Journal of Applied Veterinary Sciences, 10 (2): 64-74 (April, 2025).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Effect of Dietary *Bacillus subtilis* Supplementation on Lymphoid Organ Weights and Antibody Production Against the Newcastle Disease Vaccine in Broiler Chicks

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ABSTRACT

This study investigated the impact of *Bacillus subtilis* supplementation on lymphoid organ weights and antibody production in response to the Newcastle Disease Vaccine (NDV) in Ross 308 chicks. A total of 120 one-day-old chicks were divided into four groups with three replicates of 10 birds each, receiving different concentrations of *Bacillus subtilis* (Bs): Control (Bs-0), 0.05% (Bs-0.05), 0.10% (Bs-0.1), and 0.15% (Bs-0.15) for six weeks. Newcastle disease, infectious bronchitis and Gumboro disease vaccines were administered in drinking water on days 3, 11 and 15 of age, respectively. Blood and serum samples were harvested on day 21 to assess antibody production against NDV and lymphoid organ weights on day 42. On day 21, the antibody titers against the NDV vaccine were significantly higher (P≤0.05) in all chick groups supplemented with *Bacillus subtilis* compared to the control; the highest titer was observed in the Bs-0.15 group. By day 42, the final body weight (FBW) was significantly (P≤0.01) higher in the supplemented group Bs-0.15 than in others. The supplemented group, Bs-0.05, showed a numerically higher FBW than Bs-0.1 and the control group. The absolute and relative weights of lymphoid organs (spleen, thymus and bursa of Fabricius) were significantly higher (P≤0.01) in the supplemented groups than in the control. The highest organ weights were recorded in chicks receiving the highest concentration of *Bacillus subtilis* (Bs-0.15). These findings demonstrate that dietary supplementation with *Bacillus subtilis* at 0.15% enhances BW and the immune response and organ development associated with immunity in chicks. This supplementation level improves immune system health, bolsters disease resistance, and inspires and motivates further exploration of its potential utility in poultry nutrition strategies.

Original Article:
DOI:https://dx.doi.org/10.21608/javs.2025.352341.1513
Received : 12 January, 2025.
Accepted: 01 March, 2025.
Published in April, 2025.

Keywords: *Bacillus subtilis*, Broilers, Dietary supplementation, Immune response, lymphoid organs.

J. Appl. Vet. Sci., 10(2): 64-74.

INTRODUCTION

Poultry provides an affordable protein source, essential for reducing poverty and improving national food security. It plays a significant role in the animal food market by increasing production to meet global demand and supporting community livelihoods (**FAO, 2025**). Additionally, poultry is recognized as an efficient and environmentally sustainable meat option (**Neeteson *et al.*, 2023**). By 2032, poultry is projected to account for 41% of protein consumption from all meat sources (**OECD-FAO, 2022**).

Feed supplements are crucial in small amounts to prevent toxicity and residues. Strict regulations govern these additives, which are vital for enhancing feed quality and animal health, thereby improving performance and reducing morbidity and mortality (**Wang *et al.*, 2024**). The use of antibiotics in poultry for pathogen control has raised concerns about resistance, disrupted microflora, and potential gene transfer to humans (**Abreu *et al.*, 2023**). Despite banning antibiotics as growth promoters, infection rates have increased, prompting interest in alternatives like probiotics and prebiotics. Probiotics can help prevent diseases, improve growth and feed efficiency, boost immunity, and regulate gut microflora (**Chandrasekaran *et al.*, 2024**). The role of probiotics in regulating intestinal flora to enhance host immunity has recently received widespread attention.

Newcastle disease (ND) is a highly contagious viral infection caused by the Newcastle disease virus

(NDV) of the *Paramyxoviridae* family, affecting poultry worldwide, especially broiler chickens, and posing significant economic challenges to the poultry industry (**Alexander, 2000; Dimitrov *et al.*, 2016**). The disease manifests in three clinical forms: lentogenic, mesogenic, and velogenic, depending on the virus's virulence and the host's susceptibility, with symptoms ranging from respiratory distress (gasping and coughing) and neurological disorders (torticollis and paralysis) to digestive issues (greenish diarrhea) and high mortality rates in velogenic cases (**Ross *et al.*, 2022**). Diagnosis relies on clinical observations, laboratory tests such as RT-PCR and hemagglutination inhibition (HI), and postmortem findings like gastrointestinal hemorrhage (**Mayo, 2002; Oberländer *et al.*, 2020**). Preventive strategies include vaccination with live attenuated and inactivated vaccines, strict biosecurity measures, and active surveillance to ensure early detection and control of outbreaks (**Dimitrov *et al.*, 2016; Oberländer *et al.*, 2020**). Given the severe economic impact of production losses, high mortality, and trade restrictions, effective prevention and management of ND are essential for sustaining poultry health and mitigating financial losses in the industry.

Bacillus subtilis, as feed probiotics, is a potential alternative for antibiotic growth promoters capable of enhancing production and improving the innate immune response of broiler chicks (**Ramlucken *et al.*, 2020; Mohamed *et al.*, 2022**). Previous studies reported by **Sikandar *et al.*, (2022)** proposed that due to its effective probiotic capability, *Bacillus subtilis* can be potentially endorsed for utilization in the poultry industry as an effective probiotic capable of enhancing performance and immunity (**Dong *et al.*, 2020; Qiu *et al.*, 2021**).

Immunomodulation is one of the important modes of action of *Bacillus subtilis*. Humoral immunity, such as lymphocyte proliferation and serum antibody titer, increased significantly in *Bacillus subtilis*-fed broiler chicks. Including *Bacillus subtilis* as a probiotic in broiler diets has enhanced immune responses through multiple mechanisms, including the modulation of gut-associated lymphoid tissues (GALT), stimulation of cytokine production, and improved gut microbiota balance (**Ayana and Kamutambuko, 2024**). Studies report that *Bacillus subtilis* increases the activity of innate immune cells such as macrophages and heterophils while enhancing adaptive immunity by boosting immunoglobulin levels (IgA, IgG, and IgM) and improving antibody responses to vaccines like Newcastle disease and infectious bronchitis (**Jeong and Kim, 2014; Lee *et al.*, 2020**). The probiotic also helps mitigate stress-induced immunosuppression and supports gut barrier integrity, reducing pathogen translocation and enhancing overall immune surveillance (**Wang *et al.*, 2018**). These findings highlight *Bacillus subtilis* as an effective dietary supplement for improving broiler health and immunity, reducing reliance on antibiotics, and supporting sustainable poultry farming.

Lymphoid tissue is essential for the immune response and consists of central and peripheral components (**Ratcliffe *et al.*, 2005**). The central components include the thymus, spleen, and bursa of Fabricius, which play significant roles in immunoglobulin synthesis and antibody production.

The spleen, a primary immune organ, is essential for filtering blood and mounting immune responses. It serves as a blood filter, removes aged red blood cells and pathogens, and initiates immune responses by producing lymphocytes. Its functions commence on day one and continue to grow and develop its immune capabilities until six weeks (**Lillehoj, 1991**). The bursa of Fabricius is the primary lymphoid organ in broiler chickens (**Cooper *et al.*, 1966**). It is responsible for B-cell maturation, essential for antibody production, and supports the development of humoral immunity. The bursa develops rapidly in the first week, peaks at four weeks, and regresses after six weeks (**Glick, 1991; Ratcliffe, 2005**). The size and mass of the bursa provide important insights into the maturation and structure of the immune system, as it is the site for the maturation of T and B lymphocytes (**Park and Kim, 2014; Cheng *et al.*, 2023**). The thymus is the site of T-cell maturation and differentiation, contributing to cell-mediated immunity. It is most active during the first few weeks, peaking at 4 to 6 weeks before gradually regressing (**Cooper *et al.*, 1966**).

The poultry industry in Sudan encounters numerous challenges, primarily related to feeding costs. Therefore, developing an appropriate diet that meets beneficial nutritional requirements and enhances immune system function is essential.

Few studies have used probiotics to improve broiler chickens' lymphoid organ weights and bolster their immunity. Therefore, this study aimed to evaluate the potential effects of dietary supplementation of a mono-species commercial probiotic *Bacillus subtilis* (Biogen S®) on antibody titer against the NDV and lymphoid organ weights in Ross 308 broiler chicks.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Sudan Veterinary Council Ethics Committee (EA/0023-2018).

Experimental design and management

The experimental study, designed with a comprehensive approach, involved 120 one-day-old

unsexed Ross 308 strain broiler chicks, each with a similar body weight (mean = 47.5 g), divided into four groups, each receiving different concentrations of *Bacillus subtilis*: control (Bs-0), 0.05% (Bs-0.05), 0.10% (Bs-0.1), and 0.15% (Bs-0.15) for six weeks. The chicks were housed in an animal house made of iron posts, reinforced brick (25 cm high), and wire netting, with a concrete floor and corrugated iron sheet roof. The facility contained 12 pens arranged in three rows and four columns, each measuring 1.0 meters in length and width and 0.75 meters in height and accommodating 10 birds each. Each pen had a tubal feeder, a plastic drinker, and a 100-watt bulb lamp. The area was cleaned with formaldehyde, and dry wood shavings were used as litter to a depth of about 3 cm. Continuous lighting was provided for 24 hours, with natural sunlight during the day and artificial light at night. Water and diet were available *ad libitum*. Vaccinations for Newcastle disease, infectious bronchitis, and Gumboro disease were administered via drinking water on days 3, 11, and 15.

The supplemented level was adjusted according to the manufacturer's recommendation (0.10%). However, both lower and higher levels of supplementation (0.05% and 0.15%) were employed to examine the effect of variations in concentration on the parameters investigated in broiler chicks.

Experimental diet and chemical analysis

The basal diet was formulated to meet **NRC (1994)** requirements during the starter (1–21 days) and finisher periods (22–42 days). The broiler chicks were fed isonitrogenous and isocaloric diets during the experimental period. Three diets were formulated to meet their nutritional needs: pre-starter, starter, and finisher. Importantly, these diets were free from antibiotics and growth promoters. The pre-starter diet (Intraco Broiler Pre-star®) was administered during the chicks' first week of life and continued until the end of the third week. It contains 23% crude protein, 6% fat, 7.5% ash, 3.5% crude fiber, 35% starch, 0.9% calcium, 0.14% sodium, and phosphorus (0.6% total, 0.34% digestible, and 0.38% available). The amino acid profile is carefully balanced for optimal digestibility, including 1.4% total lysine (1.22% digestible), 0.59% total methionine (0.55% digestible), and 0.95% total methionine plus cystine (0.82% digestible), as well as 0.9% total threonine (0.75% digestible) and 0.28% total tryptophan (0.24% digestible). The formulation also includes 0.2% premix, 100 mg/kg of the antioxidant Butylated hydroxytoluene (BHT), 375 FTU/kg of phytase enzyme, 150 mg/kg of wheat xylanase enzyme, 65 units of Salinomycin (anti-coccidiostatic), along with an anti-mould agent and a preservative. After this, the finisher diet replaced the starter diet, starting from the beginning of the fourth week and continuing until the end of the six-week experimental period (**Tables 1 and 2**). The feed ingredients were mixed manually until they achieved final homogeneity in the mash mixture, resulting in the following experimental diets:

Bs-0 (Control): basal diet without Bs
Bs-0.05: basal diet + 0.05% Bs
Bs-0.1: basal diet + 0.1% Bs
Bs-0.15: basal diet + 0.15% Bs

Table 1: Ingredients and nutrients of the basal diet used to feed the broiler chickens**.**

Ingredients	Bs-0		Bs-0.05		Bs-0.1		Bs-0.15	
(%)	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher
Sorghum	71.8	69.3	71.25	68.95	70.9	68.7	70.65	68.45
Groundnut cake	21	23.5	21.5	23.8	21.8	24	22	24.2
Dicalcium phosphate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lysine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium bicarbonate	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Broiler concentrate*	5	5	5	5	5	5	5	5
Premix**	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Salt	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Bacillus subtilis	0	0	0.05	0.05	0.1	0.1	0.15	0.15
Total	100	100	100	100	100	100	100	100

* The broiler concentrate comprises vegetable proteins, fishmeal, dicalcium phosphate, limestone, vitamins, trace elements and antioxidants.

**Premix contents: Vitamins: A 12 500 IU/kg; D3 2 500 IU/kg; E 90 mg/kg; K3 2.5 mg/kg; B1 1.5 mg/kg; 6 B2 mg/kg; B3 17.5 mg/kg; B6 4 mg/kg; B12 0.03 mg/kg; Niacin 30 mg/kg; Folic Acid 3 mg/kg; Biotin 0.3 mg/kg; Choline Chloride 550 mg/kg; Trace minerals: Fe 150 mg/kg; Cu 15 mg/kg; Mg 50 mg/kg; Zn 120 mg/kg; I 1.25mg/kg; Se 0.4 mg/kg

Table 2: Proximate analysis of basal diet used to feed the broiler chickens.

Ingredients (%)	Starter (1-21 days)	Finisher (21-42 days)
Dry Matter (DM)	95.57	94.43
Fat	3.45	4.28
Crude protein (CP)	21.65	19.13
Crude fiber (CF)	4.83	4.9
Ash	9.27	10.19

The chemical analysis was conducted at the Faculty of Agriculture, University of Khartoum, Sudan. Additionally, Biogen S®, a mono-species commercial probiotic produced by Samu Median Co. LTD in Korea (Lot No.: 101068), was utilized. This product contains *Bacillus subtilis* at a concentration of 1.0×10^{11} colony-forming units (CFU) per kilogram. The recommended level of supplementation was 0.10%, although variations at lower (0.05%) and higher (0.15%) levels were also tested to evaluate their impact on the parameters studied.

Blood and serum collection

Blood samples were drawn from five birds in each group on days 21 and 42, using disposable 5 ml syringes fitted with a 22-gauge needle (**Kelly and Alworth, 2013).** The brachial wing vein (*vena cutanea ulnaris*) was first scrubbed with a disinfectant (70% ethanol). Three ml of blood were drained in plain vacutainers and allowed to stand at room temperature for 3 hours before being centrifuged at 3000 r.p.m. for 15 minutes (Gallenkamp Junior, UK). Haemolysis-free serum samples were carefully separated and transferred to clean plastic vials immediately frozen at -20°C for subsequent immunological analyses.

Final body weight (FBW) and lymphoid Organ Weights

On day 42, Five chicks from each experimental group were randomly selected and individually weighed using the electronic digital balance (Mettler Toledo, ME204) to obtain the FBW. Subsequently, the chicks were scarificed and the internal lymphoid organs, including the thymus, the spleen, and the bursa of Fabricius, were carefully dissected and determine their absolute weight (g). The relative organ weight (% of BW) was calculated by dividing the respective organ weight (g) by the BW and multiplying it by 100.

Antibody titer against NDV vaccine

The effects of *Bacillus subtilis* supplementation on the humoral immunity of broilers were evaluated at 21 days by measuring NDV antibody titers using the ELISA technique at the Detassy laboratory in Khartoum State, Sudan. On day eleven, seven chicks from each group were vaccinated with the NDV vaccine (live freeze-dried virus strain Clone 30). The ProFLOK® NDV-C ELISA Kit (https://www.zoetisdiagnostics.com/us/assets/Resources/PDF/proflok_ndv_plus__chicken_.pdf) detects NDV antibodies in the serum of vaccinated chicks. The serum is diluted and added to a plate coated with NDV antigen, allowing antibodies to bind. After washing away unbound substances, a goat anti-chicken IgG (H+L) peroxidase conjugate is added.

Following a 5-minute incubation and an additional wash, a substrate with the chromogen ABTS is introduced, resulting in a color change from clear to green-blue, which indicates the presence of the enzyme. The intensity of this color, measured after 15 minutes, reflects the NDV antibody levels compared to controls. A stop solution was added, and the plate was read at 405 nm.

ELISA test procedure

To prepare the test plate, 50 µl of dilution buffer was added to each well, followed by 50 µl of diluted NDV-positive control serum. After discarding the pipette tips, 50 µl of each diluted serum and the normal control serum were transferred to the corresponding wells. The plate was incubated at room temperature for 30 minutes; then, the liquid was discarded into a bleach vessel. Each well was filled with about 300 µl of wash solution, soaked for three minutes, and discarded. This washing step was repeated twice more.

Addition of anti-chicken IgG conjugate, substrate, and stop solution

A 100 µl dilution of the conjugate was added to each well and incubated at room temperature for 30 minutes. After three washes, 100 µl of diluted substrate solution was added and incubated for 15 minutes, followed by 100 µl of the diluted stop solution.

Data processing

The plate was analyzed at 405 nm using an ELISA reader, recording the average absorbance for positive and normal control serums. The corrected positive control value was obtained by subtracting the normal control average from the positive control average. The sample-to-positive (*Sp*) ratio was calculated by subtracting the normal control absorbance from each sample's absorbance and dividing by the corrected positive control value.

$$Sp = \frac{(\text{Sample absordance}) - (\text{Average normal control adsorbance})}{\text{Corrected postive control absorbance}}$$

The NDV titer was calculated using the following equation:

$$\log_{10} titer = (1.464 \times \log_{10} Sp) + 3.740$$

$$TITER = Anti - log(log10(TITER))$$

Statistical analysis

The experimental data were analyzed using standard statistical methods with the Statistical Package for the Social Sciences (SPSS) software for Windows, version 23.0 (**SPSS Inc., 2015**). A one-way analysis of variance (ANOVA) test was conducted to assess the significance of the results. Differences among the means of the dietary treatment groups were compared using the LSD test. A threshold value of P ≤0.05 was established to indicate statistical significance.

RESULTS

Table 3 reveals that the final BW was significantly (P≤0.01) higher in the supplemented group Bs-0.15 than in the other groups. In contrast, the supplemented group Bs-0.05 exhibited a numerically higher final BW than Bs-0.1 and the control group. The results of dietary *Bacillus subtilis* supplementation on the weights of lymphoid organs of broilers at day 42 of age are presented in **Table 3 and Fig. 1**.

Table 3: Effect of dietary *Bacillus subtilis* supplementation on the lymphoid organ absolute (g) and relative weight (g/g) weights of broilers on day 42 (n = 5).

Body weight (BW) Lymphoid Organ	Bs-0	Probiotic supplementation			P-value
		Bs-0.05	Bs-0.1	Bs-0.15	
Final BW	1567±26^{b}	1593±276^{b}	1525±29^{b}	1766±308^{a}	0.009
Spleen					
Absolute	1.53±0.59	2.00±0.60	2.14±0.77	2.44±0.84	0.28
Relative	0.14±0.05^{c}	0.18±0.08^{b}	0.18±0.06^{b}	0.22±0.06^{a}	0.03
Thymus					
Absolute	1.16±1.05^{b}	3.38±1.12^{a}	4.40±2.05^{a}	4.88±1.24^{a}	0.004
Relative	0.10±0.10^{c}	0.30±0.18^{b}	0.37±0.09^{b}	0.44±0.11^{a}	0.001
Bursa of Fabricius					
Absolute	1.66±0.97^{b}	1.88±0.66^{b}	2.64±0.72^{b}	3.88±0.51^{a}	0.001
Relative	0.15±0.09^{c}	0.160.06^{c}	0.22±0.06^{b}	0.35±0.05^{a}	0.001

Data are expressed as mean ± standard deviation (SD). Body and absolute lymphoid organ weights are in grams; relative weight: organ weight to body weight ratios (%) is represented in (g) organ weight/(g) body weight. Means within the same row with different superscripts significantly differ at (P≤0.05).

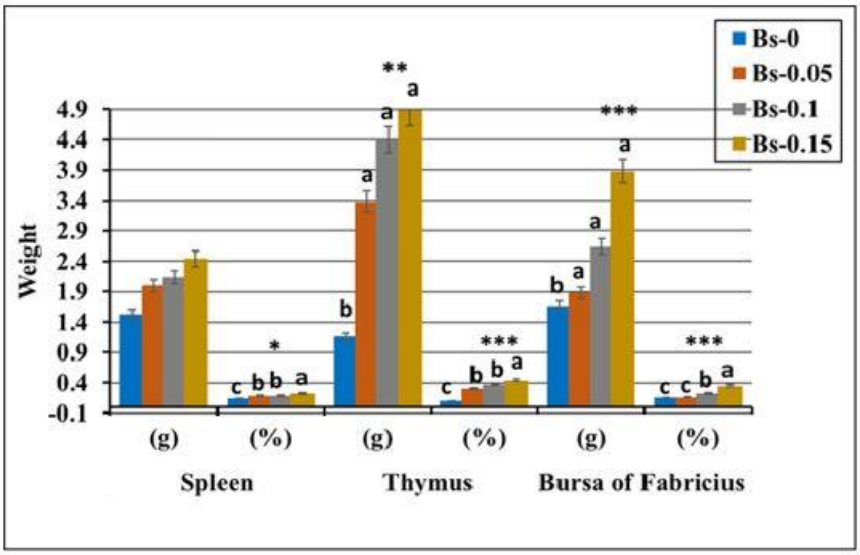

Fig. 1: The absolute (g) and relative (%) weights of lymphoid organs in Ross 308 broilers chicks on day 42 (n = 5). Bars with different superscripts significantly differ at * P≤0.05), **P≤0.01, ***P=0.001.

The absolute weight of the spleen was numerically higher in Bs-0.15 compared to Bs-0, Bs-0.05 and Bs-0.1. Supplemented group Bs-0.15 exhibited the highest spleen weight (P≤0.01), followed by Bs-0.1 and Bs-0.05; the lowest weight was observed in the control group (Bs-0). The relative weight of the spleen was significantly (P≤0.05) higher in the supplemented groups Bs-0.05, Bs-0.1 and Bs-0.15 compared to Bs-0. The absolute and relative weights of the thymus were significantly higher in the supplemented groups compared to Bs-0 (P≤ 0.01, 0.001). The highest weight was observed in the Bs-0.15 group.

The absolute weight of the bursa of Fabricius was significantly (P≤0.001) higher in the supplemented groups compared to the control group; the Bs-0.15 group exhibited the highest weight compared to Bs-0.1 (P≤0.01) and Bs-0.05% and Bs-0 (p≤0.001). The relative weight of the bursa was significantly (P≤0.001) higher in the supplemented groups compared to the control group.

The results of the effect of dietary *Bacillus subtilis* supplementation on the antibody titer against the NDV vaccine at day 21 of age are presented in **Table 4 and Fig. 2**. The antibody titer against the NDV vaccine was significantly (P≤0.05) higher in the supplemented group Bs-0.15 compared to Bs-0, followed by Bs-0.1 and Bs-0.05. In contrast, the control group showed the lowest value.

Table 4: Effect of dietary *Bacillus subtilis* supplementation on antibody titer against NDV vaccine of broilers on day 21 (n = 7).

Parameter	Bs-0	Probiotic supplementation			P-value
		Bs-0.05	Bs-0.1	Bs-0.15	0. 05
Anti-NDV titer	1384±258^{d}	1615±779^{c}	2367±445^{b}	2452±1448^{a}	

Data are presented as the mean ± standard deviation (SD). Means within the same row with different superscripts significantly differ at (P<0.05)

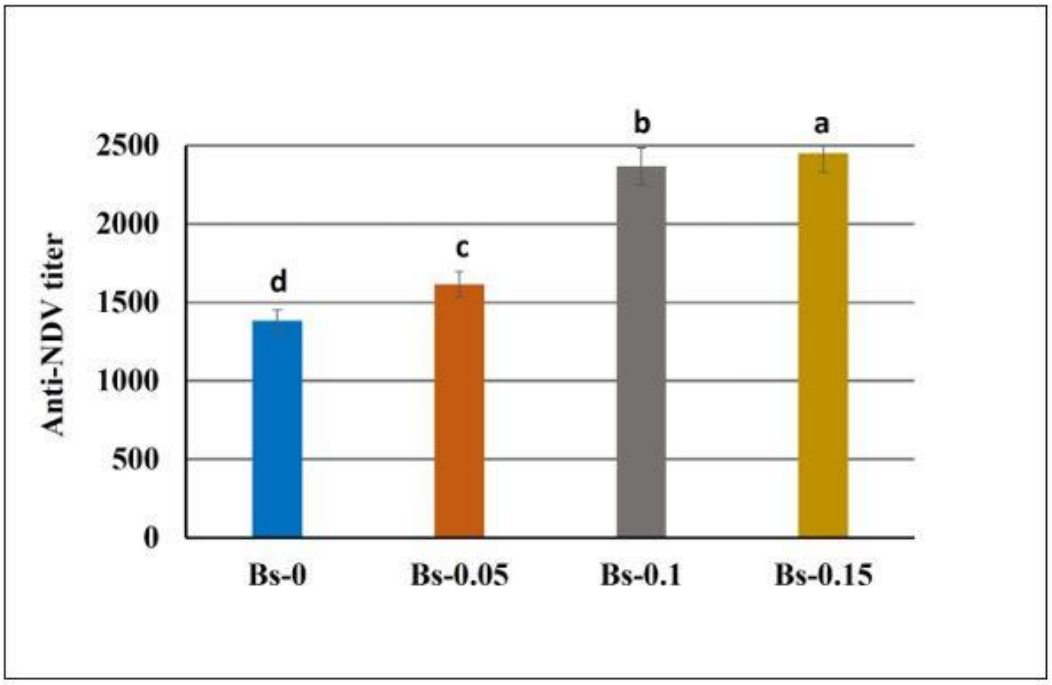

Fig. 2: The antibody titer against NDV vaccine in Ross 308 broilers chicks on day 21 (n = 7). Bars with different superscripts significantly differ at * P≤0.05).

DISCUSSION

The findings of this study underscore the positive effects of *Bacillus subtilis* supplementation on the final body weight and the growth and functional development of lymphoid organs and immune response in broilers. The observed increases in the absolute and relative weights of the spleen, thymus, and bursa of Fabricius across the supplemented groups reflect stimulation of the immune system, likely linked to the immunomodulatory properties of *Bacillus subtilis*. This agrees with previous studies highlighting the role of probiotics in enhancing immunity by modulating gut microbiota, which subsequently influences systemic immune responses **(Ramlucken *et al.*, 2020; Qiu *et al.*, 2021; Sandvang *et al.*, 2021; Mohamed *et al.*, 2022**; **Sikandar *et al.*, 2022; Chandrasekaran *et al.*, 2024; El-Sayed *et al.*, 2024; Khongthong *et al.*, 2025)**.

The effectiveness of probiotics is influenced by several interconnected factors, including the characteristics of the host organism, the specific bacterial strain used, the dosage, and the application method. Different probiotic strains have varying abilities, such as producing antimicrobial agents, and their effectiveness relies on intrinsic factors like their ability to adhere to the intestinal wall and resist gastrointestinal secretions. These factors are crucial for the probiotics' function and survival within the gastrointestinal tract (**Timmerman *et al.*, 2004; Shim *et al.*, 2010; Rhayat *et al.*, 2019**). Determining the optimum dosage is essential to avoid over- and under-dosing, as this can disrupt the balance of gut microbiota. Additionally, the application method, whether through feed, water, or encapsulation techniques, dramatically impacts the viability of probiotics until they reach the target site. It is also important to consider the strain's survival under specific conditions, including temperature and storage, to preserve potency and ensure a suitable substrate is available for growth. Therefore, careful management is crucial for optimizing probiotics' performance and achieving the desired outcomes.

In the present study, Tables 1, 2, and 3 demonstrated a well-balanced nutrient profile that supports optimal growth, health and performance. The inclusion of high crude protein, fiber, fat, and starch levels provides essential energy, promoting growth, digestive health, muscle development and the utilization of fat-soluble vitamins. Additionally, digestible amino acids enhance protein utilization, reducing waste and improving feed conversion ratios. The formula also contains appropriate vitamins and trace minerals that support metabolism, immune function, and development. Enzymes such as phytase and wheat xylanase were incorporated to enhance nutrient availability and digestibility in plant-based diets. Butylated hydroxytoluene was added as a recommended antioxidant to ensure feed stability, while salinomycin helps protect against common poultry diseases. This meticulously formulated diet effectively satisfies the nutritional requirements of broiler chicks under investigation, promoting their final BW (Table 4), health and growth.

The results indicated that the dietary inclusion of *Bacillus subtilis* at levels of 0.05%, 0.10%, and 0.15% in broiler feed significantly enhanced the final BW, particularly in chicks receiving 0.15% compared to the control and other groups. This improvement is likely attributed to *B. subtilis* promoting feed consumption of the balanced nutrient feed provided (Tables 1, 2 and 3). It is well documented that *Bacillus subtilis* boosts digestive enzyme activity and secretes protease, lipase, and amylase, which decompose complex carbohydrates, improve nutrient digestibility, reduce NH_3 production, alter bacterial metabolism, and support beneficial microbial populations (**Ramlucken *et al.*, 2020; Ciurescu *et al.*, 2020; Arif *et al.*, 2021**). It also offers broilers an extra source of nutrients and enzymes, stimulating the synthesis of B-group vitamins and promoting the growth of nonpathogenic bacteria, leading to the production of compounds that inhibit harmful bacteria, boosting resistance to enteric pathogens and enhancing the uptake of dissolved organic materials (**Dong *et al.*, 2020; Mohamed *et al.*, 2022; Khongthong *et al.*, 2025**). The study's findings align with previous research, indicating that *Bacillus subtilis* enhanced the growth performance of broiler chicks by improving the final BW, BW gain and feed conversion ratio (**Ciurescu *et al.*, 2020; Dong *et al.*, 2020; Arif *et al.*, 2021; Qiu *et al.*, 2021; Oladokun and Adewole, 2023; EL-sayed *et al.*, 2024; Li *et al.*, 2024; Khongthong *et al.*, 2025).**

In the current study, dietary supplementation of *Bacillus subtilis* significantly increased the absolute weights of the thymus and bursa of Fabricius in 42-day-old broilers (Table 5). The spleen also exhibited a numerical weight increase in chicks given diets containing 0.05%, 0.10%, and 0.15% *Bacillus subtilis*, particularly when these weights were expressed as a body weight ratio. The relative weights of all lymphoid organs were notably higher in the supplemented groups compared to the control group. Furthermore, the highest concentration of *Bacillus subtilis* (0.15%) was associated with increased weights of the lymphoid organs.

The significantly higher relative spleen weights in supplemented groups, especially Bs-0.15, suggest enhanced immune functionality. This is consistent with earlier research showing that dietary probiotics can improve lymphoid organ development,

contributing to improved disease resistance in poultry (**Gao *et al.*, 2017**). Similarly, **Park and Kim (2014)** found that the absolute weights of the thymus, spleen and bursa of Fabricius were significantly increased in chicks fed *Bacillus subtilis*. In contrast, **Reis *et al.* (2017)** demonstrated that *Bacillus subtilis* supplements did not influence the relative weight of the spleen.

In the present study, the most pronounced effects were observed in the Bs-0.15 group, indicating a dose-dependent response to supplementation. These findings are consistent with studies by **Apata (2008)** and **Mountzouris *et al.*, (2010)**, who reported that probiotics enhance the structural integrity and functionality of immune organs in poultry. In this study, the inclusion of *Bacillus subtilis* in the diet led to an increase in the absolute weight of the bursa of Fabricius by day 42. This observation aligns with results reported by **Mohamed *et al.*, (2022)** who also found that the weight of the bursa of Fabricius in broilers fed diets containing *Bacillus subtilis* was significantly higher compared to the control group. Additionally, **Hatab *et al.*, (2016)** found that probiotics as dietary supplementation increased the thymus weight in layer chicks. Conversely, **Zhang *et al.*, (2013)** reported that the dietary addition of *Bacillus subtilis* did not affect the weight of the bursa of Fabricius in broiler chicks. Both the thymus, which is essential for T-cell maturation, and the bursa of Fabricius, which is crucial for B-cell development, exhibited significant weight increases in the supplemented groups.

Furthermore, the present study demonstrated an increase in the relative weights of lymphoid organs, specifically the spleen, thymus and bursa of Fabricius; the observed increase in this study aligns with findings by **Mohamed *et al.*, (2022).** Their research indicated that dietary supplementation with *Bacillus subtilis* promotes the growth of lymphoid organs and positively influences the immune system of broilers. Additionally, this study suggests that the observed effects may be linked to the enhancement of body weight and weight gain in broiler chickens due to *Bacillus subtilis* supplementation. The results regarding lymphoid organ weights are also consistent with previous studies **(Zhang *et al.*, 2013; Hatab *et al.*, 2016)**, which reported that chicks fed diets supplemented with *Bacillus subtilis* had heavier bursa of Fabricius, thymus, and spleen compared to the control group. **Awad *et al.*, (2009)** also reported that the relative weights of the thymus, spleen, and bursa increased by adding 1 kg of *Bacillus subtilis* per ton of broiler diets. It is well known that measuring the weight of lymphoid organs is an important method for evaluating the immune status in chickens (**Ceccopieri and Madej, 2024**). Therefore, in this study, the notable increase in the relative weights of the bursa of Fabricius, thymus, and spleen in broilers fed 0.05%, 0.10%, and 0.15% levels of *Bacillus subtilis* could indicate a positive signal for the development of the immune system in broilers.

The study evaluated the effects of dietary supplementation with *Bacillus subtilis* on the antibody titers of broilers against the NDV vaccine at 21 days of age (Table 4). The results indicated significant increases in antibody titers for broiler chicks fed *Bacillus subtilis* at concentrations of 0.05%, 0.10%, and 0.15%. The observed values were 1615, 2367, and 2452, respectively, compared to 1384 in the control group. The highest antibody titer was found in the group receiving the highest concentration (Bs-0.15). This increase can be attributed to two main factors: the notable enlargement of lymphoid organs (Table 3) and the enhanced activity of B-lymphocytes, which has been documented in previous studies (**Ma and Suzuki, 2018**). This indicates that the immune system is exhibiting a heightened response, likely due to the increased size of these important organs and the improved functioning of B-lymphocytes, which are crucial for the body's immune defense mechanisms (**Chi *et al.*, 2024**). These findings are consistent with research by **Hatab *et al.*, (2016)** who reported higher NDV antibody titers in the Hy-Line layer chicks fed various concentrations of *Bacillus subtilis*. Additionally, chicks that received a diet containing a commercial *Bacillus subtilis* probiotic (0.01%) or a multi-strain probiotic combined with vitamins and minerals (0.5%) showed increased antibody titers compared to those without any additives (**Sugiharto *et al.*, 2018**).

Research has shown that administering *Bacillus subtilis* can enhance the specific systemic antibody response in chickens and positively influence both humoral and cellular immune responses **(Larsberg *et al.*, 2023; Oladokun and Adewole, 2023; Ergün *et al.*, 2024).** Additionally, recombinant *Bacillus subtilis* probiotics significantly improve gut integrity, enhance innate immunity, and boost growth performance in broiler chicks **(Arif *et al.*, 2021).**

Recent studies emphasize the beneficial effects of *Bacillus subtilis* supplementation on broiler health and immune responses, particularly in enhancing the efficacy of the NDV vaccine by modulating immune function **(Li *et al.*, 2023)**. Research by **Dong *et al.*, (2020) and Li *et al.*, (2024)** demonstrated that dietary inclusion of Bacillus subtilis ACCC 11025 and BYS2 improved growth performance and increased NDV antibody titers in broiler chicks. Similarly, **Kiarie *et al.*, (2022)** reviewed the roles of probiotics and postbiotics in poultry production, highlighting their positive impact on gut health and vaccine response. **Ahmed and**

Siddiqui (2021) reported improved NDV antibody titers in broilers receiving probiotic supplementation. These findings align with the results of the present study, underscoring the potential of *Bacillus subtilis* as a dietary intervention to enhance immunity and optimize poultry production systems.

CONCLUSION

This study demonstrates that dietary supplementation with *Bacillus subtilis* at 0.15% offers significant growth- and immune-related benefits to broiler chicks, including the promotion of final body weight and the enhancement of growth and development of key lymphoid organs (spleen, thymus, and bursa of Fabricius), as well as increased antibody titers against the NDV vaccine. These findings suggest that *Bacillus subtilis* effectively enhances immune response and disease resistance, particularly under Sudan conditions. The research provides strong evidence for using *Bacillus subtilis* as a practical and sustainable feed additive, aligning with previous studies and contributing valuable insights for improving poultry health, productivity, and antibiotic-free farming practices.

Acknowledgements

The authors thank and acknowledge the support of their respective universities and the Ministry of Higher Education and Scientific Research, Sudan.

Conflict of interest

The authors declare that there is no conflict of interest.

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How to cite this article:

Bashir, A.H., Abdelatif, A.M., and Elkhair, N.M., 2025. Effect of Dietary *Bacillus subtilis* Supplementation on Lymphoid Organ Weights and Antibody Production Against the Newcastle Disease Vaccine in Broiler Chicks. Journal of Applied Veterinary Sciences, 10 (2): 64-74. **DOI:https://dx.doi.org/10.21608/javs.2025.352341.1513**