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The Influence of Short-term and Continuous Administration of *Lactobacillus casei* on Basic Haematological and Immunological Parameters in Gnotobiotic Piglets

ROBERT HERICH, ALOJZ BOMBA, RADOMÍRA NEMCOVÁ AND SOŇA GANCARČÍKOVÁ

Research Institute of Veterinary Medicine, Hlinkova 1/A, Košice, 040 01, Slovak Republic

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Two experiments were carried out on gnotobiotic piglets by model infection with the strain Escherichia coli *O:8 K88$^+$ Ent$^-$ to investigate the influence of preventive, short-term and preventive continuous long-term administration of the* Lactobacillus casei *strain on basic haematological and immunological parameters. In each of the experiments, the animals were divided into experimental (EG1 and EG2, respectively) and control groups (CG1 and CG2, respectively). The pigs in the experimental groups were treated by* L. casei *(EG1, for 3 days; EG2, for 10 days) and the* E. coli *strain when 5 days old; the pigs in the control groups (CG1, CG2) were inoculated only with* E. coli *at the age of 5 days. In the first experiment, no significant differences in either of the parameters investigated were detected between the animals of the experimental (EG1) and the control group (CG1). In the second experiment, a significant difference ($p < 0.05$) in the percentage proportion of neutrophils (%Ne) and of phagocytic activity (%PA) was observed in the EG2 on day 7 of age. The percentage of lymphocytes (%Ly) was, on the contrary, significantly lower in the EG2 group ($p < 0.05$). Out of the haematological values observed, we detected significantly higher values of haematocrit ($p < 0.05$) and concentrations of haemoglobin (HG) ($p < 0.05$) in the experimental group EG2. Also, the index of phagocytic activity (IPA) in the EG2 group was 1.5-fold higher than that in the control group CG2. On day 5 after the administration of* E. coli, *i.e. on day 10 of age, significant differences were recorded between the groups in %Ne ($p < 0.05$) in favour of EG2 and in %Ly in favour of CG2 ($p < 0.05$). The EG2 group exhibited 2.5-fold higher percent of PA and more than twofold higher IPA. The comparison of both experimental groups (EG1 and EG2) showed a significant difference in %PA ($p < 0.05$), IPA ($p < 0.01$) and HG ($p < 0.05$) in the EG2 animals during the continuous inoculation with* L. casei. *The comparison presented indicates a more favourable effect of 10-days continuous administration of lactobacilli in comparison with 3-day short-term administration.*

Keywords: *Lactobacillus casei, immunity, probiotic, gnotobiotic piglets*

Correspondence to: Robert Herich, Tel: +421 95 6337429; Fax: +421 95 6331853; E-mail: saruman55@yahoo.com

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INTRODUCTION

The bacterial flora residing in the intestinal microenvironment is a very effective barrier against the action of various pathogenic and conditionally pathogenic bacteria which can be found in the external environment or directly in the gastrointestinal tract. This microflora also participates in the more effective utilization of nutrients contained in the food (Fuller, 1989). According to numerous studies, the lactobacilli form one of the dominant groups of intestinal and faecal microflora, and the unfavourable action of pathogenic organisms can be minimized by supporting the intrinsic intestinal lactobacilli flora (Shahani & Ayebo, 1980).

The influence of different factors on animals, such as bad animal hygiene conditions, insufficient feed or its low quality, stress, extensive productive load, uncolonized digestive tract in newborns and unsubstantiated antibiotic therapy, can affect significantly their balance and result in the outbreaks of diseases. Restoration of the microenvironmental balance can be achieved by supplementing suitable probiotic strains.

The high degree of effectiveness has been ascribed to the preventive utilization of lactobacilli or other probiotic microorganisms. However, opinion on the suitable length of probiotic administration differs. It is important to select appropriate strains and to determine the dose of probiotic in such a way as to result in the maximum supporting and stimulative effects required.

According to Fuller (1992), the favourable effect of administration of probiotic can be reflected in better growth of animals, utilization of feed and productivity, and in the health of animals. The good health state includes the resistance to infectious diseases which can be ensured on the principle of direct antagonism or immunostimulation.

The substances which affect the immune system or stimulate the immune response can affect the non-specific protective mechanisms of the host, activate the cells involved in the specific immune response or lead to a systemic increase which activates the host immune system as a whole.

The immunostimulative action of different lactobacilli strains has been known for a longer time. Definite proof of the stimulative action of *Lactobacillus acidophilus* on peritoneal macrophages (Perdigón *et al.*, 1986a) and the mononuclear phagocytic system (Perdigón *et al.*, 1986b) has been presented. Similar effects of *Lactobacillus plantarum* on mononuclear phagocytes and NK cells (Bloksma *et al.*, 1981) have been observed. Marteau & Rambaud (1993) have confirmed that some lactobacilli are capable of inducing the activation of macrophages and increasing the immune response.

Because the changes induced in the immune system in healthy conventionally reared animals are not always significant, we used gnotobiotic piglets delivered under sterile conditions free of microbial contamination.

The immunological parameters were selected with respect to the method of rearing and the age of the animals, to enable the most advantageous observation of the non-specific immune response manifested by polymorphonuclear phagocytes and monocytes which play a primary role in the host defence response to penetrating pathogenic microorganisms.

MATERIALS AND METHODS

Experiment 1

The experiment was carried out on six gnotobiotic piglets delivered by a modified method of open hysterotomy. The animals were divided into two groups, three animals in each, and were reared in isolators in a microbe-free environment. The ration consisted of autoclaved sterile milk which was supplied to animals four times daily. The composition of the dried milk (per 100 g) was as follows: 25.3 g fat, 27.3 g proteins, 38.3 g lactose, 6.1 g mineral substances, and 3.0 g water. On days 2, 3 and 4 of life, the animals of the experimental group (EG1) were treated once a day with *Lactobacillus casei* at a dose of 1×10^8 CFU in 2 ml inoculum. The control group was not inoculated during the first 4

days of life. On day 5 of age, all animals were administered *Escherichia coli* O:8 $K88^+$ Ent^- strain at a dose of 1×10^5 CFU in 2 ml inoculum.

Samples of blood for haematological and immunological examination were taken on days 7 and 10 of age. Because of microbiological sampling of the gut, the number of animals in both groups after the first sampling decreased to one. The blood was sampled from the infraorbital plexus venosus in the isolators, by means of a blunt needle, into a tube with heparin in such a way that the resulting concentration of heparin was 5 U per 1 ml blood. The blood samples were examined for the following: haematocrit, haemoglobin, number of erythrocytes (Er), number of leukocytes (Le), differential blood picture, percentage of phagocytic activity (%PA), and the index of phagocytic activity (IPA).

Determination of haematocrit value. Haematocrit was determined by the routine micro-haematocrit method. A drop of blood was drawn into a heparinized capillary. The capillary was closed with plasticine and centrifuged at 12 000 rpm for 15 min. After the centrifugation, the height of the haematocrit column was read on the evaluator and the haematocrit value was expressed as $1l^{-1}$.

Determination of haemoglobin concentration. The concentration of haemoglobin in the blood of the animals examined was determined with Sahli's haemoglobinometer (Benzamine, 1961).

The number of erythrocytes. Hayem's solution 4.975 μl was pipetted into a test tube and 25 μl heparinized blood added quantitatively. The pipette used was rinsed two or three times with the Hayem's solution and the tube contents were mixed thoroughly. A Bürker cell was used for counting the erythrocytes. They were counted at magnification $10 \times 1.25 \times 20$. The number of erythrocytes was expressed as Tl^{-1}.

The number of leukocytes. To a volume of 475 μl Turk's solution in a test tube, a 25 μl aliquot of heparinized blood was added quantitatively. The content of the test tube was mixed thoroughly. A Bürker chamber was used for counting the leukocytes. The prepared mixture was transferred to the Bürker chamber and leukocytes were counted at magnification $10 \times 1.25 \times 20$. The total number of leukocytes was expressed as Gl^{-1}.

Differential blood picture. The differential blood picture was obtained by means of a blood smear prepared from 10 μl heparinized blood. After 24 h, the blood smear was stained by a routine panoptic staining method according to Pappenheim using two staining solutions. The blood smear was stained and dried up, and the percent 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 $10 \times 1.25 \times 100$.

Percent of phagocytic activity and the index of phagocytic activity. The parameters % PA and IPA were determined by means of microspherical haematological particles (Artim Prague, Czech Republic). Within 1 h of sampling, a 0.1 μl aliquot of heparinized blood was incubated with 0.05 μl microspherical particles solution prepared according to manufacturer's instructions. The incubation lasted for 1 h at 37°C in a plastic test tube at regular mixing. After the 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 $10 \times 1.25 \times 100$ magnification. Each potentially phagocytizing cell (monocytes, neutrophil granulocytes, eosinophils, basophils) which contained three and more phagocytized particles was considered to be a phagocytizing cell.

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

Experiment 2

The second experiment was performed on nine gnotobiotic piglets delivered by the same method as that used in the first experiment. The rearing and feeding of the piglets was also ensured in the same way. Two groups of piglets were formed, the experimental group (EG2) consisting of five animals at the first sampling and of three animals at the second. The control group (CG2) comprised four animals at the first sampling and two at the second. *L. casei* was administered to the animals of the experimental group (EG2) during the entire experiment (10 days) using a dose of 1×10^8 CFU in 2 ml inoculum once a day. The CG2 group was not treated up to day 5 of life. On day 5, all piglets were inoculated with *E coli* O:8 $K88^+$ Ent^-, using a dose of 1×10^5 CFU in 2 ml inoculum.

Samples of blood were taken on days 7 and 10 of age in the same way as that used in the first experiment, and the same haematological and immunological parameters were determined employing the methods already described in the experiment with preventive short-term administration of lactobacilli bacteria.

RESULTS

The comparison of results obtained in the first experiment showed differences in the haematocrit value on day 7 of age, which reached $0.21\,l\,l^{-1}$ in EG1 and $0.15\,l\,l^{-1}$ in CG 1, and in the number of erythrocytes which amounted to $4.37\,T\,l^{-1}$ in CG1 and $3.17\,T\,l^{-1}$ in the experimental group. The concentration of haemoglobin in the blood taken at that sampling was the same in both groups. The percentage values of PA and IPA were higher in EG1; however, the difference was insignificant. Despite the higher activity of potentially phagocytizing cells in the group subjected to preventive treatment with *L. casei* (EG1), the percentage proportion of neutrophils (%Ne) in the differential blood picture was only 29.33%, while in the control group (CG1) it reached 42.00% on average. A contradictory situation was observed for the percentage of lymphocytes (%Ly), which reached, on average, 69.67% in EGI and 58.00% in CG1. At that time, the total mean number of leukocytes was $12.67\,G\,l^{-1}$ in EGI and $8.33\,G\,l^{-1}$ in the control group (Table 1).

On day 10 of age of piglets, i.e. 5 days after inoculation with *E. coli*, was the sampling done only on one animal of each group. The results could not be statistically analyzed. A decrease in haematocrit value to $0.19\,l\,l^{-1}$ was observed in EG1, while the haemoglobin concentration was maintained at the same level and the number of erythrocytes in EG1 increase to $3.3\,T\,l^{-1}$. A similar situation was observed also in the control group (CG1); however, an increase in the number of Er to $5.00\,T\,l^{-1}$ in the control animal was accompanied by a simultaneous increase in the concentration of haemoglobin to $88.80\,g\,l^{-1}$. The total number of leukocytes (Le) increased in both groups; however, the increase was more pronounced in EG1, rising to the value of $21.00\,G\,l^{-1}$ and exceeding the upper physiological limit. An increase in the number of white blood cells was not accompanied with increasing phagocytic activity of potentially phagocytizing cells. A decrease in this parameter was recorded in both groups. The decrease was more pronounced in EG1, in which a five-fold decrease to 2.00% was recorded for %PA in comparison with the first sampling. The IPA value was at the same level in both groups (1.3 in EG1 and 1.2 in CG1). The comparison of differential blood pictures showed an increase in the number of lymphocytes and a decrease in the number of neutrophil granulocytes in both groups (EG1 and CG1) (Table 1).

The second experiment was evaluated on the basis of the same haematological and immunological parameters. The main methodical difference consisted of the way of administration of *L. casei* applied continuously during the entire experiment. Significant differences in several parameters were observed in 7-day-old piglets. Out of the haematological parameters determined (Table 2), significant differences ($p < 0.05$) were observed in the haematocrit values and concentrations of haemoglobin. A significantly higher ($p < 0.05$) percentage of neutrophils (52.8%) was observed in EG2 in comparison with the control (21.0%). The %PA in EG2 was significantly higher ($p < 0.05$) than that in the control

TABLE 1. Differences between experimental and control groups in selected haematological and immunological parameters in the first experiment with 3-days-long application of *L. casei*

Age	Group	Haematocrit ($l l^{-1}$)	Haemoglobin ($g l^{-1}$)	Erytrocytes ($T l^{-1}$)	Leukocytes ($G l^{-1}$)	Lymphocytes (%)	Neutrophils (%)	%PA	IPA
7 days	EG1	0.21 ± 0.01	79.47 ± 2.49	3.17 ± 0.98	12.67 ± 5.56	69.67 ± 9.78	29.33 ± 9.78	11.00 ± 8.67	2.00 ± 0.8
	CG1	0.15 ± 0.04	79.47 ± 3.56	4.37 ± 0.51	8.33 ± 0.89	58.00 ± 6.00	42.00 ± 5.33	8.67 ± 10.22	1.53 ± 1.58
10 days	EG1	0.19	79.00	3.30	21.00	73.00	26.00	2.00	1.30
	CG1	0.14	88.80	5.00	11.50	68.00	32.00	7.00	1.20

EG1, Experimental group 1; CG1, control group 1; %PA, percentage of phagocytic activity; IPA, index of phagocytic activity.

TABLE 2. Differences between experimental and control groups in selected haematological and immunological parameters in the second experiment with 10-days-long application of *L. casei*

Age	Group	Haematocrit ($l l^{-1}$)	Haemoglobin ($g l^{-1}$)	Erytrocytes ($T l^{-1}$)	Leukocytes ($G l^{-1}$)	Lymphocytes (%)	Neutrophils (%)	%PA	IPA
7 days	EG2	0.26 ± 0.03*	95.68 ± 7.94*	4.22 ± 0.58	10.80 ± 3.24	45.00 ± 17.92	52.80 ± 18.24*	29.60 ± 8.48*	11.42 ± 2.06
	CG2	0.19 ± 0.03	64.00 ± 11.20	3.15 ± 0.55	10.75 ± 2.25	77.50 ± 9.75*	21.00 ± 10.50	11.25 ± 7.75	6.95 ± 2.45
10 days	EG2	0.25 ± 0.03	98.13 ± 13.16	3.83 ± 0.44	21.17 ± 5.78	58.00 ± 4.00	38.67 ± 4.89*	12.67 ± 3.78	7.10 ± 2.07
	CG2	0.19 ± 0.04	70.40 ± 14.40	2.90 ± 0.40	15.50 ± 5.50	81.00 ± 1.00*	14.00 ± 4.00	5.00 ± 2.00	3.00 ± 1.40

EG2, Experimental group 2; CG2, control group 2; %PA, percentage of phagocytic activity; IPA, index of phagocytic activity.
*$p < 0.05$.

group (29.6 and 11.25%, respectively) (Table 2). On the contrary, significantly higher ($p < 0.05$) %Ly was observed in the control group (CG2, 77.5%) in comparison with the experimental one (EG2, 45.0%). The difference in IPA between the groups was insignificant with respect to the values determined, despite the fact that the value determined in EG2 was more than 1.5 times that found in CG2 (Table 2).

The sampling on day 10 of age showed an insignificant decrease in phagocytic activity of potentially phagocytizing cells. The values of %PA and IPA determined in EG2 were, however, more than twice as high as those found in the control group (CG2). Significant differences were recorded in the percentage of lymphocytes and neutrophils ($p < 0.05$), while %Ly was higher in the control group and %Ne in the experimental group. All haematological parameters, determined in 10-day-old piglets, were higher in the EG2 group; however, the differences were insignificant (Table 2).

The comparison of the both experimental groups (EG1, EG2) showed significant difference ($p < 0.05$) by haemoglobin concentration. The haematocrit value and number of erytrocytes were higher in experimental group with 10-day-long application of lactobacilli (EG2) (Table 1 and 2). The number of leucocytes was not significant higher in EG2, but was more than 1.5-fold higher than in animals with short-term inoculation of *L. casei*. %Ne was higher in EG2, on the contrary to %Ly which was higher in EG1. The significant differences between experimental groups were obtained by %PA and IPA. Both parameters were significant higher in the group with 10-day-long inoculation of *L. casei*: %PA ($p < 0.05$) and IPA ($p < 0.01$).

DISCUSSION

Many authors have proved the favourable influence of lactobacilli bacteria on the host (Ebringer *et al.*, 1995; Perdigón *et al.*, 1995; Pouwels *et al.*, 1996). It involves the positive influence on the processes of digestion, increased resistance to diseases, improvement in the overall health state, stimulation of immunity and anti-tumorous action. According to Fuller (1989), the determination of the correct dose and length of administration is essential. Perdigón *et al.* (1995) determined the relationship between the length of administration of lactobacilli bacteria and their effect on the immune system of the animals tested. On the basis of present knowledge, we can speak about two principal ways of administering lactobacilli and other probiotic microorganisms. The aim of the preventive short-term administration is to induce the positive development of intestinal microflora immediately after birth by adding suitable species-specific starting probiotic strains. Due to the short period of administration and continuous contamination with microorganisms from the external environment, the action of lactobacilli after a short initial period is only marginal, if at all. The preventive continuous administration of lactobacilli differs from the previous one in its positive action, not only on the local level in the intestine itself, but, due to prolonged administration and the possible higher concentration, also on other systems in the organism, particularly the immune system. In this process, as stated by Berg (1983), lactobacilli are even capable of leaving the intestine and, according to Bloksma *et al.* (1979), survive for many days in the spleen, liver, and lungs.

How long this period of continuous administration should last remains an open question. Svozil (1995) recommended a 10-day continuous administration of the probiotic micro-organism *Enterococcus faecium* M-74 to calves. A similar view was presented by Koudela (1995), who recommended the continuous administration of the probiotic preparation and described a wide range of its positive biological effects in poultry.

Perdigón *et al.* (1995) mentioned different doses and lengths of administration for individual probiotic microorganisms. They found that an optimum dose for mice of *L. casei* and *L. acidophilus* was 2.4×10^9 administered for 2 days, and of yoghurt in feed after a 7-day administration. On the contrary, long-term administration of *L. acidophilus* decreased the total number of lymphoid cells in the intestinal mucosa.

The aim of our study was to compare the advantages and possible shortcomings of short-term (3 days) and long-term (10 days) administration of a species-specific lactobacillus strain to gnotobiotic piglets. All other experimental conditions, i.e. animal species, delivery, and rearing of animals, rations, and *L. casei* and *E. coli* strains were identical in both the experiments. The selected parameters of the red blood picture provided information about the physiological status of the animals investigated. The differential blood picture and basic immunological parameters which were included in the experiment are important for the evaluation of the response of an organism to stress factors, bacterial infection, and other antigens.

The results of the first experiment (short-term administration of *L. casei*) showed that there were differences between the groups in the parameters investigated, although they were insignificant. Table 1 shows that of all the haematological parameters investigated, only haematocrit was higher in EG1. Despite the fact that the colonization of the digestive tract results in better utilization of nutrients from the feed in comparison with the sterile intestinal microenvironment (Fuller, 1989). More favourable haematological parameters were not reached in the group treated with *L. casei* in comparison with the control group (CG1) uncolonized by bacteria during the first 4 days of life.

The immunological parameters were selected with the aim of pointing to the activity of the non-specific defence system in which the polymorphonuclear cells represent the first defensive line against numerous bacterial species capable of inducing acute infections. Such bacterial species also include *E. coli*, which was used in both experiments as a model pathogen in gnotobiotic newborns. *E. coli* O:8 $K88^+$ Ent^- was used to induce model enteritidis also due to the fact that no pronounced changes in the activity of the healthy host immune system could be detected because such a system is always in equilibrium with other systems, e.g. the nervous and endocrine systems. Two days after the inoculation with *E. coli*, a positive influence of *L. casei* on the total number of leukocytes, %PA and the PA index were observed, reflected in higher values in EG1, although the differences were insignificant. However, an untypical differential blood picture was obtained (Table 1).

This indicated that the short-term (3 days) administration stimulated the activity of potentially phagocytizing cells (mainly of neutrophils); however, their percentage proportion in the differential blood picture, characteristic of acute bacterial infectious diseases including colienteritidis, was not increased. Such a state was observed only in the experiment with 3-day administration of lactobacilli. The continuous 10-day administration resulted in an increase in both activity and percent of potentially phagocytizing cells in the experimental group. The difference in degree of action on the immune system was most likely caused by the different length of administration of *L. casei* (Figure 1).

Even more obvious was the insufficient action of lactobacilli bacteria at sampling on day 10 of age, i.e. after the last administration of *L. casei* to animals of the experimental group (EG1) and 5 days post-inoculation of *E. coli* to all animals included in the experiment. Table 1 shows that of all the haematological parameters determined, only haematocrit was higher in EG1. The insufficient immunostimulative effect of short-term administration of *L. casei* was demonstrated by the higher %PA in animals of the control group. The difference in IPA in favour of the experimental group was negligible. The results from second sampling of the first experiment could not be statistically analyzed. They were used because animals were reared in specific and defined gnotobiotic conditions.

Our observations showed that, although some effects of the lactobacilli strain used on the non-specific immune system were observed, it was of low intensity and short duration, as indicated by a decrease in the immunological parameters in relation to the increased lapse of time from the last inoculation of the experimental animals (EG1) with *L. casei*. Similar results were published by Votava & Svozil (1995), who applied radioactively marked bacteria *Streptococcus faecium* C-68 to chickens and observed their gradual elimination from the digestive tract within 3–4 days following the single administration, always down to the original state of intestinal microenvironment. On the contrary, continuous administration of

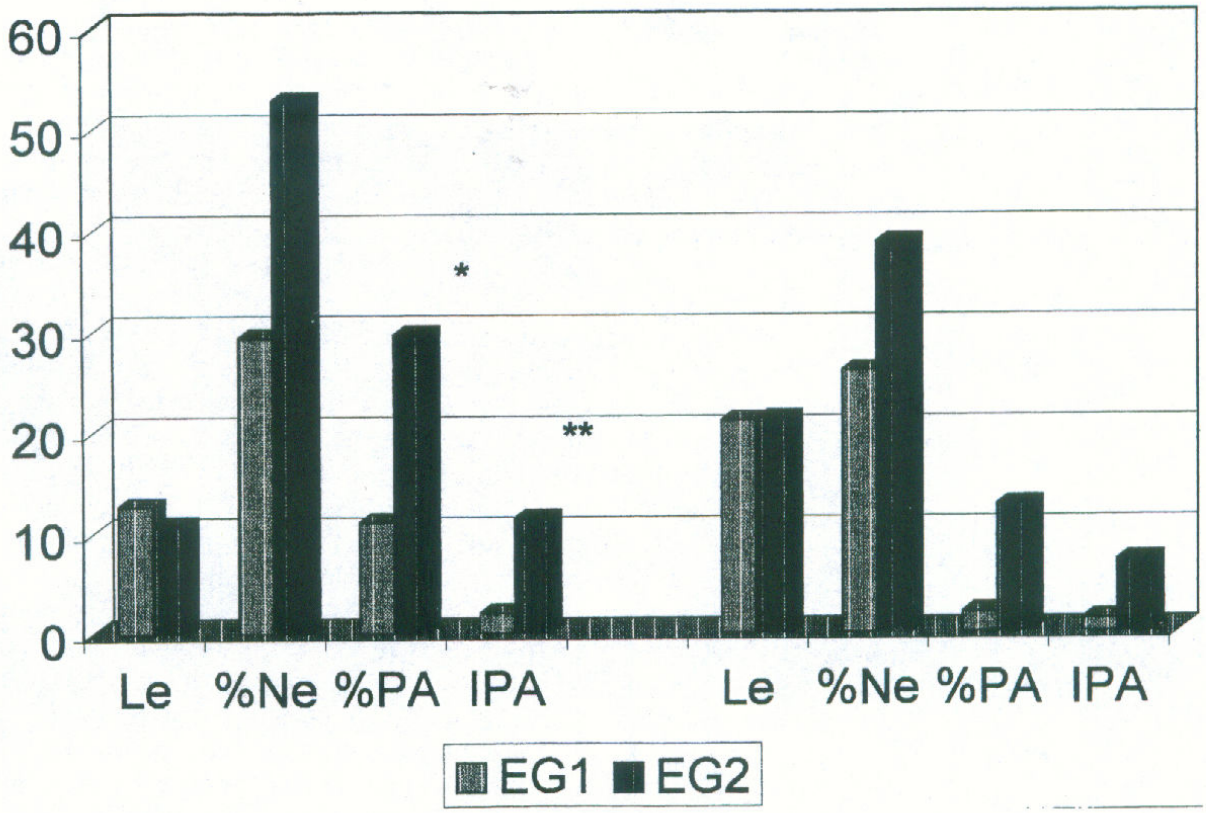

FIG. 1. Comparison of selected immunological parameters determined on days 7 and 10 of age in the experimental groups of piglets treated for 3 days (EG1) and 10 days (EG2) with the *L. casei* strain. $*p<0.05$, $**p<0.01$.

the microorganisms mentioned resulted in a moderate increase in their adhesion to the walls of the digestive tract of chickens.

The experiment with continuous administration of the same lactobacilli strain, lasting 10 days, revealed significant differences in several parameters at the age of 7 days, i.e. 2 days after the inoculation with *E. coli*. Significantly higher ($p<0.05$) haematocrit value, concentration of haemoglobin, %PA and percentage proportion of neutrophilic granulocytes (%Ne) was observed in EG2 (Table 2). The direct comparison of both experimental groups (EG1 and EG2) showed significant differences in %PA and IPA ($p<0.05$ and $p<0.01$, respectively) (Figure 1). The results point to the more intensive and longer-lasting immunostimulative effect of long-term administration of *L. casei*.

The 10-day-long administration of probiotic strain appears to be a more convenient period for reaching a sufficient number of lactobacilli bacteria in the intestinal microenvironment which are then capable of affecting the host organism more effectively. The short-term administration (3 days) did also result in stimulative and supporting action of lactobacilli; however, the manifestations were time-limited and the short-term protective barrier created was liable to disruption.

According to Kishi *et al.* (1996), the effect of lactobacilli on the immune system depends on the dose. A higher intake of bacteria resulting in a superior response compared with a lower intake. In our study, we maintained the same dose of *L. casei* (1×10^8 CFU in 2 ml inoculum) in both experiments. The activity of lactobacilli that colonized the gastrointestinal tract of gnotobiotic piglets was then influenced by period of administration. We could suggest influencing the immunomodulation properties of probiotic bacteria by dose factor and/or by the factor of the length of administration. It is necessary to determine these factors with respect to possibility to induce the tolerance. Administration of too high doses or too long a period of application could provoke this phenomena.

On the basis of our results, we can conclude that it is more effective to regulate the period of administration. Too short a period of application requires too high doses of bacteria to produce the same effects and this could lead to immunotolerance with negative influence on the immune system of the host. Our results obtained with the *L. casei* strain specified proved the advantage of 10-day administration of that respective strain in comparison with the 3-day period.

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