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RESEARCH ARTICLE

Evaluating the Potential of Three Novel Probiotic Isolates as Antibiotic Alternatives in Improving Growth Performance, Immunity, and Producing Safe Meat in *Salmonella*-Challenged Broiler Chickens

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Abstract

The overuse of antibiotics in poultry farming underscores the need for safe, effective alternatives to produce clean meat. This study aimed to isolate and evaluate probiotic bacteria with strong antibacterial activity for broiler production. Thirty bacterial isolates were obtained from broiler feces samples, and three isolates were selected and identified using MALDI-TOF MS as *Paenibacillus polymyxa* EB7, *Bacillus licheniformis* EB14, and *Bacillus mycoides* EB26. These isolates were screened for their strong inhibitory activity against pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Among these, *P. polymyxa* EB7 emerged as the most promising, combining high antibacterial and antioxidant activities with exceptional tolerance to acidic pH and bile salts. Importantly, EB7 was sensitive to major antibiotics (tetracycline, azithromycin, erythromycin, and gentamicin) and showed no hemolytic or cytotoxic activity, confirming its safety profile. *In vivo* broiler trials confirmed its effectiveness. Dietary supplementation with EB7 at 200 mg/kg significantly improved growth performance (body weight gain and feed conversion ratio) and mitigated the negative effects of the *Salmonella* challenge. EB7 supplementation improved blood biochemistry by lowering liver and kidney stress markers and oxidative damage (malondialdehyde), while increasing antioxidant enzymes (SOD, GSH, and CAT) and immune markers (IgG and IgA). It also modulated the gut microbiota by reducing pathogenic loads (*E. coli*, *Salmonella*) and increasing beneficial lactic acid bacteria. Furthermore, EB7 helped balance the immune response to *Salmonella* infection by modulating key immune-related genes (TLR4, IL-6, and AvBD6), reducing excessive inflammation while maintaining host defenses. The findings demonstrated that *P. polymyxa* EB7 is a safe, multifunctional probiotic that enhances growth, strengthens immunity, and improves gut health in broilers.

Keywords: Probiotics, Human health, Gut microbiota, *Salmonella* challenge, Pathogenic bacteria

INTRODUCTION

Salmonellosis remains one of the most significant infectious diseases in poultry, responsible for considerable economic losses, public health risk, and persistent challenges in global poultry production [1]. *Salmonella enterica* serovars cause poultry salmonellosis, which manifests primarily as enteritis and septicemia, leading to high flock morbidity/mortality and contamination of chicken meat and eggs. Recent systematic reviews report *Salmonella* prevalence in poultry at 12-18%, varying by geographic region and production system [2,3]. Outbreaks are not limited to industrial settings but have also been reported in backyard flocks, underscoring widespread vulnerability. In addition to direct flock loss, the economic toll of *Salmonella* on the poultry industry in major producing countries exceeds $2.8 billion annually, mainly due to reduced productivity and food safety recalls [4].

Salmonella infection in poultry leads to reduced growth, impaired feed conversion, increased susceptibility to secondary infections, and elevated mortality-sometimes up to 50% in poorly managed operations [2]. The zoonotic nature of *Salmonella* presents serious foodborne illness risks to consumers, often resulting in large outbreaks and hospitalizations worldwide [5]. Contaminated poultry products are a leading source of human salmonellosis, prompting strict regulations and surveillance in many jurisdictions [1].

Historically, antibiotics have been used not only to treat *Salmonella* outbreaks but also as growth promoters and

prophylactics in broiler production. However, misuse and overuse have instigated high levels of antimicrobial resistance (AMR) in *Salmonella*, with recent studies reporting 100% resistance to some commonly used drugs among isolates sampled from chicken environments [1]. This dire scenario has necessitated the urgent search for effective, safe alternatives capable of curbing infection without perpetuating AMR [6,7]. Several strategies, including vaccination, improved biosecurity, organic acids, and notably, probiotics, have emerged as promising tools [8].

Among probiotic candidates, *Bacillus* and *Paenibacillus* species have garnered considerable attention [9]. These spore-forming bacteria are resilient to gastrointestinal conditions, exhibit broad-spectrum antimicrobial activity, and have demonstrated improvements in growth, immunity, and gut health in poultry. Both genera produce extracellular enzymes, competitive exclusion factors, and antimicrobials (such as bacteriocins and lipopeptides) that inhibit not only *Salmonella* but also other pathogens such as *E. coli* and *Staphylococcus* [10].

Recent *in vitro* and *in vivo* studies show that carefully selected *Bacillus* and *Paenibacillus* isolates from chicken feces can yield strong inhibitory effects against *Salmonella*, improve antioxidant status, and support gut barrier functions in broilers [11]. These isolates also possess high survivability in the avian gut though their persistence may be transient, necessitating regular supplementation to maintain their probiotic effect [12].

Bacillus and *Paenibacillus* species exhibit antimicrobial activity by producing multiple bioactive compounds, including peptides and organic acids, that reduce *Salmonella* colonization and shedding. Their ability to modulate the immune response, reinforce gut integrity, and suppress oxidative damage adds essential layers of protection against the adverse effects of infection, as evidenced in recent trials. The functionality of these isolates extends to the competitive exclusion of pathogens, the modulation of the microbiota, and the improvement of both nutritional absorption and systemic health in broiler chickens [9-12].

Despite extensive research into probiotic alternatives to antibiotics, several gaps remain. Most notably, the strain-specific effects of *Bacillus* and *Paenibacillus* spp., their synergistic action, and their ability to confer consistent protection against multidrug-resistant *Salmonella* in broilers have not been thoroughly explored, especially using indigenous isolates from regional poultry systems. Few studies focus on the molecular identification, gene profiling, and detailed biological characterization of these candidates as next-generation antibiotic alternatives [9,10].

This study aims to address these gaps by isolating, identifying, and evaluating the safety and efficacy of three indigenous *Bacillus* and *Paenibacillus* isolates (EB7, EB14, and EB26) as alternatives to antibiotics in combating *Salmonella* infection in broiler chickens. The main aim is to characterize the antioxidant and antimicrobial activities of EB7, EB14, and EB26 against *Salmonella* and other poultry pathogens. The objectives of the study are to assess the probiotic properties and survivability of these isolates in simulated gastrointestinal conditions. Also, to evaluate their influence on broiler health, growth performance, and immune responses during experimental *Salmonella* challenge and to profile their impact on gut microbiota composition and resistance gene transmission. The study seeks to contribute to the development of safe, effective, and sustainable biopreparations to enhance poultry health and reduce reliance on conventional antibiotics.

Material and Methods

Ethical Approval

The animal study has been reviewed and approved by ZU-IACUC committee. was performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory Animals (2025). Ethical code number ZU-IACUC/3/F/521/2025.

Isolation, Screening, and Identification of Selected Isolates

Bacterial isolates were obtained from freshly voided chicken feces collected in sterile containers from poultry farm cages and delivered to the microbiology laboratory within 24 h. About 10 g of fecal samples were homogenized in 90 mL of peptone buffer, yielding a 10^{-1} dilution. Subsequent tenfold serial dilutions were performed to 10^{-7}. Aliquots from each dilution were spread onto Luria-Bertani (LB) agar and incubated for 24 h at 37°C. Isolates displaying pronounced inhibitory effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected for further analysis [13].

Initial identification of the promising bacterial candidate was based on morphological, biochemical, and physiological profiling in accordance with Bergey's Manual of Systematic Bacteriology. For definitive species-level identification, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was employed, utilizing the Microflex LT/SH system (Bruker Daltonics, Bremen, Germany), representing a methodological strength due to its high accuracy (>99%), speed, and reproducibility for probiotic *Bacillus*/*Paenibacillus* identification compared to 16S rRNA sequencing. Bacterial colonies were processed in a suitable extraction buffer, and samples were applied to the target plate, followed by the addition of matrix solution. The generated mass spectra were compared against the Bruker

Biotyper database, allowing rapid and accurate species identification as described by Kluz et al.[14]. Indicator pathogens *Salmonella Typhimurium* and *Escherichia coli* were specifically selected due to their prevalence as major enteric pathogens in poultry, causing economic losses *via* diarrhea and mortality, and their relevance to antibiotic-free production challenges [14].

Safety and Probiotic Properties

To assess the probiotic characteristics of bacterial isolates EB7, EB14, and EB26, acid and bile salt resistance assays were performed based on the method described by Sahadeva et al.[15]. For the acid tolerance assay, 1 mL aliquots of bacterial culture were inoculated into 9 mL of LB broth adjusted to pH 2.5, then incubated at 37°C for 3 h. The optical density (OD) of each sample at 650 nm was determined hourly using a spectrophotometer. OD (A650) was adjusted to 0.08±0.05 to normalize bacterial concentrations across samples.

Following the acid-resistance analysis, bile-salt tolerance was tested by inoculating 100 µL of overnight-cultured bacteria into freshly prepared LB broth supplemented with 0.3% bile salts. To evaluate bacterial viability under bile stress, 100 µL samples were withdrawn at 0, 1, 2, 3, and 4 h post-inoculation and plated onto LB agar. Viability was assessed based on the presence (positive) or absence (negative) of colony growth after incubation. The rates of acid tolerance and survival were determined using the following formula:

% Survival rate = Absorbance after treatment/Absorbance before treatment x100

To determine the safety of the selected bacterial isolates, antibiotic sensitivity testing was performed. Isolates were plated on a suitable solid nutrient medium at a final concentration of 10^6 colony-forming units (CFU) per gram (CFU/g). Standard antibiotic discs, including tetracycline (30 µg), azithromycin, erythromycin, ceftriaxone, and gentamicin, were then placed on the media. Results were recorded after incubation for 48 h at 42°C.

Biological Activities

Antioxidant: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *Bacillus* suspension (10, 20, 40, 80, 160, and 320 µg/mL) was evaluated according to Abdel-Moneim et al.[16]. The reaction was initiated by incubating 0.5 mL of ethanolic DPPH with 1 mL of *Bacillus* suspension in the dark for 30 min, after which the absorbance at 517 nm was measured using a spectrophotometer (JENWAY, UK). The half-maximal inhibitory concentration (IC_{50}) value reflects the minimum concentration required to scavenge 50% of the DPPH radical [17]. The percentage of DPPH scavenging activity was calculated using the formula:

% Antioxidant activity = (Control absorbance - Sample absorbance)/ control absorbance x 100

Antibacterial: Antibacterial activity was assessed using the distinct bacterial isolate by preparing suspensions at concentrations of 50, 100, and 200 µg/mL. For each concentration, sterile 8-mm filter paper discs were immersed in the corresponding bacterial suspension for 30 min to ensure complete saturation. These treated discs were then placed onto agar plates previously inoculated with pathogenic bacteria relevant to poultry health, including *S. aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes, Salmonella typhi*, *E. coli*, and *Klebsiella pneumoniae*. After disc placement, the plates were incubated under optimal conditions for bacterial growth. After incubation, the diameter of the inhibition zones around each disc was measured in millimeters to determine antibacterial efficacy [18,19].

Experimental Design

A total of 550 Indian River broiler chicks were allocated to eleven experimental groups in a randomized design based on initial body weights on day one. Each group comprised five replicates, with eleven chicks per replicate. This experiment was designed to evaluate the probiotic effects of three soil-derived bacterial isolates (EB7, EB14, and EB26), administered at different concentrations, and their potential to counteract *Salmonella* infection in broiler chickens. The study followed a completely randomized design (CRD) with 11 experimental treatments, as outlined below. Healthy broiler chicks of uniform body weight at 10 days old. The experimental duration was 35 days. The basal diet is formulated to meet the National Research Council (NRC nutrient requirements for broilers, without antibiotic growth promoters. The composition and nutrient analysis of the basal diet were detailed in the study of Al-Quwaie [20]. The treatment groups were as follow: T1; Negative control delivered basal diet without additives (non-infected control), T2; EB7 (low dose delivered basal diet supplemented with *Paenibacillus polymyxa* EB7 at 50 mg/kg feed), T3; EB7 (high dose delivered basal diet supplemented with *P. polymyxa* EB7 at 200 mg/kg feed), T4; EB14 (low dose delivered basal diet supplemented *Bacillus licheniformis* EB14 at 50 mg/kg feed), T5; EB14 (high dose delivered basal diet supplemented with *B. licheniformis* EB14 at 200 mg/kg feed), T6; EB26 (low dose delivered basal diet supplemented with *Bacillus mycoides* EB26 at 50 mg/kg feed), T7; EB26 (high dose delivered basal diet supplemented with *B. mycoides* EB26 at 200 mg/kg feed): T8; Positive control (infected broilers challenged with *Salmonella* spp. (no supplementation): T9; EB7 + broilers infected with *Salmonella* and treated with EB7 at 200 mg/kg): T10; EB14 + infected broilers and treated with EB14 at 200 mg/kg): T11; (EB26 + infected broilers and treated with EB26 at 200 mg/kg).

EB7 *(P. polymyxa),* EB14 *(B. licheniformis),* and EB26 *(Bacillus mycoides)* were cultured in nutrient broth for 24 h at 37°C, centrifuged, and adjusted to 10^8 CFU/mL before dietary inclusion. Each bacterial suspension was uniformly mixed into the formulated diets at designated concentrations (50 mg/kg and 200 mg/kg). On day 15, birds in groups T8-T11 were orally challenged with *S. enterica* (10^7 CFU/mL) to induce intestinal infection [21]. Birds were housed in battery cages featuring three tiers and automated watering systems, with ad libitum access to feed and water throughout the study.

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 using the methodologies of Saad et al.[22] and Brody and Lardy [23]:

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

At day 35, five chicks from each treatment were anesthetized using an R550 Multioutput laboratory small animal anesthesia machine. Each anesthesia channel operated independently, allowing precise control of gas flow to the induction box in the range of 0-2.0 L/min. Blood samples were collected from the hepatic portal vein for biochemical analyses and transferred into heparinized tubes. The blood samples were centrifuged at 5.000 rpm for 10 min to separate the serum. The activities of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and the AST/ALT ratio were estimated following Cheng et al[24]. The liver was excised, rinsed in chilled 0.9% saline solution (w/v), weighed, and stored at −70°C. The superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and malondialdehyde (MDA) contents were assessed according to the protocol of Alatawi et al.[25]. Total antioxidant capacity (TAC) was measured as described by Pappas et al.[26]. Serum concentrations of immunoglobulins (IgG, IgA, and IgM) were quantified using a sandwich ELISA, with OD measured at 450 nm using a microplate reader (BioTek 800, USA), as outlined by Erhard et al.[27].

Gene Expression

Total RNA was isolated from chick intestinal tissue, and the resulting RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA purity and concentration were determined spectrophotometrically by measuring the 260/280 nm absorbance ratio, as described by Saif and Khan [28]. For semiquantitative reverse transcription-PCR, 3 µg of RNA was used as a template. The RNA was denatured at 70°C for 5 min in a Bio-Rad T100TM thermal cycler. cDNA synthesis was performed using 0.5 ng of oligo (dT) primers, 2 µL of 10X RT buffer, 2 µL of 10 mM dNTPs, and 1 µL of 100 U reverse transcriptase. The reaction was incubated at 42°C for 1 h, followed by enzyme inactivation at 70°C for 10 min. Gene expression levels were quantified by real-time PCR using the 2^−ΔΔCT method, with actin as the endogenous reference gene. Densitometric analysis was performed to assess mRNA expression, and specific primers *(Table 1)* were used for amplification. The cycle threshold (Ct) values were used to compare gene expression levels across samples, following established comparative quantification protocols.

Intestinal Microbial Quantification

Post-mortem, intestinal digesta samples were aseptically collected, homogenized in sterile glass containers, and stored at 4°C until analysis. Microbial enumeration included total viable bacteria, *E. coli*, coliforms, and *Lactobacillus* spp., using selective media as described by Abd El-Wahab et al.[29]. Results were standardized and reported as log10 colony-forming units (CFU/g) per gram of digesta.

Statistical Analysis

Statistical analysis was performed using SPSS (Version 17.0, IBM, USA). Results were presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and group means were compared using Fisher's least significant difference (LSD) test when appropriate. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Isolation, Screening, and Identification of the Selected Isolates

A total of 30 bacterial isolates were recovered from fecal samples and coded as EB1-EB30. These isolates were preliminarily screened for antibacterial activity using a dual-culture agar diffusion method against two indicator pathogens, *S. aureus* and *Pseudomonas aeruginosa*. Three isolates, EB7, EB14, and EB26, were screened for the largest inhibition zones against both bacteria. Following this, all 30 isolates were biochemically profiled using standard Bergey's Manual protocols, and the top three were further identified at the species level by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). The high antibacterial isolates (EB26, EB14, and EB7, with inhibition zones ranging from 25.9 to

Table 1. *Primer sequences and characteristics for selected defense-related genes*

Target Gene	Primer Sequence (5′→3′)	Product Length (bp)	Melting temperature (Tm °C)		GenBank Reference
			F	R	
TLR4	F: GAACATGCGGCTGAGTGGA R: TGGCTTCTCCACATGGAGAA	146	62.2	60.4	NM_001030693.2
TLR1LA	F: ATGTGGCTGAGGTGGTGTTT R: GCAGGATGACCTTGGAGAA	130	55.3	58.0	NM_001305826.1
AvBD6	F: CTTGCAGTGCTCCTGTCAGT R: CTTCAGCAACCTGCTTCCTG	110	59.9	59.9	NM_204478.3
IL-1B	F: TGCCAGAAGGAAATGCCAA R: GTCAAGGAGCAGGGTTTGG	164	58.0	58.4	NM_204524.2
IL-6	F: ACAACACGACTCCCACCAA R: AGGTGAGTGGCTGTCTGTGT	112	60.2	58.7	NM_204628.2
IFN-γ	F: GAGCCAGATTGACCAGAGC R: CCTTTTGCCCATCCAGGAGT	130	55.8	58.7	NM_205149.1
TGF-β1	F: AGGAATCGGCTGACACAAA R: TTCCAGGTCACTGGTCATCA	125	62.2	57.6	NM_205149.2
MHC	F: GCTCAGACACCCGGAGACTT R: GCCCTCGTCGTCTTCCTCT	140	59.6	62.0	NM_205823.1
cLEAP-2	F: ATTCTGCTTCCCTGAGGCTG R: TCAAGGCAGGGTCCACTCTC	120	59.9	59.9	NM_001277318.1
ACSL1	F: GATTGCCCAGTTCCTTTGTC R: GAGGACAGTGAGGTGCAGG	150	62.2	60.4	NM_001006323.2

TLR4: Toll-like receptor 4; TLR1LA: Toll-like receptor 1 type A; AvBD6: Avian β-defensin 6; IL-1B: Interleukin 1 beta; IL-6: Interleukin 6; IFN-γ: Interferon gamma; TGF-β1: Transforming growth factor beta 1; MHC: Major histocompatibility complex (class II, B); cLEAP-2: Chicken liver-expressed antimicrobial peptide 2; ACSL1: Acyl-CoA synthetase long chain family member 1. F: Forward primer, R: Reverse primer, bp: Base pairs. PCR was performed using SYBR Green-based qPCR with an annealing temperature of 58°C (optimized based on primer Tm values ranging 55–62°C), initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C/15 s, 58°C/30 s, 72°C/30 s, and melt curve analysis

32.0 mm against *S. aureus* and 21.7 to 26.9 mm against *P. aeruginosa*, were selected.

Based on morphological and biochemical tests, all isolates were Gram-positive, motile, spore-forming, rod-shaped cells observed singly under light microscopy. The colonies on LB agar were flat, round, and translucent with irregular edges and a pale cream color. Based on the results, EB7 corresponded to *P. polymyxa,* EB14 to *B. licheniformis,* and EB26 to *B. mycoides*. Functionally, all three isolates demonstrated multiple beneficial activities: solubilizing tricalcium phosphate within seven days; producing indole-3-acetic acid (IAA) in the presence of tryptophan; fixing nitrogen as confirmed by acetylene reduction assays; utilizing 1-aminocyclopropane-1-carboxylic acid (ACC) as its sole carbon source; and forming dense biofilms on glass surfaces.

MALDI-TOF MS analysis successfully categorized the isolates as follows: EB7 was identified as *P. polymyxa*, EB14 as *B. licheniformis*, and EB26 as *B. mycoides*. Each isolate's spectral profile was matched against an established microbiological reference library with high log-score confidence values (>2.0), indicating precise species-level identification.

Paenibacillus polymyxa (EB7) was characterized by its large mass peak values consistent with polymyxin-type peptide biosynthesis, supporting its broad-spectrum antimicrobial activity and known probiotic functions. The identification of *B. licheniformis* (EB14) matched published spectral libraries, showing characteristic peaks associated with the production of lipopeptides, such as lichenysin, a biosurfactant detected at mass-to-charge (m/z) ratios 1015-1087, often used as a biochemical marker of this species. This bioactivity underpins its strong antibacterial activity. *B. mycoides* (EB26) displayed unique filamentous colony morphology and a distinctive m/z spectral profile typical of spore-forming capability and environmental adaptation.

Overall, the MALDI-TOF MS confirmed that all three isolates belong to spore-forming, Gram-positive genera with established probiotic and biocontrol potential. The differentiation accuracy parallels earlier findings showing that MALDI-TOF MS is particularly reliable for species separation within the *Bacillus subtilis, B. licheniformis*, and *Paenibacillus* groups when proper protein extraction and culture conditions are used.

Probiotic Properties

Low pH and Bile Salt Survival: The isolates (EB7, EB14, and EB26) that exhibited the highest zone of inhibition

against *S. aureus* and *P. aeruginosa* also demonstrated strong tolerance to both acidic (pH 2.5) and bile salt (0.3%) conditions, with survival rates indicating their resilience under gastrointestinal tract conditions *(Table 2)*. Specifically, EB7 showed survival rates of 84.1% at pH 2.5 and 77.4% in bile salt (0.3%).

Antibiotic Resistance: Among the three tested isolates, EB7 was sensitive to all tested antibiotics, with inhibition zones of 23.0 mm for tetracycline, 25.1 mm for azithromycin, 26.5 mm for erythromycin, and 30.5 mm for gentamicin, each exceeding the Clinical and Laboratory Standards Institute (CLSI) susceptibility cutoff of 19.0 mm. The other isolates, EB14 and EB26, produced inhibition zones below the susceptibility threshold for tetracycline, azithromycin, erythromycin, and gentamicin. For ceftriaxone, none of the isolates achieved the sensitivity breakpoint, as all measurements fell within the intermediate or resistant range *(Table 3)*.

Table 2. *Screening the selected isolates based on their inhibition zones against S. aureus and P. aeruginosa, and survival at low pH and bile salt (0.3%)*

Isolate	Inhibition Zone (mm)		Survival Rate (%)	
	S. aureus	*P. aeruginosa*	pH 2.5	Bile Salt (0.3%)
EB7	32.0±1.2^{c}	26.9±1.3^{b}	84.1±2.5^{a}	77.4±2.1^{a}
EB14	28.3±0.9^{b}	26.2±1.0^{b}	78.3±2.7^{b}	68.5±2.4^{b}
EB26	25.9±1.1^{a}	21.7±1.2^{a}	69.0±2.8^{c}	65.9±2.3^{c}

Data are presented as mean ± SD. Different lowercase letters in the same column indicate the significant differences (P<0.05). S. aureus, P. aeruginosa

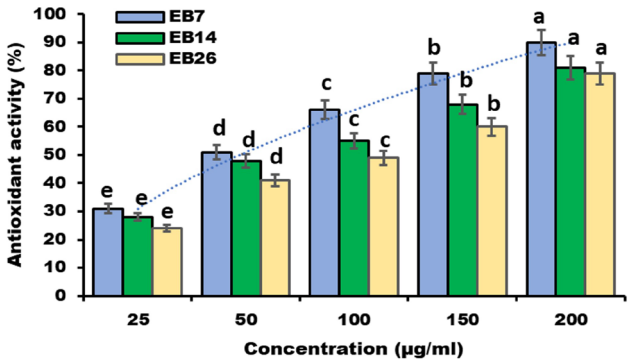

Fig 1. Antioxidant activity of EB7, EB14, and EB26 isolates against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. Lowercase letters above columns indicate significant differences (P<0.05)

Biological Activities

Antioxidant: Fig. 1 clearly shows a strong, dose-dependent increase in antioxidant activity (%) for all three isolates (EB7, EB14, and EB26) as the concentration increases from 25 to 200 µg/mL. At every tested concentration, EB7 consistently demonstrated the highest antioxidant activity compared to EB14 and EB26, with statistically significant differences, especially from 50 µg/mL upward (indicated by non-overlapping error bars and greater separation of bars). At the maximum dose (200 µg/mL), EB7 approached or surpassed 90% activity, while EB14 and EB26 reached about 80% and 75%, respectively.

Based on where each curve intersects, EB7 reached IC_{50} at a lower concentration (approximately 48 µg/ml), compared to EB14 (~58 µg/mL) and EB26 (~67 µg/ml). Lower IC_{50} values indicate higher antioxidant potency. Therefore, EB7 was the strongest antioxidant isolate, requiring the lowest concentration to reach 50% activity, whereas EB14 and EB26 were significantly less potent.

Antibacterial: *Table 4* reveals significant differences among the isolates (EB7, EB14, and EB26) and concentrations (50, 100 and 200 µg/mL) for all tested pathogenic bacteria. In each case, higher concentrations result in larger inhibition zones, from 50 to 200 µg/mL. EB7 consistently exhibited the strongest antibacterial activity *(Table 4)*. At 200 µg/mL, EB7 produces the largest inhibition zones across all pathogens, such as 32.0 mm for *S. aureus*, 31.2 mm for *S. pyogenes*, and 30.8 mm for *E. coli*, all significantly surpassing EB14 and EB26. EB14 typically showed intermediate activity (for example, 28.3 mm, 27.6 mm, and 27.5 mm for the same three pathogens, respectively), while EB26 was the least effective but still demonstrated notable increases at higher concentrations (30.5 mm for *S. aureus*, 29.4 mm for *S. pyogenes*, and 29.1 mm for *E. coli* at 200 µg/mL).

The statistical letters confirmed that, for each tested pathogen, the differences among the three isolates at the

Table 3. *Antibiotic resistance profiles of selected isolates*

Antibiotic (30 µg)	EB7	EB14	EB26	CLSI Interpretation
Tetracycline	23.0±1.1^{a}	17.9±1.1^{b}	16.3±0.7^{b}	S (≥19 mm)
Azithromycin	25.1±1.1^{a}	18.7±1.2^{b}	15.1±1.1^{c}	S (≥19 mm)
Erythromycin	26.5.4±1.6^{a}	21.3±0.8^{b}	20.5±1.1^{b}	S (≥19 mm)
Ceftriaxone	16.9±0.9^{a}	15.0±0.5^{ab}	12.4±0.9^{b}	I (15-18 mm), R (≤14 mm)
Gentamicin	30.5±1.6^{a}	23.2±1.4^{b}	21.9±1.1^{b}	S (≥19 mm)

Data are inhibition zone diameters (mm ± SD; n=3). Statistical letters within a row indicate significant differences (P<0.05). CLSI: Clinical and Laboratory Standards Institute; S: Susceptible; I: Intermediate; R: Resistant

Table 4. *Antibacterial activity of the selected isolates at concentrations (50, 100, and 200 µg/mL) against pathogenic bacteria*

Pathogen	EB7 (µg/mL)			EB14 (µg/mL)			EB26 (µg/mL)		
	50	100	200	50	100	200	50	100	200
S. aureus	14.2±1.0^{c}	16.5±0.9^{b}	32.0±1.3^{a}	12.1±0.8^{c}	15.0±1.0^{b}	28.3±1.1^{a}	13.8±0.9^{c}	16.2±1.0^{b}	30.5±1.2^{a}
S. pyogenes	12.9±0.9^{c}	15.4±1.0^{b}	31.2±1.4^{a}	11.8±0.8^{c}	14.5±1.1^{b}	27.6±1.2^{a}	13.0±0.8^{c}	15.7±1.0^{b}	29.4±1.1^{a}
L. monocytogenes	13.3±0.8^{c}	15.0±0.9^{b}	26.7±1.3^{a}	12.5±0.7^{c}	14.8±0.9^{b}	24.2±1.2^{a}	13.6±0.8^{c}	15.9±1.0^{b}	25.3±1.0^{a}
S. typhi	12.5±0.9^{c}	14.4±1.0^{b}	28.6±1.1^{a}	11.2±0.7^{c}	13.9±0.8^{b}	25.9±1.0^{a}	12.8±0.8^{c}	15.2±0.9^{b}	27.1±1.2^{a}
E. coli	13.0±0.8^{c}	15.1±1.0^{b}	30.8±1.2^{a}	12.0±0.8^{c}	14.0±1.1^{b}	27.5±1.2^{a}	13.5±0.9^{c}	15.4±1.0^{b}	29.1±1.3^{a}
K. pneumoniae	12.7±0.9^{c}	14.6±1.1^{b}	28.2±1.3^{a}	11.5±0.8^{c}	13.6±0.9^{b}	25.0±1.1^{a}	12.9±0.9^{c}	15.3±0.9^{b}	26.9±1.1^{a}

Data are mean inhibition zone diameters (mm ± SD; n = 3). Different superscript letters (a-c) within rows denote significant differences between concentrations (P<0.05). S. aureus, S. pyogenes, L. monocytogenes, S. typhi, E. coli, and K. pneumoniae

same concentration were significant in most cases, with EB7 achieving the highest activity in almost all cases

In vivo Experiment

Growth Performance of Salmonella-challenged Broilers: The data in *Table 5* demonstrate the effects of dietary supplementation with isolates EB7, EB14, and EB26 at two concentrations (50 and 200 mg/kg) on the growth performance of broilers under both normal and *Salmonella*-challenged conditions. Supplementation with EB7 at 200 mg/kg (T3) resulted in the greatest improvements in all growth parameters compared with the control and other treatments. T3 birds exhibited the highest final body weight (FBW: 2418.0 g), body weight gain (BWG: 2370.0 g), performance index (PI: 153.5), and optimal feed conversion ratio (FCR: 1.54), all significantly superior to T1 (negative control) and *Salmonella*-infected groups (T8–T11), as shown by distinct superscript letters. EB14 at 200 mg/kg (T5) also markedly improved metrics (FBW: 2374.0 g, BWG: 2323.0 g, PI: 150.1) versus controls, performing intermediately between EB7 and EB26 treatments. Lower doses (50 mg/kg; T2, T4, T6) yielded moderate, significant gains over controls, but 200 mg/kg doses excelled consistently; *Salmonella* challenge alone (T8) severely reduced all parameters (lowest FBW/BWG/PI, highest FCR).

Notably, post-challenge treatments with EB7 (T9), EB14 (T10), and EB26 (T11) at 200 mg/kg partially mitigated the harmful effects of *Salmonella* infection, as evidenced by improved BWG and PI and a lower FCR compared to T8. *Table 5* clearly demonstrates, supported by statistical groupings, that the probiotic candidates, especially EB7 at 200 mg/kg, can significantly boost broiler growth efficiency and resistance to *Salmonella* challenge.

Blood Biochemistry Markers: The results in *Table 6* clearly show significant, dose-dependent, and isolate-specific effects of the tested treatments on serum kidney and liver function, oxidative stress markers, and immune parameters in broilers. The *Salmonella* challenge group

Table 5. *Effect of dietary EB7, EB14, and EB26 at 50 and 200 mg/kg on growth performance parameters of Salmonella-challenged broilers*

Treatment	LBW (g)	FBW (g)	BWG (g)	FI (g)	FCR	GR	PI
T1	45.5±0.3^{c}	2240.0±2.5^{bc}	2195.0±2.6^{bc}	3665.0±3.0^{bc}	1.66±0.01^{c}	193.0±1.1^{bc}	135.9±0.8^{bc}
T2	46.2±0.3^{bc}	2305.0±2.5^{ab}	2260.0±2.6^{ab}	3709.0±3.0^{ab}	1.60±0.01^{bc}	194.0±1.1^{ab}	145.7±0.8^{ab}
T3	47.9±0.3^{a}	2418.0±2.5^{a}	2370.0±2.6^{a}	3715.0±3.0^{a}	1.54±0.01^{a}	197.0±1.1^{a}	153.5±0.8^{a}
T4	46.0±0.3^{bc}	2292.0±2.5^{ab}	2250.0±2.6^{ab}	3701.0±3.0^{ab}	1.61±0.01^{bc}	194.0±1.1^{ab}	144.0±0.8^{ab}
T5	47.1±0.3^{ab}	2374.0±2.5^{a}	2323.0±2.6^{a}	3706.0±3.0^{a}	1.57±0.01^{ab}	196.0±1.1^{a}	150.1±0.8^{a}
T6	45.7±0.3^{c}	2277.0±2.5^{bc}	2244.0±2.6^{bc}	3669.0±3.0^{bc}	1.63±0.01^{c}	192.0±1.1^{bc}	141.9±0.8^{bc}
T7	46.5±0.3^{bc}	2310.0±2.5^{b}	2260.0±2.6^{b}	3683.0±3.0^{b}	1.60±0.01^{bc}	193.0±1.1^{b}	143.6±0.8^{b}
T8	42.1±0.3^{d}	2035.0±2.5^{d}	1996.0±2.6^{d}	3686.0±3.0^{d}	1.84±0.01^{d}	186.0±1.1^{d}	110.2±0.8^{d}
T9	45.2±0.3^{c}	2202.0±2.5^{c}	2170.0±2.6^{c}	3662.0±3.0^{c}	1.70±0.01^{c}	190.0±1.1^{c}	134.4±0.8^{c}
T10	45.1±0.3^{c}	2196.0±2.5^{c}	2161.0±2.6^{c}	3644.0±3.0^{c}	1.72±0.01^{c}	188.0±1.1^{c}	132.9±0.8^{c}
T11	44.9±0.3^{c}	2189.0±2.5^{c}	2148.0±2.6^{c}	3632.0±3.0^{c}	1.75±0.01^{c}	187.0±1.1^{c}	132.1±0.8^{c}

Data are presented as mean ± SE. Different superscript letters within each row indicate significant differences between means (P<0.05). Parameters include live body weight (LBW), final body weight (FBW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), growth rate (GR), and performance index (PI). Treatments included T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with Salmonella but no probiotic. T9/T10/T11: Broilers challenged with Salmonella and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

Table 6. *Effect of treatments on serum kidney, liver function, oxidative stress, and immunity markers in broilers*

Treatment	AST (U/L)	ALT (U/L)	Creat (mg/dL)	Uric Acid (mg/dL)	MDA (nmol/mL)	SOD (U/mL)	GSH (umol/L)	CAT (U/mL)	IgG (mg/dL)	IgA (mg/dL)	T3 (ng/dL)	T4 (ng/dL)
T1	248±3^{c}	3.20±0.1^{c}	0.32±0.01^{b}	5.4±0.2^{b}	5.8±0.2^{b}	32.5±1.2^{d}	45.2±2.1^{c}	28.1±1.0^{c}	958±4.5^{c}	177±1.1^{c}	2.34±0.01^{b}	134±3^{b}
T2	242±3^{c}	2.90±0.1^{c}	0.34±0.01^{b}	4.8±0.2^{b}	4.9±0.2^{b}	35.9±1.2^{c}	50.2±2.1^{c}	31.1±1.0^{c}	1012±4.5^{c}	185±1.1^{c}	2.33±0.01^{b}	138±3^{b}
T3	220±3^{a}	2.10±0.1^{a}	0.33±0.01^{a}	4.3±0.2^{a}	3.8±0.2^{a}	40.2±1.2^{a}	56.3±2.1^{a}	36.5±1.0^{a}	1091±4.5^{a}	205±1.1^{a}	2.32±0.01^{a}	143±3^{a}
T4	239±3^{c}	2.80±0.1^{c}	0.34±0.01^{b}	4.9±0.2^{b}	4.7±0.2^{b}	34.8±1.2^{c}	52.0±2.1^{b}	32.0±1.0^{b}	1030±4.5^{b}	186±1.1^{b}	2.33±0.01^{b}	137±3^{b}
T5	225±3^{b}	2.20±0.1^{b}	0.33±0.01^{a}	4.4±0.2^{a}	4.1±0.2^{a}	38.7±1.2^{a}	54.1±2.1^{a}	35.0±1.0^{a}	1081±4.5^{a}	200±1.1^{a}	2.32±0.01^{a}	142±3^{a}
T6	244±3^{c}	2.90±0.1^{c}	0.35±0.01^{b}	5.1±0.2^{b}	5.0±0.2^{b}	33.6±1.2^{d}	48.6±2.1^{c}	29.7±1.0^{c}	990±4.5^{c}	180±1.1^{c}	2.33±0.01^{b}	135±3^{b}
T7	233±3^{b}	2.70±0.1^{b}	0.34±0.01^{a}	4.6±0.2^{a}	4.5±0.2^{a}	36.9±1.2^{b}	51.5±2.1^{b}	33.1±1.0^{b}	1045±4.5^{b}	189±1.1^{b}	2.33±0.01^{a}	138±3^{a}
T8	269±3^{d}	3.90±0.1^{d}	0.37±0.01^{c}	6.2±0.2^{c}	7.0±0.2^{c}	29.2±1.2^{d}	38.3±2.1^{d}	25.9±1.0^{d}	835±4.5^{d}	140±1.1^{d}	2.20±0.01^{c}	120±3^{c}
T9	242±3^{b}	2.50±0.1^{b}	0.31±0.01^{a}	4.5±0.2^{a}	4.0±0.2^{a}	38.5±1.2^{a}	55.3±2.1^{a}	34.2±1.0^{a}	1066±4.5^{a}	197±1.1^{a}	2.31±0.01^{a}	141±3^{a}
T10	241±3^{b}	2.40±0.1^{b}	0.32±0.01^{a}	4.4±0.2^{a}	4.1±0.2^{a}	38.0±1.2^{a}	54.9±2.1^{a}	33.9±1.0^{a}	1061±4.5^{a}	196±1.1^{a}	2.30±0.01^{a}	140±3^{a}
T11	240±3^{b}	2.30±0.1^{b}	0.32±0.01^{a}	4.4±0.2^{a}	4.0±0.2^{a}	37.9±1.2^{a}	54.7±2.1^{a}	33.8±1.0^{a}	1058±4.5^{a}	195±1.1^{a}	2.29±0.01^{a}	139±3^{a}

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) within a column indicate significant differences (P<0.05). Values are presented as mean ± SE. Treatments included T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with Salmonella but no probiotic. T9/T10/T11: Broilers challenged with Salmonella and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

(T8) showed the most notable increases in AST (269±3) and ALT (3.9±0.1), along with higher creatinine and uric acid levels, indicating clear hepatic and renal impairment. All probiotic-supplemented groups, especially at the 200 mg/kg dose (T3, T5, and T7), significantly lowered AST, ALT, and renal indices compared to both the control (T1) and the *Salmonella* group (T8). T3 was the most effective in lowering AST (220±3a) and ALT (2.1±0.1), and in positively affecting creatinine and uric acid levels.

MDA, a key indicator of oxidative damage, was highest in the *Salmonella*-only group (T8: 7.0±0.2) and lowest in the EB7 200 mg/kg group (T3: 3.8±0.2). EB14 and EB26 at 200 mg/kg also demonstrated clear protective effects. The antioxidant activities of SOD, GSH, and CAT were significantly elevated following probiotic supplementation, with the highest increase observed in EB7 (T3) (SOD: 40.2±1.2; GSH: 56.3±2.1a; CAT: 36.5±1.0) in comparison to both unchallenged and *Salmonella*-challenged controls.

Immunity Markers: Supplemented groups demonstrated clear improvements in humoral immunity. IgG and IgA reached peak levels in EB7 (T3 and T9), EB14 (T5 and T10), and EB26 (T7 and T11) treated birds at the highest dose, even under *Salmonella* challenge, while the challenge group (T8) showed the lowest values. Probiotics also tended to restore thyroid hormone (T3 and T4) levels toward control levels, counteracting the suppression caused by *Salmonella*. The findings confirmed that probiotic supplementation, especially EB7 at 200 mg/kg, significantly reduced hepatic and renal stress, decreased oxidative damage, and enhanced immunity in broilers, both in healthy conditions and under pathogen challenge. Probiotic effects depend on the isolate and dose, with statistically significant improvements compared to both the control and infection groups.

Gene Expression Against Salmonella Infection: The *Salmonella* challenge (T8) caused a remarkable increase in TLR4 (3.50-fold), TLR1LA (3.20-fold), AvBD6 (3.00-fold), IL-1B (2.90-fold), IL-6 (3.50-fold), IFN-γ (3.20-fold), TGF-β1 (2.70-fold), and cLEAP-2 (3.10-fold) compared to control (T1) (P<0.01 for all, a vs c). This robust innate immune activation and antimicrobial peptide production reflect the acute inflammatory response to infection, accompanied by a significant decrease in major histocompatibility complex (MHC, 0.70-fold), indicating suppression of adaptive antigen presentation. Supplementation with EB7 at 200 mg/kg (T3) before or after challenge (T9) significantly increased the expression of Toll-like receptors (TLRs), avian β-defensins (AvBDs), cytokines (IL-1β, IL-6, IFN-γ), and antimicrobial genes (e.g., TLR4: 2.50±0.14; cLEAP-2: 2.20±0.11), though to a lesser extent than the *Salmonella*-only group-suggesting immunomodulation without excessive inflammation.

Table 7. *Effects of EB7, EB14, and EB26 treatments on gene expression profiles in Salmonella-challenged broilers*

Treatment	*TLR4*	*TLR1LA*	*AvBD6*	*IL-1B*	*IL-6*	*IFN-γ*	*TGF-β1*	*MHC*	*cLEAP-2*	*ACSL1*
T1	1.00±0.12^{c}	1.00±0.15^{c}	1.00±0.11^{c}	1.00±0.15^{c}	1.00±0.11^{c}	1.00±0.15^{c}	1.00±0.11^{c}	1.00±0.10^{c}	1.00±0.14^{c}	1.00±0.11^{c}
T2	1.80±0.15^{c}	1.60±0.14^{c}	1.70±0.12^{c}	1.30±0.15^{c}	1.40±0.13^{c}	1.60±0.10^{c}	1.30±0.14^{c}	1.20±0.12^{b}	1.50±0.12^{c}	1.20±0.10^{c}
T3	2.50±0.14^{a}	2.20±0.11^{a}	2.30±0.12^{a}	1.90±0.14^{a}	2.30±0.12^{a}	2.40±0.11^{a}	1.60±0.10^{a}	1.80±0.11^{a}	2.20±0.11^{a}	1.70±0.13^{a}
T4	1.60±0.13^{c}	1.40±0.11^{c}	1.50±0.14^{c}	1.20±0.12^{c}	1.30±0.13^{c}	1.50±0.10^{c}	1.20±0.15^{c}	1.10±0.14^{c}	1.30±0.14^{c}	1.10±0.12^{c}
T5	2.20±0.11^{b}	2.00±0.11^{b}	2.10±0.11^{b}	1.70±0.10^{b}	2.00±0.13^{b}	2.30±0.12^{b}	1.50±0.14^{b}	1.60±0.13^{a}	2.00±0.14^{b}	1.50±0.13^{b}
T6	1.50±0.11^{c}	1.30±0.12^{c}	1.40±0.13^{c}	1.10±0.13^{c}	1.20±0.11^{c}	1.30±0.12^{c}	1.10±0.11^{c}	1.00±0.12^{c}	1.20±0.13^{c}	1.00±0.15^{c}
T7	2.00±0.10^{b}	1.80±0.13^{b}	1.70±0.13^{c}	1.40±0.12^{b}	1.80±0.15^{b}	1.90±0.11^{b}	1.30±0.10^{c}	1.40±0.10^{b}	1.60±0.14^{b}	1.30±0.11^{b}
T8	3.50±0.14^{a}	3.20±0.12^{a}	3.00±0.10^{a}	2.90±0.11^{a}	3.50±0.14^{a}	3.20±0.14^{a}	2.70±0.14^{a}	0.70±0.12^{a}	3.10±0.12^{a}	1.80±0.12^{a}
T9	2.10±0.13^{b}	1.80±0.11^{b}	2.00±0.13^{b}	1.70±0.12^{b}	1.90±0.15^{b}	2.00±0.12^{b}	1.40±0.14^{b}	1.60±0.12^{a}	1.70±0.13^{b}	1.50±0.14^{b}
T10	2.00±0.14^{b}	1.70±0.13^{b}	1.80±0.11^{b}	1.60±0.10^{b}	1.80±0.14^{b}	1.90±0.11^{b}	1.40±0.14^{b}	1.50±0.14^{b}	1.60±0.12^{b}	1.40±0.11^{b}
T11	1.90±0.10^{b}	1.60±0.11^{c}	1.70±0.10^{c}	1.50±0.15^{b}	1.70±0.13^{b}	1.80±0.13^{b}	1.30±0.14^{c}	1.40±0.13^{b}	1.50±0.10^{c}	1.30±0.10^{b}

Different superscript letters (a,b,c) within a column indicate significant differences (P<0.05). Values are presented as mean ± SD. Treatments included: T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with Salmonella but no probiotic. T9/T10/T11: Broilers challenged with Salmonella and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

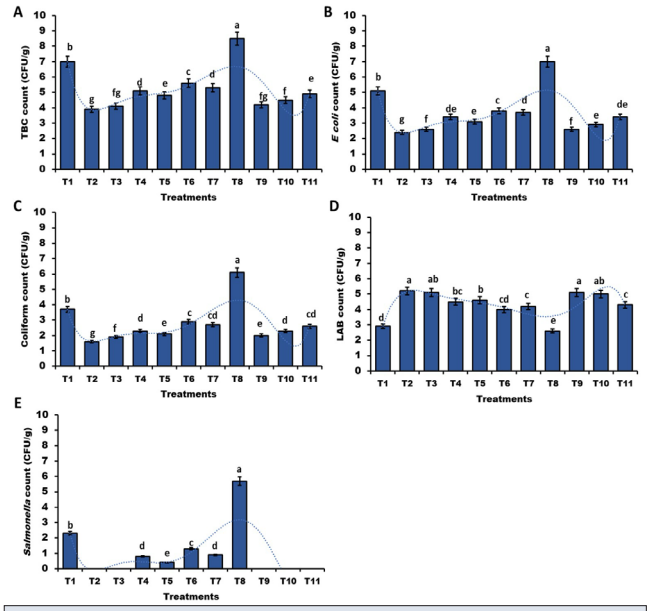

Fig 2. Microbial counts (total bacterial count (TBC), *E. coli*, coliform, lactic acid bacteria (LAB), and *Salmonella*) in broiler guts affected by different concentrations of EB7, EB14, and EB26 on *Salmonella*-challenged broilers. Treatments included: T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with *Salmonella* but no probiotic. T9/T10/T11: Broilers challenged with *Salmonella* and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

Probiotic treatments with EB14 and EB26 induced moderate increases in gene expression, but consistently lower than with EB7 or *Salmonella* alone *(Table 7)*. The results showed strong *Salmonella*-induced upregulation of immune genes, with targeted probiotic treatments reducing excessive pro-inflammatory responses and supporting a balanced, protective immune profile in broilers. The largest fold-changes were observed for genes related to pathogen recognition (TLRs), cytokines (IL-1β, IL-6, IFN-γ), and antimicrobial peptides *(AvBD6* and *cLEAP-2),* but only EB7 at a high dose achieved significant, strong upregulation while avoiding excessive immunopathology.

Microbial Count: *Fig. 2* shows that T8 *(Salmonella* challenge) experienced a significant increase in pathogenic bacteria *(E. coli* and *Salmonella)* and total bacterial load (TBC), along with a decrease in beneficial lactic acid bacteria (LAB), indicating severe dysbiosis. All probiotic treatments (T2-T7, T9, and T11) significantly reduced *E. coli*, coliforms, *Salmonella,* and TBC compared to T1 and T8, with the most notable reductions seen in T3 (EB7 high dose) and T9 *(Salmonella* + EB7). LAB counts increased substantially across all probiotic groups, especially in high-dose and combination treatments (T3, T5, T9, and T10), supporting targeted enrichment of beneficial microbiota. Groups treated with EB7 (T3, and T9) showed the lowest pathogenic counts and the highest LAB recovery, closely followed by EB14 (T5, and T10), demonstrating the dose-dependent and strain-specific ability of the probiotics to modulate microbiota. Overall, these results showed that probiotic supplementation, especially at higher doses, effectively restores gut microbial balance, inhibits enteric pathogens, and improves microbiological safety and gut health of broilers.

Discussion

Salmonellosis is one of the most important infectious diseases affecting poultry worldwide, both in terms of

production losses and public health concerns. Caused mainly by *S. enterica* subspecies *enterica*, this disease causes significant economic damage, including slower growth rates, poor feed efficiency, increased mortality (which can reach 20-50% in endemic areas), and greater vulnerability to other illnesses. Outbreaks in both large-scale and backyard systems have led to major food recalls, higher healthcare costs, and supply chain disruptions, sometimes costing millions of dollars each year in leading poultry-producing countries [30,31].

The persistence of *Salmonella* is fueled by multiple risk factors: large flock sizes, poor biosecurity, mixing of birds from different sources, floor housing, inadequate sanitation, and the widespread use of antibiotics as growth promoters and for infection control. Although various antibiotics have initially been effective, prolonged use has led to a sharp increase in multidrug-resistant *Salmonella* strains and other resistant bacteria in poultry settings. This trend now endangers not only flock health but also consumer safety and the sustainability of poultry industries worldwide [1,30].

As regulatory agencies and producers navigate control measures, including new standards, incentives, and advanced diagnostics, recent scientific and industry consensus has shifted toward prevention. Prebiotics, probiotics, and strict on-farm management are now recognized as essential elements in any sustainable *Salmonella* control plan. *Bacillus* and *Paenibacillus* species, in particular, have become leading probiotic options due to their durability, safety, and ability to fight pathogenic bacteria [7].

The isolation and identification of potent *Bacillus* and *Paenibacillus* strains support a growing body of evidence that these genera are promising probiotic candidates with significant antimicrobial properties for poultry applications. Their distinct antibacterial activity against important pathogens such as *S. aureus* and *Pseudomonas aeruginosa* aligns with findings reported by Alagawany et al.[9]; Zhou et al.[12], in which *Bacillus* and *Paenibacillus* isolates showed broad-spectrum inhibition of poultry pathogens, reinforcing their potential to reduce bacterial load in commercial flocks. The accuracy of MALDI-TOF MS identification, consistent with these studies, confirms the reliability of proteomic methods for species-level microbial classification, aligning with Calderaro and Chezzi [32], who emphasized MALDI-TOF MS as a powerful tool for differentiating closely related *Bacillus* species based on unique spectral profiles.

The triple isolates demonstrated multiple plant growth-promoting traits, including solubilizing phosphate, producing indole acetic acid, nitrogen fixation, and ACC utilization, which not only endorse their environmental adaptability but also align with observations by previous researchers [12,33], who described the biostimulant and stress mitigation potential of *Bacillus* and *Paenibacillus* strains in agriculture and animal husbandry. These multifunctional properties indicate that such probiotics benefit not only pathogen control but also promote intestinal homeostasis and resilience.

The strong tolerance of the isolates to acidic pH and bile salts emphasizes their ability to survive in the harsh gastrointestinal environment. This key trait is supported by recent studies, including those by Zhang et al.[34] and Alagawany et al.[9], which have confirmed that *Bacillus*-based probiotics can effectively colonize and exert effects within the avian gut. The exceptional survivability of isolate EB7, especially under pH and bile salt challenges, also exemplifies the robustness typical of *P. polymyxa* AM20, a trait linked to improved broiler health and pathogen resistance.

Notably, the antibiotic sensitivity profile of isolate EB7 indicated it is a safe candidate for probiotic use, with susceptibility to major antibiotics. This finding aligns with concerns about the transfer of resistance genes from probiotic strains [35]. It also supports the caution expressed by Khairunnesa et al.[36], who emphasized the importance of confirming antibiotic susceptibility before using probiotic strains harboring resistance. The multidrug resistance observed in other isolates but not in EB7 further supports its use to help prevent the spread of antibiotic resistance while offering therapeutic benefits.

In terms of antioxidant capabilities, the dose-dependent increase in radical scavenging capacity and the significantly lower IC_{50} values for EB7 confirm enhanced free radical neutralization-a key feature in maintaining gut and systemic health during infection-related oxidative stress. These findings align with observations by Zhou et al.[12]; Saeed et al.[37], who documented increased antioxidant enzyme activities and serum antioxidant status following dietary supplementation with *Bacillus* and *Paenibacillus* probiotics. Such antioxidant effects can reduce cellular damage and inflammation associated with *Salmonella* infection, supporting immune homeostasis.

The antibacterial assays showed that EB7 not only maintained strong effectiveness against a wide range of pathogens but also demonstrated dose-responsive inhibition, consistent with other recent studies where *Bacillus subtilis* and *P. polymyxa* strains significantly reduced pathogenic bacterial colonization in *vitro* and in poultry models [9,38]. The notable suppression of *Salmonella typhi, E. coli,* and *Staphylococcus* spp. highlights the broad-spectrum potential of these isolates, reinforcing their role as potent biological control agents in food animal production.

The *in vivo* efficacy of these isolates in improving growth performance during a *Salmonella* challenge demonstrated their ability to counteract infection-induced growth retardation, within the limits of pathogenic stress. This aligns with the conclusions of Zhang et al.[34] and Nam et al.[39], who reported improved feed conversion ratios, body weight gains, and health indices in broilers receiving *Bacillus* and mixed probiotics during a bacterial challenge. The dose-dependent improvements, especially at 200 mg/kg for EB7, highlight the importance of optimal dosing to maximize probiotic benefits.

Serum biochemical improvements, especially in liver and kidney markers, antioxidant enzymes, and immunoglobulin levels, align with extensive literature showing that probiotic supplementation reduces oxidative stress and liver inflammation caused by pathogen exposure [9,12]. The increased activities of SOD, GSH, and CAT further demonstrate how these isolates can regulate oxidative balance and inflammatory responses. Additionally, immune markers such as IgG and IgA secretion indicate a direct enhancement of systemic immunity. In parallel, the restoration of thyroid hormone levels reflects improved endocrine homeostasis, suggesting that the treatment confers coordinated immune and thyroid benefits, similar to those reported in previous studies [36,37].

Gene expression profiling demonstrated probiotic-mediated immunomodulation, reducing excessive pro-inflammatory cytokine overexpression while boosting pathogen recognition receptors and antimicrobial peptides. These dynamic immune adjustments are consistent with recent high-resolution transcriptomic studies, which depict *Bacillus* and *Paenibacillus* strains as fine-tuners of innate and adaptive immunity, balancing effective pathogen clearance with controlled tissue damage [9,35]. The increased expression of MHC-II and regulatory cytokines after probiotic supplementation also indicates improved antigen presentation and immune resolution, which are essential for maintaining health during infectious challenges.

Microbiological analyses showing decreased pathogenic bacterial loads and increased beneficial LAB reflect the well-known mechanism of competitive exclusion and gut microbial modulation by probiotics, as reported by Zhou et al.[12]; Nam et al.[39]. This microbial balance directly supports gut integrity and resistance to disease [40]. The dose-dependent improvements in LAB populations, especially with EB7 treatment, highlight that potency and strain specificity are crucial factors in probiotic effectiveness.

The superior profiles seen with EB7 confirm its potential for further development as an alternative to antibiotics in broiler production systems. This study not only supports existing knowledge but also addresses significant gaps in the detailed biological characterization and comparative assessment of local isolates to inform integrated *Salmonella* control strategies in poultry. In conclusion, this study illustrates the potential of selected probiotic isolates-EB7 *(P. polymyxa),* EB14 *(B. licheniformis),* and EB26 *(B. mycoides)* as natural alternatives to antibiotics for enhancing health and resistance to *Salmonella* in broiler chickens. Phenotypic and proteomic analyses demonstrated multifunctional benefits, including robust antibacterial and antioxidant activities, gastrointestinal resilience, safety -exhibiting no virulence or antibiotic sensitivity- and modulation of immunity and gut microbiota. EB7 proved particularly effective, corroborating previous research that indicates *Bacillus/Paenibacillus* supplementation enhances growth, resistance to enteric disease, antioxidant capacity, and immunity for sustainable poultry production. These indigenous strains successfully restored productivity, promoted beneficial microbiota, and reduced pathogen loads in challenged birds, underscoring strain-specific, dose-dependent effects. Consequently, EB7, EB14, and EB26 are deserving of further investigation and commercial development as tailored, biotechnological solutions for poultry health management. While this study demonstrates the promising multifunctional benefits of probiotic isolates EB7 *(P. polymyxa),* EB14 *(B. licheniformis),* and EB26 *(B. mycoides)* as antibiotic alternatives for *Salmonella* resistance and broiler health enhancement, limitations include controlled experimental conditions limiting field extrapolation, short trial durations, reliance on targeted phenotypic and gene expression assays without comprehensive multi-omics profiling, and the absence of molecular screening for antibiotic resistance genes which precludes definitive assessment of horizontal gene transfer risks. Future studies should prioritize long-term field trials, multi-pathogen challenges, dose optimization, synbiotic combinations using metagenomics/metabolomics, and PCR-based resistome profiling for safety validation. For commercial translation, prioritize EB7 for GRAS/FDA approval via comprehensive safety-efficacy dossiers, scale-up production through fermentation-lyophilization at 10^8-10^9 CFU/kg for feed additives, targeting 3-5% FCR improvement and 10-20% mortality reduction in antibiotic-free poultry markets within 1-2 years.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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