The effect of probiotics potentiated with polyunsaturated fatty acids on the digestive tract of germ-free piglets

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ABSTRACT: Oil with higher a content of ω-3 polyunsaturated fatty acids (PUFA) was administered for 4 weeks to experimental germ-free piglets and from 21 days of age the piglets were inoculated perorally with probiotic microorganisms *Lactobacillus casei* subsp. *casei*. Control piglets were administered saline solution in identical doses and starting from the fourth week were also supplemented with probiotics. At the age of 28 days, the number of *Lactobacillus casei* subsp. *casei* that had adhered to jejunal mucosa of experimental piglets was significantly higher ($P < 0.05$) compared to that in the control group. The differences in the number of lactobacilli, which colonised mucosa of the ileum and colon of experimental and control piglets, were insignificant. With the exception of the stomach, the pH level in the digestive tract of piglets was lower in the experimental piglets. Significantly higher levels of propionic ($P < 0.05$), acetoacetic and succinic ($P < 0.01$) acids were observed in germ-free experimental piglets in colon. The differences in the level of lactic and acetic acid were insignificant. PUFA-potentiated probiotics positively affected the adhesion of lactobacilli, pH and the level of organic acids in the digestive tract of germ-free piglets. Supplementation of oil containing ω-3 PUFA significantly increased the blood level of α-linolenic, eicosapentaenoic and docosahexaenoic acids in experimental piglets at the expense of arachidonic acid in comparison with the control.

Keywords: potentiated probiotics; supplementation; polyunsaturated fatty acids; piglets

Probiotics potentiated with ω-3 PUFA have recently come into the limelight. There has been an increase in the number of studies dealing with the synergistic effect of prebiotic-potentiated probiotics, the so-called synbiotics. PUFA and their derivatives, eicosanoids, are biologically active substances of lipidic character. They act as local hormones which control important processes in an organism (Dobronova and Sajbidor, 1992).

Mechanisms of their effect in the body and their relationship to thyroid hormones and products of fat metabolism have not been explained sufficiently. Essential PUFA are important precursors of the production of prostaglandins, thromboxans and prostacyclins (McMurray et al., 1983; Das, 2002). There are two groups of PUFA, ω-3 and ω-6. The effect of these two groups on lipid metabolism and the immune system differs (Stenbek, 1984; Vaughn and Reinhart, 1996; Calder, 1998; Kankaanpaa et al., 2004). The ω-3 PUFA exhibit anti-inflammatory and anti-proliferative influences on cells of the immune system. Contrary to that, ω-6 PUFA, through arachidonic acid, have pro-inflammatory and immunoregulative effects (Calder, 1998). An increased supply of ω-3 PUFA decreases the level of triacylglycerols and cholesterol in blood plasma of experimental animals. The level of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in plasma lipids increases at the expense of arachidonic acid (AA) (Herold and Kinsella, 1986; Fritsche et al., 1993; Das, 2002; Kankaanpaa et al., 2004) with a more pronounced effect of DHA compared to that of EPA (Morisaki et al., 1983). The mechanism of action of ω-3 PUFA on plasma lipids has not

been explained sufficiently. It has been assumed that ω-3 PUFA decrease either the synthesis rate of apoprotein B (Nestel, 1986) or the production of very low-density lipoproteins (VLDL) (Harris et al., 1984; Vaughn et al., 1994). The structure of PUFA in immune system cells indicates their potential influence on biological receptors, signal transduction and lymphocyte proliferation (Vaughn et al., 1994; Kelley, 2001).

Probiotic bacteria are known for their action on the host body including its immune system (Fuller, 1992). Lactobacilli are probiotics most frequently used in the food industry and human and veterinary medicine. Despite the considerable body of knowledge about probiotics, the mechanism of their effect has not been explained completely. One of the presumed mechanisms of the inhibitory effect of probiotics on pathogens of the digestive tract is the competition for intestinal mucosa receptors (Stavric et al., 1987; Das, 2002). An important criterion for the selection of lactobacilli for probiotic purposes is their adherence properties (Nemcova et al., 1997). Ringo et al. (1998) observed that PUFA increases the colonisation of fish intestine with lactobacilli.

The aim of our study was to investigate the synergistic influence of the administration of PUFA in combination with *Lactobacillus casei* subsp. *casei* on adherence properties and selected parameters of the intestinal metabolism of germ-free piglets and the effect of potentiated probiotics on the metabolism of plasma lipids in germ-free piglets.

MATERIAL AND METHODS

The experiment was carried out on 8 germ-free piglets, divided to experimental (n = 4) and control (n = 4) groups. The piglets, obtained by hysterotomy, were kept under germ-free conditions and fed a dried-milk formula for 28 days of their life (PMV, Hradec Kralove, Czech Republic).

The chemical composition of milk was as follows (g/kg): proteins 273, fat 253, lactose 383 and Fe 0.004. The dried milk was diluted at a ratio of 1:9. Piglets were allowed to suckle 6× daily, *ad libitum*. Piglets from the control group (C) were supplied saline once a day for 4 weeks at a dose of 0.5–1.0–1.5–1.5 ml. Experimental piglets (E) were administered PUFA (Table 1) once a day for 4 weeks at a dose of 0.5–1.0–1.5–1.5 ml.

Both groups were inoculated perorally with *Lactobacillus casei* subsp. *casei* once a day during the fourth week of their life at a dose of 2 ml (1×10^8/ml). The piglets were slaughtered at the age of 28 days. Immediately after slaughter pH of the stomach content, duodenum, jejunum, ileum and colon was determined and a sample of the intestinal content and intestinal mucosa were taken. The MS-20 pH meter (Laboratorni pristroje, Czech Republic) was used for the determination of pH.

Table 1. Chemical composition of oil with an increased content of ω-3 PUFA

5 g of oil	
Total ω-6 PUFA	0.1 g
Total ω-3 PUFA	1.0 g
Total monounsaturated FA	2.6 g
Total saturated FA	0.9 g
Cholesterol	0.005 g

The level of lactic, acetic, propionic, acetoacetic and succinic acids was determined by capillary isotachophoresis (ITP) using 10^{-2} mol/l HCl + 2.2×10^{-2} mol/l ε-aminocapronic acid + 0.1 methylhydroxyethylcellulose acid (MHEC) as the leading electrolyte and 5×10^{-3} mol/l capronic acid as the terminating electrolyte.

Samples of mucosa taken from the jejunum, ileum and colon were transferred to 0.15 M-PBS (pH 7.2), cooled with water to 4°C for 30 min, washed three times and homogenised in a homogeniser at 5 000 r.p.m. The counts of *Lactobacillus casei* subsp. *casei* were determined on Rogosa agar (Imuna, Sarisske Michalany).

Preparation of samples for gas chromatography (GC)

Blood serum (0.5–1.0 ml) was mixed with 5 ml of the extraction mixture CH_3OH:$CHCl_3$ (ratio 1:2), shaken for 3 min, allowed to stand for 30 min and filtered. 1 ml of saline (0.9% NaCl) was added to the filtrate. After shaking, the mixture was allowed to stand for a minimum of 3 hours or better overnight. The bottom layer was separated in a separating funnel, evaporated until dry on a water bath at 60°C under nitrogen. After evaporation, the residue was extracted quantitatively with 3 ml of *n*-hexane. The solvent was evaporated under nitrogen and the residue was hydrolysed.

Hydrolysis and esterification of lipids

50 µl of benzene was added to the residue in a test tube, mixed with a mixer and 2 ml of 0.5 mol/l KOH in an ethanol-water mixture (ratio 9:1) was added. The test tube was closed, mixed and the mixture was allowed to hydrolyse at 80°C for 1 hour. After cooling to laboratory temperature, 0.5 ml distilled water was added and mixed again. After adding 3 ml *n*-hexane, the hydrolysed lipids were extracted for 5 min by shaking and the content of the tube was centrifuged for 5 min at 5 300 G. After the removal of the upper hexane layer by means of a water pump, the bottom layer was re-extracted with an additional 3 ml of *n*-hexane acidified with 0.35 ml 3 mol/l HCl. Then another 5 ml portion of *n*-hexane was added and after extraction the mixture was centrifuged. The upper extraction phases were transferred to a tube with a Teflon stopper. The sample was prepared for esterification this way. *n*-hexane was evaporated under nitrogen, the dry residuum dissolved in 1 ml of hexane and 0.1 ml of trans-esterificating reagent was added and shaking mixed the mixture. After 20 min, a red layer of reagent sediment formed on the bottom. The mixture was neutralised with 0.7 ml methanolic solution of HCl (after mixing the red colour disappears) and allowed to stand at laboratory temperature for 45 minutes. The upper hexane layer was transferred to a clean tube and hexane was evaporated under nitrogen. The residue was dissolved in 100 µl hexane and injected onto a GC column.

Conditions of GC analysis

Fatty acids were determined chromatographically using a Carlo Erba (Italy) gas chromatograph. The capillary used was 30 m long (2b-WAX), internal diameter 0.53 mm. The stationary phase was polyethyleneglycol, pressure of the carrier gas 0.8×10^5 Pa, flow rate of hydrogen 28 cm^3/min and of air 500 cm^3/min, detector temperature 250°C and the temperature of working flow-through column 180°C. A flame-ionisation detector (FID) was used. The sample volume injected onto the column was 2 µl (approx. 5% solution of methylesters of FA). APEX-CSW1.7 computer software was used for integration.

RESULTS

The counts of lactobacilli that colonised jejunal mucosa of the experimental piglets (5.1 log 10/cm^2) were significantly higher ($P < 0.05$) in comparison with the control (4.55 log 10/cm^2). The numbers of lactobacilli that colonised mucosa of the ileum and colon of experimental (4.44 log 10/cm^2 and 4.95 log 10/cm^2) and control piglets (4.45 log 10/cm^2 and 5.05 log 10/cm^2) differed insignificantly (Figure 1).

The pH of the digestive tract of experimental piglets was insignificantly lower in comparison with control piglets with the exception of the stomach (Figure 2). The biggest difference was observed in the colon (6.18 in experimental and 6.78 in control piglets).

We observed a significantly higher level of propionic acid ($P < 0.05$), acetoacetic and succinic acid ($P < 0.01$) in the digestive tract (colon) of experimental piglets (Figures 3, 4, 5). No significant differences were observed in the level of lactic and acetic acid in the content of digestive tract of these animals. The level of acetic acid in the entire digestive tract of the experimental piglets was higher.

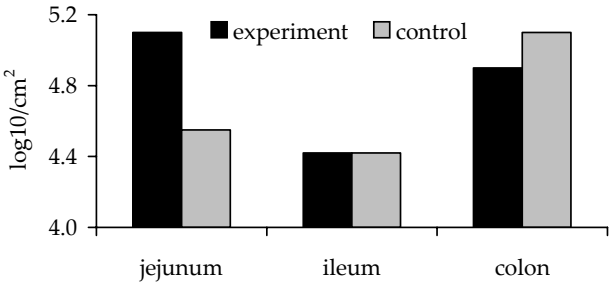

Figure 1. Colonisation of jejunal, ileal and colonic mucosa of germ-free piglets with *Lactobacillus casei* subsp. *casei* after the administration of PUFA

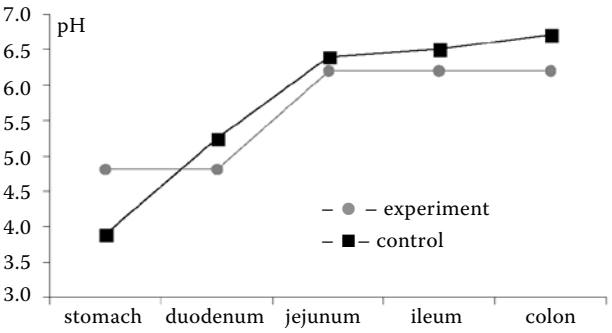

Figure 2. Acidity of the digestive tract content of germ-free piglets after the administration of PUFA

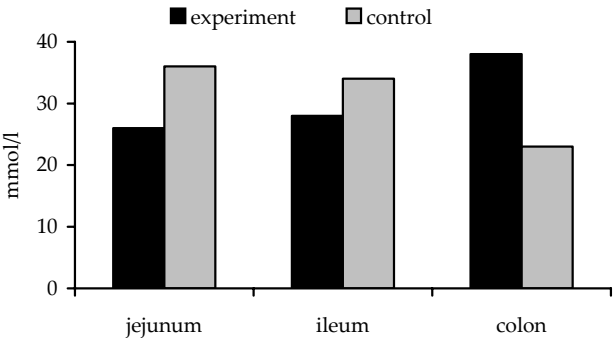

Figure 3. Concentration of propionic acid in the content of the jejunum, ileum and colon of germ-free piglets after the administration of PUFA

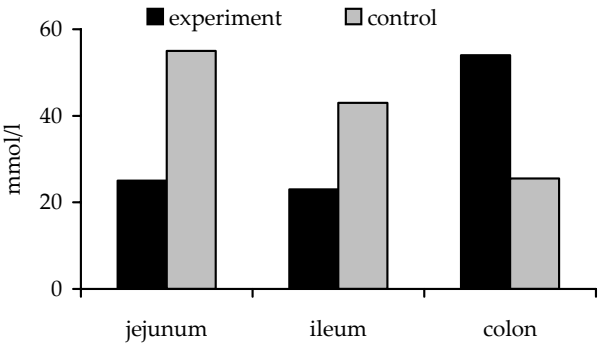

Figure 4. Concentration of acetoacetic acid in the digestive tract of germ-free piglets after the administration of PUFA

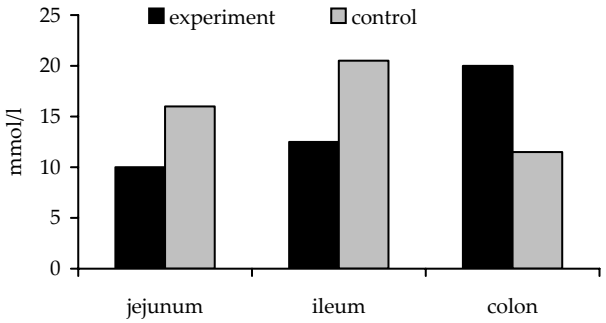

Figure 5. Concentration of succinic acid in the content of the digestive tract of germ-free piglets after the administration of PUFA

Supplementation of oil with a higher content of ω-3 PUFA to experimental piglets increased the level of ω-3 PUFA in their blood compared to the control animals (Figure 6). The blood level of α-linolenic acid (ALA) was higher at the second sampling in experimental piglets (1.53 ± 0.14 g/100 g total FA) compared to control animals in which it reached 1.48 ± 0.15 g/100 g total FA. The level of EPA in the experimental group also increased from 0.42 ± 0.04 g/100 g to 0.68 ± 0.11 g/100 g total FA. A more pronounced significant increase was observed in the concentration of DHA, the level of which was higher in experimental piglets compared to control animals (0.75 ± 0.13 g/100 g and 0.45 ± 0.05 g/100 g total FA, respectively). The concentration of arachidonic acid was lower in the experimental group compared to the control (1.59 ± 0.17 g/100 g and 2.11 ± 0.49 g/100 g total FA, respectively) at the expense of ω-3 PUFA.

DISCUSSION

The results obtained are in agreement with those of Ringo et al. (1998) who observed a positive effect of PUFA on the colonisation of the digestive tract of fish with lactobacilli. Our results point to the favourable effect of PUFA on adhesion of lactobacilli in the jejunum. Such effect was not observed in the lower parts of the digestive tract. The mechanism of the effect of PUFA will be a subject of further

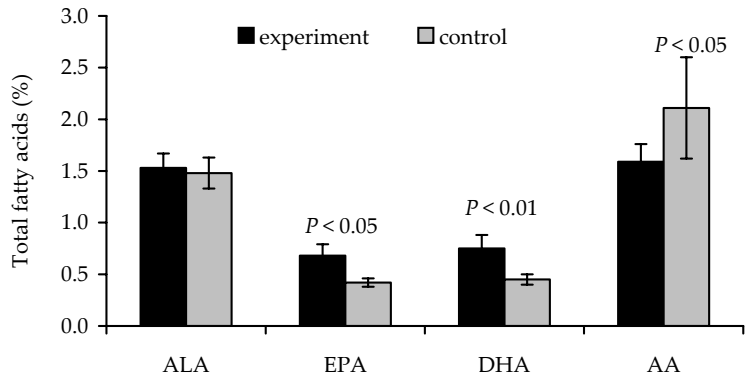

Figure 6. Effect of ω-3 PUFA supplementation on the level of polyunsaturated fatty acids in blood serum of piglets on Day 21 of the experiment. ALA = alpha linolenic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, AA = arachidonic acid

research on the level of the digestive tract microstructures. With regard to the potential location of the positive effect of PUFA on the adhesion of lactobacilli, we assume that this effect was limited due to their metabolism in this section of the digestive tract.

One of the positive effects of probiotics on the intestinal metabolism is based on a decrease in acidity and an increase in the production of organic acids (Piard and Desmazeaud, 1991). Our study showed a decreased pH of the digestive tract content and a significant increase in the level of propionic, acetoacetic and succininc acids. From the viewpoint of optimisation of the digestive environment, one should also evaluate very positively the increased level of acetic acid throughout the digestive tract and of lactic acid in the jejunal content of experimental piglets. According to Wong and Chen (1988), the inhibitory effect of acetic acid is the highest from among all of the organic acids. The inhibitory effect of both acids mentioned increases with decreasing pH and a synergistic effect of lactic and acetic acid on *E. coli* and *Salmonella enteritidis* becomes apparent (Adams and Hall, 1988). Lactic acid decreases the pH of the medium, which results in an increased toxicity of acetic acid.

Supplementation of oil with an increased content of ω-3 PUFA increased the concentration of plasma lipids, ALA, EPA and DHA at the expense of AA concentration in the blood of experimental piglets in comparison with the control group. Comparable results were obtained in conventional piglets (Kastel et al., 1999). The decrease in the level of arachidonic acid in experimental animals has been ascribed to the production of prostaglandins, such as in the experiments by Fritsche et al. (1993). EPA and other ω-3 PUFA displace AA from membrane phospholipids and thus affect the metabolism of lipids. This fact was described in some species of mammals (cats, rodents, rats and some higher species) including humans (Fritsche et al., 1993; Das, 2002).

CONCLUSION

Results of our study indicated a positive effect of PUFA on colonisation of lactobacilli and intestinal metabolism in the digestive tract of germ-free piglets. Of the biochemical blood serum parameters, we observed increased concentrations of ALA, EPA and DHA and a parallel decrease in the level of AA. ω-3 PUFA positively affect the metabolism of fatty acids and synthesis of prostaglandins. This may play an important role in the therapy of inflammatory processes and decreasing the risk of infectious diseases in young suckling animals. It may result in the development of more efficient probiotic preparations – potentiated probiotics for use in human and veterinary medicine.

REFERENCES

Adams M.R., Hall C.J. (1988): Growth inhibition of food borne pathogens by lactic and acetic acids and their mixtures. International Journal of Food Sciences and Technology, 23, 287–292.

Calder P.C. (1998): Fat chance of immunomodulation. Immunology Today, 19, 244–247.

Das U.N. (2002): Essential fatty acids as possible enhancers of the beneficial actions of probiotics. Nutrition, 18, 786–789.

Dobronova S., Sajbidor J. (1992): Biochemical characteristics and physiological effect of eicosanoids (in Slovak). Biochemicke Listy, 54, 1–2.

Fritsche K.L., Alexander D.W., Cassity N.A., Huang S.C. (1993): Maternally-supplied fish oil alters piglet immune cell fatty acid profile and eicosanoid production. Lipids, 28, 677–682.

Fuller R. (ed.) (1992): Probiotics. Chapman and Hall, London. 118 pp.

Harris W.S., Connor W.E., Inkeles S.B., Illingworth D.R. (1984): Dietary omega-3 fatty acids prevent carbohydrate-induced hypertriglyceridemia. Metabolism, 33, 1016–1019.

Herold P.M., Kinsella J.E. (1986): Fish oil consumption and decreased risk of cardiovascular disease: A comparison of findings from animal and human feeding trials. The American Journal of Clinical Nutrition, 43, 566–598.

Kankaanpaa P., Yang B., Kallio H., Isolauri E., Salminen S., (2004): Effects of polyunsaturated fatty acids in growth medium on lipid composition and on physicochemical surface properties of lactobacilli. Applied and Environmental Mikrobiology, 70, 129–136.

Kastel R., Revajova V., Magic D., Pistl J., Levkut M., Bindas L., Sajbidor J., Horvath M. (1999): Effect of oil containing ω-3 polyunsaturated fatty acids (PUFA) on the immune response and growth factors in piglets. Acta Veterinaria Hungarica, 47, 325–334.

Kelley D.S. (2001): Modulation of human immune and inflammatory responses by dietary fatty acids. Nutrition, 17, 669–673.

McMurray C.H., Rice D.A., Kennedy S. (1983): Nutritional myopathy in cattle: From a clinical problem to experimental models for studying selenium, vitamin E and polyunsaturated fatty acid interactions. In: Ssuttle N.F., Gunn R.G., Allen W.M., Linaker K.A., Wiener G. (eds.): Trace Elements in Animal Production and Veterinary Practice. Occasional publication 7. British Society of Animal Production. 61–73.

Morisaki N., Shinomiya M., Matsuoka N., Satto Y., Kumagai A. (1983): *In vivo* effects of cis- 5, 8, 11, 14, 17–20:5 (ω-3) and cis- 4, 7, 10, 13, 16, 19–22:6 (ω-3) on serum lipoproteins, platelet aggregation and lipid metabolism in the aorta of rats. The Tohoku Journal of Experimental Medicine, 141, 397–405.

Nemcova R., Laukova A., Gancarcikova S., Kastel R. (1997): *In vitro* studies of porcine lactobacilli for possible probiotic use. Berliner und Munchener tierarztliche Wochenschrift, 110, 413–417.

Nestel P.J. (1986): Fish oil attenuates the cholesterol induced rise in lipoprotein cholesterol. The American Journal of Clinical Nutrition, 43, 752–757.

Piard J.C., Desmazeaud M. (1991): Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. Le Lait, 71, 525–541.

Ringo E., Bendiksen H.R., Gausen S.J., Sundsfjord A., Olsen R.E. (1998): The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and from faecalia of Arctic charr, *Salvelinus alpinus* (L.). Journal of Applied Microbiology, 85, 855–864.

Stavric S., Gleeson T.M., Blanchfield B., Pivnick H. (1987): Role of adhering microflora in competitive exclusion of *Salmonella* sp. from young chicks. Journal of Food Protection, 50, 928–932.

Stenbek E.I. (1984): The influence of human plasma lipoproteins and fatty acids in immunological reactions. Allergy, 39, 1–11.

Vaughn D.M., Reinhart G.A. (1996): Blood coagulation parameters in dogs. Recent research. Proceedings of the Jams International Nutrition Symposium, 243–255.

Vaughn D.M., Reinhart G.A., Swaim S.F. (1994): Evalution of dietary ω-6 to ω-3 fatty acid ratio on leukotriene B synthesis in dog skin and neutrophils. Journal of Veterinary Internal Medicine, 8, 188–198.

Wong H.C., Chen Y.L. (1988): Effects of lactic-acid bacteria and organic-acids on growth and germination of *Bacillus cereus*. Applied and Environmental Microbiology, 54, 2179–2184.

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