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RESEARCH ARTICLE

Investigations on the Immunomodulatory Effects of *Streptomyces* and *Bacillus*-Based Probiotics in Broilers

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ABSTRACT

This study was planned to evaluate the immunomodulatory potential of *Streptomyces* and *Bacillus*-based probiotic formulations in broiler chickens. Day-old (n=90) broiler chicks were randomly allocated to six experimental groups (n=15 each), including: Group A served as a negative control; Group B was administered with *Streptomyces* sp. @ 1×10^9 CFU/kg; Group C was administered with *Bacillus* sp. @ 1×10^8 CFU/kg; Group D was administered a commercial probiotic (Probio™) @ 2g/kg; Group E was administered with *Streptomyces* sp. and *Bacillus* sp. @ 1×10^9 CFU and 1×10^8 CFU/kg, respectively, and Group F was administered with *Streptomyces* sp., *Bacillus* sp. and *E. coli* Challenge @ 1×10^9 CFU/kg, 1×10^8 CFU/kg and 3×10^7 CFU/mL of broth culture, respectively. The treatments were given in feed from day seven till the Day 42 of age. The results indicated more pronounced immunostimulatory effects in the *Streptomyces-Bacillus* combination (Group E) those were evident by significant increase ($P<0.05$) in the absolute weight of the immune organs as compared to the control. Cellular immunity based on phagocytic activity in the carbon clearance assay revealed two folds higher values as compared to control in the probiotic supplemented groups and lymphoproliferative response was also sustainable in these groups. Humoral immune responses also revealed that the probiotic combination enhanced both primary (IgM) and secondary (IgG) antibody responses, with 37% higher IgG titers against sheep RBCs and 25% higher NDV vaccine titers ($P<0.05$). Furthermore, the probiotic combination conferred better protection against *E. coli* challenge and prevented the lymphoid organ atrophy typically associated with bacterial infection. Histopathological examination confirmed these functional improvements, revealing well-developed germinal centers (white pulp) in the spleen, cortical hyperplasia in the thymus, and follicular hypertrophy in the bursa of probiotic-fed birds. These findings collectively demonstrate that the *Streptomyces-Bacillus* probiotic combination effectively enhances both innate and adaptive immunity in broilers.

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INTRODUCTION

In the poultry sector, the infectious enteric illnesses are of the biggest concern, as they cause increased mortality, reduce production, and pose a serious public health risk through the contamination of meat and eggs (Maqbool *et al.*, 2023). Different antibiotics have been used to control infectious diseases for a long time, leading to the development of antibiotic resistance in both poultry birds and humans (Muhammad *et al.*, 2020). Antibiotic growth promoters used in animals may affect human health through antibiotic residues in eggs and meat, as well as by spreading antibiotic resistance genes to human infections. The addition of antibiotics to the animal feed for growth enhancement eliminates beneficial bacteria residing in the gastrointestinal tract of animals. According to Maqbool *et al.* (2023), the prevalence of resistant factors in commensal bacteria is a

driver of new resistance development among pathogenic bacterial strains.

These days, feed additives and nutritional supplements are gaining popularity in the poultry sector and healthcare systems due to their numerous beneficial impacts, such as boosting growth and productivity, improving immune function, and maintaining good health. A wide range of feed additives is used in poultry diets, including organic acids, oligosaccharides, probiotics, antibiotics, and enzymes (Abd El-Hack *et al.*, 2020). Living microorganisms that provide health benefits to hosts upon sufficient consumption are defined as probiotics by the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO). The characteristics which are necessary for a probiotic to be considered functional include: the bacteria should belong to the intestinal microflora, exhibit resistance to stomach acidity, and possess exceptional intestinal tissue anchoring ability and the ability to sustain optimal microbiome levels (Krysiak *et al.*, 2021; Magnoli *et al.*, 2024).

The probiotics have a broad spectrum of antagonistic activity against pathogenic and opportunistic microorganisms and have no effect on the development of bacterial resistance. They create multiple positive results for the body by reducing tissue barrier permeability to toxins and detoxifying compounds originating from pathogens (Poberezhets and Kupchuk, 2021; Isgro *et al.*, 2024). Since probiotics administration has shown positive effects on animal performance, protection against microbial toxins, intestinal microbial equilibrium, immune regulation, and vitamin and digestive enzyme synthesis abilities, they have been suggested as promising substitutes for antibiotics in livestock farming as preventative, medicinal, and growth-enhancing drugs (Cuozzo *et al.*, 2023). Different bacteria, including Lactococcus spp., Bacillus spp., Lactobacillus spp., Streptococcus spp., Bifidobacterium spp., and yeasts like Candida spp., are the most widely used microorganisms to produce probiotics (Krysiak *et al.*, 2021; Bidura *et al.*, 2024; Fredrick *et al.*, 2024; Du *et al.*, 2025).

Streptomyces represent the Gram-positive bacteria, which belong to the phylum Actinobacteria, and show a unique cellular developmental pattern as a distinguishing feature. The specific *Streptomyces* species derived from animal origins demonstrate exceptional adaptation capabilities in animal gastric conditions. Previous studies have revealed that *Streptomyces* species that originated from poultry droppings exhibited resistance when exposed to 2.3mg/mL pepsin in combination with 1mg/mL pancreatin and 0.3% bile. The ability of *Streptomyces* species to form spores, together with their high tolerance to bile acids, makes them viable alternative probiotic bacteria compared to bacterial strains that do not produce spores (Maqbool *et al.*, 2023; Ryandini *et al.*, 2024).

According to Wang *et al.* (2018), *Lactobacillus plantarum* demonstrates a strong capability to stop *Salmonella typhimurium* while simultaneously decreasing inflammatory processes and intestinal membrane disruptions. Similarly, Bilal *et al.* (2021) and Usman *et al.* (2024) have demonstrated that *Bacillus*-based probiotics affect different attributes of the host through specific effects unique to each strain. Distinct *Bacillus subtilis* probiotic strains exhibit an improved feed conversion ratio (FCR) alongside other growth performance parameters.

The worldwide rise of antimicrobial resistance requires immediate investigation into antibiotic alternatives that would protect poultry safety and poultry market profits across developing and developed nations. The present study was planned to investigate the beneficial effects of *Streptomyces* and *Bacillus* species collected from local soil on the growth and immunological functions of commercial broiler chickens. It was hypothesized that *Streptomyces* and *Bacillus*-based probiotics would enhance innate and adaptive immune responses in broiler chickens more effectively than single-strain or commercial probiotics and confer protection against pathogenic challenge.

MATERIALS AND METHODS

Sample collection: The Streptomyces and Bacillus species were isolated from soil samples taken at depths ranging from just below the surface to 20cm deep, from a variety of settings, such as agricultural fields and plant rhizospheres during the year 2021-22. Through microbiological characterization, Streptomyces, along with Bacillus, were identified, isolated, and then added to the broiler feed.

Experimental plan: The trial was conducted on day-old broiler chicks at the experimental poultry shed in the Pathology Department, University of Agriculture, Faisalabad, Pakistan, from February to April 2023. Before the arrival of chicks, the shed was thoroughly cleaned, disinfected, and fumigated to avoid any contamination from the previous flock. Day-old broiler chicks (n=90) were purchased from a nearby hatchery and kept in an experimental room where an ambient temperature of 32–35°C and a relative humidity of 50-70% were maintained. After an acclimatization period of seven days, these birds were randomly divided into six groups (n=15 each). Birds were given *ad libitum* basal feed supplemented with probiotic strains of *Streptomyces, Bacillus*, and commercial probiotics in treatment groups, except that the control group A was given basal feed only. The treatments were given from day eight till the end of the experimental trial (Day 42 of chicken age). Group A served as the control group and was offered a basal diet containing 17% proteins and drinking water. The basal feed was composed of maize, wheat byproducts, soybean meal, fats, minerals, vitamins, and amino acids. Group B was administered with *Streptomyces* sp. @ 1×10^9 CFU/kg; Group C was administered with *Bacillus* sp. @ 1×10^8 CFU/kg; Group D was administered a commercial probiotic (Probio^{TM}) @ 2g/kg; Group E was administered with *Streptomyces* sp. and *Bacillus* sp. @ 1×10^9 CFU and 1×10^8 CFU/kg, respectively, while Group F was administered with *Streptomyces* sp., *Bacillus* sp. and *E. coli* challenge @ 1×10^9 CFU and 1×10^8 CFU/kg of feed and 3×10^7 CFU/mL of broth culture. These birds were vaccinated according to the broiler vaccine schedule recommended by the National Disease Control Committee of Pakistan Poultry Association.

Immunological parameters evaluation: Different immunological parameters were recorded to find out potential beneficial effects of treatments on immune system functions including antibody titers against Newcastle Disease Virus (Timms and Alexander, 1977), and level of

antibodies against sheep red blood cells on 14th, 21st, 28th and 35th day of chicken age, as per the procedure described by Delhanty and Solomon (1966), phagocytic activity by carbon clearance assay after 3 and 15 minutes (Sarker *et al.,* 2000), avian incompetence with avian tuberculin at 24, 48 and 72h (Corrier and DeLoach, 1990). A total of 3 birds were taken from each group for slaughtering on each specified day. Spleen, thymus, and bursa samples were collected and preserved in 10% formalin solution for histological study, as described by Bancroft and Gamble (2008), and the weights of these immune organs were also recorded on 28, 35, and 42nd day of chicken age.

Statistical analysis: Mean values (±SD) of various parameters for birds of each group were computed. In order to ascertain the magnitude of variation in these parameters among birds of different groups, the data were subjected to ANOVA. Tuckey's test was applied for multiple means comparisons, where necessary. The level of significance was set at P≤0.05.

RESULTS

Absolute weight of immune organs: The analysis of the thymus absolute weight revealed significant treatment effects (P<0.05) on 35th and 42nd days, when compared to the control group. Group E demonstrated significantly greater control over the control group with the most pronounced thymotropic effect, achieving peak absolute weight (3.67±0.58g) by day 42. Group D showed significant secondary efficacy, while Groups B, C and F exhibited non-significant intermediate effects when compared with control by day 42. By day 35, birds of only Group E showed significantly higher thymus weight compared to the control group (P<0.05). The thymus weight of all other treatment groups differed non-significantly from that of the control group (Table 1).

Table 1: Absolute weight (g) of immune organs, including the thymus, spleen, and bursa of Fabricius at different experimental days in all experimental groups

Experimental Groups	Experimental Days		
	28th (Post treatment 20th day)	35th (Post treatment 27th day)	42nd (Post treatment 34th day)
Thymus			
A (Control)	1.33 ±0.58^{a}	1.33±0.58^{b}	2.00±0.00^{c}
B	1.67± 0.58^{a}	1.67±0.58^{b}	2.67±0.58^{bc}
C	1.33±0.58^{a}	1.00±0.00^{b}	2.00±0.00^{c}
D	2.33±0.58^{a}	2.00±0.00^{ab}	3.00±0.00^{ab}
E	2.67±0.58^{a}	3.00±0.00^{a}	3.67±0.58^{a}
F	1.67±0.58^{a}	1.67±0.58^{b}	2.00±0.00^{c}
Spleen			
A (Control)	0.83± 0.29^{b}	1.33±0.58^{c}	1.67±0.58^{a}
B	1.33 ±0.58^{ab}	3.00±0.02^{ab}	2.33±1.16^{a}
C	1.00 ±0.01^{ab}	2.00±0.03^{bc}	1.67±1.20^{a}
D	1.67 ±0.60^{ab}	2.33±0.57^{abc}	2.67±0.58^{a}
E	2.00 ±0.01^{a}	3.33±0.60^{a}	3.33±0.60^{a}
F	0.67 ±0.28^{b}	1.67±0.57^{c}	2.00±0.03^{a}
Bursa of Fabricius			
A (Control)	1.00 ±0.01^{c}	1.33±0.58^{b}	1.67±0.58^{b}
B	2.00± 0.01^{abc}	2.33±0.60^{ab}	3.00±1.00^{ab}
C	1.67±0.58^{bc}	1.67±0.58^{b}	2.00±0.01^{ab}
D	2.33±0.58^{ab}	2.67±0.58^{ab}	2.67±0.58^{ab}
E	3.00±0.02^{a}	3.33±0.57^{a}	3.67±0.60^{a}
F	1.33±0.60^{bc}	2.00±0.01^{ab}	2.00±1.00^{b}

Values (Means±SD) having similar alphabets in a column for each organ are statistically non-significant (P>0.05): A=Control, B=*Streptomyces* sp., C=*Bacillus* sp., D=Commercially probiotic (Probio™), E=*Streptomyces* sp. + *Bacillus* sp., F=*Streptomyces* sp. + *Bacillus* sp.+ *E. coli* Challenge.

The assessment of spleen absolute weight revealed non-significant treatment effects (P<0.05) compared to control (Group A) by day 42. Group E did not significantly demonstrate the most pronounced effect, achieving peak absolute weight (3.33±0.60g) by day 42nd. Birds of Groups B and E showed significantly (P<0.05) higher spleen weight than control at day 35. At day 28, Group E exhibited significantly (P<0.05) higher spleen weight than the control. The control group exhibited a gradual increase in spleen weight as the study was advanced (Table 1).

The absolute weight measurements of the bursa revealed significant (P<0.05) treatment effects in group E compared to control across the experimental timeline. Group E demonstrated the most substantial bursal growth, achieving the highest absolute weight (3.33±0.57 and 3.67±0.60g) by day 35th and 42nd. Birds of Groups D and E showed significantly higher bursa weight than those of the control group by day 28th. Control group A tended to maintain its normal weight throughout the study period (Table 1).

Lymphoproliferative response to Avian Tuberculin: The lymphoproliferative response to avian tuberculin revealed significant treatment-dependent variations across all time points (24, 48, and 72h). Birds of Group D and Group E consistently demonstrated the strongest immune reactivity, maintaining peak responses at the three measured intervals (0.68±0.02 to 0.34±0.05mm for Group D; 0.68±0.01 to 0.35±0.04mm for Group E). These groups showed significantly (P<0.05) higher lymphoproliferation rates compared to the control (Group A), suggesting enhanced cell-mediated immunity. Groups D and E showed the highest stimulation indices at 24h. Group B exhibited moderate but consistent responses (0.55±0.01 to 0.29±0.04mm) and showed better maintenance of proliferation at later points compared to control. Groups A (control) and C displayed baseline reactivity patterns; an expected temporal decline in proliferation rates was observed, as shown in Fig. 1a. Group F showed a decrease in lymphoproliferative response compared to other groups.

Carbon clearance assay: The carbon clearance assay demonstrated significant treatment-enhanced macrophage activity across all experimental groups. Control birds (Group A) showed characteristic slow clearance rates (OD values) (103.35±3.14 at 3 minutes, declining to 73.51±2.83 at 15 minutes), while treatment groups exhibited markedly accelerated clearance kinetics. Birds of Group E achieved maximal clearance (66.41±2.98 to 33.45±2.95) and Group D showed comparable efficacy (66.57±2.88 to 35.46±3.07) to control at 15 minutes. A significant (P<0.05) improvement over control was recorded (67.81±2.93 to 37.57±2.79) for Group B and maintained consistent clearance patterns. Group F displayed significantly reduced efficacy, as shown in Fig. 1b. The data revealed two distinct phases: Initial Phase (3-min), when all treatment groups already showed significantly (P<0.05) lower carbon levels than control. Immediate treatment effects were evident. Secondary Phase (15-min), when the clearance rates diverged further. Group E maintained significantly (P<0.05) strongest activity (Fig. 1b).

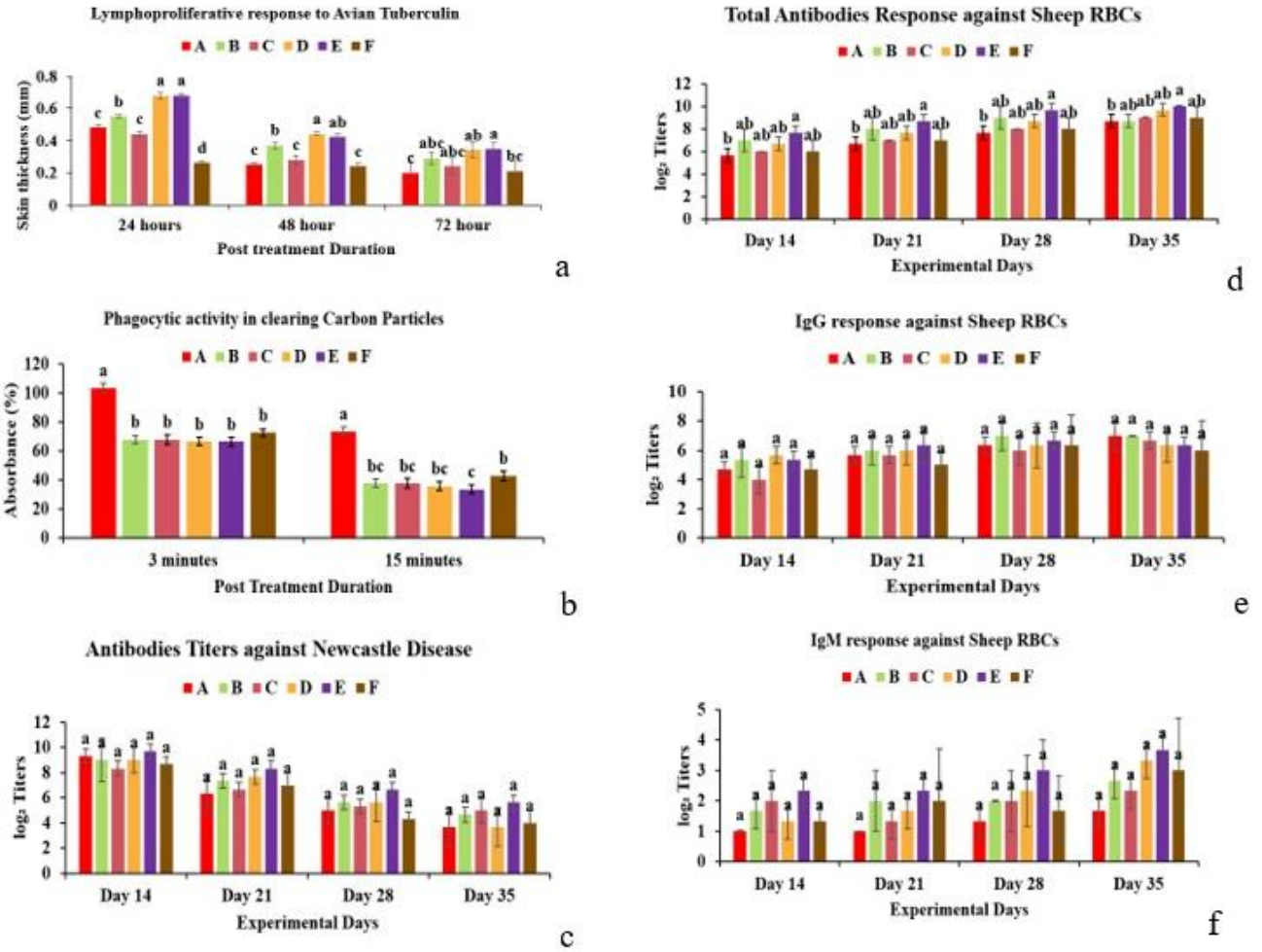

Fig. 1: Effects of treatments on immunological parameters of birds, including a): avian tuberculin, b): Phagocytic activity, c): Newcastle disease vaccine, and d-f): sheep RBCs, total IgG, and IgM. Group A=Control, B=*Streptomyces* sp., C=*Bacillus* sp., D=Commercially probiotic (Probio^{TM}), E=*Streptomyces* sp.+ *Bacillus* sp., F=*Streptomyces* sp.+*Bacillus* sp.+*E. coli* Challenge.

NDV-specific antibody response: The NDV-specific antibody response demonstrated distinct temporal patterns across experimental groups, revealing non-significant treatment effects on vaccine-induced immunity. Control birds (Group A) exhibited characteristic antibody decline from 9.33±0.57 (Day 14) to 3.67±1.53 (Day 35), representing typical post-vaccination kinetics. Superior responder (Group E) maintained the highest non-significant titers throughout the study period, achieved peak response (9.67±0.58) at Day 14, and demonstrated gradual decline (6.67±0.58 at Day 28 and 5.67±0.58 at Day 35). At Peak Response Phase (Days 14-21), all groups showed non-significantly comparable high NDV antibody titers (Fig. 1c).

Total antibodies against sheep RBCs: The anti-sheep RBC antibody response (Fig. 1d) revealed significant treatment-dependent enhancement across all measured time points in Group E (days 14-35). Control birds (Group A) demonstrated baseline antibody production (5.67±0.58 to 8.67±0.58), while treatment groups exhibited non significantly elevated and sustained humoral responses compared to control, except group E. Group E maintained significantly better antibody titers throughout (7.67±0.58 to 10.00±0.03) than the control. Group E demonstrated ≈11% higher final titers than the control ($P<0.05$). All groups showed progressive antibody accumulation through Day 35. Treatment effects became increasingly distinct over time. Group E demonstrated both rapid onset and sustained maintenance (Fig. 1d).

IgG and IgM response against sheep RBCs: The IgG antibody response against sheep RBCs demonstrated non-significant progressive enhancement in probiotic-treated groups compared to controls, with all treatment groups showing elevated titers by Day 35. Groups B, D, and E exhibited non-significantly strong responses, peaking at Day 28, with Group E reaching the highest titer (6.67±0.58). However, control birds showed expected immune development (4.67±0.57 to 7.00±1.00), as shown in Fig. 1e.

The IgM antibody response against sheep RBCs showed non-significant distinct temporal patterns among treatment groups, with Group E demonstrating the most robust and sustained primary immune response. Group E maintained non-significantly elevated titers throughout the study, peaking at 3.67±0.58 by Day 35. This enhanced primary response suggested more effective early antigen recognition and B-cell activation in Group E. Group E uniquely maintained this elevated response through Day 35 (Fig. 1f).

Histological examination of immune organs: The histological examination of lymphoid organs from broilers supplemented with a combination of *Streptomyces-Bacillus* probiotics revealed microstructural improvements compared to controls. The thymus exhibited cortical

hyperplasia with dense lymphocyte populations and well-defined corticomedullary junctions, supporting the 83% greater thymic weight and explaining the improved lymphoproliferative responses in group E and moderately defined cortex, medulla and lobules in other treatment groups. Group A showed normal thymic architecture with distinct cortical and medullary regions, densely packed lymphocytes in the cortex, and a thin connective tissue capsule. Group B showed intact lobular structure with mildly increased lymphocytic density and enhanced corticomedullary definition, suggesting improved immune activity. Group C showed compact thymic lobules, prominent lymphocytic population in the cortex, and clear medullary regions, indicating lymphoid proliferation and functional stimulation. Group D showed well-developed lobules with hypercellular cortex and well-defined medulla, reflecting probiotic-induced thymic activation and improved lymphopoiesis. Group E showed the most organized thymic architecture with thick cortex, abundant lymphocytes, and distinct corticomedullary junctions, indicating synergistic immunomodulatory effects. Group F showed mild cortical depletion, slight congestion, and thinning of lobular boundaries; however, partial structural preservation indicates a protective role of probiotics under infectious stress (Fig. 2).

In the spleen, Group E showed expanded white pulp with prominent germinal centers, indicating enhanced B- and T-cell activity, along with increased red pulp cellularity that correlates with the observed 2.2-fold greater macrophage phagocytic activity. Group A showed normal splenic architecture with well-organized white pulp (lymphoid follicles) and red pulp regions, intact capsule, and healthy trabeculae. Group B showed prominent lymphoid follicles with slight expansion of white pulp and increased lymphocytic proliferation, indicating mild immunostimulatory activity. Group C showed distinct periarteriolar lymphoid sheaths (PALS) and densely packed lymphocytes with well-maintained white and red pulp demarcation, suggesting enhanced immune response. Group D showed well-developed white pulp with hyperplastic lymphoid follicles and uniform tissue organization, reflecting improved splenic immune competence. Group E showed highly organized splenic structure with extensive lymphoid cell proliferation, expanded white pulp, and active germinal centers, indicative of synergistic probiotic immunostimulant. Group F showed mild lymphoid depletion, congestion in red pulp, and few degenerative changes; however, partial preservation of follicular structure suggests protective probiotic effects against infection (Fig. 3).

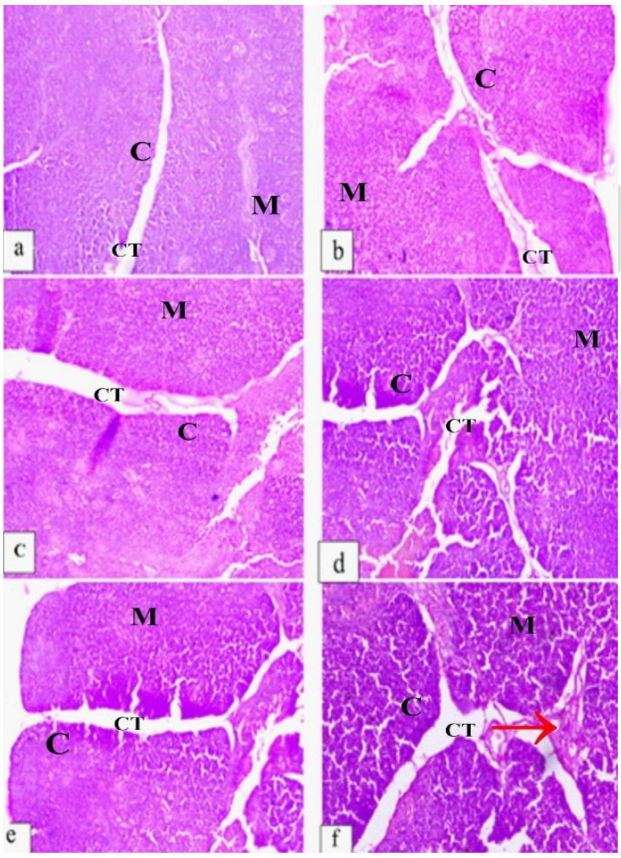

Fig. 2: Photomicrograph of thymus showing: (a): normal thymic architecture with distinct cortical (C) and medullary regions (M), densely packed lymphocytes in the cortex, and a thin connective tissue (CT) capsule. (b): Mildly increased lymphocytic density and enhanced corticomedullary definition. (c): compact thymic lobules, prominent lymphocytic population in the cortex, and clear medullary regions. (d): well-developed lobules with hypercellular cortex and well-defined medulla. (e): the most organized thymic architecture with thick cortex, abundant lymphocytes, and distinct corticomedullary junctions. (f): mild cortical depletion (Red arrow), slight congestion, and thinning of lobular boundaries; H&E, 40X.

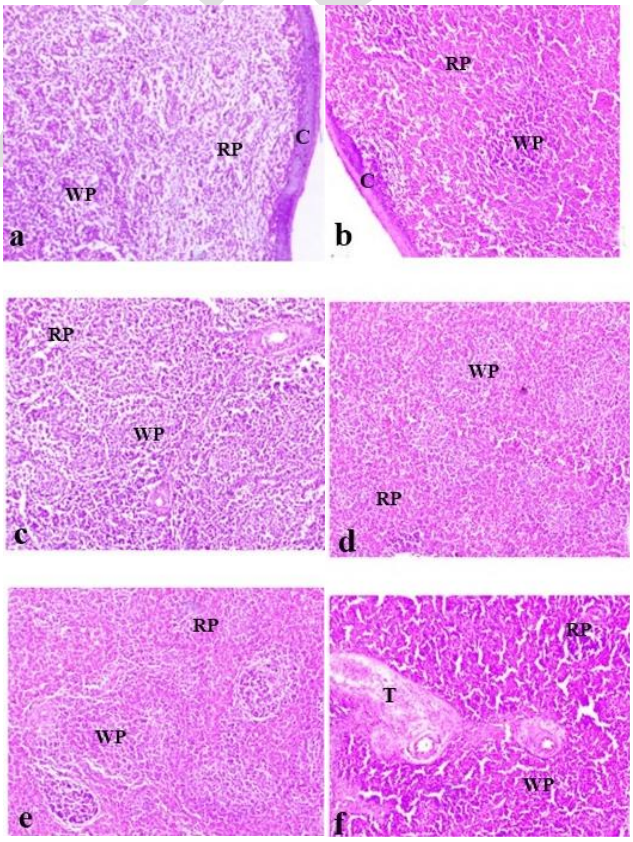

Fig. 3: Photomicrograph of spleen showing: a): normal splenic architecture with well-organized white pulp (lymphoid follicles-WP) and red pulp (RP) regions, intact capsule, and healthy trabeculae. b): prominent lymphoid follicles with slight expansion of white pulp and increased lymphocytic proliferation. c): densely packed lymphocytes with well-maintained white and red pulp demarcation d): well-developed white pulp with hyperplastic lymphoid follicles and uniform tissue organization. e): highly organized splenic structure with extensive lymphoid cell proliferation, expanded white pulp, and active germinal centers. f): mild lymphoid depletion, congestion in red pulp, and few degenerative changes; (H&E, 100X).

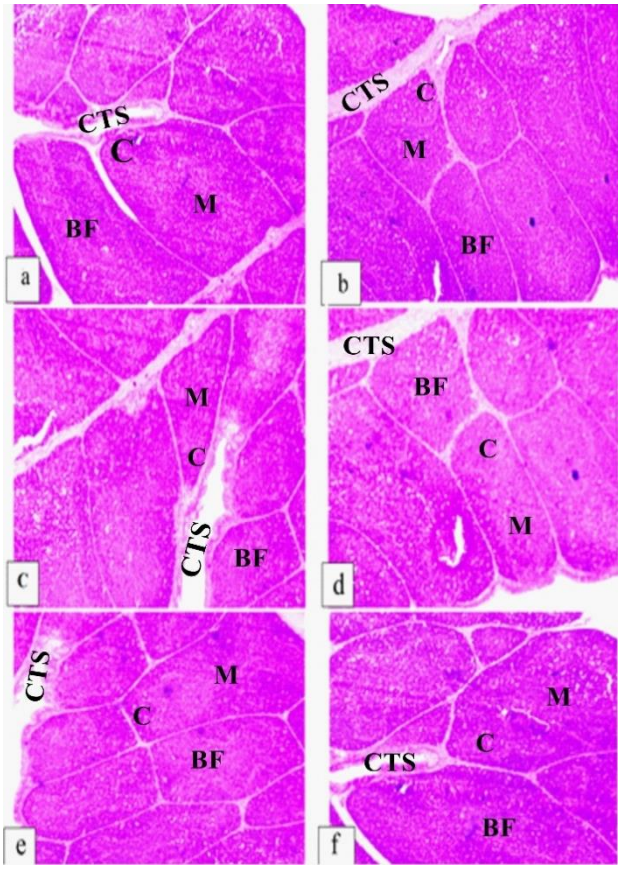

Fig. 4: Photomicrograph of Bursa showing: a): normal histoarchitecture with well-defined bursal follicles (BF), distinct cortical (C) and medullary (M) zones, and intact interfollicular epithelium. b): slightly increased lymphoid cell density. c): dense bursal follicles with compact lymphocytes and intact epithelial covering. d): moderate lymphoid hyperplasia and well-maintained cortical-medullary demarcation. e): prominent bursal follicular development. f): mild to moderate lymphoid depletion and disrupted follicular boundaries. (H&E, 40X). (Note: CTS stands for connective tissue septum).

Bursal follicles displayed preserved epithelial integrity and well-defined cortex and medulla in Group E compared to control. At the same time, all other groups also showed well-defined microstructures. Group A showed normal histoarchitecture with well-defined lymphoid follicles, distinct cortical and medullary zones, and intact interfollicular epithelium. Group B showed normal follicular structure with slightly increased lymphoid cell density, indicating mild immunostimulatory activity. Group C showed dense lymphoid follicles with compact lymphocytes and intact epithelial covering, suggesting enhanced immune cell proliferation. Group D showed healthy follicles with moderate lymphoid hyperplasia and well-maintained cortical-medullary demarcation. Group E showed prominent follicular development, abundant lymphoid cells, and well-organized epithelial structure, indicating synergistic probiotic effects. Group F showed mild to moderate lymphoid depletion and disrupted follicular boundaries; however, partial preservation of structure suggests protective effects of probiotic co-administration (Fig. 4).

These histological findings provided structural evidence for the functional immune enhancements observed in humoral and cellular immunity assays, with Group E consistently showing superior preservation and activation of lymphoid microarchitecture that suggested its immunomodulatory efficacy. The probiotic's ability to maintain lymphoid organ integrity during challenge conditions suggested its value as a prophylactic agent in poultry production systems.

DISCUSSION

Global poultry industry is facing serious threats due to massive increase in antimicrobial resistance (AMR) and there is a dire need for the immediate and innovative solution. Keeping in view the demands of the industry for the alternative approaches this study was planned and executed. The results of this research demonstrated that *Streptomyces-Bacillus* probiotic combinations can be a solution for the sustainable and safer poultry production strategies. the current study revealed comprehensive immunomodulatory effects through innate, cellular, and humoral immunity, being a more effective and promising alternative to antibiotics, addressing a priority research area identified by the World Organization for Animal Health (de Mesquita *et al.*, 2022) for antimicrobial stewardship in livestock production.

The immunomodulatory effects of probiotics are increasingly recognized as critical to their mode of action. Research indicates that probiotics can stimulate both innate and adaptive immune responses by interacting with gut-associated lymphoid tissue (GALT) and systemic immune organs (Cristofori *et al.,* 2021; Bidura *et al.,* 2024). In the present study, the *Streptomyces-Bacillus* blend (Group E) significantly increased thymus and bursa weights compared to controls at 35th and 42nd days of experimental trials, suggesting enhanced T- and B-cell development. These findings align with those of Cristofori *et al.* (2021), who reported that *Streptomyces*-derived metabolites promote lymphoid organ hyperplasia through Toll-like receptor (TLR) activation. Similarly, current study observation of improved macrophage phagocytosis (2.2-fold increase) supports the findings of Macpherson and Uhr (2004), who demonstrated that Bacillus probiotics enhance myeloid cell function via the interleukin-1β (IL-1β) signaling pathway.

Recent advances in microbiome science have revolutionized current study understanding of host-microbe interactions in poultry (Broom, 2019). The gut-immune axis, in particular, has emerged as a key mediator of probiotic effects, with the avian cecal microbiome playing a crucial role in immune system development (Broom and Kogut, 2018). Thus, the *Streptomyces-Bacillus* probiotics can systemically influence immune organ development and function. The 83.5% increase in thymus weight in birds of Group E compared to those of control group at 42nd day of age observed in the present study suggests profound effects on T-cell maturation, potentially mediated through microbial metabolite signaling to thymic epithelial cells (TECs), as recently described by Nanjundappa *et al.* (2022). This thymotrophic effect of *Streptomyces-Bacillus* probiotics could have far-reaching implications for poultry health, given the central role of the thymus in establishing immune competence during early development (Larsberg *et al.,* 2024).

The increase in bursal weight in this trial is particularly noteworthy in light of discoveries about avian B-cell biology (Scott, 2004). The latest genomic analyses reveal

that the bursa serves not just as a site for B-cell development, but also as a reservoir for regulatory B-cells that modulate systemic immunity (Ratcliffe and Hartle, 2022). In this study, observation of elevated IgG responses may therefore reflect both quantitative and qualitative improvements in B-cell function induced by the probiotic combination. This aligns with cutting-edge research demonstrating that specific microbial metabolites can directly influence immunoglobulin switching through epigenetic modifications in B cells (Calciolari *et al.,* 2022).

The humoral immune responses observed in current study further emphasize the potential of *Streptomyces-Bacillus* probiotics as immunomodulators. The 37% increase in IgG titers against sheep red blood cells (SRBCs) are consistent with a recent report suggesting that probiotics can act as vaccine adjuvants (Gharajalar *et al.,* 2020).

At the cellular level, the 2.2-fold increase in macrophage activity observed in this study aligns with breakthroughs in understanding avian macrophage biology (John *et al.*, 2024). Single-cell RNA sequencing studies have revealed significant heterogeneity in chicken macrophage populations, with distinct subsets specialized for pathogen clearance versus immune regulation (Zhang *et al.*, 2025). Current study findings suggest that the Streptomyces-Bacillus combination may be preferentially activating the phagocytic subsets, possibly through stimulation of pattern recognition receptors (PRRs), as described by Ankati and Podile (2018). This targeted immune modulation could explain the excellent protection against *E. coli* challenge without causing excessive inflammation (Fitriadi *et al.*, 2024).

The vaccine adjuvant effects observed in this experimental trail (25% higher NDV titers) gain particular significance considering recent industry challenges. The global avian influenza pandemic highlighted critical gaps in poultry vaccine efficacy (Domínguez-Odio *et al.,* 2025), creating urgent demand for immune-enhancing feed additives. This study results suggest that *Streptomyces-Bacillus* probiotics could help bridge this gap by amplifying vaccine responses through multiple mechanisms recently elucidated by Lynn *et al.* (2022), including dendritic cell activation and cytokine milieu modulation.

From an ecological perspective, the environmental sustainability of *Streptomyces*-based probiotics deserves special consideration. Life cycle assessments of poultry production systems indicate that antibiotic alternatives must demonstrate not just efficacy but also environmental compatibility (Kheiralipour *et al.,* 2024). Fortunately, *Streptomyces* species are naturally abundant soil organisms with well-established roles in nutrient cycling (Shepherdson *et al.,* 2023), suggesting minimal ecological disruption from their use in poultry production. This environmental profile represents a significant advantage over some synthetic alternatives currently under development (Chauhan *et al.,* 2023).

The economic implications of current study findings warrant careful examination. Recent modeling by Guerrand (2018) suggests that widespread adoption of effective antibiotic alternatives could save the global poultry industry $3-5 billion annually in productivity losses and antimicrobial costs. Current study *Streptomyces-Bacillus* combination, with its dual benefits of immune enhancement and pathogen protection, appears well-positioned to contribute to these savings. However, as noted in recent techno-economic analyses by Al-Shaibani *et al.* (2021), successful commercialization will require optimization of production processes and demonstration of consistent field performance.

Looking ahead, several promising research directions emerge from current study findings. The burgeoning field of microbial consortia engineering (Parvin and Sadras, 2024) offers tools to further optimize current study probiotic combination, potentially by incorporating additional functional strains. Advances in precision fermentation (Nazir *et al.,* 2024) could enhance the production of key immunomodulatory metabolites. Meanwhile, emerging technologies like gut-on-a-chip systems (Nugmanova *et al.*, 2024; Yang *et al.*, 2025) could provide novel platforms for mechanistic studies of host-probiotic interactions.

The societal implications of this research extend beyond poultry production. As noted by Kasimanickam *et al.* (2021), reducing agricultural antibiotic use is critical for addressing the broader AMR crisis. This study demonstrates that carefully designed probiotic combinations can maintain animal health while reducing reliance on antibiotics contributes directly to several Sustainable Development Goals, particularly SDG-3 (Good Health and Well-being) and SDG-12 (Responsible Consumption and Production). Recent surveys of poultry producers (Leistikow *et al.,* 2022) have identified several barriers to probiotic adoption, including cost concerns and variable efficacy. Current study future work should therefore focus on developing cost-effective formulations and establishing clear management protocols for implementing antibiotic alternatives in livestock systems.

Conclusions: Current study investigation of *Streptomyces-Bacillus* probiotics illuminates a promising path forward for sustainable poultry production. The multi-faceted immune enhancements evaluated in this study, combined with significant protection against bacterial challenge, position these probiotics as a viable cornerstone for antibiotic-free production systems. As the industry navigates the complex transition away from routine antibiotic use, solutions like using probiotics that simultaneously address animal health, economic viability, and environmental sustainability will be essential. Future research should build on these findings to fully realize the potential of microbial solutions in meeting the global demand for safe and sustainable poultry proteins.

Authors' contribution: Haotian Chen, Namra Kanwar and Shazia Khaliq planned, executed the research. Muhammad Kashif Saleemi, Naima Waheed and Ahrar Khan provided help to execute this project through materials and finances and also in the editing of the draft. All the members were actively involved in write up and editing of the manuscript.

Ethical approval: This study was conducted keeping in view all the considerations for animal welfare (husbandry and euthanasia) and it was duly approved by the Graduate studies and research board (GSRB), University of

Agriculture, Faisalabad, Pakistan vide letter No./DGS/6561-6, dated: 11-06-2025.

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