Morphological Responses of the Small Intestine of Broiler Chicks to Dietary Supplementation with a Probiotic, Acidifiers and their Combination

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SUMMARY

This study was conducted to investigate the effects of dietary supplementation of a probiotic (*Bacillus subtilis*), acidifiers and their combination on the mucosa of the broiler small intestine. Two hundred and twenty straight run 1-day-old broiler chicks (Ross 308) were distributed into 4 experimental treatments with 5 replicates per treatment (11 birds per each replicate) as a 2x2 factorial arrangement. The factors were probiotic and a blend of acidifiers with two levels: 0.0% and 0.05 % for probiotic, and 0.0% and 0.2% for acidifiers. On day 42, tissue samples from the duodenum, jejunum and ileum of five birds from each group, were collected and processed for histology, immunohistochemistry, and scanning electron microscopy (SEM). Compared to the jejunum and ileum, the duodenal mucosa displayed the most prominent morphological changes in response to the feed additives. Duodenal villus height (VH) increased ($P \leq 0.05$) when probiotic was added to the diet. Chickens fed the probiotic had a significantly higher ($P \leq 0.05$) percentage of intact villi compared to the other groups. The jejunum showed an increased VH in the group of birds supplemented with acidifiers, while an increased ($P \leq 0.05$) crypt depth (CD) was observed in the group of birds received the probiotic. The ileum showed decreased ($P \leq 0.05$) VH and CD when probiotic was added to the diet, and increased VH when given acidifiers. Dietary supplementation of probiotic only or in combination with acidifiers showed a reduction ($P \leq 0.05$) in the number of somatostatin immunoreactive cells (SIC) in the duodenal villi. A decrease in the number of SIC was also noted in the jejunal villi of birds receiving acidifiers, and the jejunal crypts of birds receiving probiotic. In conclusion, dietary supplementation with the probiotic, *Bacillus subtilis,* or acidifiers improved the mucosal morphology of the small intestine in the broilers used in the present study. The two additives, however, did not show any synergistic effect on the intestinal morphology.

DESCRIPTION OF PROBLEM

It is well known that the improvement of gut structural morphology leads to increased digestive and absorptive function of the intestine due to an increased absorptive surface area [1]. The small intestine is a crucial part of the digestive system due to its involvement in nutrient absorption. Hence, the healthy development of this digestive region is essential to broiler health and performance [2]. In this respect, the intestinal histology is found to be closely related to intestinal absorptive function [3].

The use of feed additives and supplements, such as probiotics and acidifiers, have generally resulted in beneficial changes to gut morphology and growth performance of poultry species [4, 5]. However, some controversial and inconsistent results have emanated from the use of probiotics and acidifiers, indicating the existence of a knowledge gap regarding the precise mechanisms or modes of action of these feed additives [4, 6]. Nevertheless, the evidence of benefits presented in relation to the use of these feed additives necessitates the continuity of research on this subject with the objective of expanding the knowledge base on their beneficial and immunomodulatory effects. In addition, the methods that aid the maintenance of probiotic and acidifier viability in animal feed need to be investigated.

Enteroendocrine cells are specialized epithelial cells dispersed among mucosal cells of the gastrointestinal tract [7]. Some of these endocrine cells are known to secrete the hormone, somatostatin [7, 8]. This hormone has been reported to inhibit the release or action of several gut hormones, which are involved in the regulation of gastro-intestinal function [8]. Directly or indirectly, somatostatin affects both epithelial transport and intestinal motility [8]. Despite the knowledge gained from the previous studies [7, 8], there is still a lack of information concerning

the influence of feed additives on somatostatin enteroendocrine cells in the small intestines of broiler chicks.

The aim of the present study was to investigate the effects of dietary supplementation with the probiotic, *Bacillus subtilis*, and a blend of acidifiers, either administered singly or in combination, on the number of somatostatin enteroendocrine cells and the histometry, as well as epithelial integrity of villi in the small intestine of broiler chicks.

MATERIALS AND METHODS

Experimental Site, Duration and Design

The experiment was carried out in an open-sided experimental housing unit (Faculty of Animal Production, University of Khartoum). The duration of the experiment was 42 d. All procedures performed were in accordance with guidelines presented in Guide for the Care and Use of Agricultural Animals [9]. Two hundred and twenty straight run 1-day-old broiler chicks [10], vaccinated against Marek disease were used in this study. A randomized complete design arranged as a 2 X 2 factorial was used to evaluate the effects of the probiotic and a blend of acidifiers with two levels: 0.0% and 0.05 % for probiotic, and 0.0% and 0.2% for acidifiers. Birds were assigned to 4 treatments with 5 replicates for each treatment (11 birds per replicate). Birds for each replicate were weighed to determine the average starting weight. The birds were then placed into a pen (1 x 1m^2) with wood shavings on the floor. Feed and water were provided to the birds *ad libitum.* Each pen was equipped with a tube feeder and fountain drinker. Continuous lighting was provided by natural sunlight during the day-time and artificially during the night. During the experiment, birds were vaccinated against Newcastle disease, Infectious Bronchitis and Infectious Bursal disease.

Table 1: Composition and analysis of the starter (0-21) and finisher (22-42) diets of the four experimental groups (A, B, C and D) of broiler chickens.

Ingredient %	Starter (0-21)				Finisher (22-42)			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**
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[a,b]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
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
Premix[b]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Antimycotoxins	0.20	0.20	0.20	0.20	0.10	0.10	0.10	0.10
Probiotic[c]	-	0.05	-	0.05	-	0.05	-	0.05
Acidifiers[d]	-	-	0.20	0.20	-	-	0.20	0.20
Total	100	100	100	100	100	100	100	100
Calculated values								
ME (Kcal/kg)	3155	3155	3155	3155	3200	3200	3200	3200
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

A= 0.0% probiotic and 0.0% acidifiers, B= 0.05% probiotic and 0.0% acidifiers, C= 0.0% probiotic and 0.2% acidifiers, D= 0.05% probiotic and 0.2% acidifiers.

[a] The analysis of super concentrate: Metabolizable energy 8.79 MJ/Kg; Crude protein 36%; Crude fiber 5%; Crude fat 2.5%; Lysine 11%; Methionine 4.5%; Methionine+cystiene 5%; Threonin 2.3%; Tryptophane 0.2%; Total phosphorus 2%; Available phosphorus 4.2%.

[b] The addition of premix and super concentrate provide (per kg of mixed feed): 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.

[c] Product powder (GalliPro) contained *Bacillus subtilis* $1.6X10^9$ CFU/gm; Chr. Hansen, Hørsholm, Denmark.

[d] Product powder (Citrinal) contained Citric Acid, Fumaric Acid, D-L Malic Acid, Lactic Acid and Orthophosphoric Acid; Dex Ibérica, Vila-seca, Spain.

Experimental Diet

The starter 0-21 and finisher 22-42 (Table 1) mash diets of the four treatment groups, which were approximately isocaloric and isonitrogenous, were formulated according to guidelines provided by the NRC [11]. The four groups were fed the experimental diets as follows: group (A) received a basal diet only (0.0% probiotic and 0.0% acidifiers); group (B) received a basal diet + probiotic (*Bacillus subtilis*) [12] (0.05% probiotic and no acidifiers); group (C) received a basal diet + blend of acidifiers [13] (0.2 % acidifiers and no probiotic) and group (D) received a basal diet + probiotic and acidifiers with the same inclusion rate utilized in the B and C (0.05% probiotic and 0.2% acidifiers).

Sample Collection

At the end of the experiment, one bird from each pen (5 birds for each group) was selected randomly for tissue sampling. Chickens were successfully restrained before slaughtering using a metal cone. Afterwards, a very sharp knife with a straight surface (approximately 20 cm in length) was used to make a single ventral cut in the neck. Subsequently, arteries, veins, trachea and oesophagus within the neck were cut as well. Birds were permitted to bleed out before further work was conducted [9]. Tissue samples from different regions of the small intestine were then immediately collected.

For histological and immunohistochemical investigations, three tissue samples from each bird were collected from the middle regions of the duodenum, jejunum and ileum. Hence, fifteen specimens were collected from each dietary group resulting in a total of sixty tissue samples. Samples were then washed in phosphate buffer saline (pH 7.4) and fixed in 10% neutral buffered formalin for 24 hours. Tissues were then processed using routine histological techniques and

embedded in paraffin wax. Sections of 5-µm thickness were then cut using a rotary microtome and placed on glass slides coated with 0.1% poly-L-Lysine.

Sixteen birds were sampled for scanning electron microscopy (SEM), resulting in 48 samples. The SEM samples, which were approximately 5 mm^2 in size, were collected from the middle regions of the duodenum, jejunum and ileum. Each group was represented by four birds, which were randomly selected from five birds used for histological and immunohistochemical investigations described earlier.

Histometry

Sections for histometry were stained with haematoxylin and eosin (H & E). A light microscope, connected to an image analyser [14], was used to measure villus height (VH) and crypt depth (CD). The VH was measured from the tip of the villus, distally to the level of the crypt opening. Ten villi and ten crypts were measured per tissue sample with a total of 100 evaluations of each intestinal segment per group. Therefore, a total of 1200 measurements were carried out. Villus height to crypt depth ratio (V:C) was then calculated.

Immunohistochemistry

Tissue sections were processed for immunohistochemistry as described by Madekurozwa [15] using a polyclonal rabbit anti-somatostatin primary antibody [16] at a dilution of 1:650. In each section, somatostatin immunoreactive cells (SIC) within cross sections of intestinal crypts, in fifteen light microscope fields were counted using a 60× stage objective lens. The data were then expressed as the average number of SIC per microscopic field. SIC within 10 well-oriented villi, with the *lamina propria* present, were also counted using a 40× stage objective lens. Data were then expressed as the average number of SIC per intestinal villus.

Scanning Electron Microscopy

Samples collected for SEM were washed with 0.1 M phosphate buffer saline (pH 7.4) and fixed in 2.5% gluteraldehyde buffered with 0.2 M cacodylate (pH 7.4) for 24 hours. Samples were then processed as described by Pelicano *et al.* [17] and then viewed under a scanning electron microscope [18]. Three electron photomicrographs of the same magnification (X150), representing three different fields of each sample, were used to count the number of intact villi using a modification of the method described by Gomide *et al*. [19]. Villi, which were completely covered by epithelial cells or exhibited epithelial loss only at their tips were regarded as intact villi (Fig. 1a). Villi that showed varying degrees of epithelial loss, which extended to a level below the tip, or villi displaying complete epithelial loss were regarded as non-intact (Fig. 1b & c). The percentage of intact villi (IV) was then calculated from the total population of the villi presented in the electron photomicrographs.

Statistical Analysis

Data were analyzed by the GLM procedure for 2x2 factorial arrangement using SPSS version 21 [20]; both main effects and interaction were examined. Treatment means were compared by Duncan's multiple-range test when a significant interaction had existed. The percentage data were first transformed to arc sine before conducting analysis and then displayed as the actual scale after back transformation. A P value of ≤ 0.05 was considered statistically significant.

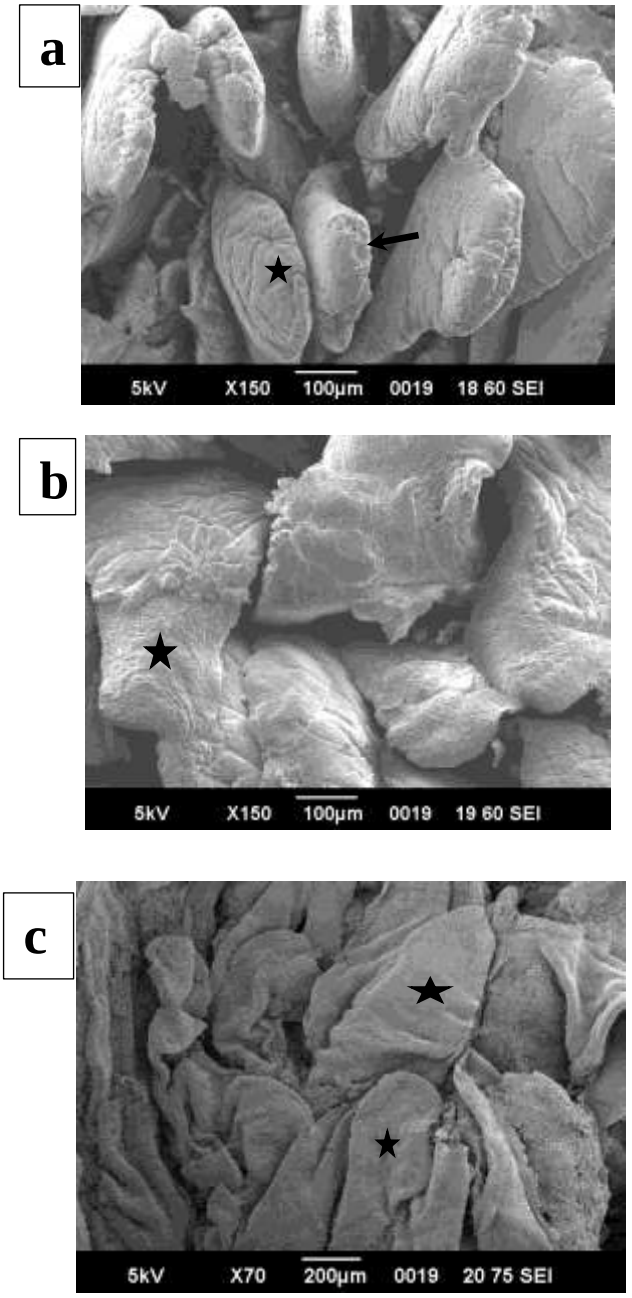

Fig. 1: Scanning electron photomicrographs of the duodenum in a broiler chick. (a) Probiotic group showing intact villi completely covered by epithelium (star) or with epithelial loss at the tip (arrow). (b) Control group with epithelial detachment from the upper part of a non-intact villus (star). (c) Control group showing non-intact villi with total epithelial loss, but no detachment of the villi (stars).

RESULTS AND DISSCUSION

Table 2 shows the effect of the probiotic, acidifiers and their combination on VH, CD and V:C in the three segments of broiler small intestine. In the duodenum, regarding the main effects, VH were significantly increased ($P \leq 0.05$) while CD was significantly decreased ($P \leq 0.05$) when using the probiotic. This is in line with the findings of Sen *et al*. [21] who reported an increase in VH after the use *Bacillus subtilis* as a probiotic in broilers. However, Pelicano *et al.* [17] reported that *Bacillus subtilis* had no effect on duodenal VH when administered singly, but the VH in this intestinal region increased when the probiotic was used in combination with the prebiotic, *Mannan oligosaccharide.* In addition, in the current study, there was no significant effect on VH or CD of the duodenum when acidifiers were administered. This is in agreement with reports by Vieira *et al.* [22] and Houshmand *et al.* [23] who did not observe significant effects of organic acids on the duodenum. On the contrary, earlier findings have indicated that the use of acidifiers increases duodenal VH [24, 25]. These variations in the beneficial effects of acidifiers on the duodenal mucosa may be due to the utilization of acidifier blends composed of different ingredients. Additionally, there was a significant interaction ($P \leq 0.01$) between the probiotic and acidifiers on V:C. Thus, birds fed either probiotic, acidifiers or their combination had greater ($P \leq 0.05$) V:C than those fed neither of the two additives. It has been demonstrated that an increase in V:C is directly related to a rise in epithelial turnover [21].

Table 2: Effect of dietary supplementation of a probiotic, acidifiers and their combination on the villus height (µm), crypt depth (µm) and villus height to crypt depth ratio in the three segments of the small intestine of 42 d broiler chickens

Probiotic	Acidifiers	Duodenum			Jejunum			Ileum		
		VH	CD	V:C	VH	CD	V:C	VH	CD	V:C
0.0%	0.0%	1161.6	145.2	8.7^{b}	831.0^{b}	151.6	6.2^{b}	808.8	150.8^{a}	6.2
0.05%	0.0%	1357.5	123.8	12.5^{a}	922.7^{ab}	187.7	6.0^{b}	684.9	123.5^{b}	5.9
0.0%	0.2%	1250.4	130.9	10.6^{a}	991.6^{a}	131.3	8.7^{a}	964.2	166.2^{a}	6.7
0.05%	0.2%	1282.7	116.8	11.7^{a}	904.5^{ab}	167.8	6.1^{b}	755.6	105.3^{b}	7.6
SEM		43.3	6.5	0.6	32.1	9.8	0.4	42.9	7.0	0.5
Main effects										
Probiotic										
	0.05%	1320.1	120.3	12.1	913.6	177.8	6.1	720.2	114.4	6.8
	0.0%	1206.0	138.1	9.6	911.3	141.4	7.5	886.5	158.5	6.4
Acidifiers										
	0.2%	1266.5	123.9	11.1	948.0	149.6	7.5	859.9	135.6	7.2
	0.0%	1259.5	134.5	10.6	876.8	169.6	6.2	746.8	137.1	6.1
SEM		30.6	4.6	0.43	22.7	6.9	0.3	30.4	4.9	0.3
P value										
Probiotic		*	*	*	NS	*	*	*	*	NS
Acidifiers		NS	NS	NS	*	*	*	*	NS	*
Probiotic X Acidifiers		NS	NS	**	**	NS	**	NS	*	NS

n=50
VH= villus height
CD= crypt depth
V:C= villus height to crypt depth ratio
[a-b] Means with different superscript within the same column are significantly different (P≤0.05)
* P≤0.05
**P≤0.01

The probiotic and acidifiers in the current study appeared to interact (P≤0.01) with each other in respect of their effects on VH and V:C of the jejunum. When compared to VH of birds fed neither the probiotic nor acidifiers (831 µm) the probiotic, *Bacillus subtilis*, did not influence jejunal VH when administered alone (922 µm) or in combination with acidifiers (904.5 µm). In previous work, on the contrary, an increase in the jejunal VH by the same *Bacillus subtilllis* strain has been observed (26). In addition, an increased VH was noted in chickens administered the

acidifiers (991 µm) rather than the basal diet alone. This finding is in agreement with the results of Samanta *et al*. [27] who stated that organic acid supplementation resulted in a remarkable increase in jejunal VH. Furthermore, in the current study, significant increase in jejunal V:C was observed in birds received acidifiers only as compared to birds in other groups. This could be attributed to the aforementioned increased VH in chickens supplemented with acidifiers. On the other hand, as a response to the main effects, jejunal CD increased when the probiotic, *Bacillus subtilis*, was added to diet, while the CD decreased when acidifiers were administered to the birds. This is in agreement with the findings of Pelicano *et al.* [28] who observed a greater CD in the intestinal mucosa of broilers fed probiotics based on *Bacillus spp*.

Dietary supplementation with acidifiers, in the current study, resulted in an increased VH ($P<0.05$), as well as V:C ($P<0.05$) in the ileum. Similar observations have been reported by several researchers [24, 25, 30]. On the other hand, the ileal VH, in the current study, decreased drastically when chicks were supplemented with the probiotic only ($P<0.05$).Furthermore, when compared to birds fed either acidifiers, or the basal diet only, a decrease in ileal CD was observed in broilers that were administered the probiotic alone or in combination with acidifiers. Taking into account the influence of the probiotic on the VH and CD, within the three intestinal regions, it seems plausible to suggest that the effect of *Bacillus subtilis* on such structures gradually decreased from the proximal parts of the small intestine towards the distal parts. However, in contrast to the probiotic, the increase in VH and CD, presumably caused by the acidifiers, appeared to be more marked in the jejunum and then gradually decreased towards the ileum.

In the present study, probiotic supplementation had a significant effect on the maintenance of villous integrity in the duodenum ($P\leq0.05$), as 98% of the villi in this intestinal region were intact (Table 3). In contrast, when compared to birds not receiving dietary additives (64%), no

effect (P≥0.05) was noted on the percentage of duodenal IV in birds administered acidifiers, either alone (67%) or in combination with the probiotic (66%).

Table 3: Effect of dietary supplementation of a probiotic, acidifiers and their combination on the percentage of intact villi (with lower and upper 95% CI in brackets) per small intestinal segment of 42 d broiler chickens

Probiotic	Acidifiers	IV %		
		Duodenum	Jejunum	Ileum
0.0%	0.0%	64[b] (35-84)	88[ab] (67-98)	69 (36-90)
0.05%	0.0%	98[a] (88-100)	60[b] (26-83)	91 (70-100)
0.0%	0.2%	67[b] (39-86)	78[ab] (52-94)	85 (59-98)
0.05%	0.2%	66[b] (38-86)	96[a] (81-100)	83 (57-97)
Main effects				
Probiotic				
	0.05%	87 (75-95)	82 (66-93)	87 (72-96)
	0.0%	65 (46-80)	83 (68-93)	78 (58-91)
Acidifiers				
	0.2%	67 (48-81)	88 (75-97)	84 (67-95)
	0.0%	87 (74-95)	76 (58-89)	81 (64-93)
P value				
Probiotic		*	NS	NS
Acidifiers		*	NS	NS
ProbioticXAcidifiers		*	*	NS

n=12
IV= Intact villi
[a-b] Means with different superscript within the same column are signify cantly different (P≤0.05)
*P≤0.05

In addition, the jejunal region of chicks fed either the probiotic, acidifiers or their combination, showed no significant differences in the percentages of IV compared to birds fed the basal diet.

In the ileal region, no significant differences or interaction were noted in the percentage of IV of chickens supplemented with either the probiotic or acidifiers.

Samanya and Yamauchi [31] attributed the retention of villous integrity to the reduction in the levels of ammonia in the contents of the small intestine of chicken when probiotics containing *Bacillus subtilis natto* were administered. It is known that in the rat ammonia is toxic to the gastric mucosa [32] and causes severe mucosal damage in the colon [33].

It has been shown that the use of a blend of probiotics (*Bacillus mesentericus, Clostridium butyricum* and *Streptococcus faecalis*) reduced mucosal damage in chickens caused by coccidial infections [34] and anti-coccidial vaccines [35]. This may be applicable in the current study because of the positive effect of *Bacillus subtilis* on villous integrity, particularly in the duodenum.

In the present study, somatostatin immunoreactive cells (SIC) were observed in the mucosal layers of the duodenal, jejunal and ileal regions of the broiler small intestine, in each of the experimental groups. The SIC, which displayed centrally located nuclei, were basally located in the epithelia of the villi and intestinal crypts (Fig. 2). The shape of these immunoreactive cells varied from oval (Fig. 2 & 3) to pyramidal. In addition, the SIC displayed long cytoplasmic processes, which extended apically towards the luminal surface of the villi and intestinal crypts (Fig. 4). Generally, most of the samples studied showed a gradual decrease in the number of SIC, in the crypts and villi, from the duodenum towards the ileum.

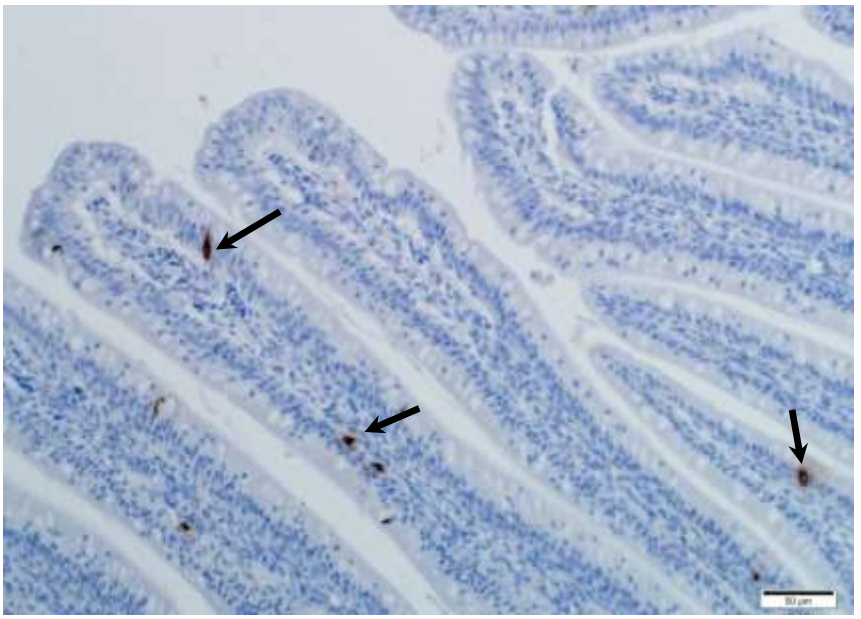

Fig. 2: Light photomicrograph of the duodenum in a broiler chick showing the distribution of SIC (arrows) in the epithelia of villi (V).

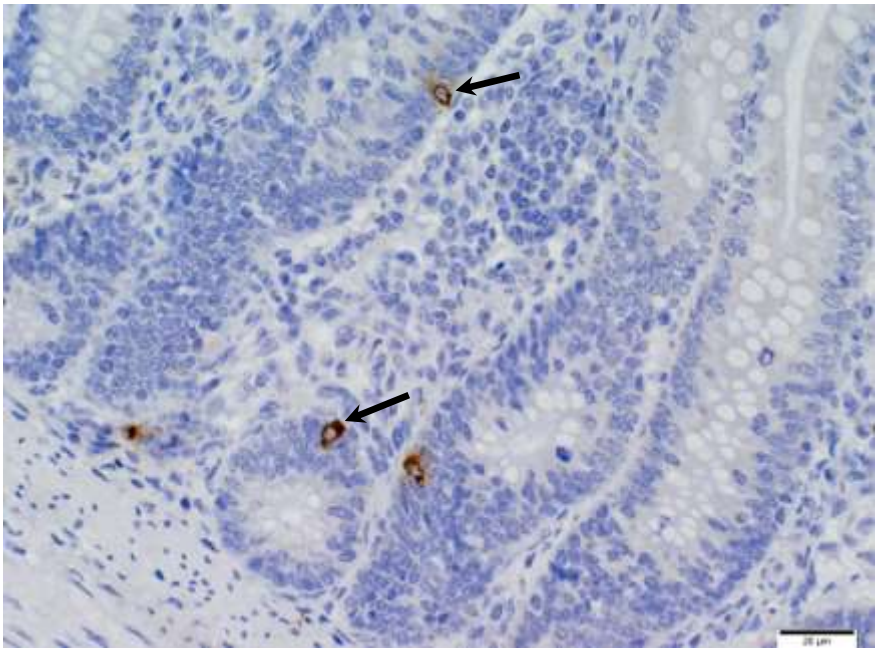

Fig.3: Light photomicrograph of the duodenum in a broiler chick showing SIC (arrows) within the epithelial lining of intestinal crypts.

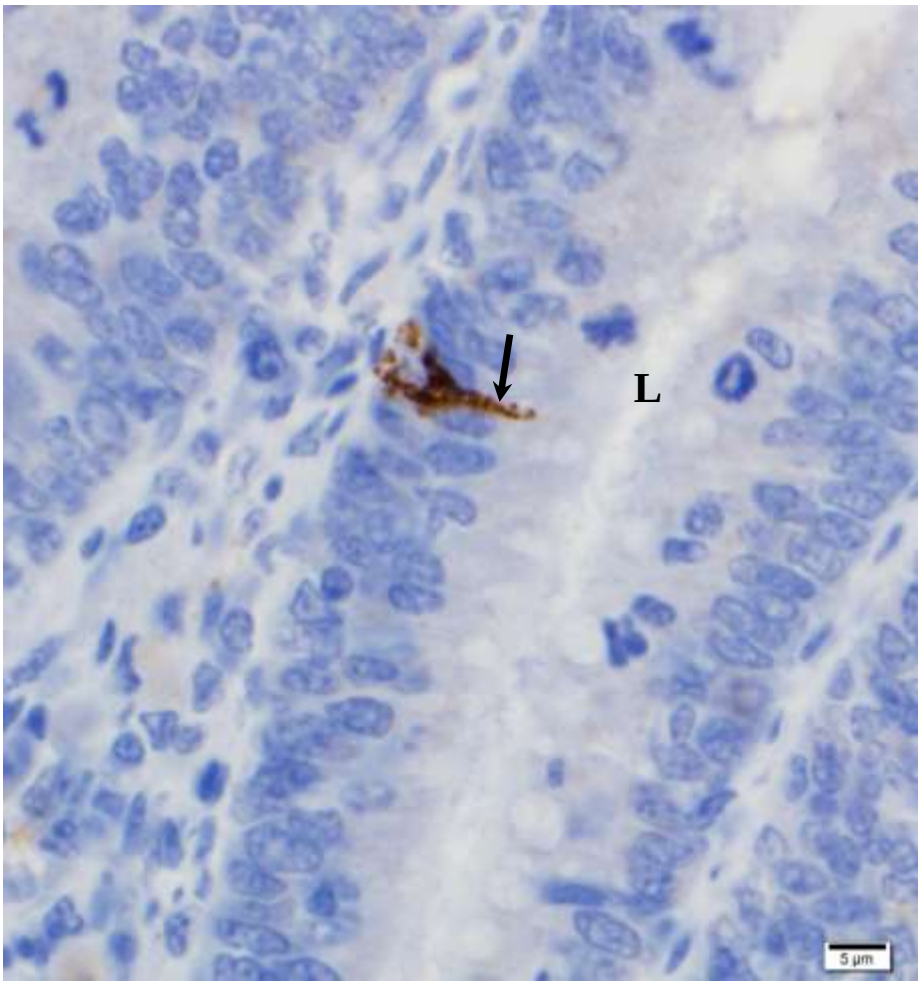

Fig.4: Longitudinal section of the ileum in a broiler chick showing a SIC with a long apical cytoplasmic process (arrow) extending towards the lumen of the intestinal crypt (L).

The mean number of SIC in the intestinal crypts, as well as in the villi of the three intestinal regions of the broiler chickens are shown in Table 4. A marked decrease in the number of SIC within the duodenal villi was noted in chickens supplemented with either the probiotic, acidifiers or their combination ($P \leq 0.05$) compared to chickens supplemented with neither additive. It has been stated that somatostatin inhibits the growth of the gastrointestinal mucosa, and that this effect is mediated by either an indirect mechanism (inhibition of other trophic hormones) or directly through the interaction with somatostatin receptor subtype 2 [36]. Furthermore, it is known that

somatostatin has an inhibitory effect on the immune response [37, 38]. In particular, somatostatin negatively affects the synthesis of immunoglobulins and cytokines [39, 40]. In broiler chicks, it has been stated that cysteamine, an immunostimulating agent, could improve the proliferation of Ig-A producing cells and intraepithelial lymphocytes by reducing the number of SIR within the duodenal mucosa. [41].

Table 4: Effect of dietary supplementation of a probiotic, acidifiers and their combination on the number of somatostatin immunoreactive cells per crypts and villi in the mucosa of the small intestinal segments of 42 d broiler chickens.

Probiotic	Acidifiers	Duodenum		Jejunum		Ileum	
		Crypts[1]	Villi[2]	Crypts[1]	Villi[2]	Crypts[1]	Villi[2]
0.0%	0.0%	1.91	6.04 [a]	1.92	3.90	0.89 [c]	1.64[c]
0.05%	0.0%	1.99	4.10 [b]	1.56	3.34	1.81 [a]	3.10[b]
0.0%	0.2%	2.32	4.88 [b]	1.30	1.96	1.25 [bc]	4.02[a]
0.05%	0.2%	1.88	4.72 [b]	1.45	1.99	1.39 [b]	1.08[c]
SEM		0.17	0.41	0.15	0.34	0.15	0.28
Main effects							
Probiotic							
	0.05%	2.1	4.80	1.51	2.35	1.34	2.55
	0.0%	1.95	5.07	1.61	3.62	1.32	2.37
Acidifiers							
	0.2%	1.93	4.41	1.38	2.65	1.58	2.09
	0.0%	2.11	5.46	1.74	2.93	1.07	2.83
SEM		0.12	0.29	0.11	0.24	0.11	0.20
P value							
Probiotic		NS	*	*	NS	*	*
Acidifiers		NS	NS	NS	*	NS	NS
Probiotic X Acidifiers		NS	*	NS	NS	**	**

[1] n= 75
[2] n= 50
[a-b] Means with different superscript within the same column are significantly different (P≤0.05)
*P<0.05
**P<0.01

In the present investigation, the jejunum exhibited a significant decrease in the number of SIC within the intestinal crypts of birds supplemented with the probiotic ($P<0.05$), as well as in the villi of chicks supplemented with acidifiers ($P<0.05$). As previously mentioned for the duodenum, the decreased number of SIC could also provide an explanation for the increased jejunal CD when the probiotic was administered and VH when the blend of acidifiers was added to diet.

With regards to the number of ileal SIC, the present investigation revealed a significant interaction ($P\leq0.01$) between the probiotic and acidifiers. The number of SIC within the ileal villi increased ($P\leq0.05$) when the probiotic (3.10) and acidifiers (4.02) were administered, compared to when only the basal diet was fed (1.64). An increase ($P\leq0.05$) in the number of SIR in the ileal intestinal crypts was observed in birds fed the probiotic, either alone or in combination with acidifiers, when compared to those that received the basal diet. The results could explain the findings of the present study in which ileal VH and CD decreased when only the probiotic was added to the diet, as well as the decrease in CD when a combination of the probiotic and acidifiers were administered. Furthermore, the increased number of SIC in the ileum when the probiotic was administered would presumably increase the amount of somatostatin released in this intestinal segment. As somatostatin has been shown to decrease gut motility [42, 43]**,** it is possible that the probiotic administered in the present study had an inhibitory effect on ileal motility. Therefore, feed may be retained for a longer period in the ileal lumen, resulting in an increase in nutrient absorption.

Overall, the results of the present study have shown that dietary supplementation with the probiotic, *Bacillus subtilis*, remarkably influenced the number of SIC within the mucosa of the small intestine. It would appear that the decrease in the number of SIC resulted in a reduction in

growth inhibition, which in turn led to the increase in villus height and crypt depth. The findings of the present study indicate that further research is required to elucidate the interaction between probiotics and the enteroendocrine system.

CONCLUSIONS AND APPLICATIONS

1. Dietary supplementation with the probiotic, *Bacillus subtilis*, has improved the mucosal morphology and structural integrity of the small intestine, especially that of the duodenum. The acidifiers have also improved the morphology of the mucosal layer of the small intestine, particularly that of the jejunum and ileum. Therefore, the use of these additives may consequently increase nutrient absorption within the small intestine of broiler chickens.
2. The tested probiotic and acidifiers, under the conditions of this study, did not show any synergistic effect on the intestinal morphology. Further investigation with different types of organic acids will be needed to confirm possible interactions with *Bacillus subtilis* in broilers.
3.

REFERENCES AND NOTES

1 Awad, W.A., Ghareeb, K., Abdel-Raheem, S. and Böhm, J. (2009). Effects of dietary inclusion of probiotic and symbiotic on growth performance, organ weights and intestinal histomorphology of broiler chickens. Poultry Science, **88**:49-55.

2 Kawalilak, L.T., Ulmer Franco, A.M. and Fasenko, G.M. (2010). Impaired intestinal villi growth in broiler chicks with unhealed navels. Poultry Science, **89**(1):82-87.

3 Sittiya, J. and Yamauchi, K. (2014). Growth performance and histological Intestinal alterations of Sanuki Cochin chickens fed diets diluted with untreated whole-grain paddy rice. Journal of Poultry Science, **51**: 52-57.

4 Ajuwon, K.M. (2016). Toward a better understanding of mechanisms of probiotics and prebiotics action in poultry species. Journal of Applied Poultry Research, **25**: 277-283.

5 Samanta, S., Haldar, S. and Ghosh, T.K. (2008). Production and carcase traits in broiler chickens given diets supplemented with inorganic trivalent chromium and an organic acid blend. British Poultry Science, **49**(2): 155-163.

6 Kim, J.W., Kim, J.H. and Kil, D.Y. (2015). Dietary organic acids for broiler chickens: a review. Revista Colombiana de Ciencias Pecuarias, **28**: 109-123.

7 Rawdon, B.B. and Andrew, A. (1999). Gut endocrine cells in birds: an overview, with particular reference to the chemistry of gut peptides and the distribution, ontogeny, embryonic origin and differentiation of the endocrine cells. Progress in Histochemistry and Cytochemistry, **34**(1): 3-82.

8 Dharmsathaphorn, K. (1985). Intestinal somatostatin function. Advances in Experimental Medicine and Biology, **188**: 463-73.

9 Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010). 3rd ed. Federation of Animal Science Societies. Champaign, Illinois.

10 Ross 308, Inmaa for Poultry and Feed Co., Khartoum, Sudan.

11 NRC (1994). Nutrient Requirements of Poultry. 9th revised edition. National Academy Press, Washington, DC.

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

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

14 Cell Sens, Version 510, Olympus Corporation, Tokyo, Japan.

15 Madekurozwa, M.C. (2014). Immunolocalization of intermediate filaments and lamininin the oviduct of the immature and mature Japanese quail (*Coturnix coturnix japonica*). Anatomia, Histologia, Embryologia, **43**: 210-220.

16 DakoCytomation, Glostrup, Denmark. Product code A0566.

17 Pelicano, E.R.L., Souza, P.A., Souza, H.B.A., Figueiredo, D.F. and Amaral, C.M.C. (2007). Morphometry and ultra-structure of the intestinal mucosa of broilers fed different additives. Brazilian Journal of Poultry Science, **9**: 593-597.

18 JEOL, JSM-6390LA analytical scanning electron microscope, Tokyo, Japan.

19 Gomide Junior, M.H., Sterzo, E.V., Macari, M. and Boleli, I.C. (2004). Use of scanning electron microscopy for the evaluation of intestinal epithelium integrity. Revista Brasileira de Zootecnia, **33**(6):1500-1505.

20 IBM SPSS Statistics for Windows, Version 21. IBM corp. Armonk, NY, USA.

21 Sen, S., Ingale, S.L., Kim, Y.W., Kim, J.S., Kim, K.H., Lohakare, J.D., Kim, E.K., Kim, H.S., Ryu, M.H., Kwon, I.K. and Chae, B.J. (2012). Effect of supplementation of *Bacillus subtilis* LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. Research in veterinary science. **93**(1): 264-268.

22 Vieira, S.L., Oyarzabal, O.A., Freitas, D.M., Berres, J., Peña, J.E.M., Torres, C.A. and Coneglian, J.L.B. (2008). Performance of broilers fed diets supplemented with sanguinarine-like alkaloids and organic acids. Journal of Applied Poultry Research, **17**: 128-133.

23 Houshmand, M., Azhar, K., Zulkifli, I., Bejoand, M.H. and Kamyab, A. (2012). Effects of non-antibiotic feed additives on performance, immunity and intestinal morphology of broilers fed different levels of protein. South African Journal of Animal Science, **42**: 22-32.

24 Pelicano, E.R.L., Souza, P.A., Souza, H.B.A., Figueiredo, D.F., Boiago, M.M., Carvalho, S.R. and Bordon, V.F. (2005). Intestinal mucosa development in broiler chickens fed natural growth promoters. Brazilian Journal of Poultry Science, **7**(4): 221-229.

25 Paul, S.K., Halder, G., Mondal, M.K and Samanta, G. (2007). Effect of organic acid salt on the performance and gut health of broiler chicken. The Journal of Poultry Science, **44**: 389-395.

26 Al-Baadani, H.H., Abudabos, A.M., Al-Mufarrej, S.I. and Alzawqari, M. (2016.). Effects of dietary inclusion of probiotics, prebiotics and synbiotics on intestinal histological changes in challenged broiler chickens. South African Journal of Animal Science, **46**(2): 157-165.

27 Samanta, S., Haldar, S. and Ghosh, T.K. (2010). Comparative efficacy of an organic acid Blend and bacitracin methylene disalicylate as growth promoters in broiler chickens: Effects on performance, gut histology, and small intestinal milieu. Veterinary Medicine International, Article ID 645150, 8 pages.

28 Pelicano, E.R.L., Souza, P.A., Souza, H.B.A., Oba, A., Norkus, E.A., Kodawara, L.M. and Lima, T.M.A. (2003). Morfometria e ultra-estrutura da mucosa intestinal de frangos de corte alimentados com dietas contendo diferentes probióticos. Revista Portuguesa de Ciências Veterinárias, **98**(547): 124-134.

29 Adil, S., Banday T., Bhat, G.A., Mir, M.S. and Rehman, M. (2010). Effect of dietary supplementation of organic acids on performance, intestinal histomorphology, and serum

biochemistry of broiler chicken. Veterinary Medicine International, Article ID 479485, 7 pages.

30 Agboola, A.F., Omidiwura, B.R.O., Odu, O., Popoola, I.O. and Iyayi, E.A. (2015). Effects of organic acid and probiotic on performance and gut morphology in broiler chickens. South African Journal of Animal Science, **45**(5): 494-501.

31 Samanya, M. and Yamauchi, K. (2002). Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis var. natto*. Comparative Biochemistry and Physiology, part A **133**: 95-104.

32 Warzecha, Z., Dembiński, A., Brzozowski, T., Ceranowicz, P., Pajdo R, Niemiec, J., Drozdowicz, D., Mitis-Musioł, M., and Konturek, S.J. (2000). Gastroprotective effect of histamine and acid secretion on ammonia-induced gastric lesions in rats. Scandinavian Journal of Gastroenterology, **35**: 916-924.

33 Lin, H.C. and Visek, W.J. (1991). Colon mucosa cell damage by ammonia in rats. The Journal of Nutrition, **12**: 887-893.

34 Hayakawa, T., Masuda, M., Tsukahara, T., Nakayama, K. and Maruyama, K. (2014). Morphometric and histopathological evaluation of a probiotic and its synergism with vaccination against coccidiosis in broilers. Animal Science Letters, **1**(1): 33-49.

35 Luquetti, B.C., Furlan, R.L., Alarcon, M.F.F. and Macari, M. (2012). *Saccharomyces cervisiae* cell wall dietary supplementation on the performance and intestinal mucosa development and integrity of broiler chickens vaccinated against coccidiosis. Brazilian Journal of Poultry Science, **14**(2): 71-58.

36 Yamada, Y., Post, S.R., Wang, K., Tager, H.S., Bell, G.I. and Seino, S. (1992) Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proceedings of the National Academy of Sciences of the United States of America, **89**: 251-255.

37 Fais, S., Annibale, B., Boirivant, M., Santoro, A., Pallone, F. and Fave, G. D. (1991). Effects of somatostatin on human intestinal lamina propria lymphocytes modulation of lymphocyte activation. Journal of Neuroimmunology, **31**: 211-219.

38 Ferone, D., Lombardi, G. and Colao, A. (2001). Somatostatin receptors in immune system cells. Minerva Endocrinologica, **26**: 165-173.

39 Aguila, M.C., Dees, W.L., Haensly, W.E. and Mc Cann, S.M. (1991). Evidence that somatostatin is localized and synthesized in lymphoid organs. Proceedings of the National Academy of Sciences of the United States of America, **88**(24): 11485-11489.

40Van Hagen, P.M. (1996). Somatostatin receptor expression in clinical immunology. Metabolism, **8** (Suppl. 1): 86-87.

41Yang, Q., Lian, G. and Gong, X. (2007). Enhancement of mucosal immune responses in chickens by oral administration of cysteamine. Poultry Science, **86**(7): 1323-1328.

42 Barnett, P. (2003). Somatostatin and somatostatin receptors physiology. Endocrine, **20**(3):255-264.

43 Van Op den Bosch, J., Adriaensen, D., Van Nassauw, L. and Timmermans, J.P. (2009). The role(s) of somatostatin, structurally related peptides and somatostatin receptors in the gastrointestinal tract. A review. Regulatory Peptides, **156**(1-3): 1-8.