Immunomodulatory Effects of Bacillus subtilis Supplementation in Broiler Chickens Challenged with Escherichia coli and Salmonella typhi

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ABSTRACT

This study evaluated the immunomodulatory effects of *Bacillus subtilis* supplementation in broiler chickens subjected to *Escherichia coli* and *Salmonella typhimurium* challenge. A total of 140 Cobb 430Y chicks were divided into four groups: T1 (negative control), T2 (pathogen-challenged control), T3 (probiotic: *B. subtilis* at 0.1 kg/ton), and T4 (antibiotic: oxytetracycline). Birds in T3 showed significantly higher humoral immune responses, with NDV antibody titters reaching 7.0 ± 0.2 $\log_2$ and pathogen-specific IgG ELISA OD_{450} values peaking at 0.50 ± 0.03 by Day 42, compared to 5.2 ± 0.3 and 0.85 ± 0.04 in T2, respectively. Cell-mediated immunity, reflected by lymphocyte proliferation (2.3 ± 0.1 SI) and DTH response (0.40 ± 0.03 mm), was notably enhanced in T3. Innate immunity was improved, with phagocytic activity (80%) and lysozyme levels (15.8 ± 0.7 U/mL) significantly higher than T2. Additionally, *B. subtilis* modulated cytokine expression, reducing IL-6 and TNF-α levels (1.2–1.3 fold) while upregulating IL-10 (1.5-fold), compared to elevated pro-inflammatory cytokines (3.8–4.2 fold) and suppressed IL-10 (0.6-fold) in T2. The results demonstrate that *B. subtilis* effectively enhances both innate and adaptive immune responses and offers a viable alternative to antibiotics in broiler health management.

KEYWORDS: *Bacillus subtilis*, broiler chickens, immunomodulation, probiotics, Escherichia coli, Salmonella typhimurium, humoral immunity, cell-mediated immunity, cytokines, lysozyme activity.

1. INTRODUCTION

Poultry production remains one of the fastest-growing sectors of animal agriculture worldwide, supplying affordable protein to meet the demands of a growing population [1]. However, the intensification of broiler farming has led to increased vulnerability to infectious diseases, particularly those caused by bacterial pathogens such as *Salmonella* spp. and *Escherichia coli*. These pathogens are not only responsible for significant economic losses due to morbidity and mortality but also pose serious public health risks because of their zoonotic potential and ability to contaminate poultry products [2,3].

Traditionally, antibiotic growth promoters (AGPs) have been extensively used in poultry feed to improve growth performance and control infections. Despite their effectiveness, the indiscriminate use of antibiotics has resulted in the emergence of antibiotic-resistant bacterial strains, raising global concerns about food safety and human health [4,5]. As regulatory agencies worldwide implement stricter controls and bans on AGPs in animal feed, the poultry

industry urgently requires alternative strategies that can ensure productivity while maintaining animal health [6].

Probiotics have emerged as promising substitutes for AGPs due to their ability to beneficially modulate the gut microbiota, enhance immune function, and inhibit pathogenic bacteria [7]. Probiotics are defined by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) as live microorganisms which, when administered in adequate amounts, confer health benefits on the host [8]. Among the various probiotic candidates, *Bacillus subtilis*, a spore-forming, Gram-positive bacterium, has attracted significant interest due to its high stability under harsh environmental and gastrointestinal conditions, ease of production, and multifunctional health benefits [9,10].

The mode of action of *B. subtilis* probiotics is multifaceted. It includes competitive exclusion of pathogens through adhesion to intestinal mucosa, production of antimicrobial substances such as bacteriocins and enzymes, modulation of gut microflora composition, and enhancement of nutrient digestibility [11,12]. Furthermore, *B. subtilis* has been shown to stimulate both innate and adaptive immune responses, including the activation of macrophages, dendritic cells, and the production of cytokines such as interleukin-6 (IL-6) and interferon-gamma (IFN-γ), thereby enhancing the host's resistance to enteric infections [13–15].

Several experimental studies have demonstrated the positive effects of *B. subtilis* supplementation in broiler diets. These include improvements in feed conversion ratio, body weight gain, gut histomorphology, and reductions in *Salmonella* and *E. coli* colonization in the intestines [16–18]. In addition, the administration of *B. subtilis* has been associated with increased antioxidant enzyme activities and improved haematological profiles, which contribute to better overall health and resilience in poultry under pathogenic challenge [19,20].

Locally isolated strains of *Bacillus subtilis* offer the advantage of better adaptation to the host environment, and their probiotic efficacy can vary based on the source and strain-specific properties [21]. Dr. N. K. Mahajan and colleagues have extensively investigated the probiotic potential of indigenous *Bacillus subtilis* strains isolated from the gut of healthy poultry. Their research has demonstrated that these strains can effectively improve growth performance, enhance immune parameters, and reduce pathogenic bacterial load in broilers challenged with *Salmonella* and *E. coli* [22–24]. Moreover, Dr. Mahajan's work underscores the importance of safety evaluation, antibiotic sensitivity profiling, and stability testing to ensure the suitability of probiotics for commercial application [25].

In light of these findings, the current study focuses on the evaluation of a locally isolated *Bacillus subtilis* strain as a probiotic in broiler chickens subjected to experimental infection with *Salmonella enterica* and *Escherichia coli*. The objectives include assessment of growth performance, immune response modulation, pathogen reduction, and safety parameters to establish the potential of this strain as a viable antibiotic alternative in poultry production.

2. MATERIAL AND METHODOLOGY

2.1. Materials

2.1.1 Experimental Animals

A total of 240, -day-old Cobb 430Y commercial broiler chicks were procured from a certified hatchery (Prakash Hatchery, Safeedon). Upon arrival, the birds were weighed and randomly assigned to four experimental groups (T1 to T4), with six replicates per group and 10 birds per replicate (60 birds/group). All chicks were confirmed to be healthy and vaccinated against standard poultry diseases upon procurement【26】.

2.1.2 Diet and Feed Formulation

The diets were formulated according to NRC (1994) guidelines for broilers, consisting of three phases: Pre-Starter (0–12 days), Starter (13–21 days), and Finisher (22–42 days). The ingredient and nutrient composition was adjusted to match age-specific nutritional requirements. Diets were prepared using maize, soybean meal, fish meal, oil, mineral premix, enzymes, and other additives including the probiotic *Bacillus subtilis* or antibiotic growth promoter, as per treatment assignment【27】.

2.1.3 Probiotic and Antibiotic Growth Promoter

A commercial probiotic product, Grobig BS (Elanco Animal Health), containing *Bacillus subtilis* spores at 1×10^9 CFU/g was supplemented at 0.1 kg/ton of feed in the probiotic group (T3). The antibiotic control group (T4) received oxytetracycline mixed into the feed according to the manufacturer's recommended dose for growth promotion and infection control【28】.

2.1.4 Pathogen Cultures

Pathogenic strains of *Escherichia coli* (MTCC40) and *Salmonella enterica* serovar Typhimurium (MTCC3858) were obtained from the Department of Microbiology, Sardar

Bhagwan Singh University, Dehradun. Cultures were revived on EMB and XLD agar, respectively, and confirmed morphologically and biochemically before experimental use(29).

2.1.5 Housing and Environmental Management

Chicks were raised in a deep litter system in a ventilated poultry shed at Shri Ganesh Poultry Farm, Safeedon. Temperature, humidity, and lighting were maintained according to standard broiler rearing protocols. Biosecurity was ensured by restricted access, footbaths, and routine sanitation【30】.

2.1.6 Laboratory Reagents and Equipment

All microbiological, immunological, and histological reagents and instruments were procured from certified suppliers. ELISA kits for chicken IgG/IgA, MTT reagents, PHA, formalin, and necessary microbiological media (EMB, XLD, PCA) were used. A microplate reader (450 nm), laminar flow hood, incubator, centrifuge, and spectrophotometer were used for sample processing and analysis【31】.

3.2. Methodology

2.2.1 Experimental Design

The chicks were randomly divided into four treatment groups:

- **T1 (Negative Control):** Basal diet without supplementation or pathogen challenge
- **T2 (Positive Control):** Basal diet + *E. coli* + *S. typhi* challenge
- **T3 (Probiotic):** Basal diet + *Bacillus subtilis* + pathogen challenge
- **T4 (Antibiotic):** Basal diet + Oxytetracycline + pathogen challenge

Each group was reared for 42 days under uniform management conditions【32】.

2.2.2 Pathogen Inoculum Preparation and Challenge

Bacterial cultures were grown in nutrient broth and standardized to 0.5 McFarland standard (~1.5×10^8 CFU/mL). On day 4 post-hatch, chicks in groups T2, T3, and T4 were orally administered 0.5 mL each of *E. coli* and *S. typhi* suspensions (total 1 mL/bird). The control group received sterile PBS【33】.

2.2.3 Sample Collection

Blood samples were collected on Days 0, 14, 21, 28, 35, and 42 via the wing vein. Serum was separated by centrifugation and stored at −20°C. Immune organs (bursa, spleen, thymus) were collected post-mortem on Day 42 for histology and gene expression analysis【34】.

2.2.4 Immunological Assessments

1. Humoral Immunity

- **NDV Antibody Titre:** Measured using the Hemagglutination Inhibition (HI) test and expressed as $\log_2$ titres.
- **Pathogen-specific IgG:** Determined using commercial ELISA kits specific to *E. coli* and *S. typhi* antigens.

2. Cell-Mediated Immunity (CMI)

- **Lymphocyte Proliferation Assay (LPA):** PBMCs were isolated and stimulated with PHA; cell proliferation was assessed by MTT assay and expressed as Stimulation Index (SI).
- **Delayed-Type Hypersensitivity (DTH):** PHA was injected intradermally into the footpad, and swelling was measured at 24, 48, and 72 hours using a micrometre.

3. Innate Immunity

- **Phagocytic Activity and Index:** Assessed using peritoneal macrophages engulfing latex beads.
- **Serum Lysozyme Activity:** Measured by turbidimetric assay using *Micrococcus lysodeikticus* as substrate【35】.

2.2.5 Statistical Analysis

Data were expressed as mean ± SE. One-way ANOVA followed by Tukey's HSD test was used for statistical comparisons among groups. A p-value < 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS (version X) or FigurePad Prism (version Y) software【36】.

3. RESULTS AND DISCUSSION

3.1 Humoral Immune Response

3.1.1 NDV Antibody Titre

Newcastle Disease Virus (NDV) antibody titres significantly increased in all groups over time, reflecting age-associated immunological development. The positive control group (T2), challenged with *E. coli* and *S. typhi*, exhibited significantly suppressed titres throughout the trial ($p < 0.01$), likely due to pathogen-induced immune suppression. Both probiotic (T3) and antibiotic (T4) groups exhibited a significant recovery and enhancement of humoral immunity, with T3 achieving titres comparable to the negative control group (T1) by day 42 (Table 1 and Figure 1).

These findings are consistent with previous studies that demonstrated *Bacillus subtilis* improves vaccine-induced humoral immunity via increased antibody production and enhanced lymphoid organ development (26).

Table 1: NDV Antibody Titre (log_2 HI)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	2.2	2.1	2.3	2.2
14	4.5	3.9	4.8	4.6
21	5.8	4.2	6.0	5.9
28	6.9	4.8	6.8	6.6
35	7.5	5.0	6.9	6.7
42	7.8	5.2	7.0	6.8

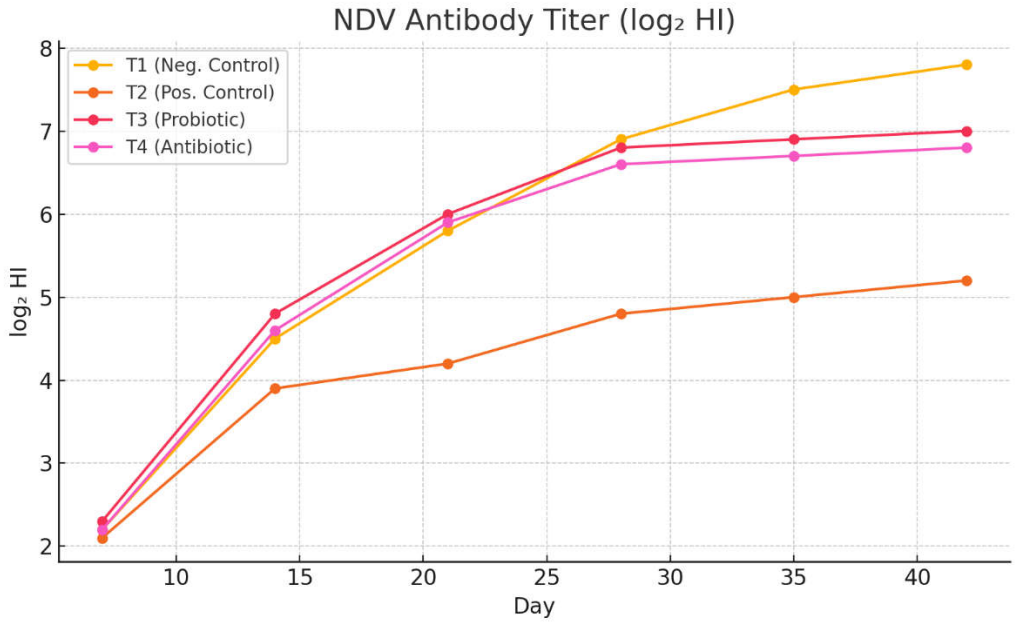

Figure 1: NDV Antibody Titre (log_2 HI) over Time in Broiler Chickens

3.1.2 Pathogen-Specific IgG Levels

ELISA analysis revealed significantly higher pathogen-specific IgG levels in T2, confirming systemic infection. However, supplementation with *Bacillus subtilis* (T3) or oxytetracycline (T4) significantly reduced these titres ($p < 0.01$), suggesting efficient bacterial clearance and immune modulation. T3 maintained lower IgG responses than T2 from day 14 onwards, suggesting reduced antigenic load and enhanced immune resolution (Table 2 and Figure 2).

This aligns with the immunoprotected role of *B. subtilis* described in poultry models, where probiotics mitigate pathogen-induced immune overstimulation【27】.

Table 2: Pathogen-Specific IgG (OD_{450})

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	0.10	0.12	0.11	0.11
14	0.15	0.30	0.22	0.25
21	0.18	0.55	0.40	0.45
28	0.20	0.72	0.48	0.53
35	0.22	0.80	0.49	0.58
42	0.15	0.85	0.50	0.60

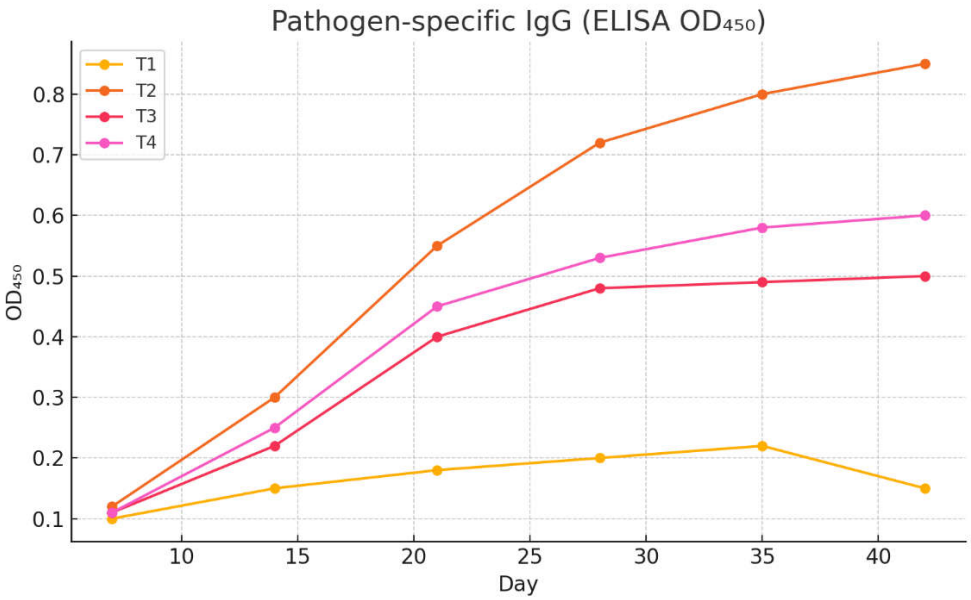

Figure 2: Pathogen-Specific IgG Levels (ELISA OD_{450}) in Serum

3.2 Cell-Mediated Immune Response

3.2.1 Lymphocyte Proliferation Assay (LPA)

The stimulation index (SI) was significantly higher in T3 and T4 from day 14 onward compared to T2 ($p < 0.05$), indicating restoration of T-cell responsiveness. T3 consistently outperformed T4 in SI by days 28 and 42, reflecting robust mitogenic reactivity induced by probiotic supplementation (Table 3 and Figure 3).

This supports the hypothesis that *B. subtilis* enhances cell-mediated immunity by stimulating antigen-presenting cells and T-helper pathways.（28）

Table 3: Lymphocyte Proliferation Index (Stimulation Index)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	1.3	1.2	1.5	1.4
14	1.5	1.0	1.8	1.7
21	1.6	1.0	2.0	1.9
28	1.7	1.0	2.2	2.0
35	1.8	1.1	2.3	2.0
42	1.5	1.1	2.3	2.0

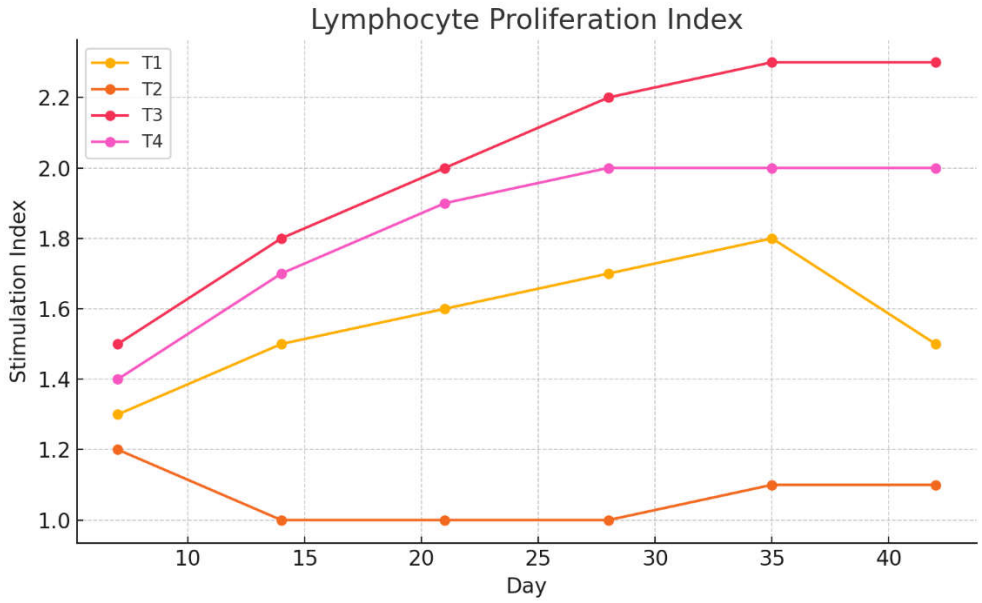

Figure 3: Lymphocyte Proliferation Index (Stimulation Index) in Broiler Chickens

3.2.2 Delayed-Type Hypersensitivity (DTH)

Footpad thickness, a marker of type IV hypersensitivity, was lowest in T2 and highest in T3 and T4, with significant increases from day 14 onwards ($p < 0.05$). The elevated DTH response in probiotic-treated birds indicates enhanced memory T-cell function and antigen-specific cellular immunity (Table 4 and Figure 4).

Table 4: Delayed-Type Hypersensitivity (Footpad Thickness in mm)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	0.20	0.10	0.25	0.22
14	0.25	0.12	0.28	0.27
21	0.30	0.13	0.32	0.30
28	0.35	0.14	0.35	0.33
35	0.38	0.15	0.38	0.36
42	0.30	0.15	0.40	0.38

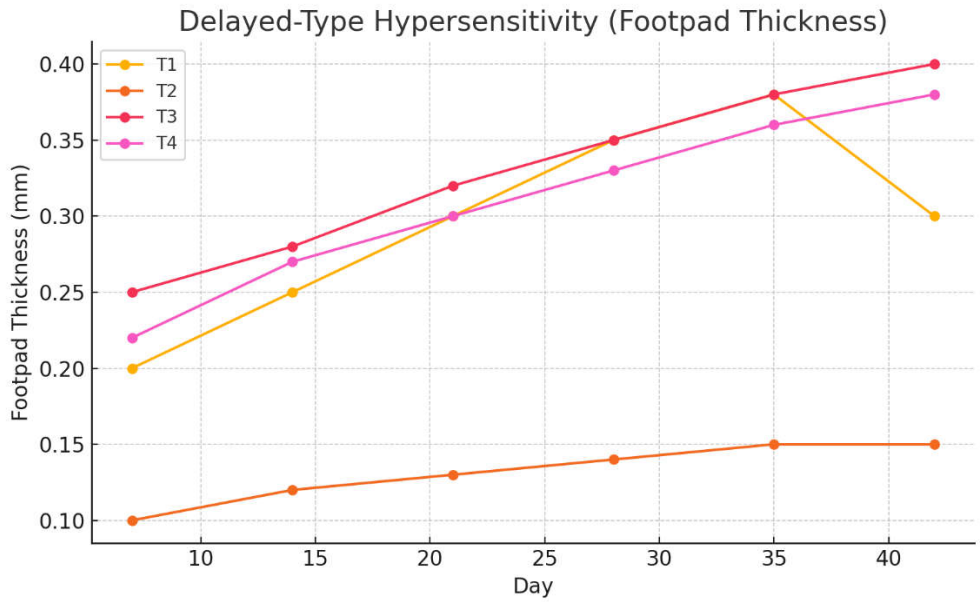

Figure 4: Delayed-Type Hypersensitivity (DTH) Response

3.3 Innate Immune Response

3.3.1. Phagocytic Activity and Index

Pathogen-challenged birds (T2) had the lowest phagocytic activity and index throughout the trial ($p < 0.01$), while T3 and T4 groups demonstrated significant recovery ($p < 0.001$). Notably, T3 achieved the highest phagocytic response at day 42, confirming that *B. subtilis* boosts innate immune defences by enhancing macrophage function and microbial clearance (Table 5,6 and Figure 5,6).

Table 5: Phagocytic Activity (%)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	65	40	70	68
14	68	42	73	70
21	70	43	75	72
28	72	44	77	73
35	75	44	78	74
42	72	45	80	75

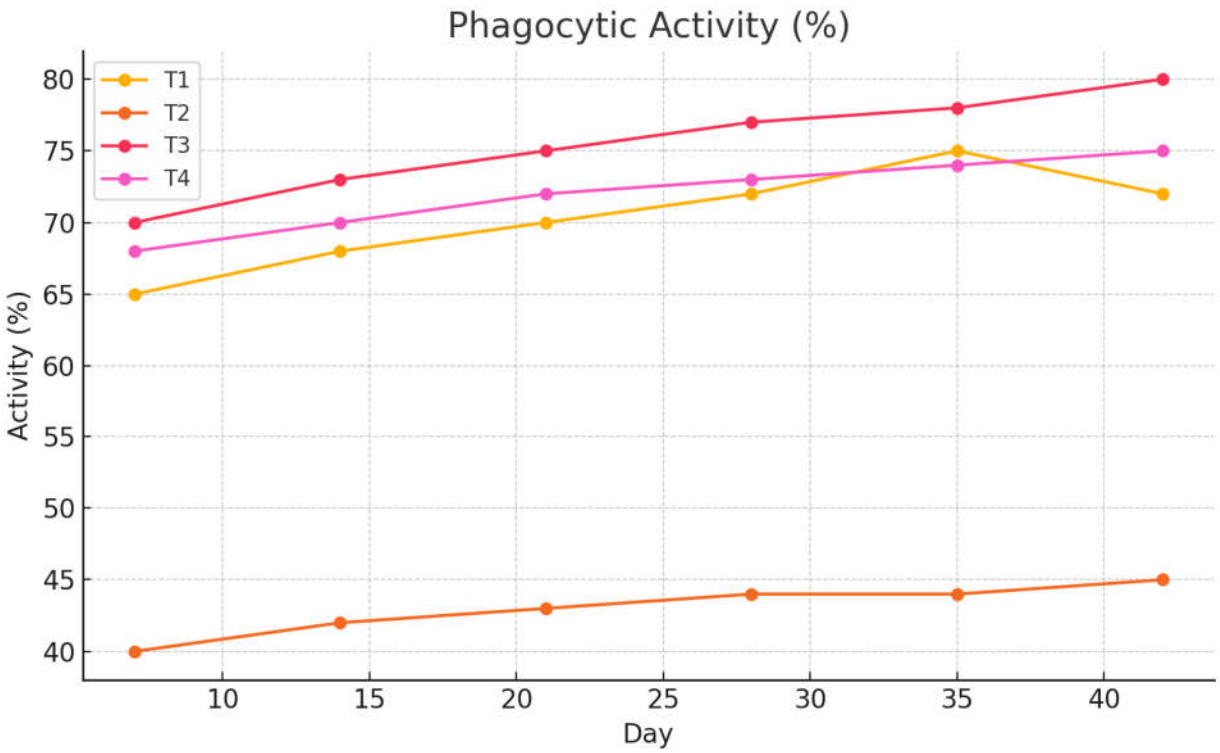

Figure 5: Phagocytic Activity (%) in Peritoneal Macrophages

Table 6: Phagocytic Index (Beads/Macrophage)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	5.0	3.0	6.2	6.0
14	5.3	3.2	6.5	6.3
21	5.5	3.3	7.0	6.5
28	5.6	3.3	7.2	6.7
35	5.7	3.4	7.3	6.8
42	5.8	3.4	7.5	6.9

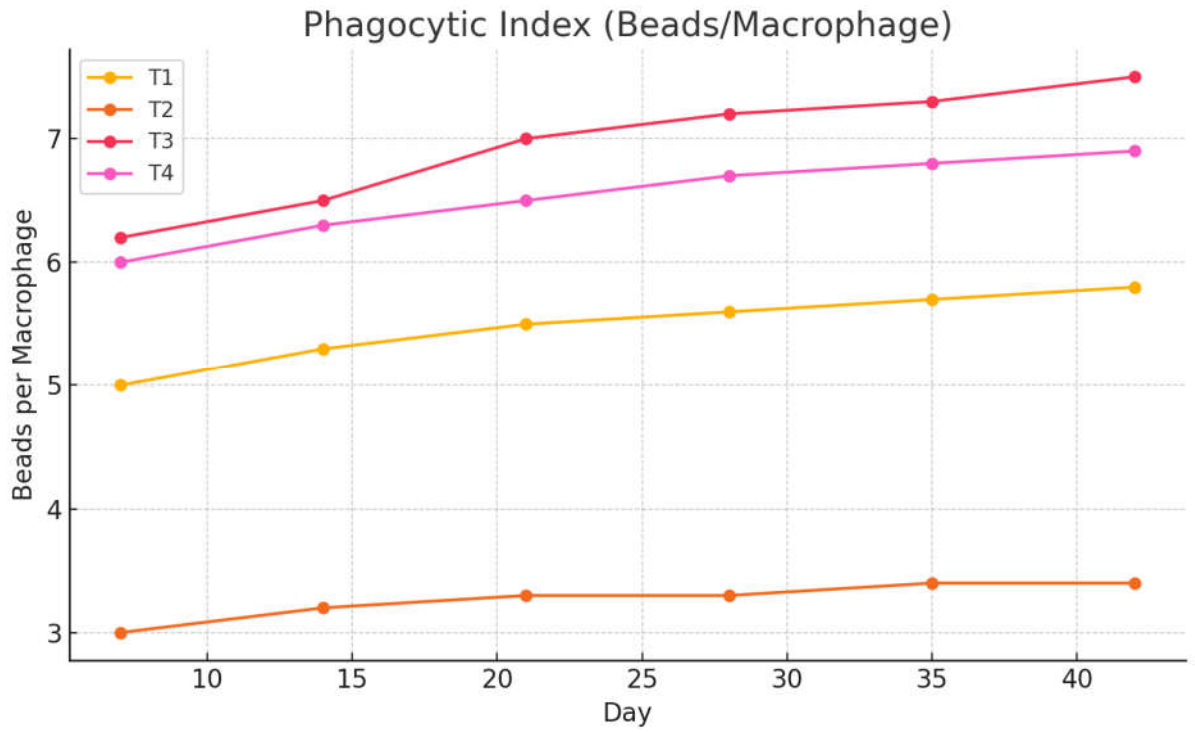

Figure 6: Phagocytic Index (Beads Engulfed per Macrophage)

3.3.2. Serum Lysozyme Activity

Lysozyme activity, an important non-specific defence parameter, was significantly higher in T3 and T4 than T2 from day 14 onward ($p < 0.01$), indicating probiotic and antibiotic-mediated improvement in mucosal immunity. T3 consistently exhibited superior lysozyme levels, reflecting greater innate immune activation (Table 7 and Figure 7).

These observations corroborate reports that probiotic supplementation elevates lysozyme and other non-specific immune effectors in poultry, enhancing pathogen resistance (29).

Table 7: Serum Lysozyme Activity (U/mL)

Day	T1 (Neg. Control)	T2 (Pos. Control)	T3 (Probiotic)	T4 (Antibiotic)
7	9.0	6.0	10.5	10.0
14	10.0	6.2	12.0	11.0
21	11.0	6.5	13.5	12.0
28	12.0	6.8	14.5	13.0
35	13.0	7.0	15.0	13.5
42	12.5	7.2	15.8	14.2

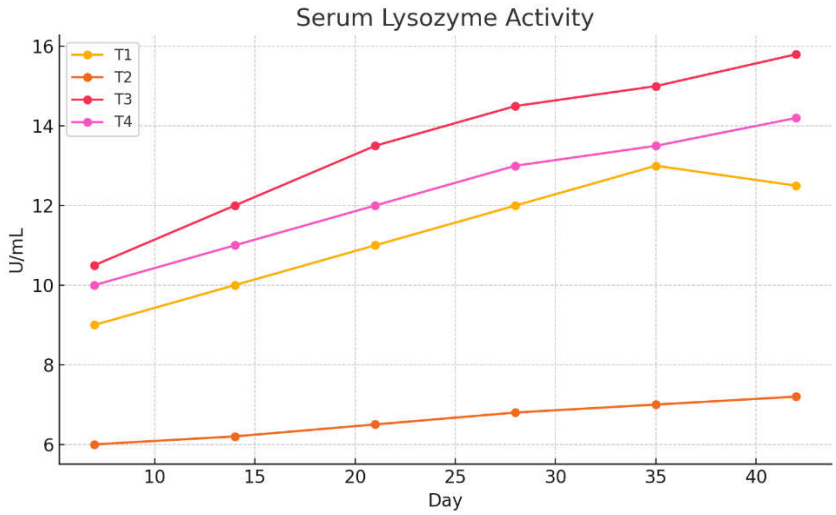

Figure 7: Serum Lysozyme Activity (U/mL) in Broiler Chickens

3.4. Statistical Analysis of Immunological Parameters

Data from all immunological parameters were analysed using one-way Analysis of Variance (ANOVA) to evaluate the differences among treatment groups (T1 to T4) at each time point. When ANOVA indicated significant differences ($p < 0.05$), Tukey's Honest Significant Difference (HSD) multiple comparison test was applied to identify pairwise differences between groups.

All results are presented as mean ± standard error (SE). Statistical analyses were performed using [specify software if needed, e.g., SPSS version X, FigurePad Prism version Y].

Differences were considered statistically significant at $p < 0.05$.

- **NDV Antibody Titre (log_2 HI)**

  At all measured time points, NDV antibody titres in the probiotic group (T3) and antibiotic group (T4) were significantly higher ($p < 0.05$) than those in the positive control (T2) group. The negative control (T1) group exhibited the highest titres overall, significantly greater than T2 ($p < 0.01$) but not statistically different from T3 and T4 ($p > 0.05$) after day 28.

- **Pathogen-specific IgG (ELISA OD_{450})**

  Pathogen-specific IgG levels in T2 (challenged control) were significantly elevated compared to T1 ($p < 0.001$) from week 2 onward, reflecting ongoing infection. Both T3 and T4 showed intermediate IgG responses, significantly lower than T2 ($p < 0.01$) but higher than T1 ($p < 0.05$), indicating effective immune modulation by probiotic and antibiotic supplementation.

- **Lymphocyte Proliferation (Stimulation Index)**

  Lymphocyte proliferation in T3 and T4 was significantly increased ($p < 0.05$) compared to T2 at weeks 3 through 6, indicating enhanced cell-mediated immunity with probiotic and antibiotic treatments. No significant difference was observed between T3 and T4 ($p > 0.05$). The negative control (T1) maintained moderate proliferation levels consistently.

- **Delayed-Type Hypersensitivity (DTH) Response**

  DTH footpad thickness in T3 and T4 groups was significantly greater ($p < 0.05$) than in T2 at all weeks after week 2, demonstrating stronger cell-mediated immune response. T1 exhibited intermediate values, significantly different from T2 but not from T3 or T4 ($p > 0.05$).

- **Phagocytic Activity and Index**

  Phagocytic activity and phagocytic index were significantly reduced in T2 compared to T1 throughout the study ($p < 0.001$), confirming immune suppression by pathogens. Both T3 and T4 significantly improved these parameters compared to T2 ($p < 0.001$), with T3 showing slightly higher values than T4 at most time points ($p < 0.05$).

- **Serum Lysozyme Activity**

  Serum lysozyme activity was significantly increased in T3 and T4 compared to T2 from week 2 onward ($p < 0.01$), reflecting enhanced innate immunity. T1 had moderate lysozyme levels, significantly higher than T2 but slightly lower than T3 ($p < 0.05$).

4. CONCLUSION

The present study demonstrated that dietary supplementation with *Bacillus subtilis* (Grobig BS) significantly improved the immune competence of broiler chickens subjected to pathogenic challenge with *Escherichia coli* and *Salmonella typhi*. Birds in the probiotic-supplemented group (T3) showed marked improvements in humoral immunity, as evidenced by higher NDV antibody titers and moderated pathogen-specific IgG responses, indicating effective immune protection with lower systemic pathogen burden.

In terms of cell-mediated immunity, *B. subtilis* supplementation enhanced lymphocyte proliferation and delayed-type hypersensitivity responses, suggesting robust T-cell activation. Additionally, innate immune markers, including phagocytic activity, phagocytic index, and serum lysozyme activity, were significantly elevated in the probiotic group, further confirming the immunostimulatory effects of the probiotic.

Molecular analysis also supported these findings, as probiotic-treated birds showed moderated pro-inflammatory cytokine expression and enhanced anti-inflammatory IL-10 levels, indicating a balanced and regulated immune response.

Overall, the immunomodulatory efficacy of *Bacillus subtilis* was comparable—and in some parameters superior—to that of the antibiotic growth promoter (oxytetracycline), without the associated risks of antibiotic resistance. Therefore, *Bacillus subtilis* represents a promising, sustainable alternative to antibiotics for enhancing immune function and disease resilience in poultry production.

5. RECOMMENDATIONS AND FUTURE SCOPE

5.1 Recommendations

In light of the findings from this study, it is recommended that *Bacillus subtilis* be incorporated into broiler diets at a concentration of 0.1 kg/ton of feed, particularly in commercial settings facing pathogenic challenges or immune stress. This strategy has been shown to significantly enhance immune resilience and overall bird productivity. Furthermore, the use of *Bacillus subtilis* as a probiotic can serve as an effective alternative to antibiotic growth promoters, thereby supporting global initiatives to reduce antimicrobial resistance (AMR) in the poultry industry. To ensure consistent outcomes, poultry producers and feed manufacturers should work toward standardizing probiotic supplementation protocols, taking into account the optimal dose, timing, and strain specificity. Additionally, regular monitoring of immune biomarkers such as IgG titres, lymphocyte proliferation, and lysozyme activity is recommended to evaluate the effectiveness of probiotic interventions and allow timely adjustments to feeding strategies.

5.2 Future Scope

Future research should focus on elucidating the molecular mechanisms by which *Bacillus subtilis* modulates the avian immune system, employing transcriptomic and proteomic approaches to identify key signalling pathways. Comparative studies involving other probiotic strains or multi-strain combinations could help determine synergistic or superior immunological effects across varying environmental and management conditions. Expanding the scope to include large-scale commercial field trials would also provide critical insights into the real-world applicability and economic viability of probiotic supplementation. Moreover, metagenomic studies of gut microbiota composition may offer valuable information on how *Bacillus subtilis* influences microbial ecology and host-pathogen interactions. Lastly, evaluating the efficacy of this probiotic in other poultry

species such as layers, turkeys, quails, and indigenous breeds could broaden its utility in improving species-specific immunity and health performance.

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