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Effect of dietary supplementation of *Bacillus subtilis, Lactobacillus plantarum* and mannan oligosaccharides on immune response in broiler chicken

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Abstract

A study was conducted to study the influence of dietary supplementation with Lactobacillus plantarum, Bacillus subtilis, mannan-oligosaccharides (MOS) and their combinations on intestinal immune response in broiler chicken. A total of 216-day-old Cobb broiler chicks were assigned to six treatment groups with each group comprising three replicates of 12 birds each, ensuring a completely randomised design (CRD): T1 (control), T2 (L. plantarum), T3 (B. subtilis), T4 (MOS), T5 (L. plantarum + MOS) and T6 (B. subtilis + MOS). The humoral immune response was assessed by measuring antibody titres against the Newcastle disease vaccine using the haemagglutination inhibition (HI) test at 7, 14, 21, 28, and 35 days of age. Significant differences in antibody titres were observed only on the 28th day, with the group receiving B. subtilis and MOS (T6) exhibiting the highest titre. The results suggest that B. subtilis, particularly in combination with MOS, enhanced the vaccine-induced immune response at specific time points. The cell-mediated immune response was evaluated through cutaneous hypersensitivity reactions to 2,4-dinitrochlorobenzene (DNCB) at 24, 48 and 72 hours post-injection, though numerical differences were noted, particularly in the T6 group at 48 hours, the variations were not statistically significant. The findings highlighted that dietary supplementation with probiotics and prebiotics had enhanced immune responses during specific periods but with consistent results. Future research may focus on optimising supplementation strategies and exploring mechanisms to maximize the immunomodulatory potential of probiotics and prebiotics in broilers.

Keywords: *Bacillus subtilis, Lactobacillus plantarum*, Mannan-oligosaccharide, immune response, broiler

In modern poultry production, optimising the immune response of broiler chicken is critical for enhancing health, growth performance, and resistance to disease. With increasing global efforts to phase out antibiotic growth promoters (AGPs), alternative strategies such as probiotics and prebiotics have garnered significant attention in recent years. These feed additives inhibit pathogenic bacterial load, improve immune response, reduce inflammatory reactions and improve gut health in poultry (Vineetha and Shamna, 2024). Among the most promising functional feed additives,

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Bacillus subtilis (*B. subtilis*), *Lactobacillus plantarum* (*L. plantarum*) and mannan-oligosaccharides (MOS) have been shown to improve gut health and boost immunity by modulating the gut microbiome and reinforcing intestinal integrity. *Lactobacillus plantarum* exhibits strong probiotic potential due to its high acid and bile tolerance, cell surface hydrophobicity, autoaggregation and coaggregation abilities (Anija *et al.*, 2023).

Bacillus subtilis, a robust spore-forming probiotic, promotes the secretion of antimicrobial peptides, enhances the production of short-chain fatty acids, and outcompetes pathogenic bacteria, thereby supporting gut and immune health (Gadde *et al*., 2017; Al-Fataftah *et al*., 2014; Park *et al*., 2020). *L. plantarum*, a lactic acid bacterium, strengthens the intestinal barrier, reduces inflammation, regulate the innate and adapted cellular and humoral immunity (Zhou *et al*, 2023; Kim *et al*., 2024; Wang *et al.*, 2015). Meanwhile, MOS, a prebiotic derived from yeast cell walls, prevents harmful bacteria from adhering to the intestinal lining, enhances macrophage activity, and modulates cytokine expression to promote a stronger immune response (Lu *et al*., 2022).

The combined supplementation of *B. subtilis, L. plantarum* and MOS is hypothesised to yield synergistic effects, amplifying immune benefits more effectively than individual use. By supporting both innate and adaptive immunity, these additives reduce disease incidence and mortality in broilers, offering a sustainable alternative to AGPs. This study investigates the effects of these supplements, individually and in combination, on broiler chickens' immune response, aiming to provide insights into advancing sustainable and efficient poultry production practices.

Materials and methods

The experiment was conducted at Department of Poultry Science, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (KVASU), Mannuthy, Thrissur. A total of 216-day-old broiler chicks (Cobb 430Y) were procured from Ayisha Hatchery, Koduvayoor, Palakkad for the experiment. The chicks were wing-banded for dentification and weighed to record their initial body weight. The chicks were then randomly assigned to six treatment groups with each group comprising three replicates of 12 birds each, ensuring a completely randomised design (CRD). The experiment was conducted over a period of 42 days, from day old to 42 days of age.

Experimental diets

Standard basal diets were formulated to meet the nutrient requirements of broilers as per BIS (2007) guidelines. The birds were fed with a pre-starter diet up to seven days of age, a starter diet from eight to twenty-one days of age and a finisher diet up to 42 days of age. The ingredients composition of the basal diet is presented in Tables 1. The experimental diets were prepared by incorporating the respective supplements into the basal diets.

Table 1. Ingredient composition of the basal diets (%)

Ingredients	Pre-starter	Starter	Finisher
Yellow maize	51.97	52.72	57.34
Soyabean meal	41.50	39.2	33.6
Rice bran oil	2.64	4.10	5.10
Dicalcium phosphate	1.80	1.80	1.80
Calcite	1.40	1.40	1.40
Salt	0.38	0.36	0.31
L-Lysine	0.14	0.24	0.20
DL -Methionine	0.17	0.18	0.25
Total	100	100	100

Treatment groups

The treatment groups and their diets are described in Table 2. Each treatment group received their respective diets throughout the experimental period.

Antibody titre against Newcastle disease vaccine

The chicks were vaccinated with live attenuated Newcastle disease vaccine at 7 and 21 days of age. Blood samples from three birds in each treatment were collected at seventh day (before vaccination) and then at 7, 14, 21 and 28 days post-vaccination to assess the humoral immune response. The antibody titre in the serum against Newcastle Disease was tested using the standard Haemagglutination Inhibition test (HI) as per OIE (2021).

Cutaneous hypersensitivity reaction

At 28 days of age, three chicks from each treatment group were inoculated with 0.25 mL of 2,4-dinitrochlorobenzene (DNCB) in the interdigital space between the third and fourth toes of the right foot by intradermal injection. In the same interdigital space of the

Table 2. Treatment groups and their respective diets

Treatments	Diets
T1	Basal diet (Control)
T2	Basal diet supplemented with *L. plantarum* (10^8 CFU/kg)
T3	Basal diet supplemented with *B. subtilis* (10^8 CFU/kg)
T4	Basal diet supplemented with MOS (1g/ kg)
T5	Basal diet supplemented with *L. plantarum* (10^8 CFU/kg) and MOS (1 g/kg)
T6	Basal diet supplemented with *B. subtilis* (10^8 CFU/kg) and MOS (1 g/kg)

left foot of the same bird, 0.25 mL acetone was injected as a control. Just before inoculation and at 24, 48 and 72 h post-challenge, the cell reaction was evaluated by measuring skin thickness at the injected area with a vernier calliper. Based on the above measurements, the following calculations were made (Sajadifar *et al.,* 2013).

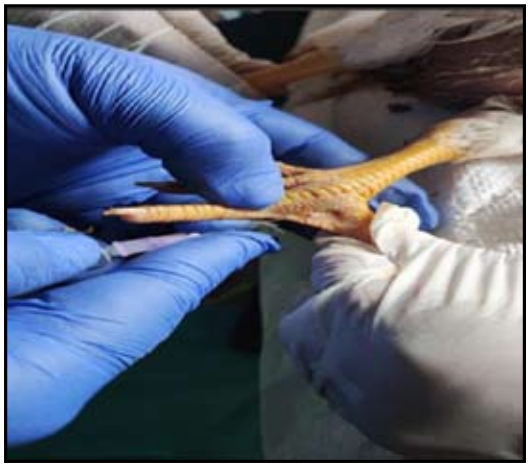

Fig.1. Inoculation of DNCB in the interdigital space

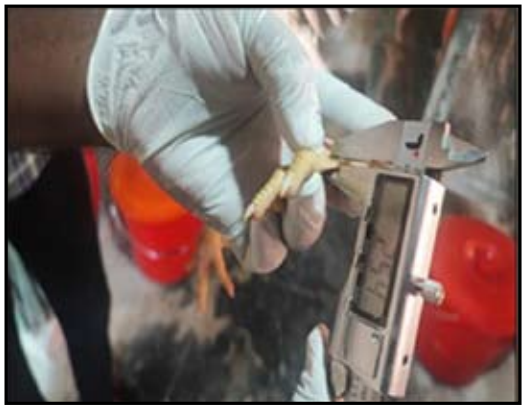

Fig.2. Measuring skin thickness

DNCB response is calculated as the difference between thickness of the right foot web before and after DNCB inoculation (mm). Acetone control response is calculated as thickness of the left foot after acetone inoculation and thickness of the left foot before DNCB inoculation (mm). The cutaneous hypersensitivity at each evaluation time was calculated as the difference between DNCB and acetone response.

Result and discussion

The present study assessed the antibody titre (log2) against the Newcastle disease vaccine in broilers subjected to various dietary treatments, using the haemagglutination inhibition (HI) test to evaluate the immune response at different days post-vaccination. The results revealed no statistically significant differences in antibody titres at most intervals, with the exception of day 28, where significant differences were observed among the treatment groups (Table 3). These findings suggest that the dietary treatments containing *B. subtilis* and MOS, enhances the immune response to the Newcastle disease vaccine at specific time and the overall impact was not consistent.

On 7th day of vaccination, all treatment groups exhibited similar baseline antibody titres, indicating comparable initial immune status across the groups before the vaccine-induced response. These findings are in line with study by Malik *et al.* (2016), which showed no significant differences in serum antibody titres against Newcastle disease virus on day 7 post-vaccination, suggesting that the dietary treatments did not influence the birds' baseline immune response.

By day 28 (seven days after the booster dose), statistically significant differences in antibody titres were observed among the treatment groups. The T6 group (*B. subtilis* + MOS) exhibited the highest antibody titre, which was significantly greater than that of the control (T1) and some other treatment groups. The T3 group (*B. subtilis*) also showed relatively high antibody titres, though not significantly different from T6. These results suggest that *B. subtilis*, particularly in combination with MOS, may enhance the immune response to the Newcastle disease vaccine. This is consistent with findings by Rahimi (2009), who reported that dietary supplementation with a probiotic mixture containing *B. subtilis* significantly improved serum antibody titres against Newcastle disease virus and Al-Sultan *et al.* (2016) who observed that supplementation with MOS (1 g/kg feed) *E. faecium* and their combination significantly ($p < 0.05$) enhanced the post-vaccination Newcastle disease vaccine antibody levels in serum at 3 weeks post-vaccination. Mohsin *et al.* (2022) also reported that the probiotic supplemented groups showed

Table 3. Antibody titre against Newcastle disease vaccine

Age (Days)	Treatments						p-value
	T1 (Control)	T2 (*L. plantarum*)	T3 (*B. subtilis*)	T4 (MOS)	T5 (*L. plantarum* + MOS)	T6 (*B. subtilis* + MOS	
7	4.67±0.33	4.67±0.33	5.00±0.58	5.00±0.58	5.67±0.88	5.33±0.88	0.86
14	5.33±0.33	7.00±1.15	5.33±0.33	6.67±0.33	5.00±0.00	6.00±0.00	0.10
21	5.67±0.33	6.67±1.20	5.67±0.67	5.33±0.33	6.33±1.33	5.67±0.33	0.85
28	6.00^{c}±0.58	7.33^{abc}±0.67	7.67^{ab}±0.33	6.33^{bc}±0.33	6.33^{bc}±0.33	8.00^{a}±0.00	0.03
35	5.67±0.33	6.33±0.88	5.33±0.33	6.00±0.00	6.00±0.58	6.33±0.33	0.68

*Different superscript within a row differs significantly

significantly higher humoral immunity with higher antibody titres.

In present study the T6 group (*B. subtilis* + MOS) exhibited the highest antibody titre, seven days after second immunisation, which was significantly greater than that of the control, which is in line with the report of Yin *et al. (*2023) demonstrated that the antibody potency on the seventh day after secondary immunisation was significantly ($p \leq 0.05$) higher in the group supplemented with probiotics compared to the control group.

The potential mechanism behind this immune-enhancing effect may be related to the role of *B. subtilis* in modulating gut microbiota and stimulating the immune system, as noted by Ebeid *et al.* (2021).

In contrast, at other time points (days 7, 14, 21and 35), no statistically significant differences in antibody titres were observed among the treatment groups (Table 3). The lack of significant differences in these intervals suggests that the dietary treatments did not consistently influence the birds' immune response throughout the study. These findings are similar to those of Rehman *et al.* (2020) who found that the development of humoral immunity against the Newcastle disease vaccine was not significantly affected by the supplementation of MOS.In additional Malik *et al.* (2016) reported that probiotics such as *B. subtilis* could improve humoral immunity, but the effects were not always significant across all time points. Similarly, Al-Khalaifa *et al.* (2019) found that MOS can improve humoral immune responses in broiler.

For day 35, the final day of measurement, the T6 (*B. subtilis* + MOS) and T2 (*L. plantarum*) groups had the highest antibody titres, but the differences were not statistically significant. This suggests that the immune-enhancing effects of certain dietary treatments may diminish over time, or that the birds' immune response reaches a plateau. Similar trend was also observed by Hassanpour *et al.* (2013), who reported that synbiotic supplementation led to improved immune responses.

Cutaneous hypersensitivity reaction

The effect of dietary supplementation with *B. subtilis*, *L. plantarum*, MOS and their combinations on cell-mediated immunity in broilers, measured through the cutaneous hypersensitivity response at 24, 48 and 72 hours post-injection at 28 days of age is shown in Table 4. Results revealed no statistically significant differences in hypersensitivity responses among the treatment groups at any time point. At 24 hours, all the treatment exhibited similar low-level responses, with *B. subtilis* (T3) showing slightly higher values compared to the control, but this difference was not significant. This indicates that dietary interventions had minimal influence on the early-phase immune response, aligning with previous studies suggesting limited immediate effects of probiotics and prebiotics on hypersensitivity reactions.

At 48 hours, the *B. subtilis* + MOS (T6) group exhibited the highest response, while the *L. plantarum* + MOS (T5) group had the lowest. However, these differences were not statistically significant, suggesting that probiotics and prebiotics did not consistently enhance mid-phase cell-mediated immunity. This is in contrast with findings of Mohsin *et al.* (2022) who reported an improved cell-mediated immune response to DNCB in all probiotic-supplemented groups ($p < 0.05$), compared control group and Al-Khalaifa *et al.* (2019) who observed improved cell-mediated immune responses on MOS supplementation in broiler chicken.

By 72 hours, the *L. plantarum* (T2) and *B. subtilis* (T3) groups showed the highest responses, though differences remained statistically non-significant. The absence of a strong delayed-type hypersensitivity response suggests that the dietary treatments did not substantially enhance long-term cell-mediated immunity. This aligns with the studies of Karimi *et al.* (2010), where probiotic supplementation failed to significantly impact hypersensitivity responses to DNCB in broilers. However, Biswas *et al.* (2019), reported significant improvements in cell-mediated immunity with MOS supplementation. The lack of significant results in this study could be attributed to factors such as dosage, duration of supplementation, or variability in immune response among broilers. In contrast to the present findings of Ebeid *et al.* (2021), found that probiotics such as *L. plantarum* and *L. acidophilus* could enhance cell-mediated immunity.

The discussion highlights the complexity of modulating cell-mediated immunity through dietary interventions. While some studies have demonstrated

Table 4. Mean (±SE) value of cutaneous hyper sensitivity reaction in broilers in different dietary treatments at 28 days of age

Time interval (h)	Treatments						p -value
	T1 (control)	T2 (*L. plantarum*)	T3 (*B. subtilis*)	T4 (MOS)	T5 (*L. plantarum* + MOS)	T6 (*B. subtilis* + MOS	
24	0.11±0.04	0.25±0.16	0.27±0.10	0.14±0.09	0.18±0.09	0.21±0.07	0.86
48	0.83±0.58	0.63±0.16	0.88±0.46	0.78±0.54	0.40±0.07	1.03±0.54	0.94
72	0.23±0.16	0.74±0.40	0.71±0.12	0.63±0.27	0.19±0.03	0.18±0.07	0.24

the potential of probiotics and prebiotics to enhance immune responses, the effects are often variable and context-dependent. The absence of significant effects in this study suggests that *B. subtilis*, *L. plantarum* and MOS may require longer periods of supplementation, higher dosages, or specific immune challenges to yield measurable improvements. Additionally, interactions between probiotics and host immunity may differ based on environmental factors, gut health, and baseline immune status, further contributing to the variability in outcomes. The lack of a significant effect on cell-mediated immune responses could be due to several factors, including the nature of the immune challenge, the type of probiotics and prebiotics used, or the duration of supplementation.

Conclusion

The study demonstrated that dietary supplementation with *Bacillus subtilis* and MOS, individually or in combination, can enhance the humoral immune response to the Newcastle disease vaccine at specific time points, with the T6 group (*B. subtilis* + MOS) showing the highest antibody titres on day 28. However, these effects were not consistent across all time intervals, and no significant improvements were observed in the cell-mediated immune response, as measured by cutaneous hypersensitivity reactions. These findings suggest that while certain dietary treatments may temporarily boost immune responses, their overall impact on broiler immunity is variable and context-dependent. Further research is needed to optimize supplementation strategies for consistent immunomodulatory effects.

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Conflict of interest

The authors declare that they have no conflict of interest

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