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Effects of orally administered probiotic *Pediococcus acidilactici* on the small and large intestine of weaning piglets. A qualitative and quantitative micro-anatomical study

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Summary. Probiotic research has been approached, above all in recent years, by widely differing points of view, both for human and animal uses. Lactic acid bacteria release bacteriocins, and some of them may function as probiotic. The aim of the present study was to investigate the effects of dietary supplementation with the probiotic *Pediococcus acidilactici* on the piglet intestine, on circulating lymphocytes, and on aspects of piglet performance during the first 42 days after weaning.

Sixteen female piglets were at weaning assigned to two dietary groups: Control (Ctr, 8 animals) and *Pediococcus acidilactici* supplemented (Pa, 8 animals). Piglets' growth was monitored from weaning to the end of the trial. On day 42 post-weaning, the piglets were slaughtered and small specimens from both ileum and cecum were examined with haematoxylin/eosin staining to ascertain structural details. Histometry was performed by villi and crypts measurements, as well as GALT measurements. Histochemical analyses were performed to investigate the intestinal mucins. Immuno-histochemical analyses were used to visualize proliferating as well as apoptotic mucosal cells, and to identify mucosal macrophages and IgA producing cells. Intra-epithelial CD8+ T lymphocytes were identified and counted. Subsets of circulating T lymphocytes were analyzed by flow cytometry.

Pediococcus acidilactici supplementation positively influenced weight and post-weaning average daily gain of treated piglets. Histometry showed an increase in villi height and crypts depth in Pa animals in comparison with controls. Treated piglets showed a larger number of proliferating enterocytes than controls. Intra-epithelial CD8+ T lymphocytes were scarcer in treated than in control piglets, likely in relation with catarrhal enteritis shown in the latter.

We conclude that the studied supplementation was possibly able to protect the piglet small intestinal mucosa, improving local resistance to infections in the stressful weaning period.

Key words: Villi and crypts, Adherent mucous gel, GALT, Lymphocyte subsets, Histometry

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Introduction

The alimentary canal, by virtue of the enormous numbers of micro-organisms it contains, offers the greatest potential for the induction of changes in its microhabitat, with the aim of possibly obtaining significant positive effects on the overall health of farm animals, similarly to what has been shown in humans (Rastall et al., 2005; Sanders, 2006). These changes can be obtained by either qualitative or quantitative modifications of the diet, the addition of viable non-pathological bacteria (either with the diet or by a separate oral administration), the inclusion of antimicrobial substances in the diet, the latter however leading to resistance development in micro-organisms.

The comprehension of the role of the diet in animal health and well-being has profoundly changed as the science of nutrition has evolved. Researchers' interest is currently directed towards the improvement of defined physiological functions beyond the obvious nutritional impact of feed in reared animal species, including the potential reduction in the risk of diseases, as well as the health of human consumers. This is also the focus for probiotic research, which, above all in recent years, has been approached from different points of view for both human and animal uses, after the banning from CE countries of the use of chemotherapeutics as growing

substances. Even if the action mechanisms are in part unclear, probiotic micro-organisms may be reputed viable nutritional agents, which are especially able to confer high levels of resistance to diseases, not only gut-associated diseases, possibly decreasing toxic microbial activities and improving host local defensive properties (Fuller, 1992; Sanders, 2003; Vandenbergh, 2003; Duncker et al., 2006).

A large body of scientific studies seems to indicate a need for a better than previously obtained characterisation of probiotic strains and species to be used in farm animals: some probiotics adhere with high efficacy to the small intestine, while some others bind specifically to different parts of the large intestine (Jin et al., 2000). Even closely related probiotics have been shown to possess different *in vitro* properties (Ouwehand et al., 1999; Laukova et al., 2004), and this possibly explains differences in clinical effects for some of them reported (Majamaa and Isolauri, 1997; Sartor, 2005). Probiotics with low total in vitro binding capacity may display an *in vivo* high competitive exclusion of pathogens, or vice versa (Lee et al., 2000; Ibnou-Zekri et al., 2003; Bogovic Matijasic et al., 2006). In addition, species-specific differences, as well as age-related differences, may be detected when testing the efficacy of different probiotic strains and species (Vitini et al., 2000; Dogi and Perdigon, 2006; Di Giancamillo et al., 2007).

The intestinal microflora of the pig has been shown to be capable of resisting the establishment of intestinal pathogens (Hillman et al., 1994; Collier et al., 2003; Casey et al., 2004). This is possibly related to the presence of certain lactic acid bacteria within the pig intestinal microflora, which possess an inhibitory activity towards coliform bacteria. More in general, lactic acid bacteria-derived components are able to influence mucosal defensive properties (Menard et al., 2005). The addition of large numbers of lactic acid bacteria to the porcine micro flora results in an *in vitro* consistent and reproducible increase in the rate of removal of the pathogen (Hillman et al., 1995; Davilla et al. 2006; Larsen et al., 2007; Mayrhofer et al., 2007). However, the application of these isolates to live animal studies generally produces highly variable results.

Recent works of our group (Bontempo et al., 2006; Di Giancamillo et al., 2007) and of other authors (Baum et al., 2002; Van Heugten et al., 2003) have shown that dietary administration of the probiotic *Saccharomyces cerevisiae* ssp *boulardii* (live yeast) displays potentially beneficial effects upon the piglet gut structure in a period, that of weaning, which is extremely critical because of the abundance of environmental stressors. It is well known that when stressors are abundant, morbidity rates increase and growth performances decrease, both of which are in turn sustained by the not fully mature immune system of piglets at weaning (Pluske et al., 1997; Brown et al., 2006).

The aim of the present study was to investigate the effects of dietary supplementation with the probiotic *Pediococcus acidilactici* on morpho-functional aspects of the piglet intestine, on circulating lymphocytes, and on aspects of piglet performance during the first 42 days after weaning. This lactic acid bacterium is known to produce the Pediocin PA-1 bacteriocin (Schved et al., 1993; Beaulieu et al., 2006), and a potential role towards a prophylactic activity in the pig farm is conceivable.

Material and methods

Animals

The study was conducted in a conventional farm, in which a liquid feed administration was used. At the beginning of the trial 16 (Landrace x Large White) x Duroc female weaning piglets, whose average weight was 7.0±0.2 kg and age 25±2 days, were allocated for 42 days to compare the effects of either a control diet (Ctr, n=8) or the same diet supplemented with *Pediococcus acidilactici* (Pa, n=8) (see below). Animals were equally distributed in the two groups on the basis of the individual weights and of variance between groups. The piglets were allowed an *ad libitum* access to water, and the feed was supplied as a meal mixed with water to provide a dry matter concentration of 255 g/kg^{-1} (water:dry feed ratio was 2.5:1, w/w). All animals were treated in accordance with the European Community guidelines approved by the Italian Ministry of Health.

Diet

All piglets received a starter liquid diet that was either control (Ctr, no added lactobacilli) or (Pa) containing added lactobacilli, 1g/kg dry feed ($2x10^9$ CFU/g of dry feed). *Pediococcus acidilactici* was a single live strain (MA18/5M) (Bactocell, Lallemand, France). Feed was automatically dispensed to the piglets 3 times per day by a liquid feed delivery system. Diets were fortified to meet or exceed nutrient requirements (NRC 1998) for all nutrients (Table 1). Antibiotics as growth-promoting agents were absent. Individual live weights, feed intake, and feed efficiency (at 0, 14, and 42 d post-weaning) were recorded for the study.

Micro-anatomical analyses of the gut

Histology and histometry

At the end of the trial (42 days), the 16 piglets were slaughtered, and small fragments of both the ileum and cecum were collected from each animal immediately after the sacrifice. The samples (total n=32) were immediately fixed in 4% para-formaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Serial microtome sections (4 µm-thick) were obtained from each sample and stained as follows.

The sequential Haematoxylin/eosin (HE) stain was performed for the evaluation of the structural aspects of both ileum and cecum, and for histometry.

For histometry, on HE-stained ileum sections the

height of intestinal villi (V) (10 villi measured per section), the depth of intestinal crypts (C) (10 crypts measured per section), and the ratio of villi and crypts values (V:C ratio) were measured and calculated. In addition, the GALT (gut-associated lymphatic tissue) was examined with attention paid to both the lymphatic area of individual lymphatic follicles (LF) which composed the Peyer's patches (PP), and their different components. For this latter purpose, the lymphatic follicles were divided into their defined compartments (the cortical region, the germinal centre, the dome region) according to Makala et al. (2000). Such compartment areas were measured (μm^2) in five LFs in randomly selected fields of each tissue sample.

On HE-stained cecum sections, the depth of intestinal crypts (C) (10 crypts measured per section) was measured.

Histochemistry and histometry

The ileum and cecum mucin profile was determined by staining ileum and cecum sections with: (a) the Alcian blue 8GX pH 2.5-periodic acid Schiff (AB-PAS) sequence, which reveals neutral (PAS-reactive, purple stained) and acid (AB-reactive, azure stained) glycoconjugates, and (b) the high iron diamine-Alcian blue 8GX pH 2.5 (HID-AB) sequence, which demonstrates sulphated (diamine-positive, brown-black stained) and sialylated (AB-reactive, azure stained) glycoconjugates respectively.

These histochemical reactions selectively evidence both the intestinal mucous cells and the adherent mucous gel, which is synthesized by the mucous cells and lies upon the mucosal epithelial layer. The thickness of the adherent mucous gel, determined as the distance from the outermost layer of adherent mucous gel itself to the luminal surface of the mucosal epithelial layer, was measured at 10 randomly selected points in each AB-PAS stained-section from both ileum and cecum, according to Matsuo et al. (1997).

Immunohistochemistry and histometry

Sections from both ileum and cecum were processed as follows.

a) Some sections were processed to reveal mucosal cells, which were in S-phase of the cell cycle by immunostaining (peroxidase anti-peroxidase method, PAP) with monoclonal antibodies against proliferating cell nuclear antigen (PCNA; clone PC10, Sigma, Italy) (dilution, 1:3000), as described in detail elsewhere (Domeneghini et al., 2004; DiGiancamillo et al., 2005, 2007; Bontempo et al., 2006). The PCNA-immuno-reactive nuclei will for brevity be designed as belonging to "proliferating" cells.

b) Other sections were processed to identify mucosal cells, which were in apoptosis. Apoptotic nuclei were identified using a modified TdT-mediated dUTP nick end labeling technique (DeadEndTM Colorimetric TUNEL System, Promega, USA) and identified with streptavidin labeled with horseradish peroxidase as previously described in detail (Domeneghini et al., 2004; Di Giancamillo et al., 2005, 2007; Bontempo et al., 2006). The TUNEL-reactive nuclei will for brevity be designed as belonging to "apoptotic" cells.

Apoptotic cell number (A) as well as proliferating cell number (M) were evaluated by counting the nuclei of enterocytes (in 10 villi and 10 crypts for the ileum; in 10 crypts for the cecum) which were either or TUNEL-reactive or PCNA-immunoreactive.

In addition, the numbers of apoptotic and proliferating lymphocytes in the germinal center of three lymphatic follicles were evaluated in the ileum, according Burrin et al. (2000). For each section, the lymphocytes whose nuclei were either TUNEL-reactive or PCNA-immuno-reactive were counted in 10 fields (at x400 each field represented a tissue section area of about 0.036 mm^2) (Sozmen et al., 1996).

c) Other sections of both ileum and cecum were immuno-histochemically (PAP) processed to identify mucosal macrophages using monoclonal anti-human macrophage antibodies (clone LN-5, Sigma) diluted 1:400 in TBS (Tris-buffered saline).

Macrophagic index was evaluated in the diffuse lymphatic tissue, which was present in the intestinal lamina propria. For each section, cells were counted in 10 fields (each field representing a tissue section area of

Table 1. Composition of piglet diet, as-fed basis.

Ingredients	%
Maize	35
Barley	19.3
Whey powder, spray-dry	16.7
Soybean meal (48% CP)	13
Fish meal, menhaden	6.2
Wheat middlings	5.2
Maize oil	2
Dicalcium phosphate	1
Limestone, round	0.7
Zinc oxide	0.3
Vitamin–mineral premix[a]	0.27
Salt	0.1
Copper sulphate	0.08
l-Lysine HCl	0.08
dl-Methionine	0.06
Tryptophan	0.01
Pediococcus acidilactici[b]	±
Calculated composition	
ME (metabolic energy) (kcal/kg)	3250
CP (crude protein)	20.5
Lysine (g/kg)	1.25
Ca (g/kg)	0.9
P (g/kg)	0.75

[a]: Supplying a minimum per kilogram complete diet of: 13,500 IU Vitamin A; 1200 IU Vitamin D; 135 IU Vitamin E; 92 µg Vitamin B_{12}; 9 mg riboflavin; 48 mg pantothenic acid; 37 mg niacin; 4.2 mg folic acid; 0.26 mg biotin; 145 mg Fe; 150 mg Zn; 16 mg Cu; 32 mg Mn; 0.60 mg I; and 0.28 mg Se. [b]: *Pediococcus acidilactici*, (Bactocell, MA18/5M; Lallemand, France) was added to treated diets at 1 g/kg of feed at the expense of maize (calculated as $2x10^9$ CFU/g of feed).

about 0.036 mm^2 at X400) (Sozmen et al., 1996).
d) Other sections of both ileum and cecum were processed for the visualization of the IgA producing mucosal cells, by immunostaining (PAP) with policlonal antibodies against IgA (Dakocytomation, Italy). These sections were treated with 0.05% pronase before incubating them with the primary antiserum diluted 1:200 overnight.

IgA-forming plasma cells index was evaluated counting immuno-reactive cells in the intestinal lamina propria. For each section, cells were counted in 10 fields (each field representing a tissue section area of about 0.036 mm^2 at X400) (Sozmen et al., 1996).

e) Other sections of both ileum and cecum were processed for the visualization of the intra-epithelial CD8+ T lymphocytes (IETL-CD8+), by immunostaining (PAP) with a monoclonal primary antibody to CD8 (76-2-11, VMRD, Pullman, WA, USA). These sections were treated with 0.05% pronase before incubating them with the primary antiserum diluted 1:200 overnight.

Intra-epithelial CD8+ T lymphocytes index was evaluated counting immuno-reactive cells in the intestinal epithelium. For each section, immuno-reactive cells were counted in 10 villi and crypts.

Controls

We tested the specificity of immuno-staining (anti-PCNA, anti-macrophage, anti-IgA, anti-CD8 immuno-histochemical reactions) by incubating other ileum and cecum sections with normal mouse (anti-PCNA, anti-macrophage, anti CD8 immuno-histochemistry) or rabbit (anti-IgA immuno-histochemistry) serum (Dakocytomation) instead of the primary antibodies, which always gave negative results. As positive controls, alimentary canal samples from calf and dog were tested: in all cases the expected positive reactions were observed.

All the observations were conducted by a blind observer utilizing an Olympus BX51 microscope equipped with a digital camera and DP software (Olympus, Italy) for computer-assisted image acquirement and managing.

Isolation and staining of circulating lymphocytes

The 16 piglets were bled at 0, 14 and 42 days of the trial. Blood samples were collected at jugular veins. The fresh heparinised blood samples which were used for determination of lymphocyte subsets were rotated at room temperature until immuno-staining, which was performed after collection. Contaminating erythrocytes were lysed with 0.155 M NH_4Cl pH 7.2, followed by centrifugation at 1500 r.p.m. for 10 min at 4°C. The leukocyte pellets were washed twice with PBS before re-suspension in PBS with 5 % foetal calf serum. The leukocyte pellets were treated during a 30min incubation with commercially available monoclonal primary antibodies to CD3 (8E6, VMRD, Pullman, WA, USA) and CD8 (76-2-11, VMRD). The cells were then washed and incubated with phycoerythrin (PE)-conjugated (Becton Dickinson, Franklin Lakes, NJ, USA) and FITC-conjugated (Caltag Laboratory Products, Italy) secondary antibodies. After incubation, the cells were washed, re-suspended and finally fixed with 1% para-formaldehyde in PBS until flow cytometry analysis.

Flow cytometry analysis

Stained cells were washed twice with PBS and quantified in a FACSCalibur flow cytometer (Becton Dickinson) by the collection of forward and orthogonal light scatter and FITC and PE fluorescence. In each sample, 30000 cells were recorded. Lymphocytes were gated by size and granularity with forward and orthogonal light scatter and further analysed for FITC and/or PE staining. The results were evaluated with region analysis using the Cellquest software (Becton Dickinson). The numbers of CD3+ and CD8+ lymphocytes were evaluated and expressed as percentages of CD3+, and CD3- CD8+ T lymphocytes in the three different periods.

Statistical analyses

Statistical analysis of the quantitative data was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC, 2000).

Data from variables measured over time (growth parameters and flow cytometry) were analysed for time, treatment and time-treatment interactions using the REPEATED statement within the general linear models procedure of SAS.

Histometrical analyses (histology, histochemistry and immunohistochemistry) were analyzed by ANOVA using the PROC MIXED of the SAS package. The mixed model included the fixed effects of treatment and the random effect of the piglet.

The individual piglet values were considered to be the experimental unit of all response variables. The data were presented as least squared means ± pooled SE. Differences between means were considered significant at $P<0.05$.

Results

Data referring to piglet performances are shown in Table 2. Piglets from Pa group turned out to be heavier than Ctr animals at the end of the trial (P=0.002) (day 42 post-weaning). Treated piglets were heavier than controls at day 15 post weaning also, but the different values were not significant. ADG (average daily gain) was higher in the Pa animals than in controls (P=0,009).

Micro-anatomical analyses of the gut

The histological and structural aspects of the ileum (Fig. 1a) and cecum in *P. acilactici*-supplemented animals was judged to be fully normal. The GALT (gut-associated lymphoid tissue) was prominent in ileum of

both treated and control piglets, in which it was especially developed in the form of Peyer's patches (Fig. 1b). On the contrary, the ileum (Fig. 1c) and cecum (Fig. 1d) sections in the control animals revealed the presence of mucosal inflammatory signs that can be referred to as chronic catarrhal enteritis .

Histology and histometry

Oral feeding with *P. acilactici* resulted in an increase in villi (V) height (P=0.010) and crypt (C) depth (P=0.009) of the ileum, and a consequent decrease in V:C ratio (P=0.63) in comparison with control animals (Table 3). Crypt depth was also greater in cecum of treated piglets than in controls (P=0.050) (Table 3). Gut-associated lymphoid tissue (GALT) was present and similarly organized in both control and treated piglets. Defined region measurements of LF compartments within the Peyer's patches were summarized in Table 3. No differences between the two groups were observed in

Table 2. Least mean square of piglets growth performance (Ctr=controls, Pa=*Pediococcus acidilactici* treatment).

	Ctr	Pa	Pooled SE	P values
At weaning (kg)	7.05	7.15	0.103	0.928
Day 15 post-weaning (kg)	8.77	10.15	0.208	0.107
Day 42 post-weaning (kg)	19.35	22.03	0.397	0.002
Average daily gain (g)	406	425	0.329	0.009

Values are means ± pooled SE, n=16.

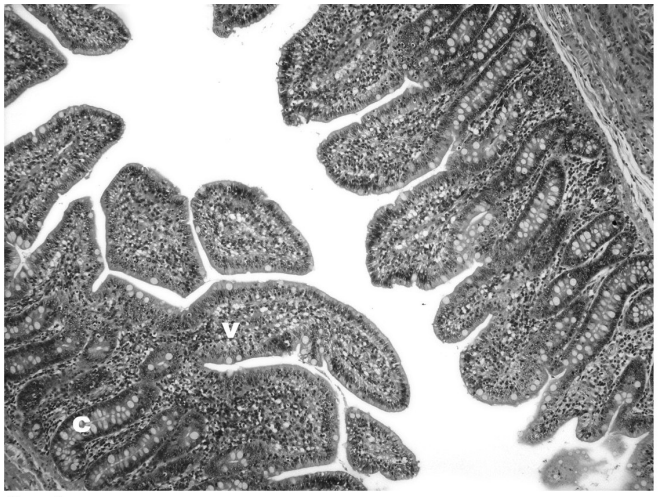

fig. 1a

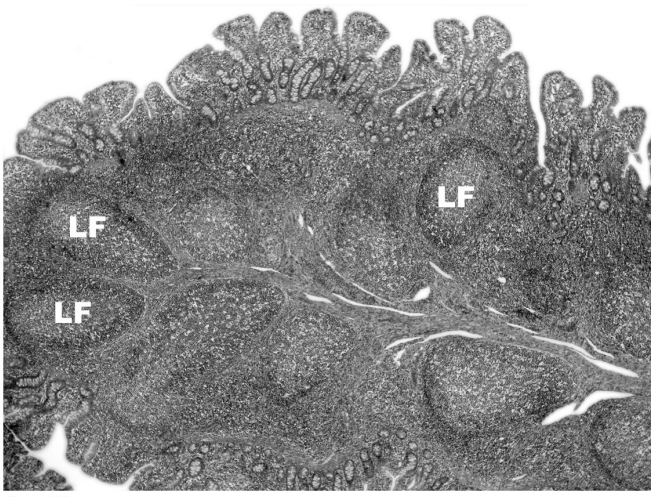

fig. 1b

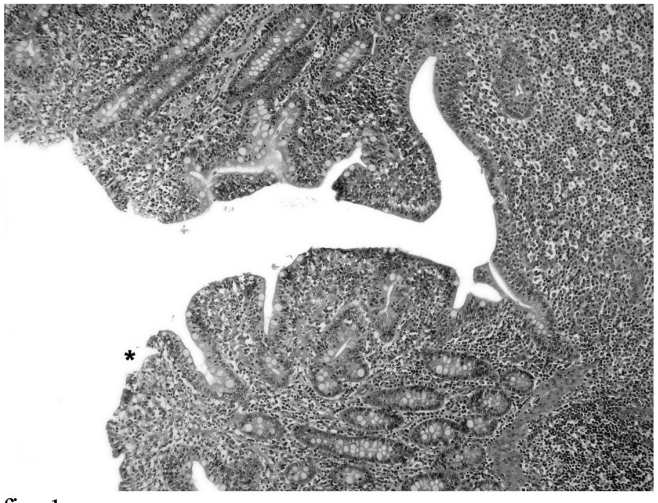

fig. 1c

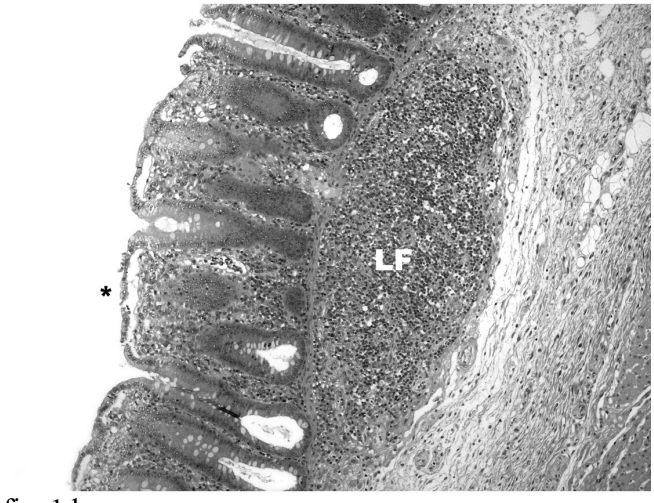

fig. 1d

Fig. 1.a. Ileum of a dietary treated (*Pedicoccus acidilactici*, Pa) piglet, haematoxylin/eosin staining. The small intestine structure is regularly organized in villi (V) and crypts (C). **b.** Ileum of a Pa piglet, haematoxylin/eosin staining. Several lymphatic follicles (LF) are evident in GALT. **c.** Ileum of a control piglet, haematoxylin/eosin staining. Aspects are evident referring to chronic catarrhal enteritis (asterisk). **d.** Cecum of a control piglet, haematoxylin/eosin staining. Aspects are evident referring to chronic catarrhal enteritis (asterisk), LF: lymphatic follicle. x 100

the area of each lymphatic follicle and their defined compartments (cortical region, germinal centre, dome region).

Histochemistry and histometry

The AB/PAS sequential staining showed that intestinal mucous cells contained mixtures of neutral and acidic glycoconjugates in both ileum (Fig. 2a) and cecum (Fig. 2b) of either control or treated piglets. In the ileum of both treated and control piglets, sulphated glycoconjugates containing mucous cells predominated in the villi, and sialo-glycoconjugates containing mucous cells occurred mainly at the bases of crypts (Fig. 2c). In cecum of both control and Pa piglets, sulphated glycoconjugates were only detected in mucous cells (Fig. 2d). The adherent mucous gel after the AB/PAS staining showed a mixed reactivity. It was shown to be thicker in control than in treated piglets, but this difference was significant in the ileum ($P<0.001$), not in the cecum ($P=0.88$) (Table 3).

Immunohistochemistry and histometry

Ileum

The anti-PCNA immuno-reactivity was prominent in ileum sections from both groups. Several immuno-

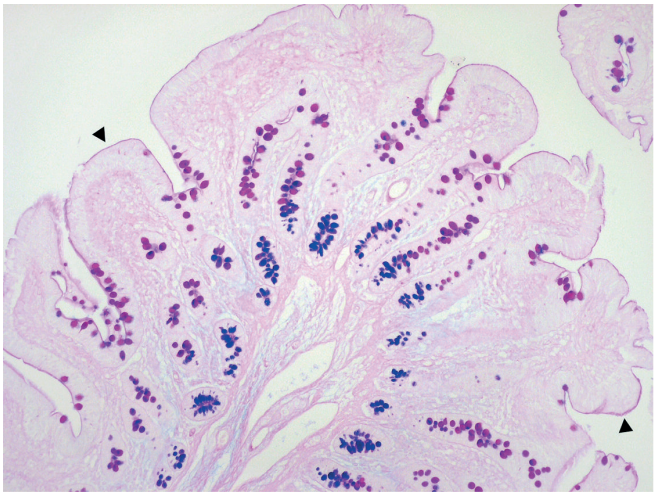

fig. 2a

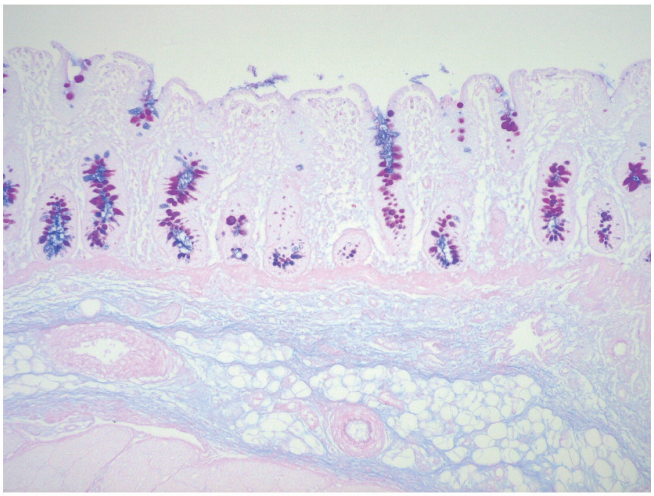

fig. 2b

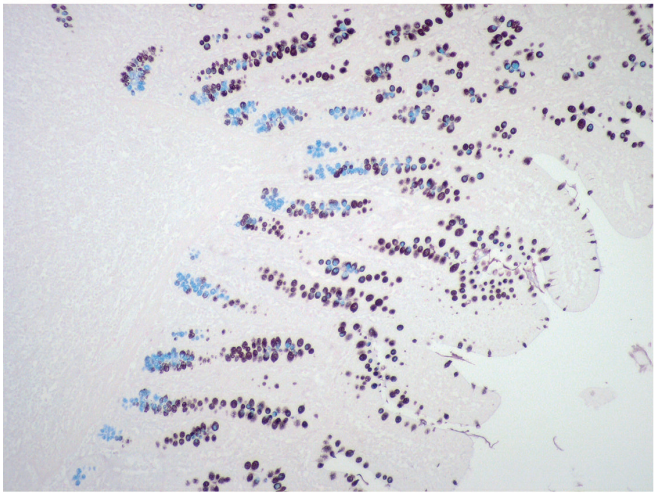

fig. 2c

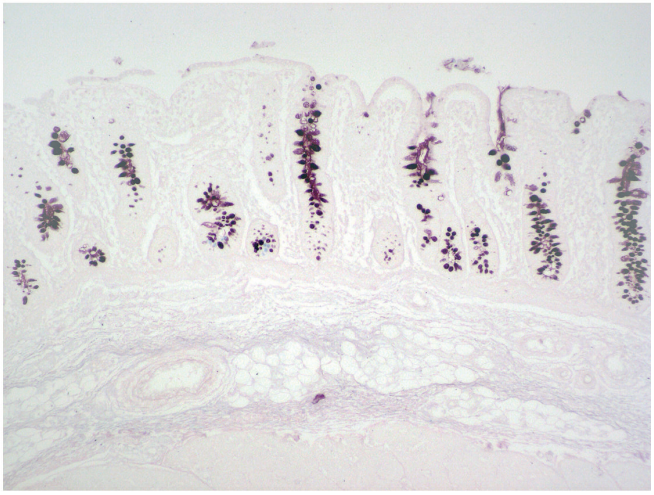

fig. 2d

Fig. 2. a. Ileum of a Pa piglet, AB/PAS staining. Several mucous cells are detected containing either acidic (blue-stained) or neutral (magenta-stained) glycoconjugates. The adherent mucous gel is also evident with a mixed reactivity (arrowheads). **b.** Cecum of a control piglet, AB/PAS staining. Several mucous cells are detected containing either acidic (blue-stained) or neutral (magenta-stained) glycoconjugates. **c.** Ileum of a Pa piglet, HID/AB staining. Several mucous cells are detected containing either sialylated (azure-stained) or sulphated (brown-stained) glycoconjugates. **d.** Cecum of a control piglet, HID/AB staining. The mucous cells contain sulphated glycoconjugates. x 100

stained nuclei were detected in both epithelial cells of intestinal crypts and villi (Fig. 3a), and mucosal cells of GALT (Fig. 3b), of which the latter were prevalently lymphocytes. Nuclei of apoptotic cells were TUNEL-labeled in epithelial localizations (at the apex of intestinal villi), in both Ctr and Pa piglets (Fig. 3c). TUNEL-labeled apoptotic bodies could be detected in nuclei of the GALT cells (presumably macrophages) both in control and treated piglets (Fig. 3d).

Histometrical analyses aimed at a quantitative evaluation of apoptotic (A) and proliferating cells (M), in both epithelial localizations and lymphatic follicles (germinal centre), are summarized in Table 4. The epithelial proliferating cells were more numerous in the ileum crypts of treated piglets than control ones

Table 3. Effect of *Pediococcus acidilactici* (Pa) on histometrical analyses related to histology and histochemistry of piglet ileum and cecum (Ctr=control animals). LF=lymphatic follicle.

	Ctr	Pa	Pooled SE	P values
Villi height (V), ileum, µm	300	327	7.52	0. 010
Crypts depth (C), ileum, µm	247	287	10.31	0.009
V:C ratio	1.25	1.15	0.03	0.063
LF area, μm^2	343450	360350	41533	0.86
LF germinal centre, μm^2	182905	189905	24958	0.70
LF dome region, μm^2	47127	63062	6992	0.11
LF cortical region, μm^2	99839	107383	12819	0.67
Crypts depth, cecum, µm	387.85	423.32	13.07	0.050
Mucous layer, ileum, µm	2.95	2.35	0.07	<0.001
Mucous layer, cecum, µm	1.54	1.50	0.10	0.88

Values are means ± pooled SE, n=16.

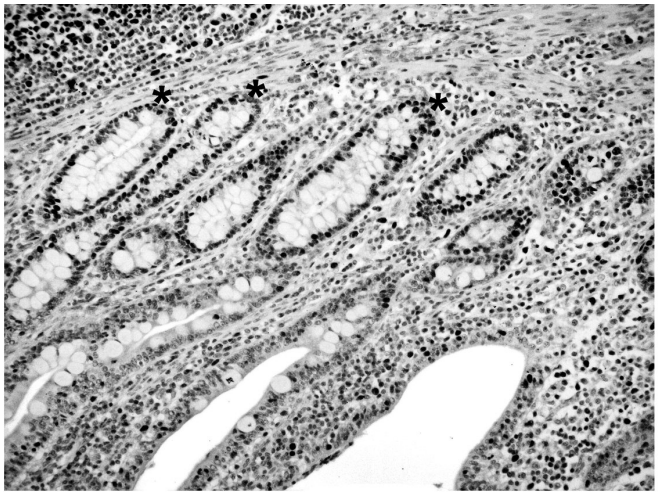

fig. 3a

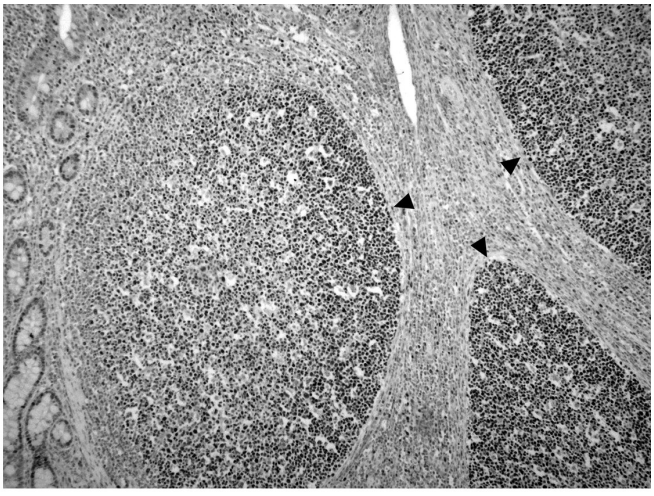

fig. 3b

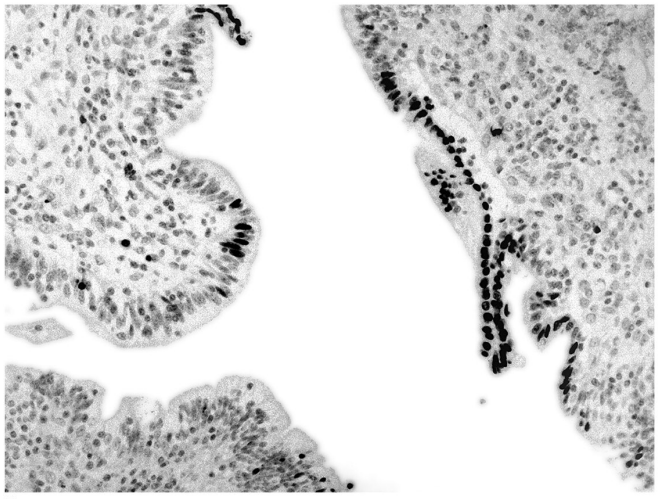

fig. 3c

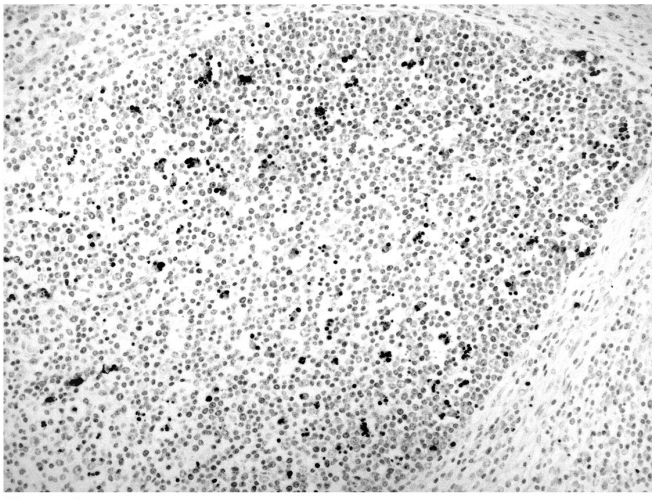

fig. 3d

Fig. 3. a. Ileum of a Pa piglet, anti-PCNA IHC (immuno-histochemistry). Several nuclei are PCNA-immunoreactive in enterocytes of intestinal crypts (asterisks). **b.** Ileum of a control piglet, anti-PCNA IHC. Several nuclei are PCNA-immuno-reactive in lymphatic follicles (arrowheads), which compose GALT. **c.** Ileum of a Pa piglet, TUNEL staining. Some enterocytes at the apex of intestinal villi contain apopotic nuclei. **d.** Ileum of a Pa piglet, TUNEL staining. Some nuclear apopotic bodies are evident in a lymphatic follicle. x 200

(P=0.0026). The proliferating cells were more numerous also in ileum villi and the germinal centre of lymphatic follicles in treated than in control piglets, but these differences were not significant. Differences in apoptotic cell values (in both epithelial and lymphatic localizations) showed a decrease in treated piglets in comparison with controls, but these values were not significant.

Immuno-staining to detect mucosal macrophages picked out numerous round immuno-reactive cells in GALT (Fig. 4a). Counts of mucosal immunoreactive macrophages were not significantly different between Ctr and Pa piglets, even if in the latter a tendency towards a decrease was present. Similarly, a decreased number of the mucosal IgA-immuno-positive mucosal cells (Fig. 4b) was detected in treated in comparison with control piglets, but this difference was not significant (Table 4).

CD8+ T lymphocytes were detected in intra-epithelial localizations in the ileum of both control and treated piglets. Histometry revealed that they were more abundant in control than in treated piglets (Table 4).

Cecum

The PCNA- and TUNEL-immuno-reactivities were evident in cecum sections from both groups. The PCNA-immuno-positive nuclei were detected in enterocytes

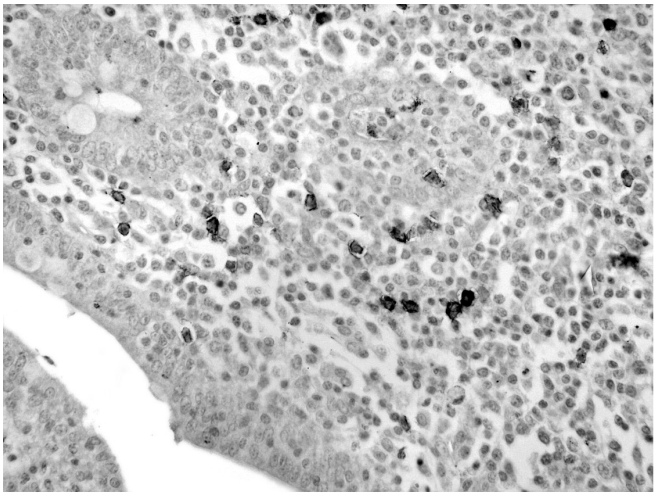

fig. 4a

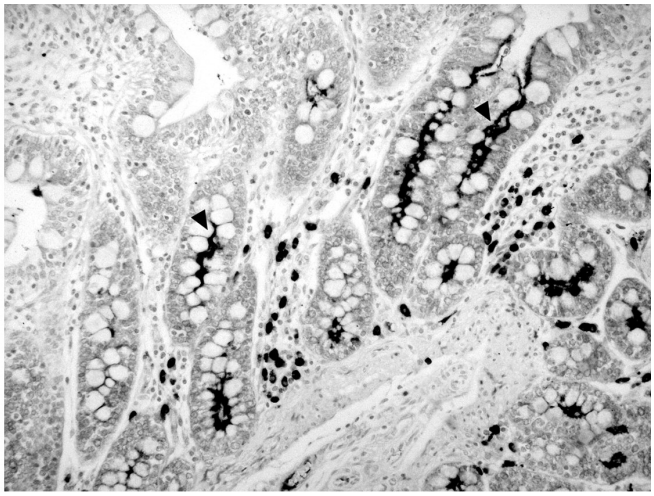

fig. 4b

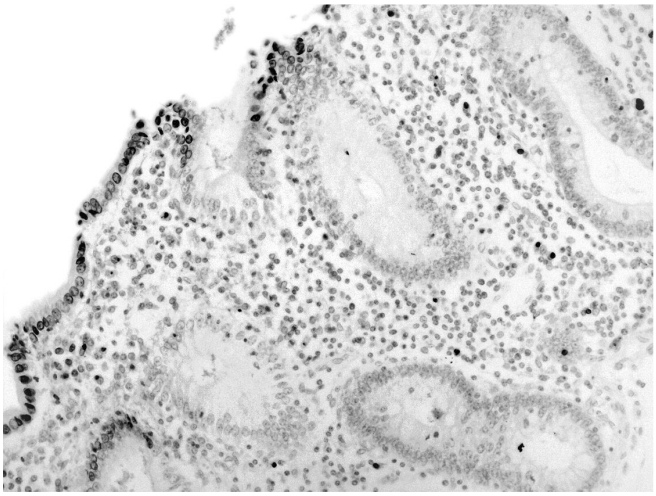

fig. 4c

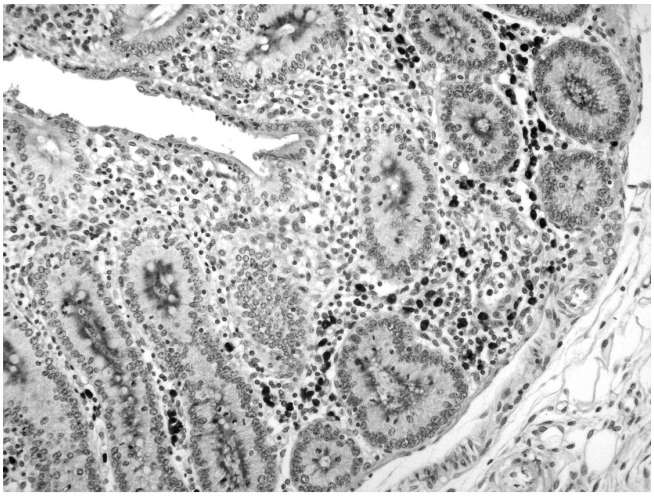

fig. 4d

Fig. 4. a. Ileum of a Pa piglet, anti-macrophage IHC. Macrophages are immuno-positive in the diffuse lymphatic tissue of the intestinal lamina propria. **b.** Ileum of a control piglet, anti-IgA IHC. Ovoidal mucosal IgA-immuno-reactive cells are evident. A secretory immuno-reactive material (arrowheads) is also evident in the lumen of intestinal crypts. **c.** Cecum of a control piglet, TUNEL staining. Some enterocytes in the surface epithelium contain apoptotic nuclei. **d.** Cecum of a control piglet, anti-IgA IHC. Ovoidal mucosal IgA-immunoreactive cells are evident. a, x 400; b-d, x 200

Table 4. Effect of *Pediococcus acidilactici* (Pa) on histometrical analyses related to immunohistochemistry of ileum (Ctr=control animals). LF: lymphatic follicles; IEL: intra-epithelial lymphocytes.

Ileum	Ctr	Pa	Pooled SE	P values
Proliferating cells, numbers				
In crypts (mean cells nr: 70)	56.94	64.82	2.19	0.0026
In villi (mean cells nr: 100)	8.14	12.28	1.45	0.067
In LF germinal centre (area: 0.36 mm2)	1039	1050	88.73	0.928
Apoptotic cells, numbers				
In crypts (mean cells nr: 74)	0.53	0.39	0.29	0.87
In villi (mean cells nr: 100)	26.00	23.40	6.30	0.77
In LF germinal centre (area: 0.36 mm2)	34.26	31.00	1.71	0.19
Macrophages, numbers (area: 0.36 mm2)	5.05	4.34	0.64	0.43
IgA+ forming cells, numbers (area: 0.36 mm2)	25.68	17.96	6.25	0.42
CD8+ IEL (area: 0.36 mm2)	462	332	33.03	0.013

Values are means ± pooled SE, n=16.

Table 5. Effect of *Pediococcus acidilactici* (Pa) on histometrical analyses related to immuno-histochemistry of cecum. Ctr: control animals; IEL: intra-epithelial lymphocytes.

Caecum	Ctr	Pa	Pooled SE	P values
Proliferating cells, numbers				
In crypts (mean cell nr: 92)	89.36	93.94	0.41	0.319
Apoptotic cells, numbers				
In surface epithelium (means cell nr: 62)	1.71	1.25	0.36	0.24
Macrophages, numbers (area: 0.36 mm2)	6.87	5.50	1.97	0.62
IgA+ forming cells, numbers (area: 0.36 mm2)	67.81	50.17	8.37	0.14
CD8+ IEL (area: 0.36 mm2)	193	163	8.03	0.099

Values are means ± pooled SE, n=16.

localized at the basis of intestinal crypts, and TUNEL-reactive nuclei were observed in enterocytes localized in the surface epithelium (Fig. 4c). Histometry revealed that there were no significant differences between groups of treatment for apoptosis and mitosis within cecum epithelial cells (Table 5).

Immunostaining to detect cecum mucosal macrophages and IgA-positive mucosal cells (Fig. 4d) found no significant differences between groups.

CD8+ T lymphocytes were detected in intra-epithelial localizations in the cecum of both control and treated piglets. Histometry revealed there was a tendency towards a diminution in treated vs control piglets (Table 5).

Table 6. T lymphocyte subsets in peripheral blood in control (Ctr) and *Pediococcus acidilactici* (Pa) treated piglets at 0, 15, and 42 day after-weaning.

T cells subsets (%)	Ctr	Pa	Pooled SE	P values
T lymphocytes (CD3+)				
At weaning	47.33	49.49	10.05	0.365
Day 15 post-weaning	46.83	47.34	9.34	0.994
Day 42 post-weaning	50.86	51.03	10.50	0.862
NK–cells (CD3- CD8+)				
At weaning	7.88	8.04	1.55	0.240
Day 15 post-weaning	7.80	8.05	1.43	0.530
Day 42 post-weaning	11.76	11.53	3.21	0.236

Values are means ± pooled SE, n=16.

Flow cytometry analyses

Data referring to the T lymphocyte subsets distribution in peripheral blood are shown in Table 6. CD3+ lymphocytes were not present in different values between control and treated piglets at either weaning, or day 15, or day 42 post-weaning. Similarly, CD3-, CD8+ T lymphocytes were not detected in different quantities between control and treated piglets at either weaning, or day 15, or day 42 post-weaning. No differences were thus detected in the studied lymphocyte subsets referring

to either treatment or time-treatment.

Discussion

During post-weaning, piglets fed the diet integrated with *Pediococcus acidilactici* had a final greater weight and showed a greater average daily gain than control piglets. Similar improvements in piglet average daily gain as a result of lactobacilli supplementation have been reported by some studies (Abe et al., 1995; Shim et al., 2005; Taras et al., 2005). However, it should be underlined that the effects of a probiotic dietary administration in the pig farm are extremely variable, depending on individual age and sanitary conditions, composition of diet, and quantity and type of micro biota added to the diet. In addition, good growing performances in litters are not the primary effects to be expected when testing a probiotic (Zani et al., 1998; Alexopoulos et al., 2004; Bohmer et al., 2006).

Nutritional stress may contribute to the poor growth rate of piglets after weaning (Pluske et al., 1997), either directly by a diminished feed intake, or by inducing the villous atrophy and local inflammatory responses, which have been observed in the small intestine (Van Beers-Schreurs et al., 1998; Mc Cracken et al., 1999; Vente-Spreeuwenberg et al., 2003). These important detrimental changes in the piglet intestinal structure lead to post-weaning impaired digestion and absorption and marked morbidity rates (Pluske et al., 1997; Burrin et al., 2000).

Histometric analyses performed in this study showed that ileum villi height and crypts depth values were greater (P=0,010 and P=0.009 respectively) in treated piglets compared to controls. The same histometric analyses conducted in cecum showed that crypt depth values were greater (P=0.050) in treated piglets than in controls. The longer the villi and crypts are, the more enterocytes are present in them, which function in digesting and absorbing food molecules (Hampson, 1986). Baum et al. (2002) and Bontempo et al. (2006) also found that villi length was greater in the small intestine of piglets fed probiotic yeast (*Saccharomyces cerevisiae* ssp boulardii) than controls, and these results are similar to the ones found in this study.

We have in addition histochemically examined the mucin content of intestinal mucous cells and the thickness of the intestinal adherent mucous gel. Mucus acts not only as a physico-chemical barrier, but also as a lubricant and a stabilizer of the intestinal microclimate, an important interface between the lumen contents and the intestinal epithelium, as well as, when digested, as a source of energy for the resident microflora (Deplancke and Gaskins, 2001). In healthy animals, the adherent mucous gel produced by mucous cells prevents pathogens in the gut lumen from invading the mucosa (Neutra and Forstner, 1987; Laukova et al., 2004; Sartor, 2005). Mucous secretion is stimulated in laboratory mammals by bacterial infections (Cohen et al., 1983; Mantle et al., 1989), bacterial toxins (Roomi et al., 1984) and parasite infestation (Miller et al., 1981).

In the present study, the AB-PAS staining to reveal neutral and acid glycoconjugates showed that the mucous layer was thicker in control piglets than in treated animals, and that this difference was significant in the ileum (P<0.001). Also, when piglets were dietary administered with yeast, the adherent mucous gel was thicker in control than in treated animals (Bontempo et al., 2006; Di Giancamillo et al., 2007). We suggest that this may indicate an increased presence of potential pathogens in the gut lumen of control animals in comparison with *P. acidilactici*-supplemented animals. The control animals of this study showed the constant occurrence of catarrhal enteritis in the small and large intestine. It is well known that probiotics are capable of synthesizing antimicrobial substances (Talarico et al., 1988; Menard et al., 2005), and so our hypothesis about a protecting effect of probiotics towards pathogen invasion is fully plausible. A correlation between a thick adherent mucous gel and a possible increased presence of intestinal potential pathogens has been drawn in post-weaning piglets by Tang et al. (1999), too.

The thicker mucus layer in control animals in comparison with treated piglets may limit the diffusion of nutrients to the apical surface of epithelial cells, thereby reducing absorption. Thus, the thinner mucus layer in *P. acilactici*-treated piglets may have contributed to the better-feed efficiency here presented, as also suggested by Patience et al. (1997) in a previous study.

We have in addition found that proliferating enterocyte counts were significantly higher in the ileum crypts of treated piglets than in the control group (P=0.0026). Ichikawa et al. (1999) have reported findings, which were similar to the ones here presented, in rats fed *Lactobacillus casei* and *Clostridium butyricum* as probiotic preparations, whereas Baum et al. (2002) found no differences in the number of proliferating cells between control weaned piglets and those treated with either yeast (*S. boulardii*) or *Bacillus cereus* var. Toyoi. We recently found that the small intestine enterocytes with PCNA-immunoreactive nuclei were more numerous in piglets dietary administered with *Saccharomyces cerevisiae* ssp boulardii than in control piglets (Bontempo et al., 2006). We suggest that the higher proliferation rate of the intestinal epithelial cells in the ileum crypts of treated piglets in comparison with controls may be due to the enhanced endoluminal release of polyamines linked to the presence of probiotic bacteria (Buts et al., 1994; Costalos et al., 2003). Polyamines are necessary for cell growth and differentiation, and the demand for them is presumably high in rapidly proliferating tissues with a high rate of cell turnover, such as the intestinal mucosa of a young animal, instances in which, in fact, their intracellular concentrations increase (Tabor and Tabor, 1984; Alarcon et al., 1987; Costalos et al., 2003).

Probiotics have been shown to influence selected aspects of immune functions, particularly those ones locally displayed (Erickson and Hubbard, 2000).

Although several *in vitro* and *in vivo* studies on probiotic effects on immunity have been reported, the specific mechanisms of the observed changes in defensive cells remain unclear (Bai et al., 2004; Menard et al., 2005; Rastall et al., 2005; Duncker et al., 2006; Galdeano and Perdigon, 2006). Moreover, many probiotic preparations have been tested in several separate laboratories with diverse and sometimes contradictory results. One of the most common methods used for assessment of immune function is the evaluation of lymphocyte proliferation, the principal actor in intestinal local immunity, whose anatomic counterpart was the GALT (gut-associated lymphoid tissue). In this study we have evaluated lymphocyte proliferation in lymphatic follicles which constitute the ileum Payer's patches, utilizing the quantification of PCNA-immunoreactive nuclei: we have not observed differences referring to the treatment. Similarly, no difference was observed between the two groups of treatment referring to the histometry of measured defined regions of lymphatic follicles. Likely, *P. acidilactici* did not stimulate the local immunity effectors components, at least in this species and at the studied age. In agreement, a recent paper by Duncker et al. (2006) shows that the number and distribution of intestinal immune cells were not influenced in young pigs by a dietary probiotic.

A further fundamental defensive activity of the intestinal mucosal surfaces is the production of secretory IgA by specific mucosal B lymphocytes and plasma cells. These antibodies act in both an inflammatory and non-inflammatory intestinal context, neutralizing potential pathogens inhabiting the intestinal lumen, thus contributing to the building of the mucosal barrier (Corthesy, 2007). Similarly to mucosal macrophages, IgA-forming mucosal cells showed in this trial a tendency to be more numerous in control (in both ileum and cecum) than in treated piglets ($P>0.05$), but the observed different values were not significant. In conventional mice also the number of IgA forming mucosal cells were not influenced by dietary probiotics (*Lactobacillus* spp) (Dogi and Perdigon, 2006). Human small intestinal mucosa was, on the contrary, affected by dietary lactic acid bacteria, which induced a greater number of IgA-positive mucosal cells in comparison with controls (Vitini et al., 2000).

The intestinal mucosal barrier is in addition composed of CD8+ intraepithelial T lymphocytes, which are specifically aimed at eliminating epithelial cells which have been invaded by pathogens, at least in rodents and humans (Jabri and Ebert, 2007). In these species they can be triggered by non-classical epithelial antigen-presenting cells, and can thus mediate mucosal immunity and intestinal immunopathology (Spiller et al., 2000). We found in this trial that this subset of intraepithelial T lymphocytes were more abundant in the ileum of control than treated piglets ($P=0.013$). Also, in the cecum of control animals the number of CD8+ intraepithelial T lymphocytes was larger than in treated piglets, even if the observed difference was not significant. These observations are likely to be in relation with the presence of catarrhal enteritis in controls, in both ileum and cecum. It is conceivable that the intestinal mucosal barrier of *P. acidilactici* treated piglets may be more efficient in contrasting the adhesion of bacterial pathogens than control piglets, at least in ileum. Our observation appears in ideal agreement with the results by Piva et al. (1995), who showed in an in vitro trial that Pediocin A did not display influences upon pig cecal micro flora. It is conceivable that *P. acidilactici* exerts in piglets its protective effects with regards to competition with potential pathogens, above all in the ileum (and only to a limited extent in cecum), possibly via the intervention of Toll-like receptors (TLRs), which are essential for the control of intestinal host-microbial interactions and protection from the adhesion of microbial pathogens (Rakoff-Nahoum et al., 2004).

Taking into account that the local defensive elements are profoundly linked to general ones (via the intestinal lymphatic vessels), we have finally approached the quantitative analysis of the CD3+ T lymphocytes as well as CD3-, CD8+ T lymphocytes (possibly NK cells) in the blood of both treated and control piglets. These subsets of circulating lymphocytes are in some instances influenced by stressful conditions (Spreeuwenberg et al., 2001; Bao et al., 2006; Borghetti et al., 2006). The quantitative values we have found in this trial are quite similar to those identified in piglets by Terzic et al. (2002). In this part of the trial the results were similar between the two treatment groups, so that we can hypothesize that the piglet general immunity processes have not been influenced by the studied dietary treatment, at least with regards to innate immunity processes.

However, owing to the assessed capabilities of probiotics to compete with possible pathogens in the adhesion to the intestinal mucosa, we can with reason hypothesize that *P. acidilactici* prevented, in this trial, by more than one mechanism of action, the enteric pathologies observed in the control animals, and this without modifying with detrimental or pathological aspects the structure of the intestinal mucosa. This might be of a potentially prophylactic significance, due to the assessed frequent occurrence of ileitis in pig farms (Kyriakis et al., 2002; Chouet et al., 2003), and might be helpful with the aim of designing a diet potentially able to limit the gut disorders so frequently occurring in pig farms around weaning.

In conclusion, when dietary administered to piglets, *Pediococcus acidilactici* has been shown to positively influence the length of small intestine villi and crypths, as well as the length of cecum crypts, in this way probably assuring an early restoration of the intestinal mucosal thinning that often occurs at weaning, as well as influencing the better growing performances of the treated piglets. In addition, they probably have been able to contrast the invasion of micro biota responsible for the aspects of catarrhal enteritis observed in both the

ileum and cecum of not-treated piglets.

Based on the present and other studies from both our group and other authors, we can confirm that probiotic bacteria are likely able to prevalently act at a local level on the intestinal mucosa, possibly in different manners in different species. It is important, consequently, to know what structural aspects are modified in a certain species by their presence in the gut lumen, and what are the putative mechanisms for those modifications, if present. This in turn enables the researcher to determine the optimal use of probiotics for either prophylaxis, or treatment of a food animal species, like the pig, and to possibly improve the body of knowledge of the use of probiotics for humans.

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