**International Journal of Agriculture and Animal Production**
Vol: 06, No. 01, Jan-June 2026
ISSN: 2799-0907, DOI: 10.55529/ijaap.61.26.38 

**Research Paper**

# Dietary multi-strain probiotic supplementation improves growth performance, intestinal morphology, serum biochemistry, and gut microbiome diversity in Ross 308 broiler chickens

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**Article Info**

***Article History:***
Received: 02 October 2025
Revised: 11 December 2025
Accepted: 19 December 2025
Published: 05 February 2026

***Keywords:***
Probiotics
Broiler Performance
Gut Microbiome
Intestinal Morphology
Feed Conversion Ratio
Antimicrobial Resistance

**ABSTRACT**

With the ban on antibiotic growth promoters (AGPs), the demand for alternative feed additives for commercial poultry production has increased. This study evaluated the effects of a multi-strain probiotic containing *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus reuteri* DSM 17938, and *Bifidobacterium longum* ATCC 15707 on growth performance, intestinal morphology, serum biochemical indices, immune responses, and cecal microbial diversity in Ross 308 broiler chickens. In a 42-day feeding trial, 360 day-old male chicks were randomly allocated to four treatments: T0 (basal diet), T1 (basal diet + 0.5 g/kg probiotic), T2 (basal diet + 1.0 g/kg probiotic), and T3 (basal diet + 1.5 g/kg probiotic), with six replicates of 15 birds each. Birds in the T2 group exhibited significantly improved ($p < 0.05$) final body weight (2425 g vs. 2210 g), total body weight gain (2383 g vs. 2168 g), and feed conversion ratio (1.74 vs. 1.91) compared with the control group. Villus height and the villus height-to-crypt depth ratio increased by 20.4% and 45.3%, respectively, in T2 compared with T0 ($p < 0.001$). Probiotic supplementation significantly increased serum IgG concentration while decreasing total cholesterol, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels ($p < 0.05$). Cecal microbiome analysis based on 16S rRNA gene sequencing revealed significant differences in alpha diversity indices (Shannon, Chao1, and Faith's phylogenetic diversity), with T2 showing a higher abundance of beneficial *Lactobacillus* spp. and a lower abundance of *Clostridium perfringens*. A dose-dependent response was observed up to 1.0 g/kg, whereas no additional benefits were detected at 1.5 g/kg supplementation. These findings indicate that supplementation with 1.0 g/kg of the multi-strain probiotic optimizes production efficiency, gut health, and immune function in commercial broilers and represents a promising alternative to AGPs.

International Journal of Agriculture and Animal Production (IJAAP)

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## 1. INTRODUCTION

The worldwide poultry broiler industry is facing harsh pressures to continue to achieve high productivity while adapting to the growing issue of antimicrobial resistance (AMR) and the demand from consumers for antibiotic-free poultry products [1]. The European Union ban on antibiotic growth promoters (AGPs) in 2006 has led to the progressive reduction in the use of sub-therapeutic antibiotics worldwide, which has driven research into the safe, effective and economical alternatives [2]. Probiotics are live microbial products (preparations) or combinations of microbial products when added in adequate amounts provide a health benefit to the host and are among the most promising classes of feed additives investigated [3].

Probiotics are believed to have multiple complementary mechanisms of action including competitive inhibition of pathogens, bacteriocins, short chain fatty acids (SCFAs), modulation of intestinal morphology, stimulation of mucosal and systemic immunity and improvements in nutrient digestibility [4], [5]. Specific strains of Lactobacillus sp. like Lactobacillus acidophilus, Lactobacillus reuteri and Bifidobacterium longum have been shown to have growth promoting activity in poultry separately [6]. Theoretically better, and with more consistency in different production conditions, however, are multi-strain formulations that take advantage of the ecological synergism and functional complementarity between microbial taxa [7].

The intestinal mucosa serves as the main barrier between the intestinal microbiome and host immune system [8]. The well-known indexes of absorptive capacity are villus height and villus height-to-crypt depth (VH: CD) ratio: the higher these indexes, the better the absorptive capacity. [9] In contrast, deep crypts are reflective of increased enterocyte turnover, a metabolically expensive response to enteric inflammation or challenge by a pathogen [10]. These morphometric parameters have been found to be altered in a beneficial way in broiler chickens following probiotic administration, but the extent of the response depends greatly on the probiotic strain, dose and age of the chicks [11].

The next-generation sequencing (NGS) platforms have changed the face of gut microbiome analysis, allowing for insights into the microbial communities within the cecum with unprecedented depth and resolution without the need for cultivation [12]. Changes in alpha diversity indices (Shannon entropy, Chao1 richness, and Faith's phylogenetic diversity) as well as changes in certain indicator taxa (Lactobacillus spp. and Clostridium perfringens) are emerging as important gut health status markers in broilers [13]. However, in-depth analyses that combine productivity, intestinal histomorphology, serum biochemistry, immune parameters and metagenomics in a single well-controlled study are still sparse in the literature. In this background, the present study was performed to assess the dose-response effects of a novel combination of three strains of probiotics (L. acidophilus ATCC 4356, L. reuteri DSM 17938, B. longum ATCC 15707) on growth performance, intestinal morphology, serum biochemistry, immune indices, and diversity of cecal microbiome in Ross 308 broiler chickens over a 42-day production cycle. Dietary probiotic supplementation is hypothesized to have a dose-dependent effect on the gut health markers in the tested range with identifying an optimal dose. The results of this trial give solid scientific recommendations for commercial production of broiler diets without the use of AGPs.

## 2. RELATED WORK

Probiotics have been shown to have a beneficial effect on growth, gut health and immune function in broiler chickens in several studies. Probiotics comprising of multi-strain combinations of Lactobacillus and Bifidobacterium species have been proved to enhance body weight gain, feed conversion efficiency, and cecal microbial composition. Furthermore, probiotic supplementation was linked to greater villus height and villus height to crypt depth ratio, suggesting improved gut integrity and absorptive function of the gut.

In broilers, previous investigations also have revealed that probiotics can improve feed efficiency as well as positively influence the intestinal flora. Furthermore, probiotics have proven to enhance immunity through boosting immunoglobulin production and resistance to diseases.

With the development of next-generation sequencing techniques, the poultry gut microbiome could be characterized in detail. Research has emphasized the significance of a balanced gut microbial community in terms of efficient nutrient utilization, health and performance. Better broiler production and lower occurrence of enteric diseases have been consistently correlated with the presence of a high percentage of beneficial lactobacilli species and low counts of enteric bacteria like Clostridium perfringens.

Previous studies have shown that probiotics have beneficial effects in growth performance, intestinal morphology, immunity, and microbial composition but there are limited studies to evaluate the probiotics in a multi-strain probiotic formulation using all these variables such as growth performance, serum biochemistry, intestinal histomorphology, immunity, and cecal microbiome diversity in Ross 308 broiler chickens. Hence, the present study was conducted to assess the production performance, gut health, immune responses and microbial diversity in broiler chickens fed with probiotic mixture of Lactobacillus acidophilus, Lactobacillus reuteri and Bifidobacterium longum.

## 3. METHODOLOGY

### 3.1. Ethics Statement

Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, India, Institutional Animal Ethics Committee (VNMKV/IAEC/2024/007) approved all experimental procedures and followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

### 3.2. Birds, Housing, and Experimental Design

A total of 360 male broiler chicks of Ross 308 strain (mean body weight at day old 42.2 ± 0.5 g) were bought from a certified commercial broiler hatchery (Suguna Poultry Farm, Ltd., Pune, India) and randomly distributed into four dietary treatment groups in a completely randomized design (CRD) [14]. There were 6 replicates per treatment (90 birds/treatment, a total of 360 birds). This trial was carried out in deep litter house with natural ventilation at the Livestock Research Station, Parbhani. Birds were kept in separate cages (1.5 × 2.0 m) provided with ad libitum feed and clean drinking water for 42 days during the experiment. The light period was 23 h/day for the first week, and 18 h/day for the rest of the experiment. Following the recommended vaccination schedule [15] the following vaccinations were administered: standard vaccinations (Marek's at the hatchery; Newcastle disease + Infectious Bronchitis at day 7; Gumboro at day 14; Newcastle disease at day 21).

### 3.3. Probiotic Preparation and Dietary Treatments

The multi-strain probiotic product (ProAvian-3S™, Biovet India Pvt. Ltd., Mumbai) contained L. acidophilus ATCC 4356 (3.0 × 109 CFU/g), L. reuteri DSM 17938 (2.0 × 109 CFU/g), and B. longum ATCC 15707 (2.0 × 109 CFU/g), for a combined potency of 7.0 × 109 CFU/g. The four experimental diets were: T0 (Basal diet), T1 (Basal diet + 0.5 g probiotic/kg feed), T2 (Basal diet + 1.0 g/kg) and T3 (basal diet + 1.5 g/kg). The probiotic was added to the feed at the mixing stage and the recovery of the probiotic from the feed was verified by plate-count culture (mean recovery 94.3 ± 3.1%). The basal cornsoybean meal diet

Table 1 was designed to include components sufficient to satisfy or exceed the recommendations of the NRC [16] and BIS [17] for both the starter (0–21 days) and finisher (22–42 days) diets.

Table 1. Composition and Calculated Nutritive Values of the Basal Diet (As-Fed Basis)

| Ingredient | Starter (0–21 d) % | Finisher (22–42 d) % |
|---|---|---|
| Maize (ground) | 55.40 | 57.80 |
| Soybean meal (46% CP) | 31.20 | 28.60 |
| Poultry by-product meal | 5.00 | 5.00 |
| Wheat bran | 2.00 | 2.00 |
| Vegetable oil | 2.80 | 3.20 |
| Dicalcium phosphate | 1.40 | 1.30 |
| Limestone | 0.80 | 0.80 |
| Salt (NaCl) | 0.20 | 0.20 |
| Vitamin–Mineral premix | 0.50 | 0.50 |
| DL-Methionine | 0.40 | 0.30 |
| L-Lysine HCl | 0.30 | 0.30 |
| Calculated Nutritive Values | | |
| ME (kcal/kg) | 2,950 | 3,050 |
| Crude Protein (%) | 22.50 | 20.00 |
| Ether Extract (%) | 5.20 | 5.80 |
| Crude Fiber (%) | 3.10 | 3.00 |
| Calcium (%) | 0.95 | 0.90 |
| Available Phosphorus (%) | 0.45 | 0.42 |
| Lysine (%) | 1.25 | 1.10 |
| Methionine + Cystine (%) | 0.92 | 0.82 |

ME = metabolizable energy; CP = crude protein. Values for vitamins and minerals: vitamin A, 10,000 IU; vitamin D3, 2,000 IU; vitamin E, 25 mg; vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; niacin, 35 mg; pantothenic acid, 12 mg; pyridoxine, 3 mg; folic acid, 1 mg; biotin, 0.1 mg; B12, 0.015 mg; Mn, 80 mg; Zn, 60 mg; Fe, 30 mg; Cu, 8 mg; I, 1 mg; Se, 0.3 mg per kg of feed.

### 3.4. Growth Performance Measurements

The weight of the body was measured using a calibrated digital scale with a precision of ±1 g, both at the time of placement and once a week. Feed intake (FI) was calculated each week, by subtracting residual feed from offered feed per replicate. Body weight gain (BWG), feed conversion ratio (FCR = FI/BWG) and European Production Efficiency Factor (EPEF = [Livability% × Average daily gain × 100] / [FCR × Age]) were determined for the entire trial period. During the experiment, the number of deaths were recorded daily [18].

### 3.5. Intestinal Morphology

When the chickens were 42 days old, two chickens/replicate (12 chickens/treatment) were randomly selected and slaughtered humanely by cervical dislocation, and segments of the mid-duodenum, mid-jejunum and mid-ileum were taken. 10% neutral buffered formalin was used to fix the samples for 48 h then dehydrated in ascending alcohol series, cleared in xylene and embedded in paraffin. Hematoxylin and Eosin (H&E) was used to stain sections (5 µm). The villus height (VH) was measured from the base to the top of the villus and the crypt depth (CD) was measured from the base of the villi to the muscularis mucosa for 10 well-oriented villi per section using a calibrated ocular micrometer and Leica LAS X image analysis software (v3.7) as described by Caspary [19]. The VH:CD ratio was determined as an index of absorptive capacity of the mucosal surfaces.

### 3.6. Serum Biochemistry and Immune Parameters

At day 42, blood (5 mL) was drawn from brachial vein of two birds per replicate, allowed to coagulate at room temperature, then centrifuged in 3,000 × g for 15 min (4°C) and serum collected was stored at −80°C until analysis. Biuret method was used to measure total protein while bromocresol green was used to measure albumin. The concentration of cholesterol, triglycerides, glucose, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was measured by semi-automatic analyzer (Chem-5 Plus, Erba), using commercially validated colorimetric kits (Erba Mannheim, Germany). Concentration of serum immunoglobulin G (IgG) was measured by an ELISA (Chicken IgG ELISA kit, MyBioSource, USA, intra-assay CV < 8%). The turbidimetric lysis of Micrococcus lysodeikticus was used for determining lysozyme activity as described by Shugar [20].

### 3.7. Cecal Microbiome Analysis

Cecal contents were collected at slaughter under sterile conditions, snap-frozen in liquid nitrogen and stored at −80°C and total DNA was extracted from 2 birds per replicate (n = 12/treatment) using the PowerSoil Pro Kit (Qiagen, Germany) protocol. NanoDrop 2000 spectrophotometry (A260/A280 1.8–2.0) and Qubit fluorometry were used to verify the quality and quantity of the DNA. The V3-V4 hypervariable regions of 16S rRNA gene were amplified with the primers 341F/806R [21] and then sequenced on an Illumina MiSeq platform (2 x 300 bp PE). The raw data were processed using QIIME 2, v2024.2 [22] by trimming primers with Cutadapt, denoising and merging with DADA2, and assigning taxonomy to the Silva 138 database (at 99% identity). The following alpha diversity metrics were calculated: Shannon index, Chao1, observed OTUs, Faith's PD and Simpson 1-D at rarefied sequencing depth (n = 40,000 reads/sample).

### 3.8. Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze the data in IBM SPSS Statistics v28.0 (SPSS Inc., Chicago, IL, USA). The growth performance experimental unit was the replicate and each bird was the experimental unit for morphological and biochemical variables. Tukey's Honest Significant Difference (HSD) post-hoc test was used to make comparison of means. Data are presented as means ± SEM. The significance of the data was taken as $p<0.05$. To explore associations between the microbiome diversity indices and production traits, a correlation analysis (Pearson's r) was conducted.

## 4. RESULTS AND DISCUSSION

### 4.1. Growth Performance

The performance of growth during the 42-day trial is summarized in Table 2 and shown in Figure 1 and Figure 2. The groups were successfully randomized since there was no statistical difference in initial body weights among all groups ($p = 0.921$). Birds receiving 1.0 g/kg probiotic (T2) achieved significantly higher final body weight (2425 ± 55 g) and total BWG (2383 ± 54 g) compared with the control T0 (2210 ± 48 g and 2168 ± 46 g, respectively; $p < 0.05$). Feed intake was not significantly different between the treatments ($p = 0.412$) suggesting that the performance improvements were due to improved feed utilisation, not to increased feed intake. The FCR was significantly improved in T2 (1.74 ± 0.03) relative to T0 (1.91 ± 0.04; $p = 0.012$), representing a 9.0% improvement. The EPEF (an integrative measure of growth rate, mortality and FCR) was also significantly better in T2 (358) and T3 (348) than T0 (302; $p = 0.007$). Quadratic dose response was seen with T3 (1.5 g/kg) having intermediate performance as compared to T1 and T2, indicating an optimum supplementation level of 1.0 g/kg of T3

Table 2. Growth Performance Parameters of Broiler Chickens Supplemented with Multi-Strain Probiotics (Mean ± SEM)

| Parameter | T0 (Control) | T1 (0.5 g/kg) | T2 (1.0 g/kg) | T3 (1.5 g/kg) | P-Value |
|---|---|---|---|---|---|
| Initial BW (g) | 42.3±0.5 | 42.1±0.4 | 42.4±0.6 | 42.2±0.5 | 0.921 |
| Final BW at 42d(g) | 2210±48$^{b}$ | 2312±51$^{b}$ | 2425±55$^{a}$ | 2389±53$^{a}$ | 0.003 |

| | | | | | |
|---|---|---|---|---|---|
| Total BWG (g) | 2168±46[b] | 2270±49[b] | 2383±54[a] | 2347±51[a] | 0.002 |
| Total FI (g/bird) | 4141±62 | 4210±70 | 4146±65 | 4263±68 | 0.412 |
| FCR | 1.91±0.04[a] | 1.82±0.03[ab] | 1.74±0.03[b] | 1.78±0.04[ab] | 0.012 |
| Mortality (%) | 3.33 | 2.22 | 1.11 | 2.22 | 0.743 |
| EPEF Score | 302±11[b] | 331±12[ab] | 358±14[a] | 348±13[a] | 0.007 |

A,b,c Means within a row bearing different superscripts differ significantly ($p < 0.05$). BW = body weight; BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio; EPEF = European Production Efficiency Factor.

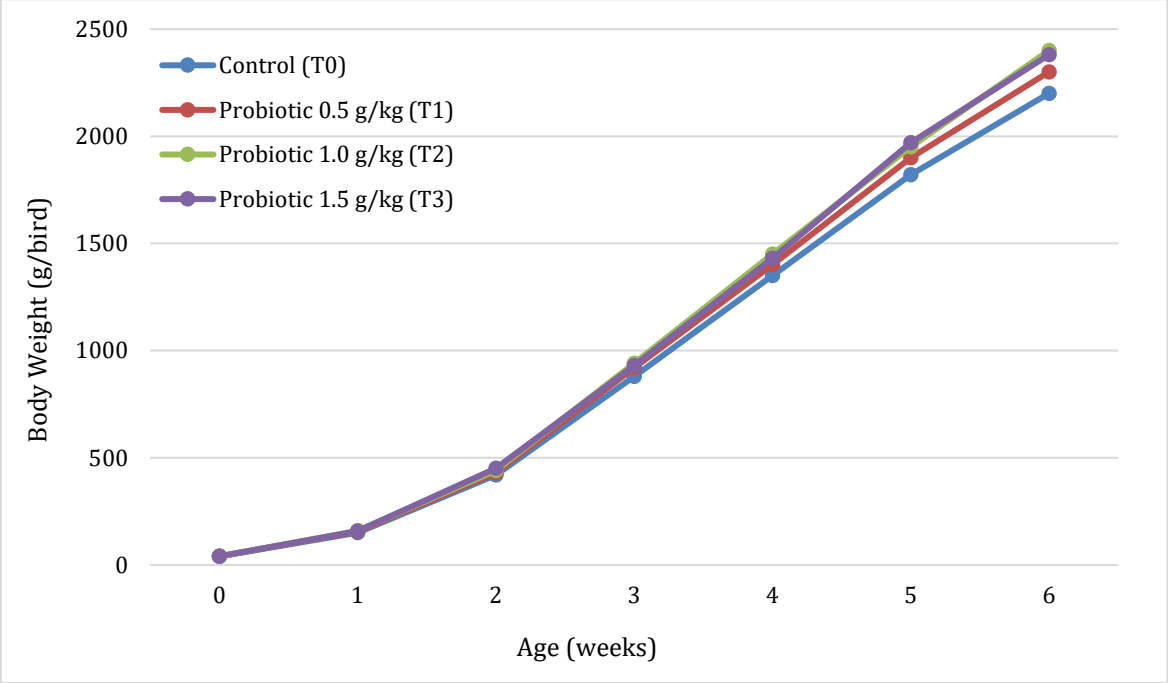



Figure 1. Weekly Body Weight Progression (g/bird) Across Treatment Groups (Mean ± SEM)

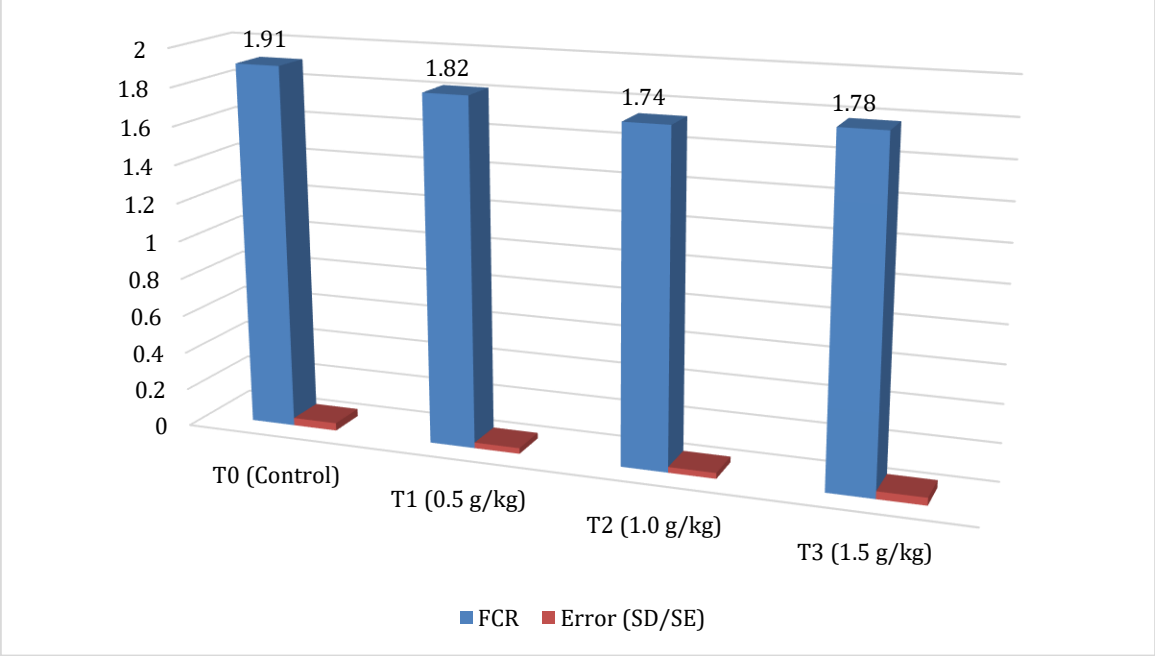



Figure 2. Feed Conversion Ratio (FCR) at 42 Days (Mean ± SEM)

### 4.2. Intestinal Morphology

The intestinal morphometric parameters were improved significantly in all the three intestinal segments in the probiotic supplemented group Table 3, and Figure 3. In the duodenum, T2 birds exhibited a 20.4% increase in villus height (1149 vs. 954 µm) and a 17.1% reduction in crypt depth (165 vs. 199 µm) relative to the control ($p < 0.001$). Consequently, the VH:CD ratio in the duodenum was 45.3% higher in T2 (6.96) than T0 (4.79). The same type of trends were noted in jejunum and ileum. The morphometric data of T3 birds were intermediate between that of T1 and T2 birds and the dose-response relationships remained. The structural changes indicate increased mucosal surface area and decreased enterocyte turnover in probiotic fed broilers, which both can be associated with better absorptive efficiency.

Table 3. Intestinal Morphometry of Duodenum, Jejunum, and Ileum at 42 Days (Mean ± SEM, µm)

| Parameter (µm) | T0 Control | T1 0.5 g/kg | T2 1.0 g/kg | T3 1.5 g/kg |
|---|---|---|---|---|
| Duodenum – Villus Height | $954±18^{c}$ | $1033±20^{b}$ | $1149±23^{a}$ | $1102±20^{ab}$ |
| Duodenum – Crypt Depth | $199±6^{a}$ | $182±6^{b}$ | $165±5^{c}$ | $172±6^{bc}$ |
| Duodenum – VH:CD Ratio | $4.79±0.18^{c}$ | $5.68±0.21^{b}$ | $6.96±0.28^{a}$ | $6.41±0.25^{ab}$ |
| Jejunum – Villus Height | $882±16^{c}$ | $961±18^{b}$ | $1074±21^{a}$ | $1032±19^{ab}$ |
| Jejunum – Crypt Depth | $185±5^{a}$ | $172±5^{ab}$ | $155±4^{c}$ | $163±5^{bc}$ |
| Jejunum – VH:CD Ratio | $4.77±0.17^{c}$ | $5.59±0.19^{b}$ | $6.93±0.26^{a}$ | $6.33±0.23^{ab}$ |
| Ileum – Villus Height | $763±15^{c}$ | $841±17^{b}$ | $948±20^{a}$ | $912±18^{ab}$ |
| Ileum – Crypt Depth | $168±5^{a}$ | $156±4^{ab}$ | $141±4^{c}$ | $149±4^{bc}$ |

A,b,c Means in the same row with different superscripts are significantly different ($p < 0.05$). VH:CD = villus height-to-crypt depth ratio.

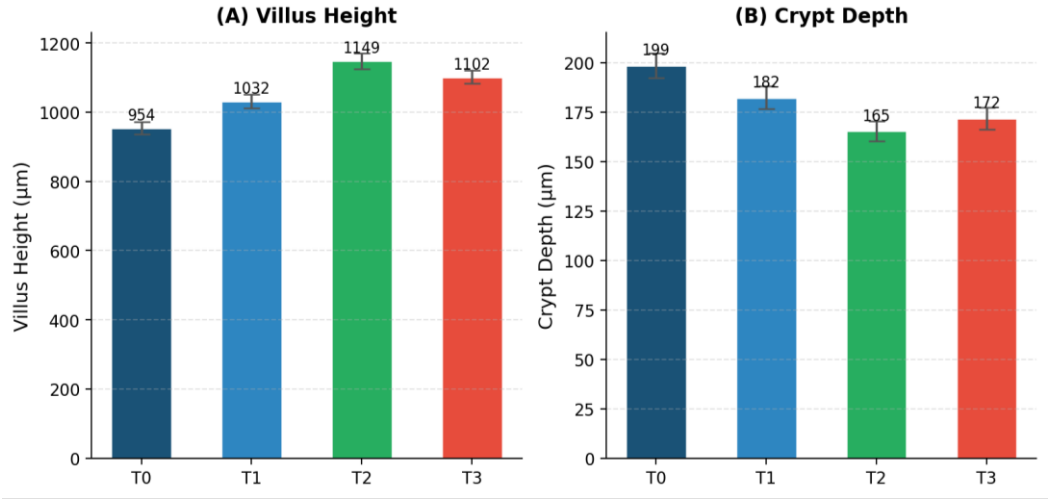



Figure 3. Intestinal Morphology Parameters at 42 Days (Mean ± SEM)

### 4.3. Serum Biochemistry and Immune Parameters

The results of the biochemical and immune tests are given in Table 4 and Figure 4. There was a significant increase in the concentrations of total protein in T2 (6.53 g/dL) compared to T0 (5.82 g/dL; $p = 0.018$), which reflects increased digestibility and absorption of protein. The numerical increase in T2 was observed in Albumin which was not statistically significant ($p = 0.067$). The mean levels of total cholesterol and triglycerides were significantly reduced in T2 versus T0 (151 vs 182 mg/dL; 118 vs 145 mg/dL, $p < 0.05$) showing beneficial effects on lipids. The activities of the enzymes (ALT and AST) were significantly decreased in T2 ($p<0.05$) indicating hepatocyte integrity. Notably, the concentration of serum IgG was 33.4% higher in T2 (6.43 mg/mL) than in T0 (4.82 mg/mL; $p < 0.001$) and there was an increase in lysozyme activity of 35.4% (11.02 vs. 8.14 µg/mL; $p < 0.001$), reflecting greater humoral and innate immune responses.

Table 4. Serum Biochemical and Immune Parameters at 42 Days (Mean ± SEM)

| Parameter | T0 | T1 | T2 | T3 | p-Value |
|---|---|---|---|---|---|
| Total Protein (g/dL) | 5.82±0.18$^{b}$ | 6.14±0.20$^{ab}$ | 6.53±0.22$^{a}$ | 6.38±0.21$^{a}$ | 0.018 |
| Albumin (g/dL) | 3.21±0.10 | 3.42±0.12 | 3.65±0.13 | 3.54±0.11 | 0.067 |
| Total Cholesterol (mg/dL) | 182±8$^{a}$ | 168±7$^{ab}$ | 151±6$^{b}$ | 158±7$^{ab}$ | 0.024 |
| Triglycerides (mg/dL) | 145±7$^{a}$ | 131±6$^{ab}$ | 118±5$^{b}$ | 124±6$^{ab}$ | 0.031 |
| Glucose (mg/dL) | 221±12 | 218±11 | 211±10 | 215±11 | 0.784 |
| ALT (U/L) | 28.4±1.6$^{a}$ | 24.7±1.4$^{b}$ | 21.3±1.2$^{c}$ | 22.8±1.3$^{bc}$ | 0.005 |
| AST (U/L) | 38.2±2.1$^{a}$ | 33.6±1.9$^{ab}$ | 29.4±1.7$^{b}$ | 31.1±1.8$^{ab}$ | 0.012 |
| Serum IgG (mg/mL) | 4.82±0.18$^{c}$ | 5.61±0.22$^{b}$ | 6.43±0.25$^{a}$ | 6.12±0.21$^{ab}$ | <0.001 |
| Lysozyme (µg/mL) | 8.14±0.31$^{c}$ | 9.35±0.36$^{b}$ | 11.02±0.42$^{a}$ | 10.47±0.38$^{ab}$ | <0.001 |

A,b,c Rows with different superscripts differ significantly ($p < 0.05$). ALT = alanine aminotransferase; AST = aspartate aminotransferase; IgG = immunoglobulin G.

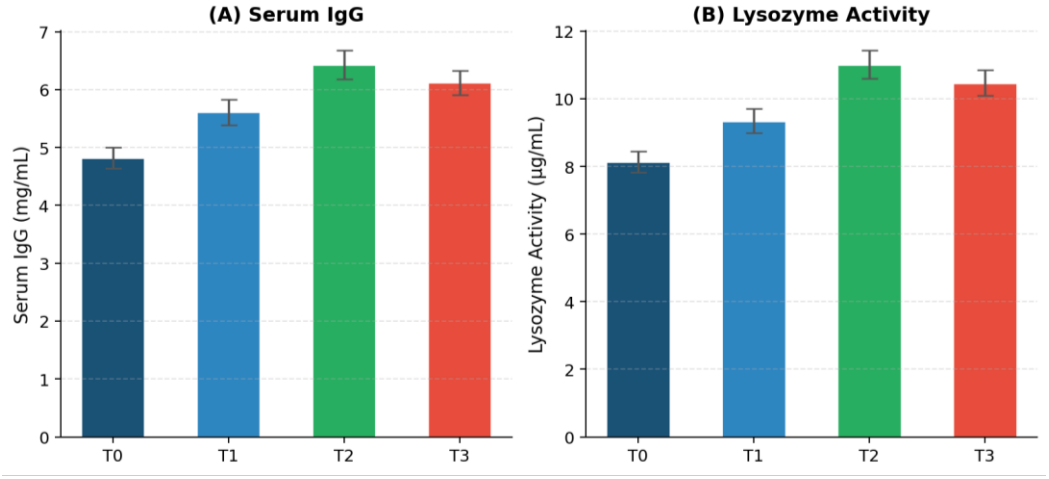



Figure 4. Serum Immune Parameters at 42 Days (Mean ± SEM)

### 4.4. Cecal Microbiome Diversity

Overall, 14.4 million reads with a high quality were obtained (300,000 ± 22,000 reads/sample) and clustered into 1,204 amplicon sequence variants (ASVs). Table 5 and Figure 5 show microbiomes diversity data. Probiotics supplementation significantly increased alpha diversity in the cecum based on all measures. T2 exhibited the highest Shannon index (3.67 ± 0.16), observed OTUs (427 ± 24), and Chao1 richness (201.4 ± 10.3), all significantly greater than T0 ($p < 0.001$). Faith's phylogenetic diversity was also similar (20.8 compared to 14.2 for T2 and T0). The Firmicutes:Bacteroidetes (F:B) ratio is also a gut dysbiosis marker and was significantly different between T2 (1.84) and T0 (2.41; $p = 0.007$) indicating a more balanced community at the phylum level. Lactobacillus relative abundance was 71.7% higher in T2 (31.6%) than T0 (18.4%), while Clostridium perfringens prevalence was reduced by 49.2% (1.94 vs. 3.82%; $p < 0.001$). Shannon diversity was significantly correlated with final body weight and FCR improvement, with r values of 0.78 and −0.71, respectively, and all p values < 0.001, according to the Pearson correlation analysis.

Table 5. Cecal Microbiome Alpha Diversity Indices and Key Taxa at 42 Days (Mean ± SEM)

| Diversity Metric | T0 Control | T1 0.5 g/kg | T2 1.0 g/kg | T3 1.5 g/kg (p) |
|---|---|---|---|---|
| Observed OTUs | 312±18$^{c}$ | 368±21$^{b}$ | 427±24$^{a}$ | 408±23$^{ab}$ |
| Chao1 Index | 142.3±8.2$^{c}$ | 168.7±9.1$^{b}$ | 201.4±10.3$^{a}$ | 189.2±9.6$^{ab}$ |
| Shannon Index (H') | 2.84±0.12$^{c}$ | 3.21±0.14$^{b}$ | 3.67±0.16$^{a}$ | 3.49±0.13$^{ab}$ |
| Simpson Index (1-D) | 0.874±0.012$^{c}$ | 0.901±0.013$^{b}$ | 0.931±0.014$^{a}$ | 0.924±0.014$^{a}$ |

| | | | | |
|---|---|---|---|---|
| Faith's PD | 14.2±0.9$^{c}$ | 17.1±1.1$^{b}$ | 20.8±1.3$^{a}$ | 19.4±1.2$^{ab}$ |
| Firmicutes:Bacteroidetes ratio | 2.41±0.14$^{a}$ | 2.12±0.12$^{b}$ | 1.84±0.10$^{c}$ | 1.93±0.11$^{bc}$ |
| Lactobacillus (% relative) | 18.4±1.2$^{c}$ | 24.7±1.8$^{b}$ | 31.6±2.1$^{a}$ | 28.9±2.0$^{ab}$ |
| Clostridium perfringens (%) | 3.82±0.28$^{a}$ | 2.71±0.21$^{b}$ | 1.94±0.16$^{c}$ | 2.24±0.18$^{bc}$ |

A,b,c Means in the same row with different superscripts are significantly different ($p < 0.05$). OTUs = Operational Taxonomic Units; PD = phylogenetic diversity.

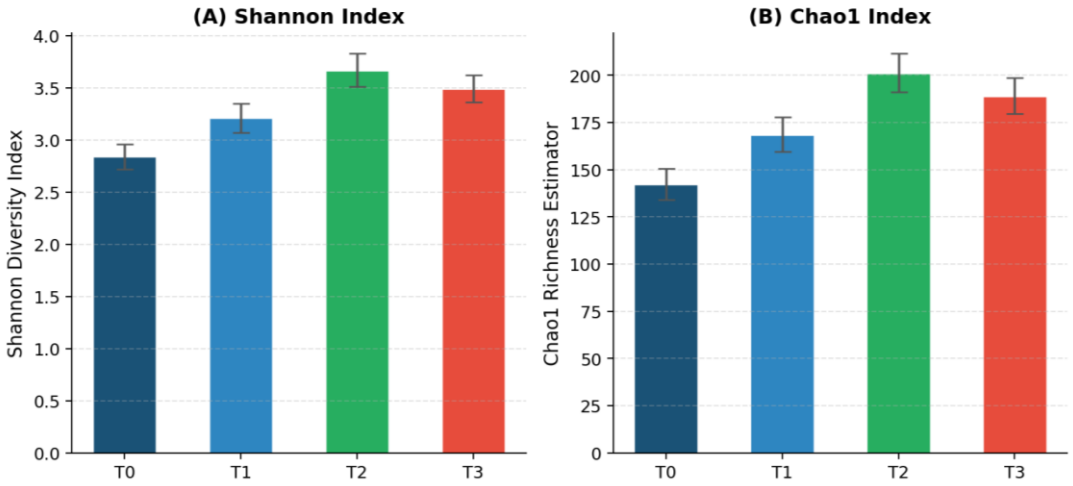



Figure 5. Alpha Diversity Indices of Cecal Microbiota

### 4.5. Discussion

The present study revealed that dietary supplementation of a combination of multiple strains of probiotics at 1.0 g/kg feed significantly increases the production efficiency, gut mucosal integrity, immune competence and cecal microbial community structure of commercial broilers. The results are in agreement with and greatly contribute to some of the lately published studies involving probiotic supplementation in poultry [5], [23].

The reported results of the probiotic dose of 1.0 g/kg regarding the final BW and FCR are in agreement with the meta-analysis findings [7] which reported the improvements of final BW and FCR as 6–10% across the studies used Lactobacillus-based probiotics. This mechanism is likely to be the production of SCFA (mainly acetate, propionate and butyrate) which decreases the pH of the intestinal lumen, suppresses the proliferation of pathogenic bacteria, induces differentiation of goblet cells and directly supports the oxidative metabolism of colonocytes [4], [24]. Butyrate especially downregulates the pro-inflammatory NF-κB signaling and upregulates tight junctions proteins (claudin-1, occludin, ZO-1) leading to decreased intestinal permeability and endotoxin translocation [25].

The VH improvement shown in Table 3 and Figure 3, and the decrease in CD, and the increase in VH:CD ratio, are mechanistically consistent with the performance data. The increase in the number of villi in taller villi increases absorptive surface in the lumen and the decrease in crypt size suggests a decrease in turnover of enteroendocrine cells, which results in a decrease in metabolic costs of mucosal renewal [9], [10]. The 3.7% increase in VH:CD in T2 was one of the highest reported digestive intakes of 45.3% in single product probiotic trials in broilers, which is most likely due to the synergistic effect of the three complementary strains aiming at different niches in the intestine [8]. These morphological effects were consistently observed throughout the whole duodenum, jejunum and ileum which highlighted overall gut-level effects as a result of administration of multi-strain products.

Of particular interest is the decrease in blood lipids (serum cholesterol and triglycerides) in T2. The ability of lactobacilli to deconjugate bile salts with bile salt hydrolase (BSH) activity decreases enterohepatic bile acid recycling and steers cholesterol into de novo bile synthesis [6]. This mechanism is very well established in humans but not in birds as probiotics with Lactobacillus have been used in human

subjects [26] and the present study demonstrates this mechanism in avian hosts. The high decrease of ALT and AST activities in T2 points to the fact that the probiotic-induced decrease in hepatic lipid deposition and in endotoxin load in the lumen were both effective in decreasing metabolic stress in the hepatocyte [27].

The immunostimulatory activity seen, 33.4% increase in IgG and 35.4% increase in lysozyme activity, is in line with the well documented effects of intestinal microbiota on host immunity. Lactobacillus spp. has been demonstrated to activate dendritic cells by stimulating the Toll-like receptor 2 (TLR2) and Toll-like receptor 9 (TLR9) pathways, which leads to the production of both IgA and IgG, through both T cell dependent and T cell independent processes [28]. The innate antimicrobial enzyme lysozyme, the major source of which is the Paneth cells, is upregulated in response to increased mucosal colonization of Lactobacillus, and is the most important first line of defence against infection [20]. The practical implications of the current findings include the ability for birds with greater mucosal and humoral immunity to better resist commercial disease challenge and thus less reliance on therapeutic antibiotics.

Mechanistic detail is gained from the phenotypic observations and given by the 16S rRNA metagenomic data. The greater Shannon and Chao1 index diversity in T2 suggests a more complex and even microbial community, typically linked with higher metabolic redundancy and resilience to dysbiosis-causing stressors [12], [13]. The lower F: B ratio from T0 to T2 (2.41 to 1.84) agrees with other published reports in healthy poultry that decreased F: B ratios relate to increased body fatness and improved feed efficiency [13], [29]. The significant rise in the relative abundance of Lactobacillus and decrease in C. perfringens in T2 (71.7% and 49.2%, respectively) is notable since necrotic enteritis is one of the most economically important enteric diseases in global broiler production, caused by infection with C. perfringens [30]. Probiotic Lactobacillus strains are also capable of inhibiting this pathobiont through nisin production, hydrogen peroxide secretion and acidification of the intestinal environment which are important mechanisms in disease prevention without the use of AGPs [24].

The observed quadratic dose response (T2 (1.0 g/kg) was more effective than T3 (1.5 g/kg) across most parameters), is an important practical consideration. Microbial crowding" [4] refers to the phenomenon of competition of supplemented strains over the native beneficial bacteria and can actually decrease diversity indices when probiotics are super-optimal dosed. Thus, the above returns decrease as rates of inclusion increase, indicating that there is no economic benefit to using higher rates of inclusion and potentially an added cost for feed additives.

A drawback of this study is that it took place in a single climatic and biosecurity setting, and the recommendations presented here would benefit from validation in various environments and disease challenge scenarios in the commercial setting. Future studies should also include the characterization of the metabolome (SCFA profile, bile acid pools) in conjunction with the microbiome to significantly advance elucidation of the mechanistic pathways that lead to the observed phenotypic improvements.

## 5. CONCLUSION

The study presents thorough data on the growth performance, intestinal mucosal integrity, serum biochemistry, immune response and cecal microbiome diversity improving in Ross 308 broiler chickens fed multi-strain probiotic (L. acidophilus, L. reuteri, B. longum) at 1.0 g/kg feed throughout a 42-day production journey. The recommended dosage was 1.0 g/kg and 1.5 g/kg did not provide any further advantage. It is clear from the positive correlation of microbial alpha diversity with important production parameters that good production relies on having a balanced gut microbiome to turn feed inputs into productive outputs. Together, these results help to establish the scientifically, commercially and microbiologically proven practice of using 1.0 g/kg of multi-strain probiotics for modern broiler production, which is antibiotic free. The use of this formulation in production systems without the use of antimicrobials could help achieve the global goal of limiting the spread of antimicrobial resistance and maintaining production efficiency.

### Acknowledgments

The authors have no specific acknowledgments to make for this research.

## Funding Information

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.


## Author Contributions Statement

| Name of Author | C | M | So | Va | Fo | I | R | D | O | E | Vi | Su | P | Fu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Madhusmita Swain | ✓ | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | ✓ | ✓ | | | |

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

## Conflict of Interest Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Informed Consent

All participants were informed about the purpose of the study, and their voluntary consent was obtained prior to data collection.

## Ethical Approval

Not Applicable.

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to Cite:** Madhusmita Swain. (2026). Dietary multi-strain probiotic supplementation improves growth performance, intestinal morphology, serum biochemistry, and gut microbiome diversity in Ross 308 broiler chickens. International Journal of Agriculture and Animal Production(IJAAP), 6(1), 26-38. https://doi.org/10.55529/ijaap.61.26.38

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