Histological Changes in the Small Intestinal Epithelium in Fattening Pigs Fed Selected Feed Additives

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Abstract

The production experiment was conducted on 48 fattener pigs fed complete diets supplemented with the antibiotic favomycin (group C), the probiotic Bactocell® (*Pedicoccus acidilactici,* strain MA18/5M) at the amount of 0.01% in the first and second stage of fattening (group E1), and the prebiotic BIO-MOS® (*Saccharomyces cerevisiae*, strain 1026) at the amount of 0.1% in the first stage of fattening (group E2). Serial sections of the duodenum, jejunum and ileum were prepared *post mortem.* Haematoxylin and eosin and immunohistochemical (Ki-67, PCNA) staining procedures were performed. The effect of the feed additives on the morphological characteristics and proliferation capacity of small intestinal crypt epithelium varied when mitotic indices in groups were compared. In comparison with group C, the enterocytes were higher in group E2 in the jejunum ($P \leq 0.01$) and smaller in the ileum ($P \leq 0.01$). Administration of the Ki-67 antibody resulted in fewer positive reactions in the jejunum in group E1 than in group C ($P \leq 0.01$). Enterocyte proliferation in crypt epithelium decreased after the administration of the probiotic or the prebiotic vs. the antibiotic, but the absence of significant differences between the groups may suggest that these feed additives have no adverse effect on the mucosal epithelial cells.

Small intestine, mitotic index, PCNA, Ki-67

Morphometric and functional changes in the intestinal mucosa are caused by nutritional factors (Schweiger et al. 2003; Domeneghini et al. 2004; Babinska et al. 2005). Various bacterial populations in the gastrointestinal tract are capable of modifying intestinal microstructures and the immune system (Vitini et al. 2000; Lata et al. 2006). Histological research investigates the effect of various diets (e.g. milk diet, diet with a varied share of faba beans) and dietary systems (restricted or *ad libitum*) on the morphometric characteritics of the intestines (Mroz 2001). It was found that feed additives (probiotics and prebiotics) do not have an adverse effect on the morphometric characteristics of the intestines (Budiño et al. 2005; Reiter 2006). Dietary factors increase the height of intestinal villi as well as the number and depth of crypts (Mroz 2001). Crypts are responsible for the proliferation of epithelial cells, the production of defensins - antibacterial immunity agents, and endocrine substances, such as chomogranin A. Defence reactions are stimulated in crypts, supporting antibody production and phagocytosis (Manning and Gibson 2004). Diet components, fibre content and the applied dietary method and feeding system affect intestinal microflora (Rekiel and Gajewska 2006), as well as the proliferation and secretory activity of intestinal crypt enterocytes (McCullogh et al. 1998; Ekelund and Ekblad 1999; Schweiger et al. 2003; Piel et al. 2005; Van Nevel et al. 2005).

The objective of this study was to determine the impact of antibiotics, probiotics and prebiotics on the morphometric characteristics and proliferation activity of crypt enterocytes in the small intestine of fatteners.

Materials and Methods

The experiment was conducted on 48 hybrid fatteners [(Polish Large White × Polish Landrace) × Duroc and (Polish Large White × Polish Landrace) × Belgian Landrace) (1:1); gilts and young hogs (1:1)] divided into three

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equal groups: control (C) and experimental - E1 and E2 (each n = 16). The animals were clinically healthy and had been treated for parasites prior to fattening. They were kept individually throughout the experiment. During a two-stage fattening process (21-56 kg, 56-100 kg), the feed was dosed individually in line with the observed standards (Polish Norm of Pigs Nutrition 1993). Group C pigs were fed a complete diet supplemented with 5% favomycin premix (100 mg per kg), and group E1 and E2 pigs were fed a premix without the antibiotic (Table 1). At the first and second stage of fattening (103 days), group E1 pigs received mixed feed containing 0.01% of the probiotic Bactocell® (Lallemand Animal Nutrition) (*Pedicoccus acidilactici,* strain MA18/5M). Group E2 pigs received mixed feed with the addition of 0.1% of the prebiotic BIO-MOS® (Alltech) (*Saccharomyces cerevisiae*, strain 1026) at the first stage of fattening (49 days). The chemical composition of the feed components and diets was analysed (AOAC 1990), and their nutritive value was determined. The energy value of diets administered at the first and second stage of fattening was 12.3 and 12.2 MJ ME/kg respectively, and the protein content of diets for groups C, E1 and E2 was: stage 1 – 165, 168, 166 g/kg, stage 2 – 143, 145, 149 g/kg, respectively.

Table 1. Composition of dites

Item (per cent)	Group					
	Control		Experimental I		Experimental II	
	Period					
	I	II	I	II	I	II
Ground barley	53.15	58.74	53.23	58.73	53.05	58.74
Ground wheat	25	25	25	25	25	25
Soybean meal	11.5	6	11.5	6	11.5	6
Meat bone meal	5	5	5	5	5	5
Premix with antibiotic[1]	5	5	-	-	-	-
Premix without antibiotic[2]	-	-	5	5	5	5
Bactocell*	-	-	0.01	0.01	-	-
BIO-MOS**	-	-	-	-	0.1	-
L-Lysine	0.3	0.26	0.3	0.26	0.3	0.26
DL-Methionine	0.05	-	0.05	-	0.05	-

Composition of 1 kg of premix Lidermix T 5%: [1]vitamins: A 210000 IU; D_3 40000 IU; E 2000 IU; B_1 30 IU; B_2 80 IU; B_3 400 mcg; B_6 45 mg; B_{12} 500 mcg; K 32.5 mg; H 500 mcg;
choline 1500 mg; folic acid 9.0 mg; Synthetic amino acids: methionine 7.50 g; lysine 36.0 g; threonine 5000 mg; mineral components: Mn 1500 mg; Zn 2250 mg; Co 8.0 mg; Se 6.0 mg;
Cu 500 mg; Fe 1800 mg; J 20 mg; Mg (total) 1.0 g, P (total) 27.5 g; Na (total) 25.0 g, Ca (total) 122.0 g; Other components: antioxidant 300 mg; Beta fn S_1 3750 mg, Flavomycin 100 mg;
Ca panthotenate 200 mg; Pigor 757 – 4000 mg; brans – 4969000 mg. [2] as above, without Flavomycin,
* Bactocell – contains strain *Pedicoccus acidilactici* MA18/5M
** BIO-MOS – contains cellular walls of *Saccharomyces cerevisiae* yeasts, strain 1026;

Pigs were slaughtered at the completion of fattening. Duodenum, jejunum and ileum sections (5 × 20 mm each) were sampled immediately after slaughter. Tissue samples were rinsed with a 0.9% saline solution, fixed with a 10% buffered formalin solution and embedded in paraffin (Paraplast-Sigma). Paraffin sections of 4 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin (HE), and the presence of PCNA and Ki-67 antigens was determined immunohistochemically (Table 2).

Proliferation was determined by an immunohistochemical method (EnVision + two-stage process) with the use of monoclonal antibodies: Monoclonal Mouse Ant-Human Ki-67, Clone Ki-S5 (Ki-67), and Monoclonal Mouse Anti-Proliferating Cell Nuclear Antygen (PCNA), Clone PC10 (PCNA). Samples were evaluated with a BX50 Olympus light microscope at × 400 magnification.

The mitotic index (MI), i.e. the number of enterocytes in crypts, was determined by counting each time 100 cells in three replications. The height of epithelial cells in crypts was determined with the use of the AnalySIS 3.0 image analysis system. The results are presented as mean values for 10 measured cells. Ki-67 positive cells and PCNA positive cells were counted in three replications of up to 100 cells each. The results are presented as means over replications.

Based on the above results, the statistic calculations were carried out, using one-way analysis of variation. The significance of differences between the groups was calculated based on Duncan's multiple test. The computer package SPSS 10.0 pl was used for calculation.
The following equation was used:

$y_{ij} = \mu + a_i + e_{ij}$

y_{ij} – observed value,
μ – overall mean,
a_i – effect of i-th feed addition,
e_{ij} – random residual effect.

The study was approved by the institutional Ethics Committee.

Results

No significant differences in MI between the investigated groups were observed (Table 3, Plate V, Fig. 1). Compared to group C, the height of enterocytes in crypts in group

Table 2. Experimental material

Intestinal section/ investigated properties/ staining	Group		
	C	E1	E2
	Number of analysed samples		
Duodenum			
MI*, enterocyte height: H-E	30	23	12
Proliferation: Ki-67	24	20	15
PCNA	27	21	15
Jejunum			
MI, enterocyte height: H-E	45	32	24
Proliferation: Ki-67	24	21	26
PCNA	48	42	48
Ileum			
MI, enterocyte height: H-E	33	18	12
Proliferation: Ki-67	15	9	15
PCNA	21	9	18

*MI – mitotic index
H-E – haematoxylin - eosin

Table 3. Mitotic index (MI), veigth and proliferation of enterocytes in intestinal crypts[1]

Investigated properties /intestinal section	Group			Total average	
	C	E1	E2	LSM	SEM
Mitotic index (IM), %					
Duodenum	5.7	6.1	5.0	5.6	0.930
Jejunum	9.8	8.4	7.1	8.4	1.010
Ileum	12.1	11.7	5.0	9.6	1.569
Enterocyte height, µm					
Duodenum	18.65	18.71	18.85	18.74	0.298
Jejunum	19.50 A	20.77	21.74 B	20.67	0.280
Ileum	25.99 A	24.78	23.67 B	24.81	0.357
Ki-67, %					
Duodenum	52.67	45.25	37.93	45.28	2.409
Jejunum	55.46 A	42.91 B	51.85	50.07	1.857
Ileum	40.67	49.78	34.73	41.73	2.690
PCNA, %					
Duodenum	72.22	66.43	60.87	66.51	2.050
Jejunum	67.81	70.55	65.77	68.04	1.268
Ileum	59.91	48.56	53.28	53.91	2.465

C – control group, (antibiotic - Flavomycin); E1 – experimental group, (probiotic – Bactocell);
E2 – experimental group, (prebiotic - BIO-MOS); A, B – signi f cant differences at $P \leq 0.01$
[1] least square means – LSM; total average – average for all (three) groups; SEM – standard error of mean

E1 was greater in the jejunum (11.49%) ($P \leq 0.01$) and smaller in the ileum (8.93%) ($P \leq 0.01$) (Table 3).

In the present study, the percentage of positive reactions during Ki-67 and PCNA labelling was not indicative of a regular dependency, and the obtained reactions were clearly pronounced (Table 3) (Plate V, Fig 2, Plate VI, Fig. 3). In group C, the highest percentage of Ki-67 positive cells was found in the jejunum. A smaller percentage of those cells was determined in the duodenum, and Ki-67 positive cells were least likely to occur in the ileum. In comparison with group C, the Ki-67 reaction was less noticeable in groups E1 and E2. Signi f cant differences ($P \leq 0.01$) in proliferation activity were determined in the jejunum between groups C and E1. Proliferation activity was limited in group E1 in comparison with group C, which can be regarded as an undesirable phenomenon (Table 3).

In the current study, the number of PCNA positive cells in groups E1 and E2 was below that reported in group C. Enterocyte proliferation in intestinal crypts was lower in the experimental groups than in the control group (with the exception of the jejunum in group E1), but the differences between groups E1, E2 and C were not statistically veri f ed (Table 3). Regular dependencies were not determined with respect to the proliferation activity in crypts in the investigated groups and segments of the small intestine. In group C, the number of PCNA positive cells in the jejunum and the ileum was 6.11% and 17.95% lower than in the duodenum, respectively. In the group administered the probiotic (E1), the proliferation activity of enterocytes in the jejunum was 6.20% higher than in the duodenum, and it was 26.9% lower in the ileum than in the duodenum. Similar dependencies were reported in

the group administered the prebiotic (E2) where proliferation was 8.05% higher in the jejunum than in the duodenum and 12.46% lower in the ileum than in the duodenum (Table 3).

Discussion

In a study investigating weaned piglets, Budiño et al. (2005) administered feed without additives as well as feed containing an antibiotic, a probiotic, a prebiotic and a synbiotic to discover that the density and length of duodenal villi was higher in pigs fed a diet with the prebiotic than in those receiving a diet with the probiotic. The above authors also concluded that in comparison with diets containing other additives, the structure and efficiency of intestinal villi was restored at a much faster rate in pigs administered the probiotic. Reiter (2006) observed no effect of the supplementation of diets for sows and piglets with a probiotic (*Enterococcus faecium* SF 68 NIMB 10415) on the morphology of the small intestine in young pigs. Nutritional factors, such as short-chain organic acids, may directly affect intestinal morphology, stimulating the proliferation of intestinal epithelial cells (Sakata et al. 1995; Ichikawa et al. 2002). In studies involving *Lactobacillus acidophilus* and *Bifidobacterium* spp., probiotic strains had no adverse effect on the morphology of the digestive tract, the liver and the pancreas (Babinska et al. 2005). Changes in glucose absorption were reported in young, growing pigs fed a probiotic-supplemented diet (Lodemann et al. 2006). The administration of probiotic strains enhanced glucose absorption. The authors are of the opinion that the above results confirm a positive effect of probiotics on animals.

Apoptosis and proliferation can be determined in the intestinal epithelium by immunohistochemical methods (Reed 1994; Kelman 1997). Ki-67 antigen expression is observed during all phases of the cellular cycle (G_1, S, G_2 and M phase), and it does not take place in the quiescent (G_0) phase and in inactive cells. The antigen is decomposed when the cell enters the non-proliferative state (Scholzen and Gerdes 2000).

The PC10 monoclonal antibody facilitates the labelling of proliferating cells in healthy tissues (Hall 1990). This protein's expression is observed at the final stage of G_1 phase and at the initial stage of S phase. PCNA significantly contributes to the continuity of the cellular cycle. Oligonucleotides directed against PCNA inhibit the transition of cells to phase S of the cellular cycle. PCNA plays an important role in the life and death of cells, being an element of the DNA replication and repair mechanism. The absence or the low level of functional PCNA may lead to cell apoptosis (Kelman 1997; Paunesku et al. 2001). In cells, PCNA is nearly completely limited to the nucleus, it occurs in diffused, granular or mixed form (Hall 1990).

Micromorphometrical and immunohistochemical (PCNA) evaluation after the application of L-glutamine and/or nucleotides has shown that supplementation exerts a beneficial effect on the morphological and functional properties of intestinal mucosa (Domeneghini et al. 2004). Short-chain fatty acids were found to stimulate the proliferation of epithelial cells to a various degree: n-butyric acid > propionic acid > acetic acid. The source of those acids may be the fermentation process, but they can also be administered orally, or by intravenous, gastric and intestinal infusion (Sakata et al. 1995).

A morphological and immunohistochemical evaluation of the small intestinal mucosa of animals whose diets were supplemented with antibiotics, probiotics and prebiotics indicated that the administered additives had a varied effect on the mitotic index, enterocyte height and the proliferation capacity of crypt epithelium. Decreased enterocyte proliferation in crypt epithelium following the administration of probiotics or prebiotics necessitates further research in this area, although the absence of significant differences between groups (C, E1 and E2) may suggest that probiotics and prebiotics have no adverse effect on mucosal epithelial cells.

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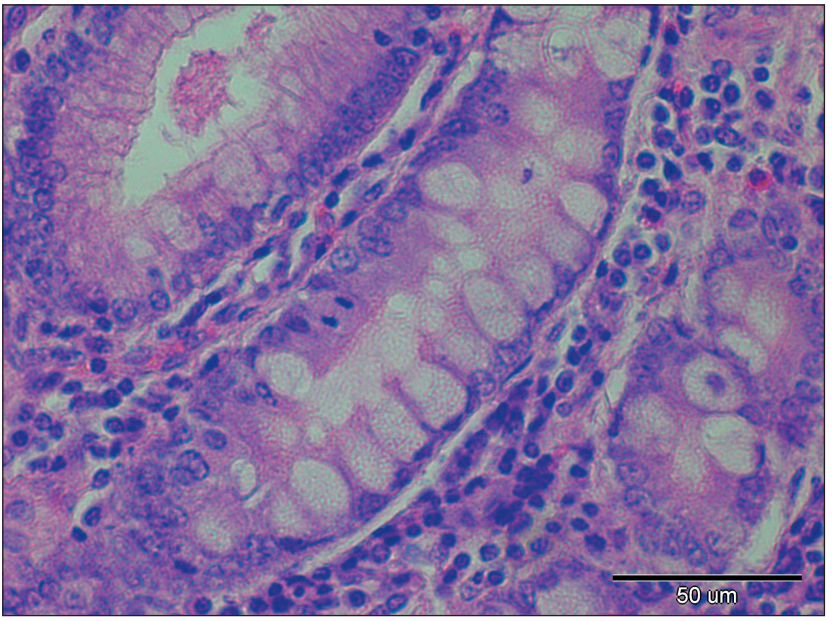

Fig. 1. Histological image of duodenal mucosa of E1 group of pigs. HE staining, magnification × 200

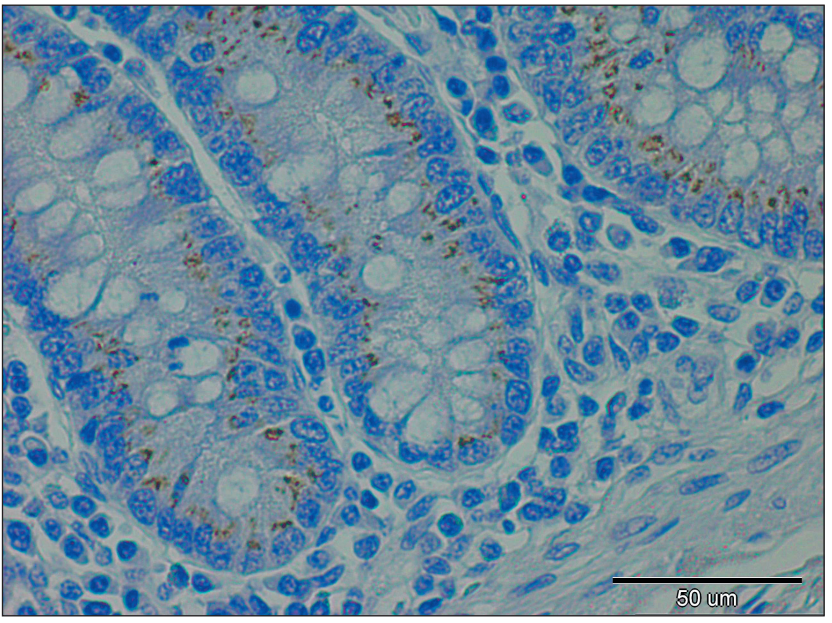

Fig. 2. Presence of Ki-67 positive cells in jejunal mucosa of E1 group of pigs. Immunohistochemical staining with the use of Ki-S5 monoclonal antibodies. Magnification × 200

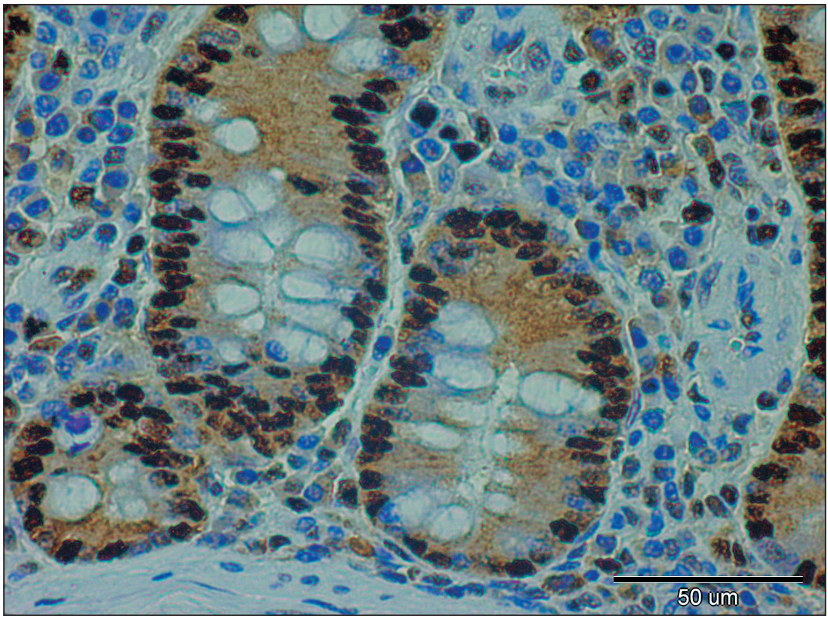

Fig. 3. Presence of PCNA positive cells in jejunal mucosa of E1 group of pigs. Immunohistological staining with the use of PC 10 monoclonal antibodies. Magnification × 200