Effect of Probiotic and Prebiotic Inclusion in Weaned Piglet Diets on Structure and Ultra-structure of Small Intestine

Fábio Enrique Lemos Budiño, Maria Cristina Thomaz[*], Rodolfo Nascimento Kronka, Laura Satiko Okada Nakaghi, Fernanda Marcussi Tucci, Alessandro Luís Fraga, Antônio João Scandolera and Rizal Alcides Robles Huaynate
Faculdade de Ciências Agrárias e Veterinárias; Universidade Estadual Paulista; Rod. Paulo Donato Castellane, s/n; 14700-000; thomaz@fcav.unesp.br; Jaboticabal - SP - Brasil

ABSTRACT

Forty-four piglets weaned were distributed in a complete randomized blocks in factorial scheme. Experimental diets were: T1 = Basal Diet (BD); T2 = BD + Antibiotic; T3 = BD + Probiotic; T4 = BD + Prebiotic; T5 = BD + Symbiotic (T3+T4). As control, four piglets were slaughtered at weaning. Seven and 14 days after weaning, four animals per treatment were slaughtered. Small intestine segments were sampled from each animal for electron and light microscopy evaluation. The evaluated micro-ingredients affected intestinal histology. It was observed higher villous density in duodenum of piglets fed diet with prebiotic in relation to those fed diets with probiotic. In jejunum of piglets fed diets with prebiotic was found higher villous density, but this difference was not significant. In relation to BD, symbiotic increased duodenal micro-villous height at the 14th day after weaning. Piglets supplemented with probiotic had better recovering of micro-villous density in relation to those fed with other diets.

Key words: Crypts, fructo-oligosaccharides, micro-villi, swine, villi

INTRODUCTION

Piglet weaning triggers stress events changing small intestine morphology and resulting intake and digestive disorders, consequently, animal performance is prejudiced during the first weeks after weaning (LI et al., 1990). Two primary cytological events are associated to intestinal mucous development. One is the cell turn-over, a result of mitotic divisions of totipotent cell in crypt and along the villous; the other is the usual cell losses by sloughing in villous top (UNI et al., 1998). Thus, the villous atrophy, commonly observed at post-weaning, may be caused by increasing of sloughing rate or reducing of turn-over rate. When the reason is the increase of sloughing rate, villous reduction is associated to the increase of crypt depth and cell proliferation. Also, villous atrophy might be related to cell division reduction in crypt (TUCCI, 2003).

At weaning, besides physiological unbalance, microbial changes may affect intestinal mucous morphology due to toxins production by pathogenic bacteria. In the last decades, the utilization of antibiotic as growth promoters has been an alternative for reducing these cited negative effects on intestinal development.

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[*] Author for correspondence

Recently, micro-ingredients, as probiotic and prebiotic, have been studied as an option for replacing chemical therapy (MENTEN, 2001). Probiotics are alive microorganisms composed by specific bacteria or yeast for improving intestine microbiota balance by reducing pathogenic agents and stimulating host immune system (WALKER and DUFFY, 1998).

Prebiotic may be a diet nutrient or ingredient, which is indigestible but is selectively fermented by animal digestive system, stimulating growth and, or the activity of specific groups of health bacteria (ROY and GIBSON, 1998). There are some evidences that prebiotics may provoke health changes in digestive anatomical traits. HOWARD et al. (1993) verified increase in cell density and in number of stained cell in cecal mucosa of piglet fed diets with fructo-oligosaccharides prebiotic. Also, higher crypt length, increase in proliferation area (stained cell/cell density), higher number and length of stained cell in proximal and distal colon were observed. MACARI and MAIORKA (2000) related significant increase in villous height in the three segments of small intestine of seven day-old broiler chickens receiving 0.2% manan-oligosaccharides (MOS) prebiotic in diet. In general, these results showed that the use of prebiotics increases area for nutrient absorption in intestinal mucosa.

The goal of this study was to evaluate the development of structure and ultra-structure of intestinal mucosa of newly-weaned piglets fed diets with probiotic and, or prebiotic.

MATERIAL AND METHODS

Animals and diets

Trial was carried out at Sector of Swine Breeding of Zootecnia Department, College of Veterinarian and Agrarian Sciences (FCAV), UNESP, Jaboticabal City, São Paulo State, Brazil. Products evaluated were: 15% Zinc Bacitracin (antibiotic), Bio-Plus 2B® of CHR – Hansen (*Bacillus licheniformis* + *Bacillus subtilis* – 3.2 x 10^9 UFC.g^{-1}) (probiotic), Fructo-oligosaccharide of Brazilian Corn Products (prebiotic) and a mixture of probiotic + prebiotic (symbiotic). Forty-four piglets, homogeneous in relation to line and weight, weaned with 21 days of age and with 5.40 ± 0.73

kg of live weight, were placed in individual pens with 2.55 m^2 (1.50 x 1.70 m) for avoiding animal contact. The experimental diets T1 (Basal Diet = BD), T2 (BD + Antibiotic at 40 ppm), T3 (BD + 0.13% Probiotic), T4 (BD + 0.65% Prebiotic) and T5 (BD + Symbiotic = T3+T4 in the same concentrations cited above) were formulated according to animal requirements of NRC (1998) (Table 1). Diet composition was calculated based on tables published by ROSTAGNO et al. (2000).

Slaughter and sampling

For control, four piglets were slaughtered at weaning. Seven and 14 days after weaning, four piglets per treatment were slaughtered after 16 hours of diet fasting. Just after slaughter, samples of duodenum, mid-portion of jejunum and ileum (± 3 cm) were collected for analysis in light microscopy. For electron microscopy, samples were taken from duodenum and mid-portion of jejunum. Samples for light and scanning electron microscopy analysis were prepared, respectively, at Histology Laboratory of Animal Morphology and Physiology Department and at Electron Microscopy Laboratory of FCAV, UNESP, Jaboticabal City. Samples for transmission electron microscopy analysis were prepared at Electron Microscopy Center of Bioscience Institute, UNESP, Botucatu City, São Paulo State, Brazil.

Light microscopy

Immediately after slaughter, small intestine (duodenum, mid-portion of jejunum and ileum) samples were washed in 0.1M phosphate buffer (pH 7.4). Samples were opened by mesenteric border, stretched through serosa layer and fixed in Boiun solution during 24 hours. Then they were washed in run water and 70% ethyl alcohol for releasing the excess of fixation solution. For dehydration, washings in graded (from 70 to 100%) alcohol series were done. Diaphanization was done in xylene before embedding in paraffin. Microtomy was done for obtaining from 12 to 14 semi-serial cuts of 5µm of each segment per animal. Eosin-hematoxilin was used for staining. Morphometric analysis of intestinal ephitelium were carried out at Laboratory of Light Microscopy of Morphology Science Department, UFRGS, Porto Alegre City, Rio Grande do Sul State, Brazil. An image analyzer system composed by Nikon Eclipse E-600 microscopy, Samsung video-camera and Image

Pro-Plus 4.1 soft-ware was used with zoom of 125 times for estimating villous height (VH, μm) and crypt depth (CD, μm). Thirty readings per sample for each parameter were done for calculating VH:CD ratio.

Scanning Electron Microscopy (SEM)
After washing in 0.1 M phosphate buffer (pH 7.4), 1-cm samples of duodenum and mid-portion of jejunum were fixed in 3% glutaraldehyde, washed many times in the buffer and post-fixed in 2% osmium tetroxide per 2 hours and washed again in phosphate buffer. Then they were dehydrated in graded ethanol serial washings, dried in CO_2 critical point dryer, assembled, metalized with gold/palladium and viewed. Electron-micrographs were taken using JEOL, JSM-5410 scanning electron microscopy (operated at 15 kV) from five areas of each sample for estimating villous density (villous number per cm^2).

Transmission Electron Microscopy (TEM)
Samples of small intestine (duodenum and mid-portion of jejunum) were washed in 0.1M phosphate buffer (pH 7.4), fixed in 3% glutaraldehyde, washed in the same buffer and post-fixed in 1% osmium tetroxide per 2 hours at 4^oC.

Table 1 - Percentual composition and nutritional levels of experimental diets.

INGREDIENTS	%
Soybean meal	26.60
Corn	48.71
Milk Product*	20.00
Bicalcic Phosphate	1.20
Limestone	0.85
Vehicle**	2.00
BHT antioxidant	0.01
DL – Metionin 98%	0.04
L – Lisin HCl 78%	0.23
L – Threonin 98%	0.06
Common Salt	0.20
Mineral and vitamin supplement***	0.10
TOTAL	100.00
Metabolizable Energy (kcal.kg^{-1})	3,317
Lactose (%)	8.00
Crude Protein (%)	21.1
Lisin (%)	1.41
Metionin + Cistin (%)	0.79
Metionin (%)	0.36
Threonin (%)	0.90
Triptophan (%)	0.28
Calcium (%)	0.81
Available Phosphorus (%)	0.47

* Nuklospray K10 – 40% lactose;
** Microingredients tested in this study were included in substitution to vehicle (caulim) in proportions cited before;
*** Mineral and vitamin supplements did have any kind of growth promoter or antibiotic. Guarantee levels per kg of ration: Vit. A – 4,000 I.U.; Vit. D_3 – 220 I.U.; Vit. E – 22 mg; Vit. K – 0.5 mg; Vit. B_2 – 3.75 mg; Vit. B_{12} – 20 μg; Calcium pantotenate – 12 mg; Niacin – 20 mg; Choline – 60 mg; Iodine – 140 μg; Selenium – 300 μg; Magnesium – 10mg; Zinc – 100 mg; Copper – 10 mg; Iron – 99 mg.

After washing in distillated water and dehydration in serial crescent acetone solutions (50, 70, 90 and 100%), material was filtrated in a 1:1 acetone:resin mixture during 24 hours.
This mixture was rejected and the material was inserted in resin and maintained in oven at 60^oC per 48 hours for polymerization. Blocks were trimmed for removing resin excess and for preparation of semi-thin cuts with 0.5μm of thickness. Staining

was done with toluidine blue. Areas were chosen for ultra-thin cuts done in an ultramicrotomy with diamond knives, assembled in cooper grids, contrasted with uranyl acetate and lead citrate. Electron micrographs (17.000X) of microvilli were taken in JEOL -1010 transmission electron microscopy. An Image Pro-Plus 4.1 software were used for estimating these following morphometric parameters: microvillous height and width (μm), apical diameter of enterocytes (distance between two joint complexes, in μm) and microvillous density (number of microvilli per μm^2).

Apical surface of enterocytes (S) was calculated using apical diameter of enterocytes (C) according to FERRER et al. (1995), and amplification factor of microvilli (AFM) was estimated following this formula:

$$S = \pi \times C^2/4 \times AFM$$

Where:
AFM = π x A x B x D + 1
A = microvillous height (μm);
B = microvillous width (μm);
D = microvillous density (number/μm^2).

Experimental Design
A completely randomized block design in 5 x 2 + 1 factorial scheme (five diets x two slaughter times + control group) was used for controlling weight difference. Variance analysis were performed using GLM proceedings of SAS (SAS® Institute, 1998) and means were compared by Tukey test at 5% of significance level.

RESULTS AND DISCUSSION

Light Microscopy
Table 2 shows the values of villous height (VH), crypt depth (CD) and VH:CD ratio of duodenum, jejunum and ileum. Significant interactions (P<0.05) among some variables, and unfolding results are presented in Table 3. Interactions (P<0.05) between slaughter ages and diets occurred for VH of the three intestinal segments, for CD of duodenum and ileum and for VH:CD ratio of duodenum. Higher (P<0.05) crypt depth in jejunum was observed at 14th day after weaning when it was compared to the values of the other sampling

periods (0 and 7 days after weaning). Similar values were reported by THOMAZ (1996) and TUCCI (1999). The VH:CD ratio decreased (P<0.05) in jejunum and ileum from weaning to further periods, which reflected the VH reduction and CD increase in these regions.

Other authors (THOMAZ, 1996; THOMAZ et al., 2002) registered VH reduction, which was a common response to weaning stress. CERA et al. (1988), repaired VH reduction due to feed intake decrease and due to diet changes. According to PLUSKE et al. (1997), villous atrophy after weaning was caused by higher cell losses and, or smaller cell turn-over rate.

From seventh to 14th day post-weaning, greater decreases (P<0.05) in villous height of the three small intestine regions (duodenum = -22%, jejunum = -16% and ileum = -18%) were observed in animals fed basal diet. At 14th day post-weaning, there was significant (P<0.05) changes in duodenal villous height, being the smaller and the higher means found in animals from basal diet and probiotic treatments, respectively (Table 3).

At seventh day post-weaning, jejunal villous height was affected (P<0.05) by different diets. The higher height was observed in animals fed basal diet; while animals supplemented with prebiotics and symbiotics had smaller heights. But, at 14th day post-weaning, animals from prebiotics and symbiotics treatments showed signals (P>0.05) of villous recovering. Also, ileal villous height was affected (P<0.05) at seventh day post-weaning; being the smaller height found in animals supplemented with prebiotic and the higher height in animals from the symbiotic treatment. Instead of lacking significance, piglets fed prebiotic showed ileal villous recovering at 14th day post-weaning.

These results disagreed to those of THOMAZ et al. (2002), who did not find significant differences in small intestinal villous height of weaned piglets fed diets with 0.2% of MOS prebiotic. However, SANTOS et al. (2002a) observed that 0.2% of MOS prebiotic in diet offered to piglets at nursing phase increased significatively duodenal villous height. In other trial, SANTOS et al. (1998) reported smaller VH in piglets fed diets without prebiotic than those receiving *Lactobacillus*.

During the studied period, piglets fed diets with probiotic showed higher (P<0.05) duodenal and ileal CD. But, SANTOS et al. (1998) observed

higher crypt depth in small intestine of 63 day-old piglets fed diet with antibiotic (zinc bacitracin) than those fed diets with probiotic. Higher crypt depth is related to higher cell proliferative activity for allowing adequate epithelial turn-over rate and compensate losses in height of villi (PLUSKE et al., 1997).

Table 2 - Means of villous height (VH, μm), crypt depth (CD, μm) and villous/crypt (VH/CD) ratio in duodenum (D), jejunum (J) and ileum (I) in function of days after weaning experimental diets.

Factors	Villous Height[1]			Crypt Depth			Villous/Crypt Ratio		
	D	**J**	**I**	**D**[1]	**J**	**I**[1]	**D**[1]	**J**	**I**
Days									
0	355	365	241	197	178[B]	196	1.97	2.19[A]	1.26[A]
7	243	262	179	222	203[B]	209	1.20	1.36[B]	0.89[B]
14	235	268	185	223	230[A]	227	1.17	1.24[B]	0.85[B]
F test					*			*	*
Diet									
Basal	242	293	183	216	224	211	1.26	1.39	0.90
Antibiotic	234	284	171	241	227	220	1.07	1.33	0.80
Probiotic	241	256	178	212	208	207	1.23	1.30	0.91
Prebiotic	239	254	167	225	196	217	1.16	1.36	0.79
Symbiotic	240	238	209	219	227	233	1.21	1.12	0.94
F test					NS			NS	NS
VC (%)	12.2	20.6	25.2	20.0	16.0	13.6	20.9	24.3	22.5

[1] There was significant interaction among factors;
[A,B] Means followed by the same letter within column are similar by Tukey test (5 %);
NS = non significant; *(P< 0.05).

Table 3 - Means of villous height (VH, μm), crypt depth (CD, μm) and villous/crypt (VH/CD) ratio.

Variable[1]	Basal	Antibiotic	Probiotic	Prebiotic	Symbiotic
VH					
Duodenum					
Day 7	273.0[A]	225.4	237.4	239.9	241.0
Day 14	212.5[Bb]	242.8[ab]	245.7[a]	238.5[ab]	238.9[ab]
Jejunum					
Day 7	319.2[a]	287.0[ab]	267.4[ab]	220.4[b]	220.2[b]
Day 14	267.9	282.9	244.6	287.9	257.2
Ileum					
Day 7	202.4[ab]	169.3[ab]	172.4[ab]	132.1[b]	218.5[a]
Day 14	164.6	173.4	184.4	202.3	201.1
CD					
Duodenum					
Day 7	198.3	250.5	187.0[B]	241.2	235.1
Day 14	233.6	232.5	237.7[A]	210.3	203.5
Ileum					
Day 7	210.1	218.4	182.9[B]	203.9	229.8
Day 14	213.6	222.9	232.0[A]	230.0	237.5
VH/CD					
Duodenum					
Day 7	1.48[a]	0.94[b]	1.37[a]	1.08[ab]	1.15[ab]
Day 14	1.04	1.21	1.10	1.24	1.27

[1] Means followed by the same minuscule (capital) letter within line (column) are similar by Tukey test (5%).

In relation to VH:CD ratio, animals from basal diet and probiotic treatments were better (P<0.05) than those fed diet with antibiotic (Table 3), in accordance with results obtained by SANTOS et al. (1998) in a trial where 63 day-old pig receiving the same antibiotic (zinc bacitracin) had smaller VH:CD ratio in relation those fed diet without antibiotic. A good VH:CD ratio was considered when villi were high (similar to fingers) and crypts were flat (little depth), consequently, nutrient absorption was better (SANTOS et al., 2002b).

Scanning Electron Microscopy
Table 4 shows villous density in small intestine of piglets in function of post-weaning days and different diets. There was no effect of age on villous density in duodenum, but in jejunum there was reduction (P<0.05) in villous density at 14th day post-weaning in relation to seventh day post-weaning. Reduction in villous density with increase of age was also found by other authors in weaned piglets (HANNAS, 2003; TUCCI, 2003) and in

broiler chickens (SILVA, 2001; MAIORKA, 2002).
Piglets from prebiotic treatment showed higher (P<0.05) villous density in duodenum than piglets from probiotic treatment. Instead of lacking statistical significance (P>0.05), animals fed diets with prebiotic also showed higher villous density in jejunum. These results suggested that FOS prebiotic should have had effect on intestinal mucosa, provoking increase in absorption area, since higher intestinal mucosa region was exposed to intestinal lumen. In contrast, TUCCI (2003) did not found changes in duodenal and jejunal villous density of weaned piglets supplemented with 0.2% MOS prebiotic.
Intestinal mucosa development is due to two primary related cytological events: cell turn over and losses (extrusion). Balance between these both events determines a constant cell renovation, which means maintaining of villous size (MAIORKA, 2002). Following this thought, apparently, animals supplemented with prebiotic showed better balance.

Table 4 - Villous density (number of villi.cm^{-2}) in small intestine of piglets in function of days post-weaning and experimental diets.

Factors	Segment of Small Intestine	
	Duodenum	Jejunum
Days		
0	9,087	8,994[AB]
7	9,046	11,746[A]
14	7,902	9,686 [B]
F test	NS	**
Diet		
Basal	8,530[AB]	10,331
Antibiotic	7,974[AB]	9,986
Probiotic	7,629[B]	10,077
Prebiotic	9,829[A]	12,848
Symbiotic	8,556[AB]	10,431
F test	**	NS
VC (%)	20.7	26.9

[A,B] Means followed by the same letter within column are similar by Tukey test (5%).
NS = non significant; ** (P<0.01).

Transmission Electron Microscopy

Table 5 shows values of height (µm), width (µm), microvillous density (n° of microvilli/µm^2) and enterocyte apical surface area (µm^2) of piglet small intestine in function of different days after weaning and experimental diets. Microvillous density and

enterocyte apical areas in duodenum of animals were higher (P<0.05) at day 0 than at seven and 14 days after weaning. In jejunum, enterocyte apical area was higher (P<0,05) at day 0 than 14 days after weaning. These data demonstrated that these might be some extrusion process on the surface of

these enterocytes, probably due to diet changing (from liquid to solid).

Duodenal microvillous height and jejunal microvillous density showed significant (P<0.05) interaction. Thus, treatment unfolding was performed (Table 6). Fourteen days after weaning, symbiotic utilization caused higher (P<0.05) duodenal microvillous height in relation to basal diet (Table 6). CERA et al. (1988) related in piglets reduction in jejunal microvillous height from three to seven days after weaning, which indicated that diet with symbiotic in this study improved morphometric features of small intestine. In most recently research TUCCI (2003), did not find duodenal and jejunal microvillous height changes in weaned piglets supplemented with prebiotic. Animals of probiotic treatment had higher (P<0.05) density of microvilli at the 14th post-weaning than the seventh day post-weaning.

Table 5 - Means of height (µm), width (µm), density of microvilli (nº of microvilli.μm^{-2}) and enterocyte apical surface area (μm^2) of small intestine in function of days post-weaning and experimental diets.

	Duodenum				Jejunum			
Factors	**Height**[1]	**Width**	**Density**	**Area**	**Height**	**Width**	**Density** [1]	**Area**
Days								
0	1.16	0.11	138^A	1107^A	1.00	0.12	111	873^A
7	0.79	0.13	108^B	697^B	0.95	0.12	92	681^{AB}
14	0.82	0.12	104^B	714^B	0.94	0.12	95	617^B
F test		NS	*	*	NS	NS		*
Diet								
Basal	0.79	0.13	107	704	0.99	0.13	92	759
Antibiotic	0.75	0.12	106	614	0.96	0.13	85	576
Probiotic	0.81	0.13	101	857	0.90	0.12	94	632
Prebiotic	0.71	0.12	112	581	0.92	0.12	96	618
Symbiotic	0.96	0.12	105	773	0.94	0.12	100	659
F test		NS	NS	NS	NS	NS		NS
VC (%)	19.8	10.6	12.4	34.8	31.1	8.1	14.4	32.0

[1] There was significant interaction among factors;
^{A,B} Means followed by the same letter within column are similar by Tukey test (5%);
NS = non significant; * (P< 0.05).

The results showed that microingredients added to weaned piglet diets affected small intestinal histology (villous height and crypt depth). In relation to probiotic, prebiotic caused increase in duodenal villous density. In relation to basal diet, diet with symbiotic increased duodenal microvillous height 14 days after weaning. Animals supplemented with probiotic showed better microvillous density recovering 14 days after weaning in relation to those from the other treatments.

Table 6 - Means of height (µm) and density of microvilli (n° of microvilli.μm^{-2}) of weaned piglet intestinal epithelium.

Variable	Basal	Antibiotic	Probiotic	Prebiotic	Symbiotic
Height					
Duodenum					
Day 7	0.88	0.75	0.76	0.67	0.87
Day 14	0.70^b	0.75^{ab}	0.87^{ab}	0.75^{ab}	1.05^a
Density					
Jejunum					
Day 7	98.3	82.0	86.4^B	94.9	100.6
Day 14	86.4	89.5	101.8^A	98.5	100.2

^{a,b} Means followed by the same minuscule (capital) letter within line (column) are similar by Tukey test (5%).

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RESUMO

Foram utilizados 44 leitões desmamados, distribuídos em um delineamento em blocos casualizados, em esquema fatorial onde foram avaliadas cinco dietas: T1 – Dieta Basal; T2 - Dieta Basal + Antibiótico; T3 - Dieta Basal + Probiótico; T4 - Dieta Basal + Prebiótico; T5 - Dieta Basal + Simbiótico (T3+T4). Inicialmente foi abatido um grupo de quatro leitões ao desmame. Nos dias sete e 14 pós-desmame foram abatidos quatro leitões de cada tratamento. Foram coletados segmentos do intestino delgado, de cada animal, para análise microscópica de luz e eletrônica. Os microingredientes testados influenciaram a histologia intestinal dos leitões. Foi observada maior densidade de vilos na porção duodenal dos leitões que consumiram ração com prebiótico em relação aos que consumiram dieta com probiótico. Na porção do jejuno dos leitões recebendo ração contendo prebiótico houve, embora não significativa estatisticamente, maior densidade de vilos. Os animais recebendo ração com simbiótico apresentaram maior altura de microvilos aos 14 dias no duodeno em relação aos que consumiram a dieta basal. Nos leitões suplementados com probiótico houve melhor recuperação na densidade dos microvilos em relação às outras dietas que não mostraram diferenças entre as idades estudadas.

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