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The Effect of *Lactobacillus paracasei* and Raftilose P95 Upon the Non-specific Immune Response of Piglets.

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This work was focused on the effects of administration of Lactobacillus paracasei *and the fructooligosaccharide (FOS) Raftilose P95 upon the immune system of piglets 10 days after birth and 10 days after weaning under standard farming conditions. The piglets were divided into three groups of 30 animals: F – combined application of* Lc. paracasei *and Raftilose P95; L – application of* Lc. paracasei *only; C – control group without treatment. In the first phase of the experiment, group L revealed a significantly higher value in total absolute leukocyte (Le) counts ($P < 0.01$) as well as in neutrophil counts (Ne), $CD2^+$, $CD4^+$ T lymphocyte and B lymphocyte counts ($P < 0.05$) compared to group F. Significant differences ($P < 0.05$) were obtained in phagocyte activity of leukocytes (%PA Le) and in phagocyte activity of neutrophils (%PA Ne) when groups C and F were compared. At the time of weaning in comparison with newborn piglets a tendency to increase the parameters under observation was seen in group F with a simultaneous decrease of most indices in group L and in the control group. A significant difference ($P < 0.05$) in favour of the combined treatment compared to simple lactobacilli application was observed in absolute numbers of $CD4^+$ T lymphocytes and B lymphocytes. In the second phase of the experiment – application after weaning – the counts of total anaerobes (TAn) and total aerobes (TA) in the faeces of animals receiving the combined treatment (F) were significantly higher ($P < 0.05$) when compared to the lactobacillus-only treated piglets (L) and to untreated piglets (C). In the case of total lactobacilli (TLc), the value in group F was significantly higher ($P < 0.05$) compared to group L ($P < 0.01$) and compared to the control group. Application of FOS Raftilose P95 seems to be a suitable method for fortification of the beneficial effects of probiotic bacteria especially during changes of the intestinal microflora composition, e.g. during weaning.*

Keywords: *Fructooligosaccharides, lactobacilli, immune system, pig*

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INTRODUCTION

The original microflora of the gastrointestinal tract has a pronounced effect upon the anatomical and physiological development of the host. It stimulates the immune system to a faster response to infection and, by bacterial antagonism, it inhibits colonization of the gastrointestinal tract by pathogenic microorganisms. In a stable gastrointestinal ecosystem, all sites are occupied by microorganisms and each new bacterial species from food, water or any part of the host gastrointestinal system either gets attached to or is passed through the digestive tract. However, there is a whole scale of natural and artificial factors that can influence the composition and activity of the digestive tract microflora in piglets: composition of the feed ration; use of antibiotics to stimulate growth; probiotics; specific carbohydrates; organic acids; and fermented feed (Jensen, 1998).

Under normal circumstances, the fetus in the uterus is sterile. During birth it is contaminated with a heterogenous collection of microorganisms from the birth canal and the outer environment surrounding it at that time. In the course of subsequent days many of these organisms are eliminated and quantitative and qualitative changes in the gastrointestinal microflora occur. Lactic acid bacteria and coliforms predominate in the first period of life; later, at the time of weaning, microflora composition changes drastically in favour of the obligatory anaerobic bacteria.

Recently, specific bacterial strains capable of survival in different gut environments have evoked interest. Theoretically these bacteria may react with other ones or with the mucosa cells of the host and thus induce or modulate different biological activities that can be of benefit to the host. Such microorganisms were named probiotics (Fuller, 1989). Intestinal bacteria like lactobacilli can penetrate the intestinal mucosa and survive for many days in the spleen or other organs where they can stimulate phagocyte activity (Deitch *et al.*, 1991).

Oligosaccharides present a new approach to the use of microorganisms in manipulating the gut microflora and intestinal metabolism of monogastric animals. A general feature of the former is that they resist the influence of digestion enzymes and are not directly metabolized by the host. If used at a restricted dose as feed supplement (under 1%), oligosaccharides may increase the weight gain and improve the health state of animals (Monsan and Paul, 1995). A similar approach was chosen in our trials where animals of one of the experimental groups received a combination of Fructooligosaccharide FOS and a probiotic strain. The work was carried out under standard farming conditions and was aimed at comparing the effects of the combined application of FOS and lactobacilli on the immune system with those of the classical monoapplication of a probiotic strain.

MATERIAL AND METHODS

Clinically healthy piglets (n = 90) – landrace, yorkshire and piétrain crossbreds – were included in the experiment and divided into three groups (F, L and C) each counting 30 animals. After birth the piglets were suckling their mothers. After weaning at the age of 36 days they were housed in nine pens with 10 animals each and reared under standard farming conditions. The animals were weighed after birth, after weaning and four weeks after weaning. For 10 days following birth and weaning, respectively, the animals of group F had been given 1.10^9 CFU/g of *Lc. paracasei* in powdered milk at a dose of 2 g per animal and day perorally. Simultaneously they received 3 g of the FOS Raftilose P95 (Raffinerie Tirlemontoise, Belgium) per animal and day. In group L an identical application scheme was employed, however, these piglets received only 1.10^9 CFU/g of *Lc. paracasei* in powdered milk at a dose of 2 g per animal and day. To animals from the control group powdered milk as a placebo was applied. Sampling of the biological material was carried out in 10 randomly chosen animals of each group at 10 and 46 days of age, respectively.

Blood Sampling
For leukocyte (Le), differential blood picture (DBP), percentage of phagocyte activity (%PA) and index of phagocyte activity (IPA) determination blood was taken from the suborbital venose plexus into a heparinized syringe using a blunt needle. T lymphocyte subpopulation (CD2, CD4, CD8), B lymphocyte and macrophage counts were determined by flow cytometry using blood samples taken into syringes containing 1.5% solution of EDTA (0.1 ml EDTA per ml of blood). Concentration of total imunoglobulins was measured in the sera.

The Number of Leukocytes
The number of leukocytes was estimated by a routine haematological method by counting in a Bürker chamber. The total number of leukocytes was expressed as g l^{-1}.

Differential Blood Picture
The differential blood picture was obtained by means of a blood smear stained by a routine panoptic staining method according to Pappenheim using two staining solutions (May Grünwald and Giemsa-Romanowski). Per cent proportion of lymphocytes, monocytes, neutrophil granulocytes, eosinophils and basophils of the total number of 100 cells was determined in each of the samples at magnification 10 × 1.25 × 100.

Concentration of Total Immunoglobulins (TIg)
After precipitation the sera were centrifuged at 3500 rpm. for 30 min Total immunoglobulins were determined turbidimetrically according to the method of McEwan *et al.* (1970).

Per cent of Phagocyte Activity (%PA) and the Index of Phagocyte Activity (IPA)
The parameters mentioned above were determined by means of microspherical synthetic hydrophilic particles (MSHP, Artim Prague, Czech Republic). Within 1 h of sampling, a 0.1 μl aliquot of heparinized blood was incubated with 0.05 μl of the microspherical particles prepared according to the manufacturer's instructions. The incubation lasted for 1 h at 37°C in a plastic test tube with regular mixing. After incubation the blood smear was prepared, dried for 24 h and stained by panoptic staining according to Pappenheim. Then %PA and IPA were determined for 200 cells from all samples using 10 × 1.25 × 100 magnification. Each of the potentially phagocytising cells (monocytes, neutrophil granulocytes, eosinophils, basophils) which contained three and more phagocytized particles was considered to be a phagocytizing cell (Větvička *et al.*, 1982).

Flow Cytometry
Primary monoclonal antibodies (MAbs) CD2, CD4 and CD8 were kindly provided by Dr Trebichavsky (Institute of Microbiology, Czech Academy of Science, Prague, Czech Republic), IgM and SWC3 by Dr Haversson (University of Bristol, UK).

Specificity	MAbs	Isotype	Diluted
CD2	MSA4	IgG2a	1:25
CD4	74–12–4	IgG2b	1:25
CD8	76–2–11	IgG2a	1:25
IgM (μ chain)	K 139.3E1	IgG2a	1:25
SWC3	74–22–15	IgG1	1:25

Fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG (whole molecule) at a dilution 1:128 (Immunochemicals, Sigma, Germany) was used as secondary antibody. Venous blood samples were collected from all animals and put into ethylenediamine

tetra-acetic acid (EDTA). Lymphocytes were separated by Ficoll-Hypaque gradient sedimentation (Boyum, 1974). After the separation, the lymphocytes were washed twice with phosphate-buffered saline (PBS) supplemented with 0.2% sodium azide and once in immunofluorescent medium (RPMI 1640 supplemented with 5% fetal calf serum and 0.2% sodium azide). 50 μl of cellular suspension (1×10^6 lymphocytes in immunofluorescent medium) and 50 μl of specific or control MAbs were mixed with 25 μl of FITC-conjugated anti-mouse immunoglobulins and incubated as described above, in the dark. The working dilution of monoclonal and FITC-conjugated anti-mouse immunoglobulins was determined by titration and the calculation of a resolution index. After being stained, the cells were washed twice in the IFM and once in PBS supplemented with 0.2% sodium azide. The cells were resuspended in 0.5 ml of the same buffer. The FACS system (Becton Dickinson, Germany) was provided with a 15 mW argon ion laser.

Microbiological Examinations

Faecal samples were taken from five animals of each group in order to determine total anaerobe (TAn), total aerobe (TA) and total lactobacilli (TLc) counts. Faeces (lg) was placed in sterile polyethylene Stomacher Lab Blender bag (Seward Medical Limited, London, UK) with 9 ml sterile anaerobic diluent (0.4 g $NaHCO_3$, 0.05 g L-cysteine HCl, 1 ml resazurin (0.1%), 7.5 mineral solution I (0.6% K_2HPO_4), 7.5 ml mineral solution II (1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, 0.12% $CaCl_2$, 0.25% $MgSO_4$) and 84 ml distilled water, pH 6.8) and stomached (Stomacher Lab Blender 80, Seward Medical Limited, London, UK) for 2 min under a CO_2 atmosphere. Series of 10-fold dilutions (10^{-2} to 10^{-8}) were made in the same diluents. From appropriate dilutions, 0.1 ml aliquots were spread onto two nonselective agar plates: trypticase soy blood agar with 10% sheep blood (BBL, Microbiology systems, Cockeysville, USA) for aerobes, Schaedler agar with 1% vitamin K_1 – hemin solution (BBL) for anaerobes and Rogosa agar (Imuna, Šarišské Michalany, Slovakia) for *Lactobacillus*. For anaerobes, plates containing the media were kept in the anaerobic jars for 24 h before analysis. Incubation of the inoculated media for anaerobic bacteria was carried out at 37°C for 4 days under anaerobiosis (Gas Pak Plus, BBL). Plates for the enumeration of aerobic bacteria were incubated for 2 days at 37°C. Colonies were counted and bacteria were Gram stained and visualized under the microscope for morphological characterization. The anaerobic counts were corrected by evaluating aerotolerance of the different colony types.

Statistical Analysis

Results were evaluated statistically by means of the Student's *t*-test.

RESULTS

At 10 days of age when the first phase of the experiment was finished, most parameters peaked in the group receiving only *Lc. paracasei* and in the control group. Total absolute counts of leukocytes, lymphocytes, $CD4^+$ T cells and B cells were highest in group L (Table 1). Significant differences between two experimental groups (L and F) were found in the counts of leukocytes ($P < 0.01$), lymphocytes, neutrophils, $CD2^+$ and $CD4^+$ T cells, B cells and macrophages ($P < 0.05$). The absolute counts of $CD8^+$ T lymphocytes in group L was twice of that of the group receiving combined treatment (L – 17.72 vs F – 8.55 g l^{-1}). The parameters characterising the activity of non-specific phagocytes when comparing both experimental groups were higher in group L (Table 2). Phagocyte activity of leukocytes (%PA Le) and neutrophils (%PA Ne) were significantly lower ($P < 0.05$) in animals of group F compared to untreated animals in the control group. Index of phagocyte activity of leukocytes (IPA Le) and neutrophils (IPA Ne) were also lower in both experimental groups when compared to group C. Concentration of total immunoglobulins (TIg) in the sera was very similar in all three groups (Table 2).

TABLE 1. Total counts of white blood cells (g l^{-1}) after 10 days of application of *Lc. paracasei* and Raftilose P95 after birth (F/1, L/1, C/1) and after weaning (F/2, L/2, C/2)

Group	Leukocytes	Lymphocytes	Neutrophils	$CD2^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	B cells	Macrophages
F/1	17.76 ± 0.5[a]	15.88 ± 0.48[b]	1.60 ± 0.35[c]	13.20 ± 1.04[d]	3.95 ± 1.23[e]	8.55 ± 1.57	1.96 ± 0.85[f]	1.77 ± 0.61[g]
L/1	34.10 ± 6.52[a]	31.04 ± 7.18[b]	2.74 ± 0.87[c]	21.83 ± 3.68[d]	7.69 ± 1.93[e]	17.72 ± 6.54	4.28 ± 1.36[f]	3.35 ± 0.87[g]
C/1	33.80 ± 12.88	29.26 ± 8.85	4.19 ± 1.53	24.54 ± 7.39	6.28 ± 2.41	19.84 ± 6.34	3.56 ± 0.82	3.64 ± 1.52
F/2	34.50 ± 10.0	24.74 ± 7.67	9.24 ± 2.79	16.10 ± 5.62	6.61 ± 1.72[h]	11.66 ± 4.26	3.25 ± 1.05[j]	2.38 ± 0.91
L/2	23.20 ± 7.76	17.34 ± 5.50	5.33 ± 1.98	12.47 ± 3.13	4.01 ± 1.99[h]	8.86 ± 1.73	2.19 ± 1.31[j]	1.71 ± 0.51
C/2	30.70 ± 8.36	23.10 ± 6.41	7.12 ± 2.86	17.08 ± 5.51	4.97 ± 2.56	12.77 ± 3.71	2.43 ± 1.30	2.17 ± 0.91

[a] $P < 0.01$. [b–j] $P < 0.05$.

TABLE 2. Phagocyte activity of leukocytes (%PA, IPA), neutrophils (%PANe, IPANe) and total immunoglobulins concentration (Ig) after 10 days of application of *Lc. paracasei* and Raftilose P95 after birth (F/1, L/1, C/1) and after weaning (F/2, L/2, C/2)

Group	%PA	IPA	%PANe	IPANe	Ig
F/1	2.40 ± 1.12[a]	1.76 ± 0.58	22.63 ± 8.56[b]	2.03 ± 0.93	15.43 ± 2.33
L/1	3.40 ± 1.12	2.40 ± 0.76	28.55 ± 6.49	2.42 ± 0.70	14.09 ± 2.41
C/1	4.20 ± 1.36[a]	2.61 ± 0.97	37.10 ± 10.32[b]	2.59 ± 1.08	13.53 ± 2.18
F/2	10.00 ± 6.00	3.16 ± 2.77	34.86 ± 18.38	3.11 ± 1.05	22.82 ± 3.03
L/2	5.20 ± 2.96	2.17 ± 1.61	23.13 ± 16.32	2.27 ± 1.79	22.18 ± 1.75
C/2	5.75 ± 2.75	1.77 ± 0.20	21.36 ± 6.63	1.72 ± 0.23	24.14 ± 4.21

[a,b] $P < 0.05$.

The total counts of selected groups of bacteria – TAn, TA and TLc – were at approximately the same level in all groups with values oscillating around 9.0 log per g faeces (Table 3).

After finishing the second phase of the experiment, the sampling of the biological material was carried out at age of 46 days after second treatment with probiotics and fructooligosaccharides lasting 10 days. In animals from the group receiving simultaneously *Lc. paracasei* and Raftilose P95 all values were increased when compared to postnatal treatment. The most notable increase was seen in absolute Le counts (17.76 vs 34.50 g l^{-1}), Ne and $CD4^+$ T lymphocyte numbers (1.60 vs 9.24 g l^{-1} and 3.95 vs 6.61 g l^{-1}, respectively). Significant differences between the experimental groups (L and F) were observed in $CD4^+$ T cell and B lymphocyte counts ($P < 0.05$) (Table 1). The group F dominated in these kind of immunological parameters at age 46 days except for $CD2^+$ and $CD4^+$ T cell numbers.

The phagocyte activity of non-specific immune cells by second sampling in group F revealed the following increases: %PA from 2.40 to 10.00%, IPA from 1.76 to 3.16, %PANe from 22.63 to 34.86% and IPANe from 2.03 to 3.11 and this group dominated over group C and L. No similar trend could be observed in the untreated group (C) and in the group treated with lactobacilli only (L) and with exception of %PA all other values were decreased (Table 2).

All groups showed an increase of total immunoglobulin concentration in the sera, but differences among them were very low. After the second administration of FOS and lactobacilli in both experimental groups increased bacterial counts in the faeces (except for TA counts in group L) (Table 3) were observed. Counts of total anaerobes, total aerobes and total lactobacilli in the faeces of animals in group F were significant higher ($P < 0.05$) when

TABLE 3. Total counts of selected bacteria groups (log g^{-1}) in the faeces of animals after 10 days of application of *Lc. paracasei* and Raftilose P95 after birth (F/1, L/1, C/1) and after weaning (F/2, L/2, C/2).

Group	Total anaerobes	Total aerobes	Lactobacillus
F/1	9.372 ± 0.462	9.332 ± 0.274	8.899 ± 0.570
L/1	9.293 ± 0.305	8.719 ± 0.173	9.063 ± 0.547
C/1	9.073 ± 0.099	8.817 ± 0.866	8.891 ± 0.046
F/2	10.175 ± 0.159[a,b]	9.278 ± 0.654[c,d]	10.261 ± 0.108 [e,f]
L/2	9.807 ± 0.255 [a]	8.294 ± 0.198 [c]	9.854 ± 0.278[f]
C/2	9.805 ± 0.212[b]	7.975 ± 0.505[d]	9.896 ± 0.142[e]

[a-d,f] $P < 0.05$.
[e] $P < 0.01$.

F to L was compared. Significant difference ($P < 0.01$) was estimated by total lactobacilli counts and ($P < 0.05$) by TAn and TA when F to C was compared (Table 3).

Average weight of pigs after birth was – 1.62 kg in group F, – 1.39 kg in group L and – 1.37 kg in group C. After weaning these average values were obtained: F – 7.54 kg, L – 6.56 kg and C – 6.68 kg. Four weeks after weaning the average weights reached were: F – 15.03 kg, L – 14.04 kg and C – 13.62 kg.

DISCUSSION

Several authors have confirmed the positive effect of different probiotic bacteria on the immune system (Saito *et al.*, 1983 Kato *et al.*, 1984 Perdigon *et al.*, 1993, 1995). Oral administration of lactobacilli lead to macrophage and lymphocyte stimulation (Perdigon *et al.*, 1986) and enzyme release from peritoneal macrophages (Perdigon *et al.*, 1986). The mechanisms enabling lactobacilli to access the immune system and perform their immunostimulating effects are not clear. Lactobacilli are probably absorbed by M cells and transported into the deeper lymphatic follicles where they are inspected by immunocompetent cells. Lactic acid bacteria and their products may also be transported into systemic lymphatic tissues like the mesenterial lymph nodes or the spleen. This theory is also supported by the work of Classen *et al.* (1995) who confirmed that lactobacilli adhere to or are accepted by M cells of the Peyer's patches and to occur later in the mesenteric lymph nodes.

Some oligosaccharides are used as specific substrates for potentially beneficial intestinal bacteria like bifidobacteria and are not utilized by pathogenic or potentially harmful microorganisms. These properties are well characterized namely in the case of FOS and their interaction with bifidobacteria (Hidaka *et al.*, 1986, 1990; Modler *et al.*, 1990; Hidaka and Hirayama, 1991). Fructooligosaccharides are one of the types of oligosaccharides; they consist of a linear chain of β-D-fructofurane units connected by 1,2 glycoside bonds. According to Fukuyasu and Oshida (1988), addition of 0.25 or 0.5% FOS to the diet of weanlings increased the weight gain and reduced the problems with pig diarrhoea. Bailey *et al.* (1991) observed the combined use of faecal microflora and FOS to have a positive effect upon chicken susceptibility to *Salmonella sp.* infections.

In the first part of the experiment the results indicated no stimulatory effect of application of *Lc. paracasei* on the immune system of the animals. The values in the control group and in group L are almost on the same level or even lower in group L (Table 1). Results obtained in group F indicated that there is no co-stimulating effect of FOS Raftilose P95 upon the applied strain of lactic acid bacteria and on the immune system of newborn piglets. It is known that after birth the digestive tract microflora undergoes gradual development and new organisms gradually replace the bacterial species encountered in the first weeks of life. We suppose that addition of Raftilose P95 also stimulated the growth of other bacterial species able to utilize oligosaccharides as a growth factor (*Bacteroides spp.*, bifidobacteria) that do not positively affect the immune system. When both the experimental groups are compared the significant differences ($P < 0.01$, resp. $P < 0.05$) in group L are the counts of leukocytes, neutrophils, lymphocytes, $CD2^+$ and $CD4^+$ T cells, B cells and macrophages (Table 1). This coincides with the findings of Chesson (1993) who confirmed the systemic effects of lactic acid bacteria upon the immune response. The low difference between L and C, or lower numbers of the specific cells in group L are probably influenced by the fact, that to obtain significant stimulation of the immune response by a healthy organism is not easy, because in particular of the balance of the immune system with other systems including the nervous and endocrine systems.

As a consequence of feed ration changes, the status and composition of intestinal microorganisms are altered, thus predisposing the spread of pathogenic bacteria. Lactic acid bacteria and coliforms bacteria are predominant in the first period of life. By the time of weaning drastic changes in composition of microflora occur and the dominant class are

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obligatory anaerobes. For orally administered bacteria, it is more difficult to exercise their effects during this period of life. It is the stimulating effect of some probiotic additives (as substrates suitable for lactic acid bacteria, among them FOS) that can improve the overall effect of probiotics administered at the time of weaning when a wide range of bacterial species occur. The supporting effect of FOS will positively influence the ability of lactobacilli to survive in the gut environment and adhere in greater numbers. Even under the conditions of broad microbial competition, when coliforms, clostridia, eubacteria and enterobacteria present in the intestine at weaning are not able to utilize this substrate. In addition, oligosaccharides themselves exert the same effect upon the immune system as do probiotic bacteria, which possibly use the signal system on the memory cell level in the *lamina propria* and the Peyer's patches. Oligosaccharides may also interfere with this system and significantly activate the immune response at low peroral doses. It seems to be possible that oligosaccharide molecules pass the intestinal barrier and stimulate the immune system.

In the second phase of the experiment (after weaning) the maximum of almost all immune parameters was seen in group F compared to group C and group L (Tables 1 and 2). The supporting effect manifested itself to its full extent and combined administration stimulated the immune system more than the single administration of lactobacilli. The absolute numbers of selected immune cells and their activity were increased in group F. Significant differences ($P < 0.05$) between experimental groups were observed in total counts of $CD4^+$ T cells and by B cells. In groups L and C, most of the values at 46 days of age were decreased. Concentration of total immunoglobulins was increased in all groups, that is in corelation with the age of the animals and with the activity of their immune system. There was no influence of treatment recorded.

According to Svozil (1995) probiotics stimulate natural physiological functions and they increase the productive potential of animals. The highest average weight during the whole experiment in group F was recorded. The beneficial effect of FOS on colonization of the gut by lactobacilli was presented not only by increased immune functions and counts of total lactobacilli in the faeces but also in better utilization of the feed.

The results suggest a specific action of probiotic strain on cellular immune response by supporting activity of FOS Raftilose P95. Supplementation with these additives thus enables probiotics use not only in the prevention of diseases by increasing the resistance of animals to pathogens but also in the treatment of digestive tract diseases, mainly diarrhoea of bacterial origin.

CONCLUSIONS

On the basis of the results we can assume that FOS, in our case Raftilose P95, is an additive that is suitable for improving the immune stimulating effect of lactobacilli by increasing survival and intestinal adherence, particularly in the period of weaning when the gut microflora is unstable or has a composition that is unsuitable for the host.

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