Growth performance, immune status and organ morphometry in broilers fed *Bacillus subtilis*-supplemented diet

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Abstract

The present research aimed to investigate the effects of *Bacillus subtilis* (*B. subtilis*) on performance, immune system, gut, and lymphoid organs' microarchitecture in broilers. A total of 120 day-old broiler chicks were randomly distributed into four groups. The birds were fed a corn-soy-based basal diet (BD) (control) or the same BD supplemented with 10% zinc bacitracin (ZnB), 0.05 g/kg or 0.1 g/kg of *B. subtilis* (BS). The broilers fed 0.1 g/kg of *B. subtilis* had superior mean bodyweight and lower feed conversion ratio compared with the non-supplemented or ZnB-fed groups. The BS-0.1 group registered higher antibody titer against the Newcastle disease (ND) virus. Cell-mediated immune response post Phytohaemagglutinin-P (PHA-P) injection was attained by both BS-0.1 and BS-0.05 groups. Histomorphological study revealed increased thymus cortical width, and cortex/medulla ratio in BS-0.1 group compared with control. Area of bursal follicles and germinal centres of spleen also improved in BS-0.1 group. Compared to ZnB and control, higher villus height (VH) and villus crypt ratio of the duodenum and jejunum were recorded on day 21, and higher VH of duodenum and ileum was noted on day 35 in BS-0.1 and BS-0.05 groups. In conclusion, *B. subtilis*-type probiotics contributed positively to better growth performance, improved immune system and modulated morphology of lymphoid organs and gut mucosa in broilers.

Keywords: Immunity, intestinal mucosa, poultry, probiotics
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Introduction

Proper digestion, absorption, and conversion of feedstuff into valuable end products are important factors in animal growth performance. Healthy gut development and its mucosal modulations are of substantial importance. The small intestine provides a prime location for digestion and absorption of available nutrients. The important histological component in this portion is the villus (Ragaa & Korany, 2016). An increase in villus length results in an enlargement of the contact surface with the nutrients and improves absorption (Awad *et al*., 2009), which may be linked to improved growth performance. Certain environmental and managemental stressful factors may lead to poor growth performance through suppression of the immune system (Schwartzman & Ruby, 2016). The immune system performs a valuable function in protecting the body. Incompetency of the immune system leads to various infections (Madej *et al*., 2015). For immune modulation, synthetic antibiotics have been used as feed additives in the poultry industry to control the growth of pathogens and thus act as antibiotic growth promoters (AGP) for birds (Kabploy *et al*., 2016). Following the ban on the use of dietary antimicrobials in Europe (Lee & Lillehoj, 2016), products such as probiotics, organic acids and phytobiotics are of great importance in replacing AGPs, and have improved gut health.

Probiotics are orally fed living cultures of microorganisms (yeast and bacteria) that reveal favourable outcomes once consumed. Variable findings have been reported about the effects of probiotic-supplemented diets on growth performance and immune-associated organs of broilers. Probiotics have been found to stimulate gut-associated lymphoid tissues (Haghighi *et al*., 2006), and are assumed to affect other lymphoid organs through mucosal-associated lymphoid tissue. Probiotics are also active against enteropathogens in several ways, including improved immunity-based elimination, competing for mucosal attachment and crucial nutrients, and producing antimicrobial complexes (Patel *et al*., 2015). They colonize in the gut and its

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antigenic fragments have the ability to cross the mucosal barrier (Dobson *et al*., 2012). In lamina propria mucosae, the dendritic cells present these fragments to T and B lymphocytes in the germinal centres of the immune organs (Jiang *et al*., 2004). Through this process, probiotics modulate immune responses in the animals. Probiotics flourish in the gut of hosts and enhance the growth of intestinal beneficial commensals (Lee *et al*., 2016), minimizing the destruction triggered by pathogens (Park *et al*., 2014), supporting the integrity of gut mucosa and maintaining stability and balance of normal microflora (Lei *et al*., 2015). They are known to improve relative weight of spleen. However, a comparable effect on bursa is controversial (Zhang *et al*., 2012).

B. subtilis, as spores or direct-fed microbials (DFM) as vegetative cells, is likely to fortify the performance of the immune system by lowering oxygen, releasing proteases and decreasing the pH of the gut. These changes are brought about through an increase in the population of beneficial intestinal microflora, including *Lactobacillus,* and may lead to reduced growth of pathogenic bacteria in the gut (Park *et al*., 2014). Direct-fed microbials are known to decrease the intestinal luminal pH and maintain the equilibrium, stability and enhancement of other gut microbiota (Lei *et al*., 2015). It has been suggested that *B. subtilis* should be offered continuously to broilers in feed (Latorre *et al*., 2014). Based on non-toxigenic and beneficial effects, European Food Safety Authority (EFSA) declared that *B. subtilis* is a safe animal feed additive for veterinary use (EFSA, 2015). *B. subtilis* is reported to have the ability to improve digestion and promote growth (Tactacan *et al*., 2013), and food conversion ratio (FCR) (Teo & Tan 2007; Lei *et al*., 2015) in host animals. Based on reported advantageous features, the authors hypothesized that *B. subtilis* may be a potential replacement for AGP in broilers. Therefore, the present study reports the effects of *B. subtilis* supplementation on growth performance, cellular and humoral immune responses, relative weight and morphometry of lymphoid organs, and gut mucosal microstructural modulations.

Materials and methods

The study was executed in an environmentally controlled research shed at the Department of Poultry Production, University of Veterinary and Animal Sciences-Lahore, Pakistan. A total of 120 day-old broiler chicks (Hubbard M77) were reared for 35 days under standard husbandry conditions approved by the Ethical Review Committee for the Use of Laboratory Animals of the University (Notification No. DR/ 257 dated 13 April 2015). The temperature and relative humidity (RH) at the beginning were maintained at 33 ± 2 °C and 70 ± 5%, respectively, and were decreased at around 3 °C per week until they reached 24 ± 2 °C, with RH 65 ± 5%. Thereafter, the temperature was kept constant for the remaining period of the study. On day 1, chicks were divided randomly into four groups of 30 chicks in each group (BS-0.1, BS-0.05, ZnB and control) with three replicates (n = 10) each. The birds in each group were offered a corn-based basal diet (Table 1) in starter (day 1 to day 21) and grower (day 22 to day 35) phases.

Diet and water were supplied *ad libitum* throughout the study. The birds in the groups BS-0.1 and BS-0.05 were supplemented with *B. subtilis* QST-713 (Agraquest® Bayer, Davis, Calif, USA) at the rate of 0.1 g/kg and 0.05 g/kg (2.0×10^{10} colony forming unit (CFU)/g) feed, respectively. Group ZnB consisted of 10% ZbN (Hubei Yuancheng Tech. Develop. Co. Ltd. China), and were given 0.01% of feed, while the control group served as negative control and were maintained on the basal diet only. On day 1 and day 9, the chicks were vaccinated with Newcastle disease vaccine (Nobilis ND LaSota, Intervet International B.V. Boxmeer, Holland) via the ocular route and boosted on day 16 and day 23 via drinking water.

The cumulative bodyweight of treated chicks was compared with that of the control group. Average bodyweight (ABW), weekly weight gain (WWG), average feed intake (AFI) and FCR were studied to record growth performance (Kiczorowska *et al*., 2016).

The humoral response was assessed in terms of determination of antibody titer against NDV and sheep RBCs antigens as described earlier (Sikandar *et al*., 2017). Briefly, two birds from each treatment replicate (group of n = 6) were randomly selected on day 14 and wing tagged. The selected birds were inoculated with 5% sheep red blood cells (SRBCs) antigen (sheep blood collected in Alsevier's solution, washed thrice and suspended in phosphate buffered saline) in two parts (0.5 ml each, intramuscularly in both sides of the breast muscles *(Musculus pectoralis)* on day 14. Booster dose (similar) was given on day 21. Blood samples were collected on day 21 (after 7 days of primary inoculation) and day 35, and sera separated through centrifugation at 2000 x g for 10 minutes and stored at -20 °C till analysis. The immune responses to NDV and SRBCs were measured using micro-titer haemagglutination inhibition and haemagglutination assays, respectively.

Cell-mediated immune response was evaluated in terms of cutaneous basophilic hypersensitivity test produced by injecting phytohemagglutinin-P (PHA-P) (Sigma-Aldrich, USA) as described by Corrier (1990) with minor modifications. Briefly, two birds from each replicate (n = six per group) were selected randomly on day17 and wing tagged.

Table 1 Control diet composition and calculated analysis

Ingredient (%)	Starter phase	Grower phase
Corn	40.15	57.57
Rice broken	15	---
Soy meal	11.54	9.60
Sunflower meal	12.00	13.00
Canola meal	9.00	5.00
Rapeseed meal	5.00	7.60
Rice polish	---	4.00
Guar meal	1.00	---
Wheat bran	1.34	---
Molasses	2.00	---
Sodium bicarbonate	0.03	0.065
Sodium chloride	0.21	0.21
Di-calcium phosphate	1.33	1.49
L-Lysine	0.30	0.35
DL-Methionine	0.10	0.12
Vit-mineral premix*	1.00	1.00
Nutrient composition		
Calculated metabolisable energy (kcal.kg)	2750	2850
Crude protein (%)	19.6	18.5
Dry matter (%)	87	88
Crude fibre (%)	6.05	6.35
Crude fat (%)	2.16	2.35
Total ash (%)	5.77	5.40

*Vitamin mineral premix (each kg contained): ascorbic acid, 26000 IU ; retinol, 200,000 IU; cholecalciferol, 80,000 IU; tocopherol, 1072 IU; thiamine, 11666 IU ; pyridoxine, 33333 IU ; menadione, 11,333 IU; riboflavin, 54,000 IU ; niacin, 5,36,000 IU; folic acid, 13600 IU; methylcobalamin, 223 IU; biotin, 1340 IU; Ca, 195 g; K, 70 g; Na, 18 g; Mg, 6 g; Fe, 2,000 mg; Zn, 1,200 mg; Mn, 1,200 mg; Cu, 400 mg; I, 40 mg , Co, 20 mg and Se, 8 mg

The PHA-P solution was prepared in sterile phosphate buffered saline (PBS) and injected (100 µg/100 µl/ chicken) intradermally between the third and fourth digits of the right foot. The left foot was injected with 100 µl PBS and served as a control. The swelling of the injection site was evaluated with a pressure sensitive micrometer 24, 48, and 72 hours post injection. Cell-mediated immune response (foot web index) to PHA-P was analysed by subtracting the thickness of the left foot from that of the right foot.

Two chickens from each replicate were randomly selected, weighed and killed on day 21 and day 35. The small intestine, liver, thymus, spleen, and bursa of Fabricius were removed from the carcass and their relative weights were calculated. The representative samples of the organs were subsequently collected and transported to the laboratory in labelled specimen bottles with 10% neutral buffered formalin for onward procedure.

Histomorphometry of the thymus was done according to Rooney *et al.* (2003) and Madej *et al.* (2015) with minor modifications. The thymic lobules were divided into four sections by two lines crossing each other at right angles in the centre of the medulla. All the lines representing the complete width of the cortex were calculated, and average was expressed as cortical thickness. The area of the medulla was calculated by combining the measurements of the two lines representing the length and width of the medulla. Later, the ratios of cortex to medulla were calculated in three well-oriented lobules per section and the mean values, obtained from three sections per bird, were calculated.

Well-oriented germinal centre areas in the spleen were combined and expressed as a percentage of the total field of view at 10 x (Madej *et al*., 2015). Later the average values of three sections were determined.

Slides were stained with haematoxylin and eosin (H&E). The total number of lymphoid follicles in one microscopic field at 4 x magnification were noted. Length, width and area of five well-oriented bursal follicles were measured (Sikandar *et al*., 2017) per section (three sections per sample). Later the average value of the three sections was determined. The area of the follicles was measured with this equation: follicular width × follicular length.

Segments of approximately 2-cm length were excised from the duodenum (10 cm distal to duodeno-gizzard junction), jejunum (5 cm proximal to Meckel's diverticulum), and ileum (5 cm anterior to the ileo-caecal junction). Intestinal samples were processed with the paraffin-embedding technique, sectioned (5 µm) with a microtome (AMOS Scientific AEM-450, Austria), and stained with H&E, except for histochemical differentiation of goblet cells, for which the slides were stained with periodic acid-Schiff-alcian blue (Bancroft *et al*., 2008).

Three sections were collected (one section after every 10 sections) from each intestinal sample. From each section, five well-oriented villi with intact lamina propria were randomly selected for observation. Thus an average of 15 values was calculated for each sample. Finally, the mean values from six chickens were expressed as mean values for one treatment group. Slides were examined under a microscope (Olympus CX 31, Olympus USA) at 4 x magnification and operated with a digitalized live image analysis program (Olympus DP 20, Olympus USA). The histomorphological variables, including villus height (VH), villus width (VW), villus surface area (VSA), crypt depth (CD), and villus height to crypt depth ratio (VH : CD) were measured (Kiczorowska *et al.,* 2016).

Normal distribution of the data was confirmed with the Kolmogorov-Smirnov test and was presented as means ± SEM. The data were analysed with one-way ANOVA (Statistical Package for Social Sciences, SPSS for Windows, version 20, SPSS Inc., Chicago, IL, USA). Statistical differences among treatment means were determined through Duncan's multiple range tests. In all statistical analyses, P <0.05 was considered significant.

Results

Chickens fed a higher level of probiotic (BS-0.1) performed well compared with ZnB and control (Table 2). In the second week, both *B. subtilis* groups showed higher (P <0.05) ABW, WWG, and lower (P *<0.05*) FCR compared with ZnB and the non-supplemented group. Better growth performance was recorded in BS-0.1 compared with ZnB and the control group in the third week.

Average bodyweight registered better (P <0.05) in *B. subtilis*-supplemented groups compared with ZnB and control in the fourth week. Average bodyweight and WWG were higher (P <0.05) in BS-0.1 compared with control and ZnB and control, respectively, in the fifth week. Feed intake differences during the grower phase were not significant among treatment groups.

On day 21 the responses among the groups were found to be non-significant, while on day 35 the antibody response to Newcastle disease virus (NDV) was affected (P <0.05) by the level of *B. subtilis*. Antibody titer against sheep red blood cells (SRBCs) registered higher (P <0.05) in the supplemented groups. There were no significant differences in the titers between the groups that received different concentrations of probiotics (Table 3).

Lymphoproliferative responses to PHA-P were registered higher (P <0.05) in chickens treated with higher concentration of *B. subtilis* compared with ZnB and control groups.

On day 35, liver, spleen and thymus weighed more (P <0.05) in the supplemented groups compared with control (Table 4).

On day 35, the thymus cortical width in BS-0.1 and the medullary area in BS-0.05 improved (P <0.05) compared with control. Compared with control, the germinal centre area of spleen was increased (P <0.05) in BS-0.1 and BS-0.05 groups on day 35. The results showed enlarged (P <0.05) bursal follicular area on day 21, and bursal follicular length on day 35 in BS-0.1 group compared with ZnB and control. Compared with control, the bursal follicular area was greater in BS-0.1 and BS-0.05 groups on day 35 (Table 6).

On day 21, in comparison with control and ZnB, the thymus cortical width and cortex/medulla ratio increased (P <0.05) in BS-0.1 group (Table 5).

Starter phase (d 21): Compared with ZnB and control, the histomorphological study revealed higher (P <0.05) VH, VSA and VH : CD of duodenum and jejunum in both the *B. subtilis*-supplemented groups and VH and VSA of ileum in BS-0.1. Variation in the VW and CD was non-significant among treatment groups in all three segments except in the duodenum on day 21, in which the CD was found to be lower (P <0.05) in ZnB compared with all other groups (Table 7).

Grower phase (day 35): VH and VSA in duodenum and ileum increased (P <0.05) in both the *B. subtilis*-treated groups compared with ZnB and groups (Table 8).

Table 2 Weekly growth performance results of broilers

Age	Parameter	Treatments				
		BS-0.1	BS-0.05	ZnB	Control	P-value
1st week	Avg. bodyweight (g)	155.43 ± 1.02	148.17 ± 2.99	151.40 ± 1.31	152.03 ± 1.13	.114
	Weekly weight gain (g)	114.43 ± 1.02	107.17 ± 2.99	110.40 ± 1.31	111.03 ± 1.13	.114
	Avg. feed intake (g)	129.20 ± 3.64	126.37 ± 0.30	127.27 ± 1.19	134.10 ± 5.06	.119
	FCR	1.13 ± 0.02	1.18 ± 0.09	1.15 ± 0.03	1.20 ± 0.02	.090
2nd week	Avg. bodyweight (g)	432.93^{a} ± 2.94	430.63^{a} ± 3.94	412.37^{b} ± 2.02	410.43^{b} ± 3.81	.002
	Weekly weight gain (g)	277.50^{a} ± 2.14	282.46^{a} ± 0.95	260.96^{b} ± 0.84	258.40^{b} ± 2.91	.000
	Avg. feed intake (g)	346.17 ± 0.27	346.50 ± 1.42	348.70 ± 1.01	350.23 ± 0.98	.065
	FCR	1.25^{b} ± 0.01	1.23^{b} ± 0.01	1.34^{a} ± 0.01	1.36^{a} ± 0.02	.000
3rd week	Avg. bodyweight (g)	786.67^{a} ± 1.45	760.33^{ab} ± 16.91	735.50^{bc} ± 3.62	729.00^{c} ± 4.04	.007
	Weekly weight gain (g)	353.73 ± 1.51	329.70 ± 17.28	323.13 ± 5.21	318.56 ± 7.71	.131
	Avg. feed intake (g)	512.53^{b} ± 1.58	518.40^{a} ± 0.31	513.93^{a} ± 1.19	523.57^{a} ± 5.06	.076
	FCR	1.45^{b} ± 0.01	1.59^{a} ± 0.09	1.58^{a} ± 0.03	1.64^{a} ± 0.02	.091
4th week	Avg. bodyweight (g)	1212.83^{a} ± 1.57	1192.75^{a} ± 4.34	1138.20^{b} ± 6.55	1039.21^{c} ± 10.43	.000
	Weekly weight gain (g)	426.17^{a} ± 0.36	432.42^{a} ± 12.83	402.71^{a} ± 8.11	310.21^{b} ± 10.78	.000
	Avg. feed intake (g)	745.92 ± 2.75	738.29 ± 3.32	741.17 ± 6.12	733.62 ± 3.95	.291
	FCR	1.75^{b} ± 0.01	1.71^{b} ± 0.06	1.84^{b} ± 0.02	2.37^{a} ± 0.09	.000
5th week	Avg. bodyweight (g)	1782.46^{a} ± 7.38	1701.42 ^{b} ± 4.08	1672.21^{b} ± 14.36	1485.88^{c} ± 19.83	.000
	Weekly weight gain (g)	569.63^{a} ± 8.26	508.67 ± 3.91^{b}	534.00^{ab} ± 11.38	446.67^{c} ± 28.81	.004
	Avg. feed intake (g)	1133.92 ± 29.58	1053.54 ± 2.92	1062.38 ± 2.67	1039.46 ± 4.78	.076
	FCR	1.94 ± 0.07	2.07 ± 0.02	1.99 ± 0.04	2.35 ± 0.17	.063

^{abc}Means within a row marked with different superscripts were significantly different (P <0.05)
Values represent mean ± SEM
BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB: 10% zinc bacitracin, 0.01% of feed; FCR: feed conversion ratio

Table 3 Humoral immune responses in broilers during starter and finisher phases

Treatments	Antibody titre (log_2) on various days			
	NDV		SRBC	
	Day 21	Day 35	Day 21	Day 35
BS-0.1	2.21 ± 0.06	2.27^{a} ± 0.05	5.50 ± 0.22	7.33^{a} ± 0.42
BS-0.05	2.26 ± 0.09	2.21^{ab} ± 0.06	5.50 ± 0.22	7.83^{a} ± 0.30
ZnB	2.09 ± 0.12	1.98^{b} ± 0.09	5.66 ± 0.33	7.16^{a} ± 0.30
Control	1.98 ± 0.11	1.98^{b} ± 0.09	5.16 ± 0.30	6.00^{b} ± 0.36
P-value	0.332	0.035	0.638	0.011

^{ab} Means within a column marked with different superscripts were significantly different (P <0.05)
Values represent mean ± SEM.
NDV: Newcastle disease virus; SRBC: sheep red blood cells; BS-0.1, BS-0.05:(0.1 g or 0.05 g *B. subtilis* per kg; ZnB (10% zinc bacitracin, 0.01% of feed)

Table 4 Relative weight[1] of immune related organs in broilers

Treatments	Relative organs weight[1] at day-35			
	Liver	Spleen	Thymus	Bursa
BS-0.1	1.8770[a] ± 0.07	0.1729[a] ± 0.01	0.4014[a] ± 0.01	0.181 ± 0.03
BS-0.05	1.6189[a] ± 0.09	0.1395[a] ± 0.01	0.3804[a] ± 0.00	0.165 ± 0.02
ZnB	1.5971[a] ± 0.11	0.1435[a] ± 0.02	0.3857[a] ± 0.00	0.162 ± 0.02
Control	1.5753[b] ± 0.05	0.0960[b] ± 0.01	0.3184[b] ± 0.01	0.116 ± 0.02
P-value	0.015	0.008	0.000	0.277

[ab] Means within a column marked with different superscripts were significantly different (P <0.05)
[1]Relative organs weight = organ weight/bodyweight × 100
Values represent mean ± SEM. BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB (10% zinc bacitracin, 0.01% of feed)

Table 5 Results of morphology of immune organs in broilers on day 21

Organs	Parameters	Treatments				
		BS-0.1	BS-0.05	PC	NC	P-value
Thymus	Thymic cortex (µm)	363.22[a] ± 32.59	324.67[ab] ± 17.59	295.83[b] ± 12.24	288.17[b] ± 11.49	.066
	Thymic medulla (µm)^2	437.50 ± 42.88	522.67 ± 10.14	523.67 ± 26.29	494.00 ± 25.26	.146
	Cortex: medulla	0.8562[a] ±.10	0.6237[b] ±.04	0.5741[b] ±.04	0.5912[b] ±.04	.016
Spleen	Germinal centre/ field area (%)	0.7326 ± 0.05	0.6473 ± 0.09	0.6465 ± 0.04	0.6449 ± 0.03	.700
Bursa	Bursal follicular length (µm)	563.17 ± 31.26	542.50 ± 19.2	485.50 ± 29.20	468.67 ± 44.10	.152
	Bursal follicular width (µm)	188.50 ± 22.60	189.67 ± 15.59	155.50 ± 8.29	142.67 ± 8.75	.089
	Bursal follicular area (µm)^2	107182.8[a] ± 17112.9	102024.3[ab] ± 6748	75004.3[bc] ± 5042.7	66691[c] ± 7330.1	.027
	Bursal follicular number	10.17 ± 0.98	10.50 ± 0.43	8.83 ± 0.48	8.67 ± 0.71	.175

Means within a row marked with different superscripts were significantly different (P <0.05).
Values represent the mean ± SEM.
BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB (10% zinc bacitracin, 0.01% of feed).

Discussion

Replacing antibiotics with feed additives is no longer an option but a necessity. *B. subtilis*, a probiotic, has been reported to have the potential to promote gut health when supplemented in broiler feed. The current study was designed to compare the effects of probiotic and antibiotic on growth performance, immune status, and histomorphological modulations in the immune organs and small intestinal mucosa.

Dietary fed probiotics, including *B. subtilis* strains, have been reported to be potential growth promoters and immune modulator agents (Lee *et al*., 2015), but little is known about the effect of the *B. subtilis* QST-713 strain. In the current study, the chickens fed with high-concentrate probiotic gained more weight during the starter and finisher phases (Table 2) than the ZnB and control groups. This may be attributed to the nutritional benefits of *B. subtilis* (Lei *et al*., 2015), which fortified growth. Feed intake was non-significant among treatment groups and, overall, better FCR was observed in the *Bacillus*-supplemented groups. These findings are similar to the observations of Al-Fataftah and Abdelqader (2014), which explained that animals performed well after being fed *Bacillus*-type probiotics. Various kinds of genes related to feed digestion in the broiler gut are regulated by DFM (Lee *et al*., 2015), such as those for lipase (CEL), chymotrypsin (CTRC), carboxypeptidase (CPA1), amylase (AMY2A), and pancreatic lipase (PNLIP). Compared with lower concentrations, the high concentration of *B. subtilis* in diet may enhance the release of

various digestive enzymes, which results in effective digestion and absorption of useful nutrients (Al-Fataftah & Abdelqader, 2014). In the light of the current observations, it may be assumed that probiotics are beneficial when used in high concentrations in the feed. These assumptions are strengthened by the statements of EFSA (2015), which reported that *B. subtilis* can express more valuable effects if used in a minimum concentration of 1×10^8 CFU/ kg of feed.

Table 6 Results of morphology of immune organs in broilers on day 35

Organs	Parameter	Treatments				
		BS-0.1	BS-0.05	ZnB	NC	P-value
Thymus	Thymic cortex (µm)	$311.52^{a} \pm 4.09$	$282.50^{ab} \pm 16.86$	$257.67^{bc} \pm 12.96$	$234.83^{c} \pm 17.91$	.006
	Thymic medulla $(\mu m)^2$	$433.50^{ab} \pm 25.49$	$463.50^{a} \pm 35.59$	$404.67^{ab} \pm 27.33$	$364.50^{b} \pm 14.18$	.090
	Cortex: medulla	0.7326 ±.05	0.6465 ±.09	0.6473 ±.04	0.6449 ±.04	.700
Spleen	Germinal centre/field area (%)	$0.8904^{a} \pm 0.13$	$0.8722^{a} \pm 0.07$	$0.7550^{ab} \pm 0.05$	$0.5329^{b} \pm 0.09$	.045
Bursa	Bursal follicular length (µm)	$451.83^{a} \pm 29.25$	$422.83^{ab} \pm 11.35$	$373.67^{b} \pm 21.53$	$373.17^{b} \pm 17.56$	.035
	Bursal follicular width (µm)	148.00 ± 10.99	155.50 ± 8.56	134.50 ± 18.24	104.67 ± 9.97	.227
	Bursal follicular area $(\mu m)^2$	$66356.3^{a} \pm 4926.2$	$65792.8^{a} \pm 4116.0$	$50285.0^{ab} \pm 7721.8$	$38022.5^{b} \pm 3932.6$	.019
	Bursal follicular number	9.00 ± 0.58	8.83 ± 0.98	7.50 ± 0.67	7.00 ± 0.58	.167

^{ab} Means within a row marked with different superscripts were significantly different (P <0.05).
Values represent Mean ± SEM.
BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB (10% zinc bacitracin, 0.01% of feed).

Table 7 Results of morphology of small intestine in broilers on day 21

Intestinal segments	Parameters	Treatments				
		BS-0.1	BS-0.05	ZnB	Control	P-value
Duodenum	Villus height (µm)	$1529.45^{a} \pm 20.03$	$1499.63^{a} \pm 29.69$	$1019.86^{b} \pm 17.35$	$1033.25^{b} \pm 14.83$	.000
	Villus width (µm)	151.60 ± 7.73	143.20 ± 9.53	148.76 ± 17.27	129.62 ± 6.49	.521
	Villus surface area $(\mu m)^2$	$665683^{a} \pm 35889$	$626945^{a} \pm 45697$	$453289^{b} \pm 54907$	$387545^{b} \pm 19406$	.000
	Crypt depth (µm)	$141.50^{a} \pm 8.29$	$138.66^{a} \pm 4.26$	$118.30^{b} \pm 2.52$	$126.58^{ab} \pm 4.19$	.018
	VH : CD	$10.97^{a} \pm 0.59$	$10.89^{a} \pm 0.51$	$8.63^{b} \pm 0.18$	$8.21^{b} \pm 0.33$	.000
Jejunum	Villus height (µm)	$916.50^{a} \pm 14.28$	$899.00^{a} \pm 18.73$	$686.00^{b} \pm 10.77$	$658.00^{b} \pm 17.64$	.000
	Villus width (µm)	108.06 ± 3.87	107.91 ± 4.97	107.17 ± 4.29	107.53 ± 1.94	.998
	Villus surface area $(\mu m)^2$	$308927^{a} \pm 14424$	$303703^{a} \pm 10659$	$232377^{b} \pm 7662$	$221756^{b} \pm 3749$	.000
	Crypt depth (µm)	114.80 ± 4.57	113.21 ± 5.57	104.07 ± 6.36	106.68 ± 2.75	.390
	VH : CD	$8.06^{a} \pm 0.42$	$8.02^{a} \pm 0.63$	$6.76^{b} \pm 0.57$	$6.19^{b} \pm 0.23$	.009
Ileum	Villus height (µm)	$487.01^{a} \pm 20.21$	$469.73^{ab} \pm 16.41$	$422.03^{b} \pm 16.43$	$417.20^{b} \pm 17.87$	.026
	Villus width (µm)	106.33 ± 2.97	105.17 ± 3.17	102.48 ± 2.19	99.82 ± 3.33	.425
	Villus surface area $(\mu m)^2$	$163063^{a} \pm 9559$	$155360^{ab} \pm 8318$	$137514^{b} \pm 5554$	$131227^{b} \pm 9138$	.038
	Crypt depth (µm)	116.06 ± 4.20	117.90 ± 3.99	109.88 ± 3.75	110.93 ± 2.99	.383
	VH : CD	4.21 ± 0.18	3.99 ± 0.15	3.85 ± 0.17	3.79 ± 0.26	.454

^{ab} Means within a row marked with different superscripts were significantly different (P <0.05)
SEM obtained in pool, Values represent mean ± SEM
BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB (10% zinc bacitracin, 0.01% of feed); VH : CD, villus height and crypt depth ratio

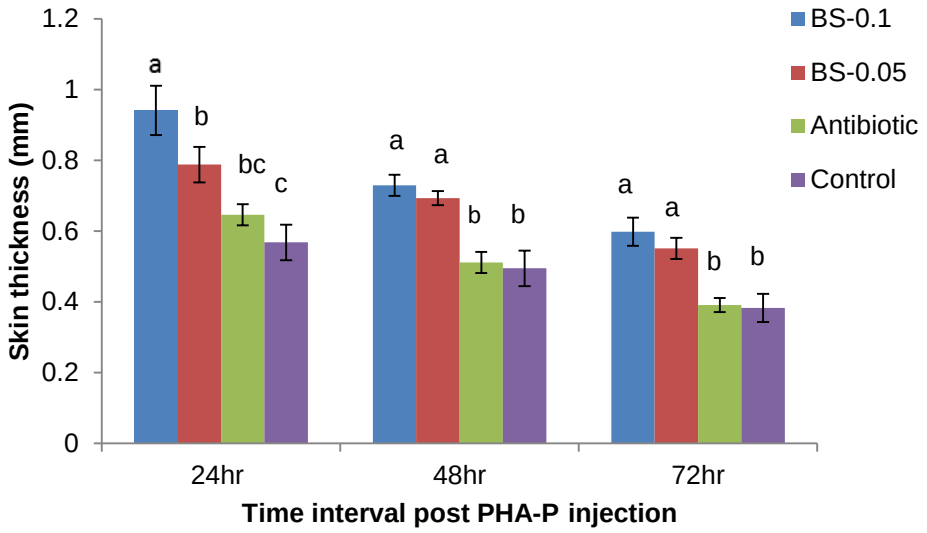
Table 8 Results of morphology of small intestine in broilers on day 35

Intestinal segments	Parameters	Treatments				
		BS-0.1	BS-0.05	ZnB	Control	P-value
Duodenum	Villus height (µm)	1462.78^{a} ± 47.61	1399.63^{a} ± 30.63	1116.70^{b} ± 27.02	1102.41^{b} ± 44.50	.000
	Villus width (µm)	150.43 ± 7.71	153.20 ± 9.53	151.60 ± 19.34	129.62 ± 6.49	.572
	Villus surface area (µm)^{2}	693732^{a} ± 51137	674774^{ab} ± 48161	531710^{bc} ± 68505	449158^{cd} ± 31243	.008
	Crypt depth(µm)	129.58 ± 4.09	124.66 ± 2.78	113.30 ± 2.20	119.85 ± 6.92	.089
	VH : CD	11.32^{a} ± 0.42	11.25^{a} ± 0.28	9.87^{b} ± 0.25	9.34^{b} ± 0.65	.007
Jejunum	Villus height (µm)	750.06 ± 56.43	728.86 ± 36.88	643.30 ± 30.77	685.15 ± 14.62	.226
	Villus width (µm)	117.95 ± 7.81	113.22 ± 7.38	112.85 ± 4.64	110.70 ± 5.69	.881
	Villus surface area (µm)^{2}	283330 ± 38433	261442 ± 28099	227965 ± 14627	238531 ± 14393	.456
	Crypt depth(µm)	122.88 ± 7.48	123.03 ± 6.04	121.79 ± 5.02	124.76 ± 9.62	.993
	VH : CD	6.26 ± 0.66	5.89 ± 0.36	5.37 ± 0.46	5.75 ± 0.52	.669
Ileum	Villus height (µm)	526.33^{a} ± 12.98	520.18^{a} ± 8.31	436.17^{b} ± 8.09	429.44^{b} ± 15.31	.000
	Villus width (µm)	112.01 ± 6.40	110.36 ± 7.62	104.85 ± 7.14	102.39 ± 5.18	.710
	Villus surface area (µm)^{2}	185479^{a} ± 12430	181053^{a} ± 14635	143299^{b} ± 9225	1382941^{b} ± 6930	.017
	Crypt depth(µm)	101.71 ± 3.13	100.05 ± 3.82	105.36 ± 3.18	106.45 ± 1.54	.507
	VH : CD	5.21^{a} ± 0.24	5.24^{a} ± 0.25	4.15^{b} ± 0.14	4.04^{b} ± 0.11	.001

^{abc} Means within a row marked with different superscripts were significantly different (P <0.05)
SEM obtained in pool
Values represent mean ± SEM
BS-0.1, BS-0.05 (0.1 g or 0.05 g *B. subtilis* per kg); ZnB (10% zinc bacitracin, 0.01% of feed); VH : CD, villus height and crypt depth ratio

In vivo evaluation of immune response to NDV and SRBCs was carried out by quantifying serum antibody titers in chickens. Vaccinations against infectious diseases, including ND, are used worldwide as prophylactic measures in poultry. SRBCs act as nonpathogenic thymus-dependent immunogens (Kundu *et al*., 1999). Probiotics have been found to modulate immunity by mediating the immune response of IgM serotype against SRBCs in birds (Haghighi *et al*., 2006). Molnar *et al*. (2011) reported higher antibody titer against NDV in birds maintained on feed supplemented with *B. subtilis* (CHCC3810-DSM17299). It is expected that birds could survive the ND challenge when they show relatively greater titers. The authors observed higher (P <0.05) antibody titers against NDV in the BS-0.1 group and in the supplemented groups against SRBCs on day 35 compared with control (Table 3), which reflects enhanced and ongoing plasma cell involvement in the production of antibodies till at least 18 days' post last antigenic exposure.

Lymphoproliferative immune response evaluation is a non lethal simple in vivo test in which the T cell mitogenic PHA-P is administered intradermally to assess the magnitude of cell-mediated immunity in the form of expanse of thickness and swelling (Corrier, 1990). The authors found that probiotic improved the cell-mediated immune response at 24, 48 and 72 hours post PHA-P injection compared with the ZnB and control groups (Figure 1). In light of these results it is indicated that birds maintained on diets supplemented with 0.1 g/kg (2.0×10^{10} CFU/g) *B. subtilis* developed relatively strong humoral and cellular responses.



Skin thickness at 24, 48 and 72 hours post PHA-P intradermal injections in chickens. Bars in the graph marked with different letters at a particular time interval were significantly (P <0.05) different

Figure 1 Effect of *B. subtilis* and zinc bacitracin on cell-mediated immune response against Phytohemagllutinin-P in broilers

Spleen, thymus, and bursa are the hub of immune cells where production and orientation of the cells occurs more efficiently in healthy animals than in immune-compromised ones. In disease-free animals, an increase in weight of immune organs correlates with enhanced proliferation of immune cells, which represents better immunity of the body (Teo & Tan, 2007). Liver participates in metabolization and contributes to immunity by synthesizing accessory proteins. The authors noted the higher (P <0.05) relative weights of lymphoid organs in the supplemented groups (Table 4), which could be the consequence of amplified B and T lymphocytes, as observed in high antibody titration and acidic natured goblet cells in the ileal mucosa. Comparable with these findings, Awad *et al*. (2009) reported higher (P <0.05) relative weight of liver and spleen and absolute weight of thymus on day 35 after feeding a *Lactobacillus*-type of probiotic. After in ovo inoculation of prebiotics and synbiotics, Madej *et al.* (2015) reported a trend of increased thymus relative weight on days 1, 7, and 35. Teo & Tan, 2007) found improved (P <0.05) indices of bursa of Fabricius in chickens fed probiotics. Similarly, in the current study the bursa weighed numerically higher in *Bacillus*-supplemented groups on day 35.

Various compartments in each lymphoid organ specify and maintain immune-related functions. The components were evaluated individually in the current study.

The histomorphological variations in cortex and medulla have a link with immune function. The larger cortex width seen in the current study (Tables 5 and 6) may be a sign of the rapid maturation rate of thymocytes, specifically T lymphocyte (Rooney *et al*., 2003). A significant increase in the cortex/medullary ratio observed in BS-0.1 on day21 indicated that hyperplastic proliferation and maturation of T cells (CD4+ or CD8+) occurred extensively in the cortex. These cells later migrate to the medulla and approach the secondary lymphatic organs via blood circulation. Histologically, it is obvious that the increased cortex : medulla ratio was because of thickening of the cortex, without any significant change in the widening of the medulla. Slawinska *et al.* (2014) reported that in ovo synbiotic-treated chickens established superior thymocyte densities in the cortex of the thymus compared with control on days 21 and 42. Madej *et al.* (2015) also reported that in ovo-delivered pre and symbiotic results in the higher cortex/medulla ratio on days 1, 7, and 35, while these were lower (P <0.05) on day 21 than in the control.

The germinal centre area is one of the important histological features in the spleen. Coupled with an increase in its relative weight (Table 4) in the supplemented groups, the authors noted increased (P <0.05) germinal centre area in spleen on day 35 (Table 6), which is similar to the findings of Madej *et al.* (2015) in symbiotic-treated chickens. It is assumed that *B. subtilis* had a stimulatory effect on B cells in the spleen, which might have potentiated the immune system by immunoglobulin synthesis. Similarly, Slawinska *et al.* (2014) reported that certain synbiotics may up-regulate the gene expression of IL-4 and IL-6 in the parenchyma of spleen, which later stimulates B cells.

The bursa is the main organ of the immune system in avian species. The dimensional increase (P <0.05) of the follicular area in BS-0.1 group (Table 5) indicates the immunomodulatory properties of *B. subtilis*. Similarly, Molnar *et al*. (2011) reported increased lymphoid follicular area in birds offered *B. subtilis*. The histomorphological improvements in various aspects of immune-related organs in chickens fed *B. subtilis* indicates that these probiotics may modulate systemic immune systems in addition to local mucosal immunity.

Most poultry producers and researchers agree that feed supplementation with probiotics from an early age helps to maintain mucosal integrity, promotes digestion and absorption, and eventually improves the overall performance of the bird (Tactacan *et al*., 2013; Latorre *et al*., 2014). However, little is available in the literature about the influence of *B. subtilis* QST-713 strain on the gut microarchitecture of broilers reared under controlled environment. Modulation of gut mucosal morphology is assumed to improve health indicators and production performance in birds (Awad *et al*., 2009). The gut mucosal barrier, which is consists of the epithelial cell membrane, which confers selective permeability and permits the entry of valuable molecules of available nutrients, also inhibits detrimental ingredients, including pathogenic bacteria and their toxins (Lee *et al*., 2015). Injury to the gut mucosal cells may provide chances for the entrance of injurious constituents, which may result in shortening of the villi and sloughing of the mucosal lining (Sikandar *et al*., 2017). The villi and microvilli increase the surface area for more efficient absorption of available nutrients in small intestine (Awad *et al*., 2009). The authors found increased VH, VSA, and villus to crypt ratio in *B. subtilis*-supplemented chickens (Table 7 and 8).

So, from this study, the authors can assume that absorptive function is increased in the modulated gut mucosa, which results in better performance in *B. subtilis*-supplemented groups compared with the control, similar to the findings of Al-Fataftah and Abdelqader (2014). Overall, lower villi heights were observed in the control group which may be linked with poor growth performance in that group.

Growing chickens rely on DFM to enhance immune system development and increase immune potential to resist infections (Lee *et al*., 2015). *B. subtilis*-type probiotic may stimulate subsets of immune cells to fabricate cytokines and antioxidants, which plays a role in the regulation of humoral and cell-mediated immune responses. In higher concentration these results were even better than those of the ZnB and control group chickens.

Conclusion

Feed supplemented with *B. subtilis* QST-713 has a positive effect on animal performance and health status through development of the microarchitecture of the gut and immune organs. These modulations have a connection with improved growth and immunity, which suggests that they may be used as alternatives to synthetic AGP in healthy broiler chickens. Further analyses are needed to reveal the mechanism(s) by which *B. subtilis* produces such effects.

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Authors' Contributions

All authors contributed equally and read the final version of the manuscript. MY,SM and HR contributed to experimental design and data analysis. AA and MS performed immunology, while AS and HZ performed the microarchitectural analysis and wrote the manuscript.

Conflict of Interest Declaration

The authors have no conflict of interest to declare.

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