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The influence of application of probiotic on the immune system of 2-3 months old calves

Summary

The study presented investigated the influence of application of a probiotic on the selected traits of the immune system (total leucocyte number - Le, differential blood picture - DBP, per cent of phagocytic activity - % PA, index of phagocytic activity - IPA, total immunoglobulins - TIg) which was affected by administration of ovalbumin with the aim to induce a model immune response. The total number of lactobacilli bacteria in the excrements was observed simultaneously. The experimental group to which a probiotic was applied (TG) exhibited uniformity of traits investigated in the course of our experiment and the application of a lactobacillus strain resulted in an increase in only some nonspecific immune functions. Animals of the control group (CG) exhibited biger variations in values of individual parameters investigated than animals treated by probiotic preparation.

Key words: probiotic, lactobacilli, immunity, calves

Zusammenfassung

Titel der Arbeit: **Einfluß der Applikation von Probiotikum auf das Immunsystem von 2 bis 3 Monate alten Kälbern**

Bei klinisch gesunden Kälbern, die unter normalen Zuchtbedingungen gehalten wurden, konnten der Einfluß der Applikation des laktobazillen Probiotikums auf die Gesamtzahl der Leukozyten, das Blutbild, die prozentuale fagozytäre Aktivität, die Indexziffer der fagozytären Aktivität und die Konzentration des Gesamtimmunoglobulins beobachtet werden. Zur Erkennung der optimalen Immunantwort erfolgte eine Beeinflussung des Immunsystems durch Ovalbumin, gleichzeitig konnte die Gesamtzahl der Laktobazillen im Kot erfaßt werden.

In der Versuchsgruppe mit der Applikation des Probiotikums wurde ein relativ höheres Gleichgewicht der speziellen Werten beobachtet und die Applikation der Laktobazillen führte zur Stimulation nur spezifischer Immunfunktionen. Bei den Tieren der Kontrollgruppe wurden größere Unterschiede bei einzelnen Werten während des Experimentes nachgewiesen

Schlüsselwörter: Probiotikum, Laktobazillen, Immunsystem, Kalb

Introduction

The use of probiotics in various farm animal species by current veterinary practice gains on importance (ŠIŠÁK et al., 1995; HEJLÍČEK and SOUKUPOVÁ, 1995; ROBERTON et al., 1995; KOUDELA, 1995). Their main advantage consists in the biological principle of their effect within which they functionally use - stimulate the natural physiological functions and by that they increase the productive potential of animals (SVOZIL, 1995).

FULLER (1989) characterized a good probiotic as such which contains live cells non-pathogenic to the host, capable of surviving and multiplicating in the intestinal environment, causes no resistance to antibiotics, it is not toxic, produces no side effects and, possibly, exerts a stimulative effect on the immune system. The stimulative effect of various probiotic strains on the immune system was confirmed by BEALMEAR et al. (1984), WADE et al. (1984) and PERDIGON et al., (1986).

The development of the immune system of calves depends on the level of colostral nutrition. The uptake of optimum doses of high-quality colostrum ensures the function of maternal antibodies during the initial three to four weeks of life. The age of 5 weeks marks the onset of organism's own immunopoiesis leading to immunological maturity. At the age of 8 weeks, the microflora of the digestive tract of calves is also stabilised and resists to variations in number and proportion of species.

The age at which the probiotic was applied to an animal is therefore an important factor which can affect its effectiveness. According to BARROW (1992) the ideal period of administration of probiotics to chickens is the short time period immediately after hatching when they still lack the so-called "protective" intestinal microflora. SVOZIL (1995) considers important to apply probiotic preparations to calves as early after calving as possible (with the first uptake of colostrum), i.e. in the period when the individual is becoming infected with the first germs from the environment which multiply and colonize the digestive tract.

Because of the mentioned the general recommendation is to use probiotics in the period immediately after birth when their preventive effect can be manifested best. The effect of administration of probiotic strains to animals the intestinal microflora of which was already stabilised has not been investigated frequently.

This experiment was aimed at the study of possible effect of administration of lactobacilli probiotic to 2-3 months old calves. At this stage of calf life the presence of intestinal bacteria population stabilised with regard to species and numbers is presumed together with termination of the development of calve's own immunopoiesis independent of the uptake of maternal colostral antibodies.

Materials and Methods

The experiment was carried out on 14 calves of average age 2 months. They were divided to two groups, 7 animals in each, and housed in groups. The experimental group (TG) was supplied a probiotic which containing *Lactobacillus casei 294/89* strain $1x10^9$ CFU in 1 g of dried powdered milk. The dose of probiotic was 2g per head and day for 4 weeks. The probiotic preparation was applied per os, individually to each of the animals. No probiotic was supplied to the control group (CG). Each animal was after zero-sampling immunized with ovalbumin (Sigma, Germany) like model antigen. The dose was 0.4 mg/10 kg of weight in 10% solution of alhydrogel. Reimmunization was done after third-sampling. Both groups received feed rations of the same composition. They consisted of feed mixture, hay and water. Blood was sampled in weekly intervals for 4 weeks in both groups, the zero-sampling taking

place before the application of the probiotic. The blood for immunological examination was withdrawn from *vena jugularis* directly into a heparinised test tube to obtain a final concentration of 5 heparin units per 1 ml of blood.

The number of leucocytes (Le)

To a volume of 475 ml of Turk's solution in a test tube a 25 ml aliquot of heparinized blood was added quantitatively. The content of the test tube was mixed thoroughly. A Bürker chamber of area 9 mm^2 and depth 0.1 mm was used for counting the leucocytes. The mixture prepared was transferred to the Bürker chamber and leucocytes were counted in 100 large squares at magnification 10 x 1.25 x 20. The total number of leucocytes was determined according to the formula $P = p.v.h.z / y$, where P - total number of leucocytes $G.l^{-1}$, p - number of leucocytes counted, v - reciprocal value of an area of the square in which the leucocytes were counted, h - reciprocal value of chamber depth, z - dilution of blood, y - number of squares in which the leucocytes were counted.

Leucogram (Ly)

The differential blood picture was obtained by means of a blood smear prepared from 10 ml of heparinized blood. After 24 hours, the blood smear was stained by a panoptic staining method according to Pappenheim using two staining solutions. The solutions were as follows: May-Grünwald stain, consisting of 1 part of the mixture of eosin and methylene blue, 50 parts of glycerin and 100 parts of methylalcohol; Giemsa-Romanowski stain, composed of 2 parts of the mixture azure II - eosin, 0.8 parts azure II., made up to 250 parts with the mixture of glycerin and methylalcohol. The blood smear was stained and dried up and 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 x 1.25 x 100.

Per cent of phagocytic activity (%PA) and the index of phagocytic activity (IPA)

The parameters mentioned above were determined by means of microspherical haematological particles (MSHP - Artim Prague, Czech Republic). Within 1 hour from sampling, a 0.1 ml aliquot of heparinized blood was incubated with 0.05 ml of microspherical particles solution prepared according to manufacturer's instructions. The incubation lasted for 1 hour 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 x 1.25 x 100 magnification. Each of potentially phagocytizing cells (monocytes, neutrophil granulocytes, eosinophils, basophils) which contained 3 and more phagocytized particles was considered to be a phagocytizing cell.

Total immunoglobulins (Tlg)

The concentration of total immunoglobulins was determined weekly in blood serum of each of the animals. After precipitation the sera were centrifuged at 3500 r.p.m. for

20 minutes. Total immunoglobulins were determined turbidimetrically according to the method of Mc EWAN (1970).

Total number of lactobacilli bacteria in poultry manure

The manure samples for microbiological examination were taken from all animals included in the experiment in weekly intervals. Zero-samples were taken from all animals before the beginning of the experiment. Manure was obtained from *ampulla recta* after stimulation of animals. The total number of lactobacilli was determined within 1 hour from sampling by cultivation on Rogosa agar.

Statistical Analysis

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

Results

The numbers of leucocytes (Le) were above the upper physiological limit in both groups during the entire experiment (Table 1). Both the experimental (TG) and the control group (CG) exhibited a marked increase in the number of leucocytes to 22.5 $G.1^{-1}$ and 21.5, resp., $G.1^{-1}$ during the first week. Then a gradual decrease was observed in TG down to the value of 16.57 $G.1^{-1}$ in the last week. After the initial increase of Le in CG during the first week their number also decreased, however, they increased to 19.71 $G.1^{-1}$ in the fourth week of the experiment.

Table 1

Values of selected non-specific immunological traits during the experiment, with marked significance (Werte der ausgesuchten unspezifischen immunologischen Merkmale, signifikante Differenzen sind gekennzeichnet)

Week	Group	Le ($G.1^{-1}$)	% Ly	% Ne	% PA	IPA	Tlg (UZST)
0	TG	12.36±4.94	90.57±4.2	8.14±4.16	3.43±1.39	4.81±1.1	21.86±7.92
	CG	12.29±7.33	89.86±3.59	8.71±4.33	2.57±1.96	5.0 ±5.37	16.67±4.87
1	TG	22.5 ±5.29	79.14±10.98	19.29±10.33	6.57±4.24	6.4 ±3.83	25.16±4.94
	CG	21.5 ±5.43	76.29±9.76	20.0 ±9.43	12.0 ±7.43	6.96±3.13	23.69±5.94
2	TG	20.0 ±5.71	79.43±10.20	19.0 ±10.29	2.57±1.96*	1.29±1.07	27.03±5.17
	CG	19.86±7.08	82.71±7.39	16.0 ±7.43	0.86±1.22	0.77±0.65	22.28±3.99
3	TG	18.21±3.67	84.86±5.31	13.71±5.31	2.57±1.96	1.29±0.84	28.54±5.66*
	CG	15.93±4.2	81.43±14.65	17.0 ±14.29	3.71±3.59	1.9 ±1.91	22.13±2.98
4	TG	16.57±4.94	87.14±4.45	7.29±4.24	2.0 ±1.14	7.21±6.16	27.18±4.5*
	CG	19.71±6.53	84.57±7.35	14.43±7.35	7.43±4.08**	10.89±5.93	20.77±5.53

* $p<0.05$, ** $p<0.01$

The concentration of total immunoglobulins (Tlg) in the serum was higher in the experimental group during the entire 4-week experimental period in comparison with the control calves. The differences between groups were significant in the third and fourth week ($p<0.05$), (Table 1). The highest average value of TIg was recorded in TG in the third week, 28.54 U ZST, while in the CG the highest value was detected in blood sampled during the first week, 23.69 U ZST.

The leucogram obtained pointed to marked lymphocytic leucocytosis in both groups during the period investigated. The average proportion of lymphocytes in per cent during the experiment was 84.22% in TG and 82.97% in CG. At the same time a

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decreased occurence of segment nucleal neutrophils was recorded. The all-experiment values ranged between 7.29 - 19.29% in TG and 8.71 - 20% in CG (Table 1).

The principal immunological traits observed were: per cent of phagocytic activity (%PA) and index of phagocytic activity (IPA) (Table 1). The %PA shows the relative number of phagocytising cells from among all leukocytes. IPA expresses the average number of phagocytised particles per one cell of potentially phagocytising cells.

The CG group exhibited an increase in %PA and IPA in the first and fourth week and maximum values of %PA, equal to 12 and 7.43, resp., and of IPA, equal to 6.96 and 10.89, resp., were reached. An increase in both values from the second week onwards was evident. Statistically significant difference in favour of the control group was recorded for %PA ($p<0.01$) in the fourth week. The TG group showed an initial increase in %PA to 6.57% in the first week and then a decrease to 2.57% in the second week which persisted more or less up to the end of the experiment. Also IPA exhibited increase from 4.81 to 6.4 in the first week. After a decrease in weeks two and three, when the average value of IPA declined down to only 1.29, an increase to 7.21 was recorded towards the end of the observation period.

Table 2
The number of lactobacilli in the excrement of animals during the experiment (Anzahl von Laktobazillen in den Ausscheidungen der Versuchstiere)

Week	Group	Total number of lactobacilli
0	TG	5.848 ± 0.338
	CG	6.184 ± 0.543
1	TG	5.103 ± 0.095
	CG	5.526 ± 0.611
2	TG	6.008 ± 0.488
	CG	6.117 ± 0.444
3	TG	5.941 ± 0.437
	CG	5.882 ± 0.363
4	TG	6.316 ± 0.370**
	CG	5.330 ± 0.397

** $p<0.01$

Table 2 shows that no significant differences in total numbers of lactobacilli, determined in excrement during the first three weeks, were recorded. In the period observed, their average numbers in TG were 5.843 $\log.ml^{-1}$ and in CG 5.808 $\log 10.ml^{-1}$. However, a significant difference ($p<0.01$) was recorded in week 4 when their numbers reached 6.316 $\log 10.ml^{-1}$ in TG and only 5.33 $\log 10.ml^{-1}$ in CG.

Discussion

A positive influence of probiotics on the digestive tract microflora and their immunostimulative effect was confirmed by a number of authors (PERDIGON et al., 1988, 1990; EBRINGER et al., 1995; POUWELS et al., 1996). The use of probiotic preparations in young animals as soon as possible after their birth appeared as most effective. It is conditional upon non-stabilised intestinal microflora, easily affected by

supplying the probiotic microorganism. Similar situation may occur in animals subjected to various stress factors, such as unsuitable hygiene conditions, antibiotic therapy, stress, and others.

The lactobacilli adherent intestinal receptors in adult animals are fully occupied by strains acquired in the natural way therefore their replacement with the probiotic strain would have to occur which necessitates a state that is difficult to induce (FULLER, 1989).

It was our intention to include in the experiment clinically healthy animals, of average age 2 months, free of negative stress factors. Increased numbers of leukocytes were recorded in both groups during the experiment. The differential blood picture showed marked lymphocytosis suggesting long-term chronic respiratory disease however no apparent clinical symptoms were observed.

The %PA showed an increase in both groups in the first week, more pronounced in CG (Table 1). This increase in the number of phagocytising cells is related to the application of ovalbumin. It was used to provoke model immune response, because the significant induction of the immune responses through the application of probiotic bacteria is by animal with stable microflora more difficult. Similar trend was observed for IPA in both groups of animals.

The samples taken in the second week showed higher values of %PA and IPA in the experimental group, however, by the third week both parameters reached higher values in the control group. Thus during weeks 2 and 3 the parameters mentioned were stable in the TG group while in the CG group an ubrupt increase in the first week was replaced with a sudden decline in %PA and IPA in the second week and a repeated moderate increase in the third week.

By the samples from the fourth week, after the repeated application of ovalbumin as an antigen in the third week, an increased reactivity of the nonspecific immune system as a secondary immune response was expected in all the animals. In reality, such a reaction was recorded only in CG to which no lactobacillus strain was applied. In the TG group an increased antigenic response was observed only in IPA while the number of activated phagocytes declined slightly. The probiotic affected directly the phagocytic ability of cells of the non-specific defence system (SCHIFFRIN et al., 1995).

The difference between the number of activated potentially phagocytising cells between the groups, determined by %PA and in comparison with IPA, suggests the capability of lactobacilli bacteria to stimulate the immune system in a very specific way which is confirmed by the total number of leukocytes during the experiment. In this case the activity of phagocytising cells was stimulated without an increase in their number. This can be related to the application of ovalbumin which was used to induce a model immune reaction of the non-specific defence system. Ovalbumin is a non-typical antigen other than, for example, bacteria mainly because it cannot cause a disease and the parenteral application is an uncommon way of possible infection of a macroorganism.

This was probably the reason why no increase in the non-specific defence response in

the form of increased number of phagocytising cells was recorded in the TG group contrary to CG animals. It is possible that in the case of common bacterial infection an increase in the number of particles phagocytised by individual cells would be accompanied by an increase in relative and absolute number of the cells. On the other hand, in this concrete case obtained results show the specific immunomodulation of the host by lactic acid bacteria. Therefore these immunomodulation effects should be study also by different influences.

The assessment of production of specific antibodies showed, however, an increase in their production in TG and the differences between groups were statistically significant (NAĎ et al., 1997). This suggests an independent action of the lactobacillus strain used on the cellular and humoral immunity and confirms the specifity of its effect.

It is interesting that rather suppressive or balancing effect on the number of activated cellular immunity cells was observed without abrupt reactions such as those recorded in the group that received no probiotic preparation.

The results of the experiment proved a predisposition effect of lactobacilli bacteria mainly on the local level in the intestinal tract and their only partially explained so-called biologically beneficial action on maintenance of homeostasis of animals without extreme changes in animal biostatus. This was reflected in the balanced health state of calves, better consumption of feed and stimulation of the immune system during the application of probiotic.

To support the proposed hypothesis it is necessary to carry out additional detailed observations aimed at older age categories of animals with regard to possible therapeutic utilization of probiotic preparations in the future.

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