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The influence of probiotic administration on selected leukocyte subpopulations and the serum amyloid A concentration in the peripheral blood of dairy cows during different lactation periods

Piotr Brodzki[1]✉, Jan Marczuk[2], Hubert Gorzkoś[3], Urszula Lisiecka[4], Marek Szczubiał[1], Adam Brodzki[5], Roman Dąbrowski[1], Leszek Krakowski[1], Katarzyna Głodkowska[1], Nikodem Brodzki[6]

[1]Department and Clinic of Animal Reproduction, [2]Department and Clinic of Animal Internal Medicine, [4]Department of Epizootiology and Clinic of Infectious Diseases, [5]Department and Clinic of Animal Surgery, [6]Student of the Faculty of Veterinary Medicine,
Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland
[3]Gorvet Private Veterinary Practice, 09-227 Sztutowo, Poland
wetdoc@interia.pl

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Abstract

Introduction: The aim of the study was to compare selected leukocyte subpopulations and the serum amyloid A (SAA) concentration in the peripheral blood of cows at different stages of lactation. The blood of cows receiving a probiotic as a dietary supplement was compared with the blood of cows not receiving it. **Material and Methods:** The research was conducted on 20 pregnant dairy cows randomly divided into two groups of 10 cows each. The experimental group consisted of cows given the probiotic as a feed supplement. The control group consisted of cows that were fed without supplementation. Blood was drawn six times for testing: 7 days before drying; 14 days before parturition; and 7, 21, 60 and 90 days postpartum. Leukocyte immunophenotyping was performed by flow cytometry. **Results:** The blood of cows administered the probiotic revealed an increased percentage of forkhead box protein 3 $(Foxp3)^+$, T $CD4^+$ and B $CD25^+$ lymphocytes and β2 $CD18^+$ and αM $CD11b^+$ integrins, and persistently low SAA levels at all time points. **Conclusion:** The activity of the immune system in cows receiving the probiotic was higher than in control cows. However, the stabilisation of the immune system of the supplemented cows may be indicated by the persistence of a low level of SAA throughout the experiment. Therefore, it can be assumed that the immune system of cows treated with the probiotic more easily adapts to changes in conditions in particular lactation periods and that these cows become more resistant to infectious diseases.

Keywords: dairy cows, integrins, leukocyte subpopulations, probiotics, serum amyloid A.

Introduction

In dairy cow breeding, maintaining a high milk yield and the profitability of its production depends on many factors: mainly the fertility and health of cows, their genetic predisposition, the appropriacy of their maintenance, the feeding method and the quality and balance of the feeding ration. The cycling of cows through pregnancy, delivery and milk production determines consecutive, correspondingly cyclical lactation periods, *i.e.* the dry period, perinatal period and lactation. Cows in a herd are functionally distributed to appropriate sections in accordance with these periods. This facilitates milking and provision of nutrition strictly adapted to the given lactation period and thus to the current needs of the cows (24). The health of cows is determined by many internal and environmental factors that may come into play in different periods of lactation (29). However, it is important to emphasise the importance for dairy cow health of the perinatal period,

which includes the last weeks of the lactation period until about 60 days postpartum (DPP). Within this time, the transition period – from three weeks before parturition to approximately three weeks after it – merits the closest attention. The numerous physiological and hormonal changes that occur in the bodies of dairy cows during the perinatal period allot a fundamental role to this period in determining the course of all subsequent lactations (14). The perinatal period has the highest prevalence of metabolic and deficiency diseases such as postpartum hypocalcaemia, hypomagnesaemia, fatty liver, ketosis, mastitis, metritis and abomasal displacement, as confirmed by numerous studies (23, 30). The cow's immune system is also temporarily suppressed during this time (19), and this state in dairy cows during the peripartum period is closely related to the metabolic diseases cited above or predisposes cows to mastitis, metritis and placental retention (15). Although constantly evolving, our knowledge of the dynamics and pathophysiology of immunosuppression during this period remains unsatisfactory. Effective strategies to improve defence mechanisms while maintaining cows' health and well-being during this critical period, as well as during other periods of lactation, remain to be devised (1).

There has been a growing interest in the use of probiotics to enhance milk production and cow health in recent years. Probiotics are natural strains of intestinal bacteria specific to the digestive tract of an animal species. Probiotic products contain live or dead microorganisms and substances produced by them. When administered orally, they colonise the intestines and prevent the overgrowth of pathogenic microorganisms, thereby improving digestion and optimising feed utilisation (5). By helping to stabilise the balance of the microbial population and enzymatic activity in the gastrointestinal tract, they have a positive effect on animal growth, development and productivity (3, 31). Probiotics may contain one or several strains of microorganisms and may be administered in the form of microbial additives, separately or in combination with other substances (32). The mechanism of action of probiotic microorganisms introduced to the gastrointestinal tract of an animal is complex and multifaceted. Primarily, these organisms compete for adhesion on the intestinal epithelium, for nutrients and for bacteriostatic substances. In addition, they inhibit the development of pathogens and stimulate systemic immunity (41). The precise mechanism underlying the immunomodulatory effects of probiotics remains poorly understood. The impact of whole probiotic bacteria cells or their fragments on T and B lymphocyte function as mediators of an immune response to antigens has been demonstrated in humans and mice. This effect is believed to occur *via* a complex formed between the bacteria and cells of the gut-associated lymphoid tissue. Probiotics also stimulate immunocompetent cells to secrete cytokines that suppress, modulate and regulate the intensity of systemic and local immune responses (12). It is currently unclear what impact probiotics have on immune system function in dairy cows; however, the majority of published research, predominantly in the form of review articles, has demonstrated a positive effect (8, 40).

The objective of the study was to conduct a comparative assessment of selected leukocyte subpopulations and the SAA concentration in peripheral blood mononuclear cells of cows administered a probiotic as a nutritional additive and these subpopulations and concentration in this blood of cows fed without the probiotic addition, during different lactation periods.

Material and Methods

Experimental animals. The study was approved by the Local Ethics Committee at the University of Life Sciences in Lublin (approval No. 41/2014). The studies were conducted in a herd of 60 Holstein-Friesian dairy cows at different stages of lactation. The cows were kept in a mixed system, chained during feeding and milking and free-range for the rest of the time. The diet was based on the total mixed ration (TMR) system. A fully mixed complete feed was used, which had a complete nutritional composition that was adapted to the physiological needs of the cows. The composition of the fodder provided to the farms included in the experiment was balanced for lactating cows with an average milk production of approximately 20 kg. Each cow with a milk yield that exceeded 20 kg also received an additional 1 kg of concentrate for every 2 kg of milk produced in excess of the average. The detailed composition of the TMR is presented in Table 1.

Table 1. Composition of the total mixed ration and dry mass (DM) daily feed ration for lactating cows (kilograms/cow/day)

Feed component	Amount per cow, daily	
	Kg	Kg DM
Maize silage	25.0	8.8
Haylage	8.0	3.2
Ensiled brewery spent grain	8.0	2.7
Wheat straw	0.8	0.7
Ensiled maize grain	2.5	1.7
Ground barley grain	1.5	1.3
Ground triticale grain	1.5	1.3
Ground rapeseeds	2.7	2.2
Extracted soyabean meal	2.0	1.7
Glycerine	0.3	0.24
Vitamin and mineral mixture	0.2	0.18
Sodium bicarbonate	0.2	0.2
Calcium carbonate	0.05	0.05
Total	52.75	24.27

Table 2. List of primary antibodies and secondary conjugates used in this study

Antibodies, dye	Cell type	Clone	Isotype
Mouse anti bovine CD4, PE	T helper cells	CC8	IgG2a
Mouse anti bovine CD8, FITC	T cytotoxic/suppressor cells	CC63	IgG2a
Mouse anti bovine CD11b, FITC	αM-integrin receptor subunit	CC126	IgG2b
Mouse anti-dog CD18, purified	β2-integrin receptor subunit	CA1.4E9	IgG1
Goat anti-mouse IgG (Fc), FITC	secondary antibody	polyclonal	polyclonal IgG
Mouse anti-bovine CD21, FITC	B lymphocytes	CC21	IgG1
Mouse anti-bovine CD25, RPE	Interleukin-2 receptor alpha chain	IL-A111	IgG1
Mouse anti-bovine Foxp3^{+}, FITC	T regulatory cells	FJK-16s	IgG2a, kappa

FITC – fluorescein isothiocyanate; RPE – R-phycoerythrin; PE – phycoerythrin

The vitamin and mineral mixture consisted of calcium carbonate, sodium chloride, sodium-calcium phosphate, magnesium oxide and magnesium sulphate (23% calcium, 2.2% phosphorus, 9% sodium and 4.5% magnesium). The parameters per kilogram of concentrate were as follows: vitamin A 450,000 IU, vitamin D_3 45,000 IU, vitamin E 6,000 mg, vitamin K 400 mg, vitamin C 1,000 mg, vitamin B_1 120 mg, vitamin B_2 60 mg, vitamin B_6 30 mg, vitamin B_{12} 300 µg, nicotinic acid 6,000 mg, pantothenic acid 120 mg, biotin 75,000 µg, choline chloride 6,000 mg, zinc 6,000 mg, manganese 4,000 mg, copper 1,200 mg, iodine 120 mg, cobalt 40 mg and selenium 20 mg. The milk yield of the cows for the 305-day lactation period ranged from 7,200 to 8,720 kg per cow. The cows were fed according to their actual milk yield and gestation period, so the composition of the feed was changed according to the lactation period of the cows. Regular monthly check-ups of the reproductive system were carried out by rectal examination combined with ultrasonography. A synchronisation protocol of oestrus and ovulation (the presynch-ovsynch protocol) and artificial insemination (AI) with frozen semen were applied to cows that showed no complications during parturition and no signs of inflammation. However, cows with evidence of uterine inflammation were treated appropriately and were subsequently subjected to the synchronisation protocol of oestrus and ovulation and next to AI. Cows with ovarian cycle disorders were treated individually in accordance with the recognised cause.

Twenty pregnant cows, aged 3–5 years, were selected for the current research. All selected cows were in the last lactation period before the drying period. Their body condition was good (body condition score 3.0–3.5). The study included a health assessment and parasitological examination to confirm that all the cows were healthy. The selected animals were divided into two groups of 10 cows each to receive or not receive the probiotic. The experimental group consisted of cows administered the probiotic product, 1mL of which contains 5×10^3 colony-forming units (CFU) of *Saccharomyces cerevisiae* and 5×10^6 CFU of each of *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Rhodopseudomonas palustris*. The product was administered in liquid form as an additive to standard feed at the dose recommended by the manufacturer (200 mL of product per cow per day), during the period from before drying to the 12th week after birth (90 DPP). The probiotic was administered to each cow individually, poured over the given TMR feed dose. The control group consisted of cows that were not administered any medication throughout the experiment and from which only the material used for studying was collected. These cows were on the same diet as the experimental group, but without the addition of the probiotic. The research procedures were identical for both groups of cows. The description of the cow herd and the selection of animals for experimental groups is similar to that in the work by Brodzki *et al.* (5), because it is a continuation of research.

Material. Peripheral blood was used as the material for cytometric analysis. The test material was collected six times: the first time on the day the animals were selected at 7 days before drying (DBD), and then at 14 days before parturition (DBP) and 7, 21, 60 and 90 DPP. Blood samples of 9 mL in volume were collected from the external jugular vein in ethylenediaminetetraacetic acid or heparinised tubes (Vacutest Kima, Arzergrande (PD), Italy). The biological material collected was sent to the laboratory for tests within 1 h (5).

Lymphocyte phenotyping by flow cytometry. Cytometric analysis was performed within 4 h of sampling. Lymphocyte immunophenotyping was performed with an Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA). Daily calibration was performed using Flow Check fluorospheres (Beckman Coulter). In both the investigated and the control groups the following receptors were investigated: CD4 (T helper (Th) cells), CD8 (T cytotoxic/suppressor (T) cells), CD11b (αM-integrin receptor subunit), CD18 (β2-integrin receptor subunit), CD21 (B lymphocytes), CD25 (interleukin (IL)-2 receptor alpha chain) and forkhead box protein 3 (Foxp3) (T regulatory cells). All antibodies were purchased from Serotec Immunological Excellence (Oxford, UK). Conjugated primary antibodies were used for all tests except

the CD18 test, where indirect labelling was applied. The cross-reactivity of the CD18 antibody was confirmed by Brodersen *et al.* (4). The details of the specific primary antibodies and secondary antibody conjugate used in this study are listed in Table 2. Before the experiment, optimal dilutions of antibodies were established by titration. For staining of peripheral blood, cell samples were incubated with appropriate antibodies for 30 min following a set protocol. Erythrocytes were removed from the analysis using ammonium chloride lysing solution. Leukocyte subsets were gated according to their size and granularity using forward scatter and side scatter parameters. At least 10,000 leukocytes were collected per tube. For Foxp3 intracellular staining, lymphocytes were stained first with extracellular antibodies (CD4 and CD25) (Table 2). Then peripheral blood cells were fixed and permeabilised using IntraPrep permeabilisation reagent (Immunotech/Beckman Coulter, Marseille, France) following a set protocol. In the next step, the samples were incubated with FITC-labelled anti-murine Foxp3 monoclonal antibody (clone FJK-16s; eBioscience, San Diego, CA, USA) for 30 min. Erythrocytes were removed from the analyte using ammonium chloride lysing solution. At least 30,000 leukocytes were collected per tube. $Foxp3^+$ cells were gated within the $CD4^+$ lymphocyte subpopulation, and appropriate controls were applied to assist gating decisions. Controls were run under exactly the same conditions as experimental samples. Validation procedures were conducted using Flow-Check fluorospheres (Beckman Coulter) and Immuno-Trol cells (Beckman Coulter). All analyses were conducted on the same, unchangeable protocol, the same instrumental settings, and with the same voltages applied. Daily compensation procedures were applied for each sampling point. All samples were run at low flow rate.

Measurement of serum amyloid A in blood serum. Blood serum SAA levels were measured using a commercial ELISA kit (Tridelta Development, Maynooth, Kildare, Republic of Ireland). The inter- and intra-assay coefficients of variation for SAA analysis were <12.1% and <7.5%, respectively. The procedures were carried out according to the manufacturer's instructions and the methods in the literature (38). Absorbance readings and the subsequent calculations of final concentrations were performed on an automatic microplate reader (Asys Expert Plus; Biochrom, Cambridge, UK) at 630 nm as a reference for SAA. Lyophilised bovine acute-phase serum was used as a standard and calibration was performed according to the European Union concerted action on standardisation of animal acute-phase proteins (APPs) (No. QLK5-CT-1999-0153 (36)).

Statistical analysis. Statistica software (version 10.0) (StatSoft, now TIBCO, Palo Alto, CA, USA) and one-way analysis of variance were used for statistical analysis. All values are expressed as mean ± SEM. Results were compared between the control and experimental groups for statistical significance using Student's *t*-test, probability P-value ≤ 0.05. One-way analysis of variance and Tukey's and Duncan's post-hoc tests were used to calculate statistical differences between results for material collected at different times in the group, with probability P-value ≤ 0.01 assumed as indicative of significance.

Results

Changes in lymphocyte subpopulations in cows during the research. The percentages of cells in individual leukocyte subpopulations in both studied groups of cows in the course of the experiment are presented in Table 3. The presented data show that the percentages of β2 integrins ($CD18^+$) on all leukocytes (lymphocytes, granulocytes and monocytes) were significantly higher in the cows from the experimental group throughout the entire period of the experiment, except for the first test period at 7 DBD, in which the obtained values were similar in both groups of cows. In the course of the experiment, an increase in the percentage of CD18 receptors from the 7 DBD baseline was observed on lymphocytes of the experimental group at 7 DPP (with decreases before and after) and on granulocytes and monocytes at 60 and 90 DPP (with decreases before) (P-value ≤ 0.01). In the control group, the percentage of CD18 receptors on lymphocytes fell on consecutive study dates and remained at a low level until the end of the experiment. After decreasing, these percentages on granulocytes and monocytes increased at 60 and 90 DPP to values higher than the 7 DBD baseline values.

The experimental group's percentages of $CD11b^+$ lymphocytes were significantly higher on 7 DBD, 14 DBP and 60 and 90 DPP than those of the control group (P-value ≤ 0.01 for all time points except 7 DBD – P ≤ 0.05). The granulocyte percentage which was $CD11b^+$ fluctuated very little within the experimental and the control groups and between them over the course of the experiment, and was only significantly different once: it was lower in the supplemented cows' blood than the control cows' blood at 7 DBD (P ≤ 0.01). The percentage of $CD11b^+$ monocytes was significantly higher on 14 DBP and 60 DPP in the experimental group than in the control group (P-value ≤ 0.01).

The percentage of $CD21^+$ lymphocytes was significantly higher at 14 DBP (P-value ≤ 0.05), and 60 DPP (P-value ≤ 0.01) in cows from the control group than in those from the experimental group. In contrast, the 90 DPP value was higher in the experimental group than in the control group (P-value ≤ 0.01). Within the group, these values remained at a similar level throughout the experiment; only in the experimental group was an in-group statistically significant difference observed, which was the described increase at 90 DPP (P-value ≤ 0.01).

Table 3. Phenotyping of leukocytes in the peripheral blood of experimental (E) and control (C) cows during the experiment

		Leukocyte percentage					
Subpopulation	Group	7 DBD	14 DBP	7 DPP	21 DPP	60 DPP	90 DPP
$CD18^+$ lymphocytes	E	6.0 ± 0.6 ^{a}	5.1** ± 0.6 ^{b}	7.22** ± 2.9 ^{a}	4.6** ± 1.5 ^{b}	4.7** ± 1.2 ^{b}	4.2* ± 0.9 ^{b}
	C	6.6 ± 0.9 ^{a}	3.2 ± 0.6 ^{b}	2.8 ± 0.5 ^{b}	2.6 ± 0.8 ^{b}	2.8 ± 0.6 ^{b}	3.3 ± 0.3 ^{b}
$CD18^+$ granulocytes	E	12.5 ± 3.9 ^{a}	11.7** ± 3.2 ^{a}	6.8* ± 1.6 ^{b}	11.0** ± 2.3 ^{a}	23.0** ± 1.6 ^{c}	24.7** ± 2.5 ^{c}
	C	14.8 ± 3.0 ^{a}	6.2 ± 1.7 ^{b}	4.9 ± 1.7 ^{b}	4.4 ± 1.5 ^{b}	11.6 ± 2.6 ^{c}	18.3 ± 4.6 ^{a}
$CD18^+$ monocytes	E	11.7** ± 1.4 ^{a}	4.5 ± 1.5 ^{b}	8.6* ± 2.9 ^{c}	9.5** ± 1.8 ^{c}	18.0** ± 1.4 ^{d}	16.1* ± 1.8 ^{d}
	C	5.8 ± 1.4 ^{a}	5.2 ± 0.9 ^{a}	6.3 ± 0.6 ^{a}	4.6 ± 0.8 ^{a}	10.9 ± 1.4 ^{b}	13.4 ± 1.4 ^{c}
$CD11b^+$ lymphocytes	E	23.1* ± 4.4 ^{a}	23.7** ± 4.0 ^{a}	14.2 ± 1.2 ^{b}	15.5 ± 2.2 ^{b}	21.8** ± 2.1 ^{a}	30.4** ± 3.3 ^{c}
	C	19.5 ± 2.6 ^{a}	16.0 ± 2.1 ^{b}	13.2 ± 2.8 ^{b}	14.1 ± 2.0 ^{b}	17.0 ± 3.0 ^{ab}	25.2 ± 3.1 ^{c}
$CD11b^+$ granulocytes	E	98.5* ± 0.9	98.9 ± 0.9	99.7 ± 0.1	99.7 ± 0.2	99.9 ± 0.0	99.6 ± 0.1
	C	99.5 ± 0.3 ^{a}	98.1 ± 0.9 ^{b}	99.8 ± 0.1 ^{a}	99.7 ± 0.1 ^{a}	99.8 ± 0.1 ^{a}	99.9 ± 0.1 ^{a}
$CD11b^+$ monocytes	E	76.1 ± 4.2 ^{a}	78.1** ± 4.1 ^{a}	74.4 ± 5.8 ^{a}	61.8 ± 5.0 ^{b}	70.3** ± 4.2 ^{ab}	69.3 ± 4.1 ^{ab}
	C	73.3 ± 4.9 ^{a}	69.8 ± 7.1 ^{a}	71.2 ± 6.6 ^{a}	61.0 ± 8.9 ^{b}	56.0 ± 8.7 ^{b}	65.9 ± 4.1 ^{b}
$CD21^+$ lymphocytes	E	23.4 ± 3.78 ^{a}	21.14* ± 4.56 ^{a}	24.72 ± 3.86 ^{a}	24.74 ± 4.62 ^{a}	22.28** ± 4.38 ^{a}	33.32** ± 2.76 ^{b}
	C	24.88 ± 1.68	25.40 ± 3.02	25.62 ± 1.32	25.56 ± 3.32	25.50 ± 1.38	25.50 ± 1.64
$CD25^+$ lymphocytes	E	10.76 ± 2.28 ^{a}	8.13** ± 0.6 ^{a}	14.9** ± 2.19 ^{b}	20.4** ± 2.62 ^{c}	17.86** ± 3.0 ^{bc}	23.22** ± 2.94 ^{c}
	C	9.26 ± 1.28 ^{a}	6.51 ± 1.45 ^{b}	6.63 ± 1.66 ^{b}	10.75 ± 2.28 ^{a}	11.78 ± 1.18 ^{a}	15.8 ± 2.77 ^{c}
$CD4^+$ lymphocytes	E	28.96 ± 2.12 ^{a}	32.80 ± 2.66 ^{b}	34.04 ± 2.92 ^{bc}	35.72* ± 2.32 ^{bc}	36.62** ± 1.32 ^{c}	34.94** ± 1.66 ^{bc}
	C	26.64 ± 2.52 ^{a}	33.40 ± 1.72 ^{b}	33.72 ± 0.32 ^{b}	33.90 ± 0.52 ^{b}	32.62 ± 2.32 ^{bc}	30.72 ± 0.96 ^{c}
$CD8^+$ lymphocytes	E	10.56 ± 1.60	11.55* ± 1.12	11.96 ± 0.97	10.47* ± 1.69	10.15 ± 1.84	11.47** ± 1.48
	C	9.69 ± 1.11 ^{a}	13.18 ± 1.56 ^{b}	10.87 ± 0.78 ^{a}	13.04 ± 2.27 ^{b}	10.94 ± 2.12 ^{a}	16.56 ± 0.46 ^{c}
$Foxp3^+$ lymphocytes	E	42.54 ± 4.44 ^{a}	44.34 ± 3.06 ^{a}	49.44** ± 4.18 ^{b}	42.56 ± 4.88 ^{a}	47.68** ± 3.84 ^{ab}	46.22** ± 4.42 ^{ab}
	C	44.08 ± 3.52 ^{ab}	43.98 ± 1.44 ^{ab}	40.44 ± 4.18 ^{a}	41.10 ± 2.64 ^{a}	45.78 ± 3.26 ^{b}	33.92 ± 2.12 ^{c}

DBD – days before drying; DBP – days before parturition; DPP – days postpartum * – statistically significant difference at P-value ≤ 0.05 compared to the control group parameter; ** – statistically significant difference at P-value ≤ 0.01 compared to the control group parameter; $^{a–d}$ – different superscript letters between any value pair indicate statistically significant difference between percentages in blood collected at different times in the same group (P-value ≤ 0.01)

The percentage of $CD25^+$ lymphocytes in the blood of cows from the experimental group was significantly higher at all transitional period time points when compared to the percentage in the blood of cows from the control group (P-value ≤ 0.01). Only at 7 DBD were the values in both groups similar. Within the experimental group, the percentage of $CD25^+$ lymphocytes decreased only at 14 DBP, and at the subsequent study points it increased significantly above the baseline value. In the control group, the values for $CD25^+$ lymphocytes decreased significantly at 14 DBP and 7 DPP, and then gradually increased on subsequent study days to exceed the 7 DBD value significantly.

The percentages of $CD4^+$ lymphocytes at 21 DPP (P-value ≤ 0.05) and 60 and 90 DPP (P-value ≤ 0.01) were higher in the cows from the experimental group than in those from the control group, and in the experimental cows the values were also progressively higher over the course of the study, with the exception of an insignificant decrease at 90 DPP. In the control group, the values of $CD4^+$ lymphocytes trended higher throughout the experiment, increasing significantly from the 7 DBD baseline, but contrastingly decreasing at 90 DPP (P-value < 0.01). The percentage of $CD8^+$ lymphocytes in the experimental group was lower than that in the control group at 14 DBP and 21 DPP (P-value ≤ 0.05) and at 90 DPP (P-value ≤ 0.01). The values obtained in the experimental cows' blood remained at a similar level at all time points. In the control group, these values fluctuated between being insignificantly higher and being significantly higher than at 7 DBD, the highest percentage being noted at 90 DPP, when it was significantly different to all earlier percentages.

The values for $Foxp3^+$ lymphocytes in the experimental group's blood samples exceeded those in the control group's samples at all time points except for 7 DBD (when they were lower). Statistically significant differences between these groups were noted at 7, 60 and 90 DPP (P-value ≤ 0.01).

Serum amyloid A concentration in cows during the research. The SAA concentration in experimental cows was significantly lower than that in cows from the control group throughout the experiment, except for at the 7 DBD baseline, when similar values were recorded (Fig. 1). In the cows from the experimental group, the highest SAA value was obtained in the first transitional period time point, then these values decreased and remained at a similar level until the end of the

experiment. In the control group, the values at 7 DBD were the lowest (27.09 ± 5.3), then there was an increase (44.19 ± 2.74) at 14 DPP, a decrease (34.13 ± 3.63) at 21 DPP, a subsequent increase (39.15 ± 4.44) at 60 DPP and constancy of the percentage until the end of the experiment (Fig. 1).

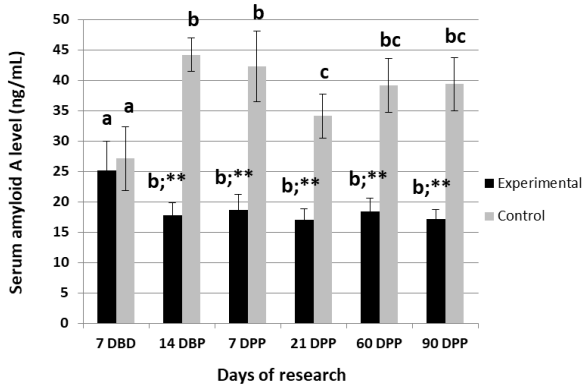

Fig. 1. The serum amyloid A level in the peripheral blood of cows at various periods of lactation
DBD – days before drying; DBP – days before parturition; DPP – days postpartum. Experimental – cows with probiotic (n = 10); Control – cows without probiotic (n = 10); ** – statistical significance at – P-value ≤ 0.01 with respect to the control; $^{a-c}$ – statistical differences between the results for the material collected at different times in the group (P-value ≤ 0.01)

Discussion

The aim of the present study was to determine whether probiotic supplementation has an effect on the function of the immune system in dairy cows and the magnitude of any effect at different stages of lactation. Selected indices of the systemic immune response in cows were assessed during the period from the last week of lactation, through the dry period and the peripartum period to the peak of the following lactation (*i.e.* 90 DPP). The effects of probiotics on specific parameters of the immune system in dairy cows have rarely been described in detail in the literature. The majority of studies on cattle made general statements regarding the immunomodulatory effect of probiotics in this animal species. Furthermore, the majority of research undertakings on probiotic support of the dairy cow immune system spanned a limited time of probiotic use and observed a short period of their effect. Additionally, this research has often been limited to the transition period (8, 28). It is evident that this is the most crucial phase of the entire lactation process, as it demands adaptation to complex metabolic transformations caused by parturition and the commencement of milk production (14, 37). However, changes in feeding conditions occur several times during lactation: firstly, before drying off, then besides the change during transition, also when adjusting the postpartum feed ration to a cow's current milk yield. The authors maintain that knowledge of the use of probiotics at different stages of lactation is important, because it allows us to decide whether probiotic use is reasonable in terms of supporting the activity of the immune system. It may be learned that the limitation of their use to the transition period is sufficient (5).

An increase in the percentage of $Foxp3^+$ regulatory lymphocytes and activated B lymphocytes ($CD25^+$) and a simultaneous decrease in the percentage of non-activated B lymphocytes ($CD21^+$) was observed in the assessed subpopulations of leukocytes in cows from the experimental group. This may mean that the increase in the percentage of regulatory lymphocytes results in the activation of B lymphocytes, which is manifested by the heavier presence of the $CD25^+$ activation receptor on these lymphocytes. This is evidence for not only the potentiated activity of cellular mechanisms (phagocytosis and phagocytic killing) previously described by Brodzki *et al.* (5), but also the activation of humoral immune mechanisms in the group of cows under study. These changes may result from the triggering of regulatory processes in the immune systems of cows receiving the probiotic. Regulatory lymphocytes contribute to the stabilisation of the cow's immune system through secretion of anti-inflammatory cytokines, such as IL-10. The appropriate mechanisms necessary to eliminate a possible threat and at the same time maintain homeostasis are activated. Lymphocytes which are $CD4^+$ Th cells, the task of which is to support cellular activities and humoral mechanisms of immunity, also contribute to this phenomenon. Specific subpopulations of activated Th lymphocytes redirect the immune response by secreting appropriate cytokines to Th1, Th2 or T17 type (16). In this study, the Th lymphocytes and hence the direction of the immune response were not precisely differentiated; however, a higher percentage of T $CD4^+$ lymphocytes in cows receiving the probiotic than in cows in the control group was demonstrated throughout the postpartum period. In addition, a rise in the percentage of lymphocytes, granulocytes and monocytes with β2 ($CD18^+$) and αM ($CD11b^+$) integrin receptors and the persistence of the percentage at an elevated level were also observed, which should be associated with a primed leukocyte state and the possibility of their adherence to the vascular endothelium blood vessels in the target tissues. The next stages of the diapedesis process initiated by integrins are the penetration through the wall of blood vessels into the tissues and combat against or removal of possible threats at the site of exposure in peripheral tissues.

Special attention should be paid to the immune mechanisms occurring in cows in the control group. The course and direction of the immune phenomena were similar to those in the experimental group, taking into account the proportions of individual leukocyte subpopulations. However, based on the obtained results, the immune system activity in this group of cows was significantly lower. Additionally, the increasing percentage of $CD8^+$ cytotoxic/suppressor lymphocytes in cows from the control group at three examination dates before and after parturition may indicate periodic

switching of the immune response mechanisms from humoral to cellular, and shows fluctuations in the activity of these mechanisms. There are too little data for a more precise assessment, and further research should be conducted; however, the immune system activity of the cows in the control group was less stable than that of the experimental group. This finding is of significant importance, as it allows the assumption that cows supplemented with the probiotic had a superior immune status prior to parturition and were more capable of adapting in the postpartum period. This is in accordance with the available literature, which indicates that during this period, the immune response of cows is weakened, increasing their susceptibility to infections, particularly those affecting the uterus and udder (30). The precise causes of immunosuppression during this period remain unclear.

A contributive factor to postpartum dairy cow immunosuppression may be the elevated levels of nonesterified fatty acids (NEFA) and ketone bodies during peripartum negative energy balance, this having been indicated by investigations of bovine blood (14, 17). Levels of NEFA and β-hydroxybutyrate acid (BHBA – a ketone body) were not evaluated in this study, but it cannot be excluded that concentrations of these metabolites were increased in these cows. The study presented by Nocek *et al.* (26) seemed to confirm these hypotheses, as it showed that the concentration of NEFA was lower in cows that were fed a diet containing *Enterococcus faecium*. Similar results with *Bacillus subtilis* natto as a dietary supplement were obtained by other authors (27). Cows supplemented with *Enterococcus faecium* had lower BHBA concentrations in the postpartum period in the study by Nocek and Kautz (25). Furthermore, Luan *et al.* (22) observed reduced postpartum ketone concentrations in cows supplemented with *Bacillus pumilus*. During periods of high energy demand at the beginning of lactation, lower concentrations of NEFA and ketones suggest that cows mobilise less energy from adipose tissue. In addition, increased glucose and insulin concentrations were observed in the blood serum of cows fed *Enterococcus faecium* in the postpartum period, in contrast to animals not fed probiotics (26). There are at least two reasons why higher blood glucose levels and lower adipose tissue mobilisation (lower NEFA and BHBA) in the postpartum period are very beneficial for cows. Firstly, more glucose can be directed to the udder for milk production, which is important for the maintenance of high milk yield. Secondly, glucose is an essential source of energy for ongoing physiological processes, for the immune system and especially for phagocytes. It may also affect the ratio of leukocyte subpopulations (10, 18).

The present study was not able to show a direct effect of the probiotics on the energy status of the cows, as the indices mentioned above (NEFA, BHBA and glucose) were not evaluated. However, the possible mode of action of probiotics as it relates to what our study did assess can be partially explained by the relationships referred to above and presented in the cited publications. The results suggest that the probiotic applied in the trial was able to improve the energy balance of the cow and that it also indirectly changed the assessed leukocyte subpopulations. Probiotics also significantly improve the digestibility of the feed consumed by cows and at the same time increase the systemic concentration of energy components, proteins, vitamins and minerals (42), which may also be important for immune cell function. According to Galdeano *et al*. (12), probiotics may also stimulate the immune system in animals through the mechanism of altering the composition of the gastrointestinal microbiota, which may shift the direction of the immune response from Th2 (humoral) to Th1 (cellular), thereby maintaining immune homeostasis in the intestines. This would mean that probiotics can stimulate appropriate immune cell subpopulations, mainly dendritic cells, T lymphocytes and plasma cells, to produce cytokines, thanks to which they can modulate the immune response (9, 20). The direction of the response depends on the probiotic used, as different probiotic strains can stimulate the secretion of different cytokines (11, 33). The mechanism for the maintenance of immune homeostasis is not fully understood. However, it is believed that it is by stimulating cytokine secretion that probiotics promote the adaptive immune response, in which T regulatory lymphocytes ($CD4^+$, $CD25^+$ and $Foxp3^+$) play a major role and producing an anti-inflammatory cytokine – IL-10 – which modulates the immune response (21, 35).

The full mechanism responsible for the changes in leukocyte subpopulations in the blood of the experimental cows cannot be demonstrated on the basis of our research. Similar research conducted by Galdeano *et al.* (13) also did not indicate a specific mechanism of immunomodulatory activity exerted by probiotics. It cannot be excluded that the modulating effect of probiotics was imparted in our research in the same way as it was in the research referred to above. In particular, the previously described increase in the number of $Foxp3^+$ regulatory lymphocytes in cows treated with the probiotic seems to be the leading mechanism regulating these changes. The increase in the percentage of $Foxp3^+$ lymphocytes in our study resulted in the presence of a greater number of $CD4^+$ Th lymphocytes, activated B $CD25^+$ lymphocytes and β2 $CD18^+$ and αM $CD11b^+$ integrins. This arrangement in leukocyte subpopulations presumably enabled the cows to use of both cellular and humoral mechanisms simultaneously. Therefore, it can be concluded that the immune system of a cow that received the probiotic was better prepared to react in the event of an emergency than the immune system of a cow that did not receive the probiotic. At the same time, despite the readiness of the immune system, its stabilisation and even equilibration was also observed. In this context, note should be taken of the constantly low level of SAA throughout the experiment in the cows in the probiotic group compared to the control cows. Blood APPs characteristic of cattle, as SAA is, are

mainly produced by hepatocytes in the liver in response to pro-inflammatory cytokines *(e.g.* IL-6 and tumour necrosis factor (TNF) and glucocorticoids (2, 39). Serum amyloid A is an apolipoprotein that appears as a first-line protein up to 24–48 h after the onset of the inflammatory factor, *e.g.* after infection, and its secretion is dependent on IL-1 and/or TNF-α (38). Changes in the secretion of SAA are observed in many conditions related to both systemic and local infections of the body, stressful situations, deficiencies, and even during ongoing physiological changes occurring in various periods of lactation (6, 7, 38). The persistently high concentration of SAA and its significant fluctuations during the study in cows that did not receive the probiotic may be manifestations of the continuous increased activity of immune cells participating in non-specific immune mechanisms, which results in the production of APPs in liver cells. This may mean that the immune system of the cows in the experimental group activated the appropriate regulatory processes preventing the immune cells from reacting too rapidly to the factors, despite their readiness. In other words, the tolerance of immune system effector cells may be much higher in cows that received the probiotic than in cows which did not. Recent reports indicate that the phenomenon of immune system tolerance is very important in maintaining homeostasis in cows (34).

Conclusion

The presented research showed an increase in the percentages of $Foxp3^+$, $CD4^+$ Th and activated B $CD25^+$ lymphocytes and β2 $CD18^+$ and αM $CD11b^+$ integrins in cows after probiotic treatment. Activation of regulatory processes in the immune system of cows receiving the probiotic allowed the simultaneous use of both cellular and humoral mechanisms. This specific pattern of leukocyte subpopulations may indicate the activation of mechanisms that are necessary to eliminate possible harmful factors and, at the same time, to stabilise the immune system. This was confirmed by the persistence of a constant, low level of SAA throughout the course of the experiment in the cows receiving the probiotic. Therefore, it can be assumed that the immune system of cows treated with the probiotic was better prepared to react to infectious or harmful agents, and that it more easily adapted to changes in conditions in different lactation periods, especially in the postpartum period. The use of probiotics can reduce the incidence of infectious diseases in dairy cows.

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