

RESEARCH ARTICLE

Impact of Dietary *Bacillus toyonensis* M44 as an Antibiotic Alternative on Growth, Blood Biochemical Properties, Immunity, Gut Microbiota, and Meat Quality of IR Broilers

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ABSTRACT

Antibiotic-resistant microorganisms may lead to treatment failure and economic losses in the poultry industry and threaten public health. One of the main factors involved in the increasing emergence of antimicrobial resistance is the non-judicious use of antimicrobial agents as growth promoters in poultry and animal production. The search for natural alternatives to growth promoters to antimicrobial agents is continuous and crucial. This investigation focused on the probiotic strains extracted from the gastrointestinal tracts of poultry reared in various habitation types. The selected strain with antioxidant properties was *Bacillus toyonensis* (BT) M44, used as a probiotic dietary supplement and antibiotic alternative, then investigated its effects on growth performance, blood biochemical parameters, immune response, gut microbiota, and meat quality of IR broilers. *Bacillus toyonensis* M44 exhibited potent antibacterial activity against MDR microorganisms, as evidenced by MICs ranging from 5% to 10%. A total of 160 IR chicks were allocated into four groups randomly, each consisting of (10 x 4) chicks. The control group was administered a basal diet; the remaining three groups were provided the control diets containing BT at concentrations of 0.4, 0.8, and 1.6 mg/kg. The results indicated that BT-supplemented groups showed increased body weights, weight gain, growth rate, and performance index and reduced feed conversion ratio during the two growth periods compared to control. The best results were obtained with BT 1.6 mg/kg for the antecedent growth metrics. Chicks receiving BT (0.4 mg/kg) had improved renal functions and lower uric acid and creatinine levels than the other groups. Moreover, ALT and AST levels $P < 0.05$ decreased with BT doses compared to the control. BT 1.6 mg/kg produced the best liver function results; the histopathological studies confirmed that BT addition didn't affect the liver and intestine structure. Adding *Bacillus toyonensis* M44 to the diet formulation improved ($P < 0.05$) lipid profile, immune response, thyroid functions, and gut microflora compared to the control group. Additionally, BT enhanced the chicken meat's juiciness and tenderness characteristics and improved its moisture and protein content; the meat color was also enhanced. Generally, supplementing IR chicken with BT could improve growth performance and blood biochemistry, modulate the gut microflora structure, and enhance the meat quality.

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INTRODUCTION

The proliferation of antibiotic-resistant microorganisms is facilitated by the selection pressure created by the broad application of antibiotics in veterinary medicine (Clement *et al.*, 2019). Although these pathogens can affect animals' and humans' health, they originate from or inhabit animals without producing illness. People can acquire these through indirect interactions with non-food-producing animals in the environment or direct contact with diseased animals or food items (Majumder *et al.*, 2020). Additionally, residues of antibiotics in poultry products such as eggs or meat harm human health. The presence of antibiotic residues in food products may result in the development of antimicrobial resistance in microbiota and pathogenic bacteria (Mancuso *et al.*, 2021). Current production practices include using probiotics as antibiotic alternatives and productive agents to promote growth (Samad, 2022).

Feed ingredients known as probiotics enhance the gastrointestinal tract's health by activating and stimulating beneficial microorganisms. The probiotic bacterial strains are resistant to the digestive enzymes of humans or non-ruminants. On the other hand, prebiotics are the substrates that help the growth of probiotic bacteria in the large intestine, such as *Lactobacillus*, *Bacillus*, *Paenibacillus*, *Bifidobacteria*, and *Bacteroides*. This may result in modifying the arrangement of bacterial collections in the digestive gut (Béghin *et al.*, 2021).

Generally, there are two kinds of microorganisms in the chicken gastrointestinal tract. The first type is allochthonous bacteria or transient microbiota. This type is exogenous and is added as a dietary complementary in feed or drinking water as a probiotic nutritional supplement (György *et al.*, 2021). Second is autochthonous or resident microbiota found in the gut by inoculation resulting from normal feeding behavior and the surrounding environment of the bird (Terzić-Vidojević *et al.*, 2020). It has been explored that allochthonous bacteria introduced by probiotics may inhibit the colonization and infection of pathogens and microbes in the gastrointestinal tract (Yousaf *et al.*, 2022). Probiotics may benefit animal/bird performance by regulating gastrointestinal tract microbiota and inhibiting the growth of potentially pathogenic microorganisms. So, it may be used as an alternative to antibiotic growth promoters for improved production performance (Kulkarni *et al.*, 2022).

Supplements containing probiotic-producing microorganisms and their metabolites have the potential to serve as nutritional supplements and antibiotic substitutes in poultry production (Ayalew *et al.*, 2022). Additionally, they improve body weight, FCR, immune response, carcass output, and digestibility of amino acids such as lysine, valine, and cysteine, increasing production and overall health (Sharma *et al.*, 2023; Fatima *et al.*, 2024).

Previous studies reported that probiotics helped in the production of H₂O₂ that damaged many harmful bacteria, dropping oxidation stress in the gastrointestinal tract, producing the necessary digestive enzymes, preventing the growth of deleterious amines and aerobic pathogens, generating (B) group vitamins, and promoting feed intake and appetite (Xu *et al.*, 2019). *Bacillus* species are found in soil, air, and sea sediments (Stenfors Arnesen *et al.*, 2008).

B. cereus can temporarily be present in mammals' stomachs; they have been investigated as probiotics for animal feeds and medications (Elshaghabee *et al.*, 2017). The soil isolates of *B. toyonensis* belong to the *B. cereus* group, which has been utilized as an animal feed additive for decades (Jiménez *et al.*, 2013).

The selection criteria for bacterial probiotics include: (a) Presence in the intestinal flora of the target animal species to ensure efficient adaptation to the gastrointestinal environment; (b) Ability to tolerate low pH conditions to survive passage through the stomach; (c) Ability to withstand the action of bile salts in the duodenum, the first portion of the small intestine; and (d) Absence of vancomycin resistance genes (VRGs) as determined by the International Scientific Association of Probiotics and Prebiotic (Tuomola *et al.*, 2001; Hill *et al.*, 2014).

Probiotic effects depend upon many factors like strain, dose, age of the bird, administration method, ability of the selected strain to survive at environmental temperature, viability, and term storage, which may be responsible for the differences in results (Aluwong *et al.*, 2013). Further, previous studies didn't find changes in antioxidant status. As a result, we separated the *Bacillus* isolates for this investigation from marine sediments, looking for the best antibacterial activity against bacteria resistant to multiple drugs. We then identified the isolates using biochemical tests and MALDI-TOF analysis. *Bacillus toyonensis* M44 was introduced to the broiler feed in place of antibiotics. They were then tested for their beneficial effects on performance, immunity, blood biochemistry, intestinal bacterial counts, and net revenue of broilers as growth promoters and meat quality enhancers.

MATERIALS AND METHODS

Bacillus isolation, screening and identification: *Bacillus toyonensis* M44 strain was used as an antibiotic alternative and growth promoter in broilers. *Bacillus* isolates with antimutagenic and antioxidant properties were isolated from healthy chicken droppings for this study. Fecal samples of chickens were obtained from cages on the farm and then transferred to the laboratory within twenty-four hours of their collection in sterile containers under refrigeration.

Using Luria-Bertani (LB) medium, intestinal bacilli were isolated. Instead of other bacteria, bacilli were chosen based on the colonies' shape, which was confirmed using a PrimoStar microscope (Jena, Germany) to identify spore-forming rods after spore staining. Specific isolates were distinguished using MALDI-TOF mass spectrometry (Bruker Daltonics GmbH, Germany) connected with Biotyper (version 3.0) software. In total, twelve genotypes of bacilli were chosen from the progeny.

Antibiotic resistance was among the potentially hazardous characteristics of each selected strain that were assessed to determine their safety. All of them were plated on blood agar and incubated for two days at 42°C to verify the hemolytic activity of each strain. The isolates were seeded to a final concentration of 10⁶ CFU/g on suitable solid nutrient media to assess antibiotic sensitivity. Standard antibiotic discs were put on the sides of the inoculated media. The following concentrations were

utilized: tetracycline (30 µg), azithromycin, erythromycin, ceftriaxone, and gentamicin. The findings were shown after a 48-hour incubation period at 42°C.

Bacterial-lux biosensors were employed to quantify bacterial fermentate's antioxidant and DNA-protective activities. As biosensors, we utilized *E. coli* MG 1655 carrying a plasmid containing luminescence genes regulated by a stress-inducible promoter, whereas *E. coli* MG 1655 pRecA-lux is reactive to DNA damage (Prazdnova *et al.*, 2015). This experiment showed *Bacillus toyonensis* M44 beneficial properties and thus it was chosen as the strain to be supplemented into feed as a potential probiotic. As a positive control, fermented milk product isolates of *B. subtilis* KATMIRA1933 tested in previous investigations were utilized (Algburi *et al.*, 2021).

The safety of isolated *Bacillus*, hemolytic activity, and mutagenicity (measured by lux biosensors) were assessed in a StatFax 4400 microplate reader (Awareness Technology Inc., Palm City, FL, USA). While none of the isolates exhibited pro-mutagenic, hemolytic, or antibiotic resistance characteristics, a subset demonstrated prooxidant activity. Using lux biosensors, the antioxidant and antimutagenic activity of the strains was assessed. One of the twelve isolated bacilli strains with the highest aggregate antimutagenic and antioxidant activities, *Bacillus toyonensis* M44, was chosen for further research.

Solid-phase fermentation prepared nutritional supplements for these isolates (Chistyakov *et al.*, 2015). Soybeans were cultivated at 42°C for two days after being inoculated with an overnight culture of the *Bacillus* strains under investigation. Following fermentation, the substrate was milled and desiccated. Using 95% ethanol, the milling apparatus was sterilized to prepare the solid-phase materials. Each feed additive contained 10⁸ CFU/g of bacteria, resulting in a final bacterial concentration of 10⁵ CFU/g in the poultry feed.

Antimicrobial activity of *Bacillus* selected isolate: Four BT concentrations of 10, 20, 40, and 80 % were prepared, and then 8 mm discs were submerged in every level for 30 min. The poultry pathogenic microorganisms, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, and *Klebsiella pneumonia* were selected to evaluate the antibacterial activity of BT concentrations. After inoculating LB plates with pathogenic bacteria, the saturated discs were put on the plates. After incubating the LB plates, the inhibition zones were measured (mm) (Saad *et al.*, 2021). The *Bacillus* isolate's minimal inhibitory concentration was assessed according to El-Saadony *et al.* (2021). The antibacterial results were compared with cephalixin (800 µg/mL).

The design of the experiment: A total of 160 newly hatched male Indian river (IR) broiler chicks with an average body weight (BW) of 44.8g were obtained from a local hatchery and assigned to four dietary treatments in a randomized complete block design with 4 replicates per treatment. Each replicate contained 10 chicks (half male and half female) housed in two cages with male and female apart. The 35-day trial spanned three phases, including the starter period (days 0–14), the grower period (days 15–28), and the finisher period (days 29–35). The *Bacillus* powder at concentrations of 0.4, 0.8, and 1.6 mg/kg were mixed in the feed mill with the dietary. The four experimental groups were as follows: G1 was the control group and was provided with the basal diet. Meanwhile, the G2, G3, and G4 were supplemented with *Bacillus* powder at concentrations of 0.4, 0.8, and 1.6 mg/kg beside the baseline feed. The birds were reared under controlled experimental conditions and received unlimited food and water. Table 1 lists the components and molecular structure of basal diets (cold pellet form) used for rearing the IR broiler during different age groups.

Table 1: Composition of the basal diet at different ages of IR broiler chicks (Barua *et al.*, 2021)

Ingredient%	Pre –starter 1-7 day	Starter 8-20 day	Grower 21-28 day	Finisher 29-35 day
Yellow Corn	55.80	57.17	61.50	68.70
Soybean Meal (44%)	28.20	28.97	25.00	19.31
Corn Gluten Meal (60%)	10.17	7.78	7.46	5.82
Soyabean Oil	1.20	1.40	2.00	2.50
L-Lysine HCL	0.50	0.50	0.50	0.50
DL-Methionine	0.13	0.18	0.14	0.16
Calcium Carbonate	1.60	1.60	1.33	1.22
Calcium Phosphate, Mono	1.73	1.73	1.40	1.12
Salt, NaCl	0.37	0.37	0.37	0.37
Vitamin and mineral mix*	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00
Calculated analysis**				
Dry Matter%	87.73	87.53	86.95	86.46
Crude Protein%	23.8	22.85	21.26	18.40
ME Kcal/Kg	3000	2990	3084	3181
Ether Extract%	2.77	2.78	2.88	3.03
Crude Fiber%	3.55	3.58	3.39	3.11
Lysine%	1.46	1.46	1.35	1.18
Methionine%	0.60	0.61	0.55	0.52
Methionine + Cysteine%	0.98	0.98	0.90	0.82
Calcium%	0.97	0.97	0.90	0.85
Available Phosphorus%	0.51	0.51	0.44	0.40
Determined				
Dry Matter%	88.5	88.7	86.8	85.6
Crude Protein%	23.89	22.77	20.85	18.29
Ether Extract %	3.00	3.00	3.00	2.95
Ash%	5.22	4.9	4.6	5.1

*Each 3.0 kg of mineral and vitamin mix contains Vit. A, 12000000 I.U.; Vit. E, 10 g; **Vit. D₃, 2500000 I.U.; Vit. K₃, 2.5 g; Vit. B₁, 1 g; Vit. B₂, 5 g; Vit. B₆, 1.5 g; Vit. B₁₂, 10g; Biotin 50 mg; Folic acid, 1 g; Nicotinic acid, 30 g; Pantothenic acid, 10 g; Choline 250000mg, Zn, 55 g; Cu, 10 g; Fe, 35 g; Co, 250 mg; Se, 150 mg; I, 1 g; Mn, 60 g; and antioxidant, 10 g. **According to NRC, 1990.

Measuring growth performance and carcass characteristics: After logging the live body weight (LBW) and feed consumption of the chicks, the body weight growth (BWG) was computed. BWG is calculated by the feed conversion ratio (FCR) and divided by feed consumption following Saad *et al.* (2022). The performance index (PI) and the growth Rate (GR) were estimated.

Body weight gain (BWG)= FBW – IBW

$GR = (LBW_{35} - LBW_1) / 0.5 \times (LBW_1 + LBW_{35})$

$PI = BWG / FCR$

At 35 days of age, a total of 24 chickens were collected from the four groups, six from each, weighed again, had their jugular veins removed, and were, after that, de-feathered and disemboweled. Following the collection of blood samples, the serum was separated (by centrifugation at 3000rpm for 15 minutes after clotting of blood samples) and kept until needed for blood analysis.

Blood biochemistry, immunity, and antioxidants: The following measurements were made on serum: low-density lipoproteins (LDL), high-density lipoproteins (HDL), and total cholesterol (Chol); according to Pirgozliev *et al.* (2023). The enzymes lipase, amylase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were tested (Wainstein *et al.*, 2022). The trypsin (Bovine Trypsin ELISA Kit MBS706461) was identified. The ELISA technique and an automated Biochrom ELISA reader were used to quantify triiodothyronine (T3) and thyroxine (T4). Commercial diagnostic kits were implied to determine all blood biochemical parameters (Biodiagnostic Company, Egypt). IgA and IgG immunoglobulin isotypes were evaluated using commercial ELISA kits (Gao *et al.*, 2023).

Histopathological studies: The liver and intestinal samples were selected, stored in formalin, and treated using an automated processor. A preliminary stage was established and subsequently desiccated. The tissue was fixed by submerging it in a 10% formalin solution for 48 hours. Afterward, the fixation solution was removed with distilled water for 30 minutes. The tissues were dehydrated by immersing them in increasing concentrations of alcohol (70, 90, and 100%) for 120 with 70% alcohol for 90 minutes, 90% alcohol for 90 minutes, and 100 % for 120 min. The utilization of repeated cycles of xylene eventually resolved dehydration. The process entailed immersing the tissue in a solution of 50% xylene and 50% alcohol for one hour, followed by a further 1.5 hours of immersion in pure xylene. Next, the specimens were fully impregnated with molten paraffin wax, enclosed, and hermetically sealed. The researchers utilized hematoxylin and eosin staining on paraffin-embedded tissue slices 4-5µm thick (Suvama and Niranjana, 2013).

Intestinal microbial counts: After slaughtering, the intestinal digesta was collected, emptied, and mixed in sterile glass bottles. The containers were stored at 4°C till the enumeration of the microbial population. The microbial population was examined for the total bacterial count, *E. coli*, total yeast and molds count (TYMC), and lactic acid bacteria, on specific media, according to Abd El-Wahab *et al.* (2022). The counts were calculated as Log₁₀ CFU/g of the digest.

Meat quality: Chicken breasts were collected and cut into pieces (3cm); the color properties of meat samples (lightness (L), redness (a), and yellowness (b)) were calculated by Hunter Lab spectrophotometer (Vista, Reston, VA, USA) following the method of Sayed-Ahmed *et al.* (2022). The oxidation of lipids was evaluated using a 2-thiobarbituric acid assay (TBA) (Sayed-Ahmed *et al.*, 2022). Total Volatile Basic Nitrogen (TVBN) is a method to assess the amount of nitrogenous molecules (ammonia, dimethyl, and trimethyl amine) in meat, showing the degree of freshness; the method was evaluated following Moosavi-Nasab *et al.* (2021). The pH value of breast samples was measured with a HANNA pH meter (Woonsocket, USA). The approximate analysis of chicken breast was estimated according to AOAC (2012) as follows: moisture content was tested by oven technique, crude protein was identified by Kjeldahl method, crude fat was calculated by the Soxhlet apparatus, and a muffin evaluated ash at 500°C. Color parameters, lightness (L), redness (a) and yellowness (b) were measured using hunter following Sayed-Ahmed *et al.* (2022). Sensory evaluation: Meat cubes (3 cm) were evaluated by ten experienced panelists. The panelists were presented with breast samples encoded with three random digits. On a nine-point hedonic scale, the panelists evaluated the sensory attributes (appearance, color, flavor, and juiciness), where a score of one indicated a strong aversion and nine signified an intense preference. In between sessions, tap water was given to alter the mouthfeel (Zhou *et al.*, 2023).

Economic efficiency: Economic efficiency of the supplemented feed was evaluated according to Kalia *et al.* (2018). Other productive expenses were ignored because they were constant, but the expense of BT was incorporated into the feed price.

Statistical analysis: The study statistics were performed by one-way ANOVA utilizing Excel (Microsoft Office, 2021). All examined means (treatments) were compared using the LSD test, ensuring significance at $P < 0.05$.

RESULTS

Antibacterial activity of *Bacillus toyonensis* M44: *Bacillus toyonensis* M44's inhibition zone diameters (IZDs) independently raised concentrations against the tested microorganisms, as summarized in Table 2. The highest *Bacillus* isolates (80%) achieved the highest inhibition zones against the tested pathogenic bacteria. *S. aureus* was the most susceptible to M44 concentration at 34 mm, followed by *S. pyogenes* at 33 mm; on the other hand, *S. Typhimurium* was the most resistant at 22 mm, followed by *K. pneumonia* at 23 mm. This experiment was conducted to investigate the effectiveness of probiotic isolate's in vivo antimicrobial activity and used different concentrations to define the most effective ones.

Growth performance: The effects of adding the *Bacillus toyonensis* M44 at three dosages (0.4, 0.8, and 1.6 mg/kg) on various growth indices during the growth period of 35 days aged IR broilers, depicted in Table 3. All levels of BT addition were significantly greater ($P < 0.01$) than those of the control group. A linear trend was observed in the

Table 2: Antibacterial activity of *Bacillus toyonensis* M44 against pathogenic bacteria presented as IZDs (mm)

Bacterial strain	Bacterial supernatant concentration (%)				Cephalexin 800 µg/mL	MIC
	10	20	40	80		
SP	17±0.0d	23±0.3c	27±0.3b	33±0.0a	32±0.2a	5
LM	16±0.2d	19±0.2c	24±0.2b	28±0.2a	27±0.1a	5
SA	22±0.8d	26±0.7c	29±0.9b	34±0.3a	34±0.3a	2.5
EC	14±0.5d	17±0.2c	21±0.1b	25±0.2a	24±0.2a	5
ST	12±0.1d	15±0.3c	19±0.7b	22±0.5a	21±0.6a	10
KP	13±0.0d	16±0.2c	19±0.2b	23±0.7a	22±0.6a	10

Values are presented as mean \pm SD, n=3, *Streptococcus pyogenes* (SP), *Listeria monocytogenes* (LM), *Staphylococcus aureus* (SA), *Escherichia coli* (EC), *Salmonella Typhimurium* (ST), and *Klebsiella pneumonia* (KP). Positive control is Cephalexin 800 µg/mL.

results for all concentrations and levels of BT. Specifically, a 1.6 mg/kg diet produced the most favorable outcomes for the investigated growth parameters. These included increases in GR (214.787), PI (148.8), BWG (2.365 kg), and LBW (2.410 kg), as well as no sense FCR (1.60) compared to control values of 204.15, 136, 2.25 kg, 2.3 kg, and 1.65 for the same prior parameters, respectively. It was discovered that broilers fed BT-containing diets outperformed those fed the basal diet in every performance indicator ($P < 0.001$).

This increase in the performance of broilers could be attributed to BT, which could be brought about by improving the digestibility of fiber and crude protein.

Blood parameters

Liver and kidney functions: The effects of varying the amount of BT (0.4, 0.8, and 1.6 mg/kg diet) on the liver and kidney functions of serum are shown in Table 4. None of the treatments looked to significantly impact creatinine levels and renal function. While BT supplementation had a positive effect on uric acid, which was 5.31 mg/dl in the control group and decreased by 20% in the BT 0.8 treated group, the other groups, BT 0.4 and BT 1.6, recorded 4.3 and 4.1 mg/dl, respectively, no sense between control and BT 0.4 group in creatinine levels; they recorded 0.34 mg/dL. The findings demonstrated a statistically significant enhancement in alanine transaminase and AST via every level of BT, comparing the non-supplemented. The most important values for liver function were observed with BT 1.6 mg/kg. There was no visible liver or kidney function variation between males and females regarding sex impact. The current data regarding liver enzymes (in serum) showed a $P < 0.05$ decrease in ALT and AST comparing the non-treated. The health of the broilers improved when they were administered BT 1.6 level. This suggests that reversal does not influence the functionality of the liver.

Lipid profile: The impact of feeding broiler chicks varying amounts of BT on total cholesterol, its components, and the proportion of abdominal fat is shown in Table 4. The most significant values in the control group were for total cholesterol and LDL, which subsequently decreased as the number of effective microbes increased. The HDL value of the control group was significantly lower than that of the BT 0.4 group, which had the maximum HDL value of 95 mg/dl via no sense of the HDL values of the other two groups (0.8 and 1.6 mg/kg). Instead of achieving control, all test groups attained significantly lower abdominal fat percentages.

Immune response and thyroid hormones: The effects of dietary BT levels on immune globulin (g/100g BW) and

blood thyroid hormones are shown in Table 4. The data clearly show that the various treatments do not affect the T3 hormone; in terms of the T4 hormone, the BT 1.6 group had the lowest value, followed by the control group, the BT 0.4 group, and the BT 0.3 group without any discernible differences. The control and BT 1.6 mg/kg results were 132, 133, 131, and 132 ng/dl, respectively. The control treatment demonstrated the least pronounced immune response to IgA and IgG compared to the alternative dietary supplements. In contrast, the BT groups demonstrated a notably higher immune response, with BT 1.6 mg/kg in IgG and IgA registering the highest levels of immune response (1087 and 199 mg/dl, respectively).

Digestive enzymes: The enzymatic concentrations exhibited a progressive increase, commencing with the non-treated group, which contained the lowest values, and reaching their maximum at BT 1.6 (510, 27, and 40 for lipase, trypsin, and amylase, respectively). Fig. 1 displays how increasing doses of BT affected the blood serum's digestive enzymes (lipase, trypsin, and amylase). Meanwhile, 0.8 mg/kg of treatment was recorded as amylase 490, lipase 25.0, and trypsin 35 U/L.

Intestinal microbial count: The total bacteria, yeast and molds, and *E. coli* are highest in the control group (7.0, 3.7, and 5.9 Log₁₀ CFU/g, respectively), as depicted in Fig. 2. Adding BT to the diet reduces the number of these microorganisms, with the lowest significant values occurring at the highest BT level (5.2, 2.7, and 3.7 Log₁₀ CFU/g, respectively). In contrast, the number of lactic acid bacteria is lowest in the control group (3.0) and increases with the addition of BT to the diet, reaching a peak at the highest BT level (4.9 Log₁₀ CFU/g).

Histopathological studies: The microscopic images of liver sections showed the usual hepatic central vein and hepatic cords in the control group as summarized in Fig. 3 depicting different sections of the liver and intestines of broilers treated with different BT levels. Also, it showed normal histological anatomy of the hepatic lobule in the BT0.4 mg/kg diet group. The broiler chickens group treated with BT 0.8 mg/kg body weight group had a standard histological structure; meanwhile, those treated with BT 1.6 mg/kg showed minor collections of immune cells (lymphocytes) around the bile ducts.

Regarding the intestines of broiler chickens, the sections of the control group showed normal intestinal villi, normal intestinal mucosa, sun mucosa, and diffuse intestinal villi within the control group. Also, the second group showed that intestinal villi seemed normal, with normal mucosa and diffuse sun mucosa within the BT 0.4 mg/kg diet. The third group (BT 0.8 mg/kg diet) showed

Table 3: Effect of dietary treatments of *Bacillus toyonensis* M44 at three levels on broilers growth performance

Treatments (mg/kg)	LBW (g)		BWG (g)	FI (g)	FCR	GR	PI
	1d	35d	1-35d	1-35d	1-35d	1-35d	1-35d
Control	44.9	2300 ^b	2255 ^b	3711 ^c	1.65	204.15 ^b	136.6 ^c
BT0.4	44.8	2320 ^{ab}	2275 ^{ab}	3730 ^b	1.64	206.70 ^b	139.5 ^b
BT0.8	44.8	2390 ^a	2345 ^{ab}	3750 ^b	1.60	212.14 ^{ab}	147.2 ^{ab}
BT1.6	44.9	2410 ^a	2365 ^a	3790 ^a	1.60	214.78 ^a	148.8 ^a
p-value	0.8	0.003	0.01	0.0013	0.93	0.012	0.001

^{a-c} Means within the same column with different superscripts differ significantly ($P \leq 0.05$). SEM¹: Pooled standard error, LBW: Live body weight BWG: body weight gain, FCR: feed conversion ratio, PI: performance index, GR: growth rate, BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

Table 4: Effect of *Bacillus toyonensis* M44 dietary treatments on broilers' serum biochemical parameters

Blood biochemistry	Treatments (mg/kg)				P value
	Control	BT0.4	BT0.8	BT1.6	
Kidney functions (mg/dl)					
Uric acid	5.33a	4.6b	4.38b	4.1c	0.001
Creatinine	0.34	0.33	0.35	0.28	0.97
Liver functions (U/L)					
AST	242a	220b	210c	185d	<0.0001
ALT	6a	5.2b	4.8c	3.5d	<0.0001
Lipid profile					
Total cholesterol (mg/dl)	133.3a	126.1b	120.7c	103d	<0.0001
HDL (mg/dl)	90.8c	92.5b	94.5a	95a	0.0012
LDL (mg/dl)	45.2a	35.2b	29.2c	21d	0.001
Abdominal fat	1.22a	0.96b	0.85c	0.75d	0.002
Thyroid hormones					
T3 (ng/dl)	2.31	2.33	2.35	2.33	0.6
T4 (ng/dl)	132.2	133.3	131.2	132.6	0.7
Immune response					
IgG (mg/dl)	955.3d	1044c	1062b	1087a	0.021
IgA (mg/dl)	178.5d	186.3c	190.1b	199.2a	0.0015

$n=3$ a-d Means within the same row with different superscripts differ significantly ($P \leq 0.05$). ALT Alanine aminotransferase; AST Aspartate aminotransferase, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, T₃: Triiodothyronine T₄: Thyroxine IgG, IgA Immunoglobulins Isotypes G, and A. BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

Table 5: Effect of *Bacillus toyonensis* M44 dietary treatments on meat quality of broiler parameters

Parameters	Treatments (Cm3/kg)				P value
Chemical composition	Control	BT0.4	BT0.8	BT1.6	
Moisture	61.2c	63.5b	66.5ab	67.8a	0.001
Protein	20.22b	20.1b	20.9b	21.8a	0.041
Fat	13.6a	10.5b	10.3b	7.8c	0.001
Ash	0.88a	0.71b	0.41c	0.35c	0.001
pH	5.7b	5.8b	6.1a	6.5a	0.021
TVBN	6.7a	5.2b	4.8c	4.5c	0.0023
TBA	0.75a	0.49b	0.44c	0.31d	0.003
Sensory properties					
Juiciness	9a	8.7b	8.4b	9a	0.025
Tenderness	8.8a	8.5b	8.6b	8.8a	0.031
Aroma	8.4a	8.2b	8b	8.3a	0.032
Taste	8.7a	8.4b	8.2b	8.8a	0.012

$n=3$ a-d Means within the same row with different superscripts differ significantly ($P \leq 0.05$). Total Volatile Basic Nitrogen (TVBN), Thiobarbituric acid (TBA). BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

intact intestinal villi, healthy intestinal mucosa, and uniform distribution of intestinal villi in the BT 0.8 mg/kg group, while normal intestinal villi, normal intestinal mucosa, sun mucosa, and diffuse intestinal villi within the control group, the group treated with a BT 1.6 mg/kg diet. The results of histopathology support the blood biochemistry results.

Meat quality: The chemical composition of chicken breast fluctuated with adding BT levels to the broiler diet is summarized in Table 5; the BT 0.8 group recorded the best value among the other groups. The moisture content increased by 20% and protein content by 10% compared to the control; the enhancement in moisture and protein in the breast significantly affected the sensory parameters (juiciness and tenderness), which they scored the highest values (9 and 8.8) in BT 0.8 group by panelists. The enhanced test was found in the control and BT 1.6 groups.

The fat and ash content decreased by adding BT levels. As for the quality parameters of meat, pH increased to 6.5, and the nitrogen compounds and TBA decreased to 4.5 and 0.31, respectively. The color parameters were significantly affected by BT addition, where the lightness of meat maintained as control and slightly decreased with BT 1.6 mg/kg addition, while *a* (redness) and *b* (yellowness) weren't affected (Fig. 4).

Economic efficiency: The data presented in Table 6 illustrates the economic efficacy of different levels of BT in the diet of broiler chickens. The findings indicate that every level of BT exhibited greater economic efficiency than the non-supplemented group. The BT 1.6 mg/kg group demonstrated the highest relative and economic efficiency values. This could be attributed to the fact that the controls achieved lower-end body weights than the experimental group.

Table 6: Effect of dietary treatments *Bacillus toyonensis* M44 on broiler diets economic efficiency

Item	Control	BT0.4	BT0.8	BT1.6
Av. Feed intake, Kg feed/ Kg meat ^a	1.65	1.64	1.60	1.60
Price Kg feed (LE) ^{*b}	25.2	25.4	25.3	25.1
Total feed cost C= (a×b)	41.58	41.65	40.48	40.16
Price/one Kg gain ^{**d}	65.00	65.00	65.00	65.00
Net revenue (L.E.) = d-c = e	23.42	23.35	24.52	24.84
Economic efficiency ^{***} (e/c)	0.56	0.56	0.61	0.62
Relative efficiency ^{****}	100.00	100.00	107.00	111.0
Viability rate (%)	100.00	100.00	100.00	100.00

*Price of Kg feed according to local market December 2022 ** Price of Kg live body weight according to the local market December 2022. ***net revenue per unit cost. ****compared to the economic efficiency of the control group BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

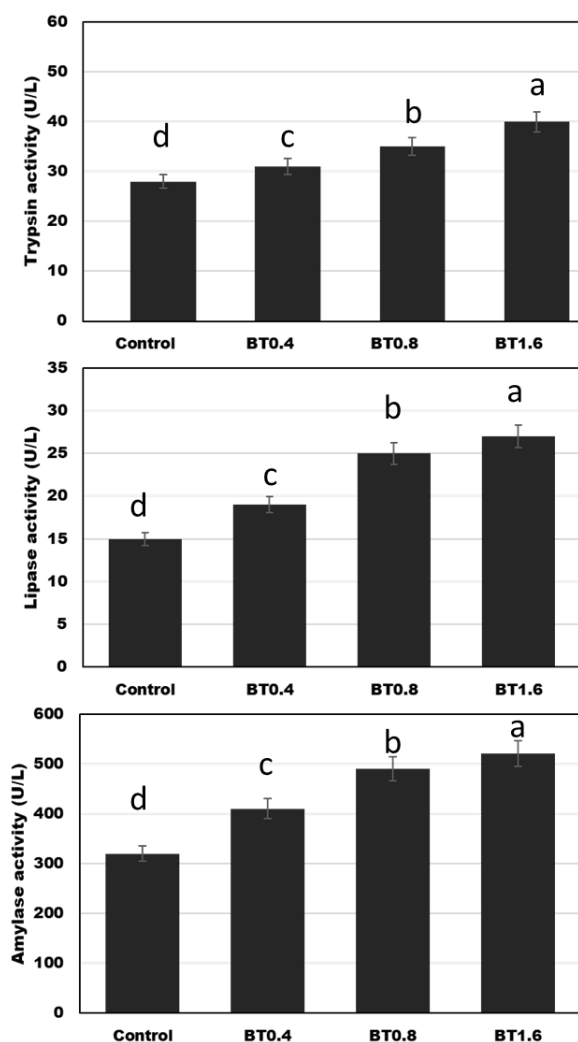


Fig. 1: Effect of dietary treatments *Bacillus toyonensis* M44 on serum digestive enzymes of broilers. ^{a- b}: Means within the same column with different superscripts differ significantly ($P \leq 0.05$). BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

DISCUSSION

The development of antibiotic resistance occurs when microbes resist the common drugs that are used for disease treatments. However, there is excessive use of antibiotics and chemical drugs in human and animal treatments (Osei Sekyere and Mensah, 2020). The biggest issue in treating infectious diseases is antibiotic resistance and the propagation of related genes among aggressive pathogen populations (Alenazy, 2022). During the early stages of antibiotic use, gene changes were the leading cause of resistance and were the specific focus of antibiotics. The

horizontal transposition of genes, or the ability of microbes to transfer genetic material, plays a crucial role in the development and spread of antibiotic resistance in harmful bacteria (Alenazy, 2022).

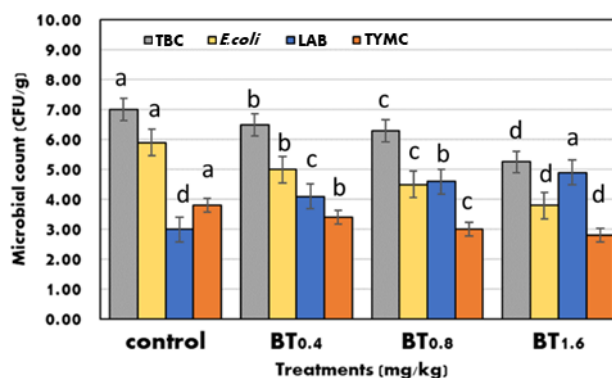


Fig. 2: Effect of dietary treatments *Bacillus toyonensis* M44 on the intestinal microbial count of broilers. TBC, total bacterial count, TYMC, total yeast and molds count, *E. coli*: *Escherichia coli* CFU/g: logarithm of colony forming unit per gram of digesta LAB: *Lactobacillus* spp. BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg.

Veterinarians and other veterinary professionals play crucial roles in regulating and supervising the use of antimicrobials in animals. They also provide expertise to animal owners and producers on the responsible application of antimicrobials. Additionally, they collaborate with the human healthcare sector to exchange data and develop strategies to address antimicrobial resistance (Xavier *et al.*, 2019). Comprehending the resistance mechanisms to create innovative countermeasures for this threat is critical. In this study, we cultured the *Bacillus* isolates from the gastrointestinal tracts of poultry reared in various habitats, identified them using biochemical tests, and analyzed them using MALDI-TOF. *Bacillus toyonensis* M44 was introduced to the broiler feed in place of antibiotics.

The addition of BT to the diet significantly improved the growth performance parameters. This is consistent with studies that used microorganism's inoculum feed additives on broilers, turkeys, quail, and partridges (Hatab *et al.*, 2016; Czech *et al.*, 2020; Ye *et al.*, 2021). According to recent studies, probiotic supplementation from 0 to 35 days of age significantly increased live-body weight (LBW), body gain, and diet intake in female chickens. Conversely, it significantly enhanced LBW and FCR in male chickens (Hatab *et al.*, 2016; Ye *et al.*, 2021; Zhang *et al.*, 2021).

Although liver cells are vital for the body's detoxification process, the toxins in food might harm them. Liver health can be evaluated by measuring blood levels of

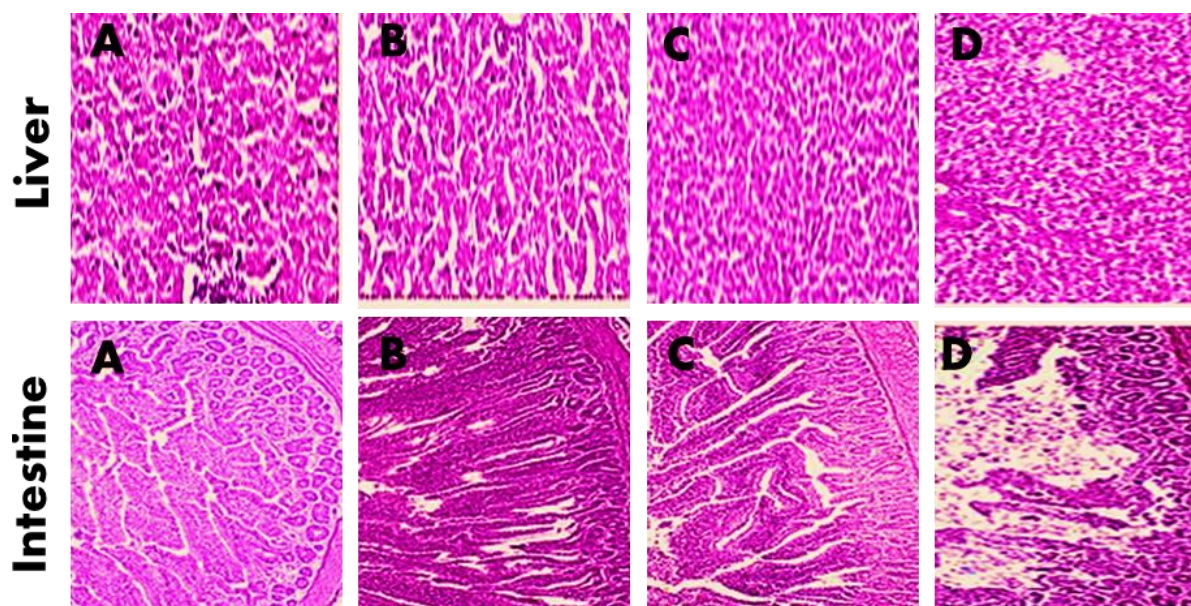


Fig. 3: Illustrations of liver sections stained with H&E (X400). **A)** the usual hepatic central vein and hepatic cords in the control group. **B)** the BT 0.4 mg/kg diet group had normal histological anatomy of the hepatic lobule. **C)** the BT 0.8 mg/kg b.w group had a standard histological structure. **D)** there are minor collections of immune cells (lymphocytes) around the bile ducts in the group treated with 1.6 mg/kilogram of body weight of broiler chickens. Photos of intestinal sections stained with hematoxylin and eosin (H&E) at a magnification of 40 times (X400) depict the following: **A)** normal intestinal villi, normal intestinal mucosa, sun mucosa, and diffuse intestinal villi within the control group. **B)** intestinal villi seem normal, with normal mucosa and diffuse sun mucosa within the BT 0.4 mg/kg diet. **C)** intact intestinal villi, healthy intestinal mucosa, and uniform distribution of intestinal villi in the BT 0.8 mg/kg group. **D)** normal intestinal villi, normal intestinal mucosa, sun mucosa, and diffuse intestinal villi within the control group, the group treated with a BT 1.6 mg/kg diet.

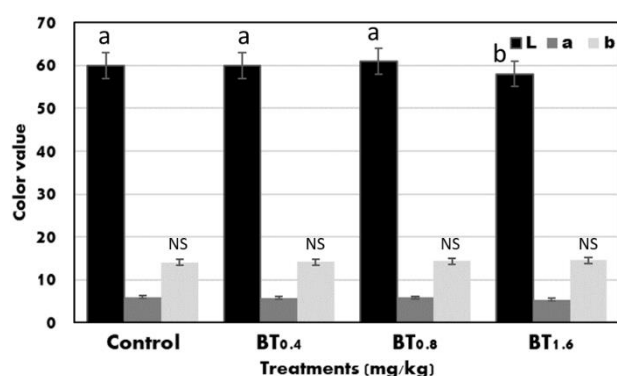


Fig. 4: Effect of *Bacillus toyonensis* M44 dietary treatments on the color parameter of broiler meat. BT0.4= basal diet + 0.4 mg BT/kg, BT0.8 = basal diet + 0.8 mg BT/kg, BT1.6 = basal diet + 1.6 mg BT/kg; lightness (L), redness (a), and yellowness (b); NS, non-significant.

AST and ALT; ALT is a more specific marker of liver cell damage than AST (Wang *et al.*, 2020). The current study stated that the addition of BT to the feed maintained the activity of liver enzymes in the broilers, agreeing with the findings of Rashidi *et al.* (2020) and Sokół *et al.* (2017) that probiotics did not influence the activity of ALT and AST in the blood. Elevated levels of liver markers in the serum indicate liver damage and the leaking of enzymes into the circulation. Furthermore, the lipid profile and the kidney and liver indicators exhibited significant reductions in all experimental groups compared to the control group. The study found no statistically significant impact of adding dietary microbes on creatinine levels.

Consistent with Musazadeh *et al.* (2022), incorporating BT into the diet, maintains organ homeostasis (liver and kidney). The probiotic addition did not affect the liver's weight; additionally, Tarradas *et al.* (2020) showed that supplementing with a single probiotic

or a combination can enhance the weight of the thymus, bursa of Fabricius, and spleen in broilers.

Probiotics can influence total cholesterol and serum triglycerides when added to broiler feed. The obtained results are consistent with those of Wajizah (2021), who noted that probiotic-containing feed may have some positive effects by lowering the levels of cholesterol and triglycerides in chicken serum. Probiotic addition to a bird's diet can lower serum total cholesterol levels (Shah *et al.*, 2021). This may be due to several mechanisms, including increased excretion of bile acids, where probiotics can degrade cholesterol into bile acids, which are then excreted in the feces, thus reducing the amount of cholesterol in the bloodstream. It also inhibited cholesterol synthesis by inhibiting the activity of an enzyme called hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is involved in the synthesis of cholesterol. Additionally, probiotics can inhibit the intestinal recycling of bile salts. This means more cholesterol is used to produce bile acids, lowering serum cholesterol levels.

There is an increase in the serum triiodothyronine level of chickens fed probiotics, and adding the dietary BT to the broiler chickens considerably boosted their immunity. Hatab *et al.* (2016) suggested a potential cause-and-effect link between T3 and T4 hormones and the impact of BT on chicken development. BT can stimulate nonspecific immunity, increase immunoglobulin secretion and proliferation of immune cells, and activate macrophage phagocytic activity (Tarradas *et al.*, 2020).

Supplementing the broiler diet with probiotics considerably boosted the immune system, i.e., IgM, IgG, and interferon γ (Fazelnia *et al.*, 2021); birds fed probiotics had higher levels of serum antibody synthesis. Consistent with the current study, Zhang *et al.* (2021) found that probiotics and probiotic-fed broiler diets significantly

increased the Immunoglobulin G and Immunoglobulin A concentrations in both male and female broilers. Lymphatic organs of poultry include the sac of Fabricius, the spleen, and the thymus; the weight of these organs is significantly correlated with the intensity of their internal immune activity (Sławińska *et al.*, 2014).

Regarding gut microbiota, adding probiotics to chicks' diet significantly reduced the count of *Salmonella* and *E. coli* in the gut and, consequently, increased the activity of digestive enzymes (protease and amylase) (Ye *et al.* 2021; Zhang *et al.*, 2021). Adding BT to the broiler diet may improve their growth performance by increasing the number of beneficial bacteria (*Lactobacillus* spp.) and reducing the number of harmful bacteria (*E. coli*) in the gut. A similar finding was reported by Liu *et al.* (2018) who demonstrated how adding microorganisms to broiler feeds improved the gut microbiome by increasing intestinal pH, intestinal bacterial composition, and digestive enzyme.

Salmonella enterica is one of the harmful microorganisms that are observed in chicken husbandry (Vieco-Saiz *et al.*, 2019). BT supplementation reduced *E. coli* number and increased *Lactobacillus* levels in this investigation. The same findings were observed in the gut of probiotic-supplemented chicks, where beneficial microbes are dominant (Zhang *et al.*, (2021). In general, adding BT to feed may increase the internal environment of the gut's competitiveness for good bacteria. Our findings suggest that supplementing broiler chicken diets with probiotics can improve economic efficiency, which aligns with Zaghari *et al.* (2020).

Conclusions: Adding probiotics to the feed improved the economic efficiency of broiler chicks. This supplementation also boosted the look of the carcass, specific blood metabolites, and enzymes. Furthermore, enhanced gastrointestinal health resulted in increased beneficial *Lactobacillus* species and decreased *Escherichia coli* in the enriched grill chicks, positively affecting the meat quality.

Competing interests: The authors declare that they have no competing interests.

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