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Original Article

The Response of Broiler Chicks to Dietary Supplementation with a Probiotic, Acidifiers Blend, and Their Combination

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Broiler; Immunity; Performance; Small intestine.

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ABSTRACT

This study investigated the effect of a probiotic (*Bacillus subtilis* DSM 17299*)*, blend of acidifiers, and their combination on the performance of broiler chicks. Two hundred and twenty unsexed one-day-old broilers (Ross 308) were randomly distributed into four groups (55 birds per group divided into 5 replicates) as 2X2 factorial arrangement including two factors, probiotic and blend of acidifiers, each of which had two levels: yes and no. Performance was determined weekly. Haemagglutination test was performed on blood samples taken on days 28 and 42 after the birds were injected twice (days 14 and 28) with 10% suspension of sheep red blood cells (SRBC). On day 42, tissue samples taken from the duodenum, jejunum and ileum were prepared for histology via scanning electron microscopy. During the first three weeks, dietary addition of probiotic significantly (p<0.05) increased body weight gain (BWG) while acidifiers significantly lowered (p<0.01) feed conversion ratio (FCR). Antibody titer against SRBC increased remarkably (p<0.01) 15 days post the first injection when probiotic was administered. The probiotic also increased (p<0.01) the number of the duodenal goblet cells, and the density of jejunal and ileal villi. Overall, the supplementation of probiotic or acidifiers enhanced the growth performance of broiler chicks, mainly during the first three weeks of age. The probiotic also improved the immune response and intestinal morphology of broilers. However, there was no evidence of synergy when probiotic and acidifiers were co-administered.

INTRODUCTION

Probiotics and acidifiers, among others, have been considered as alternatives to antibiotic growth promotors (Mountzouris *et al.*, 2010; Zhang & Kim, 2014). In general, the effect of dietary addition of either probiotics or acidifiers on broilers was investigated by many researchers (Pelicano *et al.*, 2005; Samli *et al.*, 2007; Awad *et al.*, 2009; Rodríguez-Lecompte *et al.*, 2012; Khan & Iqbal, 2016; Sikandar *et al.*, 2017).

The bacterium *Bacillus subtilis* has been widely used as commercial probiotic in poultry production because its spores are highly adapted to survive in harsh environmental conditions (Tactacan *et al.*, 2013). In addition, the spores can germinate in the gastrointestinal tract of chicks, the environment in which it becomes metabolically active (Cartman *et al*, 2008). These probiotic strains produce antibacterial compounds that can reduce the prevalence of harmful or undesirable bacteria while simultaneously creating an environment in the gastrointestinal tract in which beneficial bacteria can proliferate (Taklimi, 2012; Khan & Naz, 2013). Consequently, the dietary addition of probiotics displayed a crucial role in regulating the intestinal environment, resulting in improved broiler health and performance (Abudabos *et al.*, 2013; Manafi *et al.*, 2018).

The acidifiers used in poultry feed include inorganic and organic acids (Kim *et al.*, 2015). The latter have been commonly applied in poultry industry, although their efficacy is influenced by the combination of acids used and their presentation (Khan and Iqbal, 2016). Several modes of action have been attributed to acidifiers, but mainly they are thought to increase the digestibility of the diet by nutrient retention and suppress the growth of pathogenic bacteria by modulating the gut microbiota (Kim *et al.*, 2015; Haq *et al.*, 2017, Dai *et al.*, 2021).

Despite the positive impacts of probiotics and acidifiers on broiler chicks, the reason to continue research on these additives not only is that the results are partly contradictory but also the scarcity of information documenting their combined effects (Agboola *et al.*, 2015; Abudabos *et al.*, 2017; Rodjan *et al.*, 2017; Elhassan *et al.*, 2019). Therefore, the present study was conducted to investigate the effect of the probiotic, *Bacillus subtilis*, and a blend of acidifiers, and their combination on the growth performance, gut morphology, and immunity of broiler chickens.

MATERIALS AND METHODS

All procedures of the experiment including animal husbandry and method of slaughter were approved by the Sudan Veterinary Council (Ethical approval No. EA/0035/2019).

The feed trial was carried out in an open-sided house in the poultry experimental unit at the Faculty of Animal production, University of Khartoum, Sudan. Two hundred and twenty unsexed day-old healthy broiler chicks, purchased from the local hatchery, were reared for 42 days. The selected strain of birds was Ross308, which is widely known for its high performance in the local market.

A complete randomized design arranged as 2x2 factorial was utilized. The design included two factors (main effects), probiotic *(Bacillus subtilis* strain DSM 17299) and a blend of acidifiers (citric acid, fumaric acid, D-L malic acid, lactic acid, orthophosphoric acid) with two levels: Yes and No for each factor. On day 0 of the trial, the broiler chicks were randomly allocated to four dietary treatment groups. Each group was composed of 55 birds and was divided into 5 replicates of 11 birds each. Each replicate was represented by a pen with wood shavings (5 cm thick) on the floor having dimensions of 1x1 m. A tube feeder and fountain drinker were allocated to each pen. Feed and water were provided to birds ad libitum throughout the trial. Continuous lighting of 24 hours a day was provided naturally during the day-time and artificially during the night. Figure 1 shows the time-line chart of the different procedures applied throughout the experiment.

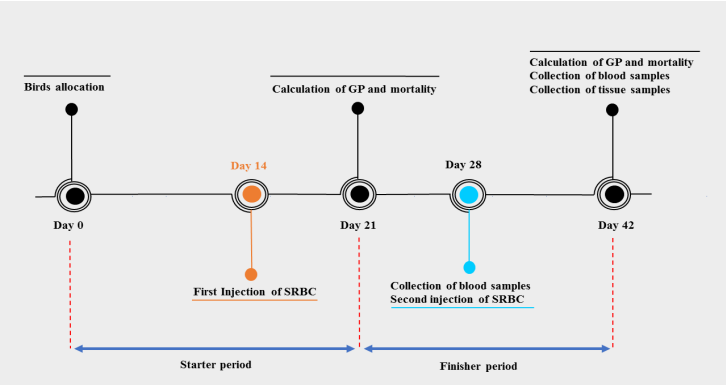

Figure 1 – Time-line chart showing the procedures and methods applied throughout the experiment duration (day 0 to day 42). GP, growth performance. SRBC, sheep red blood cells.

The experimental diet was based on sorghum and groundnut cake and formulated as mash, without anticoccidials or other medications, to meet the requirements indicated by NRC (1994). As shown in Table 1, birds were given starter feed during the first three weeks of age (0 to 21 days), and finisher feed throughout the remaining period of the experiment (22 to 42 days). Proximate feed composition was determined following the procedure given by AOAC (2005).

For growth performance, the floor pen was considered as the experimental unit. Average body weight and feed intake for each pen were recorded at weekly intervals. Feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) were determined for each treatment group at days 21 and 42.

At the end of the experiment, on day 42, after performance data were collected, the birds were fasted for 12 hours except from water. One bird from each pen was randomly selected for tissue sampling. Each treatment was therefore represented by 5 birds with a total of twenty birds. The birds were euthanized and tissue samples from the mid region of the duodenum, jejunum and ileum were immediately collected and processed for either histology or scanning electron microscopy, as will be further described later.

For the study of goblet cells density within the villi, samples were collected from the mid region of the duodenum. After washing in phosphate buffer saline (pH 7.4), samples were then fixed in 10% neutral buffered formalin for 24 hours. The tissues were then processed and embedded in paraffin wax. Cross

sections 3-4 µm thick were cut by a rotary microtome, placed on glass slides, and stained with Periodic Acid-Schiff (PAS). Fourteen villi in each section were randomly selected using a 40× stage objective lens of a light microscope (Olympus BX63-Japan) connected to a digital camera (Olympus DP72). Thus, a total of 70 villi for each group were chosen. Measurements were then carried out using video image software (Cell Sens 510 – Olympus). The epithelium on one side (lamina propria excluded) of the middle portion of each selected villus was considered as a region of interest (ROI). Using free-hand selection tool, the area (µm^2) of each ROI (epithelial area) was then determined by mouse pointer. Thereafter, only active goblet cells within each ROI were counted. The active cells were identified by their prominent cup and tail parts (Figure 2) as well as their localization closely to the edge of the villus (Brümmer, 2010). Data were then expressed as the average number of goblet cells per 1000 µm^2 of epithelial area.

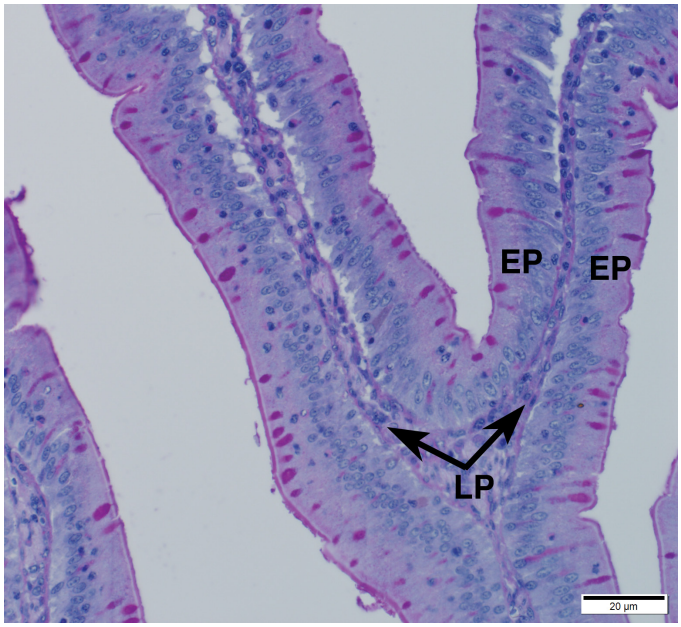

Figure 2 – Photomicrograph showing bifurcated villus in the duodenum of a broiler chick. LP: lamina propria. Arrows: bifurcation of the lamina propria. EP: epithelial layer. PAS stain.

For scanning electron microscopy, 48 samples (approximately 5 mm²) from the wall of the duodenum, jejunum and ileum at the midpoint were taken from 16 birds. Each group was represented by four birds which were randomly selected from the five birds used for histological investigations described earlier. Samples were then processed as described by Amaral *et al*., 2007. Briefly, the collected samples were washed in 0.1 M phosphate buffer saline (pH 7.4), fixed in 2.5% glutaraldehyde buffered with 0.2 M cacodylate (pH 7.4) for 24 hours. Samples were then washed with the cacodylate buffer and post-fixed in 2% osmium tetraoxide for 2 hours. Then, samples were washed again in the cacodylate buffer, dehydrated in several grades of ethanol, and coated with gold in a vacuum coater for 3 minutes. Samples were then viewed using a Jeol scanning electron microscope (JSM- 6390 LA, Japan). The villi of each sample were counted in three different microscopic fields, each of which measured 1.23 mm² (Figure 3). The villi density was then expressed as the average number of villi per 1.23 mm².

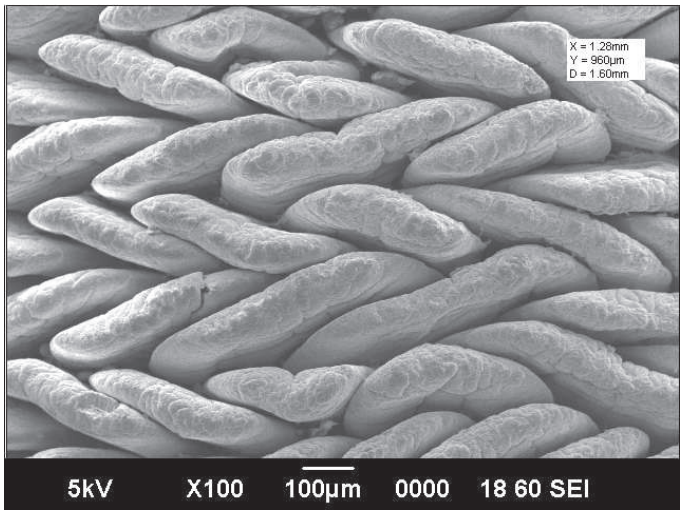

Figure 3 – Scanning electron micrograph of the luminal surface in the jejunum of a broiler chick showing the villi and the microscopic field dimensions.

In order to be used as an antigen, sheep red blood cells (SRBC) were collected and washed three times in normal saline. On day 14, three birds in each replicate (15 birds per group) were randomly selected, wing-banded and injected intramuscularly in the breast muscle with 1 ml of 10% suspension of packed SRBC in normal saline. At day 28 of age, blood samples (about 2 ml) from the brachial vein of each bird were collected into plain tubes and then birds were reinjected with 1 ml of 10% SRBC suspension and blood samples were collected for the second time at day 42. Sera were then obtained and preserved at -20°C for later analysis using the haemagglutination test to detect total antibody titer as described by Singh & Dhawedkar (1993).

Data were statistically analyzed by the GLM procedure for 2x2 factorial arrangement using a statistical software program (SPSS version 21.0, IBM Corporation, New York, USA). Both main effects and interaction were examined after confirming the normal distribution of the data. When a significant interaction had been detected, Duncan's multiple-range test was further applied to compare between means of treatment groups. p value of <0.05 was considered statistically significant.

Table1 – Composition and analysis of the starter (0-21) and finisher (22-42) diets of the four experimental groups of broiler chickens.

Ingredient %	Starter (0-21 days)				Finisher (22-42 days)			
	T1	T2	T3	T4	T1	T2	T3	T4
Sorghum	67.53	67.50	67.45	67.45	66.9	66.9	67.03	67.48
Groundnut cake	24.84	24.85	24.85	24.84	15.40	15.40	15.47	15.70
Wheat bran	-	-	-	-	8.54	8.50	8.20	7.46
Vegetable oil	-	-	-	-	2.00	2.00	2.00	2.00
Super concentrate*	5.25	5.25	5.25	5.25	5.25	5.25	5.25	5.25
Lysin	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.11
Methionin	0.15	0.15	0.15	0.15	0.1	0.1	0.1	0.1
Dicalcium phosphate	0.5	0.5	0.5	0.5	0.50	0.50	0.50	0.50
Limestone	1.03	1.00	0.95	0.95	0.80	0.80	0.80	0.80
NaCl	0.20	0.20	0.15	0.15	0.2	0.2	0.15	0.15
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.09	0.09	0.10
Antimycotoxins	0.20	0.20	0.20	0.20	0.10	0.10	0.10	0.10
Probiotic[1]	-	0.05	-	0.05	-	0.05	-	0.05
Acidifiers[2]	-	-	0.20	0.20	-	-	0.20	0.20
Total	100	100	100	100	100	100	100	100
Calculated values								
ME (MJ/kg)	13.20	13.20	13.20	13.20	13.39	13.39	13.39	13.39
Crude protein%	23.24	23.24	23.23	23.21	20.00	20.00	20.00	20.00
Crude fiber%	3.90	3.90	3.90	3.90	4.20	4. 19	4.16	4.42
Crude fat%	3.53	3.53	3.53	3.53	3.11	3.11	3.11	3.38
Lysine%	1.26	1.26	1.26	1.26	1.10	1.10	1.10	1.10
Methionine%	0.51	0.51	0.51	0.51	0.44	0.44	0.44	0.44
Methionine+Cystiene%	0.82	0.82	0.82	0.82	0.72	0.72	0.72	0.72
Calcium%	1.04	1.03	1.01	1.01	0.91	0.91	0.91	0.91
Available phosphorus%	0.41	0.41	0.41	0.41	0.42	0.42	0.42	0.41
Chemical Analysis								
Crude Protein	24.9	24.3	23.6	23.1	20.9	20.7	21.2	20.4
Crude fibre	4.1	3.66	3.60	3.57	3.68	3.94	3.89	3.69
Crude fat	3.00	3.18	3.16	3.20	4.35	4.15	4.30	4.28
Ash	5.85	6.17	5.98	5.95	5.90	5.50	6.16	5.73
Moisture	5.27	6.01	6.16	5.74	6.43	6.25	6.04	6.19

T1: treatment group received no additives; T2: treatment group received 0.5% probiotic; T3: treatment group received 2% acidifiers; T4: treatment group received 0.5% and 2% acidifiers; MJ: Mega Joule.

* Super concentrate provides each kg of mixed feed with the following amounts of vitamins and minerals: Vitamin A 12,000 IU; Vitamin D_3 3,800 IU; Vitamin E 35 mg; Vitamin K_3 2.8 mg; Vitamin B_1 2.8 mg; Vitamin B_2 8 mg; Vitamin B_6 3.3 mg; Vitamin B_{12} 0.02 mg; Niacin 40 mg, Folic acid 1.20 mg; Choline chloride 645mg; Ca 4.13 gm; Mn 100 mg; Zn 90 mg; Fe 54 mg; Mg 29 mg; Cu 19 mg; Se 0.35 mg; I 0.4 mg.

[1] Product powder contained *Bacillus subtilis* 1.6X10^9 CFU/gm (GalliPro, Chr. Hansen, Hørsholm, Denmark) and added as 500 gm/ton feed (0.05%) according to the manufacturer recommendation.

[2] Product powder contained Citric Acid, Fumaric Acid, D-L Malic Acid, Lactic Acid and Orthophosphoric Acid (Citrinal, Dex Ibérica, Vila-seca, Spain) and added as 2 kg/ton feed (0.2%) according to the manufacturer recommendation.

RESULTS

The effect of probiotic, acidifiers, and their combination on BWG, FI and FCR is shown in Table 2. On day 21, the birds that were supplemented with the probiotic showed a remarkable increase (p<0.05) in BWG (502.1 g) compared to the birds which received the diet without the probiotic (466.3 g). There was no effect of acidifier on BWG but FCR was lower (p<0.01) in the birds fed the acidifiers versus those that did not. There was no significant interaction between the two additives on BWG, FI and FCR. At 42 days of age, both of the main effects (probiotic and acidifiers) did not influence the BWG, FI and FCR (p>0.05) while there was also no significant interaction between the main effects.

The effect of probiotic, acidifiers and their combination on SRBC titer and number of duodenal goblet cells are displayed in Table 3. On day 28, the dietary supplementation with the probiotic increased the antibody titer against SRBC (p<0.01) while no effect was observed in the birds fed acidifiers alone. However, on day 42, there was no significant effect on SRBC antibody titer when the probiotic or acidifiers

Table 2 – Means of body weight gain (g), feed intake (g) and feed conversion ratio of broiler chicks supplemented with probiotic, acidifiers, and their combination.

Treatment		day 21			day 42		
Probiotic	Acidifiers	BWG	FI	FCR	BWG	FI	FCR
No	No	857.3	457.7	1.87	3384.8	1557.0	2.17
Yes	No	889.8	497.5	1.79	3549.9	1632.8	2.16
No	Yes	809.1	474.9	1.71	3239.0	1601.3	2.03
Yes	Yes	882.6	506.6	1.75	3503.2	1669.0	2.10
SEM		27.5	16.8	0.03	103.0	55.8	0.06
Main effects							
Probiotic	No	466.3	833.2	1.79	1579.2	3311.9	2.10
	Yes	502.1	886.2	1.78	1650.9	3526.6	2.13
Acidifiers	No	477.6	873.6	1.83	1594.9	3467.4	2.16
	Yes	490.8	845.9	1.70	1635.2	3371.1	2.07
SEM		16.8	19.5	0.02	39.5	72.9	0.04
p value							
Probiotic		0.04	0.07	0.59	0.22	0.06	0.52
Acidifiers		0.45	0.34	<0.01	0.48	0.36	0.07
Probiotic X Acidifiers		0.81	0.47	0.09	0.94	0.637	0.59

BWG: Body weight gain; FI: Feed intake; FCR: Feed conversion ratio.

were administered. Birds fed the probiotic showed an increase (p<0.01) in the number of goblet cells on day 42 as compared to the birds supplemented with neither of the additives. Conversely, the inclusion of acidifiers in the feed alone or in combination with probiotic had no significant effect upon the number of goblet cells.

Table 3 – Antibody titers (Log_2) against SRBC (on days 28 and 42) and the number of goblet cells per 1000 μm² of the duodenal villus epithelium (on day 42) of broiler chicks supplemented with probiotic and acidifiers.

Treatment		SRBC (Log_2)[1]		Goblet cells[2]
Probiotic	Acidifiers	day 28	day 42	day 42
No	No	2.00	3.36	3.32
Yes	No	3.21	3.54	5.09
No	Yes	2.00	3.43	3.87
Yes	Yes	2.93	3.29	4.66
SEM		0.23	0.36	0.26
Main effects				
Probiotic	No	2.00	3.39	3.59
	Yes	3.07	3.41	4.76
Acidifiers	No	2.61	3.45	4.09
	Yes	2.46	3.36	4.26
SEM		0.16	0.23	0.18
p value				
Probiotic		<0.01	0.95	<0.01
Acidifiers		0.54	0.78	0.51
Probiotic X Acidifiers		0.54	0.62	0.15

[1] n=25; [2] n=70; SRBC: Sheep red blood cells.

As shown in Table 4, the supplementation of probiotic and acidifiers had no significant effect on duodenal villi density. However, in the jejunum and ileum, the villi densities were higher (p<0.01) in birds fed the probiotic as compared to those which received either acidifiers alone or the combination of the two additives.

Table 4 – Villus density (number of villi/1.23 mm²) per small intestinal segment of broilers supplemented with probiotic and acidifiers at 42 days of age.

Treatment		Duodenum[1]	Jejunum[1]	Ileum[1]
Probiotic	Acidifiers			
No	No	18.2	23.5	27.8
Yes	No	17.9	31.8	33.3
No	Yes	18.1	23.7	29.3
Yes	Yes	22.4	30.6	39.3
SEM		1.50	2.00	2.50
Main effects				
Probiotic	No	18.3	23.6	28.6
	Yes	20.2	31.2	36.3
Acidifiers	No	18.0	27.7	30.6
	Yes	20.4	27.1	34.3
SEM		1.1	1.4	1.76
p value				
Probiotic		0.18	<0.01	<0.01
Acidifiers		0.15	0.79	1.4
Probiotic X Acidifiers		0.14	0.72	0.37

[1] n: 12

It is worth mentioning that during light microscopic investigation, some of the duodenal villi were divided approximately into two equal branches (Fig. 2). Such villi were mostly seen in birds supplemented with the feed additives. When viewed by scanning electron microscope, the branching was almost about the middle of the villus height forming a broad base and two separate branches (Figure 4).

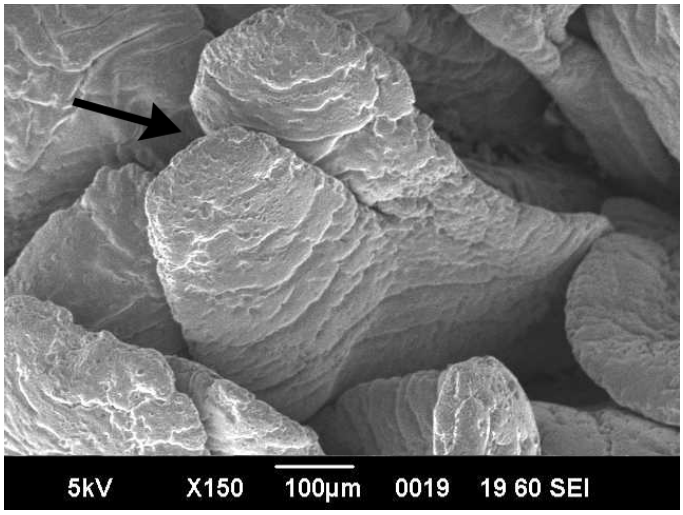

Figure 4 – Scanning electron micrograph of the luminal surface in the duodenum of a broiler chick. Arrow: branched villus with broad base and two branches.

DISCUSSION

During the first 5 weeks post hatch, it can be inferred from several studies that the growth performance of broilers has been enhanced by dietary addition of different strains of *Bacillus subtilis*, including the same strain used in the current investigation (Jeong & Kim, 2014; Akhavan-Salamat & Ghasemi, 2016). During the same period, dietary supplementation with a mixture of acidifiers (formic, phosphoric, lactic, tartaric, citric and malic acids) has also been reported to improve broiler performance (Hashemi *et al.*, 2014). On the contrary, the present study revealed that neither probiotic nor acidifiers had apparent effect on the overall growth performance of broilers.

In the current study, probiotic supplementation significantly increased the number of active goblet cells within the duodenal villi. Similar results were reported in all segments of the small intestine of turkey poults when lactic acid-based probiotics were added to the diet (Rahimi *et al.*, 2009). The density of goblet cells has been reported to correspond to the thickness of the mucus lining the intestinal wall (Specian & Oliver, 1991). Thus, the use of *B. subtilis* DSM 17299 in this study is suggested to enhance the protection of duodenal mucosa by increasing the number of goblet cells.

The blend of acidifiers used in the present study did not affect the number of duodenal goblet cells. However, a significant increase in the number of ileal goblet cells has been reported in broilers receiving a combination of probiotic and organic acids via drinking water (Rodríguez-Lecompte *et al*., 2012).

It has been reported that probiotics enhanced the production of natural antibodies in chickens (Haghighi *et al.*, 2006). Moreover, the probiotic *Bacillus subtilis* has been reported to improve humoral and cellular responses of broiler chicks (Khaksefidi & Ghoorchi, 2006; Sikandar *et al*., 2017). Similarly, dietary addition of probiotic in the present study significantly increased the antibody titre against SRBC at 14 days post first inoculation but no effect was observed two weeks post second inoculation. In agreement with the present findings, it has been stated that lactic acid producing probiotic (*Pediococcus acidilactici*) was suggested to enhance the primary systemic immune response to SRBC injection (Allahdo *et al*., 2018).

Scanning electron microscopic observations in the present study revealed that the number of jejunal and ileal villi increased significantly when *B. subtilis* was added to the diet. It is well known that the improvement of gut morphology, and subsequently increased absorptive surface area, is a prerequisite for efficient digestive and absorptive function of the intestine (Awad *et al.*, 2009). Positive changes in gut morphology, particularly villus length of broiler chickens have been reported by several authors after dietary supplementation with *B. subtilis* (Aliakbarpour *et al.*, 2012; Abudabos *et al.*, 2017). These findings plausibly accentuated the present study, in which the increase in jejunal and ileal villi density might be another process whereby probiotic elevates the intestinal surface area, and subsequently improved nutrient absorption.

The present investigations revealed the presence of branched duodenal villi, despite the fact that such finding was beyond the scope of the study. Similar structures, however, have been reported in other avian species, in particular ostrich (Bezuidenhout & Van Aswegen, 1990) and pied crow (Okpe *et al.*, 2016). The branching of the villi is thought to be a morphological modification to increase the surface area of the small intestine (Okpe *et al.*, 2016). In order to examine the beneficial effects of feed additives on the intestinal morphology of broiler chicks, several histological measurements have been adopted including the length and number of villi (Khan & Iqbal, 2016; Elhassan *et al.*, 2019). Taken together, the number of branched villi in broilers might be considered as one of the tools utilized for the quantification assessment of the intestinal histology. Further research is required to consolidate this assumption.

CONCLUSION

The present study revealed that the probiotic *Bacillus subtilis* strain DSM 17299 and blend of acidifiers (citric acid, fumaric acid, D-L malic acid, lactic acid, orthophosphoric acid) used in this study improved the

growth performance of broilers, most notably during the first three weeks of age. The *B. subtilis* DSM 17299 has demonstrated potential to increase the immune response as well as number of villi in the distal parts of the small intestine of broilers. No combined effect was observed between the probiotic and acidifiers in the current study. Nonetheless, further research is needed to identify if there are synergistic responses for different probiotic/acidifier combinations rather than those used in the current study.

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