

ORIGINAL ARTICLE

Synbiotics suppress the release of lactate dehydrogenase, promote non-specific immunity and integrity of jejunum mucosa in piglets

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ABSTRACT

The aim of our experiment was to study how synbiotics are able to deal with the problems of post-weaning piglets. *Lactobacillus plantarum* – BiocenolTM LP96 (CCM 7512), *Lactobacillus fermentum* – BiocenolTM LF99 (CCM 7514) and flaxseed (rich in n-3 polyunsaturated fatty acids) were administered to 36 conventional piglets from a problematic breed with confirmed presence of enterotoxigenic *Escherichia coli* and *Coronavirus*. The experimental piglets were supplied with probiotic cheeses and crushed flax-seed in the period starting 10 days before weaning and lasting up to 14 days post-weaning. Piglets in the control group were supplied only control cheese. The impact of such additives on the release of lactate dehydrogenase (LDH; spectroscopic and electrophoretic assay), alteration of immunity (index of metabolic activity), jejunum histology (light microscopy), and health of conventional piglets from a problematic breed (monitoring of hematology, consistency and moisture of feces and body temperature) were examined. We found significant decrease in LDH leakage in the blood serum and tissue extracts, indicating better cell membrane integrity in the individual organs of animals. Probiotics and flaxseed applied together seem to be a good source of nutrients to improve the immune status and the integrity of jejunum mucosa during infection.

Key words: gut histology, lactate dehydrogenase, non-specific immunity, piglet, synbiotics.

INTRODUCTION

Diarrheic syndrome in weanlings is a serious health and economic problem in livestock production due to high morbidity and mortality. Nutrition and feeding of pigs, together with other environmental factors (breeding, reproduction, health status, rearing management) are among the economically most important factors influencing animal performance parameters and the overall economics of pig farming.

Health and survival of neonatal piglets depend on the ability of intestinal mucosa to act as an effective barrier against various infectious agents. Enterotoxigenic *Escherichia coli* (ETEC) is the major reason for diarrhea in sucklings and weanlings, leading to excessive loss of fluids and electrolytes, resulting in profuse diarrhea, usually without histological lesions (Wilson & Francis 1986; Francis 2002). Infections caused by *Coronavirus* (Porcine epidemic diarrhoea virus, PEDV; Mole 2013) are among the other diseases causing diarrhea and vomiting in this breed of piglets. Inflammation caused by ETEC may be

effectively inhibited by the application of suitable immunomodulatory agents demonstrated in numerous experimental studies of piglets (Shirkey *et al.* 2006; Tohno *et al.* 2006; Walsh *et al.* 2008; Mizumachi *et al.* 2009; Chytilová *et al.* 2013). Diet supplementation with probiotics has been shown to: (i) reduce the frequency of post-weaning diarrhea (Manner & Spieler 1997); (ii) play an important role in the intestinal barrier against inflammatory bowel disease (Laukoetter *et al.* 2008); and (iii) improve growth performance (Scheuermann 1993) in piglets.

Furthermore, a strong immunomodulatory effect is also assigned to the n-6 and n-3 polyunsaturated fatty acids (PUFAs). The group of n-6 PUFAs have the ability to activate the immune system and act more as pro-inflammatories, whereas n-3 PUFAs have a significant

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anti-proliferative and anti-inflammatory effect (Yaqoob 2004). The growth of pigs may be enhanced by the intake of extra n-3 PUFAs (Nguyen *et al.* 2003), which have the capability to improve cell membrane fluidity (Stulnig *et al.* 2001; Russo 2009).

The recent ban on the use of natural feeds has put pressure on the development of new feeding strategies and formulations to support performance and gut health. Many studies have demonstrated mostly microbiological and immunological improvements of ongoing infections caused by the additives mentioned above. The aim of our study therefore was to examine the effect of synbiotics through flaxseed feed supplementation in combination with *Lactobacillus plantarum* – BiocenoI™ LP96 (CCM 7512) and *Lactobacillus fermentum* -BiocenoI™ LF99 (CCM 7514), on the activity of lactate dehydrogenase (LDH), alteration of immunity, jejunum histology and health of conventional piglets from a problematic breed.

MATERIALS AND METHODS

Animals, housing and diets

The experiments with piglets (weanlings) were performed at the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine and Pharmacy (UVMP) in Košice, Slovak Republic. The experiments were approved by the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2519/10–221) and the animals were handled in a humane manner in accordance with the guidelines established by the relevant commission. The experimental animals were housed in stainless steel cages fitted with a slatted floor strewn with ¾ insulating rubber and ambient temperature of 20–22°C. The animals were divided into two groups: control C ($n = 18$, control cheese, sprinkle on the surface of feed) and LF group ($n = 18$, probiotic cheeses + crushed flaxseed). Throughout the study, the animals were fed diets mixed for early weaning of piglets OŠ-02 (Spišské Vlachy, Slovak Republic; Table 1) and had *ad libitum* access to water.

The feed mixture was supplemented with crushed flaxseed (cultivar Flanders, AGRITEC, Czech Republic) as a source of PUFAs at a concentration of 10%. The fatty acid composition (percentage) of flaxseed was as follows: lipids (dry matter (DM) basis) – 45.78; palmitic FA (C16:0) – 5.1; stearic FA (C18:0) – 3.7; oleic FA (C18:1) – 18.4; linoleic FA (C18:2) – 16.1; linolenic FA (C18:3) – 56.8. In the period starting 10 days before weaning and lasting up to 14 days post-weaning, the experimental piglets in group LF were supplied with probiotic cheeses at a dose of 4 g/animal/day for each cheese, and in the same period the feed of group LF was supplemented with crushed flaxseed. Piglets in control group C were supplied control cheese at a dose of 8 g/animal/day.

Probiotic bacteria

The *Lactobacillus* probiotic strains were isolated in the laboratory of the Institute of Microbiology and Gnotobiology, UVMP in Košice, Slovak Republic. The *Lactobacillus plantarum* – BiocenoI™ LP96 (CCM 7512) strain originated from the gut contents of healthy suckling piglets. This strain was characterized by strong adherence to the epithelial cells from the porcine intestine, by inhibitory activity against *E. coli* O8:K88ab:H9 under *in vitro* conditions, and by production of hydrogen peroxide (Nemcová *et al.* 1997). The *Lactobacillus fermentum* – BiocenoI™ LF99 (CCM 7514) strain was isolated from the gastrointestinal tract of adult chickens. This strain was characterized by the growth in the presence of bile acids and gastric juice, sensitivity to antibiotics, inhibitory activity against *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Düsseldorf, and aggregation and co-aggregation ability (Nemcová *et al.* 2003).

Cheddar cheese (chemical composition per 1 kg: proteins 23.8%, sugars 2.8%, lipids 30.1%, metabolisable energy 1.62 MJ) was used as a vehicle for the probiotic strains. The probiotic cheeses contained probiotic strains (each cheese contained one probiotic strain) at 1×10^9

Table 1 Ingredients (%) and chemical composition (g/kg dry matter) of the basal diet for early weaned pigs

Ingredient	OŠ - 02	Chemical composition	OŠ - 02	Chemical composition	OŠ - 02
Wheat	27.6	Crude protein (g)	187.9	Vitamin B ₁₂ (µg)	26.4
Extracted soybean meal	22.0	Metabolizable energy (MJ)	12.8	Ca (g)	7.5
Maize (8.4% crude protein)	19.7	Fiber (g)	38.3	P (g)	6.2
Barley	17.0	Lysine (g)	11.6	Na (g)	1.9
Oat (11.2% crude protein)	5.0	Methionine and cysteine (g)	6.4	Cu (mg)	10
Calcium carbonate	1.1	Threonine (g)	7.6	Fe (mg)	163.4
Monocalcium phosphate	1.0	Tryptophan (g)	2.3	Zn (mg)	125.8
TKP OS – M var. B 0.5% vitamin/mineral premix	0.4	Choline (mg)	1352	Mn (mg)	72.7
Sodium chloride	0.4	Vitamin A (IU)	11530		
L-lysine-HCl	0.35	Vitamin D ₃ (IU)	1500		
L-threonine	0.35	Vitamin E (mg)	68.7		
DL-methionine	0.1	Vitamin B ₂ (mg)	7.1		

colony-forming units (CFU)/g of cheese (referred to as probiotic cheeses). The probiotic bacteria were added to the cheese milk together with 2% starter culture (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*) during typical Cheddar cheese production. The cheese that was used as a control was similar to cheddar cheese, but without the probiotic strains (referred to as control cheese).

Clinical observations and blood sampling

During the experimental period the piglets all underwent clinical observation. Health data were recorded twice daily (at 08.00 and 15.00 hours), specifically, body temperature (BT), consistency of feces (F) and moisture of feces (MF). Samples of feces were assessed visually using a scale from 1 to 5, where the number 1 meant solid feces, 2 paste, 3 sparse, 4 hydrous and 5 feces with an admixture of blood or mucus. The moisture of feces was determined by drying a sample of feces at 80°C to constant weight.

Blood samples from piglets were taken from the *plexus venosus suborbitalis* on Day 0 (day of weaning; $n = 6$), Day 7 ($n = 6$) and Day 14 ($n = 6$) after weaning. Blood serum was separated from blood samples by centrifugation at 4°C and $1095 \times g$ for 10 min for determination of total LDH (TLDH) and its isoenzymes (LDH 1 – LDH 5) expressed in $\mu\text{kat/L}$ and for measurement of leukocytes. On sampling days, the piglets were humanely sacrificed (euthanased) using T61 a.u.v. (Intervet International BV, Boxmeer, Netherlands) intracardiac administration of 1 mL/kg/head. Heart, liver and skeletal muscle were collected from each pig and processed (tissue homogenates or extracts) until other analyses were done. The samples were cut into small pieces, and washed in buffered saline to remove excess blood and connective tissue. Two grams of tissue were then homogenized in 20 volumes of cold buffer (0.05 mol/L Tris–HCl buffer, pH 7.3) in line with Heinová *et al.* (1999). Protein concentration was measured in accordance with Bradford (1976) and TLDH and its isoenzymes (LDH 1 – LDH 5) were expressed in international units per gram of protein (IU/g).

Hematology

Blood plasma was collected into tubes containing menadione ethylenediaminetetraacetic acid (K_3EDTA) for hematological analysis using a BC-2008 VET automatic analyzer (Mindray, Shenzhen, China).

Measuring of total LDH activity in blood serum and tissue extracts

The concentration of total LDH ($\mu\text{kat/L}$ or IU/g) activity was assessed using commercial diagnostic kits (Randox, Crumlin, UK) with an Alizé automatic biochemical analyser (Lisabio, Pouilly-en-Auxois, France) at laboratory temperature. The measurement was conducted using light absorbance at 340 nm.

Electrophoretical analysis of LDH isoenzyme fractions in blood serum and tissue extracts

For electrophoretic study, 10 μL of the blood serum and culture supernatant was used for each separation. A Hydrasys device (Sebia, Lisses, France) was used for the determination of LDH isoenzyme activities. The samples were separated using commercial Hydragel 7 ISO-LDH electrophoretic kits (Ecomed, Žilina, Slovak Republic) on alkaline-buffered (pH 8.4 ± 0.05) agarose gels (0.8g/dL). The dried gels were prepared for visual examination and densitometry to obtain accurate relative quantification of individual zones. The image of the electrophoretic migration was scanned by light transmission and automatically converted into an optical density curve presentation. Then photographs of the gels were taken. Qualitative evaluations of the gels were done directly from the electrophoretograms and the densitometric curves of the separations were created by means of Epson Perfection V 700 Photo densitometer scanning at 570 nm and evaluated using PHORESIS software (Version 5.50, 2009, Sebia, Lisses, France).

Analysis of non-specific immunity

The iodinitrotetrazolium test (INT) – 2-(4-iodophenyl)-5-fenyltetrazolium chloride – INT (Erba Lachema, Brno, Czech Republic) – was carried out on microscale in accordance with the modifications made by Procházková *et al.* (1986). The functional ability of phagocytes of metabolic processes occurring in phagocytosing leukocytes (production of microbicidal substances, particularly H_2O and O_2) based on the ratio between spontaneous activity and the activity after stimulation by zymosan (Sigma, St. Louis, MO, USA), that is, the index of metabolic activity (IMA), was assessed.

Histology of jejunum

Excisions from the jejunal mucosae (collected from piglets on Day 0 and Day 7) of 1 mm³ size were fixed by immersion in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide (both in 0.15 cacodylate buffer, pH 7.2–7.4). After dehydration in acetone, the excisions were transferred to propylene oxide, and embedded in Durcupan ACM (Fluka Chemie AG, Buchs, Switzerland). Semi-thin sections (250 nm) of specimens processed for transmission electron microscopy were cut on an LKB Nova ultramicrotome, stained with toluidine blue (Sigma-Aldrich, Bratislava, Slovak Republic) and examined under an Axio Lab. A1 light microscope (Zeiss, Jena, Germany) at 400 \times magnification.

Statistical analysis

The data were assessed using one-way analysis of variance with Tukey's *post hoc* analysis (GraphPad Prism 3.0 for Windows; GraphPad Software, San Diego, CA, USA). Values listed in the tables and figures are the

average values obtained from six samples. All data are means with standard error of mean (SEM). Differences within the group are marked with superscript letters (a, b, c) and considered to be significant at levels of $a = P < 0.05$, $b = P < 0.01$ and $c = P < 0.001$.

RESULTS

Health status of piglets

Before the experiment was done, diagnostic analysis was conducted in the herd as kept by the animals' owner and samples of biological material were collected for laboratory analyses (bacteriological examination of rectal swabs, virological and parasitological examination of feces, hematological and biochemical examination of blood) which allowed us to diagnose: hypoproteinemia, lymphocytic leucocytosis and increased activity of bilirubin and enzymes (alanine aminotransferase, gamma-glutamyltransferase). Infection with enterotoxigenic *E. coli* (Institute of Microbiology and Immunology, UVMP, Kosice, Slovak Republic) and *Coronavirus* (Vetservis, s.r.o., Nitra, Slovak Republic) was confirmed in fecal samples.

During the experiment selected health parameters of the piglets were recorded as shown in Table 2. The consistency of feces (F) in the control group of piglets deteriorated significantly ($P < 0.001$) during the experimental period, whereas in the experimental group the consistency had a more settled character. Moreover, the piglets with addition of synbiotics had better progress of infection in general. We noticed significant increase in the leukocyte count and hemoglobin ($P < 0.01$) after 14 days of the supplementation period.

Leakage of LDH after feeding with synbiotics

In the blood serum in the experimental group of piglets, significant decrease in TLDH concentration was noticed on Day 7 ($P < 0.001$) and Day 14 ($P < 0.01$) compared to Day 0 (Table 3). In the control group, without addition of probiotics and flaxseed, significant decrease was recorded on Day 14 ($P < 0.001$) compared to Day 7 after weaning. Average values of TLDH activity on Day 7 were significantly lower ($P < 0.01$) in the experimental group compared to control. Significant differences were also observed in the concentration of LDH 1 to 5 isoenzymes. All isoforms exhibited a decreasing trend at the end of the feeding period (Day 14) in comparison with the beginning of the supplementation period, and at different significance levels (see Table 3).

Tissue extracts from the piglets' hearts (Table 4) showed significant differences between the observed groups in the concentrations of TLDH ($P < 0.05$) and LDH 1 ($P < 0.01$) on Day 14 after weaning. Overall, the excretion of LDH (TLDH and LDH 1–5) was markedly reduced on Day 14 in the experimental group compared to control.

Table 2 Changes in health parameters in control and experimental groups of infected piglets

Parameter	One-way analysis of variance						t-test		
	C						P-value (C vs. LF)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Ec (T/L)	6.087 ± 0.1755	6.388 ± 0.3679	6.713 ± 0.182	6.203 ± 0.1196	6.149 ± 0.8431	7.063 ± 0.1898	NS	NS	NS
Le (G/L)	22.57 ± 3.818	25.67 ± 2.186	20.05 ± 3.45	18.63 ± 1.700 ^b	19.55 ± 2.148 ^b	31.35 ± 2.160 ^b	NS	NS	$P < 0.01$
Hg (g/dL)	10.03 ± 0.1202	10.58 ± 0.4826	9.6 ± 0.2556	10.13 ± 0.2499	9.75 ± 1.166	11.23 ± 0.3095	NS	NS	$P < 0.01$
Ht (L/L)	0.3867 ± 0.01978	0.3617 ± 0.01621	0.3267 ± 0.01202	0.315 ± 0.008466	0.29 ± 0.04747	0.355 ± 0.01118	$P < 0.01$	NS	NS
MCV (fL)	59.5 ± 1.607	51.83 ± 4.969	47.17 ± 2.182	50.98 ± 1.372	46.98 ± 3.724	50.42 ± 1.165	$P < 0.01$	NS	NS
BT (°C)	39.02 ± 0.09458	39.07 ± 0.2985	38.62 ± 0.08724	39.1 ± 0.08563	38.87 ± 0.1308	38.73 ± 0.1764	NS	NS	NS
F	1 ^c	2.667 ± 0.2108 ^c	2.833 ± 0.1667 ^c	1.333 ± 0.2108	1.833 ± 0.4773	1.667 ± 0.4216	NS	NS	NS
MF (%)	75.6 ± 3.305	87.86 ± 0.9942 ^b	70.74 ± 4.935 ^b	77.87 ± 2.823	87.48 ± 2.545 ^a	75.81 ± 3.527 ^a	NS	NS	NS

Note: C, control group; LF, group treated with probiotics and flaxseed; Day 0–14, study day after weaning; Ec, erythrocytes; Le, leukocytes; Hg, hemoglobin; Ht, hematocrit; MCV, mean cell volume; BT, body temperature; F, consistency of feces; MF, moisture of feces; data are means ± SEM; ^{a,b,c}Mean values in rows with same superscript letters are statistically significant at the levels of $a = P < 0.05$; $b = P < 0.01$; $c = P < 0.001$.

Table 3 Changes in concentration of total lactate dehydrogenase (TLDH) and its isoenzymes (LDH 1–5) in blood serum of piglets

Parameter ($\mu\text{kat/L}$)	One-way analysis of variance					t-test						
	C					LF						
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
TLDH	17.03 ± 0.9715 ^b	22.37 ± 1.477 ^{b,c}	13.93 ± 0.5308 ^c	22.62 ± 1.089 ^{b,c}	12.63 ± 1.837 ^c	15.2 ± 0.5657 ^b	P < 0.01	P < 0.01	P < 0.01	NS	P < 0.01	NS
LDH 1	8.047 ± 0.3712	8.577 ± 0.4095 ^a	7.15 ± 0.1371 ^a	8.93 ± 0.4511 ^a	6.725 ± 0.8292 ^a	7.317 ± 0.3004	NS	NS	NS	NS	NS	NS
LDH 2	1.72 ± 0.0728 ^c	3.72 ± 0.3133 ^c	1.745 ± 0.08682 ^c	4.372 ± 0.2318 ^c	1.712 ± 0.2188 ^c	2.388 ± 0.1499 ^c	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01
LDH 3	2.152 ± 0.09097 ^c	3.593 ± 0.2983 ^c	1.595 ± 0.1336 ^c	4.36 ± 0.2229 ^c	1.65 ± 0.3097 ^c	2.393 ± 0.1226 ^c	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01
LDH 4	3.14 ± 0.3129	3.47 ± 0.5193	2.155 ± 0.1624	2.892 ± 0.2479 ^{ab}	1.5 ± 0.29 ^b	2.06 ± 0.04967 ^a	NS	NS	NS	NS	P < 0.01	NS
LDH 5	1.973 ± 0.529	3.008 ± 0.4364 ^d	1.287 ± 0.1302 ^a	2.067 ± 0.3226 ^a	1.038 ± 0.2361 ^a	1.045 ± 0.05233 ^a	NS	NS	NS	NS	P < 0.01	NS

Note: C, control group; LF, group treated with probiotics and flaxseed; Day 0–14, study day after weaning; data are means ± SEM ($\mu\text{kat/L}$); ^{a,b,c,d} mean values in rows with same superscript letters are statistically significant at the levels of a = P < 0.05; b = P < 0.01; c = P < 0.001.

Table 4 Changes in concentration of total lactate dehydrogenase (TLDH) and its isoenzymes (LDH 1–5) in hearts of piglets

Parameter (IU/g)	One-way analysis of variance					t-test						
	C					LF						
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
TLDH	19360 ± 2568	29700 ± 4534	27220 ± 2724	14720 ± 4310	31940 ± 7044	15270 ± 2953	NS	NS	NS	NS	NS	P < 0.05
LDH 1	7733 ± 1098 ^d	13170 ± 1708 ^d	12350 ± 1225	5351 ± 1385	13750 ± 3894	6208 ± 1435	NS	NS	NS	NS	NS	P < 0.01
LDH 2	4183 ± 698.5	6090 ± 1283	4828 ± 848.9	2827 ± 1136	7048 ± 1640	3479 ± 737	NS	NS	NS	NS	NS	NS
LDH 3	3826 ± 471.6	4529 ± 808.2	4304 ± 655.1	3042 ± 945.8	5983 ± 1706	2611 ± 430.6	NS	NS	NS	NS	NS	NS
LDH 4	1844 ± 239.5	1931 ± 206.6	2542 ± 656.8	1592 ± 453.4	2512 ± 648.5	1171 ± 261	NS	NS	NS	NS	NS	NS
LDH 5	1774 ± 308.9	3982 ± 955.9	3200 ± 461.5	1910 ± 554.3	2647 ± 419.1	1803 ± 650.9	NS	NS	NS	NS	NS	NS

Note: C, control group; LF, group treated with probiotics and flaxseed; Day 0–14, study day after weaning; data are means ± SEM (IU/g); ^{a,b,c,d} mean values in rows with same superscript letters are statistically significant at the level of a = P < 0.05.

The piglets' livers (Table 5) exhibited markedly decreased activity of TLDH and its isoenzymes on Day 14 after weaning at different levels of significance ($P < 0.05$; $P < 0.01$; $P < 0.001$).

In the skeletal muscles of the piglets (Table 6), we noticed significant decrease in TLDH, LDH 2, 3 and 5 ($P < 0.05$) on Day 14 after weaning when comparing the observed groups. The activity of LDH exhibited a decreasing trend at the end of the feeding period (Day 14).

Status of non-specific immunity

In our experiment, significant increase in non-specific immunity (IMA) was noticed on Day 7 ($P < 0.01$) and Day 14 ($P < 0.05$) after weaning when comparing the observed groups (Fig. 1). Significant increase ($P < 0.05$) was recorded in the index of metabolic activity in the experimental group of piglets.

Light microscopic observation of jejunum

At the beginning of sample collection (Day 0 of the experiment) in the control group, the mucous layer of intestinal villi (Fig. 2) consisted of a single layer of columnar epithelial enterocytes (E) and goblet cells (GC). In the apical part of intestinal villi, small groups of dying enterocytes of irregular shape and with dark cytoplasm were present. Goblet cells did not show significant morphological changes. In the epithelium of intestinal villi numerous intraepithelial lymphocytes were present. Significant leukocyte infiltrations were observed mainly in the connective tissue of lamina propria mucosae. These findings contrasted with the histological sections of jejunum from the experimental piglets, where dying enterocytes occurred sporadically only in the apical part of intestinal villi.

Microscopic changes in the mucous layer of the small intestine epithelium indicated persistent inflammation in the control group of weanlings (Day 7; Fig. 2). Intestinal villi were broad, and low and had strongly deformed shape. The presence of dying enterocytes was enormous, and the sparse connective tissue of lamina propria mucosae was significantly infiltrated by leukocytes, in contrast to the jejunum of the experimental group, where intestinal villi were high, of regular shape, and mucosa of the jejunum did not show any structural changes.

DISCUSSION

Probiotics are live microorganisms which may contribute to the health of the host, when they are administered in appropriate amounts (FAO/WHO 2002). Lactic acid bacteria regulate intestinal microbial homeostasis and the stabilizing function of the gastrointestinal barrier, while expressions of bacteriocins (Mazmanian *et al.* 2008) have immunomodulatory effect (Salzman *et al.* 2003). They may inhibit procarcinogen enzymes and the ability of pathogens to colonize and infect the gut

Table 5 Changes in concentration of total lactate dehydrogenase (TLDH) and its isoenzymes (LDH 1–5) in livers of piglets

Parameter (IU/g)	One-way analysis of variance						<i>t</i> -test		
	C			LF			<i>P</i> -value (C vs. LF)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
TLDH	6197 ± 614.2 ^{a,b}	4023 ± 326.2 ^a	3545 ± 314.5 ^b	3314 ± 342.1	5149 ± 815.1 ^c	1499 ± 157.3 ^c	$P < 0.01$	NS	$P < 0.001$
LDH 1	1898 ± 182.6 ^{a,b}	1372 ± 75.58 ^a	1168 ± 121.3 ^b	833.5 ± 97.33 ^a	2212 ± 548.8 ^{a,b}	427.1 ± 65.05 ^b	$P < 0.001$	NS	$P < 0.001$
LDH 2	1516 ± 153.4 ^b	726.8 ± 136.6 ^b	628.7 ± 144.3 ^b	754.2 ± 97.03 ^a	630.8 ± 137.3	292.7 ± 35.45 ^a	$P < 0.01$	NS	$P < 0.05$
LDH 3	1326 ± 143.3 ^b	678.3 ± 114.7 ^b	596.8 ± 104.4 ^b	749.8 ± 81.73 ^a	669.4 ± 136.4 ^a	313.6 ± 32.82 ^a	$P < 0.01$	NS	$P < 0.05$
LDH 4	1007 ± 153.6	802.6 ± 48.78	649.6 ± 62.88	585.9 ± 72.89	956.3 ± 170.2 ^c	242.6 ± 33.49 ^c	$P < 0.05$	NS	$P < 0.001$
LDH 5	450 ± 64.62	443.4 ± 49.29	502.3 ± 67.81	390.3 ± 49.6	680 ± 184.2 ^a	223.2 ± 15.79 ^a	NS	NS	$P < 0.01$

Note: C, control group; LF, group treated with probiotics and flaxseed; Day 0–14, study day after weaning; data are means ± SEM (IU/g); ^{a,b,c}mean values in rows with same superscript letters are statistically significant at the levels of $a = P < 0.05$; $b = P < 0.01$; $c = P < 0.001$.

Table 6 Changes in concentration of total lactate dehydrogenase (TLDH) and its isoenzymes (LDH 1–5) in skeletal muscles of piglets

Parameter (IU/g)	One-way analysis of variance					t-test			
	C					LF			
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
TLDH	32440 ± 4086 ^c	80740 ± 5418 ^c	69800 ± 7034 ^c	70080 ± 12810	88280 ± 6528 ^a	49330 ± 4323 ^a	P < 0.05	NS	P < 0.05
LDH 1	5186 ± 489.7 ^{a,b}	10260 ± 951.8 ^b	8480 ± 1022 ^a	8288 ± 1391	11450 ± 1282	7416 ± 710.5	NS	NS	NS
LDH 2	4942 ± 508.2 ^{a,b}	9459 ± 905.9 ^b	8962 ± 1156 ^a	7701 ± 1392	7787 ± 1359	5574 ± 556.2	NS	NS	P < 0.05
LDH 3	5961 ± 742 ^c	13920 ± 1263 ^c	13370 ± 1157 ^c	12710 ± 2115	14600 ± 1700	9467 ± 802.9	P < 0.05	NS	P < 0.05
LDH 4	6848 ± 964.9 ^c	18020 ± 1168 ^c	15530 ± 1641 ^c	16050 ± 2918	21730 ± 1792 ^b	11230 ± 1029 ^b	P < 0.05	NS	NS
LDH 5	9507 ± 1534 ^c	29080 ± 1850 ^c	23450 ± 2733 ^c	25320 ± 5233	32710 ± 2301 ^b	15560 ± 1510 ^b	P < 0.05	NS	P < 0.05

Footnote: C, control group; LF, group treated with probiotics and flaxseed; Day 0–14, study day after weaning; data are means ± SEM (IU/g); ^{a,b,c}mean values in rows with same superscript letters are statistically significant at the levels of a = P < 0.05; b = P < 0.01; c = P < 0.001.

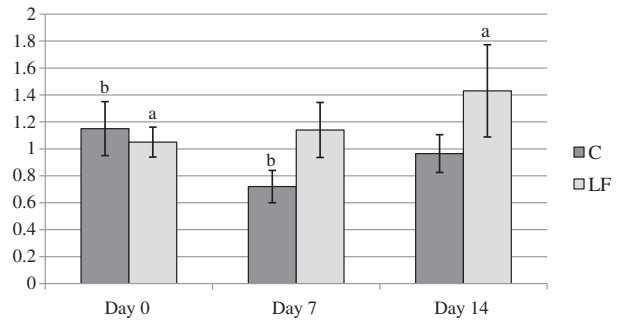


Figure 1 Changes in nonspecific immunity in control and experimental piglets during the experiment. Days 0, 7 and 14, study days of experiment; C, control group of piglets; LF, group of piglets treated with probiotics and flaxseed; Data are means ± SEM; ^{a,b}mean values between columns with the same superscript letters are statistically significant at levels a = P < 0.05; b = P < 0.01.

mucosa (Gill 2003). Probiotic microorganisms act against infections of the gastrointestinal tract by means of their own production of bioactive molecules, such as organic acids, carbon dioxide, hydrogen peroxide, other low molecular weight substances and bacteriocins (Gomes *et al.* 2012). Probiotics are able to displace pathogens such as *Salmonella* spp., *Clostridium* spp. and *E. coli* in the gastrointestinal tract of pigs through the mechanism of competitive inhibition for binding sites (Biernasiak *et al.* 2011).

The efficacy of probiotics may be potentiated by components of natural origin (so-called synbiotics) such as the essential PUFAs, which have impact on the nutrition of piglets (Tanghe & De Smet 2013; Tanghe *et al.* 2014). The presence of PUFAs may positively improve the adaptation of piglets to the rapidly changing diet at weaning (Bomba *et al.* 2005; Marcinčák *et al.* 2009; Li *et al.* 2014). They have direct regulatory action on leukocytes by binding to intracellular receptors or by modifying the release of second messengers (Klasing 1998), and on red blood cell membrane fatty acid composition (Boehm *et al.* 1996).

It is known that LDH is a highly sensitive, but not specific marker of cell membrane disruption. Intracellular LDH (EC. 1.1.1.27), L-(+)-lactate: NAD⁺oxidoreductase leakage is a possible indicator of cell membrane integrity and cell viability (Legrand *et al.* 1992). LDH catalyses the intracellular conversion of pyruvate and lactate and is involved in both the catabolism and anabolism of carbohydrates. In addition to metabolic roles in the cells, it includes a number of other biological processes (Powers *et al.* 1991). The function of specific indicators is performed by LDH isoenzymes, which allow us to identify the damage to cells and tissues by different agents. LDH leakage from various cells (e.g. hepatocytes, monocytes, lymphocytes) into the environment is used as one of the markers of cytotoxicity for monitoring tissue and cell damage in *in vitro* conditions (Kopperschlager and Kirchberger 1996; Šutiaková *et al.* 2004). Moreover,

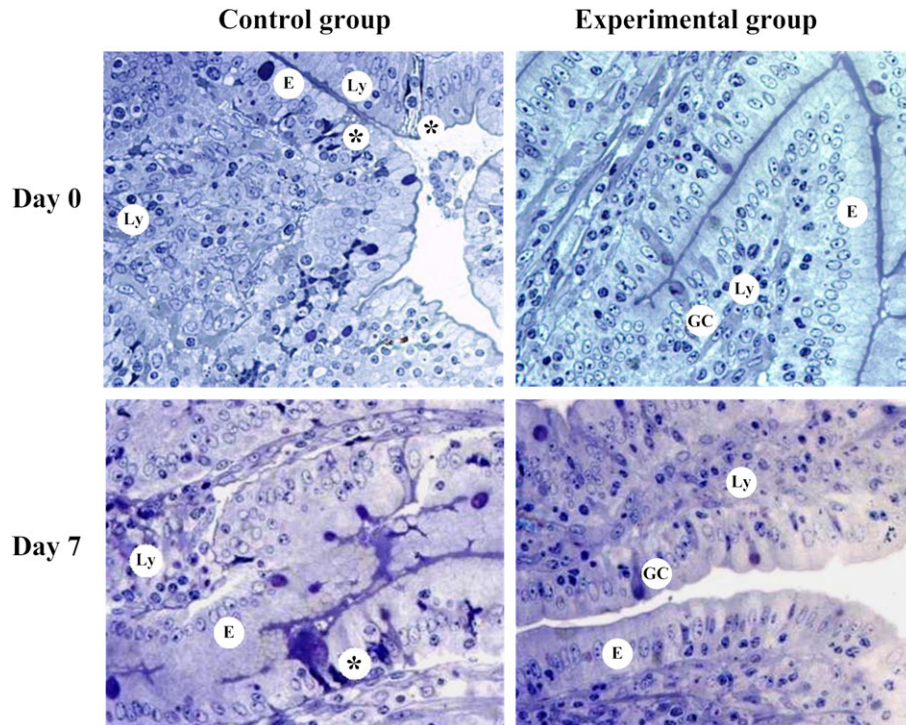


Figure 2 Light microscopy observations of pigs' jejunum. C, control group of piglets; LF, group of piglets treated with probiotics and flaxseed; Ly, lymphocytes; E, enterocytes; asterisk, necrotizing enterocytes; GC, goblet cells.

lactic acid level reflects the quantitative transformation of glycogen, and indicates typical or atypical processes of meat ripening (Koréneková *et al.* 2009).

In our experiment, leakage of LDH into the interstitial space of organs was monitored in blood serum and tissue extracts from the heart, liver and skeletal muscle in piglets with persistent infection. In the group fed with synbiotics (combination of probiotics and PUFAs in the form of flaxseed) we noticed significant decrease in TLDH leakage and isoenzymes typical for individual tissues. Ghanem *et al.* (2005) experimentally infected mice with *Schistosoma mansoni*, and they found that after 14 days of probiotic administration (yogurt containing *L. casei*, *L. plantarum*, *L. reuteri*, *L. acidophilus*), the activity of LDH decreased. Similar results were recorded in our experiment with weanling pigs. The authors described the protective effect of probiotics through non-specific stimulation of the immune system. Otherwise, Rajput *et al.* (2013) described significant increase in concentration of LDH in the blood of pigs given feed supplemented with *Bacillus subtilis*.

The concentration of TLDH and its isoenzymes in the liver of our piglets significantly decreased at the end of the feeding period, which indicated the bioprotective effect of synbiotics on hepatocytes. New studies (Imani Fooladi *et al.* 2013) have confirmed that probiotics have significant effect on the health status of the liver and the ability to treat various liver diseases.

Based on our results, it could be assumed that the temporary increase in TLDH and its isoenzymes in the

skeletal muscle of piglets, which otherwise continuously decreased, was probably due to the ongoing infection. Dauschies *et al.* (2000) noticed subsequent decrease in the activity of TLDH in muscles of calves after experimental infection with *Sarcocystis cruzi*, which may also be affected by enhancing the release of LDH into the bloodstream during ongoing infection. LDH is also produced by the parasites themselves, even though their presence in the blood of the host was not confirmed in this study. The impact of infection on serum LDH release was observed in the study by Nussinovitch *et al.* (2009), who noticed an increase in TLDH and its isoenzymes LDH 4 and 5 in the blood of people with bacterial meningitis.

It is well known that probiotics (Delcenserie *et al.* 2008; Trasino *et al.* 2013) and PUFAs (Harbige 2003) have the ability to improve the immune status of the organism. The index of metabolic activity (IMA) seems to be appropriate for testing the characteristics of non-specific immunity in animals (Spišáková *et al.* 2009; Haladová *et al.* 2011). In our study, the IMA index significantly increased in the group fed with lactobacilli and flaxseed, similar to the study of Wen *et al.* (2011), who applied probiotics alone against rotavirus disease.

The histological sections of jejunum from experimental piglets fed with synbiotics did not show any structural changes, in contrast to the control group of weanlings, where significant damage to intestinal villi was recorded, followed by lymphocyte proliferation. Many authors (Schroeder *et al.* 2006; Chandran *et al.* 2013; Núñez

et al. 2014) have ascertained that probiotics promote the health status of the gastrointestinal tract in experimental animals.

Conclusion

Currently, several studies aimed at improving the problems of pigs after weaning are based on adding health-promoting substances or additives into animal feed. In our study, we point out the beneficial effect of probiotics (*Lactobacillus plantarum* and *Lactobacillus fermentum*) in combination with PUFAs (in the form of flaxseed) on animal health. Better integrity of cell membranes in the individual organs of animals was recorded by monitoring LDH release into the bloodstream and tissue extracts. Probiotics and flaxseed significantly improved the immune response to ongoing infection. In terms of morphology, we can conclude that the additives used had a positive effect on the course of infection and renewal of the enterocyte cell membrane.

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