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RESEARCH ARTICLE Indian Journal of Animal Research

Impact of *Bacillus subtilis* and *Bacillus clausii* Probiotic Supplementation on Broiler Performance

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10.18805/IJAR.B-5621

ABSTRACT

Background: Probiotics, defined as live microorganisms offering physiological benefits to their host. Among the Probiotics, *Bacillus subtilis* and *Bacillus clausii*have been recognized as safe and effective. Study evaluated the effects of *Bacillus subtilis* and *Bacillus clausii*probiotic supplementation on broiler performance.

Methods: Total of 120 day-old broiler chicks were randomly assigned to 5 dietary treatments: control (CON), CON + antibiotic growth promoter (CONA), CON + *B. clausii* (T2), CON + *B. subtilis* (T3) and CON + *B. clausii*+ *B. subtilis* (T4). Diets were formulated for pre-starter, starter and finisher phases. Performance parameters were recorded on weekly basis. Metabolic trial, carcass evaluation, microbial counts, intestinal histomorphometry, serum biochemistry and antioxidant properties were analyzed on day 35.

Result: Showed significant ($p<0.05$) improvements in performance (body weight, feed-to-gain ratio, EPEI), protein utilization and immunological markers (IgG and IL-10) in probiotic-supplemented groups. Total protein levels were higher, while ALT and AST activities were unaffected. Antioxidant enzymes (SOD and GPX) were significantly higher and malondialdehyde (MDA) levels were lower in probiotic groups. Probiotics supplementation enhanced intestinal architecture. Probiotic supplementation also reduced cecal microbial load and improved feed cost efficiency.

Key words: *Bacillus clausii*, *Bacillus subtilis*, Broiler performance, Intestinal health, Probiotics.

INTRODUCTION

The poultry industry is one of the fastest-growing sectors globally, driven by broiler growth performance and egg production in layers. To enhance performance and reduce disease-related mortality, various feed additives, including antibiotic growth promoters (AGPs), have been widely used. However, extensive and prolonged use of AGPs has led to concerns over the deposition of antibiotic residues in animal tissues, potentially causing antimicrobial resistance in consumers.

Probiotics, defined as live microorganisms offering physiological benefits to their host, have gained prominence as potential substitutes for AGPs in poultry diets. Among the probiotics, *Bacillus subtilis* and *Bacillus clausii* have been recognized as safe and effective. *B. clausii* has been shown to improve growth performance. Despite these benefits, comparative studies evaluating the effects of *B. subtilis* and *B. clausii* from multiple perspectives remain limited. Thus, keeping that in mind the following objectives were studyto evaluate the effect of *Bacillus subtilis* and *Bacillus clausii* supplementation on the performance, nutrient utilization, serum biochemical parameters, immune response, caecalmicrobiota population, histomorphometry of intestine, carcass traits and economics for broiler production.

MATERIALS AND METHODS

In experiment 120 day-old broiler chicks were divided into five experimental groups, each comprising 6 replicates with 4 chicks in each replicate. Standard broiler diets (Con) were formulated according to commercial chick feed specifications for three growth stages: Pre-starter (0-14 days, 22.5% CP and 3000 kcal ME/kg diet), Starter (15-28 days, 21.0% CP and 3125 kcal ME/kg diet) and Finisher (29-42 days, 19.50% CP and 3250 kcal ME/kg diet). Dietary treatment ConA was identical to Con but included antibiotic growth promoter (AGP). In groups T2, T3 and T4, the AGP was replaced with *B.clausii*(@0.2 g/lit of water), *B.subtilis*(@ 250 g/ton of feed) and *B. clausii*(@ 0.1 g/lit) + *B. subtilis*(@ 125 g/ton), respectively. The nutrient composition of control diet of different stages are outlined in Table 1. Consistent environmental conditions were maintained for all groups throughout the experimental period. The experimental diets were isocaloric and isonitrogenous, prepared with the same batch of ingredients for each period, ensuring consistent composition within each growth stage.

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How to cite this article: Barde, P., Khare, A., Nayak, S., Sharma, R., Muwel, N., Sharma, P. and Khare, V. (2025). Impact of *Bacillus subtilis* and *Bacillus clausii* Probiotic Supplementation on Broiler Performance. *Indian Journal of Animal Research*. 1-9. doi: 10.18805/IJAR.B-5621.

Submitted: 08-05-2025 **Accepted:** 19-09-2025 **Online:** 03-11-2025

Growth parameters

Body weight and daily gain

The birds were weighed individually on weekly basis to know the body weight and weight gain of broilers till six weeks of age.

Feed intake

Weekly feed consumption of broilers was recorded replicate wise on the basis of feed offered and left over recorded at the end of that week.

Feed to gain ratio (F:G)

Feed consumption and weight gain for each week was worked out and FGR was calculated by following formula:

$$F:G = \frac{\text{Quantity of feed consumed (g) during the week}}{\text{Gain in weight (g) during the week}}$$

Production efficiency factor (PEF)

PEF was calculated by following formula [Pelicia *et al*., 2010].

$$PEF = \frac{\text{Daily weight gain (kg)} \times \text{Liveability (\%)}}{\text{Feed conversion ratio (FCR)}} \times 100$$

Nutrient utilization

To know the nutrient utilization of DM, CP and EE from different diets, metabolic trial was conducted on 6th wk of the experiment. During collection period quantity of feed offered and leftover were taken daily. The 100 g excreta from each replicate were collected at every 24 hours period for 3 days.

Carcass traits

At slaughter, 02 birds per replicate were bleed and their weights was recorded before and after defeathering with hot water (50-55°C). The dressed weight was calculated as:

Dressed weight = Live weight - Weight loss (blood, head, feathers, shank and wing tips)

Eviscerated weight was derived by subtracting the viscera weight from the dressed weight, while drawn weight was calculated by adding the giblet weight to the eviscerated weight. Processing losses, including blood, head, feathers, shank, separable fat and wing tips, were recorded as a percentage of live weight. The weight of organs (liver, heart, gizzard, spleen, pancreas) and lymphoid organs (bursa of Fabricius, spleen, thymus) was measured as a percentage of dressed weight.

Immunological response

The immunological response was assessed through lymphoid organ weights and serum levels of immunoglobulin G (IgG) and interleukin-10 (IL-10). Weights of the bursa of Fabricius, spleen and thymus were recorded at end of experiment to calculate relative organ weights. IgG and IL-10 concentrations were measured using chicken-specific ELISA kits (Chongqing Biospes Co., Ltd, China), with all assays conducted in duplicate as per manufacturer instructions. Absorbance (OD) was read at 450 nm to calculate concentrations using standard curves.

Caecal microbiota population count

On day 35, caecal content was aseptically collected from sacrificed birds into sterilized plastic bags, stored at -20°C and processed within 24 hours. One gram caecal content was diluted with 10 ml of normal saline in sterile test tubes. Serial dilutions up to 10^{-8} were prepared and dilutions of 10^{-4}, 10^{-5} and 10^{-6} were used for bacterial enumeration. Aliquots (100 µL) were plated on plate count agar (PCA) and incubated at 37°C for 24 hours. The standard plate technique (Quin*et al*., 2004) was followed and bacterial counts were expressed as $\log_{10}$cfu/g of caecaldigesta.

Antioxidant properties

Lipid oxidation (TBARS)

At the end of the trial, breast muscle and liver tissues were collected from sacrificed birds, stored at -20°C for 30 days and analyzed for lipid oxidation as thiobarbituric acid reactive substances (TBARS) using the method by Pikul *et al*. (1989). TBARS values were expressed as mg of malondialdehyde (MDA) per kg of tissue.

Glutathione peroxidase (GPx) estimation

GPx levels were measured using an ELISA kit (Chongqing Biospes Co., Ltd, China). Plates pre-coated with anti-GPx antibody and HRP-conjugated detection antibodies were used. Samples and standards were loaded, incubated, washed and reacted with TMB substrates. Reaction was stopped with an acidic solution and absorbance was measured at 450 nm. GPx concentrations (pmol/ml) were calculated based on optical density.

Superoxide dismutase (T-SOD) estimation

T-SOD levels were determined using a sandwich ELISA kit. Plates pre-coated with anti-T-SOD antibody were used

Table 1: Nutrient composition of experimental broiler diets.

Feed Ingredients	Experimental diets (%)		
	Pre starter (0 - 14 days)	Starter (29 - 42 days)	Finisher (15 - 28 days)
Nutrient composition analysed (%)			
Crude protein	22.59	21.22	19.41
Ca	0.82	0.81	0.84
Total P	0.63	0.61	0.64
Nutrient composition calculated (%)			
Crude protein	22.5	21.0	19.5
ME (Mcal/kg)	3.00	3.13	3.25
Total Lysine	1.44	1.34	1.20
Total methionine	0.58	0.56	0.52
Ca	0.75	0.75	0.75
Available P	0.42	0.41	0.40

with HRP-conjugated detection antibodies. T-SOD concentrations (pg/ml) were calculated from standard curves.

Serum biochemical parameters

Blood samples (2 ml) were collected in clot-activating vials during slaughter, centrifuged at 3000 rpm for 30 minutes to extract serum and stored at -20°C. Total protein, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using commercial diagnostic kits.

Activity (U/L) for AST and ALT was calculated using the difference in absorbance between test and control, standardized against blank and standard. Total protein was derived using a biuret-based standard curve.

Examination of intestinal morphology structure

Duodenum and ileo-jejunum samples were collected for histomorphometric analysis. Tissue samples (3-4 mm) were fixed in 10% formalin and processed using paraffin embedding. Sections (4-5 µm thick) were stained with haematoxylin and eosin. From each sample, five intact villi per replicate (6 replicates) were selected, yielding 60 measurements per treatment. Study assessed intestinal wall thickness, villus height, crypt depth, VH:CD ratio. Villus height was measured from the top of the villus to the lamina propria, while crypt depth from the crypt base to the transition region.

Statistical analysis

Was performed using ANOVA under a Completely Randomized design (CRD) as described by Snedecor and Cochran (1994). Duncan's multiple range test (1955) was applied for post hoc comparisons to identify significant differences among treatments. SPSS version 20.0 was used for all analyses.

RESULTS AND DISCUSSION

Performance of broilers

The results of the current study distinctly reveal a clear trend of increased live weight in broilers supplemented with Probiotics, as presented in Table 2.

Table 2: Performance in broilers fed different probiotics and their combination.

	Treatments						
Weekly	Con	ConA	T2	T3	T4	SEM	*p-value*
Performance							
Live weight	2334.32	2437.58	2594.15	2492.15	2686.10	30.95	0.04
Average weight gain	2276.32^{d}	2379.48^{cd}	2536.15^{ab}	2434.15^{bc}	2628.1^{a}	30.95	0.03
Average FI§	4070.07	4029.83	3937.77	4013.88	4147.98	34.98	0.45
Average F:G§§	1.79^{a}	1.69^{ab}	1.55^{c}	1.65^{bc}	1.58^{c}	0.02	0.01
PEI§§§	1124.85	1095.49	1070.23	1141.1	1185.32	22.2	0.57
Nutrient Utilization (%)							
DM	61.97^{c}	68.48^{b}	70.27^{ab}	70.29^{ab}	73.08^{a}	1.28	0.003
C P	62.24^{c}	67.09^{bc}	70.84^{ab}	69.22^{ab}	72.60^{a}	1.27	0.02
E E	74.35^{b}	79.94^{ab}	86.34^{a}	81.17^{ab}	87.37^{a}	1.7	0.03
Economics of broiler production							
Feed cost Rs/kg	142.65	142.41	138.29	141.57	146.39	1.05	0.19
Feed cost Rs/kg wt gain	62.70^{a}	59.02^{a}	55.31^{c}	58.26^{bc}	55.71^{c}	0.64	0.00

Means of row with different superscript showing significant difference (P<0.05); LBW= Live body weight.

§FI=Feed Intake; §§F:G= Feed to gain ratio; §§§ Production efficiency factor (PEF)= LBW(kg) × Liveability(%)/FCR × age*×100 *42 days in this experiment.

Means of row with different superscript showing significant difference (P<0.05).

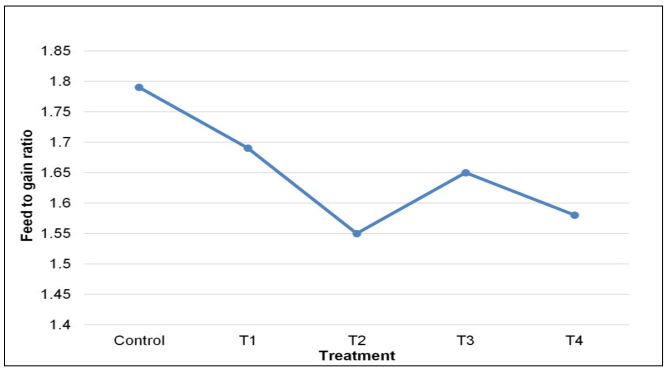


Fig 1: Cumulative feed to gain ratio of broilers in different treatment groups.

Notably, the group fed combination of *B. clausii*and *B. subtilis* (T4) exhibited the maximum and higher live weight compared to other groups. Addition of probiotic significantly influenced the weight gain of broilers when compared to the basal diet, as indicated in Table 2. Notably, the group fed with combination (T4) and *B. clausii* (T2) exhibited the maximum and higher weight gain. Moreover, minimal feed consumption was observed in broilers fed the T2 diet with *B. clausii*.

At the conclusion of the experiment, a more favorable feed-to-gain ratio was observed in broilers fed the T2 and T4 diet and there were significant difference among the groups fed with Con, ConA and T3 diets (Table 2 and Fig 1).

Production efficiency factor was lower in broilers fed the basal diet (T4) and highest in those fed with the T2 diet. Overall, these findings suggest that probiotic supplementation, particularly *B. clausii* (@ 0.2 g/lit of water), positively influences overall performance and economics (Fig 2) in broilers.

Spore-forming probiotics like *B. clausii* and *B. subtilis* exhibit resilience in harsh gastrointestinal conditions, ensuring effective colonization and activity in the gut. They improve gut microbiota balance, enhance nutrient absorption and promote enzyme production for better digestion. Their robust nature reduces pathogenic bacteria, optimizes intestinal morphology and supports efficient nutrient utilization.

Mazanko *et al*. (2022) highlighted *B. subtilis KB54* for growth performance, immune modulation and intestinal colonization, supporting its role as a probiotic alternative. Hussien *et al*. (2023) observed significantly ($p \leq 0.05$) lower feed intake and enhanced FCR in broilers supplemented with *Bacillus subtilis* and *Bacillus licheniformis* (@1 mL/L *via* drinking water), highlighting the probiotics' role in improving feed efficiency and growth performance. Cai *et al*. (2024) observed a 4.55% reduction in FCR during the starter phase with 500 mg/kg *Bacillus subtilis* supplementation.

Nutrient utilization

Findings of the study highlighted a significant impact of probiotic supplementation on Dry matter (DM), crude protein (CP) and Ether extract (EE) utilization in broilers, as outlined in Table 2.

Specifically, among all the diets, the maximum DM, CP and EE utilization was observed in broilers supplemented with probiotics *i.e.* T2, T3 and T4. This suggests that probiotic supplementation, *B. clausii*or*B. subtilis*, significantly enhances the utilization of nutrients (DM, CP and EE) by broilers. Gao *et al.* (2017) and Madhuri *et al.* (2020) observed enhanced metabolism of crude protein, fat, dry matter and organic matter in broilers supplemented with 200 mg/kg *B. subtilis*, highlighting its role in optimizing nutrient absorption and utilization.

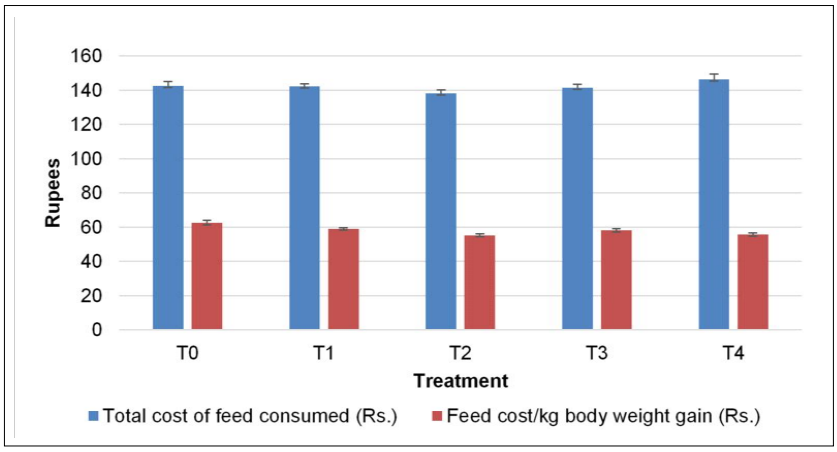
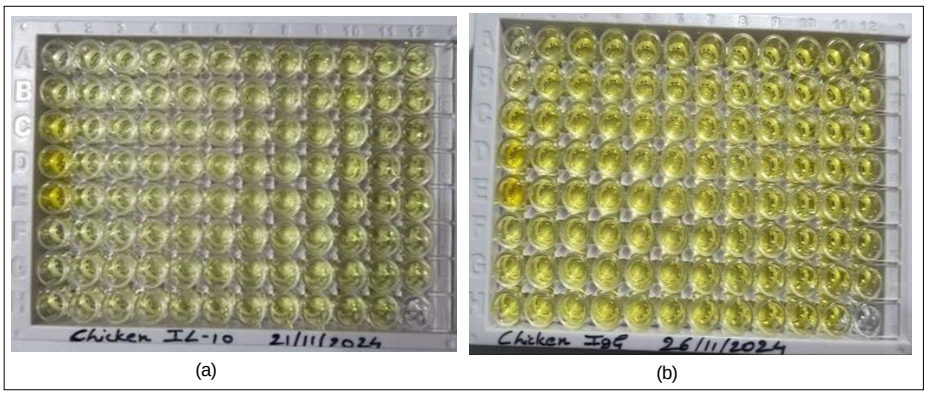


Fig 2: Economics of broiler production in different treatment groups.



(a) (b)

Fig 3: (a) IL-10 concentration of broiler in different treatment groups (b) IgG concentration of broiler in different treatment groups.

Carcass yield

Results presented in Table 3 regarding carcass yield, indicate that the eviscerated, dressing yield and drawn weight among the different treatment groups have no significant difference.

Oso *et al*. (2021) and Yadav *et al*. (2018) reported a significant increase (P<0.01) in breast muscle weight in broilers supplemented with 1 million *B.subtilis* spores/g feed. Tang *et al*. (2021) found improved carcass traits with *B.subtilis*, including increased breast muscle percentage, reduced abdominal fat (P<0.05) and enhanced fatty acid profiles (MUFAs) in breast and thigh muscles.

Immune response

ELISA analysis

Supplementation of *B.clausii* and *B. subtilis* probiotic in broiler diets significantly influenced serum IgG and IL-10 concentrations (Table 4 and Fig 3).

IgG (µg/ml)

Broilers fed diets supplemented with probiotic showed significantly higher serum IgG levels compared to the control group. Among the treatment groups, *B. clausii* (T2) showed the highest concentration, although they were statistically similar.

IL-10 (ng/l)

Serum IL-10 levels were also found higher in *B. clausii*(T2) treatment group compared to the control. Junaid *et al.* (2018) and Qiu *et al*. (2021) observed enhanced immunity and gut health in broilers fed *B.subtilis* strains, with higher serum immunoglobulins, thymus weight, lysozyme and favorable microbiota shifts. Xu *et al*. (2021) reported increased serum IgA, IL-10, antioxidant enzymes and SCFAs, alongside reduced inflammation and pathogens.

Weight of lymphoid organs

Results indicate an improvement in the weight of lymphoid organs, specifically the spleen and thymus, in broilers supplemented with *B. clausii* (T2) (Table 3). However, bursa of fabricius didn't show much change in weight with probiotic supplementation.

This suggests that probiotic supplementation, particularly at the specified concentration, has a positive influence on the development and weight of certain lymphoid organs. Spleen and thymus, which play crucial roles in the immune system, demonstrated increased weight, potentially indicating a positive impact on the immune response.

Wang *et al*. (2022) demonstrated that *B.subtilis* supplementation significantly enhanced thymus, bursa and

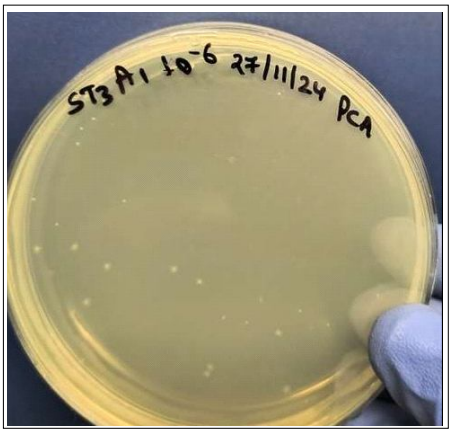


Fig 4: Standard plate count showing total viable bacterial count.

Table 3: Carcass weights (% of live weight), weight of lymphoid organ and caecal microbial count in broilers.

	Treatments						
Parameters	Con	ConA	T2	T3	T4	SEM	*p-value*
Carcass weights (% of live weight)							
Dressed weight	83.22	83.45	84.19	84.13	84.66	0.42	0.42
Eviscerated weight	74.83	75.49	76.15	76.06	75.65	0.30	0.39
Drawn weight	79.29	79.70	80.55	80.37	79.77	0.25	0.34
Weight of lymphoid organ							
Thymus	0.17	0.15	0.19	0.14	0.13	0.01	0.76
Spleen	0.1	0.11	0.14	0.14	0.12	0.01	0.86
Bursa of fabricius	0.58	0.66	0.61	0.62	0.49	0.04	0.74
Caecalmicrobiota population count							
Caecalmicrobiota count	7.56^{a}	6.98^{b}	6.27^{d}	6.60^{c}	6.52^{c}	0.15	0.02

Means of row with different superscript showing significant difference (P<0.05).

Table 4: Immune status and enzyme status of birds in different.

	Con	ConA	T2	T3	T4	SEM	p-Value
IgG µg/ml	14.13	14.80	17.21	15.08	15.52	0.35	0.73
IL-10 ng/l	7.44	8.20	10.61	8.45	8.80	0.38	0.93

IgG: Immunoglobulin G, IL-10: Interleukin-10.

spleen weights in broilers, indicating improved lymphoid organ development. Similarly, Mushtaq *et al*. (2023) found *B.clausii* (12×10^6 spores @ 0.03 ml/L) significantly increased lymphoid organ weights, villus height and immune response.

Caecal microbiota population count

Broilers fed diets supplemented with various spore-forming probiotics showed significant differences in caecalmicrobiota counts. Control group (CON) exhibited the highest count, while supplementation with probiotics led to a progressive reduction in microbial population.

Inclusion of *B. clausii* (T2) resulted in the most significant reduction, with counts markedly lower than all other treatments, including control (Table 3 and Fig 4).

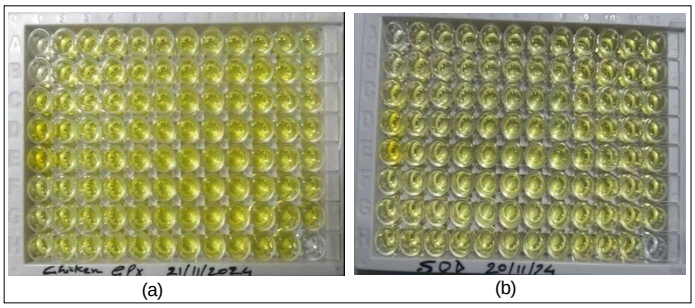
Spore-forming probiotics like *B.clausii*and *B.subtilis* produce antimicrobial compounds (*e.g.*, bacteriocins) that inhibit pathogenic bacteria in the gut. Their ability to colonize the gut and outcompete harmful microbes leads to reduction in overall microbial population. *Bacillus clausii* was particularly effective, likely due to its superior antimicrobial activity or adaptability in the gut environment. This modulation of gut microbiota enhances gut health, contributing to better nutrient absorption and overall broiler performance.

Mohamed *et al*. (2022) revealed that *B.subtilis* ATCC19659 supplementation at 1×10^8, 3×10^8 and 5×10^8 CFU/g increased beneficial caecalmicrobiota and reduced Escherichia-Shigella and Clostridia unclassified, particularly at higher doses. Hussien *et al*. (2023) demonstrated that *B.subtilis* and *B. licheniformis* supplementation (1 ml/L) significantly reduced cecalclostridial counts, emphasizing their role in enhancing gut microbial health in broilers.

Table 5: Serum biochemical parameters, and antioxidant status of broilers in treatment groups.

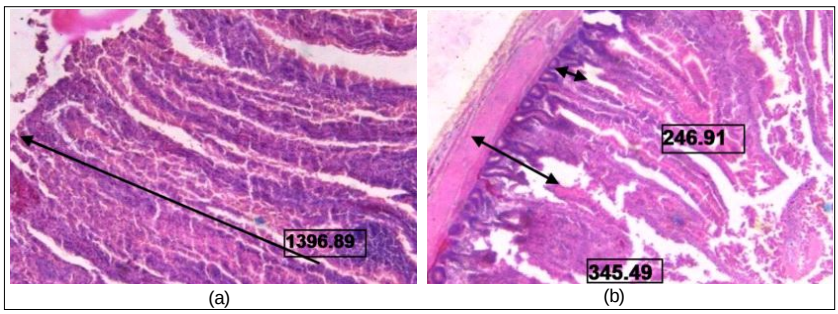
	Treatments						
Parameters	Con	ConA	T2	T3	T4	SEM	*p-value*
Serum biochemical parameters							
AST (IU/L)	288.7	273.47	333.47	221.76	272.73	18.9	0.52
ALT (IU/L)	1.99	1.67	1.86	2.36	1.96	0.35	0.99
TP (g/dL)	7.09c	7.48Gbc	8.96ab	8.50ab	9.35a	0.3	0.04
TBARS value (liver and breast muscle)							
Breast	70.2^{a}	65^{a}	28.6^{bc}	37.7^{b}	33.8^{c}	5.75	0.01
Liver	85.5^{a}	82.2^{a}	57.2^{b}	62.4^{b}	57.2^{b}	4.18	0.01
SOD (pg/ml)	7.28^{c}	7.96^{bc}	8.80^{b}	10.53^{a}	8.91^{b}	0.38	0.01
GPx (ng/ml)	2.57^{b}	2.84^{ab}	3.18^{a}	2.89^{ab}	3.09^{a}	0.08	0.06

Means of row with different superscript showing significant difference ($p<0.05$).



(a) (b)

Fig 5: GP× concentration of broiler in different treatment groups (b) SOD concentration of broiler in different treatment groups.



(a) (b)

Fig 6: (a) Photomicrograph of intestinal villus height in duodenum, H and E × 50 (b) Photomicrograph of crypt depth and intestinal wall thickness in duodenum, H and E × 50.

Antioxidant properties in broilers

Supplementation of spore-forming probiotics significantly influenced the antioxidant properties in broilers, as measured Thiobarbituric acid reactive substances (TBARS) and serum enzymes (Table 5).

Broilers on the control diet (CON) showed the highest breast MDA values, indicative of elevated lipid oxidation. Probiotic supplementation reduced these values significantly, with combination of *B. clausii* and *B. subtilis* (T4) yielding the lowest levels, suggesting enhanced antioxidant capacity. Other treatments, including *B.clausii* (T2), also reduced MDA levels significantly.

Liver MDA levels followed a similar trend. The control group (CON) had the highest values, while supplementation of probioticsand its combination achieved the lowest.

Antioxidant enzyme activity in broilers

Influence of spore-forming probiotics on antioxidant enzymes, Superoxide dismutase (SOD) and Glutathione peroxidase (GPx), in broilers were analyzed (Table 5 and Fig 5).

Superoxide dismutase (SOD)

SOD activity was significantly influenced by probiotic supplementation ($P<0.05$). The highest SOD activity was observed in T3 (*Bacillus subtilis*), followed by combination of probiotics (T4) and *Bacillus clausii* (T2). This indicates that probiotics supplementation enhanced SOD activity in broilers.

Glutathione peroxidase (GPx)

GPx activity showed numerical differences among treatments, with the highest activity recorded in *Bacillus clausii* supplemented group, followed by combination (T4). This suggests that spore forming probiotic supplementation influenced GPx activity, the effects were not pronounced among treatments.

Spore-forming probiotics like *B. clausii* and *B. subtilis* enhance antioxidant properties by producing metabolites that neutralize reactive oxygen species (ROS) and reduce oxidative stress. They stimulate antioxidant enzymes such as SOD and GPx, which protect tissues from lipid peroxidation. Their resilience ensures effective colonization and modulation of gut microbiota, reducing ROS generation by pathogens.

Tang *et al*. (2021) found that *Bacillus subtilis* supplementation (500 mg/kg feed) improved broiler meat quality by reducing oxidative stress. Xu *et al*. (2021) demonstrated that *Bacillus spp.* (1.5×10^9 CFU/kg) enhanced GPx, SOD, catalase and reduced MDA levels. Liu *et al*. (2023) highlighted *Bacillus subtilis* HC6 (5×10^8 CFU/kg) as reducing oxidative stress markers, boosting serum and liver antioxidant status and improving broiler health.

Blood biochemical indices

Results presented in Table 5 indicate that dietary supplementation of probiotics, has an effect on serum parameters. Specifically, in comparison to the control group (Con), the T4 and T2 group showed a significant increase in serum concentrations of total protein.

However, no significant effect was observed in the values of SGOT/AST (aspartate aminotransferase) and SGPT/ALT (alanine aminotransferase) across the dietary supplementation of probiotics.

Dietary supplementation of probiotics, may have a favorable impact on total proteins. The lack of significant effects on liver

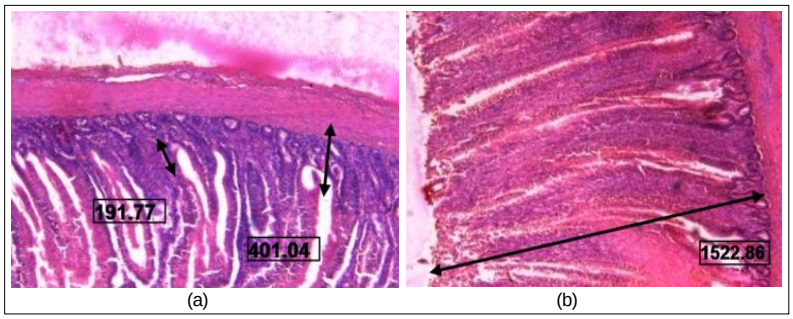


Fig 7: (a) Photomicrograph of intestinal wall thickness and crypt depth in jejunum, H and E × 50 (b) Photomicrograph of villus height in jejunum, H and E × 50.

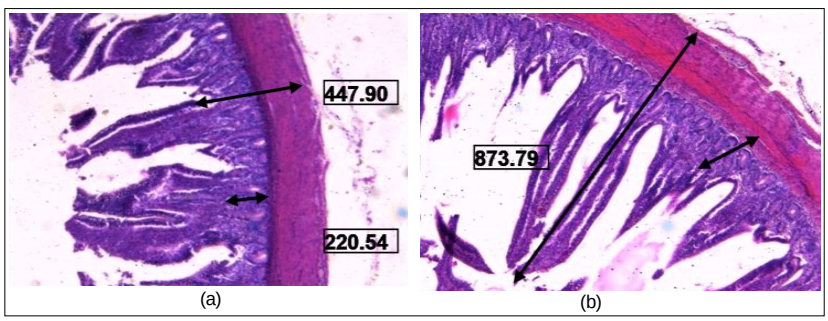


Fig 8: (a) Photomicrograph of intestinal wall thickness and crypt depth in ileum, H and E × 50 (b) Photomicrograph of villus height in ileum, H and E × 50.

Table 6: Microscopic histological parameters in different segment of small intestine in different treatment groups.

Parameters	Treatments					SEM	p-Value
	Con	ConA	T2	T3	T4		
Intestinal wall thickness (µm)							
Duodenum	345.50	349.80	358.70	360.90	358.30	3.34	0.99
Jejunum	403.60	394.50	403.00	401.00	398.30	2.03	0.83
Ileum	457.80	445.60	456.80	447.90	450.30	2.06	0.85
Villus height (µm)							
Duodenum	1388.36^{c}	1452.56^{b}	1537.84^{a}	1487.81^{ab}	1522.86^{a}	15.46	0.05
Jejunum	1236.36^{c}	1318.36^{b}	1379.26^{a}	1340.49^{b}	1366.87^{a}	13.84	0.04
Ileum	721.93^{c}	747.11^{c}	873.79^{a}	820.99^{b}	853.01^{a}	16.15	0.02
Crypt depth (µm)							
Duodenum	246.91^{a}	231.53^{b}	198.09^{c}	220.54^{b}	200.21^{c}	5.27	0.03
Jejunum	216.99^{a}	199.16^{b}	173.59^{d}	191.77^{bc}	177.40^{cd}	4.54	0.02
Ileum	201.00^{a}	193.04^{a}	151.45^{b}	162.9^{b}	159.88^{b}	5.48	0.05
Villus height to crypt depth ratio							
Duodenum	5.63^{d}	6.27^{c}	7.77^{a}	6.75^{b}	7.61^{a}	0.22	0.04
Jejunum	5.71^{c}	6.63^{b}	7.95^{a}	7.00^{b}	7.71^{a}	0.22	0.01
Ileum	3.60^{c}	3.87^{c}	5.78^{a}	5.05^{b}	5.34^{ab}	0.23	0.01

Means of row with different superscript showing significant difference ($p<0.05$).

enzymes (SGOT/AST and SGPT/ALT) suggests that probiotics supplementation may not have a notable impact on liver.

Mushtaq *et al*. (2023) demonstrated that *B. clausii* supplementation at 0.03 ml/L (12 $\times 10^{6}$ spores) for 35 days effectively supported liver health in broilers and enhancing total serum protein.

Histomorphometrical analysis of intestine

Current study (Table 6 and Fig 6, 7, 8) demonstrated that broilers supplemented with probiotics exhibited significant effects on intestinal morphology ($p<0.05$). Notably, *B. clausii* (T2) and the combination (T4) significantly improved villus height in both the duodenum and ileo-jejunum.

Spore-forming probiotics like *B. clausii* and *B. subtilis* improve intestinal morphology by stimulating the growth of villi and enhancing intestinal architecture. These probiotics promote the development of the duodenum and ileo-jejunum, where nutrient absorption is highest, by increasing villus height and the villus height-to-crypt depth ratio. The enhanced villus height increases the surface area for nutrient absorption, improving overall digestion and efficiency. Probiotic supplementation, particularly with *B. clausii*, positively impacts these structural changes, resulting in better nutrient absorption and performance in broilers.

Zhang *et al*. (2021) found that *B. subtilis* supplementation significantly improved the ileal villus height-to-crypt depth ratio, indicating enhanced intestinal structure. Mushtaq *et al*. (2023) reported improved histomorphometrical parameters, including increased villus height, villus height-to-crypt depth ratio and villus surface area ($p<0.05$) in broilers supplemented with *B. clausii*.

CONCLUSION

The research aimed to evaluate supplementation of *Bacillus clausii* and *Bacillus subtilis* based probiotics on performance of broiler chickens. Findings suggest that *Bacillus clausii*and *Bacillus subtilis*, used individually or together, effectively enhance growth, protein utilization, immunity, antioxidant status, total serum protein and intestinal architecture while reducing caecal microbial load. Probiotic supplementation significantly elevated SOD and GPx levels, especially in *B. clausii*, while reduces MDA levels.

Intestinal morphology improved with increased villus height, reduced crypt depth and better villus height-to-crypt depth ratios. Total cecal microbial counts were significantly reduced in probiotic-fed groups. Feed cost per kg weight gain was more economical in groups receiving *Bacillus clausii*, making it the most cost-effective option. Therefore, *Bacillus clausii* and its combination based probiotics can serve as a viable alternative to antibiotic growth promoters in broiler diets.

ACKNOWLEDGEMENT

The present study was supported by VvaanLifesciencePvt Ltd, Mumbai-India.

Informed consent

All experimental procedures were approved by the College animal ethical committee.

Conflict of interest

The authors declare no conflicts of interest regarding the publication of this article.

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