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Improvement of Performance, Blood Profiles, Gut Health, and Immune Response of Broilers Supplemented with Chitosan, *Bacillus subtilis*, or Their Combination

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ABSTRACT

The study aimed to investigate the effect of supplementing chitosan, *Bacillus subtilis* or blends of both on broiler growth performance, immune response, biochemical variables, and intestinal ecology of broiler chickens. Two hundred and eighty day-old chicks were distributed into 4 experimental groups, including T0 (control, basal feed), T1 (basal feed + chitosan 0.1% feed), T2 (basal feed + *B. subtilis* 0.01% feed), and T3 (basal feed + chitosan 0.1% feed + *B. subtilis* 0.01% feed). Variables measurements and sample collection were conducted on day 35. The T3 did not differ from T0 and T1, but they consumed less (p≤0.05) feed than T2 chickens. Feed conversion ratio (FCR) was lower (p≤0.05) in T1 and T3 compared to T0 and T2 groups. Leukocyte counts in T2 and T3 were higher (p≤0.05) than those in T0. The T1, T2, and T3 had higher (p≤0.05) lymphocyte counts than T0. The T3 had lower (p≤0.05) low-density lipoprotein (LDL) levels than the T0 group. The T2 had higher (p≤0.05) levels of total protein and globulin than T3 and T0. Compared to T0 and T3, serum albumin levels were greater (p≤0.05) in T1 and T2. The T3 had higher (p≤0.05) duodenal villi height than that in the other groups. The T2 and T3 chicks had higher (p≤0.05) Newcastle disease titer than T0 and T1. Compared to T2 and T3, T0 and T1 showed a worse (p≤0.05) microscopic structure of jejunum. The T2 and T3 showed lower (p≤0.05) lesion score in *bursa of fabricius* than that in the other groups. In conclusion, the blends of chitosan and *B. subtilis* resulted in improved FCR, higher leukocyte and lymphocyte counts, lower LDL, higher duodenal villi height, higher Newcastle disease titer, better microscopic structure of jejunum, and lower lesion score in *bursa of fabricius* of broiler chickens.

Keywords: Bacillus subtilis; broiler; chitosan; immune responses; intestinal ecology

INTRODUCTION

As a major source of protein for human consumption, poultry farming is an important part of the global agricultural economy (Govoni *et al.*, 2021). However, given the intricate relationships between nutrition, microbial balance, and immune function, farmers always face challenges in maintaining the health and productivity of broiler chickens. Aware of the risks associated with antibiotic resistance and consumer preferences for products free of antibiotics, researchers have been looking into non-antibiotic alternatives in an effort to improve broiler growth and health performance in recent years (Stoica & Cox, 2021).

Today, the diverse biological activities of chitosan, which is derived from the exoskeletons of crustaceans, have drawn attention. Because of its cationic properties, chitosan can interact with negatively charged gastrointestinal tract components, which may positively impact microbial balance and nutrient absorption (Li *et al.*, 2015; Egorov *et al.*, 2022). Studies have shown that chitosan not only has growth-promoting properties but also has the capacity to activate chicken immune system components (Chang *et al.*, 2020; Mohan *et al.*, 2023). In agreement, Ahmad *et al*. (2020), Miao *et al*. (2020), and Abdul-Rahman *et al*. (2023) revealed that chitosan can function as an antioxidant and elicit a range of immune responses, such as macrophage activation, increased antibody synthesis, and cytokine profile modification.

Bacillus subtilis has recently gained interest as a probiotic due to its resilience, safety, and potential health benefits for poultry (Jayaraman *et al.*, 2013). *B. subtilis* also offers stability during feed production as they are able to survive against high temperatures during pelleting (Liu *et al.*, 2020). In broilers, *B. subtilis* has shown positive effects on intestinal health, nutrient absorption, and growth performance (Abudabos *et al.*, 2019). *B. subtilis* supplementation has also been associated with the enhanced adaptive immune responses in broilers. This includes the increased antibody production, improved

T-cell function, and more robust humoral immune responses (Xu *et al.*, 2021; Pickard *et al.*, 2017). Likewise, *B. subtilis* plays a vital role in shaping long-term immunity and vaccine responsiveness in broiler chickens.

From a biological point of view, it is highly probable that active compounds will interact with one another, and hence creating a synergistic effect. It was expected in this study that chitosan and *B. subtilis* would work in concert to further enhance the health and growth of broiler chickens. The antimicrobial properties of chitosan may boost the probiotic activity of *B. subtilis*, leading to further improved intestinal conditions and functions (Jiraphocakul *et al.*, 1990). While *B. subtilis* is known for its probiotic effects, and hence supporting gut health and enhancing nutritional absorption, chitosan exhibits antimicrobial, immunomodulatory, and antioxidant properties (Li *et al.*, 2021). To the best of our knowledge, no study has investigated the potential synergistic effects of chitosan and *B. subtilis* supplementation on broiler chicken health and performance. Hence, this present study aimed to investigate the effect of supplementing chitosan and *B. subtilis* by single administration or combination on broiler performance, immune response, biochemical parameters, and intestinal ecology of broiler chickens. It was hypothesized that treatment with the blends of chitosan and *B. subtilis* resulted in better growth performance, blood biochemical parameters, gut health, and immune response in broilers when compared to control and single administration of chitosan or *B. subtilis*.

MATERIAL AND METHODS

The current experiment has been approved by the Committee of Animal Ethic of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro, under approval number 59-07b/A-16/KEP-FPP.

Animals and Experimental Diets

The study used 280 individuals of day-old Cobb broiler chicks (mixed sex) that were purchased from a local hatchery. At hatching, the chicks weighed 45.01±0.85 g. All of the chicks were raised in an open-sided broiler house using the standard broiler rearing management protocol. Rice husk was used as a litter. Throughout the study, the chicks were continuously illuminated and had unrestricted access to feed and water. From among the chicks, 4 treatment groups were randomly chosen, each consisting of 7 replicates/pens and 10 chicks in each pen. Concerning the 4 treatment groups, T0 represented the control group, which received basal feed; T1 consisted of experimental chicks fed basal feed supplemented with chitosan 0.1% feed; T2 consisted of experimental chicks fed basal feed plus *B. subtilis* 0.01% feed; and T3 consisted experimental chicks fed basal feed + chitosan 0.1% feed + *B. subtilis* 0.01% feed. The experimental chicks were fed commercial pre-starter feed upon arrival, containing 5% crude fat and 23% crude protein until day 7. From days 8-21 and days 22-35, the chicks were offered formulated starter and finisher feeds, respectively. The ingredients and composition of the starter and finisher feeds are listed in Table 1.

In this study, 200 nm-sized commercial chitosan (CV. ChiMultiguna) produced from shrimp shells was used. *B. subtilis* (>10^{12} cfu/g; Baymix Grobig), provided by PT. Bayer Indonesia was used in the study. Infectious Bronchitis (IB), Newcastle Disease (ND), Avian Influenza (AI), and Infectious Bursal Disease (IBD) vaccinations were administered to each chick. On day 4, the chicks were given injections of 0.15 mL of subcutan and eye drops of Medivac ND-IB and ND-AI. Medivac Gumboro A was administered on day 11 using drinking water.

Data Collection and Laboratory Analysis

Production variables. The chicks' feed consumption and body weight gain were all recorded at the end of the study. Body weight gain was determined by calculating the final weight of broiler chickens minus the weight of the day-old chick. Feed conversion ratio (FCR) was determined by calculating the total consumption divided by weight gain. Feed consumption was the amount of feed given to the chicks minus the amount of feed remaining at the time of feeding.

The serum and blood count profile. One male chick (to avoid gender bias) representing the average body weight of each pen/experimental unit was selected at the end of the experiment to have blood drawn from the brachial vein. One mL of blood was put in an ethylenediaminetetraacetic acid (EDTA) tube for routine blood

Table 1. Feed formulation on starter and finisher period of broilers

Items (%)	Compositions (%)	
	Starter period	Finisher period
Yellow maize	57.90	61.00
Palm oil	2.55	2.95
Soybean meal	34.8	32.00
DL-methionine	0.19	0.19
Bentonite	1.00	0.75
Limestone	1.34	1.00
Monocalcium phosphate	1.51	1.30
Premix**	0.27	0.34
Chlorine chloride	0.07	0.07
Salt	0.40	0.40
Chemical compositions:		
ME (kcal/kg) *	2900	3000
Crude protein	22.00	20.00
Crude fiber	5.50	5.55
Ca	1.04	0.97
P (available)	0.57	0.54

Note: *The Bolton formula was used to calculate the amount of metabolizable energy. Bolton formula: 40.81 {0.87 [crude protein + 2.25 crude fat + nitrogen-free extract] + 2.5}
**Premix contained (per kg of diet) of Vitamin A 7750 IU, Vitamin D3 1550 IU, Vitamin E 1.88 mg, Vitamin B1 1.25 mg, Vitamin B2 3.13 mg, Vitamin B6 1.88 mg, Vitamin B12 0.01 mg, Vitamin C 25 mg, folic acid 1.50 mg, Ca-D-pantothenate 7.5 mg, niacin 1.88 mg, biotin 0.13 mg, Co 0.20 mg, Cu 4.35 mg, Fe 54 mg, I 0.45 mg, Mn 130 mg, Zn 86.5 mg, Se 0.25 mg, L-lysine 80 mg, choline chloride 500 mg, DL-methionine 900 mg, CaCO3 641.5 mg, dicalcium phosphate 1500 mg.

testing, and 3 mL of blood was put in a non-EDTA tube to produce serum. Using a hematology analyzer (Prima Fully-auto Hematology Analyzer, PT. Prima Alkesindo Nusantara, Jakarta, Indonesia), the routine blood profile testing of the chicks was determined automatically.

The anticoagulant-free blood was centrifuged for 10 minutes at 3,000 rpm in order to separate the serum. Serum samples were frozen for later analysis of creatinine, uric acid, high-density lipoprotein (LDL) and low-density lipoprotein (LDL), total cholesterol, and triglycerides. Triglyceride levels were determined using the enzymatic colorimetric assay method developed by Werner *et al*. (1981). To precipitate low density lipoprotein, the sample was treated with magnesium ions and phosphotungstic acid (LDL). For this purpose, one part of the sample and three parts of the precipitant were used. Because of centrifugation, HDL will be present in the supernatant. The cholesterol level was determined by following the procedure described by Lopez-Virella *et al*. (1997). Total protein and albumin levels were determined using spectrophotometric assays (Sigma-Aldrich, St. Louis, USA). The data on globulin were calculated from the serum total protein minus serum albumin.

Kinetic enzymatic reaction was used to measure the activity of the SGPT and SGOT enzymes. The basic idea behind SGPT kinetic analysis is that L-alanine aminotransferase facilitates the conversion of L-alanine and α-ketoglutarate into l-glutamate and pyruvate. Nicotinamide adenine dinucleotide (NADH) was oxidized to NAD after the pyruvate was reduced to lactate by the enzyme lactate dehydrogenase (LDH). When measured photometrically at a wavelength of 340 nm, the amount of oxidized NADH resulting from the decreased absorption was directly proportional to ALT activity. The determination of SGOT was based on the catalysis of aminotransferase (AST) from L-aspartate and α-ketoglutarate to L-glutamate and oxaloacetate. Nicotinamide adenine dinucleotide (NADH) was oxidized to NAD after oxaloacetate was reduced to malate dehydrogenase enzyme (MDH). Using a wavelength of 340 nm, photometry is used to measure the amount of oxidized NADH, which is directly proportional to AST activity (Huang *et al.,* 2006). The enzymatic spectrophotometry method was used to calculate the creatinine. Following dilution with 740 µL of distilled water, 10 µL of serum sample was fortified with 500 µg/mL creatinine-D_3 solution. The mixture above was then mixed with ethanol to precipitate out the proteins. The supernatant was collected after centrifugation at 12,000 rpm for 10 minutes and vortexed for 1 minute. A creatinine level was measured and reported in milligrams per deciliter. Uric acid is determined enzymatically when uricase oxidizes the acid and produces allantoin as its substrate. At a wavelength of 293 nm, the sample was observed using the colorimetric method. ACR was obtained by dividing the albumin by creatinine level (Burtis & Aswood, 1999).

According to Kaufmann *et al*. (2017) methods, the hemagglutination inhibition (HI) assay was used to determine the antibody titers against ND and AI. LaSota antigen (4 HA units) and AI H5N1 antigen (4 AI units) were used in U-based microtiter plates for the ND and AI antibody titers test. An equal volume of ND and AI antigen was combined with the test samples' two-fold serial dilution. After adding chicken red blood cells (CRBC), the number of dilutions was counted to determine whether the hemagglutination was completely inhibited.

Gut microbiota. The gut microbiota populations were determined according to Sugiharto *et al*. (2017). The same chick from which blood samples were taken from each pen was killed, and after it had been defeathered and dissected, its internal organs were collected. The digesta from the ileum and cecum were obtained and placed in the sterile sample pots to count particular bacterial populations. After being incubated for 24 hours at 38 °C, the lactose negative enterobacteria and total coliform in ileum and caecum were counted on MacConkey agar (Merck KGaA, Darmstadt, Germany) as colorless and red colonies. De Man, Rogosa, and Sharpe agar (MRS) from Merck KGaA were used to count the lactic acid bacteria colony. This was followed by 48 hours of anaerobic incubation at 38 °C.

Lymphoid organ weight and histology variables. Following the slaughter, the lymphoid organs (*bursa of Fabricius*, spleen, and thymus) were collected and weighed. The lymphoid organ relative weight was calculated by dividing the organ's weight by the live body weight, then multiplying the result by 100%. For determining the histology variables, the lymphoid organs and pieces of small intestinal segments (around 2 cm) were placed in sterile sample pots containing 10% buffered formalin (Leica Biosystems Richmond, Inc., Richmond, USA). The organs were cut 5 µm transversely and then stained with hematoxylin-eosin (HE). An optical microscope fitted with a digital camera was used to measure the crypt depth and villus height of every intestinal segment (Leica Microsystems GmBH, Wetzlar, Germany). The villus height divided by the crypt depth yielded the villus-crypt ratio (VH/CD).

The small intestine and lymphoid organ lesions were categorized based on the degree of tissue damage. Scoring for injury distribution was based on the following factors: type of injury (focal or multifocal), loss of cilia, hypertrophy/hyperplasia of epithelial cells, inflammatory infiltration, and necrosis. Previous research (Agusetyaningsih *et al.,* 2022; Alabi *et al.,* 2020) assigned a severity score: 0 (no lesion, 0% cell damage); 1 (mild, 5%-25% of cell damage); 2 (moderate, 26%-50% of cell damage); and 3 (severe, >50% of cell damage). To determine the histopathologic score of the small intestine and lymphoid organs, the average of three observation sites per small intestine and lymphoid organ sample were calculated.

Statistical Analysis

The data in this study are analyzed using the SPSS software version 22.0. A one-way ANOVA with significance ($p \leq 0.05$) level was used to analyze the collected data statistically. To assess the differences between the treatment groups, the Duncan multiple

range test (DMRT) was employed. Histopathological lesion scores on the spleen and small intestine were analyzed non-parametrically by the Kruskal-Wallis analysis method.

RESULTS

Performance of Broilers

Table 2 shows the performance of broilers for the entire period of study. Dietary supplementation of chitosan and *B. subtilis* has been shown to influence (p≤0.05) broiler chickens' cumulative feed consumption and feed conversion but had no significant effect on the weight gain of broiler chickens. The T3 did not differ from the T0 and T1 treatment groups, but they consumed less feed (p≤0.05) than the T2 group chickens. The FCR value was lower (p≤0.05) in the T1 and T3 chicken groups compared to the T0 and T2 groups.

Blood and Serum Profile

The blood profile of broiler chickens is listed in Table 3. T1 leukocyte counts were the same as those in T0, T2, and T3, but leukocyte counts in T2 and T3 were higher (p≤0.05) than those in T0. Compared to the control group (T0), the number of lymphocytes in T1, T2, and T3 was higher (p≤0.05). Other blood profile variables (Hb, erythrocytes, hematocrit, MCV, MCH, MCHC, RDW, MPV, PDW, heterophils, eosinophils, and thrombocytes) were unaffected by the administration of chitosan and *B. subtilis*.

The blood biochemical profile of broiler chickens is shown in Table 4. The T3 chicken group had lower (p≤0.05) LDL levels than the T0 group, but there was no discernible difference between the T3 group and the T1 and T2 groups. Group T2 chickens had higher (p≤0.05) total protein and globulin levels than groups T3 and T0, but not group T1 chickens. Compared to groups T0 and T3, serum albumin levels were greater (p≤0.05) in T1 and T2. Administration of chitosan and *B. subtilis* had no effect on triglyceride, cholesterol, uric acid, creatinine, HDL, SGOT, SGPT, and ACR levels.

Intestinal Histomorphometry and Bacterial Populations

As demonstrated in Table 5, chickens in the T3 group had higher (p≤0.05) duodenal villi height than those in groups T0, T1, and T2. In the ileum and jejunum, dietary treatment had no effect on villous height, crypt depth, or the ratio of villus height to crypt depth.

Table 6 displays the data on populations of the selected bacterial populations in the ileum and caecum of broilers. Dietary supplementation of chitosan, *B. subtilis* or a combination of both showed no substantial effect on the numbers of coliform, lactose-negative bacteria, and lactic acid bacteria in the ileal and cecal contents of broiler chickens.

Table 2. Performance of broilers (days 0-35) fed diet supplemented with chitosan, *Bacillus subtilis,* or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Feed consumption (g/chick)	3091.51^{ab}	3066.76^{ab}	3168.61^{a}	2965.44^{b}	25.13	0.02
Weight gain (g)	1880.02	1920.99	1831.37	1894.36	13.19	0.10
FCR	1.68^{a}	1.56^{b}	1.69^{a}	1.53^{b}	0.01	<0.01

Note: ^{a,b,c}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, FCR= feed conversion ratio.

Table 3. Complete blood counts of broilers fed diet supplemented with chitosan, *Bacillus subtilis,* or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Hemoglobin (g/dL)	8.48	7.95	7.67	8.22	0.22	0.64
Erythrocytes (10^6/μL)	2.25	2.13	2.03	2.05	0.04	0.31
Hematocrit (%)	37.67	35.94	34.65	33.40	0.82	0.31
MCV (fl)	167.90	168.48	170.22	170.68	0.93	0.69
MCH (pg)	37.72	37.58	39.37	40.07	0.57	0.35
MCHC (g/dL)	22.02	21.88	22.62	23.08	0.32	0.55
RDW-SD (10^{-15} L)	45.92	46.20	46.98	45.98	0.41	0.80
RDW-CV (%)	9.45	9.41	9.52	9.27	0.07	0.69
MPV (10^{-15} L)	8.50	8.47	8.22	8.81	0.16	0.68
PDW (%)	14.68	8.27	8.41	10.47	1.25	0.23
Leukocytes (10^3/μL)	45.72^{b}	59.84^{ab}	79.48^{a}	73.35^{a}	3.95	<0.01
Heterophils (10^3/μL)	2.68	3.25	2.81	1.57	0.24	0.09
Lymphocytes (10^3/μL)	39.88^{c}	56.58^{b}	75.87^{a}	71.87^{ab}	3.78	<0.01
Thrombocytes (10^3/μL)	44.57	32.14	36.85	35.71	3.00	0.54

Note: ^{a,b}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, MCV= mean corpuscular volume, MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, RDW-SD= red cell distribution width-standard deviation, RDW-CV= red cell distribution-coefficient variation, MPV= mean platelet volume, PDW= platelet distribution width.

Table 4. Blood biochemical variables of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Cholesterol (mg/dL)	101.58	109.39	101.90	93.09	2.55	0.16
HDL (mg/dL)	60.42	68.71	63.42	62.00	1.86	0.44
LDL (mg/dL)	36.21^{a}	29.85^{ab}	27.88^{ab}	20.37^{b}	2.00	0.03
Triglyceride (mg/dL)	40.00	54.12	52.95	53.55	2.71	0.19
Total protein (g/dL)	2.67^{b}	3.12^{ab}	3.47^{a}	2.72^{b}	0.09	<0.01
Albumin (g/dL)	1.14^{b}	1.31^{a}	1.34^{a}	1.14^{b}	0.02	<0.01
Globulin (g/dL)	1.53^{b}	1.80^{ab}	2.12^{a}	1.57^{b}	0.07	0.01
Uric acid (mg/dL)	5.78	6.35	6.92	5.95	0.30	0.58
Creatinine (mg/dL)	0.02	0.03	0.04	0.03	0.00	0.25
SGOT (g/dL)	235.64	253.35	246.84	222.82	10.08	0.74
SGPT (g/dL)	1.52	2.18	2.88	1.98	0.18	0.07
ACR	53.29	55.22	44.37	47.63	5.74	0.91

Note: ^{a,b}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, HDL= high density lipoprotein, LDL= low density lipoprotein, SGOT= serum glutamic oxaloacetic transaminase, SGPT= serum glutamic pyruvic transaminase, MDA= malondialdehyde, ACR= albumin to creatinine ratio.

Table 5. Small intestine histomorphometry of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Duodenum						
VH (μm)	1015.05^{b}	920.11^{b}	994.47^{b}	1256.16^{a}	46.55	0.05
CD (μm)	176.28	159.35	159.82	176.93	7.58	0.76
VH/CD ratio	6.00	6.03	6.62	7.56	0.40	0.52
Jejunum						
VH (μm)	1138.42	1113.34	1162.91	1149.46	34.46	0.91
CD (μm)	220.25	185.05	158.69	184.72	8.51	0.76
VH/CD ratio	5.35	6.26	7.49	6.88	0.35	0.17
Ileum						
VH (μm)	642.13	652.37	681.86	632.15	20.56	0.86
CD (μm)	142.57	129.87	148.00	127.94	9.67	0.87
VH/CD ratio	4.85	5.40	5.05	5.70	0.33	0.83

Note: ^{a,b}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, VH= villi height, CD= crypt depth, VH/CD= villi height and crypt depth ratio.

Table 6. Ileum and caecum microbiota of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

Variables (log cfu/mg)	Treatments				SEM	p value
	T0	T1	T2	T3		
Ileum						
Coliform	<2.00	<2.00	<2.00	<2.00	0.00	1.00
LNE	<2.00	<2.00	<2.00	<2.00	0.00	1.00
LAB	3.98	4.29	4.11	3.89	0.08	0.40
Caecum						
Coliform	3.49	2.92	3.16	3.47	0.19	0.71
LNE	2.39	2.00	2.17	2.61	0.09	0.12
LAB	7.43	7.48	7.82	7.50	0.11	0.62

Note: T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, cfu= colony forming unit, LNE= lactose-negative bacteria, LAB= lactic acid bacteria. The symbol "<" indicates that some observations from which the mean was calculated had values below detection levels. When the colonies did not grow at the lowest dilution on the plates, the detection level was applied and used to make the calculations. Therefore, the real mean value may be below that reported.

Immunological Indices

The relative weights of the immune organs (i.e., spleen, thymus, and *bursa of Fabricius*) did not change in response to the dietary treatment (Table 7). The dietary treatments showed a substantial effect (p≤0.05) on the ND antibody titers, at which T2 and T3 chicks had higher titer than T0 and T1. The AI antibody titers were not affected by the treatments.

Histopathologic Scoring of the Small Intestines

Table 8 presents data indicating that the addition of *B. subtilis* and chitosan to feed modified (p≤0.05) the

jejunum's tissue structure. Compared to groups T2 and T3, groups T0 and T1 showed a worse (p≤0.05) microscopic structure. On the other hand, the duodenum and ileum intestinal segments did not react to treatment. The microscopic state of the broiler chickens' small intestine is depicted in Figures 1–12.

Histopathologic Scoring of the Lymphoid Organs

The data about the lymphoid organ lesion score are displayed in Table 9. Dietary treatment using chitosan and *B. subtilis* had no discernible effect on the lesion scores of the spleen and thymus of broiler chickens. Indeed, a significant (p≤0.05) impact on changes in the *bursa of Fabricius* lesion score was observed in the T2 and T3 groups. Figures 13-24 illustrate the microscopic condition of the lymphoid organ in broiler chickens.

DISCUSSION

This study demonstrated that, in comparison to those receiving *B. subtilis* alone, the chicks receiving chitosan and *B. subtilis* blends consumed less feed. With regard particularly to probiotic *B. subtilis*, Ayman *et al*. (2022) and Chang *et al*. (2020) suggested that *B. subtilis* may stimulate the appetite of broilers, leading to the increased feed consumption of broiler chickens. On the other hand, broiler feed consumption was decreased when *B. subtilis* and chitosan were combined. In fact, the broiler FCR values in the current study were the lowest when chitosan and *B. subtilis* were combined. This circumstance indicated that the blends of chitosan and *B. subtilis* improved nutrient digestibility, leading to the more efficient nutrient utilization by the broiler chickens. In such condition, the synergistic works between

Table 7. Immunology parameters of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

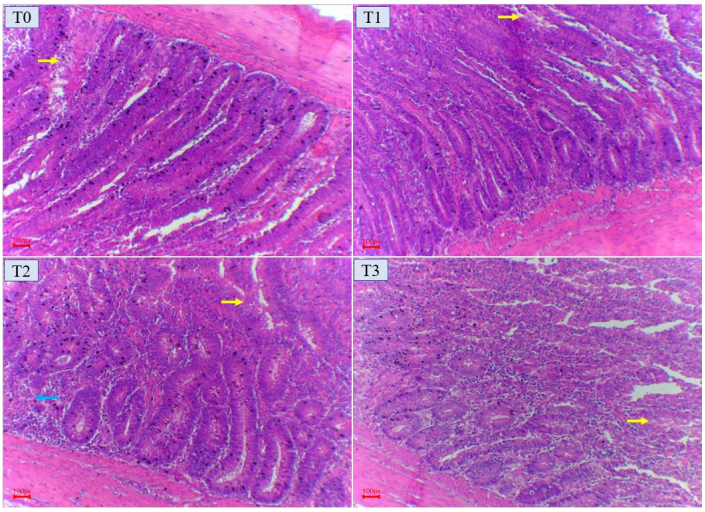
Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Relative immune organ weight (% BW)						
Bursa of Fabricius	0.03	0.04	0.03	0.05	0.00	0.16
Spleen	0.08	0.09	0.07	0.08	0.00	0.49
Thymus	0.14	0.24	0.25	0.21	0.01	0.11
Antibody titer (Log_2 GMT)						
ND	1.42^{b}	1.71^{b}	3.28^{a}	3.00^{a}	0.25	0.01
AI	1.71	2.28	2.28	3.14	0.21	0.12

Note: ^{a,b}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means, BW= body weight, GMT= geometric mean titer, ND= Newcastle Disease, AI= Avian Influenza.

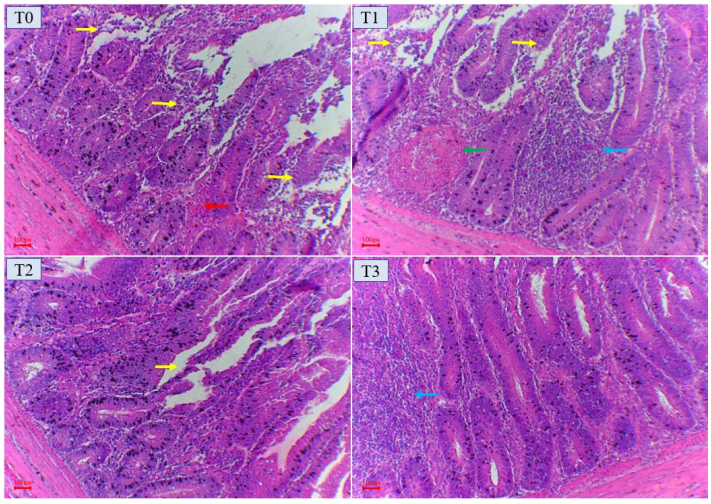
Table 8. Histopathologic scoring of the small intestines of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Duodenum	2.00	2.00	1.57	2.00	0.14	0.65
Jejunum	2.85^{a}	2.28^{ab}	2.00^{bc}	1.28^{c}	0.16	<0.01
Ileum	1.85	2.00	1.85	1.57	0.14	0.75

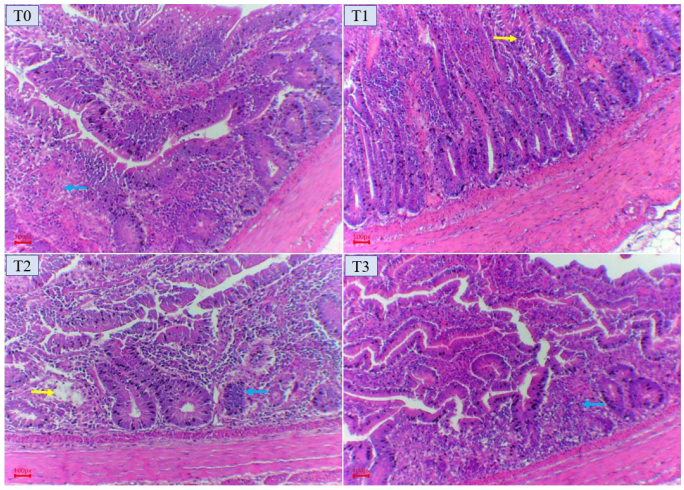
Note: ^{a,b,c}Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means.



Figures 1-4. Microscopic structure of 35 days old broiler's duodenum (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. Yellow arrow (T0, T1, T2, T3) showing light erosion of mucosal layer, blue arrow (T2) showing a very mild inclusion of lymphoid cells on submucosal layer.



Figures 5-8. Microscopic structure of 35 days broiler's jejunum (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. T0 and T1 had severe mucosal damage (yellow arrow), mild hemorrhage at submucosal layer (red arrow), accumulation of lymphoid cells on submucosal layer (blue arrow), accumulation of eosinophilic masses in the submucosa (green arrow). T2 and T3 showing mild mucosal erosion (yellow arrow), also had moderate submucosal lymphoid cells accumulation (blue arrow).



Figures 9-12. Microscopic structure of 35 days broiler's ileum (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. T0-T3 showed signs of mild submucosal and mucosal damage. Yellow arrow shows a mild erosion of mucosal layer and blue arrow showing a mild accumulation of lymphoid cells on submucosal layer. The pond's microscopic appearance is generally normal.

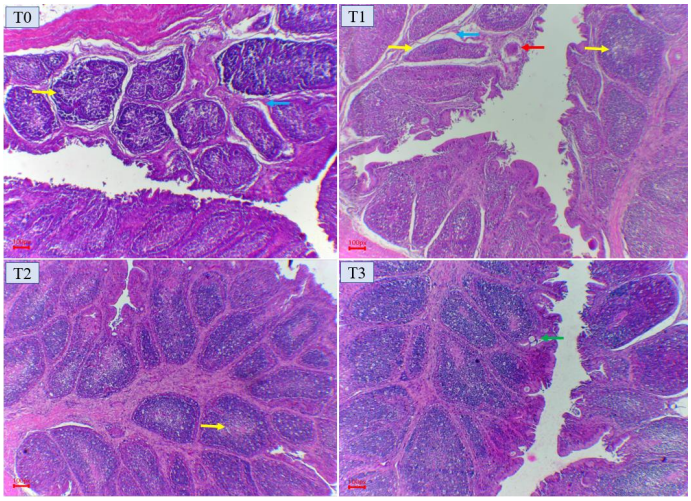
Table 9. Histopathologic scoring of the lymphoid organs of broilers fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination

Variables	Treatments				SEM	p value
	T0	T1	T2	T3		
Bursa of Fabricius	2.85[a]	2.71[a]	1.57[b]	1.57[b]	0.19	0.02
Spleen	2.85	2.71	1.85	2.00	0.21	0.26
Thymus	1.71	2.14	1.57	1.42	0.12	0.21

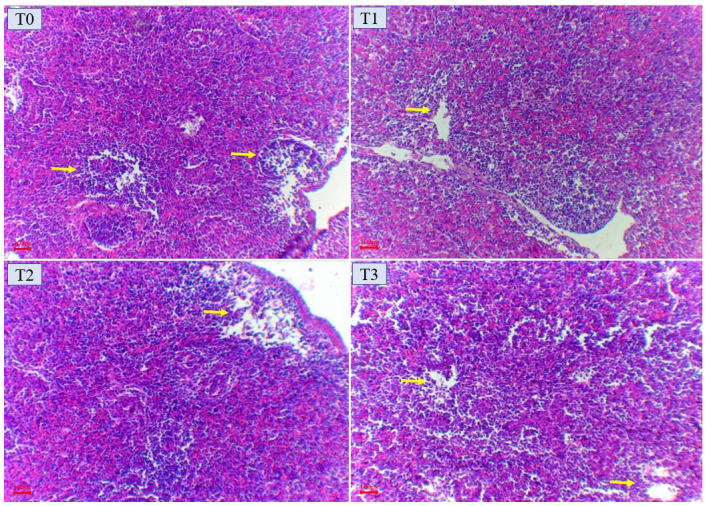
Note: [a,b]Means in the same row with different superscripts differ significantly (p≤0.05). T0= control, basal feed, T1= basal feed + chitosan 0.1% feed, T2= basal feed + *B. subtilis* 0.01% feed, T3= basal feed + chitosan 0.1% feed+ *B. subtilis* 0.01% feed, SEM= standard error of the means.

chitosan and *B. subtilis* seemed to improve the intestinal functions of broilers as represented by the enhanced villi height of duodenum and improved histopathologic scoring of the jejunum of broilers in the present study.

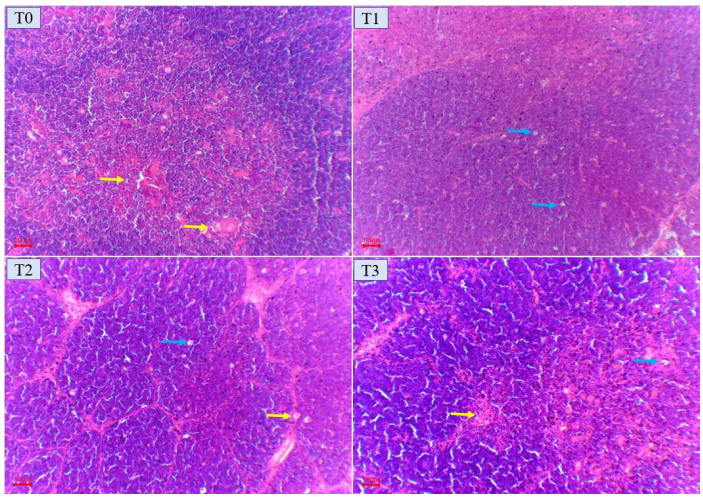
Based on data from this study, broiler chickens receiving *B. subtilis* and its combination with chitosan had higher levels of leukocytes and lymphocytes than chickens in the control group. The immune system, which defends the body against infections and foreign invaders, depends critically on leukocytes. Lymphocytes are a major class of leucocytes that are crucial to the adaptive immune response. They are involved in the development of immunological memory and the specific recognition of pathogens (Klein, 2021). The innate



Figures 13-16. Microscopic structure of 35 days broiler's *Bursa of Fabricius* (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. T0-T1 had severe follicular damage, depletion of lymphoid cells in bursa follicles (yellow arrow), decreased thickness of the interfollicular connective tissue (blue arrow), vacuolization at interfollicular surface epithelium (red arrow). The bursa structure on T2 and T3 are closed to normal, there is only mild depletion of the follicles (yellow arrow) and small vacuolization at interfollicular surface epithelium (green arrow).



Figures 17-20. Microscopic structure of 35 days broiler's spleen (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. T0-T3 show the moderate cellular change. The images show the mild depletions of lymphoid cells in the pulpa splenica alba (white pulp) and also in the pulpa splenica rubra (red pulp) (yellow arrow).



Figures 21-24. Microscopic structure of 35 days broiler's thymus (Hematoxylin-Eosin staining) fed diet supplemented with chitosan, *Bacillus subtilis*, or their combination. T0-T3 experienced mild damage of thymic lobules, vacuolization in thymic parenchyma (blue arrow) and eosinophilic connective tissue (yellow arrow).

and adaptive immune systems have been shown to be stimulated by *B. subtilis*. This entails controlling the generation of cytokines and activating a subset of white blood cells called macrophages. The overall increase in leukocyte activity may be due in part to these immune-modulating effects (Dong *et al.,* 2020). Klein (2021) stated that cytokines is signaling molecules involved in immune responses that can stimulate the proliferation and activation of white blood cells, contributing to an elevated leukocyte response. In addition, *B. subtilis* and chitosan have been known to influence lymphocyte cell response, including activation of B cells that play a role in the formation of specific antibodies due to the introduction of antigens from vaccination (Bilal *et al.,* 2021; Dong *et al.,* 2020). This is closely related to the antibody titer parameters in this present study, which indicates that broiler chickens vaccinated against ND can have higher levels of ND antibody titer when they receive *B. subtilis* either alone or in combination with chitosan. Antigens are typically proteins present on the surface of the ND virus vaccine. It has been reported that *B. subtilis* and chitosan can release specific cytokines, stimulating B cells and encouraging their activation and proliferation. Activated B cells undergo differentiation into two main cell types, plasma cell that are specialized for antibody production and memory B cells that can remember the specific antigen for faster and more robust responses upon antigen exposure (Horns *et al.,* 2020; Klein, 2021, Li *et al*., 2021).

LDL was reduced in broilers by the combination of chitosan and *B. subtilis*. In the gastrointestinal tract, chitosan was hypothesized to bind to cholesterol and prevent it from being absorbed into the bloodstream. This binding ability may aid in the decrease of LDL cholesterol. The activity of enzymes involved in the digestion and absorption of dietary fats may be affected by chitosan, which could have an impact on lipid metabolism (Fathi *et al.,* 2023). According to Ayman *et al*. (2022), chitosan may have an effect on lipase enzymes, which could change lipid metabolism and lower LDL cholesterol. *B. subtilis* is a type of probiotic bacteria that has the ability to produce bile salt hydrolase (BSH). Indeed, BSH enzymes can affect the deconjugation of bile salts, which could affect the metabolism of cholesterol. Yang *et al*. (2023) stated that bile acids are amphipathic molecules that structurally resemble cholesterol. They are produced when hepatic enzymes convert cholesterol, mostly *via* the primary synthesis pathway. By changing the makeup of bile acids, *B. subtilis* may be able to maintain cholesterol homeostasis. Probiotics like *B. subtilis* can contribute to the production of short-chain fatty acids (SCFAs) in the gut. SCFAs have been implicated in lipid metabolism and may play a role in reducing LDL cholesterol levels (Mohamed *et al.,* 2022; Xu *et al.,* 2021; Qiu *et al.,* 2021). In addition to the direct effect of BSH on cholesterol, BSH also influences lipid metabolism by promoting bile acid (BA) deconjugation and fecal excretion. This process helps to maintain hepatic cholesterol pool by reducing the plasma total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels (Duan *et al.,* 2022; Kriaa *et al.,* 2019).

In comparison to the control group, serum of broilers contained higher levels of total protein, albumin, and globulin following treatment with *B. subtilis*. It was most likely that *B. subtilis* affected the makeup and activity of the intestinal microbiota. A balanced gut microbiota is linked to several physiological functions, such as metabolism and the absorption of nutrients (Abudabos *et al.,* 2020). Moreover, probiotics contributed to the production of SCFAs in the intestine of broilers. SCFAs play a role in the absorption of nutrients and may influence protein metabolism (Markowiak-Kopec & Slizewska, 2020). Studies confirmed that total protein levels, including albumin and globulin, are influenced by liver function. Probiotics, including *B. subtilis*, may have indirect effects on liver health, potentially influencing the synthesis of proteins like albumin (Maslennikov *et al.,* 2021).

In this study, histopathologic scoring of the small intestines, particularly in the duodenum, did not show significant improvement following the treatments. Indeed, the duodenal biopsies in all treatment groups in the present study showed a low quantity of inflammatory cells in the submucosal layer. Apart from the above-mentioned condition, the combination of *B. subtilis* and chitosan increased the duodenal villous height as expected. Taken all the facts together, it seemed that there was no direct correlation between villi height and duodenal lesion score in the present study. In various studies, an increase in villi height is often associated with the presence of growth factors required to stimulate the proliferation and growth of the enterocytes, as reported by Ahmad *et al*. (2020) and (Liu *et al.,* 2020). Compared to the other treatment groups, the chickens receiving a combination of chitosan and *B. subtilis* had fewer lesion scores in the jejunum. This demonstrates that, in comparison to the other groups, the chicks receiving the combination of chitosan and *B. subtilis* had better cell structure and less cellular damage. It has been demonstrated that chitosan affects the cell cycle by affecting how cells move through various phases. It may control important proteins that affect cell integrity and proliferation during the cell cycle, including cyclins and cyclin-dependent kinases (CDKs) (Wang *et al.,* 2023). Furthermore, chitosan exhibits antioxidant properties, which may protect cells from oxidative stress. In such case, oxidative stress can negatively impact cell viability (Wasti *et al.,* 2020). Through its ability to lower oxidative stress, chitosan may improve the environment, leading to increased cell integrity. Likewise, *B. subtilis* may improve intestinal cell integrity through a variety of interactions between the probiotic bacteria and the host organism (Qiu *et al.,* 2021). It is well known that *B. subtilis* produces antimicrobial substances like lipopeptides and bacteriocins. These compounds may possess antimicrobial properties that aid in preventing the development of harmful bacteria in the gastrointestinal tract. *B. subtilis* may indirectly support intestinal cell integrity by inhibiting the overgrowth of pathogenic microorganisms, maintaining the redox balance and enhancing mucus production that plays a role in intestinal cell protection and integrity (Khan *et al.,* 2023).

In contrast to the control group of chickens and the chickens that were given chitosan alone, it was observed in this study that chickens receiving *B. subtilis* and chickens that received blends of chitosan and *B. subtilis* displayed lower lymphoid organ's histopathologic scores. This condition indicated that *B. subtilis* administration improved the condition of *Bursa of Fabricius* of broiler chickens (less damage of *Bursa of Fabricius*). It was most likely that *B. subtilis* protected the lymphoid organ by controlling the inflammatory reactions of the chickens against the invading pathogens. In this regard, Wang *et al*. (2023) reported that *B. subtilis* regulated the microbiota-modulated immunity resulting in controlled excessive inflammatory response, which may be deleterious to the lymphoid tissues and organs. In agreement with this, Klein *et al*. (2021) suggested that *B. subtilis* influences immune cells and controls cytokine and other signaling molecule production. Such immunomodulation may positively impact the development of cells in the *Bursa of Fabricius*.

CONCLUSION

The blends of chitosan and *B. subtilis* resulted in improved FCR, higher leukocyte and lymphocyte counts, lower LDL, higher duodenal villi height, higher *Newcastle disease* titer, better microscopic structure of jejunum, and lower lesion score in *Bursa of Fabricius* of broiler chickens. To optimize the health and productivity of broiler chickens, it is therefore preferable to use a combination of chitosan and *B. subtilis*.

CONFLICT OF INTEREST

The authors of this paper all declare that they have no conflicts of interest with any of the people or organizations involved in the research.

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