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Effect of *Bacillus subtilis* on the microarchitectural development of the immune system in *Salmonella*-challenged broiler chickens

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Abstract: The effect of *Bacillus subtilis* on the immune responses and morphometry of the immune organs was evaluated in broilers challenged with *S. gallinarum*. For this purpose, *Salmonella*-free birds (n = 240) were split into four groups with six replicates of ten birds each. Groups included an NC (negative control, non-infected + non-medicated), a PC-S (positive control, *Salmonella*-infected + non-medicated), an AT-S (*Salmonella*-infected + medicated with enrofloxacin), and a BS-S (*Salmonella*-infected + *B. subtilis* (2.0×10^{10} cfu/g; 0.1 g/kg) group. On day 21, the thickness of the thymus cortex and medulla, germinal centre area of the spleen, bursal follicular length and bursal follicular area increased ($P < 0.05$) in the BS-S when compared to the NC and PC-S groups. On day 35, the BS-S group exhibited a higher ($P < 0.05$) antibody titre against the Newcastle disease virus (NDV), and cortex of the thymus was thicker ($P < 0.05$) compared to the other groups. A decrease in the thymus medulla thickness, germinal area of the spleen and bursal follicular number were noted in the PC-S group when compared to the other treatment groups. In conclusion, the prophylactic use of *B. subtilis* type probiotics alleviated the stress resulting from a *Salmonella gallinarum* infection and improved the immune organs development and function in infected broilers.

Keywords: histopathology; immunity; organs; poultry; probiotic; salmonellosis

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Salmonella is a foodborne pathogen and can cause severe ailments in humans, including focal infections, bacteraemia, enteric fever, and enterocolitis. It is an active concern of the one-health approach to protect humans from it by eliminating this pathogen from food animal products. In poultry, *Salmonella* is one of the common pathogens and causes production losses (Nair and Anup 2019). Contamination of poultry products with this pathogen presents a zoonotic health hazard, i.e., salmonellosis. *Salmonella gallinarum* is one of the common pathogens causing infections in broiler chickens. Enrofloxacin has been used to contest *Salmonella gallinarum* in commercial poultry farming (Sikandar et al. 2017). The extensive practice of using therapeutic antibiotics in the poultry industry has led to a universal upsurge in bacterial resistance, the derangement of normal gut microbiota, drug residue in poultry products and resistance to treatment (Ben et al. 2019). Consequently, in-feed prophylactic antibiotics have been banned in the European Union (Kabploy et al. 2016). Additionally, intensive poultry farming has also contributed to the animal's stress, which may subsequently compromise the immune function (Ghanima et al. 2020). Due to this ban, the animal husbandry sector has been confronted with many challenges to achieve the desired growth performance (Abudabo et al. 2019). As a result of extensive research, effective substitutes, such as in-feed probiotics, have surfaced that control the pathogen and possess the growth promoting properties that are comparable to those of antibiotics (Khalique et al. 2020; Sikandar et al. 2020). Bacteria can be used as probiotics upon meeting certain criteria, such as non-pathogenicity, growth inhibition of pathogenic organisms, its tolerance in gut secretions, support for the immune system and its capability to attach to the enterocytes (Gadde et al. 2017). It has been published elsewhere that *B. subtilis* have the ability to lower the intestinal pathogen count (de Oliveira et al. 2014); however, the mode of action and associated pathways are not fully understood. Among the proposed modes of action, the immunomodulatory process has attracted attention of researchers (Guo et al. 2017) and is the focus of the current study as well.

One among the previous reported studies (Sikandar et al. 2017) proposed that due to its effective probiotic capability, the *B. subtilis* QST 713 strain can be potentially endorsed for utilisation in the poultry industry. Immunomodulation is one of the important modes of action of a potential probiotic for safeguarding the animal host against pathogenic infections.

However, it is uncertain whether the actual act of using *B. subtilis* results in maintaining the local gut immunity as well as supporting systemic immunomodulation through the development of lymphoid organ's microstructures. We hypothesise that *Bacillus* type probiotics may act as immunomodulatory agents through the development of the lymphoid organ's microarchitecture and modulation of the systemic immunity in *Salmonella*-challenged birds. There are no published data available about any of the actual achievements of *Bacillus* spp. in the development of the microarchitecture of immune organs during a *Salmonella* infection. The purpose of this study was, therefore, to investigate the activity of infeed *Bacillus subtilis* for possible use as an antibiotic alternative on one hand and their protective effect on immune organs during a *Salmonella* infection on the other.

MATERIAL AND METHODS

Selection and rearing of chicks

At the age of one day, *Salmonella*-free broiler chicks (n = 240) were assigned to four groups with six replicates of ten birds each. Groups including NC (non-infected + non-medicated) and PC-S (*Salmonella*-infected + non-medicated) worked as the negative and positive controls, respectively. The antibiotic treated group [AT-S; *Salmonella*-infected + medicated viz. 50 ppm enrofloxacin (10% solution, ISO 9001 certified; Symans Pharmaceuticals Pvt Ltd, Lahore, Pakistan) was administered at the rate of 1 ml/2 l of drinking water on day 5 for seven days followed by the ensured availability of fresh water], and the *Salmonella*-infected BS-S group (oral *B. subtilis* 2.0×10^{10} cfu/g; Agraquest® Bayer, Davis, CA, USA; 0.1 g/kg feed). The study was carried out for a period of 35 days in an experimental poultry shed. The composition of the basal diet, vaccination schedule along with all the necessary husbandry practices and biosecurity measures were observed as mentioned elsewhere (Sikandar et al. 2017). The immunisation procedures are given here in brief: On day 1 and day 9, the chicks were vaccinated with a Newcastle disease (ND) vaccine (Nobilis ND LaSota; Intervet International B.V. Boxmeer, The Netherlands) via an ocular route and boosted on day 16 and day 23 via the drinking water.

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To access the immune response against sheep red blood cell (SRBC) antigen [sheep blood collected in Alsever's solution, washed three times and suspended in phosphate buffered saline (PBS)], two apparently healthy chicks from each replicate were selected randomly on day 14 and were immunised with a 5% SRBC antigen in the *musculus pectoralis* on day 14. A similar booster dose was set on day 21. The birds were offered *ad libitum* access to water and feed. The ethics related parameters and protocols were permitted by the University of Veterinary and Animal Sciences (UVAS), Ethical Review Committee for the Use of Laboratory Animals.

Evaluation of *Salmonella* in chicks upon arrival

The caecal tonsils were aseptically isolated from three chicks, mixed with a tetrathionate broth and cultured on brilliant green agar (CM0263; Oxoid Ltd, Basingstoke, UK) for an overnight incubation at 37 °C. The absence or presence of *Salmonella* was detected on the agar plates.

Infection in the animals with *Salmonella*

Local isolates of *S. gallinarum* seed were obtained from the University Diagnostic Laboratory, UVAS, Lahore, Pakistan. The freeze-dried culture was purified in a Salmonella-Shigella agar and incubated overnight in a tryptone soy broth (Oxoid Ltd, Basingstoke, UK) prior to the chick's challenge. On day 3, a total of 0.2 ml suspension of *S. gallinarum* as a challenge medium was administered orally through gavage to all the groups except NC and similar volume (0.2 ml) of peptone water (sterile buffered; Sigma-Aldrich Inc, St Louis, Missouri, USA) was inoculated to the control group (NC) birds (Sikandar et al. 2020).

Immunological responses

The humoral immune response was evaluated in terms of the antibody determination via a microtitre haemagglutination (HA) and haemagglutination inhibition (HI) assays against sheep erythrocytes and the Newcastle disease virus vaccine (NDV) as per the methods described elsewhere (Sikandar et al. 2017).

Sampling and histology of the immune organs

One animal per replicate (n = 6/group) was humanely killed with an overdose of barbiturates [intravenously (i.v.)] on day 21 and day 35 between 6 a.m. to 2 p.m. The lymphoid organs, including the spleen, bursa of the Fabricius and thymus were eviscerated from the dead birds and their samples were consequently preserved in 10% neutral buffered formalin for the subsequent histopathological study. The histomorphometry of the thymus, spleen and bursa of the Fabricius was carried out similar to the approaches explained elsewhere (Madej et al. 2015; Sikandar et al. 2017). A total of three microscopic fields areas per section were studied and the mean values acquired from the three sections per animal were noted.

Statistical analysis

The normal distribution of the data was assessed through the Kolmogorov-Smirnov test. The values were presented as the means ± SEM and were compared using a one-way analysis of variance (ANOVA) (SPSS v21.0; IBM, Armok, USA). The differences among the treatment means were calculated through Duncan's multiple range test. $P < 0.05$ was considered significant in all the statistical analyses.

RESULTS

Immune responses

Humoral immunity against NDV in terms of the antibody titre was recorded as being higher ($P < 0.05$) in the BS-S group when compared to the NC, PC-S and AT-S groups on day 35. The titre against the SRBCs did not differ ($P < 0.05$) among the groups on day 21 (Table 1).

Organ histomorphometry

In the BS-S group, the thickness of the thymus cortex and medulla, and the germinal centre area of the spleen increased compared to the PC-S and AT-S groups. Similarly, the bursal follicular area (BFA) increased compared to all the other groups

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Table 1. Effect of *Bacillus subtilis* on the humoral immune response in the *Salmonella*-challenged birds

Parameter	Treatments				
	NC	PC-S	AT-S	BS-S	*P*-value
Titre against ND virus (day 21)	2.16 ± 0.07	1.84 ± 0.13	1.91 ± 0.12	2.16 ± 0.07	0.076
Titre against ND virus (day 35)	2.19 ± 0.14^{bc}	1.93 ± 0.07^{c}	2.05 ± 0.05^{bc}	2.45 ± 0.06^{a}	0.037
Titre against sheep RBCs (day 21)	4.33 ± 0.49	4.00 ± 0.52	4.17 ± 0.31	5.50 ± 0.22	0.065

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; ND = Newcastle disease; PC-S = *S. gallinarum*-challenged, non-medicated; RBC = red blood cell; SEM = standard error of the mean

Means with different superscripts within the same row are statistically different ($P < 0.05$); Values represent mean ± SEM

($P < 0.05$) on day 21 (Table 2). The thymic cortex was noted as being thicker ($P < 0.05$) in the BS-S group compared to the PC-S group. The thymus medulla, germinal area of the spleen, bursal follicular width, area and number decreased ($P < 0.05$) in the PC-S group compared to all the other treatment groups on day 35 (Table 3). The bursal follicular length (BFL) was observed to be greater ($P < 0.05$) in the BS-S group compared to the negative control, PC-S and AT-S groups.

Table 2. Effect of *Bacillus subtilis* on the immune organ morphology in the *Salmonella*-challenged broilers on day 21

Organs	Parameters	Treatments				
		NC	PC-S	AT-S	BS-S	*P*-value
Thymus	thymic cortex (μm)	280.17 ± 21.84^{ab}	228.00 ± 18.19^{c}	263.00 ± 11.55^{bc}	323.17 ± 16.45^{a}	0.011
	thymic medulla (μm^2)	380.17 ± 14.36^{a}	312.67 ± 28.78^{b}	322.00 ± 18.15^{b}	390.67 ± 11.56^{a}	0.006
	cortex: medulla	0.74 ± 0.05	0.76 ± 0.08	0.82 ± 0.04	0.83 ± 0.05	0.610
Spleen	germinal centre/field area (%)	0.52 ± 0.08^{a}	0.29 ± 0.02^{b}	0.31 ± 0.01^{b}	0.61 ± 0.03^{a}	0.000
Bursa	bursal follicular length (μm)	541.00 ± 22.23^{bc}	448.54 ± 18.58^{c}	518.97 ± 30.66^{c}	679.57 ± 59.02^{a}	0.002
	bursal follicular width (μm)	249.62 ± 9.54^{a}	132.12 ± 6.09^{c}	220.85 ± 8.61^{b}	247.49 ± 11.87^{ab}	0.000
	bursal follicular area (μm^2)	135.358 ± 8.621^{b}	59.124 ± 3.037^{c}	114.638 ± 7.693^{b}	165.757 ± 8.949^{a}	0.000
	bursal follicular number	6.83 ± 0.48	6.00 ± 0.26	6.83 ± 0.31	7.00 ± 0.26	0.065

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated; SEM = standard error of the mean

Means with different superscripts within the same row are statistically different ($P < 0.05$); Values represent mean ± SEM

Table 3. Effect of *Bacillus subtilis* on the immune organ morphology in the *Salmonella*-challenged broilers on day 35

Organ	Parameters	Treatments				
		NC	PC-S	AT-S	BS-S	*P*-value
Thymus	thymic cortex (μm)	238.50 ± 14.98^{ab}	200.83 ± 13.03^{b}	238.17 ± 12.46^{ab}	278.33 ± 7.86^{a}	0.003
	thymic medulla (μm^2)	312.83 ± 4.76^{a}	266.33 ± 16.23^{b}	306.67 ± 16.43^{a}	324.50 ± 12.63^{a}	0.031
	cortex: medulla	0.76 ± 0.04	0.76 ± 0.02	0.78 ± 0.04	0.89 ± 0.05	0.215
Spleen	germinal centre/field area (%)	0.65 ± 0.06^{ab}	0.33 ± 0.03^{c}	0.50 ± 0.04^{b}	0.74 ± 0.06^{a}	0.000
Bursa	bursal follicular length (μm)	532.39 ± 24.36^{cd}	451.68 ± 15.00^{d}	603.55 ± 22.98^{bc}	756.49 ± 56.61^{a}	0.000
	bursal follicular width (μm)	256.09 ± 10.40^{a}	106.99 ± 3.05^{b}	224.75 ± 15.27^{a}	253.91 ± 14.72^{a}	0.000
	bursal follicular area (μm^2)	$136\,347 \pm 8\,576^{ab}$	$48\,298 \pm 1\,907^{c}$	$136\,199 \pm 11\,621^{ab}$	$190\,756 \pm 13\,854^{a}$	0.000
	bursal follicular number	6.83 ± 0.84^{a}	5.67 ± 0.21^{b}	7.17 ± 0.40^{a}	7.50 ± 0.43^{a}	0.020

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated; SEM = standard error of the mean

Means with different superscripts within the same row are statistically different ($P < 0.05$); Values represent mean ± SEM

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Histopathology

The thymus lobules were necrosed and were mostly replaced by fibrous connective tissues (Figures 1 and 2) and germinal centre areas in the spleen were found atrophied (Figures 3 and 4) in the PC-S group. The bursal follicles were also reduced in size on day 21 (Figure 5), and most of the areas were

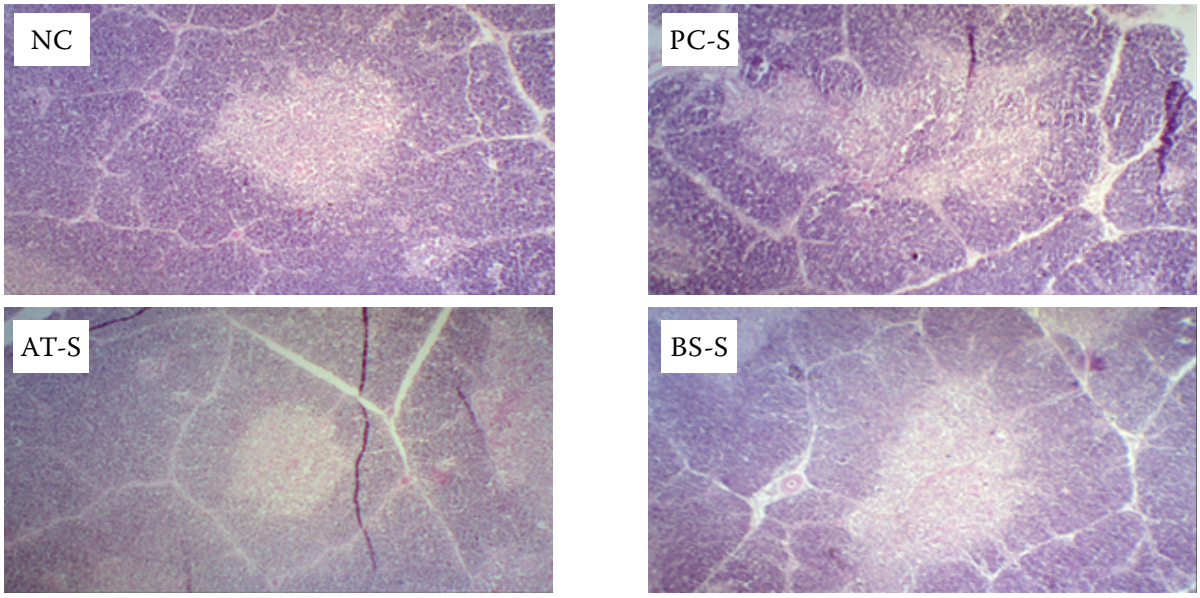

Figure 1. Microphotographs of the thymus (day 21)
Effect of *Bacillus subtilis* on the thymus morphology in the *Salmonella*-challenged broilers on day 21. Images taken at × 4, depicting the histopathological changes observed in the thymic medulla
AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

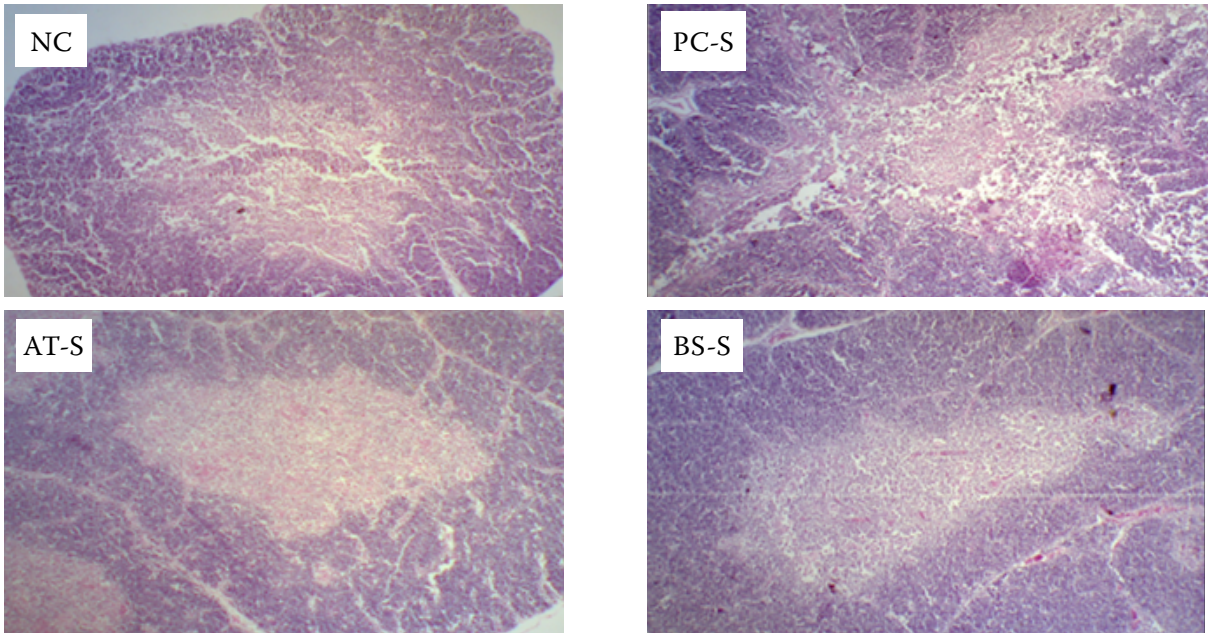

Figure 2. Microphotographs of the thymus (day 35)
Effect of *Bacillus subtilis* on the thymus morphology in the *Salmonella*-challenged broilers on day 35. Images taken at × 4, depicting the histopathological changes observed in the thymic medulla
AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

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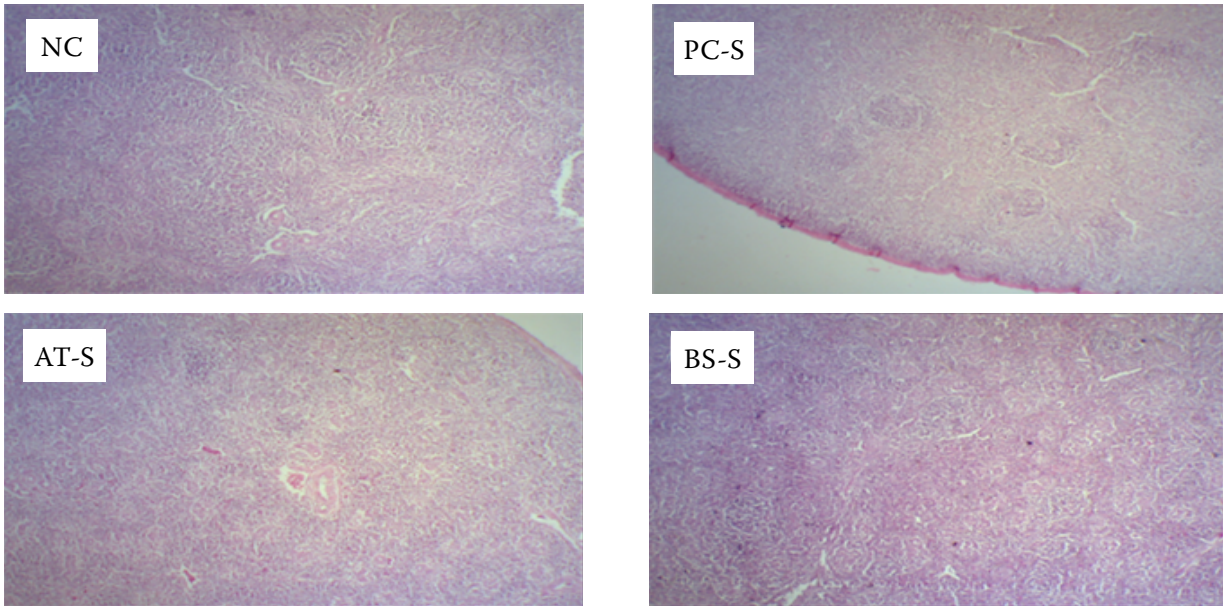

Figure 3. Microphotographs of the spleen (day 21)

Effect of *Bacillus subtilis* on the spleen morphology in the *Salmonella*-challenged broilers on day 21. Images taken at × 4, depicting the histopathological changes observed in the splenic germinal centre

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

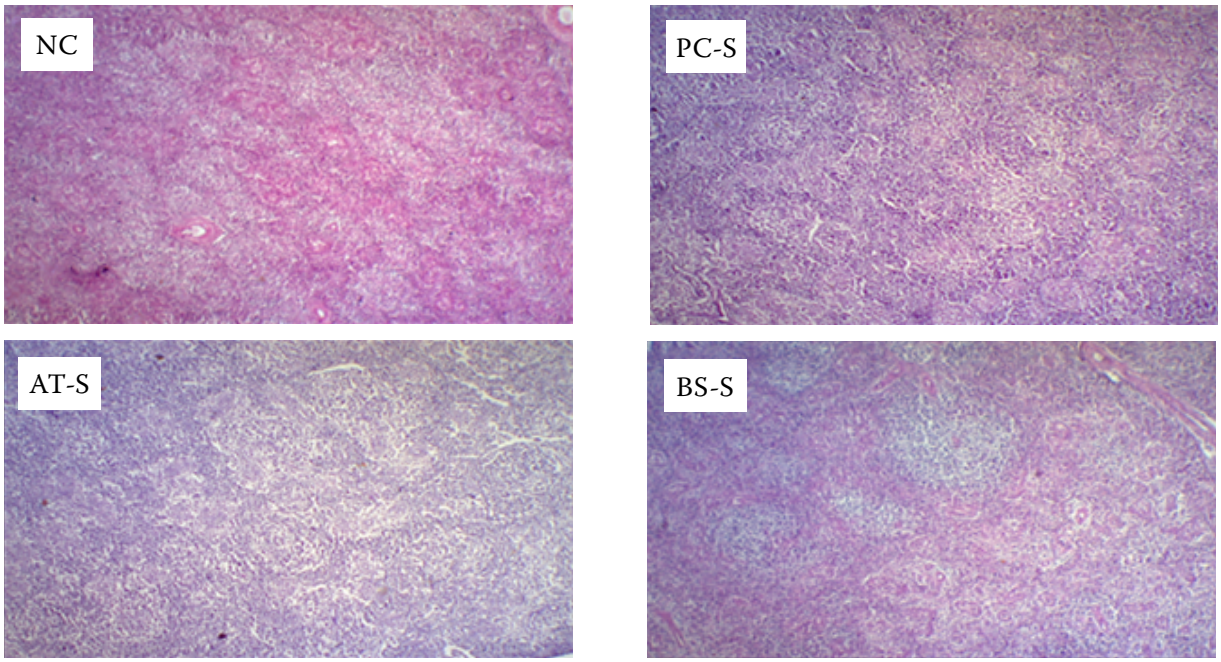

Figure 4. Microphotographs of the spleen (day 35)

Effect of *Bacillus subtilis* on the spleen morphology in the *Salmonella*-challenged broilers on day 35. Images taken at × 4, depicting the histopathological changes observed in the germinal centre area

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

replaced by stroma in the *Salmonella*-infected non-medicated group. The bursal follicular parenchyma was occupied with crypts and fundi. The bursal follicular architectural detail was preserved, but

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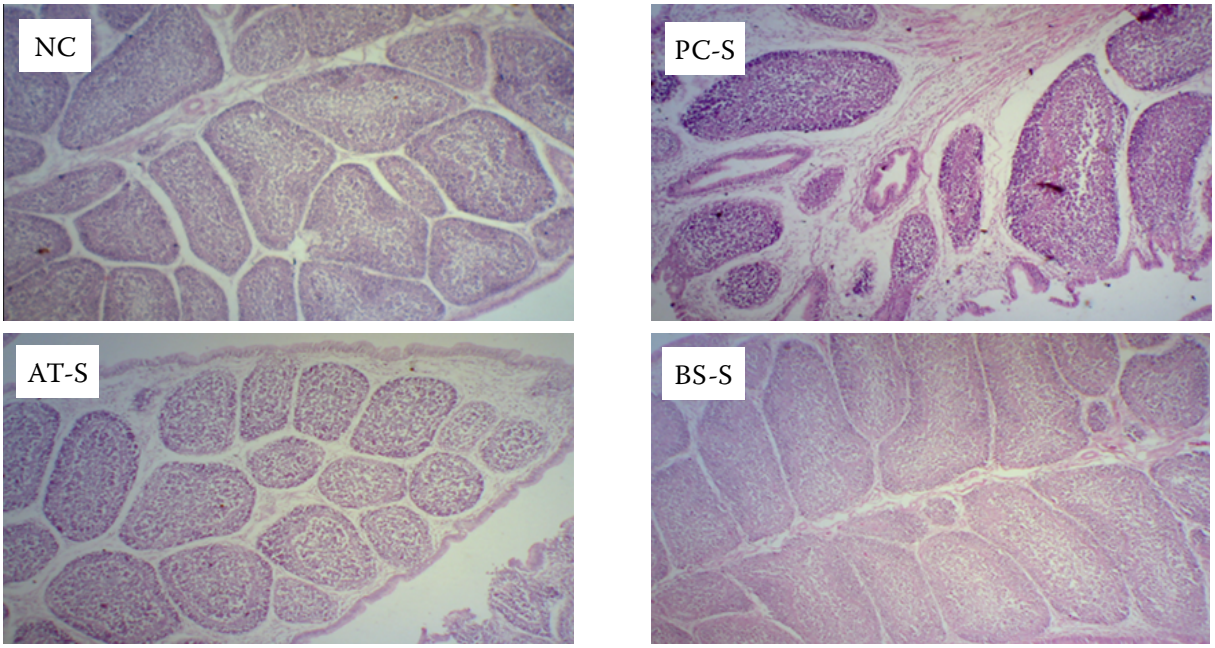

Figure 5. Microphotographs of the bursal follicle (day 21)

Effect of *Bacillus subtilis* on the bursa of the Fabricius morphology in the *Salmonella*-challenged broilers on day 21. Images taken at × 4, depicting the histopathological changes observed in the thymic medulla

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

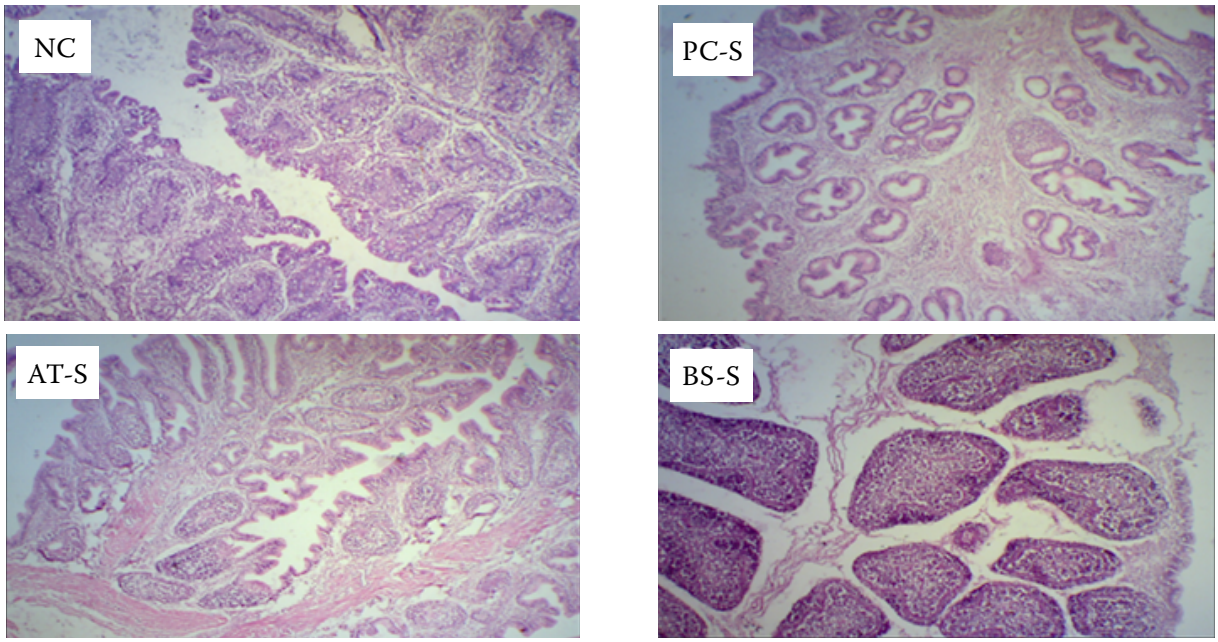

Figure 6. Microphotographs of the bursal follicle (day 35)

Effect of *Bacillus subtilis* on the bursa of the Fabricius morphology in the *Salmonella*-challenged broilers on day 35. Images taken at × 4, depicting the histopathological changes observed in the thymic medulla

AT-S = *S. gallinarum*-challenged + 50 ppm enrofloxacin; BS-S = *S. gallinarum*-challenged + 0.1 g/kg (2.0×10^{10} cfu/g) *B. subtilis*; NC = non-infected, non-medicated; PC-S = *S. gallinarum*-challenged, non-medicated

the cellular detail was lost on day 35 (Figure 6). Only rudimentary follicles were observed with very few lymphocytes. The demarcation between the cortico-medullary areas was not clear and the inter-

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follicular connective tissue proliferated extensively. The differences in the micromorphological structures in the immune organs were not significantly different in the AT-S and BS-S groups, however, the parenchyma of the bursal follicles in the BS-S group were observed to be more developed and active.

DISCUSSION

Regardless of the significance of the probiotics for the broiler growth performance and immune system, there is scant information in literature about the microarchitecture of the immune organs, their reconstitutional development and response to *Salmonella* infections. Subsequently, the current study was executed to report the effects of a *Salmonella gallinarum* infection on the histological arrangement of immune organs in broilers and to assess the protective effect a *Bacillus* type probiotic.

The probiotic antigenic fragments may cross the gut epithelial barrier and subsequently be presented to the immune system, where they play a role in modulation of both innate and acquired immunity (Dobson et al. 2012). Furthermore, stimulating the local gut immunity, the probiotics can also affect immune responses in other immune organs by the lymphoid tissue associated with the mucosa. Concerning the humoral immunity, no variation was detected among the groups in the antibody titres against ND and SRBCs on day 21 (Table 1). This insignificant response might be due to the developmental phase of the immune cells, and that RBCs are thymus dependent immunogens (Geng et al. 2015). Better geometric mean HI titres against ND in the BS-S group chickens were noted compared to the NC, PC-S and AT-S groups on day 35 (Table 1). It may be indicative of the settled plasma cells that are involved regularly in antibody production which leads the animals to subsist the ND during the challenge. *B. subtilis* is expected to modulate the systemic immune response via immunoglobulin-M against the ND and SRBCs (Haghighi et al. 2006). These probiotics were found to improve the gut integrity by expressing IL-2, IL-4, IL-10 and IL-13 in chickens (Gadde et al. 2017). *B. subtilis* has been found useful in the development of serum IgA and IgG in rabbits (Guo et al. 2017). Furthermore, such probiotic upregulates the IL-1β, IL-8 and IFN-γ. Additionally, dietary probiotic bacteria intermingle with the systemic immune system in various points, viz. proliferation of the mononuclear cells, production of cytokine, phagocytic activity, autoimmunity modulation, and boosting the immunity against enteric pathogens (Famularo et al. 1997). Probiotics are also reported to enhance the natural killer cell activity (Gill et al. 2001). The PC-S group birds reared to evaluate the antibody titre against sheep RBCs, were dead after day 21, hence the HA titre was evaluated after a single shot of RBC inoculation. Considering our results, it can be concluded that the *B. subtilis* strain that we used exhibited a role in the immune system stimulation in the grower phase of birds during an acute infection.

Organ morphometry

Various microscopic elements of the lymphoid organs conserve the immune functions (Haley et al. 2005). The thymus and bursa are considered to be central immune organs and the spleen is recorded as a peripheral organ. The intact microarchitecture of these organs confers better immunity to the body. The cortex of the thymus is an area where thorough proliferation, development, and a sorting of lymphocytes occurs, while mature $CD4^+$ or $CD8^+$ lymphocytes are present in the medulla. During the maturation processes of the T lymphocytes, the selected T-cells travel to the medullary area and have a general circulation via the post-capillaries in the medulla (Schat and Skinner 2014). In light of our study, it is difficult to explain that higher thickness in the thymus cortex in the probiotic group, it may be due to excessive the T-cell proliferation or minimal migration of cells from the cortex to the medulla or periphery. The earlier option seems more appropriate because the medullary architecture was indicated as having an optimal cell density. Improvement in the thickness of various compartments in the immune organs was found in the probiotic offered group. *Salmonella* may cause necrosis of the lymphocytes, but probiotics have been shown to prevent and/or delay *S. enterica*-induced apoptosis of the lymphocytes in mice. They are, likewise, related with the lower mRNA expression of genes, such as *Casp-2*, *Casp-12*, *Dad-1*, *Akt-1*, *Bad* that augment apoptosis. The effect of a probiotic in mice has been reported to amplify the mRNA expression for protein tyrosine phos-

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phatase receptor type-C, clusters of differentiated antigen-2, and Toll-like receptor-6 genes which are associated with the activation of B and T lymphocytes (Wagner and Jonson 2017). *B. subtilis* fortifies the α-defensin and β-defensin induction, which support the host innate immunity (Guo et al. 2017). Defensins could act upon pathogenic microorganisms directly in addition to the commencement and regulation of the adaptive immunity. The in-feed *B. subtilis* increased the number of CD-4 lymphocytes and maintained the tight junction protein mRNA expression (Gadde et al. 2017). It has been published that some of the bursal derived B-cells migrated to the periphery each day (Withers et al. 2006), where they performed their function. We cannot exclude the fact of the B-cell apoptosis, which was rapid in the *Salmonella*-challenged birds, and that the delay cell death was expected in the *Bacillus* treated birds. The thicker germinal centre area in the spleen and bursal follicular area dimension in the *Bacillus*-treated chickens (Tables 2 and 3), showed the involvement of the systemic immune system in addition to the local immunity. To the best of our knowledge, studies that outline the effect of *B. subtilis* on immune organ compartmental changes in challenged birds are not available for comparison. It can be assumed that *B. subtilis* had a stimulatory effect on the B and T lymphocytes in the spleen, which leads to immunoglobulin synthesis. Likewise, the *in ovo* administration of some synbiotics viz. *Lactococcus*, *Lactobacillus* and *Streptococcus* species expressed and upregulated the interleukin-4 and interleukin-6 genes in the spleen (Slawinska et al. 2014). The spleen, bursa and thymus are the hub of the immune cells, where cell production and positioning happen more optimally in healthy animals when compared with immune-compromised ones. The authors observed the developed compartments of lymphoid organs in the probiotic supplemented birds, which may be due to the augmented B and T lymphocytes, as observed in the high antibody titration against NDV (Table 1). Increasing ($P < 0.05$) relative weights of the immune organs were observed (Teo and Tan 2007) after feeding probiotics. The *in ovo* synbiotic inoculation in chickens established superior lymphocyte densities in the thymic cortex with control on days 21 and 42, while higher cortex/medulla ratios on days 1, 7, and 35 were observed after the *in ovo* pre- and symbiotic inoculation (Madej et al. 2015). Likewise, augmented bursal follicular areas in birds have been reported subsequent to a *B. subtilis* supplementation (Molnar et al. 2011).

The histopathological study depicts that the architecture improved with the infeed supplementation of *B. subtilis*, through a decrease in the stromal cells and an increase in the parenchymal cells in the organs. It has been reported that the increased number of parenchymal cells (lymphocytes) in the lymphoid organs contributed to a better immunity (Campbell and Ellis 2013), because these cells played an important role against the infection. In conclusion, the supplementation of *B. subtilis* product improved the systemic immune responses, amended and micro-architecturally developed the immune organs of commercial birds challenged with *S. gallinarum*.

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Conflict of interest

The authors declare no conflict of interest.

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