



ORIGINAL ARTICLE

Influence of dietary supplementation with flaxseed and *Lactobacilli* on the mucosal morphology and proliferative cell rate in the jejunal mucosa of piglets after weaning

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SUMMARY

The aim of the study was to investigate the influence of flaxseed and *Lactobacilli* supplementation to the diet of piglets during the time period between 10 days before and 21 days after weaning. The morphometry of the jejunal mucosa and proliferative ratio of both epithelial and lamina propria cells were compared with those found in a group of piglets fed with the usual diet added with sunflower oil during the same time period. The addition of flaxseed oil to the diet significantly increased the crypt depth in comparison with both groups supplemented with sunflower ($P < 0.05$ and 0.001 respectively) on the weaning day. Moreover, the flaxseed addition caused a significant decrease in villus height ($P < 0.01$) and crypt depth ($P < 0.01$) 21 days postweaning in comparison with the sunflower group. The proliferative ratio of the epithelial cells in the sunflower group on the weaning day was significantly higher than in both flaxseed groups ($P < 0.01$). Paradoxically, significantly higher proliferative activity in the mucosal connective tissue in the group with flaxseed supplementation in comparison with the sunflower group was observed on the day of weaning, as well as 3 days later ($P < 0.05$ both). A combination of flaxseed with *Lactobacilli* showed significantly lower proliferative activity in the connective tissue cells from weaning up to 7 days after weaning ($P < 0.05$ all) in comparison with the flaxseed group.

Keywords

cell proliferation, crypt depth, flaxseed, *Lactobacilli*, mucosal thickness, villus height

At weaning piglets undergo many stressful circumstances associated with change of their environment, separation from the mothers and general replacement of fluid milk nutrition by more abrasive solid food with different composition and nutrient value. As a consequence, weaning is very often accompanied by disorders in feed intake and increased susceptibility to intestinal dysfunction, including reduction of digestive and absorptive capacities (Pluske *et al.* 1997) and breakdown of intestinal barriers that, in turn, leads to increased permeability and mucosal inflammation (Moeser *et al.* 2007; Smith *et al.* 2010). The intestinal and immune system alterations result in reduced health and frequent

diarrhoea. To lower the economic burden of such intestinal dysfunctions, the antibiotic growth promoters were included in the diet of the weaning piglets a few years ago (Aarestrup 2000). Their effect depends on reduction of the bacterial growth in the intestinal tract leading to protection of nutrients against bacterial metabolism, decrease of toxin production by intestinal bacteria and reduction in the incidence of subclinical intestinal infections (Butaye *et al.* 2003). However, the present European legislation prohibits antibiotic growth promoter incorporation into animal diets, endeavouring to reduce antibiotic resistance phenomena in humans. Thus, the European breeders need to replace them using

different biological active substances, for example probiotics, prebiotics, non-specific substrates, plant extracts, organic acids or polyunsaturated fatty acids (PUFA; Bomba *et al.* 2006).

Weaning is also associated with marked changes in the morphology and biochemistry of the small intestine, such as villous atrophy and crypt hyperplasia, which cause decreased digestive and absorptive capacity while contributing to postweaning (PW) diarrhoea. It has been also shown that in this condition, villous atrophy is caused by either an increased rate of cell loss or a reduced rate of cell renewal (Pluske *et al.* 1997; Adamkov *et al.* 2014).

The immunomodulatory and anti-inflammatory properties of ω -3 PUFA suggest their possible use as a substitute for antibiotics. A combination of PUFA with probiotic has proved of benefit in the metabolism of fatty acids and synthesis of prostaglandins (Kastel *et al.* 2007), while favouring *Lactobacilli* adhesion to the intestinal mucosa, and inhibiting *Escherichia coli* K88 infection in germ-free piglets (Nemcova *et al.* 2012). Moreover, the influence of dietary supplementation with flaxseed and *Lactobacilli* on the innate immune cells in the jejunal mucosa of piglets PW has been described in a previous study from our group (Toth *et al.* 2015).

The objective of this study was to investigate the effects of active feed additives on the intestinal mucosa of weaned pigs using probiotics (*Lactobacilli*) and flaxseed oil (source of ω -3 PUFA), in terms of changes of both morphology and proliferative rate of epithelial and lamina propria cell populations. These changes were evaluated at different time points from the day of weaning to 21 days after weaning. The results were compared to the groups of piglets receiving sunflower oil as source of ω -6 PUFA.

Materials and methods

Probiotic bacteria

The *Lactobacillus* probiotic strains were isolated at the University of Veterinary Medicine and Pharmacy, Košice, Slovakia: namely the *Lactobacillus plantarum* – Biocenol™ LP96 (CCM 7512) – strain was selected from the gut contents of healthy suckling piglets and *Lactobacillus fermentum* – Biocenol™ LF99 (CCM 7514) – was isolated from the gastrointestinal tract of adult chickens. Cheddar cheese was applied as vehicle for probiotic strains at 10^9 CFU/g of cheese. The cheese without probiotic strains was used as control.

Animals, housing and diets

The study was carried out on 88 healthy piglets (cross-breds – Yorkshire \times Pietrain). The piglets were housed in typical indoor pens and divided into four groups: control group S (control cheese + sunflower oil) 19 piglets; group SL (probiotic cheeses + sunflower oil) 23 piglets; group F (control cheese + whole crushed flaxseed) 23 piglets; and group FL (probiotic cheeses + whole crushed flaxseed) 23 piglets.

The animals were fed with a meal formula of basal diet (Table 1) supplemented with whole crushed flaxseed cultivar Flanders (Agrola, Kožušice, Czech Republic) for F and FL groups and control sunflower oil for S and SL groups both at a concentration of 10% (Table 2).

The piglets were weaned at the age of 28 days. During the period of time between 10 days before and 21 days PW, the piglets in the groups SL and FL were supplied with probiotic cheeses (4 g/animal/day for each cheese, in total 8 g), while those in the groups S and F were supplied with control cheese (8 g/animal/day). The probiotic and control cheese were supplied to piglets once a day (individually in the morning) in the form of a grated cheese deposited on the surface of the meal. The animals felt the cheese and consumed it instantaneously. During the same period, the animals had *ad libitum* access to water and feed supplemented with whole crushed flaxseed or sunflower oil.

Table 1 Ingredient (%) and chemical composition (g/kg DM) of the basal diet

Ingredient %	
Wheat	27.60
Soya bean, extr.	22.00
Maize (8.4% CP)	19.70
Barley	17.00
Oat (11.2% CP)	5.00
Powdered whey	5.00
Calcium carbonate	1.10
Mono-calcium phosphate	1.00
Sodium chloride	0.40
Vitamin–mineral premix 0.5%	0.40
L-Lysine HCl	0.35
L-Threonine	0.35
DL-Methionine	0.10
Chemical composition (g/kg DM)	
CP (g)	187.9
ME (MJ)	12.8
Fibre (g)	38.3
Lysine (g)	11.6
Methionine and cysteine (g)	6.4
Threonine (g)	7.6
Tryptophan (g)	2.3
Choline (mg)	1352
Vitamin A (IU)	11,530
Vitamin D ₃ (IU)	1500
Vitamin E (mg)	68.7
Vitamin B ₂ (mg)	7.1
Vitamin B ₁₂ (μ g)	26.4
Ca (g)	7.5
P (g)	6.2
Na (g)	1.9
Cu (mg)	10.0
Fe (mg)	163.4
Zn (mg)	125.8
Mn (mg)	72.7

DM, dry matter; CP, crude protein; ME, metabolizable energy.

Table 2 Fatty acid profile (percentage)

Fatty acid	Flaxseed oil	Sunflower oil	Basal diet
Lipids (DM basis)	45.8	ND	2.2
Palmitic, C16:0	5.1	6.3	17.4
Stearic, C18:0	3.7	3.2	2.2
Oleic, C18:1	18.4	22.6	24.7
Linoleic, C18:2	16.1	67.9	51.9
Linolenic, C18:3	56.8	0	3.8

ND, not detected.

Biological samples

All pigs were sacrificed using T-61 euthanasia solution (active ingredients: embutramide, mebezonium iodide, tetracaine hydrochloride; Intervet International B.V. Boxmeer, The Netherlands, doses: 0.3 ml/kg body weight) injected intracardially on the day of weaning (28-day-old piglets) and days 3, 7 and 21 PW. The abdominal wall was immediately open, and four rings of 1–2 cm width were harvested from the first jejunal loop 10 cm downstream of the ligament of Treitz. The tissues were immediately fixed in 4% paraformaldehyde and embedded in paraffin. Serial 4- to 5- μ m-thick sections were cut, mounted on electrostatic slides (Super Frost Plus; Menzel-Glaser, Braunschweig, Germany) and dried overnight. After dewaxing and rehydration, sections were processed for both haematoxylin–eosin (HE) staining and immunohistochemistry.

Histopathological evaluation

The histopathological injury index (HII) was examined blind by an expert pathologist and graded according to the Park/Chiu scoring system adapted by Quaedackers *et al.* (2000) with a scale ranging from 0 to 8, as follows: 0 = normal mucosa; 1 = subepithelial space at villus tip, often with capillary congestion; 2 = more extended subepithelial space with moderate epithelial lifting; 3 = epithelial lifting along villus sides; 4 = denuded villi; 5 = loss of villous tissue; 6 = crypt layer destruction; 7 = transmucosal infarction; 8 = transmural infarction.

Morphometrical analysis

Morphometry was performed using an Olympus BX50 light microscope with Olympus camera SP350 (Olympus, Tokyo, Japan) and QuickPHOTO Industrial 2.3 image analyzer software (Promicra, Prague, Czech Republic). Villus height (i.e. the distance from the villus base to the tip) and crypt depth (i.e. the distance between the villus base and the muscularis mucosae) were measured in 12 axially oriented villi in at least three different quadrants of each intestinal sample. Specifically, the crypt/villous junction was defined as the position at which epithelial cell nuclei changed from being central and with a more basophilic staining to being smaller and basally located, and where the cytoplasm devel-

oped mucin vacuoles (Holt *et al.* 1984). The intestinal mucosa thickness was measured as the sum of villus height and crypt depth. All measurements were taken using magnification $\times 200$.

Immunohistochemical analysis

After blocking endogenous peroxidase activity with 3% H₂O₂ and methanol, histological sections underwent two cycles of pretreatment in a microwave oven at 600 W for 15 min each in 0.01 M citrate buffer at pH 6.0. After that, a primary Ki67 polyclonal antibody, clone MIB-5 (Millipore Bioscience Research Reagents, Billerica, MA, USA), which stains the nucleus of actively proliferating cells, was applied at 1:100 dilution with an overnight incubation at 4°C. Then, a biotinylated secondary anti-goat anti-mouse antibody IgG (H+L) (Millipore Bioscience Research Reagents) was used followed by application of the IHC Select[®] Immunoperoxidase Secondary Detection System (Millipore Bioscience Research Reagents). Positive cells were visualized with diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA), and the nuclei were counterstained using Mayer's haematoxylin. Omitting the primary antibody on a serial section was considered as the negative control. The tissue sections were examined in a blind fashion by two independent histologists and photographed using an Olympus BX50 light microscope with an Olympus SP350 camera (Olympus). Counts were performed at a constant magnification ($\times 200$) and the results expressed as the percentage of cells with the nucleus stained brown.

Statistical analysis

The statistical analysis was performed using GRAPHPAD INSTAT version 3.01 (GraphPad Software, San Diego, CA, USA). The quantitative results were determined using one-way ANOVA with a multiple-comparison Tukey–Kramer *post hoc* test. The results are expressed as mean (M) \pm standard error of the mean (SEM). *P* values less than 0.05 were considered significant.

Ethical Approval

The experimental protocol (number 2108/07-221) was approved by the State Veterinary and Food Administration of the Slovak Republic, and the animals were handled and sacrificed in accordance with the guidelines established by the respective commission.

Results

As shown in Figure 1, no relevant histopathological changes were found in the intestinal mucosa of all the piglet groups examined at any time point, except for a mild subepithelial swelling at some villus tips in the groups F (0.92 ± 0.27) and FL (1.17 ± 0.38) on the weaning day.

The morphometric analysis showed a trend towards shortening of the villi in all piglet groups (Table 3) after weaning. More precisely, the decrease in villus height was most prominent in F and FL groups, where the villi at 21 days PW were nearly 30% shorter than those at WD, while in both S and SL groups, after a transient reduction, the villus height

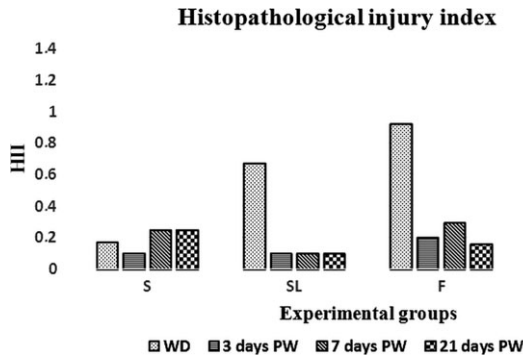


Figure 1 Histopathological injury index (HII). Values are mean ± SEM, n = 19–23.

was approximately the same, thus reaching the statistical significance in the S group in comparison with the F group (P < 0.01). As far as the crypt depth is regarded (Table 4), a robust increase in the group S was observed 21 days PW in

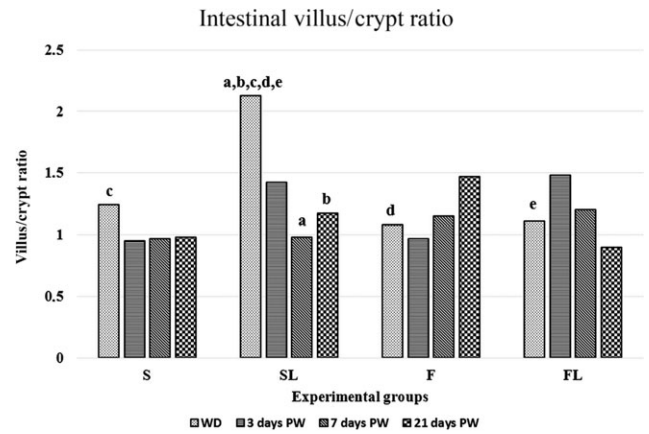


Figure 2 Intestinal villus/crypt ratio. Values are mean ± SEM, n = 19–23, a,b,c,d,e values with the same letters differ significantly; ^aP<0.01, ^{b,c,d,e}P<0.05.

Table 3 Morphometrical assessment of villus height

	Days postweaning				P value	
	0	3	7	21	AGE	DIET
S	315.2 ± 24.4	203.5 ± 12.3	181.1 ± 14.5 ^a	320.1 ± 51.9 ^{a,b}	<0.05 ^a	<0.01 ^b
SL	273.3 ± 20.8	236.2 ± 23.0	206.5 ± 30.6	265.3 ± 25.3	ns	ns
F	343.3 ± 94.4	239.1 ± 28.2	280.3 ± 45.3	236.0 ± 6.5 ^b	ns	<0.01 ^b
FL	320.0 ± 26.7	252.4 ± 11.1	282.6 ± 12.0	246.9 ± 14.1	ns	ns

Values are expressed in µm and results given as mean ± SEM, n = 19–23; AGE, differences within the animals of different age at a same diet (rows); DIET, differences within the animals at the same age with different diet (columns); ^avalues in the same row with the same letters differ significantly; ^bvalues in the same column with the same letters differ significantly; ns, not significant.

Table 4 Morphometrical assessment of crypt depth

	Days postweaning				P value	
	0	3	7	21	AGE	DIET
S	256.4 ± 15.9 ^{a,d,e,g}	214.2 ± 5.6 ^b	187.5 ± 16.1 ^c	329.7 ± 12.2 ^{a,b,c,h}	<0.05 ^a <0.01 ^b <0.001 ^c	<0.001 ^d <0.001 ^e <0.05 ^g
SL	127.8 ± 4.2 ^{d,e,f}	170.8 ± 14.6	213.5 ± 35.7	236.4 ± 39.0	ns	<0.001 ^e <0.001 ^f
F	315.5 ± 5.2 ^{a,c,g}	247.0 ± 14.8 ^{a,b}	243.3 ± 21.0 ^{a,c}	168.3 ± 14.8 ^{a,b,c,h,i}	<0.05 ^{a,b,c}	<0.001 ^e <0.05 ^g <0.001 ^h <0.001 ⁱ
FL	287.8 ± 17.0 ^{a,d,f}	180.3 ± 28.1 ^{a,b}	234.6 ± 4.2	271.3 ± 4.8 ^{b,i}	<0.01 ^{a,b}	<0.001 ^d <0.001 ^f <0.01 ⁱ

Values are expressed in µm and results given as mean ± SEM, n = 19–23; CD, crypt depth; WD, weaning day; AGE, differences within the animals of different age at a same diet (rows); DIET, differences within the animals at the same age with different diet (columns); ^{a,b,c}values in the same row with the same letters differ significantly; ^{d,e,f,g,h,i}values in the same column with the same letters differ significantly; ns, not significant.

Table 5 Morphometrical analysis of mucosal thickness

	Mucosa thickness (µm)				P value	
	Day postweaning				AGE	DIET
	0	3	7	21		
S	602.0 ± 49.2	481.8 ± 11.4	446.6 ± 87.9	652.3 ± 26.4	ns	ns
SL	426.8 ± 27.2 ^{c,d}	444.7 ± 4.3 ^d	484.7 ± 91.9	610.3 ± 52.7	ns	<0.05 ^d <0.01 ^c
F	779.1 ± 75.7 ^{a,c}	615.0 ± 40.6 ^d	616.3 ± 57.5	453.1 ± 22.5 ^a	<0.05 ^a	<0.05 ^d <0.01 ^c
FL	704.0 ± 21.1 ^{a,b,d}	478.3 ± 44.2 ^b	577.0 ± 8.5	530.5 ± 40.0 ^a	<0.05 ^a <0.01 ^b	<0.05 ^d

Values are mean ± SEM, *n* = 19–23; AGE, differences within the animals of different age at a same diet (rows); DIET, differences within the animals at the same age with different diet (columns); ^{a,b} values in the same row with the same letters differ significantly; ^{c,d} values in the same column with the same letters differ significantly; ns, not significant.

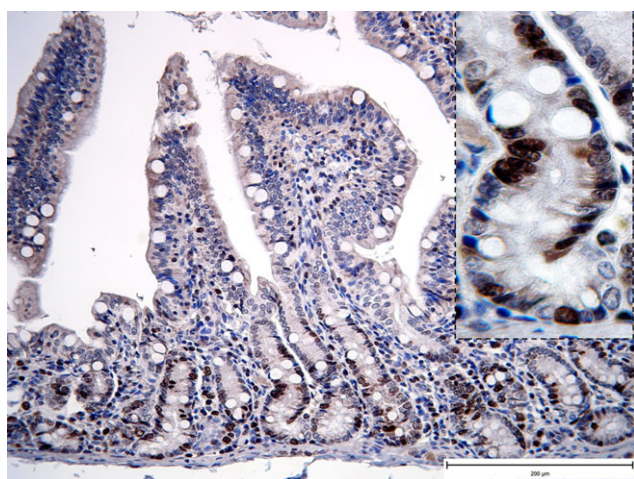


Figure 3 Representative photomicrographs illustrating the proliferative activity of the cells in the jejunal biopsy (S group – WD). Ki67 immunohistochemical staining. Significant increase in Ki67 immunoreactivity in *lamina epithelialis mucosae* (Fig. 1, ×200; detail ×1000).

comparison with the previous time periods (*vs.* weaning day *P* < 0.05; *vs.* 3 days PW *P* < 0.01 and *vs.* 7 days PW *P* < 0.001). Conversely, a significant decrease of the crypt depth was found in the F group (all time periods *vs.* 21 days PW *P* < 0.05), where it was almost 50% lower than that in S group (*P* < 0.01) at the end of the experiment. Similarly, deeper crypts were evident even in the FL group 21 days PW with respect to the F one (*vs.* F *P* < 0.01). However, the lowest crypt depth was observed in the SL group at the WD, and the difference was significant in comparison with the other groups (*P* < 0.001 for all). When considering the villus height/crypt depth ratio, no differences were observed in all the experimental groups, except for a significant increase in the SL group (2.13 ± 0.12) in comparison with the other groups at the weaning day (*P* < 0.05 for all), that underwent normalization at day 7 PW (0.98 ± 0.09; *P* < 0.01) and remained stable during the following time points (Figure 2).

A time-dependent decrease of the intestinal mucosal thickness (Table 5) was found in the flaxseed oil piglet groups (both F and FL) at day 21 PW in comparison with the weaning day (F *P* < 0.05; FL *P* < 0.05), with the FL group

Table 6 Epithelial cell proliferative rate

	Proliferative activity of cells in <i>lamina epithelialis</i>				P value	
	Day postweaning				AGE	DIET
	0	3	7	21		
S	22.3 ± 1.7 ^{e,f,g}	12.9 ± 1.8 ^a	11.7 ± 0.7	17.1 ± 1.6 ^a	<0.05 ^a	<0.001 ^e <0.01 ^{f,g}
SL	11.1 ± 0.6 ^c	10.4 ± 0.6 ^h	11.7 ± 0.3 ^{i,j}	12.9 ± 1.7 ^k	ns	<0.05 ^{h,j}
F	15.7 ± 1.0 ^{b,f}	16.1 ± 1.0 ^{c,h}	17.5 ± 1.7 ⁱ	22.7 ± 1.8 ^{b,c,k}	<0.05 ^{b,c}	<0.05 ^{i,k}
FL	14.6 ± 0.4 ^g	10.7 ± 1.0 ^d	14.5 ± 1.3 ^j	16.3 ± 1.4 ^d	<0.05 ^d	<0.05 ^j

Values are mean ± SEM, *n* = 19–23; AGE, differences within the animals of different age at a same diet (rows); DIET, differences within the animals at the same age with different diet (columns); ^{a,b,c,d} values in the same row with the same letters differ significantly; ^{e,f,g,h,i,j,k} values in the same column with the same letters differ significantly; ns, not significant.

Table 7 Lamina propria cell proliferative rate

	Lamina propria proliferative rate				P value	
	Day postweaning				AGE	DIET
	0	3	7	21		
S	15.4 ± 0.8 ^b	13.3 ± 0.8 ^{a,d}	18.2 ± 0.4 ^h	19.9 ± 2.0 ^{a,i}	<0.05 ^a	<0.05 ^{b,c,i}
SL	15.9 ± 1.9	10.9 ± 1.6 ^c	14.0 ± 1.3	11.3 ± 0.5 ^{i,j}	ns	<0.01 ^e
F	22.1 ± 2.0 ^{b,c}	23.3 ± 2.4 ^{d,e,f}	17.7 ± 1.6 ^g	22.1 ± 1.8 ^j	ns	<0.05 ^{d,i}
FL	14.7 ± 0.5 ^c	10.7 ± 1.3 ^f	12.1 ± 1.0 ^{g,h}	14.5 ± 2.3	ns	<0.01 ^f
						<0.05 ^{g,h}

Values are mean ± SEM, $n = 19-23$; AGE, differences within the animals of different age at a same diet (rows); DIET, differences within the animals at the same age with different diet (columns); ^avalues in the same row with the same letters differ significantly; ^{b,c,d,e,f,g,h,i,j} values in the same column with the same letters differ significantly; ns, not significant.

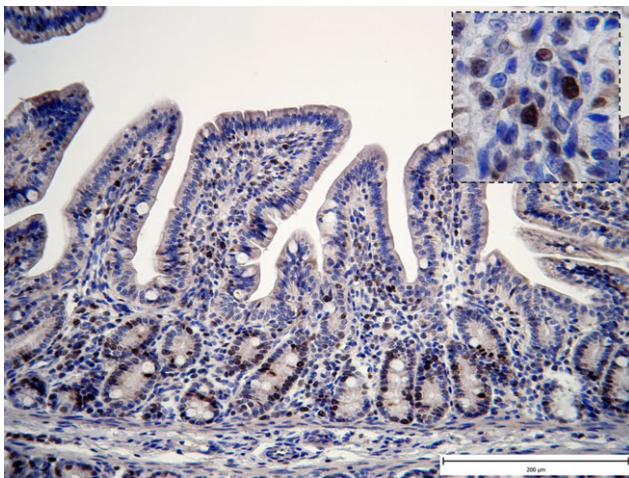


Figure 4 Representative photomicrographs illustrating the proliferative activity of the cells in the jejunal biopsy (F group – WD). Ki67 immunohistochemical staining. Significant increase in Ki67 immunoreactivity in lamina propria mucosae (Fig. 1, ×200; detail ×1000).

showing a statistically significant reduction also at day 3 PW *vs.* WD ($P < 0.01$). Furthermore, the mucosa appeared thinner in both the L (SL and FL) groups than in both groups without *lactobacilli* (S and F) on WD.

When the proliferative rate of the epithelial cell population was evaluated, the highest mitotic activity was observed in the S group at WD (Figure 3), followed by a decrease at 3 and 7 days PW, and a recovery at 21 days PW (Table 6). Even when comparing the amount of proliferating cells (Ki67-positive) with the values found in all the other groups at the weaning day, the S group displayed a significantly higher rate (*vs.* SL $P < 0.001$; *vs.* F and FL $P < 0.01$ for both). By contrast, in the F group an opposite pattern was found, with a progressive increase in the number of Ki67-positive cells that reached statistical significance at day 21 PW as compared with the WD ($P < 0.05$). When comparing the data at 21 days PW in both F and FL groups with those found at 3 days PW, again a significantly higher prolifera-

tion rate was clearly evident ($P < 0.05$ for both). Finally, in the F group the number of proliferating cells was higher at all time points from the day 3 PW onwards than those found in the SL group ($P < 0.05$ for all).

When considering the proliferative rate of the lamina propria cells (Table 7), the highest number of Ki67-positive cells was found in the F group at the WD with respect to the S and FL groups (Figure 4; $P < 0.05$ for both) and at 3 days PW with respect to all the other groups ($P < 0.05$ *vs.* S; $P < 0.01$ *vs.* both SL and FL). A high level of proliferative activity was maintained in the F group until the day 21 PW in comparison with the SL and FL groups. A lower number of proliferating cells were found in both *lactobacilli* groups reaching statistically significant differences when comparing the mean value of the FL and F groups at days 3 ($P < 0.01$) and 7 PW ($P < 0.05$). Moreover, both the *lactobacilli* groups showed the lower proliferative activity from the WD up to 21 days PW as compared to the flaxseed group.

Discussion

The intestinal tract of pigs undergoes morphological and functional modification in response to dietary changes. The intestinal villi are covered by highly differentiated epithelial cells, the enterocytes, whose brush border includes a number of specific channels and transporters devoted to absorption of a variety of nutrients. Moreover, digestive enzymes, such as disaccharidases and peptidases, are expressed on the brush border to complete the breakdown and then facilitate nutrient absorption. Villus atrophy therefore impairs pig growth performance by reduction of nutrient absorption. The presence of a positive correlation between villous height and disaccharidase activities (lactase, sucrase, maltase) was already established through all the growth stages of piglets (Tsukahara *et al.* 2013). The maintenance of villus structure to retain disaccharidase activity and carbohydrate absorption plays a pivotal role in preventing diarrhoea and ensuring an efficient growth of the piglets in the PW stage (Pluske 2001); thus, many nutritional strategies have been proposed to maintain PW villous height.

According to our experimental study, only slight damage to the intestinal mucosa in both flaxseed piglet groups was found on WD, while the morphometric analysis showed a tendency to villus shortening during the PW times in all experimental groups. Both groups receiving sunflower oil (S and SL) that is rich in linoleic acid (ω -6 PUFA) showed approximately the same villus height 21 days PW as that on WD. When this was examined in more depth, following an initial decrease by 40% in the S group, a critical increase was observed from day 7 to day 21 PW. An opposite pattern was evident in the groups supplemented with flaxseed (F and FL) that is rich in linolenic acid (ω -3 PUFA). At time of 7 days PW the decrease in villus height in both flaxseed groups was lower than that observed in the S groups, although in the F group significantly shorter villi than those in S group were found at day 21 PW. At the same time, the crypt depth decreased significantly in the F group in comparison with the S one. Our findings are extremely important if considering that the marked changes in the morphology of the small intestine associated with the weaning, such as villous atrophy and crypt hyperplasia, are responsible for decreased digestive and absorptive capacities and contribute to PW diarrhoea (Pluske *et al.* 1997). Probiotic bacteria are often applied to prevent or cure a wide range of intestinal disorders, as elegantly shown by Yoshida *et al.* (2009) who found that small intestinal villous atrophy can be alleviated by the administration of probiotic *lactobacilli* which stimulate a peculiar array of cytokines responsible for mucosal recovery. The improvement in villous atrophy was still observed an oral preparation of heat-killed and dried cells was administered (Tsukahara *et al.* 2011). Moreover, the adhesion of bacteria to the mucosa is pivotal for the beneficial effects of living probiotics, and the administration of PUFA positively affects the adhesion of *lactobacilli* to the jejunal mucosa as assessed in gnotobiotic piglets (Bomba *et al.* 2003) and germ-free piglets (Nemcova *et al.* 2012). However, at variance with these latter studies, our results did not confirm the restorative effect of *lactobacilli* addition to the piglet diet, as no significant differences in villous height were found in both *lactobacilli* groups (SL and FL) in comparison with the S groups. Significantly lower crypt depth than in S, F and FL groups was found only on WD in SL group, which led to a significant increase in the intestinal villus/crypt ratio. Conversely, the thickness of the jejunal mucosa was significantly lower in the FL group than in the F group on WD and 3 days later, as a result of reduction of both villus height and crypt depth. Similarly, no effect of ω -3 PUFA on intestinal microbial communities and performance of broiler chickens was found (Geier *et al.* 2009). The significant decrease in crypt depth in both flaxseed groups during the first week PW could contribute to reduction of the PW diarrhoea incidence (Popper *et al.* 2014). The low crypt deep could mirror a low enterocyte apoptotic rate on the villous surface, so the reduced incidence of the diarrhoea could be attributed to the anatomic-functional integrity of the whole epithelium.

In the current study we observed different effects of ω -6 and ω -3 PUFA on the proliferative activity of both the epithelial and lamina propria cells of the intestinal mucosa. The highest proliferative activity of epithelial cells in the group fed with ω -6 PUFA (S) was observed on WD, followed by a decrease 3 days later. Conversely, the highest proliferative activity in lamina propria cells in the animals with ω -3 PUFA in the diet (F) was found on WD and 3 days later. Linoleic acid and linolenic acid are two of the main representative compounds, known as dietary essential fatty acids because they prevent deficiency symptoms and cannot be synthesized by humans (Benatti *et al.* 2004). According to Viegas *et al.* (2012), PUFA regulate cell proliferation and gene expression, but cellular responses to fatty acids may depend on the differentiation status of the cell and the origin of the cell lines as well. The positive effect of ω -6 PUFA on epithelial restitution has been demonstrated in previous studies. For instance, Bliklager *et al.* (1997, 1999) showed that arachidonic acid 6-derived prostaglandins stimulate rapid recovery of gut barrier function after injury. Moreover, Hess *et al.* (2008) observed a dose-dependent relationship between dietary arachidonic acid concentration and arachidonic acid content of mucosal phospholipids, while only a negligible effect on gross villus/crypt morphology was observed. Finally, the prostanoids produced from arachidonate (via cyclooxygenase enzymes) have been implicated in accelerating recovery of gut barrier function following ischaemic injury, a mechanism thought to play a role in the pathogenesis of necrotizing enterocolitis (Bliklager *et al.* 2007).

An adequate content of essential fatty acids in the diet is necessary as their biologically active metabolites regulate gene expression and enzyme activity, dampen inflammation, modulate immune response, and affect gluconeogenesis by direct and indirect pathways, as agonists of a number of G-protein-coupled receptors (Das 2011). After ingestion, essential fatty acids undergo homogenous distribution to virtually every cell in the body, thus affecting membrane composition and cellular function. They serve in the plasma membranes as substrates for the enzymes cyclooxygenase and lipooxygenase which convert them into a number of important, very active, short-lived, hormone-like compounds referred to as 'eicosanoids'. These latter elicit changes in gene expression that precede changes in membrane composition by directly governing the activity of nuclear transcription factors (Benatti *et al.* 2004). In this regard, it should be pointed out that both the linoleic and linolenic acids compete for the same 6-desaturase in the metabolic cascade and linolenic acid is a strong suppressor of ω -6 fatty acid metabolism. These effects may not only be mediated by direct competitive mechanisms, but very likely also via regulation of the activity or abundance of desaturation and elongation enzymes at the level of expression of the corresponding gene (Jump & Clarke 1999). Fat nutrients therefore may regulate cellular functions by affecting the expression or activity of

genes in the signal transduction pathway related to the control of cell growth and apoptosis. The finding that oleic acid and ω -3 PUFA block the desaturase reaction, the first step from linoleic acid to eicosanoids, may also partially explain their inhibitory effects on cell proliferation (Benatti et al. 2004).

In conclusion, we suggest that the inhibitory effect on proliferative activity of the intestinal epithelium could be demonstrated on WD in both flaxseed groups. Three weeks PW, the villus height and crypt depth were decreased significantly in comparison with the piglets receiving ω -6 PUFA in the diet. The higher proliferative activity of the lamina propria cells in comparison with sunflower and *Lactobacilli* groups with ω -3 PUFA, which was found both on WD and 3 days later in the intestinal mucosa of flaxseed-fed piglets, was probably caused by different sensitivity of the two cellular populations. This suggestion confirms published results (Viegas et al. 2012) about different effects of PUFA on the cells of various origin and maturation stage by regulation of cell proliferation and gene expression. The cellular response to the fatty acids could be effected by the *Lactobacilli* presence, as the proliferative activity of connective tissue cells in FL group was lower significantly than that in F group from WD till 7 days PW.

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Conflict of interest

Authors declare no conflict of interest.

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