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INTESTINAL MUCOSAL MORPHOMETRY AND ANALYSIS OF CD3 LYMPHOCYTES IN THE INTESTINAL MUCOSA OF PIGLETS AFTER THE APPLICATION OF *LACTOBACILLUS*

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The distribution and number of CD3 immunocompetent cells were examined in the small intestinal mucosa of piglets after long-term application of non-host specific Lactobacilli L. casei or L. sp. strain B-5, respectively. The thickness of the lamina propria of the small intestine resulting from continuous feeding with these lactic acid bacteria was also measured. The immunohistochemical examination of the samples showed a lower number of CD3 cells in the intestinal villi of the ileum after administration of L. sp. strain B-5 and significantly fewer in the epithelium ($P<0.05$) and in the lamina propria ($P<0.01$) of the ileum after the application of L. casei. The lamina propria of the small intestine was thinner in the experimental groups of piglets in comparison with the control animals.

Key words: Lactobacillus casei, lactobacilli, CD3 positive cells, T lymphocytes, small intestine

INTRODUCTION

Large populations of *Lactobacillus* inhabit the gastrointestinal tract of pigs and other mammals. Their presence in the digestive tract may be beneficial to the host animal (Pedersen and Tannock, 1989). There is now renewed interest in using these bacteria as food additives to prevent diarrhoea and to protect the intestine against infection caused by enteric pathogens. To clarify the mechanism of the effect of probiotics, the hypothesis about the film of metabolites produced by probiotic strains covering the intestinal micosa and preventing the adherence of pathogens is often used. The results presented in the study by Bomba et al. (1996) confirm that lactobacilli produce organic acids, which form an inhibitory barrier on the small intestine mucosa against digestive tract pathogens. This phenomenon is of extraordinary importance from the viewpoint of their adherence to the intestinal mucosa. The beneficial

influence of *Lactobacilli* on gut microflora supposes changes in the response of the mucosal immune system (Cambell, 1986).

In order to assess the immune response, the number of CD3 cells was counted in the epithelium and lamina propria of the small intestine in control piglets and piglets after the application of *Lactobacillus*. The thickness of the lamina propria of the small intestine was also measured microscopically, in order to observe possible modifications in the epithelium and in the lamina propria of the gut, as a consequence of continuous feeding with this bacterium.

MATERIAL AND METHODS

Animals. Nineteen healthy piglets were included in our experiment. They were divided into two groups each coming from different litters (Table 1). Both groups consisted of control and experimental animals. In each group the control and experimental animals were kept separate. All the piglets were fed with the commercial diet ČOS-I up to weaning and ČOS-II after weaning. The pellets of the commercial diets also contained milk with *Lactobacilli*. The viable culture, which had 1.108 of germ.) was suspended in 2 ml of nutrition broth and added to 1 litre of milk, which was fermented for 24 h at 37°C. Two strains of *Lactobacillus* (L) were administered to the experimental groups: *Lactobacillus casei* - a strain that was obtained from Denmark and used for dairy produce (Fy Christan Hansen, Denmark), end *Lactobacillus* sp. strain B-5 - a non-typed strain (Kapitančik et al., unpublished data, University of Veterinary Medicine, Košice, Slovak Republic), analysis of which is in progress. The *Lactobacillus* was given twice daily to piglets from 28 days old for 28 days. All the piglets were killed when 56 days old.

Table 1. Experimental design

	Control piglets No.	No. of piglets treated with	
		L. casei (Danish) origin)	*L. sp. B-5* (non-typed)
1. litter	3	5	–
2. litter	4	–	7

Procedure for light microscopy. Duodenum, jejunum and the ileum were taken for histological processing. The material was fixed in 10% of neutral formaline and embedded in paraffin. Sections of thickness 5-6 μm were stained with Haematoxyllin eosin (HE) and by Giemsa.

Polyclonal antibodies. Polyclonal rabbit anti-human T cell CD3 antibodies raised against synthetic human CD3 epsilon chain peptide were used (Dakopats. Glostrup, Denmark) as an antiserum in solution diluted 1:300.

Immunohistochemistry. Immunohitochemical reaction was carried out on digested paraffin sections by means of the biotin-streptavidin amplified (B-SA) peroxidase detection system (Biogenex. San Ramon, CA, USA).

Tissue sections for immunohistochemical examination were placed on coated slides, deparaffinized in the sequence xylene (2 x 10 min.) 96% benzyl alcohol (2 x 8-10 min.), and 70% alkohol (5 min.). After inhibition of the endogenous peroxidase activity in 3% H_2O_2 the sections were washed in distilled water, then digested in 0,4% pepsin in 0.01 N HCl at 37^{0}C for 30 min, and washed again in distilled water. The sections were incubated with the primary antiserum (anti-CD3) in phosphate buffered saline (PBS) for 14-16 hours at 4^{0}C, followed by incubation with biotinylated anti-immunoglobulins and finally with peroxidase-labelled streptavidin in PBS. The immunological reaction was identified by diamino-benzidine. Sections were then counter-stained with Mayer's haematoxylin.

The numbers of CD3 lymphocytes were counted in twenty intestinal villi of the duodenum, jejunum and the ileum, respectively, using the Meopta micrometer system and Biolar microscope (Poland). The areas of the villi epithelium and lamina propria of the dudenum (d), jejunum (j), and ileum (i) were measured from the *lamina muscularis mucosae* to the point of the villi to a height of 25 and a breath of 15 micrometers. The count was done on twenty villi per slide in each part of the intestine at a magnitude of x 500.

Procedure for SEM. Samples of 5 mm^2 were cut from the small intestine (duodenum, jejunum, ileum) and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in a sodium cacodylate buffer. After washing in the sodium cacodylate buffer they were post-fixed in 1% osmium oxide, dehydrated through a graded series of ethanol and acetone, and dried in a critical point dryer. Specimens were then coated with gold and examined in a scaning electron microscope at an accelerating voltage of 20 kV.

Statistics. ANOVA was used to test for significant differences between the two groups of animals.

RESULTS

Under the microscope, layers of *tunica mucosa* and *tela submucosa* were insignificantly thinner in the small intestine of piglets experimentally fed with *L. casei* (Tab. 2.) and *L. sp. strain B-5* (Tab. 4.) than the same layers from piglets in the control groups.

In the experimental group, the number of CD3 cells was significantly lower in the epithelium ($P<0.05$) and in the *lamina propria* ($P<0.01$) of ileal villi after the application of *L. casei* (Fig. 1) compared with the numbers of CD3 cells in the same layers of intestines of the control group (Fig. 2.) (Tab. 3). Similarly CD3 lymphocytes were less often found in the ileal villi of the experimental group fed with *L. sp. strain B-5* compared with the control group (Tab. 4)

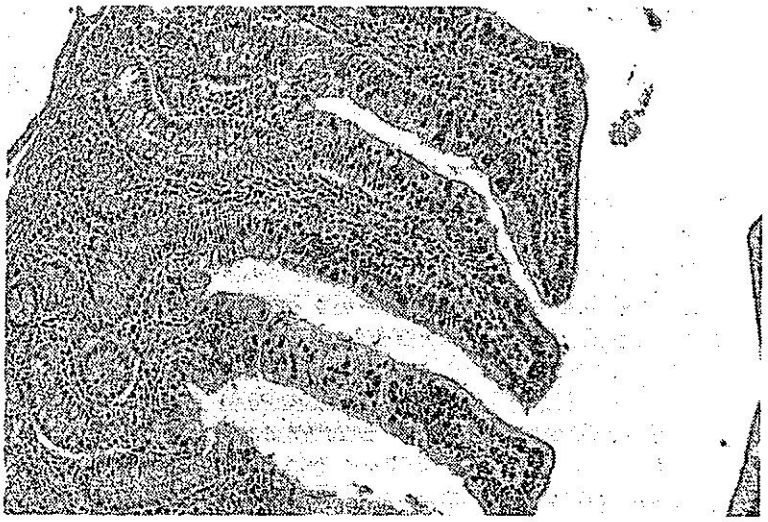

Figure 1. CD3 cells in intestinal villi of ileum after the application of *L. casei* in experimental piglets. (Biotin-streptavidin-poroxidase; x40)

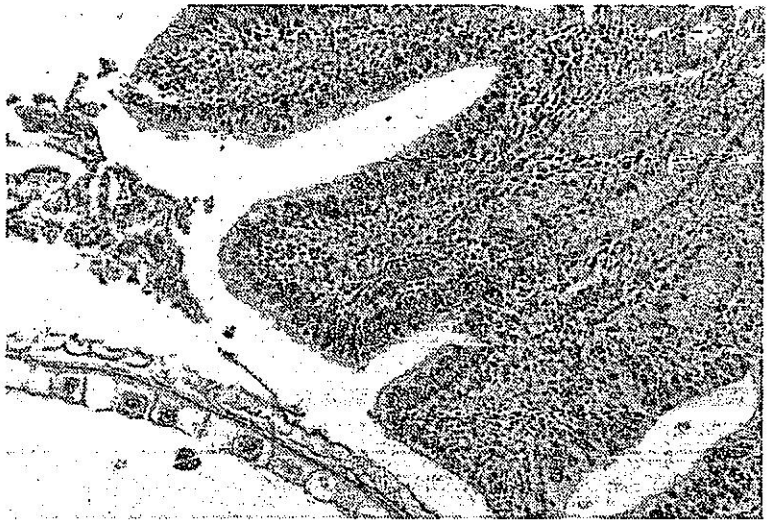

Figure 2: CD3 cells in intestinal villi of ileum in the control group of piglets. (Biotin-streptavidin-peroxidase; x 40)

Table 2. Morphometry of piglet intestinal mucosa after application of *L. casei*

		Experimental group (n_1) mean value (μm) ± SD	Control group (n_2) mean value (μm) ± SD
duodenum	mucosa	30.0 ± 5.22	37.0 ± 5.19
	submucosa	85.1 ± 21.71	94.8 ± 25.05
jejunum	mucosa	31.9 ± 7.98	33.9 ± 0.90
	submucosa	79.8 ± 19.67	106.2 ± 32.80
ileum	mucosa	33.2 ± 11.48	35.7 ± 6.17
	submucosa	82.1 ± 19.46	84.7 ± 18.69

n_1 – 5 piglets; n_2 – 3 piglets;

L. sp. strain B-5 showed adherence to the intestinal epithelium (Figure 3). By contrast, *L. casei* was found not to adhere to the epithelium in the experimental piglets. However, the counts of *Lactobacilli spp.* determined by culture from ileal content and wall (7.973 log 10 ml^{-1} and 4.741 log 10 ml^{-1}) showed a moderate increase compared with the counts of the control group (5.990 log 10 ml^{-1} and 4.193 log 10 ml^{-1}. On the contrary, the counts of *E. coli* of the experimental group in the ileal content and wall were lower (5.321 log 10 ml^{-1} and 2.134 log 10 ml^{-1}) compared with the control group (5.926 log 10 ml^{-1} and 2.474 log 10 ml^{-1}).

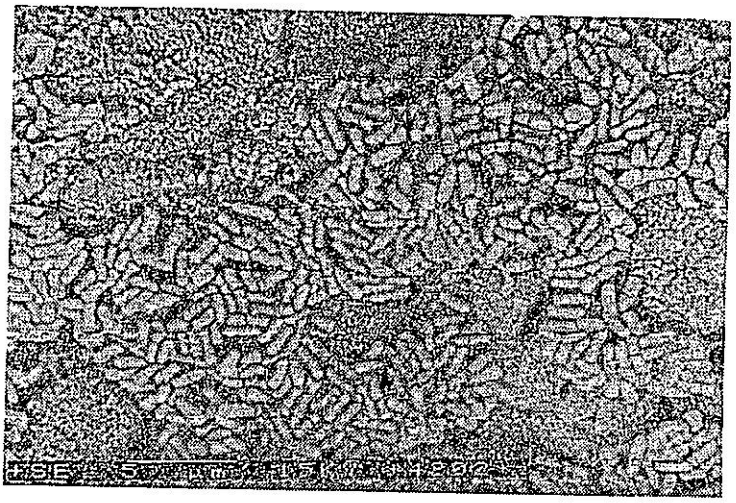

Figure 3. Adherence of *L. sp.* strain B-5 to the intestinal epithelium of the ileum in the experimental piglets (SEM; x 6000)

Table 3. Number of CD3 lymphocytes in intestinal villi after application of L. casei

		Experimental group (n_1) mean value (no.) ± SD	Control group (n_2) mean value (no.) ± SD
duodenum	epithelium	27.0 ± 3.28	26.6 ± 2.63
	lamina propria	13.4 ± 4.27	24.3 ± 7.59
jejunum	epithelium	24.2 ± 8.01	26.5 ± 10.50
	lamina propria	12.4 ± 6.25	8.0 ± 6.00
ileum	epithelium	22.0* ± 4.98	43.0 ± 8.52
	lamina propria	15.0** ± 4.15	30.0 ± 0.86

n_1 – 5 piglets; n_2 – 3 piglets; significant difference from the control group: * – ($p<0.05$); ** – ($p<0.01$)

Table 4. Morphometry of piglet intestinal mucosa after application of *L. sp.* B-5

		Experimental group (n_1) mean value (μm) ± SD	Control group (n_2) mean value (μm) ± SD
duodenum	mucosa	33.9 ± 4.73	35.1 ± 2.13
	submucosa	91.6 ± 13.96	91.9 ± 12.57
jejunum	mucosa	29.1 ± 3.99	32.0 ± 4.83
	submucosa	84.1 ± 17.78	95.2 ± 13.53
ileum	mucosa	26.1 ± 3.50	27.8 ± 4.91
	submucosa	86.7 ± 13.81	96.0 ± 9.05

n_1 – 7 piglets; n_2 – 4 piglets;

Table 5. Number of CD3 lymphocytes in intestinal villi after application of *L. sp.* B-5

		Experimental group (n_1) mean value (no.) ± SD	Control group (n_2) mean value (no.) ± SD
ileum	epithelium	16.5 ± 6.36	21.9 ± 5.35
	lamina propria	4.4 ± 2.30	9.4 ± 2.58

n_1 – 7 piglets; n_2 – 4 piglets;

DISCUSSION

Antigens such as *Lactobacilli* that enter by the oral route use the migration of cells of the gut associated lymphoreticular tissue. All the cellular elements involved in the immune response such as macrophages. dendritic cells, B-lymphocytes and regulatory cells (T-cytotoxic and T-helper lymphocytes) are present in the gut (Perdigon and Alvarez, 1992). For this study of CD3 lymphocytes, a polyclonal anti CD3 antiserum was chosen for its cross-reaction with lymphocytes of different kinds of animals (Ramos-Vara et al., 1994; Ševčikova, 1997) and for its commercial availability. It is known that the CD3

component is a part of the TCR (T-cell receptor) complex and it expresses CD4 and/or CD8 markers (Terner and Owen, 1993).

The histological sections made from the small intestine of experimental piglets fed with *L. casei* showed a significantly lower number of CD3 cells in the epithelium ($P<0.05$) and lamina propria ($P<0.01$) of ileal villi compared with the control group. Similarly, an insignificantly lower number of CD3 T-cells was found in the epithelium and lamina propria ileal villi of piglets after administration of *L. sp. B-5* (16.5 and 4.4) compared with piglets without application of *L. sp. B-5* (21.9 and 9.4).

Campbell (1986) also observed numerous changes of the lymphoid cells in the mouse gut lamina propria. This author found a decrease in the number of lymphoid cells after a previous temporary increase on day 5 of the experiment. In our experiment, piglets were killed after 28 days of twice-daily applications of *Lactobacilli.* It can only be supposed that any temporary increase in CD3 T-cells in our case was reversed by a decrease in this lymphocyte subpopulation. The decrease in CD3 T-cells can be considered a negative effect of long-term *Lactobacillus* application.

Merely morphometrical intestinal changes in the experimental group copared with the control animals can be associated also with the prolonged administration of the lactic acid bacteria.

The influence of the intestinal flora on the structure and functioning of the gut is of fundamental importance. Intestinal weight, lymphocytes, plasma cells and macrophages in the lamina propria are reduced in germ-free animals and increase when enteric flora is introduced. The gut microflora, especially gram-negative bacteria, play a significant role in the maintenance and induction of oral tolerance (Perdigon et al., 1995). Both active suppression and clonal anergy are suggested to be mechanisms of oral tolerance, depending on the dose of antigen fed (Chen et al., 1995). This author reports that oral antigen can delete antigen-reactive T-cells in Peyer's patches, in mice transgenic for the ovalbumin-specific T-cells receptor genes. The deletion was mediated by apoptosis, and was dependent on dosage and frequency of feeding. Their findings demonstrate that orally administered antigen can induce tolerance not only by active suppression and clonal energy but by extrathymic deletion of antigen-reactive Th1 and Th2 cells at very high doses.

On the other hand the activation of specific immune responses after short-term oral administration of host specific or non-host specific lactic acid bacteria to new-born animals is suggested by the increase in antibody formation (Namba et al., 1981). Specific antibodies form complexes with antigens which are phagocytic and eliminated or they bind to the cell membrane and thus activate suppressor T-cytotoxic lymphocytes and their suppression factors.

Epithelial association appears to be an important factor in the colonisation of the piglet's digestive tract (Pedersen and Tannock, 1989). In our experiment we found adherence of *L. sp. B- 5* to the intestinal epithelium, but no significant differences in the morphometry of piglet intestinal mucosa nor in the influx of

CD3 T-cells between this adherent and non-adherent strain, *L. casei* were observed. Although some authors have suggested the use of certain *Lactobacilli* as immunopotentiators (Kato et al., 1984; Yasutake et al., 1984), the elucidation of the dose for maximum effect, the host-specific and the most active strains as well as the time of administration is required.

According to Tannock et al. (1982) and Cole and Fuller (1984(the inoculation of newborn animals with *Lactobacilli* strains that colonize squamous epithelia could be more protective in ensuring the establishment of beneficial microflora in the digestive tract tham the repeated administration of probiotics to adult animals. Also, Pedersen and Tannock (1989) report that any contribution that *Lactobacilli* make to the well-being of the piglets occurs during the first week of life, when normal physiology and resistance to disease are developing.

We found that twenty-eight days application of *L. casei* significantly, and *L. sp B-5* insignificantly, decreased the number of CD3 cells in the ileal villi and caused moderate modifications in the small intestinal mucosa. It is supposed that the logn-term presence of the same *Lactobacillus* strains on the mucosal surface might have a negative influence on the density of immunocompetent cells in the intestinal mucosa. The period of administration should be taken into account in order to prevent undesirable effects in the reaction of the gut's immune system. Some models of oral tolerance are associated with the production of anti-inflammatory cytokines, others with the production of pro-inflammatory cytokines. However, in high dose regimens, the mechanisms of tolerance are believed to be energy/deletion rather than immune deviation (Thomas et al., 1995). Additional experimental studies examining more detailed identification of leukocyte surface antigens and also production of cytokines can contribute to a better understanding of this model of oral tolerance.

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MORFOLOŠKI PARAMETRI I ANALIZA CD3 LIMFOCITA U CREVNOJ SLUZOKOŽI PRASADI NAKON PRIMENE *LACTOBACILLUS*

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SADRŽAJ

U radu je ispitivana distribucija i broj CD3 imunokompetentnih ćelija u sluzokoži tankih creva kao i debljina lamine proprie kod prasadi posle dugotrajne primene nespecifičnnih Lactobacila *(L casei* ili *L. sp. soj B-5).*

Imunohistohemijskim ispitivanjem utvrđen je manji broj CD 3 ćelija u crevnim resicama i signifikantno manji u epitelu ($P<0.05$) i u lamini propri i ($P< 0.01$) ileuma posle primene *L. casei.*

Lamina propria tankih creva bila je tanja kod prasadi eksperimentalne grupe u odnosu na kontrolnu.