A and B) shows the effects of BaBS32 on the intestinal expression of IL-1b and IL-6 in both healthy and *Salmonella*-challenged broilers. In non-infected birds, BaBS32 (T1-T3) caused a dose-dependent reduction in these cytokines: *IL-1β* levels dropped by 20% (T1), 33% (T2), and 46% (T3), while IL-6 decreased by 24%, 35%, and 53%, respectively, indicating a strong anti-inflammatory effect. *Salmonella* challenge (T4) significantly increased IL-1b (38 %) and IL-6 (33 %) levels compared to the control. Notably, BaBS32 at 320 mg/kg (T5) significantly lowered IL-1b and IL-6 by 48% and 42%, respectively, compared to T4, bringing levels close to those seen in uninfected controls. Overall, BaBS32 supplementation effectively suppresses both baseline and infection-induced inflammatory cytokines, reducing IL-1b and IL-6 levels by up to 53%.

Pro-survival gene expression (Bcl-xL): Fig.s 4A and 4B illustrate how BaBS32 impacts the pro-apoptotic proteins BAX and Caspase-3. When BaBS32 was added to non-infected broilers, it caused a dose-dependent reduction in the expression of apoptosis-related genes. BAX expression decreased by 16% (T1), 23% (T2), and 29% (T3), while Caspase-3 expression was lowered by 18%, 27%, and 37%, respectively. Conversely, Salmonella challenge (T4) significantly increased apoptotic activity, with increases of 60 percent in BAX expression and 38 percent in Caspase-3 relative to control. Challenge conditions (T5) at BaBS32 mg/kg produced significant levels of apoptosis in birds, with a 30-22% reduction in *BAX* and *Caspase*-3. Whereas it remains somewhat high relative to the non-infected control, these decreases are suggestive of robust protective and anti-apoptotic actions of BaBS32.

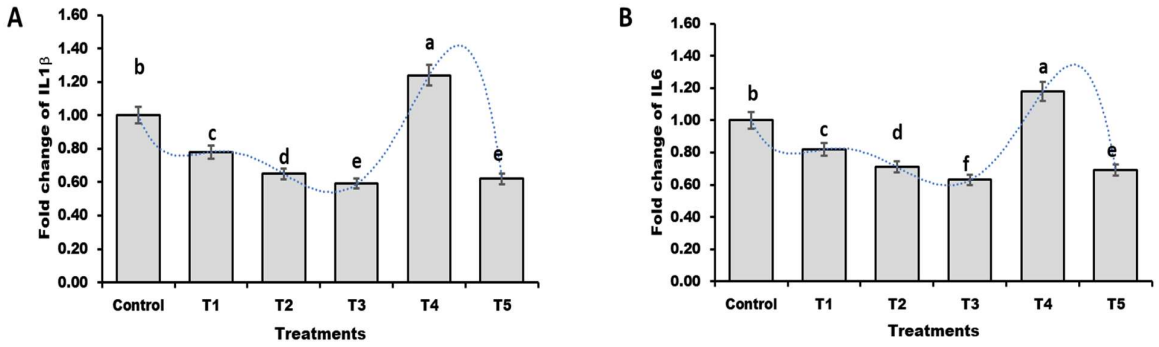

Fig. 3: Effects of dietary *Bacillus amyloliquefaciens* BS32 treatments on gene expression of proinflammatory cytokines (A) IL-1β and (B) IL-6 in *Salmonella*-challenged broilers. Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320 mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320 mg/kg). Lowercase letters above columns indicate significant differences using the LSD test at P<0.05.

Table 5: Effect of dietary *Bacillus amyloliquefaciens* BS32 on growth performance Parameters of *Salmonella*-challenged broilers

Parameters	Age (d)	Control	T1	T2	T3	T4	T5
LBW (g)	10	45±0.1	45.1±0.0	44.8±0.5	45.1±0.2	44.6±0.2	44.5±0.0
FBW (g)	35	2225.0±2.9c	2352±2.2b	2395±2.7ab	2402±1.9a	2100±1.5d	2270±2.1b
BWG (g)	10-35	2210±1.1c	2306.9±0.2b	2350.2±1.6a	2356.9±1.0a	2055.4±0.9c	2225.5±1.9c
FI (g)	10-35	3650±2.1c	3662±2.3d	3681±1.4b	3695±2.1a	3750±1.2e	3675±0.9cd
FCR	10-35	1.65±0.1c	1.58±0.2c	1.56±0.5b	1.56±0.1a	1.82±0.6e	1.65±0.2c
GR	10-35	194.7±1.1b	192.4±1.2b	192.7±1.2b	192.7±1.3a	191.6±2.1e	192.3±3.2c
PI	10-35	133.9±0.6c	145.9±1.0d	150.6±1.0b	151.0±1.5a	112.9±2.8e	134.8±2.0b

Live body weight (LBW), Final body weight (FBW), Body weight gain (BWG), Feed intake (FI), Feed conversion ratio (FCR), Growth rate (GR), Performance index (PI). Data are presented as mean ±SE. Lowercase letters within rows indicate significant differences (P<0.05). Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320 mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320 mg/kg).

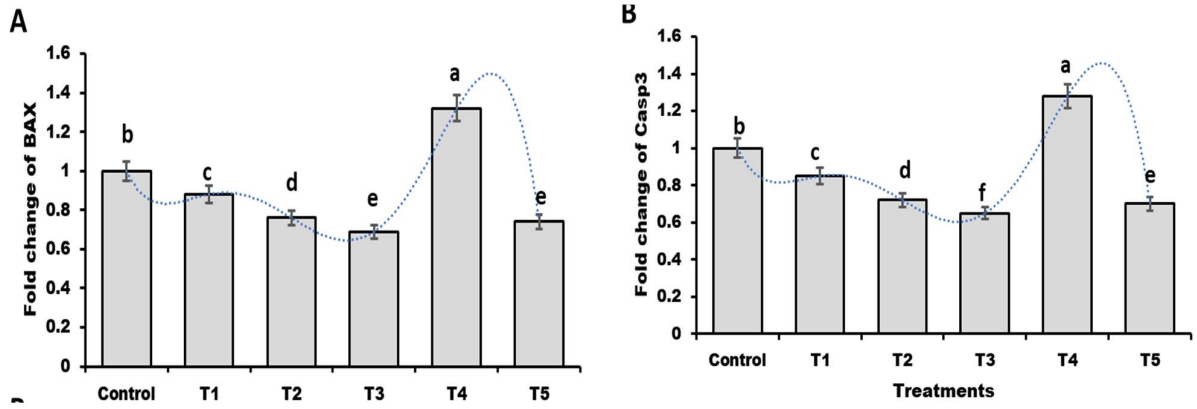

Fig. 4: Effects of dietary *Bacillus amyloliquefaciens* BS32 treatments on gene expression of precancerous markers (A) BAX and (B) Casp3 in *Salmonella*-challenged broilers' intestine. Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320 mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320 mg/kg). Lowercase letters above columns indicate significant differences using the LSD test at P<0.05.

Table 6: Effect of dietary *Bacillus amyloliquefaciens* BS32 on serum kidney and liver function, oxidative stress markers, and immunity parameters of broilers

Parameters	Control	T1	T2	T3	T4	T5
Liver and Kidney functions						
AST (U/L)	248±2.3^{a}	225±3.2^{b}	208±2.8^{c}	178±1.7^{e}	262±3.0	185±1.4^{d}
ALT (U/L)	3.2±0.3^{a}	2.9±0.2^{b}	2.2±0.2^{c}	1.9±0.1^{d}	3.4±0.2	2.1±0.2^{c}
Creat (mg/dl)	0.32±0.02	0.34±0.01	0.33±0.02	0.28±0.01	0.35±0.03	0.32±0.02
Uric acid (mg/dl)	5.4±0.4^{a}	4.65±0.5^{b}	4.42±0.3^{c}	3.8±0.4^{d}	5.5±0.6	4.5±0.4^{c}
Oxidative Stress Markers						
MDA (nmol/ml)	5.8±0.4^{a}	4.9±0.3^{b}	4.2±0.2^{c}	3.5±0.3^{e}	6.1±0.5	3.9±0.3^{d}
SOD (U/ml)	32.5±1.2^{d}	36.8±1.4^{c}	38.2±1.3^{b}	42.5±1.6^{a}	30.2±1.1	39.1±1.5^{b}
GSH (µmol/L)	45±2.1^{c}	52±2.3^{b}	54±2.0^{b}	58±2.4^{a}	42±1.9	53±2.2^{b}
CAT (U/ml)	28±1.1^{c}	32±1.3^{b}	34±1.2^{b}	38±1.5^{a}	26±1.0	33±1.4^{b}
Immunity						
IgG (mg/dl)	958±4.5^{e}	1045±5.3^{d}	1075±1.8^{c}	1098±3.4^{a}	945±3.8	1085±3.6^{b}
IgA (mg/dl)	177±1.0^{e}	185±1.2^{d}	192±1.5^{c}	205±1.3^{a}	173±2.4	197±1.8^{b}
T3 (ng/dl)	2.34±0.1	2.33±0.2	2.32±0.1	2.31±0.1	2.35±0.2	2.34±0.1
T4 (ng/dl)	134±1.2	133±2.0	135±1.0	137±0.9	132±1.6	130±1.2

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, Creat: Creatinine, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, CAT: Catalase, IgG: Immunoglobulin G, IgA: Immunoglobulin A, T3: Triiodothyronine, T4: Thyroxine. Different superscript letters (^{a,b,c,d,e}) within a row indicate significant differences (P<0.05). Values are presented as mean ± SD. Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320 mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320 mg/kg).

Table 7: Effects of dietary *Bacillus amyloliquefaciens* BS32 treatments on gene expression profiles in the broilers' intestine

Gene	Control	T1	T2	T3	T4	T5
Tight Junction Proteins						
OCLN	1.00±0.05^{c}	1.32±0.07^{b}	1.58±0.06^{a}	1.63±0.04^{a}	0.92±0.03^{d}	1.55±0.05^{ab}
MUC1	1.00±0.03^{d}	1.25±0.04^{c}	1.41±0.05^{b}	1.68±0.06^{a}	0.88±0.02^{e}	1.39±0.04^{bc}
JAM2	1.00±0.06^{c}	1.18±0.05^{b}	1.29±0.04^{b}	1.47±0.05^{a}	0.95±0.03^{cd}	1.36±0.06^{b}
Immune Markers						
CD4	1.00±0.04^{d}	1.35±0.06^{c}	1.52±0.05^{b}	1.76±0.07^{a}	0.82±0.03^{e}	1.49±0.05^{b}
CD8α	1.00±0.05^{d}	1.28±0.04^{c}	1.44±0.06^{b}	1.61±0.05^{a}	0.91±0.02^{de}	1.42±0.04^{b}
TLR4	1.00±0.07^{c}	0.85±0.03^{d}	0.72±0.04^{e}	0.68±0.03^{e}	1.32±0.06^{a}	0.78±0.05^{de}

Data presented as mean fold-change ± SEM relative to control (normalized to GAPDH). Different superscript letters (^{a,b,c,d,e}) within a row indicate significant differences (P<0.05, LSD test). *OCLN*: Occludin (tight junction protein), *MUC1*: *Mucin 1* (epithelial barrier marker), *JAM2*: Junctional adhesion molecule 2, *CD4*/*CD8α*: Cluster of differentiation 4/8α (T-cell markers). Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320 mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320mg/kg).

The microbial population analysis: Fig. 5 shows the dynamics of gut microbial populations' responses to BaBS32 supplementation, including total bacterial count (TBC), total yeast and mold count (TYMC), E. coli, Salmonella, and lactic acid bacteria (LAB). In control birds, the balance of microbial populations was stable, with LAB accounting for about 26% of the total counts. BaBS32 supplementation reduced TBC, TYMC, *E. coli*, and *Salmonella* and increased LAB by 42%. When add at 320mg/kg in the feed, the counts of *E. coli* and *Salmonella* reduced by 57% and 61%, whereas LAB abundance grew by 38 percent. *Salmonella* challenge (T4) resulted in significant dysbiosis, which augmented TBC and TYMC, and expanded *Salmonella* and *E. coli* populations by 77% and 52%, respectively. The number of LAB populations decreased by 41 % concurrently. Challenged birds (T5) fed with BaBS32 (320mg/kg) showed a significant improvement in microbial balance: *Salmonella* and *E. coli* counts were lower (48 and 53 %) as compared to T4, and LAB counts were higher (increased by 55%) than in healthy controls fed supplemented food.

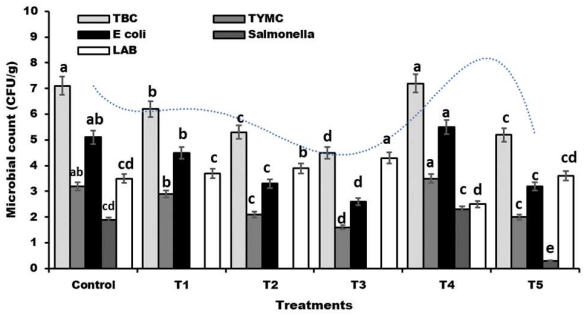

Fig. 5: Microbial count (TBC, TYMC, *E. coli*, *Salmonella*, and LAB) in broilers' gut as affected by different concentrations of *Bacillus amyloliquefaciens* BS32 on *Salmonella*-challenged broilers. Control broiler fed normal diet, T1-T3, non-infected broilers, and treated with various concentrations (80, 160, and 320mg/kg) of *Bacillus amyloliquefaciens* BS32, T4 *Salmonella*-challenged broilers, T5 *Salmonella*-challenged broilers, and treated with *Bacillus amyloliquefaciens* BS32 (320mg/kg). Lowercase letters above columns indicate significant differences using the LSD test at P<0.05.

Intestinal histomorphology: Fig. 6A-F and Table 8 illustrate the effects of BaBS32 supplementation and *Salmonella* challenge on intestinal morphology. The control group (Fig. 6A) exhibited clearly defined villi, crypts, and an intact epithelial lining. Dietary supplementation with BaBS32 (Fig. 6B-D) produced dose-dependent effects, such as elongation of villi, an increased villus-to-crypt ratio, epithelial thickening, and a higher density of goblet cells. The most significant structural enhancement was observed at the highest dose (320 mg/kg, Fig. 6D). *Salmonella* challenge (Fig. 6E) resulted in severe mucosal damage, including blunted and fused villi, shallow epithelial layers, deepened crypts, and increased inflammatory infiltration. Nonetheless, birds challenged with *Salmonella* and fed BaBS32 at 320 mg/kg (Fig. 6F) demonstrated notable histological recovery, with restoration of villus height, improved villus architecture, and maintained epithelial integrity. These findings were

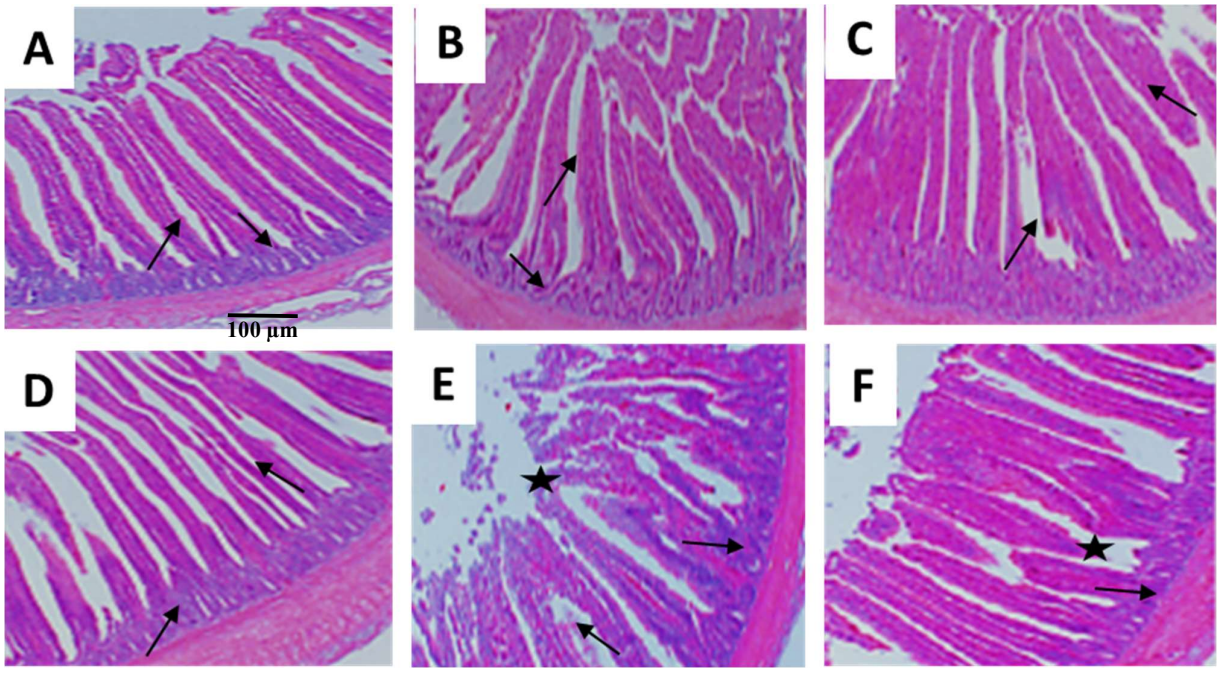

Fig. 6: Histological architecture of intestinal villi in broiler chickens from different treatment groups indicated by arrows and stars: Control (A), non-infected broilers supplemented with *Bacillus amyloliquefaciens* BS32 at 80 mg/kg (B), 160 mg/kg (C), and 320mg/kg (D); *Salmonella*-challenged broilers (E); and *Salmonella*-challenged broilers supplemented with *Bacillus amyloliquefaciens* BS32 at 320mg/kg (F). Differences in villus height, crypt depth, and mucosal integrity are visible across groups. H&E stain; scale bar = 100µm and magnification power 200x.

Table 8: Quantitative histological measurements

Treatment	Intestinal Section	Villus Height (µm)	Crypt Depth (µm)	Villus: Crypt Ratio	Epithelium Thickness (µm)	Goblet Cells/mm²
Control	Duodenum	1,468±58	291±14	5.0±0.2	42±2.1	285±16
	Jejunum	1,155±52	240±11	4.8±0.2	38±1.8	320±18
	Ileum	884±40	179±9	4.9±0.2	35±1.6	380±19
Bacillus 80mg/kg	Duodenum	1,580±62	265±12	6.0±0.3	45±2.2	305±17
	Jejunum	1,290±55	210±10	6.1±0.3	42±2.0	340±19
	Ileum	920±41	160±8	5.8±0.3	38±1.8	400±20
Bacillus 100mg/kg	Duodenum	1,740±65	208±10	8.4±0.4	48±2.3	330±18
	Jejunum	1,491±60	146±8	10.2±0.5	46±2.2	365±20
	Ileum	1,010±45	145±8	7.0±0.3	41±2.0	420±21
Bacillus 320mg/kg	Duodenum	1,820±68	185±9	9.8±0.5	52±2.5	360±20
	Jejunum	1,650±65	125±7	13.2±0.6	50±2.4	390±21
	Ileum	1,080±48	128±7	8.4±0.4	45±2.1	450±22
Salmonella	Duodenum	890±45	380±18	2.3±0.1	28±1.5	180±14
	Jejunum	720±40	320±16	2.3±0.1	24±1.4	210±15
	Ileum	580±35	250±13	2.3±0.1	22±1.3	240±16
Salmonella + *Bacillus* 320mg/kg	Duodenum	1,420±60	220±10	6.5±0.3	44±2.1	320±18
	Jejunum	1,180±55	180±9	6.6±0.3	40±1.9	350±19
	Ileum	860±42	165±8	5.2±0.3	36±1.8	380±20

corroborated by quantitative analysis (Table 8). BaBS32 supplementation increased villus height to 1,820µm and elevated the jejunal villus: crypt ratio to 13.2. Conversely, Salmonella challenge decreased villus height (to 890µm in the duodenum and 720µm in the jejunum), and the villus: crypt ratio declined to 2.3. When supplemented with BaBS32 (T5), these parameters were significantly improved, with duodenal villus height reaching 1,420µm and the villus: crypt ratio increasing to 6.5, values that approached those observed in unchallenged, supplemented birds.

DISCUSSION

Salmonella infection has become an endemic issue in global poultry production, resulting in substantial economic losses due to reduced flock performance and elevated mortality. Furthermore, it presents a significant public health concern, as contaminated poultry meat and eggs are primary sources of human salmonellosis. The widespread presence of Salmonella, its ability to survive in diverse environmental conditions, and its numerous transmission pathways—such as contaminated feed, water, litter, farm equipment, rodents, insects, and direct person-to-person contact—render its control on commercial farms particularly challenging. Among the various serotypes identified, *Salmonella enterica* serovars, including *Enteritidis* and *Typhimurium*, represent the highest risk to both avian populations and human health (Mkangara, 2023). They spread quickly in hatcheries, flocks, and processing plants because they can transmit horizontally and vertically. *Salmonella* may remain silent once it enters a flock, thereby creating silent reservoirs that persistently attack productivity and threaten the food safety of the entire production chain (Pal *et al.,* 2024).

A combination of biosecurity, vaccination, use of antibiotics, and improved farm management practices are implied to control the current threat of *Salmonella* under integrated control programs (Galán-Relaño *et al.,* 2023). The primary strategy for preventing pathogen introduction and enhancing their dissemination involves implementing rigorous hygiene standards. These include all-in/all-out production systems, heat-treated pellet feed, meticulous litter management, high-quality cleaning and disinfection protocols, and stringent pest control measures. Vaccination programs are tailored to specific serovars; however, issues such as incomplete cross-protection, variability in immune responses between flocks, and practical challenges associated with large-scale implementation often limit their overall effectiveness. Historically, antibiotic therapy has played a crucial role in controlling *Salmonella* outbreaks and providing prophylaxis. Nevertheless, the increasing global concern over antimicrobial resistance (AMR) has led to significant restrictions on the routine use of antibiotics in poultry farming (Rashid *et al.,* 2023).

The extensive use of antibiotics in poultry production has contributed to the emergence of antibiotic-resistant Salmonella and other pathogenic bacteria (Abreu *et al.,* 2023). Resistant strains pose significant threats to animal health, leading to increased morbidity and mortality. They also pose risks to human health by potentially transferring resistance genes to human-associated bacteria through food, water, or the environment. Therefore, minimizing antibiotic usage and emphasizing alternative disease-prevention strategies have become priorities for international health authorities to curb the development of antimicrobial resistance (Majumder *et al.,* 2020). Environmental surveillance has demonstrated that farms with extensive antibiotic usage harbor significantly higher populations of resistant bacteria in surrounding soil and water. Furthermore, resistance genes can transfer to natural microbial communities. Beyond treatment complications, antimicrobial resistance increases veterinary costs, results in more frequent food recalls, and prompts the implementation of more rigorous regulations, thereby challenging the sustainability of the poultry industry (Hughes *et al.,* 2021).

Probiotic supplementation, specifically with Bacillus species, has become a major focus in the search for safer alternatives as viable, natural replacements for antibiotic growth promoters (Luise *et al.,* 2022). The *Bacillus* strains, such as *Bacillus subtilis* and *Bacillus amyloliquefaciens*, are considered exemplary probiotic candidates due to their ability to form spores, which facilitates their resilience during feed processing and transit through the gastrointestinal tract (WoldemariamYohannes *et al.,* 2020). The species offers several benefits, such as better growth, higher nutrient absorption, improved gut structure, and greater resistance to infections. Multiple experiments have shown that Bacillus probiotics boost digestive enzyme production, increase villus height, deepen crypts, and enhance feed efficiency. Broilers given Bacillus also exhibit notable improvements in carcass traits and immune function. Furthermore, broilers supplemented with Bacillus tend to outperform those fed with antibiotic growth promoters (Ogbuewu *et al.,* 2022).

Furthermore, *Bacillus* supplementation has consistently been demonstrated to promote gut microbial homeostasis by increasing beneficial lactic acid bacteria and reducing pathogenic bacteria, such as *Salmonella* and *E. coli* (Khochamit *et al.,* 2020). These effects have been observed across various supplementation methods, including feed and water supplementation. They are most effective when strains are selected for high survivability, robust antagonistic activity, and effective colonization. Meta-analyses confirm that *Bacillus* supplementation favorably influences performance indicators such as body weight (BW), average daily gain (ADG), and feed conversion ratio (FCR) (Ogbuewu and Mbajiorgu, 2022). The anticipated improvements in these parameters are linked to enhanced nutrient digestibility, which results from increased secretion of amylases, proteins, and lipases. Bacillus probiotics have demonstrated effects comparable to in-feed antibiotics in the management of necrotic enteritis, maintaining growth performance and reducing intestinal lesions (Kulkarni *et al.,* 2022). They are not just promoted to grow directly, but also to enhance the intestinal barrier, regulate immune responses, and prevent colonization by pathogenic organisms. The dose-dependent antibacterial action of the BaBS32 suspension in our study is consistent with the established capacity of *Bacillus amyloliquefaciens* to form a broad selection of antimicrobial agents. These are lipopeptides and bacteriocins that prevent pathogens by disrupting membrane integrity or interfering with cellular processes. Mechanisms such as competitive exclusion are also significant because Bacillus strains compete with

pathogens for nutrients and adhesion sites, where *Salmonella* cannot successfully colonize (Lamba *et al.,* 2022). Additionally, the species of *Bacillus* naturally secretes both organic acids and hydrogen peroxide, as well as general antimicrobial agents that further weaken harmful microbes (Tran *et al.,* 2022). *Bacillus* probiotics promote the growth of beneficial microbes, such as lactic acid bacteria, creating a competitive environment that prevents pathogen survival, improves intestinal barrier function, and enhances immune defenses.

Prior studies have demonstrated that *Bacillus*-based probiotics can increase the ratio of beneficial lactic acid bacteria to harmful bacteria, as well as enhance immune response by up to 60 percent through diet supplementation with these probiotics (Hirozawa *et al.,* 2023). Several studies have shown that reducing common pathogens such as *Salmonella, E. coli,* and *Campylobacter*, as well as enhancing growth performance and overall health, was successful. It has also been demonstrated that indigenous *Bacillus* isolates with good acid and bile tolerance and colonization capacity exhibit high antibacterial activity due to the production of bacteriocins and antimicrobial metabolites (Hirozawa *et al.,* 2023).

Contemporary poultry production subjects broilers to various stressors, including dense stocking, environmental fluctuations, and exposure to pathogens. These stressors typically induce oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms. Oxidative stress further contributes to tissue damage, compromised immunity, reduced growth rate, and overall diminished performance (Niu *et al.,* 2022). The antioxidative stress response mechanism induced by *Bacillus* probiotics is attenuation of major antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Lower lipid peroxidation and better cellular integrity are reflected in reduced levels of oxidative markers such as malondialdehyde (MDA). Increased antioxidant potential beneficially alters hepatic and intestinal conditions, increases immune stability, and facilitates optimal development in adverse production environments (Riaz Rajoka *et al.,* 2021).

At the molecular level, the host immune response is also regulated by *Bacillus* species. The experimental results have shown that Bacillus supplementation of broilers' diet reduces the expression of pro-inflammatory cytokine genes such as IL-1b, IL-6, and TNF-α in intestinal and hepatic tissues, thereby reducing chronic inflammation and tissue injury under normal and Salmonella-challenged conditions (Anjum *et al*., 2020). Simultaneously, *Bacillus* increases the expression of anti-inflammatory markers, which positively influences the integrity of the intestinal barrier, curbs the entry of pathogens and toxins into the bloodstream, and reduces the inflammatory burden (Cristofori *et al.,* 2021). The supplementation also diminishes the expression of apoptotic genes, including *BAX* and *Caspase*-3, thereby preventing unnecessary epithelial cell apoptosis during infection and preserving mucosal stability. Notably, the alteration in cytokine profiles is not inherently suppressive, as *IL-1b* and *TNF*-α are commonly associated with inflammatory responses; however, recent research indicates that they also contribute to immune activation and the stimulation of regulatory T cells, which aid in maintaining a properly balanced immune response (Zadka *et al.,* 2020). Lipopeptides, notably surfactin, are among compounds derived from *Bacillus* species and play substantial roles in suppressing microbiota associated with colitis, modulating both innate and adaptive immune responses, and reducing inflammation induced by bacterial toxins. Other lipopeptides, such as iturins and fengycins, also contribute significant biological functions. Iturins exhibit potent antifungal activity by damaging fungal cell membranes and inducing oxidative stress, thereby inhibiting spore germination and hyphal growth. Fengycins are associated with antibacterial and antifungal defenses, achieved by disrupting cellular structures and attenuating pathogenic activity. Furthermore, *Bacillus* species produce antimicrobial peptides such as bacitracin and subtilin, which primarily target Gram-positive bacteria and thereby promote microbial equilibrium, immune homeostasis, and pathogen control. Collectively, these multifunctional metabolites play a vital role in enhancing the probiotic potential of *Bacillus* species by maintaining intestinal microbial stability and safeguarding the host against deleterious organisms infections. The intestinal tract is the body's largest interface with the external environment; it harbors a rich microbial ecosystem known as the gut microbiota. This microbial community is central to maintaining REDOX (reduction-oxidation) homeostasis, the process that balances pro-oxidant molecules, specifically Reactive Oxygen Species (ROS), with antioxidant defense systems (McBeth *et al.,* 2025). Oxidative stress results from exceeding the body's antioxidative capacity in response to increased ROS production. Despite the importance of ROS in some immune functions, including assisting phagocytes in the destruction of invasive pathogens, over-exposure or persistent exposure to them is detrimental. The loss of a normal REDOX balance directly impairs immune responses by interfering with vital intracellular signal transduction and promoting long-term inflammation (Bellanti *et al.,* 2025). Probiotics such as *Bacillus, Lactobacillus,* and *Bifidobacterium* species assist in this delicate REDOX balance in both direct and indirect ways. Most probiotic strains have inherent antioxidant effects that enable them to counteract ROS in the intestinal lumen. They also produce antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase, which are important for detoxifying superoxide radicals and hydrogen peroxide (Islam *et al.,* 2022). Moreover, probiotics produce potent antioxidants such as glutathione and short-chain fatty acids (SCFAs), which serve as efficient free-radical scavengers. Indirectly, probiotics have equally significant effects, as they address the underlying mechanisms of systemic oxidative stress that originate in the gut. A compromised intestinal barrier, also known as leaky gut, enables the entry of pathogens, toxins such as lipopolysaccharide (LPS), and other inflammatory agents into the bloodstream, thereby promoting inflammation throughout the body (Di Vincenzo *et al.,* 2024). Such inflammation amplifies oxidative stress as immune cells generate substantial quantities of reactive oxygen species (ROS) due to an overactive immune response. Probiotics improve the integrity of the epithelial barrier, thereby reducing the translocation of harmful substances across the intestine, suppressing chronic inflammation, and consequently alleviating overall

oxidative stress. Maintaining a stable, low-inflammatory gastrointestinal environment is crucial to prevent unnecessary immune reactions, as persistent immune stimulation leads to ongoing ROS production, tissue damage, and impaired immune function. (Srivastava and Sapra, 2022).

Conclusions: Soil-isolated *Bacillus amyloliquefaciens* BS32 emerges as a promising probiotic candidate for direct inclusion as a feed supplement to support poultry production. Incorporating BaBS32 into broiler diets offers a viable, antibiotic-free method to promote flock health, decrease enteric pathogen levels, and contribute to the global effort to curb antimicrobial resistance. Commercializing BaBS32 could help poultry producers meet regulatory restrictions on antibiotic use while enhancing productivity and health outcomes. It can also be integrated with existing vaccination protocols or combined with other probiotics to boost flock resistance and improve disease control. Further omics-based research, including transcriptomic and metabolomic studies, is recommended to optimize its application and uncover the mechanisms behind its widespread benefits. Conducting comprehensive safety, efficacy, and interaction assessments under real-farm conditions will be essential to realizing its full, evidence-based benefits for the poultry industry.

Authors contribution: Aminah Allohibi: Conceptualization, visualization, methodology, writing the original draft, writing-review, and editing.

Declaration of interests: The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Abreu R, Semedo-Lemsaddek T, Cunha E, *et al.,* 2023. Antimicrobial drug resistance in poultry production: Current status and innovative strategies for bacterial control. Microorganisms 11:953.

Ali S, and Alsayeqh AF, 2022. Review of major meat-borne zoonotic bacterial pathogens. Front Pub Health 10:1045599.

Anjum FR, Anam S, Rahman SU, *et al*., 2020. Anti-chicken type I IFN countermeasures by major avian RNA viruses. Virus Res 286:198061.

Bellanti F, Coda ARD, Trecca MI, *et al*., 2025. Redox imbalance in inflammation: The interplay of oxidative and reductive stress. Antioxidants 14:656.

Brody S, Lardy HA. 1946. Bioenergetics and growth. J Phys Chem 50:168-169.

Cristofori F, Dargenio VN, Dargenio C, *et al.,* 2021. Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body. Front Immun 12:578386.

Deng B, Wu J, Li X, *et al.,* 2020. Effects of *Bacillus subtilis* on growth performance, serum parameters, digestive enzymes, intestinal morphology, and colonic microbiota in piglets. Amb Exp 10:212.

Di Vincenzo F, Del Gaudio A, Petito V, *et al.,* 2024. Gut microbiota, intestinal permeability, and systemic inflammation: a narrative review. Inter Emerg Med 19:275-293.

Galán-Relaño Á, Valero Díaz A, Huerta Lorenzo B, *et al.,* 2023. *Salmonella* and salmonellosis: An update on public health implications and control strategies. Animals 13:3666.

Hashem ZS. 2025. Bacterial metabolites in defense: A crucial aspect of microbial interaction and host protection. In: Metabolic dynamics in host-microbe Interaction: Springer. p 101-120.

Hirozawa MT, Ono MA, Suguiura IMdS, *et al.,* 2023. Lactic acid bacteria and *Bacillus* spp. as fungal biological control agents. J App Microb 134:lxac083.

Hughes A, Roe E, Hocknell S. 2021. Food supply chains and the antimicrobial resistance challenge: On the framing, accomplishments and limitations of corporate responsibility. Envir Planning A: Econ Space 53:1373-1390.

Islam MN, Rauf A, Fahad FI, *et al.,* 2022. Superoxide dismutase: an updated review on its health benefits and industrial applications. Critl Rev Food Sci Nut 62:7282-7300.

Kasimanickam V, Kasimanickam M, and Kasimanickam R. 2021. Antibiotic use in food animal production: escalation of antimicrobial resistance: where are we now in combating AMR? Med Sci 9:14.

Khochamit N, Siripornadulsil S, Sukon P, and Siripornadulsil W. 2020. *Bacillus subtilis* and lactic acid bacteria improve the growth performance and blood parameters and reduce *Salmonella* infection in broilers. Vet World 13:2663.

Kitchens SR, Wang C and Price SB, 2024. Bridging classical methodologies in *Salmonella* investigation with modern technologies: A comprehensive review. Microorganisms 12:2249.

Kulkarni RR, Gaghan C, Gorrell K, *et al.,* 2022. Probiotics as alternatives to antibiotics for the prevention and control of necrotic enteritis in chickens. Pathogens 11:692.

Lamba S, Mundanda MD, Fanning S, *et al*., 2022. Sporulation and biofilms as survival mechanisms of *Bacillus* species in low-moisture food production environments. Foodb Path Dis 19:448-462.

Luise D, Bosi P, Raff L, *et al.,* 2022. *Bacillus* spp. Probiotic strains as a potential tool for limiting the use of antibiotics and improving the growth and health of pigs and chickens. Front Microb 13:801827.

Majumder MAA, Rahman S, Cohall D, *et al.,* 2020. Antimicrobial stewardship: fighting antimicrobial resistance and protecting global public health. Infec Drug Resis :4713-4738.

Mazkour S, Shekarforoush SS, Basiri S, *et al.,* 2022. Protective effects of oral administration of mixed probiotic spores of *Bacillus subtilis* and *Bacillus coagulans* on gut microbiota changes and intestinal and liver damage of rats infected with *Salmonella* Typhimurium. J Food Saf 42:e12981.

Mazziotta C, Tognon M, Martini F, *et al.,* 2023. Probiotics mechanism of action on immune cells and beneficial effects on human health. Cells 12:184.

McBeth A, Miller EA, Thompson B, *et al.,* 2025. Balancing oxidative stress: How the gut microbiome supports redox homeostasis and mitochondrial health. J Restorative Med 15:4.

Mkangara M, 2023. Prevention and control of human *Salmonella enterica* infections: An implication in food safety. Inte J Food Sci 2023:8899596.

Niu X, Ding Y, Chen S, *et al.,* 2022. Effect of immune stress on growth performance and immune functions of livestock: mechanisms and prevention. Animals 12:909.

Ogbuewu IP, Mabelebele M, Sebola NA, *et al.,* 2022. *Bacillus* probiotics as alternatives to in-feed antibiotics and its influence on growth, serum chemistry, antioxidant status, intestinal histomorphology, and lesion scores in disease-challenged broiler chickens. Front Vet Sci 9:876725.

Ogbuewu IP and Mbajiorgu CA. 2022. Meta-analysis of the potential of dietary *Bacillus* spp. in improving growth performance traits in broiler chickens. Open Agricul 7:618-633.

Pal M, Ragasa T, Rebuma T, *et al.,* 2024. Salmonellosis remains the hidden menace in our global food supply: a comprehensive review. Am J Med Biol Res 12:1-12.

Rashid S, Tahir S, Akhtar T, *et al.,* 2023. *Bacillus*-based Probiotics: An Antibiotic Alternative for the Treatment of Salmonellosis in Poultry. Pak Vet J 43(1): 167-173.

Riaz Rajoka MS, Thirumdas R, Mehwish HM, *et al.,* 2021. Role of food antioxidants in modulating gut microbial communities: Novel understandings in intestinal oxidative stress damage and their impact on host health. Antioxidants 10:1563.

Ringø E, Van Doan H, Lee SH, *et al.,* 2020. Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. J Appl Microb 129:116-136.

Ruvalcaba-Gómez JM, Villagrán Z, Valdez-Alarcón JJ, *et al.,* 2022. Non-antibiotics strategies to control *Salmonella* infection in poultry. Animals 12:102.

Saad AM, Sitohy MZ, Sultan-Alolama MI, *et al.,* 2022. Green nanotechnology for controlling bacterial load and heavy metal accumulation in Nile tilapia fish using biological selenium

nanoparticles biosynthesized by *Bacillus subtilis* AS12. Front Microb 13:1015613.

Srivastava RK and Sapra L, 2022. The rising era of "immunoporosis": role of immune system in the pathophysiology of osteoporosis. J Inflamm Res:1667-1698.

Su Q, Peng X, Zhang Z, *et al.,* 2025. Isolation, characterization of *Bacillus subtilis* and *Bacillus amyloliquefaciens* and validation of the potential probiotic efficacy on growth, immunity, and gut microbiota in hybrid sturgeon (Acipenser baerii♀× Acipenser schrenckii♂). Fish Shellfish Immun 157:110081.

Todorov SD, Ivanova IV, Popov I, *et al.,* 2022. *Bacillus* spore-forming probiotics: benefits with concerns? Critical Rev Micro 48:513-530.

Tran C, Cock IE, Chen X, *et al.,* 2022. Antimicrobial *Bacillus*: metabolites and their mode of action. Antibiotics 11:88.

Wibisono FM, Wibison FJ, Effendi MH, *et al.,* 2020. A review of salmonellosis on poultry farms: Public health importance. Syst Rev Pharm 11:481-486.

Woldemariam YK, Wan Z, Yu Q, *et al.,* 2020. Prebiotic, probiotic, antimicrobial, and functional food applications of *Bacillus amyloliquefaciens*. J Agricul Food Chem 68:14709-14727.

Zadka Ł, Grybowski DJ and Dzięgiel P, 2020. Modeling of the immune response in the pathogenesis of solid tumors and its prognostic significance. Cell Oncol 43:539-575.

Zhou Z, Shen B,and Bi D. 2020. Management of pathogens in poultry. In: Animal agriculture: Elsevier. p 515-530.