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RESEARCH ARTICLE

Potential Probiotic Effect of *Bacillus clausii* DM11 Isolate on Biological Parameters in Broiler Chickens

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ABSTRACT

The global movement to phase out antibiotic growth promoters (AGPs) from livestock production demands the identification and validation of effective alternative strategies. *Bacillus*-based probiotics represent a particularly promising option owing to their intrinsic resistance to environmental stressors and pronounced antagonistic activity against pathogenic microorganisms. This study investigated the probiotic potential of a selected *Bacillus* (*B.*) *clausii* isolate (DM 11) as a dietary supplement in broiler chickens through in *vitro* and in *vivo* trials. *B. clausii* DM 11 was selected from various *Bacillus* isolates for its pronounced antimicrobial activity and high tolerance to acidic pH and bile salts. In *vitro* assays demonstrated that *B. clausii* DM 11 exhibited superior antimicrobial activity and stress tolerance compared with other tested strains, with a broad, dose-dependent inhibitory spectrum. Minimum inhibitory concentrations were as low as 15 µg/mL against pathogens, including *Clostridium perfringens*. A 42-day feeding trial was conducted in which broilers were randomly assigned to six dietary treatments: a basal diet (negative control), a diet supplemented with virginiamycin (50mg/kg; AGP group), and four diets containing graded levels of *B. clausii* DM 11 (60, 120, 240, and 480mg/kg). The broilers supplemented with 240mg/kg *B. clausii* DM 11 yielded the most favorable growth response, comparable to the AGP group; broilers in this treatment attained a final average body weight of 2,720g with a feed conversion ratio of 1.86. *B. clausii* DM 11 (240mg/kg) significantly enhanced serum protein and lipid profiles, maintained balanced leukocyte populations, and showed no evidence of hepatotoxicity or nephrotoxicity. Furthermore, supplementation with *B. clausii* DM 11 upregulated the ileal expression of tight junction proteins (*OCLN, ZO-1*), nutrient transporters (*SGLT1, PepT1*), and antimicrobial peptides (*AvBD1*), while downregulating pro-inflammatory cytokines (IL-1β, TNF-α). Cecal microbiological analysis characterized by an increase in lactic acid bacteria (+0.95 log CFU/g) and a reduction in yeast and mold counts (−0.8 log CFU/g) relative to the control group. *Bacillus clausii* DM 11 (240mg/kg) may exhibit characteristics for a potential probiotic alternative to antibiotics in broilers. It enhances growth and feed efficiency by suppressing pathogens, modulating immune responses, and optimizing gut microbiota and intestinal barrier integrity.

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INTRODUCTION

The use of antibiotics in farm animals has a long history and has been practiced for two main reasons: to promote animal growth and to treat diseases. However, their overuse has led to resistance and environmental contamination, endangering the health of both humans and animals (Ali *et al.,* 2024; Yaseen *et al.,* 2025). This led to worldwide bans on antibiotic growth promoters (AGPs), and it was crucial to identify safe alternatives. Recent research explored the potential use of different alternatives, such as probiotics (Xu *et al.,* 2021), antimicrobial peptides (Yi *et al.,* 2017), phytogenic extracts (Pinheiro *et al.,* 2020; Saquib *et al.,* 2021), organic acids (Pearlin *et al.,* 2020), and essential oil compounds (Montassier *et al.,* 2021; Ayalew *et al.,* 2022). All these decisions collaboratively contribute to enhancing production, strengthening the immune system, counteracting oxidative stress, and maintaining the balance of gut microbiota (Rossi *et al.,* 2020; Luo *et al.,* 2023). The future prospects of probiotics are highly promising, given their safety and efficacy within the human body (Ningsih *et al.,* 2023). *Bacillus* species are well-known probiotic genera that are recognized as strong intestinal symbionts and that improve growth indicators and gastrointestinal performance of both healthy and impaired production systems (Kan *et al.,* 2021; Zhang *et al.,* 2021; Liu *et al.,* 2023). There has been a substantial development in the use of *Bacillus clausii*, especially in broiler production (Chang and Yu 2022; Lena *et al.,* 2022). The *Bacillus* isolate generates numerous bioactive metabolites, including digestive enzymes, bacteriocins, and host-defense peptides, which contribute to better performance and the immune system (Devyatkin *et al.,* 2021; Yang *et al.,* 2021). Previous studies have documented the positive effect of *Bacillus* on growth outcomes and reduced scouring of nursery pigs (Zong *et al.,* 2019), also improved use of structural carbohydrates and protein fractions in ruminants (Deng *et al.,* 2018), and improved antioxidant enzyme profiles (SOD, GPX). Simultaneously, the nitrogen derivatives were reduced, and microbial protein synthesis was also increased in developing lambs (Jia *et al.,* 2018). Adding *B. clausii* to poultry diets is especially important because it alters the cecal microbial ecosystem, restores population balance, and strengthens the intestinal barrier (Han *et al.,* 2023).

Nonetheless, significant strain-specific variability exists among probiotic isolates, even within *Bacillus clausii*, influencing antimicrobial efficacy, gastrointestinal survivability, metabolite profiles, and immunomodulatory potential (Ghelardi *et al.,* 2022; Söylemez-Milli *et al.,* 2025). *B. clausii* has demonstrated efficacy in promoting broiler growth by enhancing their feed conversion ratio (FCR) and villus morphology at a concentration of 4×10^6 - 12×10^6 spores per liter of drinking water (Mushtaq *et al.,* 2023) and enhancing their immunity with MOS combinations (Al-Khalaifah *et al.,* 2025). Nevertheless, the majority of existing research investigates single doses without juxtaposing them with AGP or conducting molecular mechanistic profiling. Contemporary studies predominantly focus on commercial strains (e.g., O/C, SIN, N/R, T) or multi-strain cocktails; however, they lack systematic dose-response evaluations of novel poultry-derived isolates under controlled conditions (Lirio *et al.,* 2023; Haldar *et al.,* 2025).

To date, *Bacillus clausii* DM11, a recently isolate showing initial indications of acid and bile tolerance and broad-spectrum inhibition of poultry pathogens, has not been characterized. Prior broiler studies have primarily focused on other *Bacillus* species or strains, such as *B. subtilis* DSM 32315, QST 713, ATCC 19659, *B. amyloliquefaciens* C271, *B. licheniformis* HJ0135, and *B. velezensis* BV OLS1101. These studies have demonstrated growth promotion, microbiota modulation, or immune effects, yet they have not specifically addressed DM11 or systematically linked strain identity to multiple mechanistic endpoints (Whelan *et al.,* 2018; Haldar *et al.*, 2025; Tang *et al.*, 2025).

Significant knowledge gaps remain regarding *B. clausii* DM11's ability to: (1) achieve or exceed AGP-like improvements in body weight gain and feed conversion at different inclusion levels; (2) modify the cecal microbiota, including total aerobes, lactic acid bacteria, and yeast/molds; (3) regulate intestinal barrier and immune-related genes, such as antimicrobial peptides, tight junction proteins, and cytokines, since only a few *Bacillus*-based studies have thoroughly examined host gene expression; and (4) increase the expression of nutrient transporters like *SGLT1* and *PepT1*. This study addresses these gaps through a dose–response trial of *B. clausii* DM11 (60, 120, 240, 480 mg/kg) in broilers, directly compared to an AGP control.

MATERIALS AND METHODS

Isolation, Screening, and Identification of Selected Isolates: Fresh fecal samples were aseptically collected from broilers within 24h. Peptone buffer was used to prepare a 1:10 (w/v) suspension of the sample, followed by serial tenfold dilutions up to 10^{-7}. Appropriate dilutions were spread onto Luria–Bertani (LB) agar plates and incubated at 37°C for 24h. Colonies exhibiting antagonistic activity were selected and purified for further characterization (Alagawany *et al.*, 2021).

The isolates were coded as DM1-DM40. Initial identification of the isolate was based on conventional morphological and biochemical criteria in accordance with the taxonomic standards of *Bergey's Manual of Systematic Bacteriology* (Murray and Holt, 2015). Definitive species-level assignment was subsequently achieved using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Microflex LT/SH instrument (Bruker Daltonics, Bremen, Germany), a platform widely adopted for rapid and reliable microbial identification in clinical and research microbiology.

Protein extracts were prepared following the manufacturer's recommendations, spotted onto a steel target, overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution, and analyzed in linear positive mode. The resulting mass spectra were matched against the Bruker Biotyper reference database using the Biotyper software, and species identification was accepted only when score values met the manufacturer's thresholds for reliable species-level identification.

Safety and Probiotic Properties: Four isolates, DM5, DM11, DM25 and DM40, were selected and evaluated for their probiotic potential through acid and bile salt resistance tests. Acid tolerance was assessed by inoculating 1mL of bacterial culture into 9 mL of LB broth adjusted to pH 2.5 and incubating at 37°C for 3 h. Bacterial concentration was standardized to an optical density of 0.08 at 600 nm (OD_{600}) using a spectrophotometer (Jabeen *et al.,* 2024). Bile Tolerance Test: 100 µL of the overnight culture was inoculated into LB broth with 0.3% bile salts. Aliquots were plated onto LB agar at 1-hour intervals up to 4 hours; the presence of binary growth (positive/negative) was considered indicative of viability (Mulaw *et al.,* 2019). The following expression was used to compute the survival rates:

$$Survival\ rate\ (\%) = \frac{OD\ after\ treatment}{OD\ before\ treatment} X100$$

Antibiotic sensitivity tests of the *Bacillus* isolates were performed using the disc diffusion method to assess their safety. Isolates were introduced into solid media at a density of 10^6 (CFU/g). Sensitivity to various antimicrobial agents was observed after 48 hours of incubation at 42°C.

Antimicrobial activity: The antimicrobial activity of the selected isolate was evaluated using the disc diffusion method at concentrations of 15, 30, 60, 120, 240, and 480 µg/mL. Sterile 8-mm filter paper discs were impregnated with defined volumes of each concentration and allowed to dry at room temperature for 30 min to ensure uniform loading. Test plates consisted of appropriate agar media previously inoculated with standardized suspensions (0.5 McFarland) of a panel of avian pathogens, ensuring confluent growth. The test panel included: Bacteria: *Escherichia coli*, *Salmonella* Pullorum, *Mycoplasma gallisepticum*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus zooepidemicus*. Fungi/yeasts: *Aspergillus fumigatus*, *Candida albicans*, *Microsporum gallinae*, and *Aspergillus flavus*. Impregnated discs were placed on the inoculated agar surfaces at equal spacing. Plates were incubated under species-appropriate conditions (typically 35–37°C for 18–24h for bacteria; longer where required for fungi/yeasts). After incubation, the diameter of the inhibition zones around each disc was measured in millimeters (mm) and used as an indicator of the inhibitory spectrum and relative potency of the isolate (Saad *et al.,* 2021).

For quantitative determination of the minimum inhibitory concentration (MIC), a broth microdilution assay was performed following standardized guidelines. Serial two-fold dilutions of the antimicrobial preparation were prepared in suitable broth in 96-well microtiter plates, covering a range that included and extended beyond the concentrations used in the disc diffusion test. Wells were inoculated with standardized bacterial or fungal suspensions (5×10^5 CFU/mL) and then incubated under appropriate conditions for 16–24h (Andrews, 2001).

Experimental Design: A total of 360 one-day-old Indian River broiler chicks of similar initial body weight were obtained from a commercial hatchery and used in a completely randomized design (CRD). Chicks were randomly allotted to six dietary treatment groups (T1–T6), each with five replicates of 12 birds per replicate (n = 60 birds per treatment), based on body weight at placement.

The experiment was designed to compare the probiotic activity of a soil-derived bacterial isolate (DM11) administered at graded levels (60, 120, 240, and 480mg/kg feed) with an antibiotic growth promoter (virginiamycin, 50mg/kg diet) in broiler chickens, over a 42-day feeding period, following common probiotic vs. antibiotic comparison frameworks.

All birds were reared in three-tier battery cages equipped with automatic nipple (or manifold) drinkers and manual feeders, under environmentally controlled conditions comparable to standard broiler management guides. Temperature and lighting were adjusted according to breed recommendations (initial brooding at ~33–35°C with gradual weekly reduction to ~24°C; 23L:1D after the first days), and feed and water were supplied ad libitum. The basal diet was a corn–soybean–based ration formulated to meet or exceed the NRC nutrient requirements for broilers and was free of antibiotic growth promoters. Ingredient composition and calculated nutrient levels were based on the formulation described by Al-Quwaie (2023), and all treatment diets were identical to the basal diet except for the inclusion of virginiamycin or DM11 at the specified levels, consistent with other broiler probiotic trials.

Treatment groups were as follows: T1: Control (basal diet without additives), T2: Virginiamycin-supplemented diet (50mg/kg feed), T3: *B. clausii* DM11-supplemented diet (60mg/kg feed), T4: *B. clausii* DM11-supplemented diet (120mg/kg feed), T5: *B. clausii* DM11-supplemented diet (240mg/kg feed), and T6: *B. clausii* DM11-supplemented diet (480mg/kg feed).

The DM11 isolate was cultured in nutrient broth for 24h at 37°C. After centrifugation, the bacterial biomass was resuspended and adjusted to approximately 1×10^8CFU/mL using a previously established standard curve, similar to other *Bacillus*/*Lactobacillus* probiotic preparations. The resulting suspension was thoroughly mixed into the respective treatment diets to achieve the target inclusion rates.

All procedures involving animals were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals, and the experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, consistent with ethical oversight reported in comparable broiler probiotic studies.

Growth Performance: The growth performance of the birds was assessed during the experimental period based on live body weight (LBW), feed intake (FI), and body weight gain (BWG). From these main data points, we calculated a feed conversion ratio (FCR) to evaluate feed efficiency. All calculations were performed following standardized methods established by Brody and Lardy (1946):

Body weight gain (BWG) = Final body weight (FBW) − Initial body weight (IBW)

Biochemical Parameters: Blood and tissue samples were collected at the end of the 42-day experimental period. From each treatment group, five birds (one bird per replicate; n = 5 per treatment) were selected at random and humanely euthanized using an R550 multi-output small-animal anesthesia system with isoflurane in oxygen, in accordance with AVMA guidelines and institutional ethical approval. Induction was performed in an anesthetic chamber with a controlled fresh gas flow of 1.0–2.0L/min until loss of posture and absence of reflexes were confirmed.

Immediately after deep anesthesia was confirmed, venous blood was collected from the hepatic portal vein (alternatively, brachial or jugular vein can be used using heparinized syringes and transferred to lithium-heparin tubes, which were kept on ice until processing. Blood samples were centrifuged at 5,000 × g for 15 min at 4°C to obtain plasma/serum for biochemical analysis. Activities of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and the AST/ALT ratio were determined using commercial diagnostic kits (Spinreact, Spain), following the manufacturer's instructions and the procedure described by Cheng *et al.* (2020), using an automated biochemical analyzer calibrated for poultry serum.

Liver samples were excised immediately after blood collection, rinsed in ice-cold 0.9% (w/v) physiological saline, blotted dry, and weighed. Subsamples were snap-frozen in liquid nitrogen and stored at −70 to −80°C for subsequent biochemical or molecular analyses, as rapid cooling and storage at ≤−70°C preserve enzyme and protein integrity. Serum immunoglobulin A (IgA) and immunoglobulin M (IgM) concentrations were quantified using species-specific commercial chicken ELISA kits (Chicken IgA/IgM ELISA kits, Cloud-Clone, China). Assays were performed by sandwich ELISA in 96-well plates according to the manufacturer's protocols, and optical density (OD) was read at 450nm using a microplate reader (BioTek ELx800, USA).

Gene Expression: Total RNA was extracted from intestinal tissue using an RNase-free protocol, and the final RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water to prevent RNase-mediated degradation. RNA concentration and purity were assessed spectrophotometrically by measuring absorbance at 260 and 280 nm and calculating the A260/A280 ratio, as routinely used for RNA quality control in molecular studies. Only samples with A260/A280 ratios between ~1.8 and 2.1 were used for downstream analyses to ensure adequate purity for reverse transcription and qPCR (Stoikov *et al.,* 2023; Zeng *et al.,* 2024). For first-strand cDNA synthesis, 3µg of total RNA per sample were denatured at 70°C for 5 min in a Bio-Rad T100 thermal cycler and then immediately chilled on ice. The reverse-transcription (RT) mixture contained template RNA, 0.5µg oligo(dT)18 primers, 2µL of 10× RT buffer, 2µL of 10mM dNTP mix, 20–40 U RNase inhibitor, and 100 U of reverse transcriptase in a final volume of 20µL, following standard two-step RT-qPCR protocols. The reaction was incubated at 42–45°C for 30–60 min for cDNA synthesis, followed by enzyme inactivation at 70–85°C for 5–10 min. The cDNA was stored at −20°C until quantitative PCR analysis.

Gene expression profiling was performed by quantitative real-time PCR (qPCR) using a SYBR Green–based master mix (SensiFAST SYBR No-ROX, Bioline, USA) according to the manufacturer's instructions. Each 10–20µL qPCR reaction contained 1–2µL of diluted cDNA, 200–400nM of each gene-specific forward and reverse primer (primer sequences listed in Table 1), SYBR Green qPCR mix (1X), and nuclease-free water. Thermal cycling was performed on a real-time PCR system with an initial denaturation at 95°C for 2–3 min, followed by 40 cycles of 95°C for 3–10s and 58–60°C for 20–30s for annealing/extension, with a final melt-curve analysis to verify amplification specificity. Relative mRNA expression levels were calculated using the $2^{-}\Delta\Delta$Ct comparative method with β-actin as the endogenous reference gene, following established procedures for qRT-PCR data analysis. Cycle threshold (Ct) values were used to derive ΔCt (target – actin) and ΔΔCt (treatment – control), allowing comparison of inter-sample expression levels based on validated quantification approaches.

Intestinal Microbial Quantification: After the trial, intestinal digesta samples were aseptically collected and homogenized in sterile glass tubes, then kept at 4°C and processed within 4–6h to minimize changes in microbial viability. Serial tenfold dilutions were prepared in sterile 0.85–0.9% saline or phosphate-buffered saline, and appropriate dilutions were plated for enumeration of different microbial groups following selective culture protocols adapted from (Wilson *et al.,* 2018; Abd El-Wahab *et al.,* 2022). Total aerobic bacterial count (TBC) was determined on Plate Count Agar (PCA; Oxoid or equivalent) incubated aerobically at 35–37°C for 24–48h. Total yeasts and molds (TYM) were enumerated on Sabouraud Dextrose Agar or Potato Dextrose Agar supplemented with chloramphenicol, incubated aerobically at 25–28°C for 3–5 days. Lactic acid bacteria (LAB) were quantified on de Man–Rogosa–Sharpe (MRS) agar (Difco, USA, or equivalent); plates were incubated under anaerobic or microaerophilic conditions (e.g., GasPak system) at 30–37°C for 48–72h. When necessary to suppress yeast growth, MRS agar was supplemented with cycloheximide, as commonly applied for LAB enumeration. After incubation, plates with 30–300 colonies were selected for counting. The number of colonies was multiplied by the reciprocal of the dilution factor to obtain colony-forming units per gram of digesta (CFU/g). For standardized data representation and statistical analysis, microbial counts for TBC, TYM, and LAB were transformed to log10CFU/g of digesta.

Statistical Analysis: Experimental data were processed using SPSS software (Version 17.0, IBM, USA). Results are expressed as mean ± standard deviation (SD). To evaluate treatment effects, a one-way analysis of variance (ANOVA) was performed. In instances of significant treatment variance, mean separation was conducted using Fisher's least significant difference (LSD) post-hoc test. Statistical significance was established at a threshold of $P<0.05$.

RESULTS

Antimicrobial and stress-tolerance screening of isolates: Among the four screened isolates (DM5, DM11, DM25, DM40), DM11 showed the largest inhibition zones against the indicator pathogens (Fig. 1A). Mean zones were approximately 18mm for *S. aureus*, 16mm for *E. coli* and 13mm for *C. albicans* and were significantly larger than those of DM25 and DM40 against *S. aureus* ($P<0.05$). For *E. coli* and *C. albicans*, inhibition varied by isolate, with DM11 consistently yielding the greatest zones.

All isolates tolerated acidic and bile conditions (Fig. 1B). Survival at pH 2.5 exceeded 65% for all strains, and >70% in pH 5.0 plus bile. DM11 showed the highest survival (82% at pH 2.5; 85% with bile), significantly greater than DM25 and DM40 (67–70%; $P<0.05$). DM5 was intermediate (80% at pH 2.5; 72% with bile), whereas DM40 showed the lowest stability (68%; $P<0.05$).

Minimum inhibitory concentrations of DM11 supernatant: The cell-free supernatant of *B. clausii* DM11 exhibited dose-dependent antimicrobial activity against all tested pathogens (Table 1). Gram-positive bacteria (*Clostridium perfringens, Staphylococcus aureus, Streptococcus zooepidemicus*) and *E. coli*, as well as *Microsporum gallinae*, were inhibited at 15µg/mL. *Salmonella* Pullorum*, Mycoplasma gallisepticum,* *Aspergillus fumigatus,* and *Candida albicans* required 30µg/mL, whereas *Aspergillus flavus* showed the highest MIC at 60µg/mL. A strong concentration–response relationship was confirmed (P<0.05).

Growth performance: Broiler performance from day 1 to 42 is summarized in Table 2. Dietary *B. clausii* DM11 (60–480mg/kg) increased final body weight, average daily gain (ADG) and improved feed conversion ratio (FCR) compared with the basal control, with clear dose-related trends. At 240mg/kg DM11, final body weight reached 2720g and ADG 63.7g/bird/day, representing increases of about 11–12% over the control (2450g; 57.3g/day), and approaching the antibiotic growth promoter (AGP) group (2620g; 61.3g/day). FCR decreased from 2.03 (control) to 1.86 at 240mg/kg, compared with 1.93 for AGP. These improvements between DM11 doses and control were statistically significant (P<0.05). Daily feed intake (116–119g/bird/day) did not differ among treatments (P<0.05), indicating improved feed efficiency rather than increased intake.

Hematology and serum biochemistry: Hematological and biochemical data (Fig. 2, Table 3) showed that RBC, WBC and hemoglobin remained within physiological ranges across all groups, with no significant differences between DM11-treated, control, and AGP birds (P>0.05). Platelet counts were modestly higher in DM11 groups (320 vs. 360–390 ×10^{3}/mL). Neutrophils and lymphocytes changed with dose (P=0.037 and P=0.008, respectively), but NLR remained within normal limits. Total protein and albumin were slightly increased by DM11 (e.g., 42–46 vs. 39g/L; 17–19 vs. 16g/L;P<0.05), without significant alterations in ALT, AST, urea, or creatinine, and lipid profiles remained within reference ranges (P<0.05).

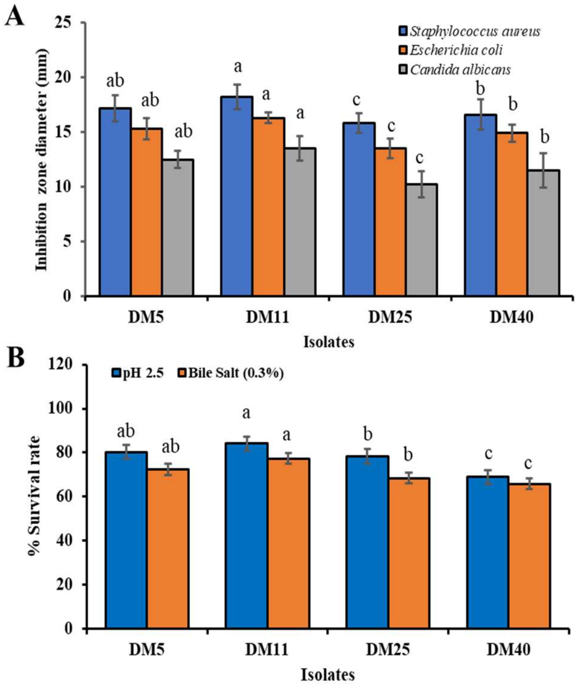

Fig. 1: (A) Screening the most active isolates based on their antimicrobial activity, (B) probiotic properties of selected isolates. Lowercase letters above columns indicated significant differences at P<*0.05*.

Table 1: Antimicrobial Activity of *Bacillus clausii* DM11 Cell-Free Supernatant at Various Concentrations (15, 30, 60, 120, 240, and 480 µg/mL) expressed as inhibition zone diameters (mm)

Pathogen	*Bacillus clausii* DM11 Concentration (µg/mL)/ inhibition zones diameters (mm)						MIC (µg/mL)	P value
	15	30	60	120	240	480		
Gram-negative								
Escherichia coli	8.2±0.8^{d}	10.5±1.0^{c}	13.1±1.2^{b}	15.7±1.4^{ab}	16.0±1.5^{a}	16.3±1.6^{a}	15±0.2	<0.0001
Salmonella Pullorum	–	9.8±0.9^{d}	12.3±1.1^{c}	14.9±1.3^{b}	15.5±1.4^{ab}	15.8±1.5^{a}	30±0.6	<0.0001
Mycoplasma gallisepticum	–	8.3±0.8^{d}	11.0±1.0^{c}	13.2±1.2^{b}	14.0±1.3^{a}	14.1±1.3^{a}	30±0.5	<0.0001
Gram-positive								
Clostridium perfringens	14.5±1.3^{d}	17.8±1.5^{c}	20.5±1.8^{b}	22.1±2.0^{ab}	22.3±2.0^{ab}	22.5±2.1^{a}	15±0.3	<0.0001
Staphylococcus aureus	10.1±1.0^{e}	12.4±1.1^{d}	15.0±1.4^{c}	17.6±1.6^{b}	18.0±1.7^{a}	18.2±1.7^{a}	15±0.3	<0.0001
Streptococcus zooepidemicus	9.8±0.9^{e}	11.9±1.1^{d}	14.5±1.3^{c}	16.8±1.5^{b}	17.2±1.6^{a}	17.3±1.6^{a}	15±0.2	<0.0001
Fungal								
Aspergillus fumigatus	–	8.0±0.8^{d}	9.5±0.9^{c}	11.0±1.1^{b}	12.5±1.2^{ab}	13.0±1.3^{a}	30±0.7	<0.0001
Candida albicans	–	9.1±0.9^{d}	10.8±1.0^{c}	12.0±1.2^{b}	13.2±1.3^{ab}	13.5±1.3^{a}	30±0.6	<0.0001
Microsporum gallinae	8.0±0.8^{e}	10.2±1.0^{d}	12.5±1.2^{c}	14.0±1.3^{b}	14.5±1.4^{ab}	14.8±1.4^{a}	15±0.4	<0.0001
Aspergillus flavus	–	–	8.9±0.9^{c}	10.5±1.0^{b}	11.8±1.1^{ab}	12.0±1.2^{a}	60±0.9	<0.0001

Values are Mean Zone of Inhibition (mm) ± Standard Deviation (n=3). A dash (-) indicates no effective zone of inhibition (<8mm). mean values with different superscript letters (a, b, c, d) are significantly different (P< 0.05). MIC values are presented as mean ± SD. (n=3).

Table 2: Effect of Dietary Supplementation with *Bacillus clausii* DM11 on Growth Performance of Broiler Chickens (1-42 days)

Treatment Group	Dosage (mg/kg diet)	Initial Body Weight (g)	Final Body Weight (g)	Average Daily Gain (g/bird/day)	Feed Intake (g/bird/day)	Feed Conversion Ratio (FCR)
Control (Basal Diet)	0	45.0±1.5	2450±7.2^{e}	57.3±1.7^{d}	116.5±3.1^{c}	2.03±0.03^{a}
Antibiotic (AGP)	50 (Virginiamycin)	44.8±1.4	2620±6.8^{c}	61.3±1.6^{b}	118.0±2.9^{ab}	1.93±0.02^{b}
B. clausii DM11	60	45.2±1.6	2555±7.0^{d}	59.8±1.7^{c}	117.2±3.0^{b}	1.96±0.03^{ab}
B. clausii DM11	120	44.9±1.5	2650±6.5^{c}	62.0±1.5^{ab}	117.8±2.8^{b}	1.90±0.02^{b}
B. clausii DM11	240	45.1±1.7	2720±6.0^{a}	63.7±1.4^{a}	118.5±2.7^{ab}	1.86±0.02^{c}
B. clausii DM11	480	45.3±1.5	2705±6.3^{b}	63.3±1.5^{a}	119.1±2.9^{a}	1.88±0.02^{c}
P-value		0.991	< 0.001	< 0.001	0.185	< 0.001

Means within a column with different superscript letters (a, b, c, d) are significantly different (P<0.05) as determined by one-way ANOVA followed by Tukey's HSD post-hoc test. Shared letters indicate no significant difference. The P-value indicates a significant overall treatment effect for all parameters.

Table 3: Effect of Dietary *Bacillus clausii* DM11 on Serum Biochemical Parameters in Broiler Chickens (Day 42)

Parameter	Control (Basal Diet)	Antibiotic (AGP)	*B. clausii* DM11 (60 mg/kg)	*B. clausii* DM11 (120 mg/kg)	*B. clausii* DM11 (240 mg/kg)	*B. clausii* DM11 (480mg/kg)	p-value
Liver Function							
ALT (U/L)	22.5±3.0^{a}	21.8±2.8^{b}	22.0±2.9^{a}	21.0±2.5^{b}	20.5±2.2^{c}	21.2±2.4^{b}	0.185
AST (U/L)	185±15^{a}	175±14^{c}	180±15^{b}	168±13^{e}	165±12^{e}	170±13^{d}	0.008
ALP (U/L)	4200±350^{a}	4100±340^{c}	4150±345^{b}	3900±320^{d}	3750±300^{e}	3850±310^{d}	0.011
Total Protein (g/dL)	3.8±0.3^{b}	3.9±0.3^{b}	3.9±0.3^{b}	4.1±0.3^{a}	4.3±0.4^{a}	4.2±0.3^{a}	0.016
Albumin (g/dL)	1.7±0.2^{b}	1.7±0.2^{b}	1.7±0.2^{b}	1.8±0.2^{ab}	1.9±0.2^{a}	1.8±0.2^{ab}	0.095
Kidney Function							
Creatinine (mg/dL)	0.38±0.04^{b}	0.40±0.05^{a}	0.39±0.04^{b}	0.37±0.04^{c}	0.36±0.03^{c}	0.37±0.04^{c}	0.102
Uric Acid (mg/dL)	6.5±0.6^{ab}	6.8±0.7^{a}	6.2±0.6^{b}	5.9±0.5^{c}	5.5±0.5^{d}	5.8±0.6^{c}	<0.001
Immunity Markers							
IgG (mg/dL)	325±35^{e}	310±32^{f}	340±36^{d}	365±38^{c}	390±40^{a}	380±39^{b}	0.007
IgM (mg/dL)	85±10^{c}	82±9^{d}	87±10^{c}	90±11^{b}	95±12^{a}	92±11^{ab}	0.078
Lipid Profile							
Total Cholesterol (mg/dL)	145±12^{ab}	148±13^{a}	140±12^{b}	135±11^{c}	130±10^{d}	133±11^{c}	<0.001
Triglycerides (mg/dL)	65±8^{b}	68±9^{a}	62±8^{b}	58±7^{c}	55±6^{d}	57±7^{c}	0.002
HDL (mg/dL)	72±7de	70±7^{e}	74±7^{d}	78±8^{c}	82±8^{a}	80±8^{b}	0.009

Means within a row with different superscript letters (a, b, c, d) are significantly different (P<0.05) as determined by one-way ANOVA followed by Tukey's HSD post-hoc test. Shared letters indicate no significant difference. The p-value indicates a significant overall treatment effect for all parameters.

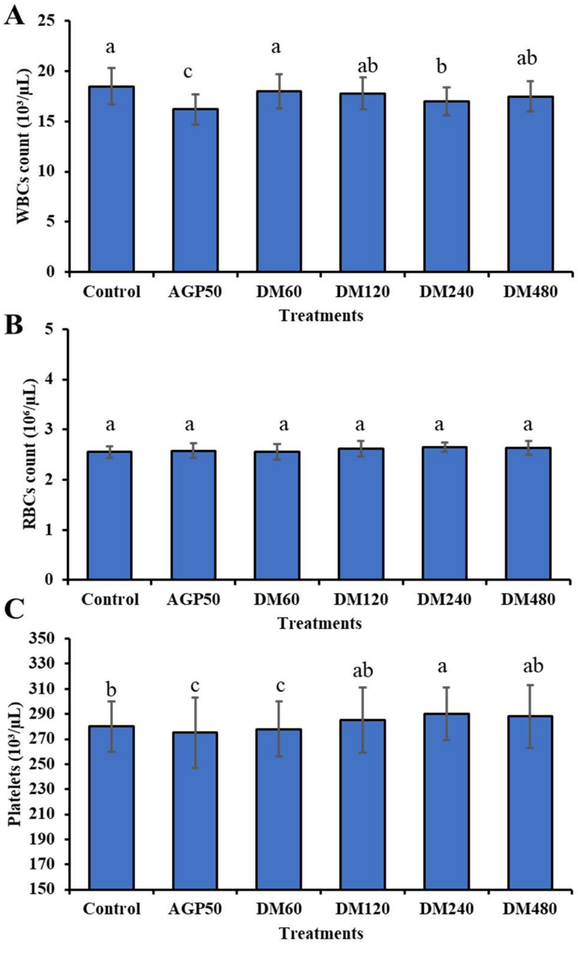

Fig. 2: The effect of diet supplementation with *Bacillus clausii* (DM11) at four concentrations (60, 120, 240, and 480mg/kg feed) compared to antibiotic on the hematology parameters (A) white blood cells count, (B) red blood cells count, and (C) platelets count of broiler chickens. Lowercase letters above columns indicated significant differences at P<0.05

Ileal gene expression: Relative mRNA expression in ileal mucosa is presented in Table 4 and Fig. 3. At 240mg/kg DM11, AvBD1 and CATHBL2 increased to approximately 1.75- and 1.80-fold of control, respectively ($p<0.05$). Tight-junction and mucus-barrier genes were elevated (OCLN 1.82-fold; ZO-1 1.48-fold; MUC2 1.60-fold at 240 mg/kg), while pro-inflammatory IL-1β and TNF-α were down-regulated to about 0.55- and 0.61-fold ($p<0.05$). Nutrient transporters *SGLT1* and *PepT1* increased up to ~1.50- and 1.88-fold at 240mg/kg (P<0.05).

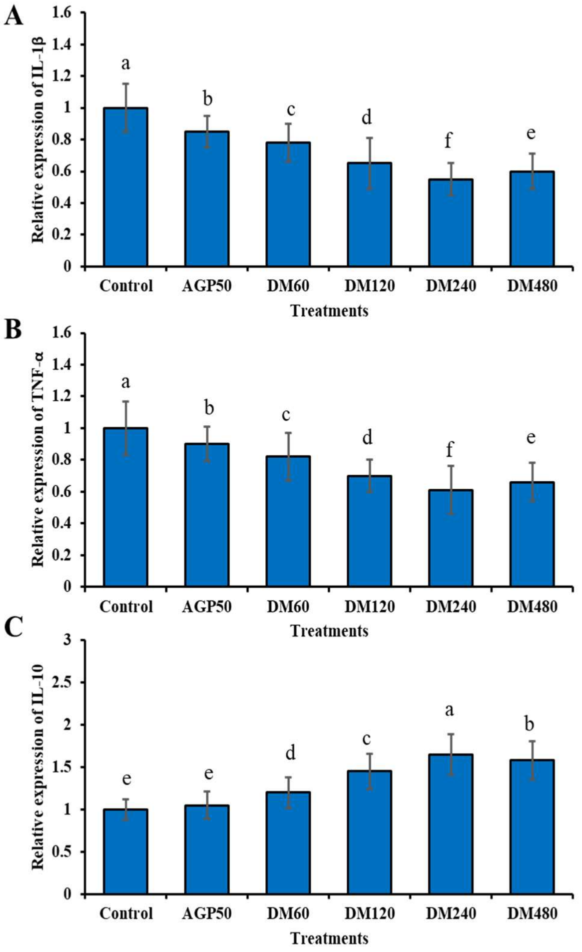

Fig. 3: The effect of dietary supplementation with *Bacillus clausii* (DM11) at four concentrations (60, 120, 240, and 480mg/kg feed) compared with an antibiotic on the regulation of inflammatory gene expression (A) interleukin-1β, (B) TNF-α, and (C) interleukin-10 in broiler chickens is not convincing. The relative expression of cytokines is less than 1 compared to the control/housekeeping genes expression, which is taken arbitrarily as 1. Lowercase letters above columns indicated significant differences at P<0.05.

Table 4: Effect of Dietary *Bacillus clausii* DM11 on Relative Gene Expression in Ileal Mucosa of Broiler Chickens (Day 42)

Gene Category / Target	Control (Basal Diet)	Antibiotic (AGP)	*B. clausii* DM11 (60 mg/kg)	*B. clausii* DM11 (120 mg/kg)	*B. clausii* DM11 (240 mg/kg)	*B. clausii* DM11 (480 mg/kg)	p-value
Gut Barrier Function							
Occludin (OCLN)	1.00±0.15^f	1.05±0.14^e	1.18±0.16^d	1.35±0.18^c	1.52±0.20^a	1.45±0.19^b	<0.001
Zonula Occludens-1 (ZO-1)	1.00±0.14^f	1.08±0.15^e	1.15±0.16^d	1.32±0.17^c	1.48±0.19^a	1.40±0.18^b	<0.001
Mucin-2 (MUC2)	1.00±0.16^f	1.10±0.17^e	1.25±0.19^d	1.41±0.21^c	1.60±0.23^a	1.55±0.22^b	<0.001
Nutrient Transport							
Sodium-Glucose Transporter-1 (SGLT1)	1.00±0.14^f	1.12±0.15^e	1.20±0.16^d	1.38±0.18^c	1.50±0.19^a	1.43±0.18^b	<0.001
Peptide Transporter-1 (PepT1)	1.00±0.15^f	1.08±0.16^e	1.22±0.18^d	1.40±0.20^c	1.58±0.22^a	1.50±0.21^b	<0.001
Antimicrobial Defense							
β-Defensin (AvBD1)	1.00±0.17^f	0.95±0.16^e	1.25±0.20^d	1.50±0.23^c	1.75±0.26^a	1.65±0.24^b	<0.001
Cathelicidin (CATHL2)	1.00±0.16^f	0.98±0.15^e	1.30±0.20^d	1.55±0.23^c	1.80±0.26^a	1.70±0.25^b	<0.001

The p-value in the final column for each gene was derived from a one-way Analysis of Variance (ANOVA). A p-value of < 0.05 indicates a statistically significant overall difference between the treatment means. Where ANOVA was significant (P<0.05), Tukey's HSD post-hoc test was applied. For clarity, specific intergroup comparison letters are omitted; however, all listed genes show a significant overall treatment effect (p<0.001). Relative Gene Expression in Ileal Mucosa of Broiler Chickens data of Occludin (OCLN), Zonula Occludens-1 (ZO-1), Mucin-2 (MUC2), Sodium-Glucose Transporter-1 (SGLT1), Peptide Transporter-1 (PepT1), β-Defensin (AvBD1), Cathelicidin (CATHL2) genes can be shown as CT values in comparison to house keeping genes.

Microbial count: Fig. 4 summarizes cecal microbial counts ($\log_{10}$ CFU/g) across treatments, showing modest but significant probiotic-related shifts rather than large absolute changes. For total bacteria (Fig. 4A), the mean count decreased slightly from 8.9±0.2 $\log_{10}$ CFU/g in the control to 8.1±0.15 in AGP50 and approximately 8.2–8.3 $\log_{10}$ CFU/g in the DM60–DM480 groups. These differences represent a reduction of about 0.6–0.8 log units, corresponding to roughly a 2–6-fold decrease in total bacterial load, not 75–85%. The reduction at DM60 and higher doses was statistically significant (p<0.05), indicating that *Bacillus clausii* DM11 can lower overall cecal bacterial counts without causing a marked collapse of the microbiota. Cecal yeast and mold counts (Fig. 4B) decreased from 4.1±0.3 $\log_{10}$ CFU/g in the control to 3.3±0.15 $\log_{10}$ CFU/g in the DM240 and DM480 groups (p<0.05). This 0.8-$\log_{10}$ reduction corresponds to about a 6-fold decrease, i.e., to 15–20% of the control level, indicating a moderate antifungal effect of DM11. AGP50 did not significantly alter fungal counts (4.0±0.2 $\log_{10}$ CFU/g), suggesting that the antifungal impact is specific to the probiotic rather than a general effect of growth promoters. Lactic acid bacteria (LAB) showed a clear dose-response (Fig. 4C), increasing from 7.8±0.15 $\log_{10}$ CFU/g in the control to 8.2±0.1 in DM120 and 8.7±0.08 $\log_{10}$ CFU/g in DM240 and DM480 (P<0.05). The ≈0.9-$\log_{10}$ increase at higher doses corresponds to about a 7–8-fold rise in LAB counts, supporting a selective enrichment of beneficial bacteria rather than a non-specific reduction of the microbiota, consistent with findings from other probiotic studies using $\log_{10}$ CFU/g enumeration.

DISCUSSION

Bacillus clausii DM11 showed strong *in vitro* antimicrobial activity and good tolerance to acidic and bile conditions, supporting its selection as a probiotic candidate for poultry. These traits are consistent with reports that *Bacillus* strains with high antimicrobial activity and gastrointestinal resistance are suitable AGP alternatives in broilers (Tran *et al.*, 2023; Tang *et al.*, 2025). The antimicrobial spectrum of DM11 against Gram-positive and Gram-negative poultry pathogens aligns with observations for other *Bacillus* probiotics, where secreted metabolites, including bacteriocin-like substances and lipopeptides, inhibit enteric pathogens and contribute to gut health (Tran *et al.*, 2023; Vieco-Saiz *et al.*, 2024). Detailed identification of DM11 metabolites and their mode of action was beyond the scope of this work and should be addressed in future mechanistic studies.

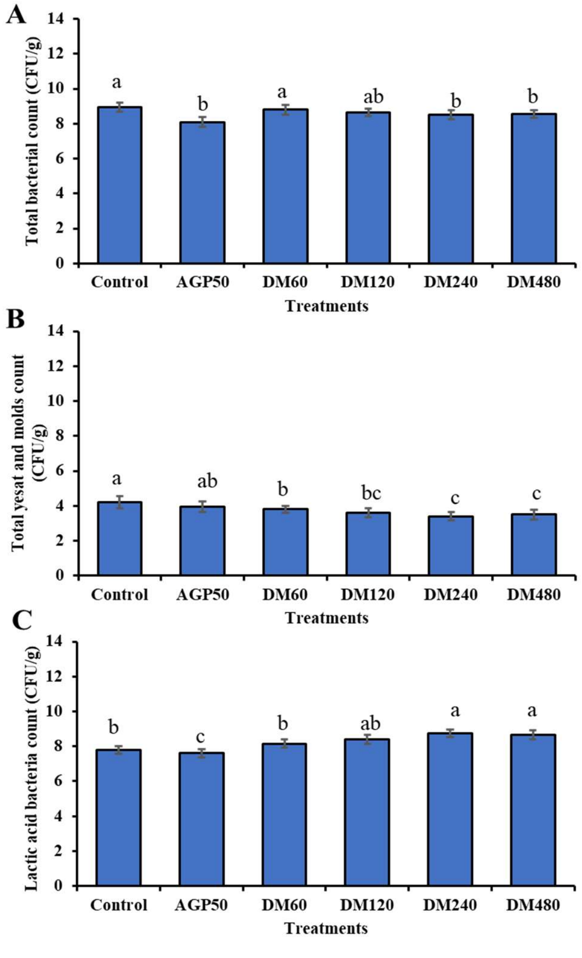

Fig. 4: Impact of varying dietary concentrations of *Bacillus clausii* (DM11) on intestinal microbial populations in broiler chickens compared to control and antibiotic growth promoter (AGP) groups. (A): Total Bacterial Count (TBC) showing the general microbial load across treatments. (B): Total Yeasts and Molds Count (YMC) illustrating the antifungal effect of the treatments. (C): Lactic Acid Bacteria (LAB) count, highlighting the proliferation of beneficial bacteria in response to DM supplementation. Values are expressed as mean log 10 CFU/g of intestinal content. Bars representing means with different superscript letters (a, b, c) indicate statistically significant differences (P<0.05).

The superior acid–bile tolerance of DM11 compared with other screened isolates is in line with findings that spore-forming *Bacillus* strains maintain high viability under simulated gastrointestinal conditions and during feed processing, offering a practical advantage over non-spore probiotics (Ogbuewu *et al.,* 2022; Söylemez-Milli *et al.,* 2025). This supports the expectation that DM11 can survive feed manufacture, storage, and passage through the upper gut, a prerequisite for *in vivo* efficacy. Dietary supplementation with DM11 improved broiler growth performance and FCR in a dose-dependent manner, with the 240mg/kg dose approaching the effects of virginiamycin. Similar performance gains have been reported for several *Bacillus*-based products, where increases in body weight (≈6–14%) and FCR improvements (≈2–5%) are comparable to AGPs when strains and inclusion levels are optimally selected (Luise *et al.,* 2022; Tang *et al.,* 2025). Recent studies show that *Bacillus subtilis* can match antibiotic treatments under enteric challenge conditions (Wang *et al*., 2021).

The lack of differences in feed intake among treatments indicates that performance benefits of DM11 are likely driven by improved feed efficiency and gut function rather than changes in appetite, consistent with work showing that *Bacillus* probiotics enhance nutrient digestibility and intestinal morphology without altering intake (Qiu *et al.,* 2021). Future studies should combine pathogen-challenge models, gut histomorphology, and economic assessments to clarify the contribution of DM11 to disease resistance, barrier integrity, and cost-effective AGP replacement. *Bacillus clausii* DM11 improved broiler growth and feed efficiency to a degree comparable with virginiamycin, supporting its candidacy as an evidence-based alternative to AGPs in line with current efforts to reduce antimicrobial resistance in poultry production (Rahman *et al.,* 2022). These findings are consistent with studies showing that *Bacillus*-based probiotics can match or approach AGP performance when appropriately dosed (Ningsih *et al.,* 2023). At 240 mg/kg, DM11 supplementation increased final body weight to 2720g and average daily gain to 63.7g/bird/day (11% above control) and improved FCR from 2.03 to 1.86, with no further benefit at 480 mg/kg. Similar magnitude improvements in body weight gain and FCR have been reported for *Bacillus clausii* and *B. subtilis* products in broilers, indicating that well-selected spore-formers can reliably enhance performance indices (Luise *et al.,* 2022; Tang *et al.,* 2025).

Feed intake did not differ among treatments ($p = 0.185$), suggesting that improved performance is driven by better feed efficiency and gut function, not altered appetite. This pattern aligns with work showing that *Bacillus* probiotics improve nutrient utilization, intestinal morphology, and microbiota balance while leaving intake largely unchanged (Grant *et al.,* 2018; Jha *et al.,* 2020).

Hematological and serum biochemical parameters remained within reference ranges across DM11 doses, with stable liver and kidney function markers. Increases in total protein, albumin, and lymphocyte proportion, together with a lower neutrophil-to-lymphocyte ratio, indicate a favorable immune and nutritional status without evidence of organ toxicity, similar to other *Bacillus*-based interventions in poultry (Ogbuewu *et al.,* 2022; Tang *et al.,* 2025). Changes in lipid profile (lower triglycerides, higher HDL) are also in line with reports that *Bacillus* probiotics can beneficially modulate lipid metabolism in poultry (Grant *et al.,* 2018). *Bacillus clausii* DM11 at 240mg/kg appears optimal, balancing performance gains, safety, and cost-efficiency, as higher inclusion did not yield additional benefits. Given the limited impact of AGPs on performance in some modern systems and their clear role in selecting antimicrobial resistance genes (Paul *et al.,* 2022), DM11 represents a practical AGP replacement that supports growth, gut health, and regulatory goals to curb AMR.

Results of current study on DM11 suggest that *Bacillus*-derived antimicrobials can strongly inhibit *Clostridium perfringens* and other Gram-positive pathogens, in line with reports that *Bacillus* spp. secrete potent bacteriocins and lipopeptides active against enteric bacteria and some multidrug-resistant strains (Xu *et al.,* 2021; Shleeva *et al.,* 2023). However, this study did not purify or characterize DM11 metabolites, so the specific molecules and their *in vivo* relevance remain uncertain. Up-regulation of tight-junction and mucus-associated genes (occludin, ZO-1, mucin-2) at 240 mg/kg DM11 is consistent with work showing that *Bacillus* supplementation enhances barrier proteins, reduces oxidative stress, and limits inflammation in poultry and *in vitro* epithelial models (Zhang *et al.,* 2022; Zou *et al.,* 2022). Improved villus architecture and barrier integrity likely contributed to better FCR and weight gain, but direct causal links were not demonstrated experimentally in this trial.

The observed increase in IL-10 expression alongside controlled levels of pro-inflammatory cytokines resembles the balanced immunomodulation reported for *Bacillus* probiotics, which can elevate IL-10 and Ig levels while attenuating excessive inflammatory responses in chickens (Larsberg *et al.,* 2023; 2024). Nonetheless, only a limited cytokine panel and one time point were assessed, restricting interpretation of longer-term or systemic immune effects. Enhanced expression of nutrient transporters such as *SGLT1* and *PepT1*, together with higher serum protein and albumin, supports a role for DM11 in improving nutrient uptake and apparent metabolizable energy, similar to findings with *B. subtilis* and *B. licheniformis* in broilers (Xu et al., 2021).

Moreover, future work should (1) chemically identify and quantify DM11 antimicrobial metabolites and test them *in vivo* under defined pathogen challenge, (2) measure SCFA profiles and GPR signaling to confirm metabolic mechanisms, (3) perform time-course and tissue-specific immune and barrier analyses, and (4) evaluate DM11 in different breeds, management systems, and multi-strain or symbiotic combinations to define dose–response, safety, and robustness across production conditions. Key limitations of the present study include single-strain focus, one challenge-free environment, lack of microbiota sequencing and metabolomics, and reliance on correlative rather than causal data for the proposed mechanistic pathways.

Conclusions: *Bacillus clausii* DM11 demonstrates considerable potential as a multifunctional probiotic and a prospective alternative to antibiotic growth promoters (AGPs). It combines extensive *in vitro* activity against important Gram-positive pathogens with *in vivo* improvements in growth performance at a dosage of 240 mg/kg, which are comparable to or surpass those observed with antibiotic controls. These advantages appear to be mediated by gut health mechanisms, such as improved barrier function, immunomodulation, and upregulation of nutrient transporters, rather than by nonspecific antimicrobial suppression, which aligns with existing evidence supporting *Bacillus*-based alternatives to AGPs in broiler chickens. Nonetheless, the antimicrobial metabolites of DM11, shifts in microbiota, and signaling pathways have not been comprehensively characterized. The current findings are derived from a single strain, specific dose range, and controlled experimental setting. Future studies should validate these findings under commercial and pathogen challenge conditions, investigate optimal dosing and safety parameters, and employ advanced omics approaches to confirm mechanisms. It may lead to positioning this probiotic as a viable antibiotic growth promoter alternative.

Authors contribution: DAHAQ: 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.

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