Effect of tylosin and prebiotics on the level of cytokines and lymphocyte immunophenotyping parameters in calves

MONIKA SZYMAŃSKA-CZERWIŃSKA[1], DARIUSZ BEDNAREK[1], BARBARA ZDZISIŃSKA[2], MARTYNA KANDEFER-SZERSZEŃ[2]

[1]National Veterinary Research Institute, Department of Cattle and Sheep Diseases, Pulawy; [2]Institute of Microbiology and Biotechnology, Departament of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland

Abstract

Introduction: *Antibiotic growth promotants (AGP) were applied until December 31, 2005 and it is now prohibited in EU. Therefore new alternatives are intensively searched in livestock nutrition. Among them, an important part plays natural compounds, such as probiotics, prebiotics and symbiotic with potential immuneprofitable properties.*

Objective: *The aim of the study was to estimate the influence of tylosin and prebiotics (β-glucans and mannan-ologosaccharides) added into the traditional ruminant feed on the level of selected cytokines (IL-1, IL-2, TNF-α, IFN-α) and alternation of peripheral blood lymphocyte subpopulations in calves (T, Th, Ts/c, B lymphocyte subsets i.e. $CD2^+$, $CD4^+$, $CD8^+$, $WC4^+$ cells, respectively).*

Material and Methods: *The calves were divided into three groups. The animals in group I were fed feedingstuffs with tylosin, in group II with the same feed suplemented with prebiotics and group III – control group (the feed without this addition). The experiment was carried out by 7 weeks and blood samples from calves were taken twice a week. The concentrations of cytokines were estimated by using ELISA test and biological methods. Immunophenothyping of lymphocytes were performed by using flow cytometry (FCM).*

Results: *The received results indicate, that prebiotics caused the significant increase of the cytokine levels and percentage of lymphocyte subpopulations in compared with the control animals. This effect was also recorded in calves treated with tylosin, however the changes were not as unequivocall and their intensity was less expressed.*

Key words: *tylosin, prebiotics, cytokines, lymphocyte subpopulations, calves.*

(Centr Eur J Immunol 2009; 34 (1): 1-6)

Introduction

Antibiotic growth promotants (AGP) were applied until December 31, 2005. At present, the antibiotic application in the previous form for livestock is completely prohibited in EU [4]. Therefore new solutions and alternatives for the animal husbandry concerning other additives in livestock nutrition are intensively searched. Natural alternatives e.g. probiotics (*Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp. etc), prebiotics, particulary such as mannan-oligosacharides (MOS) and β-glucans (1,3-1,6-β-glucan), synbiotics and organic acids are widely examinated [6, 10].

Since January 1, 2006 the selected antibiotics can be used in EU only in a form of medicated feedingstuffs manufactured on the basis of the registered commercial premixes.

Before 2006 tylosin was used as AGP and it is still used as an active substance in the medicated feedingstuffs [4]. At present, in polish veterinary market the medicated feedingstuffs with tylosin are the frequently used for poultry, swine and cattle.

Tylosin and prebiotics are considered as factors that can cause profitable changes in cellular and humoral immunity [9, 14, 15]. According to the data published in the literature

Correspondence: Monika Szymańska-Czerwińska, National Veterinary Research Institute, Department of Cattle and Sheep Diseases, Al. Partyzantów 57, 24-100 Pulawy, Poland. Phone number: +48 81 889 30 97, Email: monika.szymanska@piwet.pulawy.pl

β-glucans and tylosin are considered as powerful immunostimulators of macrophages and other immune effector cells (e.g. monocytes, neutrophiles, NK etc.). These substances stimulate the cells mainly by the binding with specific receptors situated on the immune cell surface (CR3, TLR, CD11a/CD18 etc.) [14]. Afterwards the activated cells produce and release of different cytokines such as: interleukin 1 (IL-1) and 2 (IL-2), tumor necrosis factor α (TNF-α) and interferon α (IFN-α) [7, 12-14]. Moreover, among prebiotics mannanoligosaccharides (MOS) have the confirmed regulatory effect on the desirable micro flora in the intestinal tract in different species of livestock. It is connected with the ability of MOS to bind with mannose-binding proteins presenting on the cell surface of some strains of bacteria (e.g. *Salmonella* sp.). Thereby a host organism is prevented against the intestinal tract colonization mainly by interfering with binding of microorganism to carbohydrate residues on the epithelial cell surface [5]. Apart from that tylosin and prebiotics can also stimulate cellular immunity. However, there are only a few papers presenting this problem. Therefore, the further studies are necessary and up-to-date. The present study was undertaken to explain the influence of tylosin and prebiotics (β-glucas, MOS) used as diet supplements to induce changes in the immune system of calves measured as changes in the selected cytokines and subpopulations of peripheral blood lymphocytes.

Materials and Methods

Animals

The study was performed on 18 clinically healthy, Black and White Lowland breed calves, aged 6-8 weeks and with an average body weight 75±4.12 kg. The calves were randomly divided into three equal groups. The studies were started after seven day of acclimatization. Experimental procedure and animal management protocols were undertaken in accordance with the requirements of Animal Care and Ethics Committee.

Study protocol and sample collection

The experimental calves were fed with traditional feedingstuffs (C-J feed) supplemented either tylosin, at a recommended dose of 9.4 mg of the active substance per kilo of body weight (group I), or prebiotics as a mixture of suitable amount of β-glucans (49 mg) and mannan-oligosaccharides (52 mg) per kilo of body weight (group II). The animals in group III served as controls and received the same feedingstuffs without the additives. The additives, i.e. tylosin and prebiotics were used in a form of commercialy available pharmaceutical preparations: premix Tylan (Elanco) – containing tylosin phosphoran and Alphamune (Alpharma) – source of prebiotics. The components of Alphamune derived from the cell wall of the yeast *Saccharomyces cerevisae*. The both kinds of additives were given to the calves for seven weeks. Blood samples from calves were taken twice a week by the jugular vein puncture before the calf morning feeding. The blood samples were collected into tubes containing tripotassium salt ethylenediaminetetraacetic acid (K_3EDTA) as the anticoagulant (0.07 mol/ml blood) and also in the tubes without anticoagulant. Blood was collected for 7 weeks.

Cytokine assay

The concentrations of interleukin 1 (IL-1) and 2 (IL-2) in serum were assayed by using ELISA tests (R&D Systems with own modification). This assay is based on the quantitative sandwich enzyme immunoassay technique. In the test monoclonal antibody specific for IL-1 or IL-2 is pre-coated onto a microplate. The standards and analysis samples are pipetted into the wells and any IL-1 or IL-2 is bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for IL-1 or IL-2 is used. Finally the substrate solution is added and intensivity of color depended on the amount of IL-1 or IL-2 is measured.

On the other hand, the biological technique, described earlier [3], for IFN-α and TNF-α activity determinations in calf serum was used. IFN-α activity was titrated in bovine embryonic fibroblasts (BEF) with vesicular stomatitis virus (VSV) as challenge. Dilution of the sample that protected 50% of cells against viral cytophatic effect was scored and calibrated against International IFN-α standard.

TNF-α activity was estimated by the biological method based on cytotoxic action of TNF-α on the WEHI-164 cell line. The reciprocal of the highest dilution causing destruction of 50% of the cells and compared to the TNF-α standard was defined as one unit of TNF-α.

Immunophenotyping of bovine peripheral blood lymphocytes

Immunophenotyping of bovine peripheral blood lymphocytes expressing CD2 (T-cell antigen), CD4 (T-helper antigen), CD8 (T-cytotoxic/supperessor cell antigen) and WC4 (B-cell antigen) surface marker were performed by the use of Coulter Epics 4XL Flow Cytometer (Beckman Coulter Company, USA). A panel of monoclonal antibodies (MCAs) directed against bovine leukocyte cluster of differentiation antigens (CD) was used to differentiate peripheral blood leukocyte subpopulations. It comprised those which recognize CD45 (MCA832F mouse anti-bovine CD45:FITC, clone number-CC1), CD14 (MCAl56C cross-reacting mouse anti-human CDl4:RPE-CyS, clone number – TuK4), CD2 (MCA833F mouse anti-bovine CD2:FITC, clone number – CC42), CD4 (MCAl653F mouse anti-bovine CD4:FITC, clone number – CC8), CD8 (MCA837F mouse anti-bovine CD8:FITC, clone number – CC63), WC4 (MCA1648 mouse anti-bovine WC4, clone number – CC55) it was bound additionally by the secondary F(ab')2 rabbit anti-mouse

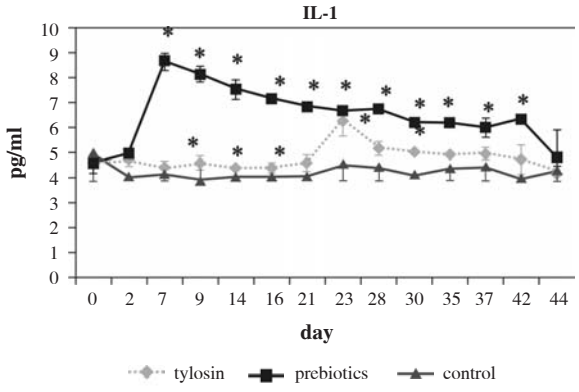
immunoglobulin FITC conjugated to FITC – STAR9B). All these monoclonal antibodies were manufactured by Serotec Ltd (Oxford, UK). Immunofluorescent analysis of peripheral blood leukocytes was performed according to Beckman – Coulter Operator's Guide Procedure. FITC-conjugated anti-CD45 and RPE-Cy5-conjugated anti-CDl4 MCAs were used together for gating the lymphocytes. The analysis of suitable surface marker expression was done directly from whole blood basing on OptiLyse Immunotech preparation standard procedure. Fifty microliters of whole blood were incubated at room temperature for 15 minutes with suitable monoclonal antibodies. Then 250 µl of lysing solution (OptiLyse C, Immunotech) was added to all the blood samples and incubated again under the same conditions. After red blood cells lysis, leukocytes were washed with PBS containing 5% fetal calf serum and resuspended in 500 µl of PBS with fetal calf serum. The cell suspension was analyzed, using a flow cytometer and a logarithmic amplifier. SYSTEM II 3.0 software for the Cytometer was used to the data acquisition (listmodes) and their cytometric analysis (histograms). Additionally, Multigraph program was used to calculate and display the data.

Statistical analysis

The statistical significance of differences between the mean values recorded in the experimental groups of animals and the controls was compared using Student's t-test at $p<0.05$.

Results

The results of cytokine analysis (IL-1, IL-2, TNF-α, IFN-α) and bovine peripheral blood lymphocyte immunophenotyping (CD2, CD4, CD8, WC4 positive cells) are summarised in Figures 1-8. The obtained results shown, that significant differences of interleukin 1 concentrations were



*The significantly different in comparison to control group at p≤0.05

Fig. 1. Effect of tylosin and prebiotics on the synthesis of IL-1

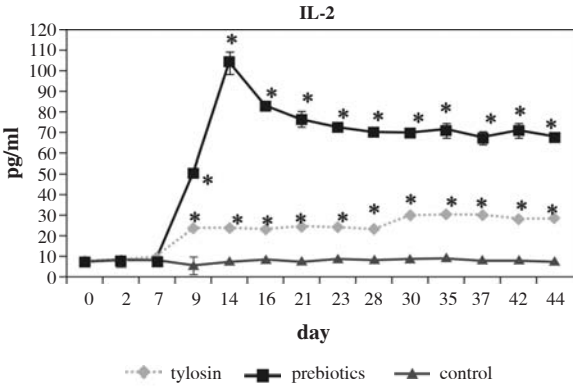


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Fig. 2. Effect of tylosin and prebiotics on the synthesis of IL-2

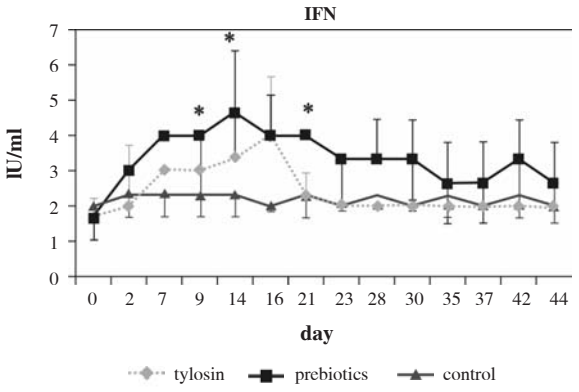

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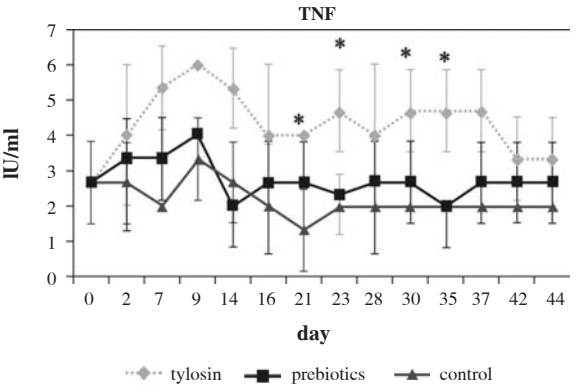
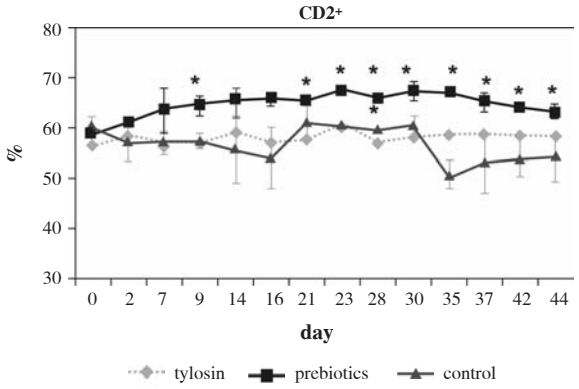
Fig. 3. Effect of tylosin and prebiotics on the synthesis of IFN-α



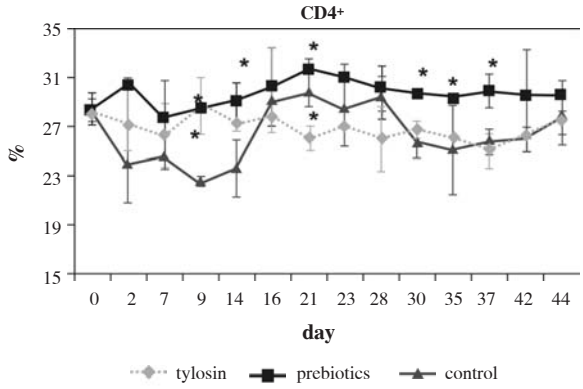
*The significantly different in comparison to control group at p≤0.05

Fig. 4. Effect of tylosin and prebiotics on the synthesis of TNF-α



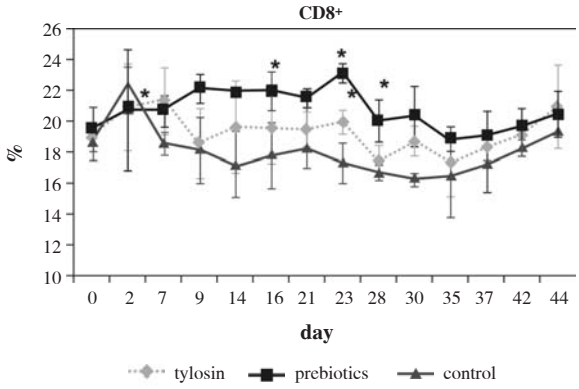
*The significantly different in comparison to control group at p≤0.05

Fig. 5. Effect of tylosin and prebiotics on the percentage of CD2+ lymphocytes



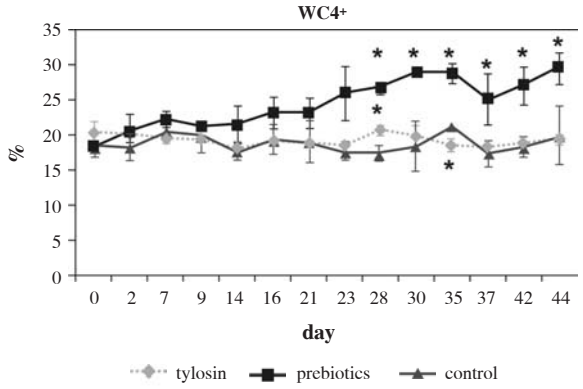
*The significantly different in comparison to control group at p≤0.05

Fig. 6. Effect of tylosin and prebiotics on the percentage of CD4+ lymphocytes



*The significantly different in comparison to control group at p≤0.05

Fig. 7. Effect of tylosin and prebiotics on the percentage of CD8+ lymphocytes



*The significantly different in comparison to control group at p≤0.05

Fig. 8. Effect of tylosin and prebiotics on the percentage of WC4+ lymphocytes

recorded in calves of group II and I from the 7th and 9th day of experiment, respectively (Fig. 1). Its highest concentration (8.63 pg/ml) was noted in group II at the 7th day of the study and then it systematically decreasing to the end of the experiment. However, it still was significantly higher in compared with the controls at the 42nd day of the study. On the other hand, in group I this highest concentration was noted at the 23th day of the experiment (6.3 pg/ml). Generally, the levels of IL-1 in group I and II of calves were higher than in the controls between the 9th and 42nd day of the observation. The received results revealed also the significant increase of IL-2 activity in the treated animals (Fig. 2). IL-2 concentration raised from 7.5 to 28.6 pg/ml in group I and from the same initial value to 104 pg/ml in group II. These mean values in experimental calves were already significantly higher in comparison to the control group from the 9th day of the study. Analysis of IFN-α concentration shown (Fig. 3), that its level in serum increased significantly in group II from 1.67 to 4.67 IU/ml. The increase was also found in group I from 1.67 to 3.33 IU/ml, however the differences in comparison to the controls were not significant. On the other hand, the different results were recorded concerning TNF-α concentration in the calves (Fig. 4). Generally TNF-α level in serum was higher in calves from group I received tylosin in compared with group II (treated with prebiotics) and the controls. In group I its mean values ranged between 2.67 and 6.33 IU/ml, and significant differences were noted at the 21st, 23rd, 30th and 35th day of the study.

Immunophenotyping of bovine peripheral blood lymphocytes shown, higher values for lymphocyte subpopulations

both in $CD2^+$ cells (T lymphocytes), with its subset ($CD4^+$ – T helper lymphocytes, $CD8^+$ – T suppressor/cytotoxic lymphocytes), and $WC4^+$ cells (B lymphocytes) in calves treated with prebiotics (Figs. 5-8). In these calves the percentage of $CD2^+$ lymphocytes (Fig. 5) was significantly higher than in the controls especially at the 9th day and from the 21st to 44th day of the experiment. It was also significantly higher in comparison to group I at the 21st day of the observation. The percentage of $CD4^+$ lymphocytes (Fig. 6) increased significantly at the 9th day of the study in the both experimental groups in compared with the controls. In addition the increase was statistically significant in group II in compared with control animals at the 14th, 21st, 30th, 35th, 37th day of the experiment. The higher values of $CD8^+$ lymphocytes (Fig. 7) were observed in the both experimental groups from the 7th day of experiment in compared with the controls. However, the values were statistically significant only at the 7th, 16th, 23rd and 28th day of study. Moreover the higher values of $WC4^+$ lymphocytes (Fig. 8) were noted from the 7th day of the study in group II in compared with both remained groups of animals. However, its values were significant in compared with the control group at the 7th day and from 28th day to the end of the experiment. In this passage it should be emphasized, that in group II changes of B lymphocytes ($WC4^+$) in peripheral blood of these calves had the increasing tendency through the whole time of the study. It was observed especially at the period between the 23rd to 44th day of experiment in comparison to group I and the controls.

Discussion

Prebiotics (β-glucans and MOS) and tylosin applied as additives into experimentally prepared feedingstuffs caused significant changes in peripheral blood of the treated calves with regard to the lymphocyte subpopulations and production of some cytokines.

Tylosin is a macrolide antibiotic produced by *Streptomyces fradiae* [2]. It is a polyketide lactone which attached three 6-deoxyhexoses (mycinose, mycaminose and mycarose). It has pharmacological effects similar like erythromycin. Moreover, it has been demonstrated recently, that tylosin can be hydrolyzed in acidic pH inside of alimentary tract and mycaminose is released from tylosin. Finally, a new antibiotic – dysmycosyn is produced from mycaminose. This new antibiotic is very effective against Gram positive and negative bacteria [2, 8].

It has been detected, that tylosin and prebiotics can influence effectively cellular immune functions in animals. According to the data in literature β-glucans and tylosin strongly activate macrophages by binding with their specific receptors (CR3, TLR, CD11a/CD18 etc.), situated on the surface of immune effector cells (e.g. leukocytes, lymphocytes, monocytes and macrophages). The activated immunological cells produce several substances such as cytokines. Among them, a particularly important cytokine is IL-1, produced largely by macrophages [1, 7]. IL-1 is responsible for the activation of T lymphocytes which then produce IL-2, and this cytokine has many different immunological functions. It should be added, that T-helper cell population is composed of two cell subsets i.e. Th1 and Th2. Generally, Th1 subpopulation responses for the cellular immunity and Th2 subpopulation for the humoral immunity. The Th1 subpopulation is especially important because it produces IL-2. Among other functions IL-2 increases cytotoxic properties of lymphocytes [13]. In our study the percentage of $CD8^+$ cells increased in the both experimental groups of animals but significant changes were observed in group II treated with prebiotics.

The activated Th2 lymphocytes are able to stimulate B lymphocytes responsible for the production of specific antibodies. The subset of $WC4^+$ cells corresponded with bovine B lymphocytes which increased significantly at the 28th day of experiment and remained on the high level to the final day of our study. The results shown, that the increase of IL-2 concentration in calves treated with tylosin (group I) and prebiotics (group II) probably could be connected with the increase of Th1 subpopulation. However, the comparison of these experimental groups of calves revealed that the higher level of IL-2 was observed in the animals treated with prebiotics than tylosin. Moreover, the activated macrophages which produce TNF as well as IL-1 can stimulate T lymphocytes. Tylosin and prebiotics caused the increase of blood TNF-α level [1, 11]. However, the higher blood TNF-α level was observed when tylosin was applied (group I). On the other hand, our study shown the significant increase of IFN synthesis in group II of calves i.e. received prebiotics. This cytokine and also TNF-α are capable to stimulation of cytotoxic lymphocytes proliferation. TNF-α stimulates collagenase activity, PGE_2 production and it is responsible for the destruction and remodeling of connective tissue in inflammatory reactions. Moreover TNF-α enhances the adherence of neutrophils to the vascular endothelium and stimulates the respiratory burst of neutrophils contributing to local tissue injury. TNF-α kills virus – infected cells and exerts an antiviral effect by inhibiting virus replication. It also has a protective role in bacterial infections [3]. The significant increase of $CD8^+$ cells observed in our study can be also consequences of biological activity of the increased TNF-α and IFN-α level. IFN-α can protect animals not only against viral infections but also against other microorganisms. This protection is thought activation of macrophages and neutrophils resulting in enhanced phagocytosis and killing of pathogens [3]. Treatment of calves with prebiotics (β-glucans and MOS) caused the statistically significant increase of $CD2^+$ and $WC4^+$ cell percentage in peripheral blood of the animals, especially in the 4th week of the experiment. Among $CD2^+$ lymphocytes the significant increase was seen in $CD4^+$ subpopulation with two peaks of the cells i.e. in the 2nd-3rd and 4-5th weeks of the experiment. Treatment of calves with

prebiotics caused also significant changes in cytokine levels. IL-1 blood concentration leaked in the 1st the week after beginning of the experiment and was on the high level to the end of the study. IL-2 contents peaked in the 2nd week of the experiment and was on the high level to the end of the observation too. Moreover, INF-α changes correlated with IL-2. In contrast to results described above TNF-α level was the higher in 1st week after starting treatment of calves with tylosin. Prebiotics did not induce TNF-α in blood of treated calves.

In conclusion, these results indicated, that prebiotics (β-glucans, MOS) exhibited immunomodulatory effect both on the cellular and humoral immunity of calves.

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