IgA production, coliforms analysis and intestinal mucosa morphology of piglets that received probiotics with viable or inactivated cells[1]

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ABSTRACT.- Rodrigues M.A.M., Oliveira D.A., Taketomi E.A. & Hernandez-Blazquez F.J. 2007. **IgA production, coliforms analysis and intestinal mucosa morphology of piglets that received probiotics with viable or inactivated cells**. *Pesquisa Veterinária Brasileira 27(6):241-245.* Departamento de Cirurgia, Setor de Anatomia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-900, Brazil. Email: fjhblazq@usp.br

Two types of probiotics were used in piglets. One product is a mixed culture of viable *Lactobacillus acidophilus*, *Enterococcus faecium* e *Bifidobacterium bifidum*. The second product is composed of inactivated *Lactobacillus acidophilus* cells. The piglets received two weekly oral doses for 30 days while a control group did not receive probiotics. All piglets were euthanized at the 30th day of life and the mesenteric lymph nodes, the small intestine, and blood samples were collected. The tissue samples were studied by light microscopy and the blood serum was analyzed by ELISA method. The treatment with the probiotic with viable cells produced higher serum levels of IgA ($P<0.05$) and more IgA expressing cells were found in the mesenteric lymph nodes than observed in the inactivated cells treatment or control groups ($P<0.05$). Also, intestinal villi were longer, crypts were deeper ($P<0.05$) and fecal coliform count was lower than found in the inactivated product ($P<0.05$). These results suggest that viable probiotics are more efficient than inactivated probiotics to induce immunostimulation and intestinal modifications in piglets, thus improving their health and development.

INDEX TERMS: Probiotics; IgA, piglets, swine, weaning, *Lactobacillus*, intestine.

RESUMO.- [Produção de IgA, análise de coliformes e morfologia da mucosa intestinal de leitões que receberam probióticos com células viáveis ou inativadas.] Dois probióticos foram usados em leitões recém nascidos: um constituído por cultivo viável misto de *Lactobacillus acidophilus*, *Enterococcus faecium* e *Bifidobacterium bifidum* e o segundo composto por células inativadas de *Lactobacillus acidophilus*. Os animais receberam duas doses orais por semana, por 30 dias; um grupo controle não recebeu probióticos. Os leitões foram abatidos aos 30 dias de idade e foram colhidos os linfonodos mesentéricos, o intestino delgado e amostras de sangue. Os tecidos foram estudados por microscopia de luz e as amostras de soro foram analisadas pelo método ELISA. O probiótico com células viáveis produziu níveis mais altos de IgA no soro sangüíneo ($P<0,05$) e mais células IgA positivas foram encontradas nos linfonodos mesentéricos que no tratamento com células inativadas ou no controle ($P<0,05$). Igualmente, as vilosidades intestinais eram mais alongadas e criptas mais profundas ($P<0,05$), com menor contagem de coliformes fecais que no tratamento com células inativadas ($P<0,05$). Estes resultados sugerem que o probiótico com bactérias viáveis é mais eficiente que o probiótico inativado para induzir imunoestimulação e modificações intestinais nos leitões, melhorando a saúde em geral e o desenvolvimento.

TERMOS DE INDEXAÇÃO: Probióticos, IgA, leitões, suínos, desmame, *Lactobacillus*, intestino.

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INTRODUCTION

Despite advancements in nutrition and management in pig farming, pathogenic microorganisms may impair efficient use of food nutrients by the animal digestive tract. Antibiotics are added to pigs' feed as growth promoters to reduce this problem; however, they are being substituted by probiotics (Vassalo et al. 1997). Probiotics are simple or mixed cultures of specific bacteria and/or yeast cells that help maintain a healthy balance of intestinal flora (Fuller 1989, Smith, 1991). They are incorporated into livestock feeds as food supplements, or they may be given separately (Ouwehand et al. 1999). The viability of the microorganisms is a necessary condition for adhesion of cells to receptors sites of the intestinal mucosa. However, even inactivated products can inhibit the action of bacteria and virus that cause diarrhea in swine as they bind to adhesion sites of the gut mucosa and prevent ulterior attachment of pathogenic cells (Coconier et al. 1993). Probiotics also promote non-specific immune response and production of a IgA that helps control intestinal infections, thus improving the animal's health (He et al. 2000, 2005). Increase in the size of intestinal villi can usually be observed. Favorable nutritional results include higher absorptive and digestive intestinal capacities (Di Nola et al. 1991).

Probiotics available in the market for use in pig farming are composed of lactic bacteria that are natural components of pig intestinal flora, either viable or inactivated. It is important to evaluate if the viability of the microorganisms is an essential condition for immunologic stimulation, IgA production and structural modifications of the intestine, or if the inactivation process applied to the bacteria reduces or inhibits some of the probiotic effects. Thus, our objective was to compare both type of products and evaluate the immune response and intestinal mucosa morphology of piglets from birth up to 30 days supplemented with a viable probiotic and an inactivated probiotic. The concentration of IgA in the blood serum was measured and the numbers of IgA expressing cells in the lymph nodes and intestine, as well as the size of the intestinal villi and crypts were measured by an image analysis software.

MATERIALS AND METHODS

Newborn crossbred piglets (Agpic x Camborough) from three litters were separated into three groups: IC (inactivated cells) group with 8 piglets, received 3.0g/animal/day of inactivated *Lactobacillus acidophilus* cells at 2.5 x 10^8CFU/g given orally with filtered water, twice a week from the first to the 30th day of life; VC (viable cell) group with 10 piglets, received 3.0g/animal/day of viable cells product, containing *Lactobacillus acidophilus, Enterococcus faecium* and *Bifidobacterium bifidum* at 3.33 x 10^6CFU/g by bacteria, and C group with 7 piglets that did not received probiotics. The piglets were kept in a pig farm with optimal sanitary conditions and were weighed at birth, at 21 days and 30 days. The live weight gain of each piglet was calculated by subtracting the weight at 21 days from weight at 30 days. All groups were fed sow milk up to the 21st day of life. After the 7th day, the milk was supplemented with a starter diet three times a day. After weaning at 21 days, they were fed starter diet up to 30 days, and euthanized by exsanguination through section of the jugular vein. The composition of the starter diet was 37% ground corn, 20% soybean meal, 3% sugar and 40% pre-starter mineral and vitaminic 400 premix (400kg/ton of food, Agroceres SA, São Paulo, Brazil). Feces samples were collected from 23 day-old piglets and kept in 9mL of saline solution at 4ºC. Total and fecal coliforms were counted by the SIM PLATE (IDEXX) method.

Blood for total serum IgA measurement from 30-day-old piglets was tested by the ELISA method. The plates were sensitized overnight at 4ºC with mouse antibody against swine IgA (Serotec, England) diluted 1:100 in PBST (phosphate-buffered saline with 0.05% Tween 20). Bovine serum albumin (BSA, Sigma, St Louis, USA) 1% in PBST for 1 hour at room temperature (RT) was the blocking step. The plate was incubated for 2 hours at 37ºC with 50ml of serum in each well at 1:40 or 1:80 dilution in 1% BSA/PBST, followed by secondary goat biotinylated antibody (Serotec) at 1:250 in 1% BSA/PBST for 1 hour at 37ºC, streptavidin/peroxidase (1:500) for 30 minutes at RT and ABTS (2,2 azino-bis 3-ethylbenzi-thiazoline-6-sulfonic acid) in 0.1M pH 5.0 phosphate-citrate buffer and H_2O_2. The plates were read in a microplates reader (Titertek Multiskan Plus, Flow Laboratories, USA) at 405nm. The results were given in optical density units.

After euthanasia, fragments of mesenteric lymph nodes, and small intestine (middle portion of duodenum, first portion of jejunum and distal portion of ileum) of 30-day-old piglets were collected for immunohistochemistry and morphometry analysis. The tissue was fixed in 10% buffered (0.1M pH 7.2 phosphate buffer) formalin for 24 hours and embedded in paraffin. Sections 5mm thick were cut and endogenous alkaline phosphatase was blocked by 8% acetic acid solution for 10 minutes (lymph node) and 30 minutes (intestine). Rabbit serum (2.5%) in TBS for 1 hour at 37ºC was used as blocking solution. The primary antibody (goat anti swine IgA 1:100 in TBS, Serotec) was incubated overnight at 4ºC. The secondary antibody (biotinylated rabbit antibody 1:100 in TBS, Serotec) was applied for 1 hour at 37ºC. Avidin-biotin-alkaline-phosphatase complex (AB/PA) at 1:100 in TBS was allowed to bind for 30 minutes at 37ºC and covered by a fast red-naftol (Sigma) solution in TBS for 3 minutes. The number of IgA-expressing cells in lymph nodes and duodenum of three animals from each group was counted in five microscopic fields by histological section/animal with a 40x objective. Fragments with 0.5cm^2 from the duodenum, jejunum and ileum were fixed in Bouin solution for 24 hours, embedded in paraffin, and 6mm transversal sections were obtained. Sections were stained by the hematoxylin-eosin method. Images of microscopic fields were digitalized with a 40X objective and height of villi and depth of crypts were measured by the Software HL Image++97" (Western Vision Software). The height (HV) of 30 villi and the depth of 30 crypts (DC) were measured in each section/animal for 3 piglets from each group.

RESULTS

The VC group had higher ($P<0.05$) live weight gain (LWG) than IC group (2.70 ± 0.18 and 2.31 ± 0.15 respectively) and C group (1.91 ± 0.24) as shown in Table 1. The average fecal coliform count after weaning of IC group was higher than that of either the VC or C groups; however no difference ($P>0.05$) was found among groups in total coliform counting (Table 2). The VC group did not develop diarrhea and kept lower levels of fecal coliform than the IC group. This group developed diarrhea in the second and third weeks. A ten times reduction of the fecal CFU value in the VC group was observed when compared with the C group, although the difference was not significant ($P>0.05$).

The IgA expressing cells were uniformly distributed in the

Table 1. Weight (W) (Kg) and live weight gain between the 21st and 30th day (LWG) means of piglets at birth, with 21 and 30 days of age treated with different types of probiotics

Groups	Days			LWG	Number of
	01	21	30		animals
C[a]	1.69±0.12[a]	6.58±0.28[a]	8.50±0.39[a]	1.91±0.24a	7
IC	1.39±0.08[b]	5.00±0.19[b]	7.31±0.30[b]	2.31±0.15b	8
VC	1.48±0.14[b]	5.11±0.18[b]	7.8±0.32[c]	2.70±0.18c	10

[a] C = control group, IC = probiotic with inactivated bacteria, VC = probiotic with viable cells. The means in the same column displaying different letters are statistically different under one-way ANOVA followed by Tukey's test (P<0.05).

Table 2. Means and standard deviation of total and fecal coliforms by gram of feces of 23-day-old piglets treated with different types of probiotics

Groups	Total coliforms (CFU/g)	Fecal coliforms (CFU/g)	Number of animals
C[a]	$6.6 \times 10^6 \pm 6.90 \times 10^{6a}$	$0.63 \times 10^6 \pm 0.67 \times 10^{6a}$	7
IC	$2.8 \times 10^6 \pm 2.02 \times 10^{6a}$	$2.35 \times 10^6 \pm 1.58 \times 10^{6b}$	8
VC	$6.8 \times 10^6 \pm 0.1 \times 10^{6a}$	$0.068 \times 10^6 \pm 0.01 \times 10^{6a}$	10

[a] C = control group, IC = probiotic with inactivated bacteria, VC = probiotic with viable cells. The means in the same column displaying different letters are statistically different under one-way ANOVA followed by Tukey's test (P<0.05).

Table 3. Average number of IgA-expressing cells by microscopic field in histological sections of lymph nodes and duodenum. Comparison between 30-day-old piglets treated with inactivated cells (IC) or viable cells (VC)

Organ	Groups*		
	Control	IC	VC
Lymph node	2.1± 0.8[a]	1.6 ± 0.3[a]	7.7± 1.9[b]
Duodenum	3.5 ± 0.5[a]	3.8± 1.1[a]	11.6±1.4[b]

* The means in the same line displaying different letters are statistically different under one-way ANOVA followed by Tukey's test (P<0.05), n=3.

Table 4. Blood serum IgA levels from 30-day-old piglets treated with probiotics composed by inactivated (IC) or live (VC) cells. The means are the followed by the standard deviation, ELISA method

Groups	Optical Density (OD)
Control (n=7)	1047.6 ± 115.5[a]
IC (n=8)	1043.9 ± 120.5[a]
VC (n=10)	1230.5 ± 119.6[b]

Means followed by different letters in the same column are statistically different under one-way ANOVA followed by Tukey's test (P<0.05).

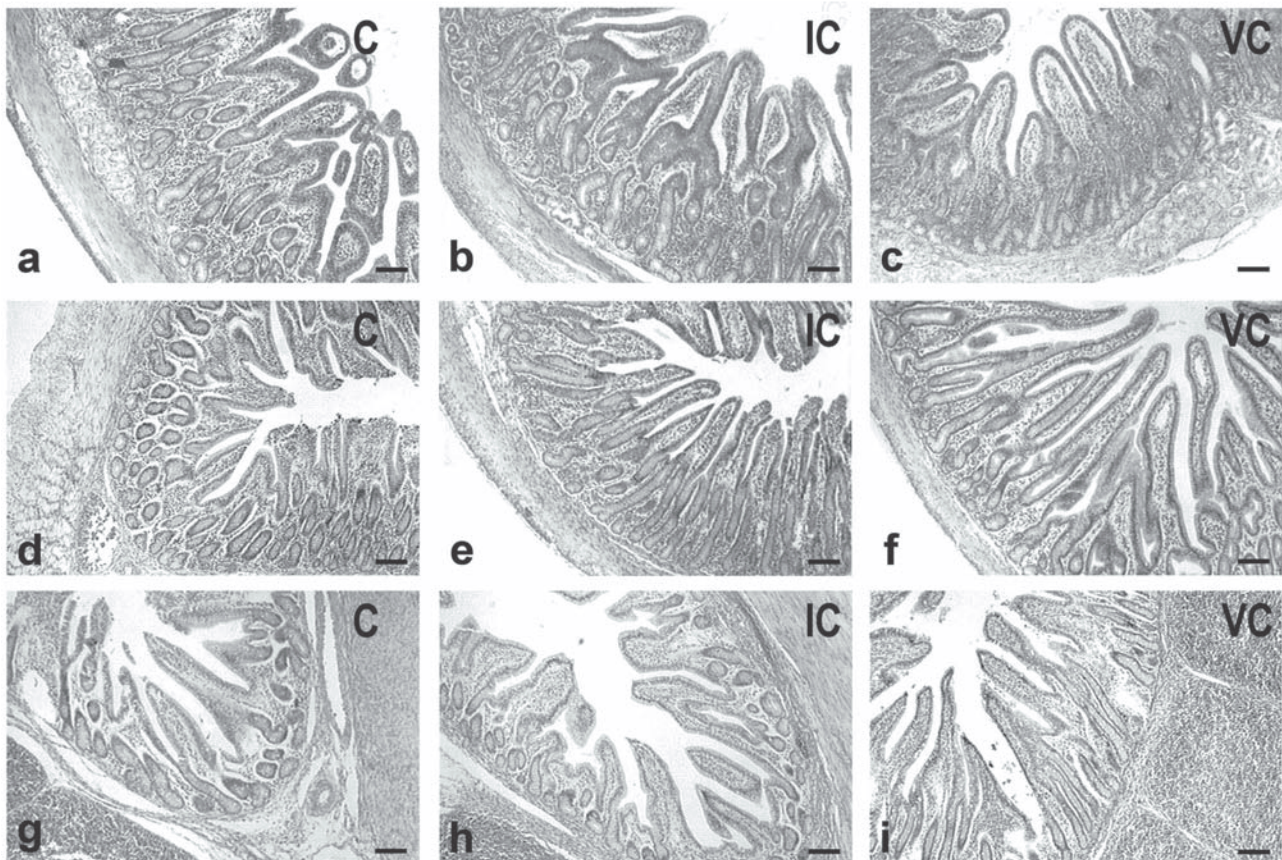

Fig.1. Histological sections of piglets small intestine stained by hematoxylin-eosin method. The treatment is indicated in the upper right of each image in uppercase: C = control, IC = probiotic with inactive cells, VC = probiotic with viable cells. **(a) (b) (c)**: images of duodenum where it may be observed that the height of the villi increases and the depth of the crypts decreases progressively from the control group to the VC group; **(d) (e) (f)**: images of jejunum, the villi and crypts follow the same patterns as it was shown in the duodenum; **(g) (h) (i)**: ileum, where it may be noted that the villi of the VC group are higher than those of the C and IC groups. Scale Bar = 100m.

Table 5. Height of villi (HV) and depth of crypts (DC) of the intestinal mucosa of the duodenum, jejunum and ileum of piglets from the 30th day after weaning. Groups IC (inactive cells) and VC (viable cells), supplemented with probiotics, and Group C (Control)

Organ	Parameter measured	Experimental groups			Increase/ decrease (VC-C)
		C(mm)	IC(mm)	VC(mm)	
Duodenum	HV	359.3±48.5^{a}	387.2±25.8^{a,b}	443.1±92.8^{b}	23%
	DC	548.1±52.9^{a}	469.3±46.2^{b}	460.6±44.7^{b}	-16%
Jejunum	HV	319.6±18.6^{a}	338.6±37.1^{a}	441.4 ± 95.5^{b}	38%
	DC	316.6±66.5^{a}	343.3±54.6^{a}	283.7±19.0^{b}	-10%
Ileum	HV	272.8±12.2^{a}	331.7±2.2^{a}	395.5±99.4^{b}	45%
	DC	293.7±64.4^{a}	247.6±17.3^{a}	270.4±21.3^{a}	-

The statistical comparison was made among the means that are in the same line, means followed by the same letter are statistically similar under the non-parametric Kruskal-Wallis multiple comparison test (P<0.05). Ninety structures (30 structures in each animal, 3 animals/group) were measured.

duodenal epithelium and lamina propria of all groups. The average number of IgA expressing cells in lymph nodes and duodenum of the VC group was more elevated than that of IC and C groups (Table 3). The IgA serum levels were measured to evaluate the comparative immunostimulating efficacy of the different probiotics. The average serum IgA level was higher in the VC group (P<0.05) than in the control or IC groups (Table 4).There was no difference (P>0.05) between the average number of IgA expressing cells in either C or IC groups in both tissues.

The results of villi height and crypts depth measurement are shown in Table 5 and Figure 1. The height of villi increased in the small intestine when the piglets received viable cells (VC group) and remained statistically unaltered (P>0.05) in piglets that received inactivated cells (IC group). The effect on VC group villi was more intense as more distally the intestinal segment was localized (+23% in the duodenum, +38% in the jejunum and +45% in the ileum), and a reverse effect occurred in crypts depth (16% in duodenum, -10% in jejunum and no changes in ileum). However, the inactivated cells reduced crypt depth in the duodenum of IC group, without changes in the jejunum and ileum. The comparison between the VC group and IC group didn't show differences between the duodenum villi and crypts sizes of both groups, although the jejunum and ileum villi were higher and jejunum crypts were shallower in VC than in IC group (P<0.05).

DISCUSSION

Results suggest that live cells probiotic was more effective to improve the biologic parameters tested than probiotic with inactivated cells. The improvement in the live weight gain of the piglets that received complete feed with probiotics, especially live probiotics, can be attributed to the balance between the natural flora and the pathogenic intestinal organisms and also to better absorption of the nutrients (Vassalo et al. 1997). The quantity of bacteria that colonize the piglets' intestinal tract can vary, depending on the sanitary conditions and the addition of antibiotics or probiotics to feed (Butler et al. 2000). In fact, the fecal CFU value of the IC group was very high (Table 2), showing that the probiotic with viable cells (VC) was more effective to reduce intestinal flora. However, a positive effect of the IC probiotic was observed as this product was able to control diarrhea in the IC group. The product with viable bacteria elicited a better response from the lymphatic tissue and immune system, as the quantity of IgA expressing cells was higher in lymph nodes and intestinal mucosa of VC group than in either the IC or C groups (P<0.05).

Viable probiotics are transferred from duodenal M cells to intraepithelial lymphocytes, thus acting as antigens that stimulate mucosal plasma cells to secrete IgA. This specific production of IgA triggers a response in mesenteric lymph nodes, which increase the number of IgA expressing cells (Tizard 1992). The results of the blood serum analysis agree with microscopic results, as the IgA levels were higher in the VC group. The viable cells given to the VC group probably acted like antigens that stimulate the IgA synthesis in the intestinal lymph tissue of the host (Ouwehand et al. 1999). This effect is beneficial because the increase of IgA in the intestine prevents local infection and allergen absorption (He et al. 2005). The inactivated organisms can still retain their capacity to inhibit pathogenic agents in the small intestine; however their IgA stimulating properties are reduced (Coconier et al. 1993), which may explain why the blood serum IgA levels of the IC group were not different from that observed in control animals.

The viable cells probiotic on villi morphology seem more effective in the lower portions of the small intestine because the villi size was higher in the ileum. The crypt depth appears to be more affected in duodenum, where they are shallow. The probiotic made from inactivated cells didn't affect mucosal morphology, except in the duodenum, were a reduction of the crypt depth was observed. The effect obtained by the viable cells product is desirable in piglet weaning management. Long villi and shallow crypts in small intestine usually provide increased superficial absorptive area and thinner lamina propria. The weaning procedure induces morphological changes of the intestinal mucosa. There is reduction of villi height due to the loss of enterocytes and an increase of crypt depth that thicken intestinal mucosa (Mahan & Cera 1993). A thicker intestinal mucosa with low villi impairs the nutrients' absorption processes (Scholten et al. 1999). These changes could be caused or aggravated by factors like diarrhea and infections followed by atrophy of the villi and intestinal dysfunction. Here is shown that live cells probiotics were more effective to induce mucosa changes that may be favorable to the absorptive function in piglet intestine, probably through the reduction or control of intestinal flora, providing a less aggressive environment to the villi and enterocytes.

It is possible that the use of a viable multispecies probiotics instead of a viable monospecies probiotics had a small influence in the differences observed between treatments, although this possibility has little support in the literature. The efficacy of viable monospecies probiotics compared against viable multispecies is still controversial, without conclusive results (Timmerman et al. 2004, 2005). Our hypo-

thesis that the differences observed among groups are mostly due to the viable condition of the cells rather than to the number of species in the probiotics is more strongly supported, because it is known that viable cells adhere to intestinal mucosa and inactivated cells do not. Adhesion to mucosa is a well know favorable condition for the protective effects of probiotics (Ouwehand et al. 2000, Roselli et al. 2005).

The product with the mixture of viable bacteria was more suitable to be used as diet additive for piglets. It was more efficient than the inactivated strain and the control food in developing both serum and local immunological response. The longer intestinal villi of piglets treated with viable cells probably increased the absorptive area and favored weight gain.

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