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# THE INFLUENCE OF COLONIZATION BY *LACTOBACILLUS SP.* AND *E. COLI* K 88+ ON LYMPHOCYTE SUBPOPULATIONS IN THE PERIPHERAL BLOOD OF GNOTOBIOTIC PIGLETS

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**Abstract**

Revajová, V., M. Levkutová, J. Pistl, R. Herich, A. Bomba, M. Levkut: *The Influence of Colonization by Lactobacillus sp. and E.coli K 88+ on Lymphocyte Subpopulations in the Peripheral Blood of Gnotobiotic Piglets.* Acta vet. Brno 2000, 69:195–199.

Flow cytometry was used to evaluate CD2, CD4, and CD8 lymphocyte subpopulations in the peripheral blood of gnotobiotic piglets after administration of *lactobacilli* and followed *E. coli K88+*. Fourteen 1-day-old white Slovak crossbreed piglets were divided into four groups. Two experimental groups were given *per os* from days 2 to 4 *Lactobacillus* – the first (n = 4) *L. salivarius* and second (n=3) *L. casei.* On day 5 the same animals were infected by enteropathogenic non-haemolytic *E. coli*. The third group was given only *E. coli* (n = 4) served as positive control and fourth group included bacteria free animals (n = 3) serve as negative control. Two blood samplings were withdrawn from the retroorbital venous sinus of the piglets' eye on days 3 and 6 after administration of *E. coli.* Indirect immunofluorescence method for flow cytometry was used. Isolated lymphocytes obtained by density gradient were incubated with primary monoclonal antibodies - mouse anti-pig CD2, CD4 and CD8 and followed FITC-conjugated sheep anti-mouse secondary antibody. Ten thousand lymphocytes were analysed by FACScan (Becton Dickinson) and relative percentage of positive cells was recalculated to the absolute numbers ($G \cdot l^{-1}$) by the differential counts of leukocytes.

Total numbers of leukocytes and absolute number of lymphocytes were significantly increased ($P < 0.05$) in both groups treated with lactobacilli, but at the different times, in sampling 1 after application of *L. casei* and in sampling 2 after *L. salivarius*. Significant increase in CD2, CD4 and CD8 positive T-cells ($P < 0.05$) were seen only in the group *L. salivarius* and *E. coli* compared to the control group.

In our experiment *L. salivarius* was revealed to be more effective in enhancing the immune response against the subsequent administration of *E. coli*.

*Lactobacilli sp., E. coli, subpopulation T-lymphocytes, gnotobiotic piglets, immune response*

Lactobacilli are a component of the regular intestinal microflora in various animal species and birds from the first days of life (Jin et al. 1996). They play an important role in ensuring the natural defence system and they have a positive effect on the general metabolism (Gilliland 1990). Some lactobacilli species prevent intestinal infection, favourably influence the intestinal microflora representation, and stabilize its constitution (Nemcová 1997). *L. acidophilus* and *L. casei* are good activators of the mononuclear phagocytic system (Perdigón et al. 1992).

Besides the positive effect of lactobacilli on the mononuclear phagocytic activity, also recorded was their effect on the increasing counts of IgA producing B cells as a result of the stimulation of T lymphocytes in the intestinal submucosa. For the above reasons, *lactobacilli* are the most productive microorganisms used for probiotic production (Nemcová 1997). On the other hand Revajová et al. (1999) referred to the decrease of CD3 cells in the intestinal mucosa after a long-term application of *L. casei*.

To find out the effect of the short-term application of lactobacilli as a protective

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microflora against *E. coli*, the study in gnotobiotic pigs was performed by identification of some lymphocyte subpopulations in the peripheral blood.

## Materials and Methods

Animals

Fourteen white Slovak crossbreed piglets obtained by hysterectomy and kept in incubators were divided into four groups (Table l). Two experimental pig groups were administered *per os* from days 2 to 4 of life with two species of lactobacilli (*L. salivarius* and *L. casei*) at infective doses of 2 ml 1x$10^8$ CFU. The enteropathogenic non-haemolytic species of *E. coli* (08:K88abH9) were administered to the same animals on day 5 of their life in a dose of 2 ml 1x$10^8$ CFU. The animals after the application of *E. coli* served only as the positive control, and negative control animals were administered with PBS. Gnotobiotic animals were fed the autoclave prepared milk (Sunar Pharmacopola, SR).

Blood sampling

The blood samples for counting the total number of leukocytes and for flow cytometry were withdrawn from the retroorbital venous sinus of the pigletęs eye. Türk solution (Medika, SR) was used for counting total number of leukocytes in Bürker chamber and examination of 100 cells per slide; staining by Haemacolor (Merck, Germany) was used for evaluation of the relative percentage of lymphocytes (%) on blood smears by light microscopy.

Table 1

| The design of the experiment | | | | |
|---|---|---|---|---|
| | *L. salivarius+E. coli* | L. casei+E. coli | E. coli | Control |
| age | | 1-day-old | | |
| No. of animals | 4 | 3 | 4 | 3 |
| 1. sampling | | 7 days of life | | |
| 2. sampling | | 10 days of life | | |

Flow cytometry

Indirect immunofluorescent method was used.

Primary monoclonal antibodies. are summarized in Table 2.

Table 2

| Primary monoclonal antibodies | | | |
|---|---|---|---|
| Monoclonal antibodies | | | |
| Specificity | MoAbs | Isotype | Diluted |
| CD2 | MSA 4 | IgG2a | 1:50 |
| CD4 | 74-12-4 | IgG2b | 1:50 |
| CD8 | 76.2.11 | IgG2a | 1:50 |

Secondary antibody. Fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG (whole molecule) was used in a dilution of 1:128 (Immunochemicals, Sigma, Germany).

Isolation of lymphocytes. Venous blood was collected into ethylenediamine tetraacetic acid (EDTA, Lachema, Brno, ČR) Lymphocytes were separated by Ficoll-Hypaque (Sigma, Germany) gradient sedimentation (Boyum 1974).

Procedure for flow cytometry (FACS). Separated lymphocytes were washed 3 times with PBS, samples were put into RPMI with 2% foetal calf serum (washing medium) and incubated with specific MoAbs at 4C for 30 minutes in the dark. For a control, the non-specific isotype control was used. After incubation the samples were washed twice with washing medium and

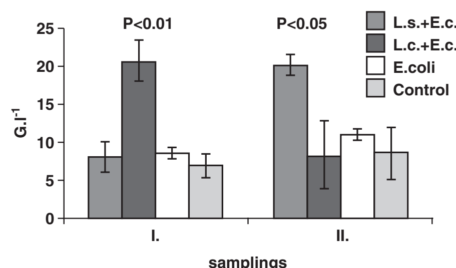


Fig. 1. The total number of leukocytes in the peripheral blood (G.l$^{-1}$).

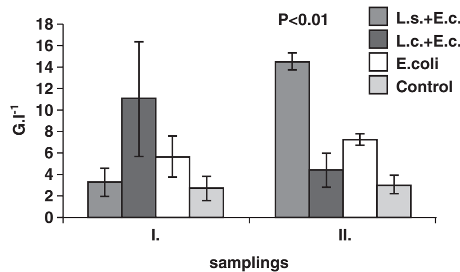


Fig. 2. The absolute number of lymphocyte in the peripheral blood ($G \cdot l^{-1}$)

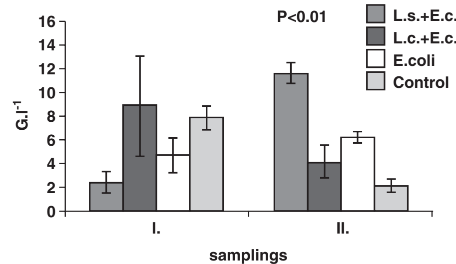


Fig. 3. The absolute number of CD2 positive cells in the peripheral blood ($G \cdot l^{-1}$)

incubation with secondary antibody followed as described above. Flow cytometry analysis was performed by FACScan (Becton Dickinson, Germany) using the Cell Quest programme. 1000 cells were collected into a gate. The relative percentage of lymphocytes was calculated to absolute numbers as follows: total number of leukocytes × the relative percentage of lymphocytes × % lymphocyte subpopulation ($G \cdot l^{-1} 1 \times 10^{9} \cdot l^{-1}$). The results were expressed as the mean ± SD and evaluated by one-way ANOVA test. A confidence level $P < 0.05$ was considered significant.

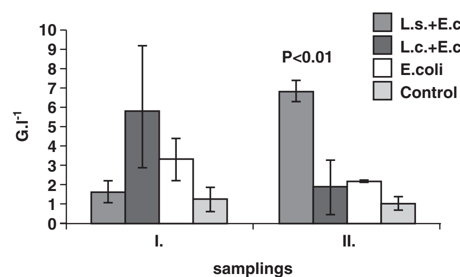


Fig. 4. The absolute number of CD4 positive cells in the peripheral blood ($G \cdot l^{-1}$)

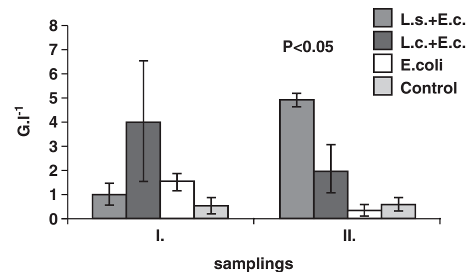


Fig. 5. The absolute number of CD8 positive cells in the peripheral blood ($G \cdot l^{-1}$)

## Results

A significant increase in leukocytes compared to the control animals was recorded in sampling 1 after application of *L. casei*, but it was recorded in sampling 2 after *L. salivarius* administration (Fig 1). Similar changes at the same time intervals were determined in the absolute number of lymphocytes in both lactobacilli (Fig 2).

The absolute number of CD2 positive lymphocytes was increased in both experimental groups and in the positive control with significance in the *L. casei* + *E. coli* group compared with the values of the control group (Fig 3).

The absolute number of CD4 positive lymphocytes showed equally high values at the same time intervals in the experimental groups in comparison with controls (Fig. 4).

A similar course as in CD4 cells was observed in the subpopulation of CD8 positive lymphocytes, but after the application of *E. coli* in sampling 2 the absolute number was lower than in the controls (Fig. 5).

## Discussion

The preventive application of lactobacilli and subsequent inoculation with *E. coli* significantly increased the total number of leukocytes and lymphocytes in weaned piglets.

The administration of *E. coli* did not cause pronounced changes in the number of cells observed in the animals. Recent works have demonstrated that the duration of the administration of lactobacilli is important for their therapeutic effect. The oral administration of lactobacilli in mice led to macrophagic and lymphocytic stimulation (Perdigón et al. 1986). As for the intestinal infection in mice, the immunostimulating effect against bacterial infection was observed only during the first days of lactobacilli application (Perdigón et al. 1990, 1993). With a long-term application Revajová et al. (1999) even observed as significant decrease in the number of CD3 lymphocytes in the intestinal mucosa of piglets.

Similarly, in both experimental groups with lactobacilli, significant changes were recorded in the number of cell of the lymphocytic subpopulations. It was very interesting to note that while the administration of *Lactobacillus salivarius* caused increases in the number of cells observed at sampling 1, the application of *Lactobacillus casei* caused increases in the number of cells at sampling 2. Some authors supposed (Perdigón and Alvarez 1992; Schriffrin et al. 1997) that heterogeneity in the structure of the cellular wall of bacteria is responsible for the different influence of individual strains of lactobacillary bacteria. The mechanism, by which bacteria of lactic fermentation are capable of reaching the immune system and performing an immunostimulating effect, is not clear. Classen et al. (1995) have found the adherence and intake of lactobacillary bacteria by M cells of Peyer's plaques in mice administered these bacteria orally. Bacteria of lactic fermentation appeared in the region of Peyer's plaques after 6 – 12 hours and in the mesenteric lymph nodes 48 hours following intake. The total number of leukocytes and absolute number of lymphocytes had a slight tendency to increase in the group of piglets with *E. coli.* Neither were pronounced changes recorded in the number of cells of individual cellular subpopulations. The cellular decrease was even recorded in CD2 positive cells at sampling 1 and CD8 positive cells at sampling 2.

The results showed that the preventive application of lactobacilli activated the immunocompetent cells after the administration of *E. coli.*

In conclusion it may be stated that the administration of *E. coli* after a previous application of either types of lactobacilli significantly increased the number of leukocytes. Significant changes in the evaluated subpopulations of lymphocytes of the peripheral blood of gnotobiotic animals were observed only after the administration of *L. salivarius* and *E. coli*. Our results demonstrated that the application of *L. salivarius* to gnotobiotic animals influenced the immune response to a greater degree after the subsequent administration of *E. coli* in comparison with *L. casei.*

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## Vplyv kolonizácie *Lactobacillus* sp. a *E. coli* K88+ na subpopulácie lymfocytov v periférnej krvi gnotobiologických prasiatok

V pokuse sme sledovali vplyv kolonizácie čreva laktobacilmi a následne *E. coli* na CD2, CD4 a CD8 bunkové subpopulácie v periférnej krvi gnotobiotických prasiatok použitím prietokovej cytometrie. Štrnásť jednodňových prasiatok plemena slovenská biela ušľachtilá sa rozdelilo do 4 skupín. Dvom pokusným skupinám sa aplikovali laktobacily *per os* – 1. skupine (n = 4) *L. salivarius* a 2. skupine (n = 3) *L. casei*. Na piaty deň tieto skupiny boli infikované enteropatogénnym nehemolytickým kmeňom *E. coli K88+*. Tretia skupina infikovaná iba *E. coli* (n = 4) slúžila ako pozitívna kontrola a 4. skupina bez aplikácie

sledovaných baktérií (n = 3) bola zaradená do pokusu ako negatívna kontrola. Krvné vzorky odobraté z očného splavu na 3. a 6. deň po infekcii s *E. coli* sa vyšetrovali nepriamou imunofluorescenčnou metódou a boli merané prietokovou cytometriou. Lymfocyty izolované na hustotnom gradiente sa inkubovali s primárnymi myšacími antiprasačími monoklónovými protilátkami – CD2, CD4, CD8 a následne s antimyšacou ovčou FITC-konjugovanou sekundárnou protilátkou. Lymfocyty v počte 10 000 boli merané prietokovým cytometrom FACScan firmy Becton Dickinson. Relatívne percento pozitívnych buniek bolo prepočítané na absolútne hodnoty podľa celkového počtu leukocytov a diferenciálneho krvného obrazu.
Celkový počet leukocytov a absolútny počet lymfocytov boli signifikantne zvýšené ($p < 0,05$) u oboch laktobacilových skupín, avšak v rozdielných časových intervaloch, v 1. odbere po aplikácii *L. casei* a v 2. odbere po podaní *L. salivarius.* Signifikantné zvýšenie subpopulácií CD2, CD4 a CD8 pozitívnych T lymfocytov ($p < 0,05$) sme zaznamenali iba v skupine s aplikáciou *L. salivarius* a *E. coli* v porovnaní s kontrolou. V našom pokuse *L. salivarius* ukázal vyššiu účinnosť v navodení imunitnej odpovede proti následnej infekcii *E. coli*.

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