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Morphometry and Ultra-structure of the Intestinal Mucosa of Broilers Fed Different Additives

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ABSTRACT

This study aimed at evaluating the effect of the use of different growth promoters on the morphometry and ultra-structure of the intestinal mucosa of 42-day-old broilers. A total number of 36 male Cobb broilers was distributed in a randomized experimental design with a 3 x 3 factorial arrangement, with 3 prebiotic and 3 probiotic sources in the feed, summing up 9 treatments, with 4 replicates each. There was a significant interaction (P<0.01) among the studied factor for villi height (VH) in all intestinal segments, and for crypt depth (CD) in the duodenum and the ileum. In the duodenum, higher villi were obtained in the control group, with the combination of *B. subtilis* and prebiotics, and with the single use of MOS+OA. No VH differences were observed between the control group and those fed prebiotics. In the jejunum, the highest villi were obtained with the use of the bacterial pool, followed by the control group, and by the use of *B. subtilis.* Higher villi were also obtained in the control group and in the groups fed MOS, when *B. subtilis* was used in combination with prebiotics, and when the bacterial pool was used individually or in combination with MOS. In the ileum, the highest villi were obtained with the individual use of *B. subtilis*, and when MOS+OA or MOS were individually used or in combination with the bacterial pool. As to duodenal CD, deeper crypts were observed in the control group and in those fed *B. subtilis* or MOS+OA. In the ileum, deeper crypts were also found in the control group and those fed *B. subtilis.* Deeper crypts were also found when the bacterial pool was individually used or in combination with MOS+OA, and with the individual use of MOS. It was concluded that the use of growth promoters was beneficial to increase intestinal villi height when *Bacillus subtilis* was used in combination with prebiotics. The other growth promoters (MOS+OA, MOS, and bacterial pool), can be individually used in most situations. The tested growth promoters did not influence intestinal villi density.

INTRODUCTION

The present production systems commonly use antibiotics, either in therapeutic doses to prevent and to control diseases, or in subtherapeutic doses, aiming at growth promotion and performance improvement. However, these applications may pose serious risk to human health when improperly used. The presence of antibiotic residues in animal products may cause many disorders in consumers – from allergy reaction to the possibility of resistance development and consequent selection of resistant microorganisms (Witte, 2000; Gomes & Demoly, 2005). In this sense, many countries, including Brazil, have been researching alternative products to replace the traditional antibiotic growth promoters, such as prebiotics and probiotics.

Probiotic action can be explained by mechanisms, such as the production of antimicrobial substances and organic acids, protection of villi and absorptive surfaces against toxins produced by pathogenic microorganisms, and immune system stimulation (Vicent, 1959; Dobrogosz *et al.,* 1991; Ewing & Cole, 1994; Walker & Duff, 1998; Pelicano *et al.*, 2002). According to Fox (1988), one of the essential requisites for the establishment of microorganisms in a changing environment, such as the gastrointestinal tract, is their ability to attach to the surface of the intestinal epithelium. This suggests that one way to prevent the colonization of this environment by pathogenic bacteria is to saturate epithelial binding sites by beneficial microorganisms, thereby preventing the attachment of pathogens. This mechanism is presently designated as the "competitive exclusion" principle.

On the other hand, prebiotics act by reducing the replication of several intestinal bacteria – pathogenic or not – due to pH reduction caused by the increase of lactic acid levels in the cecca (Choi *et al.*, 1994). Some bacteria recognize the binding sites of MOS as intestinal mucosa biding sites, therefore reducing intestinal colonization by pathogenic bacteria. Consequently, in addition to a lower incidence of infection, the mucosa is able to fully perform its secretion and nutrient digestion and absorption functions (Iji & Tivey, 1998). Several authors reported positive effects of prebiotics on the intestinal mucosa. Macari & Maiorka (2000) observed a significant increase in villi height in the three sections of the small intestine of one-week-old broilers with the addition of MOS, and Dionizio (2001) found higher duodenal villi with the use of prebiotics (FOS, lactose, MOS, and sucrose) in 21- and 42-day-old broilers.

Therefore, this study aimed at evaluating the effects of the use of probiotics, prebiotics, and their combination on histological and morphological indexes of the intestinal mucosa of 42-day-old broilers.

MATERIAL AND METHODS

Location and Experimental Period

The present study was carried out in the Optical Microscopy and Scan Electron Microscopy laboratories of the School of Agrarian and Veterinary Sciences – FCAV/UNESP, Jaboticabal, São Paulo, Brazil.

Birds and Management

The experiment initially used 1260 Cobb male broiler chicks, distributed in 9 treatments with 4 replicates of 35 birds each. Morphometry and ultra-structure analyses were carried out in 36 birds (4 per treatment), with 2.5-kg average live weight at 42 days of age.

Birds were submitted to standard management commonly adopted in commercial broiler production. Birds were housed in a conventional masonry broiler house divided into 36 pens (35 birds/pen) measuring 3.20 m x 1.46 m (bird density: 8 birds/m^2), with ± 5-cm thick wood shavings litter, and equipped with chick tube feeders, and aluminum pressure drinkers. During the first rearing weeks, chicks were brooded under infra-red lamps. After the second week of age, 20 kg capacity tube feeders, and automatic bell drinkers were introduced.

Birds were vaccinated in the hatchery against Marek's disease, and in the broilers house against Infectious Bursal disease and Newcastle disease.

Environmental temperature and air relative humidity were daily recorded, and curtains and fans were managed to ensure bird thermal comfort. Feed and water were offered *ad libitum*.

Experimental Design and Treatments

Birds were distributed in a completely randomized experimental design in a 3 x 3 factorial arrangement with 3 probiotic dietary sources (no probiotic, probiotic 1, probiotic 2) and 3 prebiotic dietary sources (no prebiotic, prebiotic 1, prebiotic 2), with a total of 9 treatments with 4 replicates of 36 birds each.

The following treatments were applied: 1 - Control (no growth promoter); 2 - *Bacillus subtilis*; 3 - *Lactobacillus acidophilus* and *casei, Streptococcus lactis* and *faecium, Bifidobacterium bifidum,* and *Aspergillus oryzae* (bacterial Pool)*;* 4 – phosphorylated mannan oligosaccharide (MOS) and Organic Acidifier (OA); 5 - MOS; 6 - *Bacillus subtilis* + MOS and OA; 7 - *Bacillus subtilis* + MOS; 8 - Bacterial pool + MOS and OA; 9 - Bacterial pool + MOS.

Growth promoter levels in the experimental feeds

Growth promoter levels were added according to the manufacturors' recommendations:

- Probiotic, based on *Bacillus subtilis,* was added at 150 g/ton feed during all rearing phases (01 - 42 days of age);
- Probiotic, based *Lactobacillus acidophilus* and *casei, Streptococcus lactis* and *faecium, Bifidobacterium bifidum,* and *Aspergillus oryzae,* was added at 1 kg/ton feed during all rearing phases (01 - 42 days of age);

- Prebiotic, based on MOS and AO, was added at 2 kg/ton feed in the starter phase (01 - 21 days) and 1.5 kg/ton thereafter (22 - 42 days of age);
- Prebiotic, based on MOS, was added at 1 kg/ton feed in the starter phase (01 - 21 days) and 0.5 kg/ton feed thereafter (22 - 42 days of age);

Experimental feeds

Feeders were filled using dedicated spoons in order to prevent cross-contamination of microorganisms among treatments. The same procedure was adopted with the material used for cleaning the drinkers.

Birds were offered water and feed *ad libitum* during the entire experimental period, which was divided in three phases. During the starter phase (01-21 days), birds were fed diets containing 3000 kcal/kg metabolizable energy, 21.4% crude protein, 1.263% lysine, 0.561% methionine, 0.960% Ca, and 0.450% available P. Grower diets (22-35 days) contained 3100 kcal/kg metabolizable energy, 19.3% crude protein, 1.156% lysine, 0.514% methionine, 0.874% Ca, and 0.406% available P. Finisher diets (36-42 days) contained 3200 kcal/kg metabolizable energy, 18% crude protein, 1.040% lysine, 0.445% methionine, 0.800% Ca, and 0.365% available P. Other nutrient levels were added according to the recommendations of Rostagno *et al.* (2000).

Statistical analysis

Data were submitted to analysis of variance using Estat 2.0 (1992) software, and means were compared using the test of Tukey at 5% significance level ($P<0.05$).

Evaluated parameters

Morphometry (Optical Microscopy)

After 12-hour fasting, birds were sacrificed, and duodenum, jejunum, and ileum sections were collected to perform optical microscopy analysis. After collection, these sections were fixed in Bouin's solution for 48 hours. Samples were then dehydrated in graded ethanol concentrations (70% up to absolute), cleared in xylol, embedded in paraffin, cut in microtome at a 5-µm sections, and stained with Masson's trichrome.

The morphometric study of intestinal villi height and crypt depth (in µm) was carried out using an image-analyzing system (Kontron Elektronik – Video Plan), coupled to a binocular microscope (Carl Zeiss). Villi height was measured from the basal region, which corresponded to the higher section of the crypts, to the apex. Crypts were measured from the base up to the crypt-villus transition region. Fifty villi height and crypt depth readings were performed per treatment/intestinal section, with a total of 2,700 readings.

Ultra-structure (Scanning Electron Microscopy)

After 12-hour fasting, birds were sacrificed, and duodenum, jejunum, and ileum sections were collected to perform scanning electron microscopy analysis. These sections were fixed in glutaraldehyde for 24 hours, fixed in phosphate buffer 0.1 M, pH 7.4, post-fixed in osmium tetroxide at 1% for 2 hours, washed in the same buffer, and dehydrated in graded ethanol series, from 30%, 50%, 70%, 80%, 95% to 100% (absolute), where samples where washed three times. Samples were then processed in a critical-point dryer with liquid CO_2, in a Bal-Tec apparatus, coated with gold/palladium in a Denton Vacuum Desk II apparatus, and observed under scanning electron microscope (Jeol-Jsm 5410).

In order to measure villi height, samples were electron-microphotographed in three different fields. The scale of each photograph was checked by measuring observation field width and length, thereby resulting in the area of the photograph. Villi were then counted in each field (324 readings/treatment), and the villi number /µm^2 was calculated.

RESULTS AND DISCUSSION

Table 1 presents the analysis of variance relative to villi height (VH), crypt depth (CD), and villi density data. VH and CD means are summarized in Tables 2 and 3. Table 1 shows that, except for the ileum with the use of prebiotics, probiotics and prebiotics significantly influenced VH and CD, but there was no effect on villi density. A significant interaction was observed among the studied factor on VH in all three intestinal segments, whereas CD was affected only in the duodenum and the ileum.

Table 2 presents VH data relative to treatment interactions of in the duodenum, jejunum and ileum of 42-day-old broilers fed different probiotics and prebiotics. In the duodenum, the highest VH was obtained in the control group, whereas the lowest VH was observed in the groups fed the in-feed probiotic containing only *Bacillus subtilis*. However, VH in these two treatments were not different from the group fed with the bacterial pool used as probiotic. Similar results were obtained by Sato (2001), who did not observed any differences in villi morphometry when more than one bacterial culture, consisting of probiotics including

Table 1 – Analysis of variance of morphometry data: probability of treatment effects.

	Villi height			Crypt depth			Villi density		
Causes of variation	*Duod.*	*Jejunum*	*Ileum*	*Duod.*	*Jejunum*	*Ileum*	*Duod.*	*Jejunum*	*Ileum*
PRO	5.21**	18.54**	10.14**	16.28**	22.78**	4.46*	3.61ns	0.13ns	0.62ns
PRE	14.59**	29.35**	2.07ns	13.60**	4.89**	45.41**	0.40ns	0.97ns	0.04ns
PRO x PRE [(1)]	5.63**	20.47**	4.13**	4.04**	0.65ns	6.60**	1.13ns	1.99ns	1.56ns
CV (%)	21.56	17.55	20.02	39.00	38.23	37.17	23.20	17.27	17.46

[(1)] The details of interactions (PRO x PRE) for villi height and crypt depth are presented in Tables 2 and 3.

Table 2 – Details of PRO x PRE interactions for villi height in the duodenum, jejunum, and ileum of 42-day-old broilers fed in-feed probiotics and prebiotics.

Treatments	*Villi height (µm)*			
	In-feed probiotics			
	No Probiotic	*Bacillus subtilis*	*Pool [(1)]*	*Mean*
In-feed prebiotics		***Duodenum***		
No prebiotics	1740 Aa*	1400 Bb	1570 ABa	1570
MOS+OA	1786 Aa	1752 ABa	1595 Ba	1711
MOS	1776 Aa	1872 Aa	1738 Aa	1795
Mean	1767	1675	1634	
		Jejunum		
No prebiotic	1529 Ba	1158 Cc	1702 Aa	1463
MOS+OA	1405 Ab	1338 Ab	1374 Ab	1372
MOS	1558 Aa	1642 Aa	1601 Aa	1600
Mean	1797	1379	1559	
		Ileum		
No prebiotic	1225 Aab	1169 Aa	1195 Aa	1196
MOS+OA	1267 Aa	1017 Bb	1163 Aa	1149
MOS	1132 ABb	1100 Bab	1214 Aa	1148
Mean	1208	1095	1190	

(*) Means followed by the same capital (small letter) in the same row (column) are not different (p>0.05) by the test of Tukey; LSM (Duodenum) = 172; LSM (Jejunum) = 122; LSM (Ileum) = 110. (1) Probiotic based on *Lactobacillus acidophilus* and *casei, Streptococcus lactis* and *faecium, Bifidobacterium bifidum* and *Aspergillus oryzae* .

Tabela 3 - Details of PRO x PRE interactions for crypt depth in the duodenum, and ileum of 42-day-old broilers fed in-feed probiotics and prebiotics.

Treatments	*Crypt depth (µm)*			
	In-feed probiotics			
	Sem Probiotic	*Bacillus subtilis*	*Pool [(1)]*	*Mean*
In-feed prebiotics		***Duodenum***		
No prebiotic	328 Aa*	284 Aa	220 Ba	277
MOS+OA	325 Aa	247 Bab	257 Ba	276
MOS	238 Ab	209 Ab	225 Aa	224
Mean	297	246	234	
		Ileum		
No prebiotic	281 Aa	259 Aa	213 Ba	251
MOS+OA	182 Ab	180 Ab	214 Aa	192
MOS	190 Ab	173 ABb	147 Bb	170
Mean	218	204	191	

(*) Means followed by the same capital (small letter) in the same row (column) are not different (p>0.05) by the test of Tukey; LSM (Duodenum) = 48; LSM (Ileum) = 36. (1) Probiotic based on *Lactobacillus acidophilus* and *casei, Streptococcus lactis* and *faecium, Bifidobacterium bifidum* and *Aspergillus oryzae*

Bacillus subtilis, Lactobacillus reuteri, and *L. johnsonii,* was added to the broiler feed, as compared to a control group. The results obtained in the present study indicate that in order to obtain better duodenal morphometry, characterized by higher villi, probiotics containing more than one bacterial culture are required. However, other literature reports show that the use of a single bacterial culture is sufficient to increase VH, such as the results described by Dobrogosz *et al.* (1991), who observed a significant increase in intestinal villi height with the use of such products.

No VH differences were observed in the duodenum between the control group and those fed only prebiotics (MOS and OA, or MOS). These results do not agree with those reported by Dionizio (2001), who found an increase in VH with the use of diets containing MOS as compared to a control group in 42-day-old

broilers. In the present study, higher duodenal VH was observed with the association of *Bacillus subtilis* with prebiotics, as well as with the individual use of MOS + OA. In the duodenum, it was deduced that the use of probiotics is more beneficial when these are associated with prebiotics, but the same is not true as to prebiotics.

In the jejunum, VH decreased according to the following order: with the individual use of the bacterial pool, followed by diets with no growth promoter inclusion, and finally by diets including only *Bacillus subtilis*. As observed in the duodenum, the use of probiotics consisting of several bacterial cultures again increased jejunal VH as compared to probiotics containing a single bacterial culture. According to Andreatti Filho & Sampaio (2000), a higher number of bacterial species seems to determine higher probiotic efficacy as compared to products with a reduced number of species. Therefore, we can deduce that the higher number of bacterial species contained in the bacterial pool ensure better protection of the villi and of the absorptive surface, having direct influence in the increase of villi height. In addition, according to Cera *et al.* (1988), maximal absorption and digestion capacity, provided by a large luminal area, with high villi and mature enterocytes, is essential for the animals' development.

Higher VH was also observed in birds fed diets containing only MOS and diets with no growth promoter as compared to those fed diets containing MOS + OA. According to Oyofo *et al.* (1989 a,b,c), MOS reduces the colonization of the intestine by enteropathogenic bacteria by blocking the binding sites of the intestinal mucosa receptors to bacterial fimbriae. Therefore, the higher VH in the groups fed MOS may result from a lower colonization by pathogenic bacteria, thereby maintaining the integrity of the intestinal mucosa, which then be able to realize its nutrient digestion and absorption functions. Also, in the jejunum, higher VH was observed when *Bacillus subtilis* was associated with prebiotics, and when the bacterial pool was used individually or associate with MOS. It was again observed that the use of probiotics is more beneficial when it is associated with prebiotics. Balog *et al.* (2007) observed that the inclusion of a symbiotic (probiotic + prebiotic) in free-range broiler diets significantly improved production performance; however, this product did not change epithelial morphometry (villi height and crypt depth) in all evaluated small intestinal segments.

In the ileum, no VH differences were observed between the groups fed only *Bacillus subtilis* or the bacterial pool as compared to the control group. These results are consistent with those obtained and reported by Bradley *et al.* (1994) and Pelicano *et al.* (2003), when including probiotics in the feed of broilers from 21 to 35 days, and from 1 to 42 days of age, respectively. In the present study, although not statistically significant, again higher ileum VH was obtained with the use of probiotics containing several bacterial species as compared to the use of individual culture.

As to the individual use of prebiotics, higher ileal VH was observed with the use of MOS associated to OA, and lower VH when MOS was individually used. There was no difference in VH when the control group was compared with those fed prebiotics. These results disagree with those reported by Loddi (2003) and Corneli *et al.* (2004), who did not observe differences in VH among groups fed different dietary prebiotics. However, these studies also showed that both additives did not promote different villi heights as compared to the control group.

In the present study, higher VH in the ileum was obtained with the individual use of *Bacillus subtilis*, when MOS + OA were individually used or associated with the bacterial pool, as well as when MOS was used. The ileum was the only intestinal segment that was beneficially affected by the individual used of the probiotic based on *Bacillus subtilis*.

Table 3 presents VH data relative to treatment interactions of in the duodenum and ileum of 42-day-old broilers fed different probiotics and prebiotics. Higher CD values were obtained in the duodenum of the birds that did not receive growth promoters and those fed the individual *Bacillus subtilis* culture, whereas the lowest values were obtained with the use of the bacterial pool. Pelicano *et al.* (2003) did not find any CD differences between the control group and groups fed probiotics in the feed (*Bacillus sp.* e *Saccharomyces cerevisiae*) and in the drinking water (*Lactobacillus sp.*) when broilers were 42 days of age. On the other hand, Santos *et al.* (2004), in 21-day-old broilers, observed higher CD values with the use of a bacterial pool (*Lactobacillus acidophilus* and *L. casei, Streptococcus lactis* and *S. faecium, Bifidobacterium bifidum,* and *Aspergillus oryzae*), and lower CD values in groups that did not receive growth promoters. In the present study, deeper crypts were observed in the control groups and in those fed only MOS + OA, whereas the shallowest crypts were obtained with the individual use of MOS. Similar results were obtained by Loddi (2003), who also found higher CD values in the duodenum of birds fed an association of MOS + OA as compared to the

individual use of MOS. Savage *et al.* (1996) observed a significant reduction in CD values in the duodenum when MOS was included in a turkey diet; however, Spring (1996) obtained higher CD values in the intestinal mucosa of broilers supplemented with synthetic MOS. In the present experiment, higher CD values were observed in the duodenum with the individual use of *Bacillus subtilis*, as well as with MOS + OA.

Consistent with the results found for the duodenum, deeper crypts were observed in the ileum of birds that did not receive growth promoters, as well in the birds fed the individual *Bacillus subtilis* culture. Pedroso (1999) also found deeper crypts in the ileum of layers with the continuous dietary inclusion of probiotics based on *Bacillus subtilis*. In the present study, the highest CD values were observed in the control groups, and the lowest in the groups receiving the individual prebiotics. Similar results, albeit non-significant, were obtained by Loddi (2003), who obtained the highest CD value in the ileum of birds in the control group. We observed higher CD values with the individual use of MOS, *Bacillus subtilis*, and bacterial pool, and also when the bacterial pool was used in association with MOS and OA.

Table 4 presents data relative to villi density per small intestine segment of broilers fed different probiotics and prebiotics. As evaluated when broilers were 42 days of age, villi density presented the following decreasing order: ileum, jejunum, and duodenum (Figure 1). These results are consistent with those found by Yamauchi & Ishiki (1991). No differences in villi density were observed among broilers fed probiotics, prebiotics, or those fed the basal diets, in all three evaluated intestinal segments.

Tabela 4 – Villi density (number of villi/1,145,306 µm²) per small intestine segment of 42-day-old broilers fed in-feed probiotics and prebiotics.

Treatments	*Villi density*		
	Duodenum	*Jejunum*	*Ileum*
In-feed probiotics (PRO)			
No Probiotics	22	32	48
Bacillus subtilis	27	31	51
Bacterial pool (1)	27	30	49
F test	3.61ns	0.13 ns	0.62 ns
LSM (%)	5.89	5.41	8.68
In-feed prebiotics (PRE)			
No prebiotic	26	32	49
MOS+OA	24	29	50
MOS	25	32	49
F test	0.40 ns	0.97 ns	0.04 ns
LSM (%)	5.89	5.41	8.68
PRO x PRE	1.13 ns	1.99 ns	1.56 ns
CV (%)	23.20	17.27	17.46

Within the same factor, means followed by the same letter in the same column are not different (p>0.05) by the test of Tukey; LSM - Least Significant Mean; CV – Coefficient of Variation. (1) Probiotic based on *Lactobacillus acidophilus* and *casei, Streptococcus lactis* and *faecium, Bifidobacterium bifidum* and *Aspergillus oryzae* .

In the duodenum, villi were more dispersed, in lower numbers, and leaf-shaped, as reported by Yamauchi & Ishiki (1991) and Sato (2001). In the jejunum, villi presented a zigzag pattern, sometimes suggesting a wave shape, which was also found by Pelicano *et al.* (2003). According to Yamauchi & Ishiki (1991), this organization of the villi may promote a more efficient structure for nutrient absorption than when villi are in parallel or randomly organized. The bolus would travel longer in a zigzag flow than in a straight flow, allowing more contact of the nutrients with the absorptive surface of the intestinal epithelium. As for the ileum, all treatment groups presented high density of tongue-shaped, making it difficult to analyze them individually.

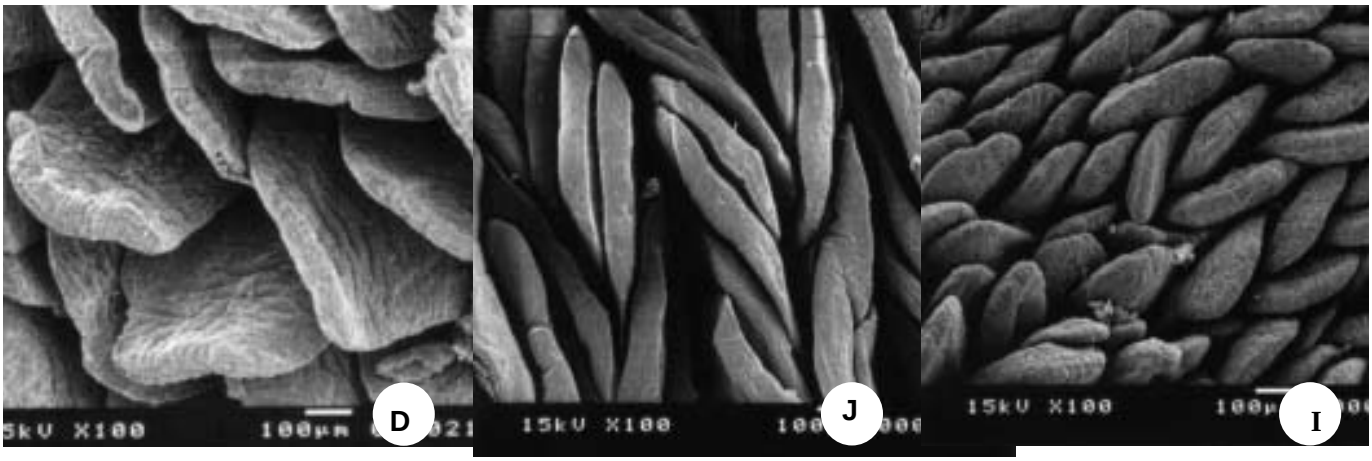

Figure1 – Scanning electron microphotograph of intestinal villi in the duodenum (D), jejunum (J), and ileum (I) of 42-day-old broilers

CONCLUSION

The use of growth promoters increased intestinal villi height when *Bacillus subtilis* was associated to prebiotics. The other tested growth promoters (MOS + OA, MOS, and bacterial pool) can be individually used in most circumstances. The tested growth promoters did not influence villi density.

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