ACTA VET. BRNO 2014, 83: 347–354; doi:10.2754/avb201483040347

The effect of Leiber Beta-S (1,3-1,6-β-D-glucan) on the phagocytic activity and oxidative metabolism of peripheral blood granulocytes and monocytes in calves

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Received December 9, 2013
Accepted September 17, 2014

Abstract

The effect of the Leiber Beta-S (1,3-1,6-β-D-glucan) dietary supplement on the phagocytic activity (Phagotest) and oxidative metabolism (Phagoburst) of peripheral blood granulocytes and monocytes in calves was determined by flow cytometry. Fourteen animals were divided into two groups: a control group without dietary supplementation and an experimental group administered 50 mg/kg body weight/day of 1,3-1,6-β-D-glucan for 60 days. At the beginning of the experiment (day 0) and on days 15, 30 and 60, blood was sampled from the jugular vein to determine and compare immunological indicators. Leiber Beta-S significantly influenced ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$) the percentages of phagocytic granulocytes (days 15 and 30) and monocytes (days 15, 30 and 60), and the percentage of bacteria engulfed by granulocytes (days 15 and 30) and monocytes (day 30). Leiber Beta-S increased the percentage of cells generating a respiratory burst in the population of granulocytes stimulated with fMLP (N-formyl-met-leu-phe) (day 15), PMA (4-phorbol-12-β-myristate-13-acetate) and *Escherichia coli* bacteria (days 15 and 30), and in the population of monocytes stimulated with fMLP (day 30), PMA (days 30 and 60) and *E. coli* (days 15, 30 and 60). The analyzed supplement increased mean fluorescence intensity in granulocytes stimulated with *E. coli* and fMLP (days 15 and 30) and PMA (days 30 and 60) as well as in monocytes stimulated with PMA, *E. coli* and fMLP (day 60). The findings of this study contribute to a better understanding of the effects of Leiber Beta-S on phagocytosis in calves, which have not been investigated to date.

Flow cytometry, calves, leukocytes

The diets of livestock animals, including cattle, are supplemented with probiotics and prebiotics, including yeast of the genus *Saccharomyces cerevisiae*, for preventive purposes and to improve the health status, body condition and productivity of animals. Whole live cells of *S. cerevisiae* yeast can be used to stabilize and stimulate rumen function. Yeast supplements are administered to calves to speed up the transition to solid feed, stimulate the development of the rumen and ruminal microflora. Yeast is a valuable source of group B vitamins and other active substances which increase weight gain, improve feed conversion, and minimize rearing loss (Lesmeister et al. 2004; Magalhães et al. 2008). The cell wall of *S. cerevisiae* yeast or its fragments are also used in cattle nutrition. The cell wall is the main structural component of yeast which stimulates the non-specific immune system, increases resistance to infections, adsorbs mycotoxins and regulates intestinal microflora. The yeast cell wall is built of 1,3-1,6-β-D-glucan polymers, mannan oligosaccharides (MOS), proteins, lipids, and chitin (Baptista et al. 2004; Yi et al. 2009). There is a broad selection of commercially available supplements containing *S. cerevisiae* yeast cells or their fragments, including the recently introduced Leiber Beta-S which contains 80% pure 1,3-1,6-β-D-glucan. No clinical studies have been conducted to investigate the biological activity of Leiber Beta-S and its influence on the immune system of cattle, in particular young calves which are highly susceptible to gastrointestinal and respiratory infections, with the exception of the author's own earlier study (Wójcik 2014). Therefore, the

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aim of this study was to evaluate the effect of the Leiber Beta-S dietary supplement on the phagocytic activity and oxidative metabolism of peripheral blood granulocytes and monocytes in calves.

Materials and Methods

Experimental design

The experiment was performed in July and August 2011, on a private farm in Księży Dwór, Poland. A total of 14, one-month-old clinically healthy Polish Holstein-Friesian calves (7 males and 7 females) were used in the experiment. The calves were fed colostrum at the dose of 2 kg/animal/day from the first hour *post partum* to 5 days of age. From day 5 to week 8, the animals received the Mlekowit milk replacer (Polmass, Poland) at a dose of 4 litres/day/animal in two portions. The animals had free access to fresh water, maize silage, meadow hay, and Kälberkost prestarter (Josera, Germany) - solid feed composed of crushed corn, barley grain, legume seeds, and protein concentrate. At one month of age, the animals were weighed and allocated to two groups (control and experimental) by the analog method. The diet for experimental group calves was supplemented with Leiber Beta-S (β-1,3/1,6-D-glucan) (Leiber GmbH, Bramsche, Germany), a commercially available formulation of natural stimulants, at 50 mg/kg body weight/day. Control group calves received standard farm-made feed without supplementation. At the beginning of the experiment (day 0) and on days 15, 30 and 60, blood samples were collected from the jugular vein to determine and compare the phagocytic activity and oxidative metabolism of peripheral blood granulocytes and monocytes by flow cytometry.

Phagocytic assay

PHAGOTEST kit (Glycotope Biotechnology GmbH, Heidelberg, Germany). The test was performed according to the manufacturer's instructions: 100 µl of heparinized whole blood was incubated for 10 min with fluorescein-labelled *E. coli* bacteria at 37 °C, while a negative control sample was kept on ice to reduce the phagocytic potential to a minimum. A quenching solution was added and, following two washing steps, erythrocytes were lysed, washed out, and DNA staining solution was added. Cellular phagocytic activity was determined in a cytometer (FACSCanto II, BD Biosciences, USA). A Phagotest was performed with the involvement of fluorescein-stained *E. coli* bacteria that are phagocytized by macrophages. Cell nuclei were also stained. The test determines the number of phagocytizing cells, granulocytes and monocytes separately, and their phagocytic activity, i.e. the number of bacteria absorbed by a single cell in terms of fluorescence intensity (PHAGOBURST kit, Glycotope Biotechnology GmbH, Heidelberg, Germany). The test was performed according to the manufacturer's instructions. Each analyzed sample of whole heparinized blood was divided into 4 test tubes of 100 µl each, and chilled to 0 ºC; 20 µl of chilled *E. coli* bacteria were added to the first sample (experimental); 20 µl of the washing solution were added to the second sample (negative control); 20 µl of fMLP (N-formyl-met-leu-phe) were added to the third sample (low control); and 20 µl of PMA (4-phorbol-12-β-myristate-13-acetate) were added to the fourth sample (high control). The contents of all test tubes were mixed and incubated for 10 min at 37 ºC (excluding the fMLP sample which was incubated for 7 min).

At the end of incubation, 20 µl of the fluorogenic substrate were added, the cells were lysed and fixed with lysing solution for 10 min at room temperature. The cells were washed and stained with DNA staining solution. Intracellular killing activity of phagocytes was determined in a cytometer (Becton Dickinson FACSCanto II). The added dihydrorodamine (123-DHR) was oxidized in mitochondria by H_2O_2 resulting from cell stimulation and was converted to cation rhodamine 123 (R123), the fluorescent emitter.

FACS acquisition and analysis

Flow cytometry was performed using a FACSCanto II cytometer (BD Biosciences, USA). Data were acquired with the FACSDiva version 6.1.3 software (BD Biosciences, USA) and analyzed by FlowJo software (Tree Star, USA). Cytometry setup and tracking beads (CST; BD Biosciences, USA) were used to initialize the photomultiplier tube (PMT) settings. Unstained control cells as well as a single stain control for every fluorochrome were prepared and used to establish flow cytometric compensation.

Statistical analysis

The obtained results were processed statistically by one-factorial analysis of variance for orthogonal designs. The significance of differences ($P < 0.05$; $P < 0.01$; $P < 0.001$) between groups was verified by Student's *t*-test and Bonferroni test with the use of GraphPad Prism 2 software.

Results

Phagotest results were processed with the use of FlowJo software (Tree Star, USA) to reveal a significant increase in the mean phagocytic activity ($P \leq 0.05$ and $P \leq 0.01$) and mean fluorescence intensity (MFI) ($P \leq 0.001$) of peripheral blood granulocytes on days

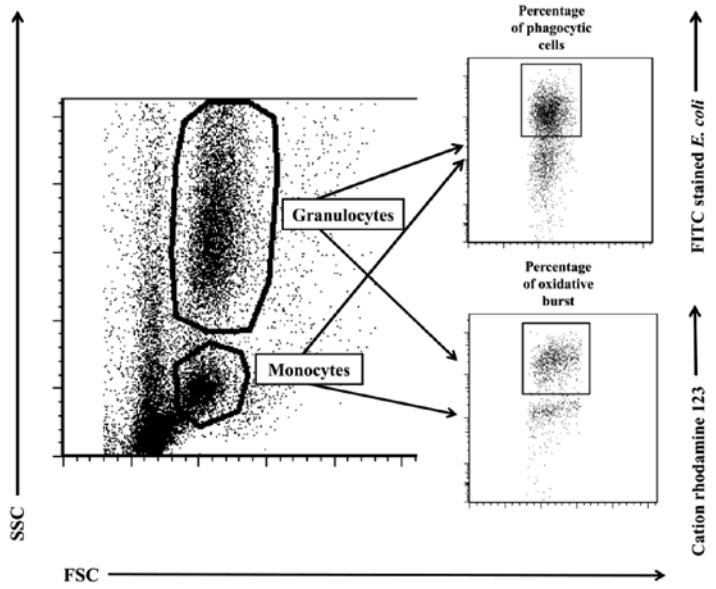

Fig. 1. Gating strategy for analysis of flow cytometry data. Granulocytes and monocytes were gated based on forward and side scatter (FSC/SSC) parameters. Each cell subset was analyzed for the relative percentage of phagocytizing cells and cells stimulated for respiratory burst (N-formyl-met-leu-phe (fMLP), 4-phorbol-12-β-myristate-13-acetate (PMA) or *E. coli* bacteria).

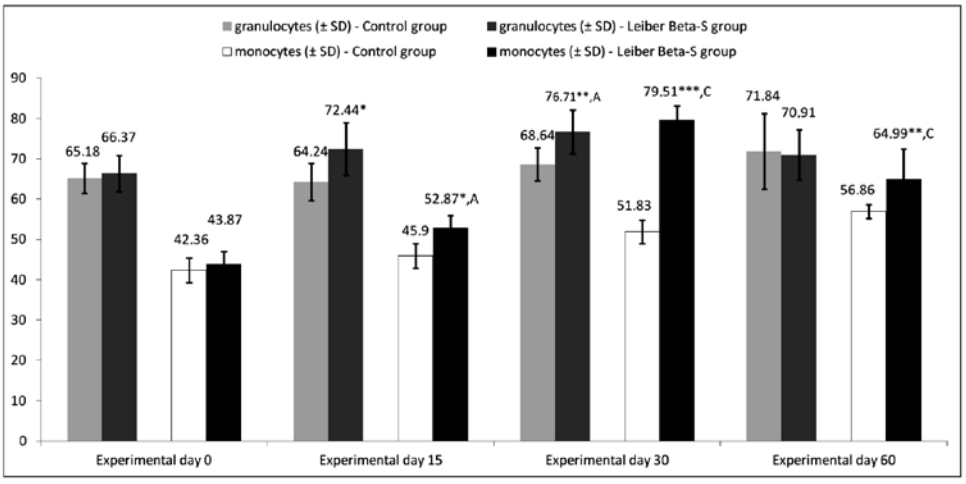

Fig. 2. Percentage of phagocytic granulocytes and monocytes in calf groups, as determined in Phagotest. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{A} $P \leq 0.05$ in comparison with experimental day 0; ^{B} $P \leq 0.01$ in comparison with experimental day 0; ^{C} $P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation.

15 and 30 in experimental group calves fed Leiber Beta-S in comparison with control animals (Fig. 1). In the experimental group, a significant increase in the mean percentage of phagocytic cells was reported on day 30 (Fig. 2) ($P \leq 0.05$) and a significant increase in

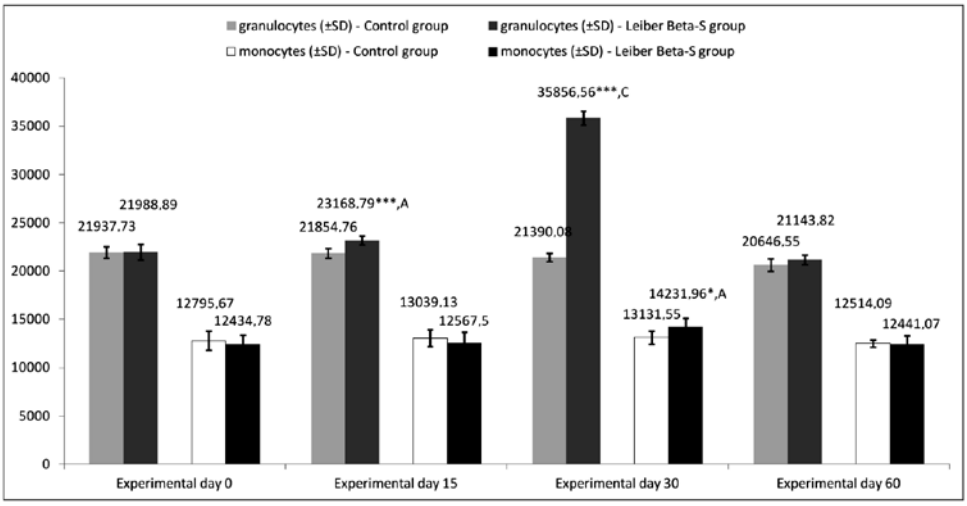

Fig. 3. Mean fluorescence intensity (MFI) of granulocytes and monocytes in calf groups, as determined in Phagotest.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{A} $P \leq 0.05$ in comparison with experimental day 0; ^{B} $P \leq 0.01$ in comparison with experimental day 0; ^{C} $P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation

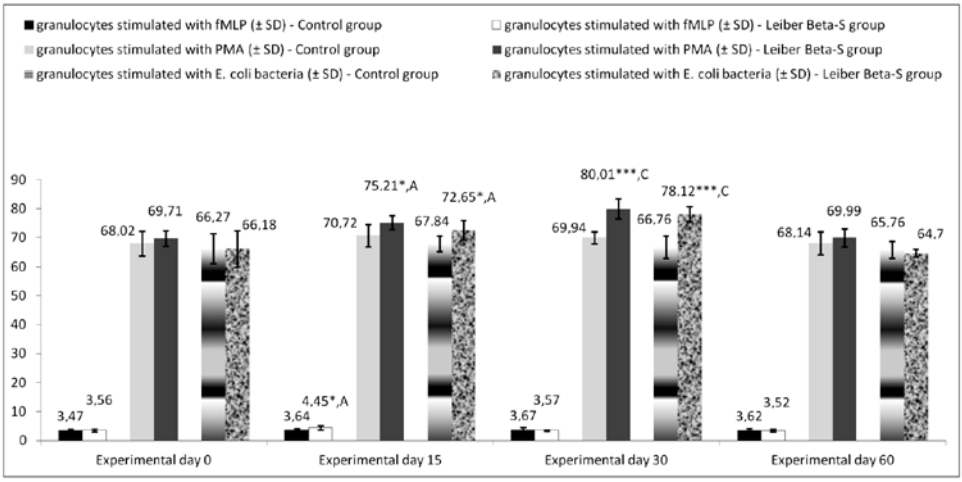

Fig. 4. Percentage of granulocytes stimulated to undergo respiratory burst in calf groups after stimulation with N-formyl-met-leu-phe (fMLP), 4-phorbol-12-β-myristate-13-acetate (PMA) and *E. coli,* as determined in Phagoburst test
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{A} $P \leq 0.05$ in comparison with experimental day 0; ^{B} $P \leq 0.01$ in comparison with experimental day 0; ^{C} $P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation.

MFI in peripheral blood granulocytes was noted on days 15 ($P \leq 0.05$) and 30 ($P \leq 0.001$) relative to the mean values noted on day 0 (Fig. 3). Monocyte responses in the experimental animals differed: a significant increase in the mean percentage of phagocytic cells was

noted on days 15, 30 and 60 ($P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.01$, respectively) (Fig. 2), whereas a significant increase in MFI relative to control group animals was observed only on day 30 ($P \leq 0.05$) (Fig. 3). The mean percentage of phagocytic monocytes increased significantly on days 15 ($P \leq 0.05$), 30 ($P \leq 0.001$) and 60 ($P \leq 0.001$) (Fig. 2), and MFI increased on day 30 ($P \leq 0.05$) in the experimental group in comparison with the mean values reported on day 0 (Fig. 3).

The ability of granulocytes to produce strongly oxidizing compounds was evaluated with the use of the Phagoburst kit and FlowJo software. In comparison with the control group, a significant increase in the percentage of stimulated cells was noted in the experimental animals on days 15 ($P \leq 0.05$) and 30 ($P \leq 0.001$). On day 15, the above increase was observed in the population of granulocytes stimulated with strong (PMA) and weak (fMLP) activators of the respiratory burst and with *E. coli*. On day 30, the observed increase was noted in granulocytes stimulated with PMA and *E. coli* (Fig. 4). On days 15 and 30, MFI, a measure of respiratory burst activity in granulocytes, increased significantly in cells stimulated with fMLP ($P \leq 0.05$ and $P \leq 0.01$, respectively) and *E. coli* ($P \leq 0.05$ and $P \leq 0.01$, respectively) in experimental calves compared to control. The use of PMA as the activator of respiratory burst led to a delayed increase in the above indicators on days 30 ($P \leq 0.001$) and 60 ($P \leq 0.001$) (Fig. 5). In the experimental group, a significant increase in the mean percentage of granulocytes stimulated with all three respiratory burst activators (PMA, fMLP and *E. coli*) was observed on day 15 ($P \leq 0.05$), and an increase in the mean percentage of granulocytes stimulated with PMA and *E. coli* was reported on day 30 ($P \leq 0.001$) in comparison with the mean values noted on day 0 (Fig. 4). The MFI of granulocytes in experimental calves increased significantly on day 30 after stimulation with PMA, ($P \leq 0.001$), fMLP ($P \leq 0.01$) and *E. coli* ($P \leq 0.01$) and on day 60 after stimulation with PMA ($P \leq 0.001$) and fMLP ($P \leq 0.01$) in comparison with the mean values reported at the beginning of the experiment (day 0) (Fig. 5).

Compared to control, the Phagoburst test produced different results in the subpopulation of monocytes where the percentage of cells generating a respiratory

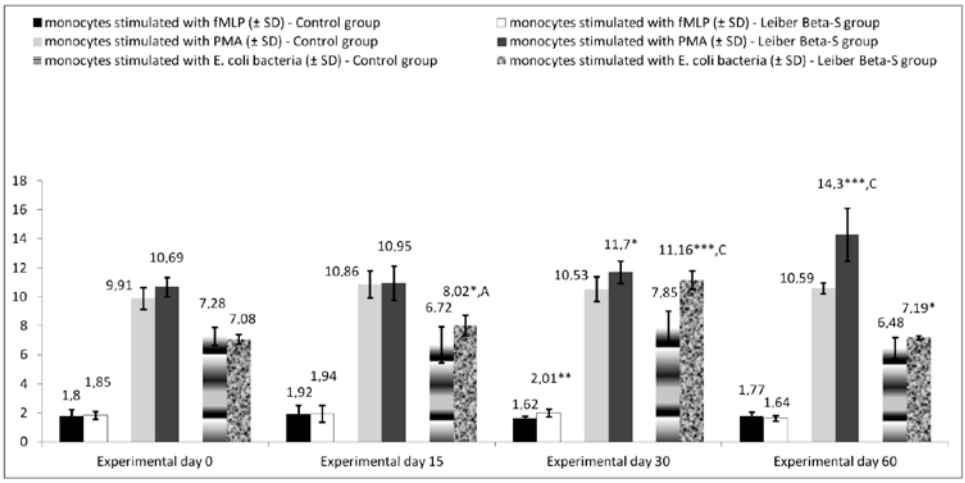

Fig. 5. Mean fluorescence intensity (MFI) of granulocytes in calf groups after stimulation with N-formyl-met-leu-phe (fMLP), 4-phorbol-12-β-myristate-13-acetate (PMA) and *E. coli,* as determined in the Phagoburst test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{A}P \leq 0.05$ in comparison with experimental day 0; $^{B}P \leq 0.01$ in comparison with experimental day 0; $^{C}P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation

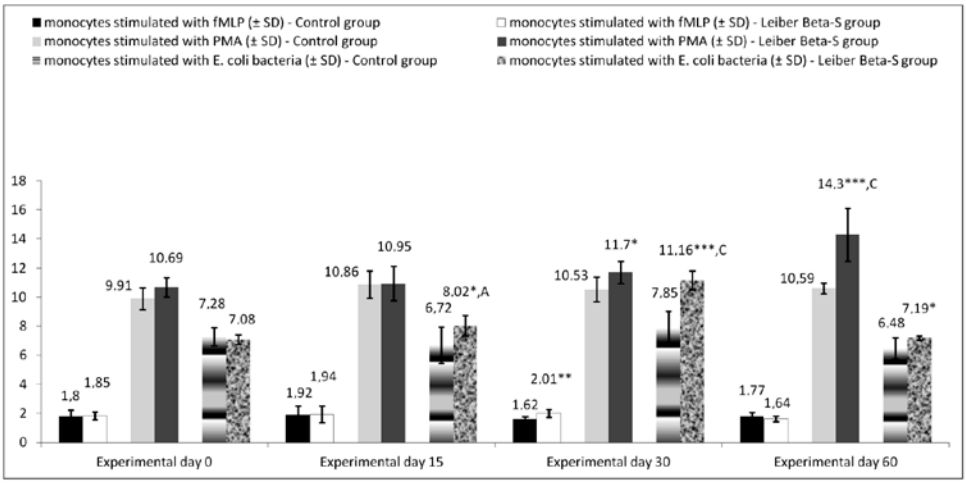

Fig. 6. Percentage of monocytes stimulated to undergo respiratory burst in calf groups after stimulation with N-formyl-met-leu-phe (fMLP), 4-phorbol-12-β-myristate-13-acetate (PMA) and *E. coli,* as determined in Phagoburst test.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{A} $P \leq 0.05$ in comparison with experimental day 0; ^{B} $P \leq 0.01$ in comparison with experimental day 0; ^{C} $P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation

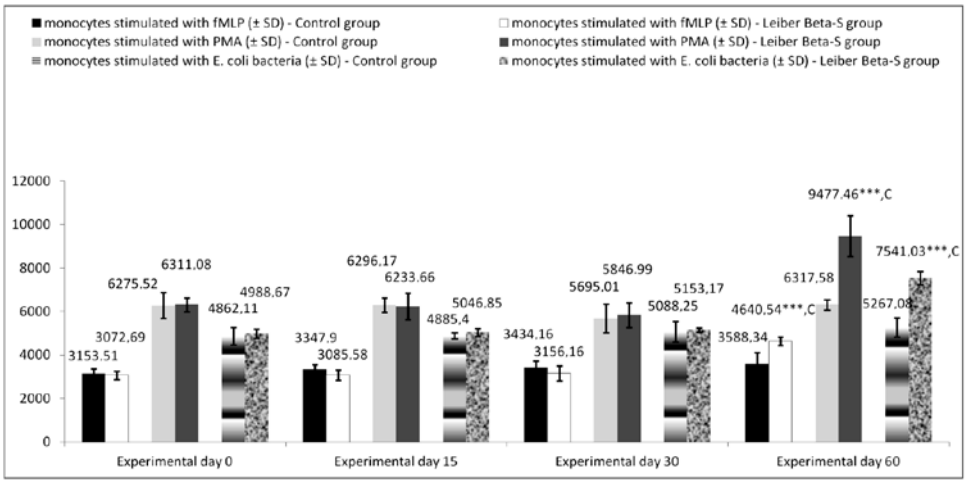

Fig. 7. Mean fluorescence intensity (MFI) of monocytes in calf groups after stimulation with N-formyl-met-leu-phe (fMLP), 4-phorbol-12-β-myristate-13-acetate (PMA) and *E. coli,* as determined in Phagoburst test.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ^{A} $P \leq 0.05$ in comparison with experimental day 0; ^{B} $P \leq 0.01$ in comparison with experimental day 0; ^{C} $P \leq 0.001$ in comparison with experimental day 0; SD - standard deviation; fMLP - N-formyl-met-leu-phe, PMA - 4-phorbol-12-β-myristate-13-acetate

burst increased significantly only on day 30 ($P \leq 0.01$) upon stimulation with fMLP, on days 30 ($P \leq 0.01$) and 60 ($P \leq 0.001$) upon stimulation with PMA, and on days 15, ($P \leq 0.05$), 30 ($P \leq 0.05$) and 60 ($P \leq 0.001$) upon stimulation with *E. coli*

(Fig. 6). The MFI increased significantly only on day 60 ($P \leq 0.001$) upon stimulation with all three respiratory burst activators in comparison with the mean values noted on day 0 (Fig.7).

Discussion

In comparison with control, the observed increase in the phagocytic activity of peripheral blood cells of experimental calves in the first month of dietary supplementation with Leiber Beta-S accounted mostly for the increase in the percentage of bacteria phagocytized by granulocytes and the percentage of phagocytizing monocytes. In our earlier study of rats, high-purity Biolex-Beta HP supplement (85% 1,3/1,6-β-D-glucan) administered at 12–19 mg/animal/day for 14 days induced growth but did not result in significant differences in the phagocytic activity of granulocytes and monocytes (Wójcik et al. 2009). A similar effect was observed in lambs where a significant increase in potential killing activity was observed in the group of animals fed 50 mg/kg BW of Biolex-Beta HP for 60 days (Wójcik et al. 2007). After oral administration of β-glucan, Suzuki et al. (1990) observed an increase in the production of H_2O_2 and IL-1 in the macrophages of mice, whose phagocytic activity was stimulated in comparison with control. In a group of lambs receiving 50 mg/kg BW of the Biolex-MB40 supplement containing β-glucan and MOS for 60 days, a significant increase in potential killing activity was observed only on day 15 (Wójcik 2010). According to Brown and Gordon (2003), the phagocytic activity of the analyzed cells is intensified when β-glucan receptors expressed on the surface of cells (dectin-1, toll-like receptor TLR-2, TLR-4, complement receptor 3, lactosylceramide) recognize and bind β-glucans, which induces intracellular mechanisms that activate the nuclear factor κB (NF-κB), increases the production of proinflammatory chemokines and cytokines (IL-1, IL-6, IL-8, IL-12, and TNF-α) and leads to feedback activation of granulocytes and monocytes (Lebron et al. 2003; Akremiene et al. 2007).

In comparison with control, an increase in the percentage of cells generating a respiratory burst and enhanced respiratory burst activity after stimulation with all three activators (*E. coli*, PMA and fMLP) were observed in granulocytes in the first month and in monocytes in the second month of dietary supplementation with Leiber Beta-S. Somewhat different results were noted in lambs whose diets were supplemented for 60 days with Biolex-Beta HP (Wójcik et al. 2007) and Biolex MB-40 (Wójcik 2010) where a significant increase in respiratory burst activity values was noted throughout the entire experiment compared to control. In our previous study of rats (Wójcik et al. 2009) administered Biolex-Beta HP for 14 days, a significant increase was reported only in the percentage of granulocytes generating a respiratory burst upon stimulation with PMA and *E. coli*, the percentage of monocytes generating a respiratory burst upon stimulation with *E. coli* and the intensity of respiratory burst in those monocytes. Kulicke et al. (1997) suggested that immunostimulating effects of β-glucans are determined by the degree of branching, length and tertiary structure of polymers, their origin, synthesis, manner of administration, dose and period of application. An increase in the concentrations of strongly oxidizing compounds (superoxide anion O_2^-, hydrogen peroxide H_2O_2, hydroxyl radical •OH, singlet oxygen $^1O_2$, chloramine NH_2Cl) generated by respiratory burst was also observed by Hino et al. (2012) in an *in vitro* study after administration of curdlan, and by Eicher et al. (2010) in an *in vivo* experiment on calves administered the EnergyPlus supplement (mannan and β-glucan). Surprisingly, in an *in vitro* study by Kim et al. (2004), the production of O_2^- and H_2O_2 by macrophages did not increase after the administration of an aqueous extract from the fruiting body and mycelia of *Phellinus linteus*.

Reduced antibiotic use in animal production and increased prevalence of antibiotic resistance prompt the search for new and more effective methods of preventing and treating

animal diseases. Immunostimulation offers a viable alternative, and the results of this study indicate that the use of dietary supplements containing prebiotics, such as β-glucans in the analyzed Leiber Beta-S supplement, deliver health benefits.

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