Functional activity of blood neutrophils and immune status of heifers under the influence of probiotics

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Abstract

The article is devoted to the effect of probiotics on the animal body. The purpose of the study is to study the effect of probiotics of individual strains on the functional activity of blood neutrophils and on individual parts of the immune system of the cattle body. Various probiotics are widely used in animal husbandry and make it possible to modernize existing animal husbandry regimens. The task of finding new approaches to optimizing the beneficial flora of the body is extremely relevant. The use of probiotics in livestock production is a cost-effective solution. It has been established that the use of probiotics *Lactobacillus spp*. and *Bacillus sb*. accompanied by activation of natural resistance mechanisms. Probiotics also help improve immune status, mainly due to the humoral component, tend to optimize the microbiocenosis of the colon, and have a positive effect on increasing animal productivity. The results of comprehensive studies of the state of the defense systems of the body of cattle and their changes under the influence of the drug expand and deepen the existing data on the effect of probiotics on the homeostasis of animals.

Key words: *beef cattle; probiotics; blood neutrophils; phagocytosis; immunity*

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Introduction

The general resistance of animals to pathogenic microorganisms and specific agents is determined by nonspecific humoral and cellular factors. Such factors include the protective properties of the mucous membranes and skin, the bactericidal activity of blood serum, saliva, tear fluid and other body fluids, which are ensured by the presence of nonspecific humoral factors in them (Smith et al., 2016).

One of the stages of phagocytosis is killing (destruction) of viable objects. The development of killing in all professional phagocytes occurs with the participation of two bactericidal systems - oxygen-independent and oxygen-dependent. Oxygen-dependent systems function with the participation of: myeloperoxidase, respiratory explosion substances (H_2O_2, hydroxyl radicals, superoxide anion radicals, halogens, singlet oxygen) that arise in phagocytes upon activation. Oxygen-independent systems also include cationic proteins, lysozyme, lactoferrin, the acidic environment of phagosomes, and alkaline phosphatase (Andrés et al., 2022).

In terms of functional properties, neutrophils in the blood of animals and humans are similar in the system of mononuclear phagocytes, despite the polymorphism of the nuclei. During the process of phagocytosis, neutrophils degranulate with the release of lysosomal enzymes. These enzymes have a damaging effect. Neutrophilic white blood cells engulf and kill bacteria. They themselves are also powerful producers of cytokines: interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-12 (IL-12), tumor necrosis factor α (TNF-α), colony stimulating factor (GM- CSF), interferon-α (IFN-α), platelet activating factor, fibroblast growth factor (Medina, 2009; Smith et al., 2016). Activation of these body defense systems can have a positive effect both in the development of pathology and in increasing the body's resistance to unfavorable environmental conditions.

Numerous studies have reported the ability of probiotics to significantly increase nonspecific resistance in cows (Milner et al., 2021; Fijan et al., 2022). The positive effect of probiotics on a living organism is based on various biomechanisms. Probiotic microorganisms produce antimicrobial substances and can have a modifying effect on toxins, preventing the development of toxic reactions. In addition, there is competition between beneficial microorganisms of the digestive tract and pathogenic bacteria for nutrients and adhesion sites (Arriel-Elias et al., 2023; Sadeghpour Heravi and Hu, 2023).

Research by Fijan et al. (2018) noted that some types of bacteria and yeast suppress the growth of pathogenic species and also reduce the adhesion of pathogens to intestinal epithelial cells. In particular, *Bifidobacterium animalis subsp. lactis BB-12 and Lactobacillus reuteri DSM 17938* inhibited the growth of *E. coli*. This has a positive effect on the resistance of animals to the effects of unfavorable environmental factors and helps to increase their productivity (Mylostyva et al., 2022; Skliarov et al., 2022).

The choice of bacteria in probiotics for ruminants is aimed at improving the processes of fiber fermentation by rumen bacteria. These microorganisms have a positive effect on cellulose breakdown and microbial protein synthesis (Uyeno et al., 2015; Plaizier et al., 2017), among which the yeast *Saccharomyces cerevisiae* is preferred. In the production of probiotics, *lactobifidobacteria*, non-pathogenic streptococci, Escherichia and spore-forming bacteria of the genus *Bacillus* can also be most often used.

For example, probiotics, which contain bacteria that secrete lactate during their life (*Enterococcus*, *Lactobacillus*), are able to maintain a constant level of lactic acid; they may be a means of reducing acidosis in animals fed higher doses of concentrates (Mobiglia et al., 2021), especially in finishing beef cattle. *Megasphaella elsdenii* or *Propionibacterium species* can be used to reduce lactate accumulation (Monteiro and Faciola, 2020; Deng et al., 2021).

The use of probiotics in feeding and veterinary medicine makes it possible to increase the economic efficiency of livestock farming, obtain high-quality products free from antibiotics, chemotherapy drugs and traces of disinfectants for a healthy nutrition system for the population (Reuben et al., 2020; Deng et al., 2022). Therefore, the purpose of the study was to study the effect of probiotics of individual strains on the functional activity of blood neutrophils and on the immune system of the body of cattle.

Materials and methods

Experimental Design

The studies were carried out on heifers of the Volyn beef breed in one of the breeding farms of the Volyn region in Ukraine. These were purebred fifteen-month-old heifers with a live weight of 360 ± 13.6 kg (mean ± sd). From these animals, 3 groups of 15 heifers were formed: a control group (not receiving a probiotic), group I, which received the *Lactobacillus spp* strain (10^7 CFU) as a probiotic, and group II, which received *Bacillus Subtilis* (10^9 CFU), by dissolving in a small amount water and drinking individually. The animals were kept loose during the winter period on deep straw bedding in a naturally ventilated room with free access to walking and feeding areas.

The diet contained all the necessary nutrients, microelements and vitamins in accordance with the feeding standards for beef cattle (Ibatullin et al., 2007). The main feeding ration consisted of concentrates, legume and cereal hay, feed straw, corn silage and haylage, and fodder beets. The animals had free access to automatic drinking bowls with electrically heated water in winter.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Commission on Bioethics of the State Agrarian and Economic University (protocol No. 1/11 dated November 14, 2023).

Methods of determining the functional activity of neutrophils and immunological indicators

Blood for research was taken in the morning from the jugular vein, before distribution of feed, in compliance with the rules of asepsis and antiseptics. Neutrophils were isolated from heparinized whole blood by centrifugation in a Ficoll-Urografin dual density gradient: p = 1.077 g/cm^3 for the isolation of lymphocytes and p = 1.119 g/cm^3 for the isolation of neutrophils. To assess natural stability, the following parameters were determined:

Phagocytic activity of neutrophils (PAN, %) was calculated as the percentage of neutrophils capable of absorbing latex particles. Phagocytic index (PI, cu), is the average number of latex particles absorbed by one active neutrophil. Absolute blood phagocytosis (AF, 10^9/l), is the total number of latex particles absorbed by neutrophils per liter of blood. Phagocytic number (PN, cu) is the average number of latex particles per active or inactive neutrophil. The functional and metabolic activity of neutrophils was assessed based on the results of the nitroblue tetrazolium (NBT) reduction reaction in NBT-positive neutrophils (+NBT, %) in two variants: spontaneous and stimulated (Klimenko and Shelest, 2013).

NBT test: 0.05 ml of 0.2% sodium chloride solution in potassium phosphate buffer (0.1, pH 7.3) and 0.05 ml of the same buffer were added to 0.1 ml of blood. The reaction mixture was thermostated in a water bath at 37°C (30–60 min), medium-thick smears were made, air-dried, fixed in ethanol (20 min), stained with an aqueous solution of neutral red (0.1%, 20 min) and microscoped under immersion.

To study the absorption capacity of neutrophils, blood was taken, stabilized with heparin (basal), and stimulated, after adding zymosan (stimulated) to the blood samples, which simulates the conditions of bacterial infection and characterizes the adaptive reserves of the absorption and microbicidal ability of neutrophil granulocytes (Freeman and King, 1972).

Neutrophil activation index (NAI) was calculated using the formula:

IAN = (0 x H0 + 1 x H1 + 2 x H2 + 3 x H3) / 100, in this case, H0, H1 and H2 are non-activated neutrophils, and H3 are activated neutrophils.

The mean cytochemical coefficient (MCC) was calculated with the formula: MCC = (0 × N0 + 1 × N1 + 2 × N2 + 3 × N3) / 100, where: N0, N1, N2, N3 are numbers of neutrophils with 0, 1, 2- and 3-point activity; N0 + N1 + N2 + N3 = 100.

The content of the T-lymphocyte population (T-cell, %) was determined by the rosette formation reaction of lymphocytes with sheep erythrocytes, B-lympho-

Table 1. Changes in the activity of neutrophils in the blood of heifers under the influence of probiotics

Indicator	Control	Experimental groups I (*Lactobacillus spp.*)	Experimental groups II (*Bacillus Subtilis*)
PAN basal, %	38.08 ± 0.69	44.67 ± 1.07*	47.08 ± 1.02*
PAN stimulated, %	46.66 ± 0.53	74.43 ± 1.09*	61.78 ± 1.43*
PI basal, a.u.	5.35 ± 0.09	6.13 ± 0.10*	6.17 ± 0.08*
PI stimulated, a.u.	6.59 ± 0.284	10.03 ± 0.15*	8.61 ± 0.15*
PN basal, a.u.	1.80 ± 0.01	2.10 ± 0.01*	1.97 ± 0.02*
PN stimulated, a.u.	2.63 ± 0.04	3.37 ± 0.07*	3.11 ± 0.04*
ABP basal, 10^9/l	2.62 ± 0.06	4.83 ± 0.18*	4.46 ± 0.16*
ABP stimulated, 10^9/l	3.26 ± 0.10	6.74 ± 0.23*	6.01 ± 0.15*
+ NBT basal, %	6.32 ± 0.20	6.30 ± 0.04	6.27 ± 0.04
+ NBT stimulated, %	45.78 ± 0.58	25.49 ± 0.45*	36.75 ± 0.79*
NAI basal	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
NAI stimulated	0.79 ± 0.01	0.64 ± 0.00*	0.70 ± 0.01*
MCC	1.41 ± 0.01	1.66 ± 0.02*	1.65 ± 0.02*

Note. PAN is Phagocytic Activity of Neutrophils; PI is Phagocytic Index; PN is Phagocytic number; +NBT is positive neutrophils; NAI is neutrophil activation index; MCC is average cytochemical coefficient; ABP is absolute blood phagocytosis. *Significant differences compared to the control group ($P<0.05$).

cytes (B-cell, %) were determined with mouse erythrocytes. Subpopulations of immunoregulatory T-lymphocytes with predominantly helper (Th3, %) and suppressor (Th3, %) activity were determined in a theophylline test (Paul et al., 1979). The content of immunoglobulins was determined by the radial immunodiffusion method according to Mancini (Mancini, 1965).

Statistical analysis

Statistical software package STATISTICA 10 (StatSoft, Inc., Tulsa, OK, USA) was used for statistical data processing. The distribution of almost all variation series did not meet the normality criteria; Therefore, nonparametric statistical methods were used in further analysis. Data are presented as mean and standard deviation. Differences between samples were determined using the Mann-Whitney U test and were considered significant at $P<0.05$.

Results

It was found that under conditions stimulating the response of neutrophil granulocytes (after adding zymosan to blood samples), there was an increase in PAN by 59.5% ($P<0.05$) in heifers treated with Lactobacillus spp (group I) and by 32.4% ($P<0.05$) in animals treated with Bacillus Subtilis (group II) compared to the control (Table 1). PI under the influence of probiotics also increased by 52.2% ($P<0.05$) and 30.6% ($P<0.05$), respectively, compared to the control group.

PN in beef heifers fed with *Lactobacillus spp* increased by 28.1% ($P<0.05$), and in those fed with *Bacillus Subtilis* by 18.2% ($P<0.05$) relative to the control. ABP increased by 2.1 and 1.8 times, respectively ($P<0.05$). At the same time, in heifers receiving probiotics there was a decrease in the level of blood factors that cause activation of neutrophils, as evidenced by a decrease in + NBT in the blood of group I by 44.3% ($P<0.05$) and group II by 19.7% ($P<0.05$). In animals after probiotics, NAI decreased by 18.9 and 11.39% ($P<0.05$), respectively, compared to the control group. At the same time, MCC values in animals receiving probiotics increased in group I by 15.1% and in group II by 14.5% ($P<0.05$) against the background of the use of probiotics.

The state of the cellular and humoral components of the immune system changed in animals receiving probiotics

Table 2. Immunological blood parameters of heifers under the influence of probiotics

Indicator	Control	*Lactobacillus spp.*	*Bacillus Sb.*
T-cell, %	54.79 ± 0.39	66.67 ± 0.73*	66.97 ± 1.03*
Th, %	29.03 ± 0.50	33.10 ± 0.51*	32.40 ± 0.68*
Th3, %	28.34 ± 0.41	30.59 ± 0.63	33.74 ± 0.89
B-cell, %	20.59 ± 0.39	21.85 ± 0.46	20.56 ± 0.34
IgG, mg ml	22.52 ± 0.23	24.64 ± 0.47*	24.85 ± 0.47*
IgM, mg ml	2.54 ± 0.021	2.80 ± 0.032*	2.75 ± 0.03*
IgA, mg ml	0.40 ± 0.006	0.41 ± 0.00	0.41 ± 0.01

Note. T-cell is T-lymphocytes, Th is helper T-lymphocytes, Th3 is suppressor T-lymphocytes, B-cell is B-lymphocytes. *Significant differences compared to the control group ($P<0.05$).

(Table 2). In heifers of groups I and II, the number of T-lymphocytes increased by 15.2 and 18.1% compared to the control (P<0.05).

In the blood of heifers under the influence of *Lactobacillus spp*. Helper T lymphocytes increased by 14.0% (P<0.05), and suppressor T lymphocytes by 7.9%. Under the influence of *Bacillus Sb*. these indicators increased by 11.6% (P<0.05) and 19.1%, respectively.

The concentration of IgG and IgM in the blood of animals of group I was higher by 9.4 (P<0.05) and 10.2% (P<0.05), and in heifers of group II by 10.4% and 8.3% respectively, compared to the control. At the same time, the use of probiotics did not lead to a significant change in the concentration of IgA in the blood.

Discussion

The irrational use of antibiotics for treatment, as well as the use of feed antibiotics, leads to increased risks for meat consumers, the demand for which is constantly increasing. Therefore, the attention of many researchers is focused on antibiotic substitutes, which are increasingly used as feed additives or probiotics (in the form of single or mixed strains) in animal nutrition (Tekce et al., 2021; Nie et al., 2022).

The effectiveness of probiotics containing live bacterial cultures is associated with favorable metabolic changes in the digestive tract, better absorption of nutrients, increased body resistance to adverse conditions, and an antagonistic effect on pathogenic and opportunistic microflora. Some studies have shown that probiotics have a positive effect not only on the microbial ecosystem of the digestive tract, nutrient synthesis (Saha et al., 2018), they also lead to increases in carcass weight (Lu et al., 2021), muscle mass, and meat quality (Liu et al., 2022). In addition, as reported by Tekce et al. (2021), supplementation with probiotics (*Lacticasebacillus rhamnosus*; *Saccharomyces cerevisiae*) for 2.5 months helped maintain microbial balance in the digestive tract of lambs at weaning.

he spectrum of opportunistic microflora is rapidly expanding; data on new pathogens of pathological processes of various etiologies appear every year (Alba et al., 2023). Since disruption of the immune system is the main cause of the unfavorable development of the pathological process, as well as relapses, transition to a chronic course and increased complications (Puggioni et al., 2019; Scott et al., 2020), in clinical practice to strengthen the immune system along with antimicrobial therapy immunomodulatory drugs must be used (Naef et al., 2021). Probiotics can modulate the production of anti- and pro-inflammatory cytokines that control inflammation in the body (Mishra and Acharya, 2021). For example, *Lactobacillus* strains can reduce inflammation by suppressing pro-inflammatory cytokines (T cells, IL-8, TNF-α, IL-6) as well as by increasing levels of anti-inflammatory cytokines (IL-10, TGF-β, IL-1Ra) in the intestine, as proven by Ding et al. (2017) in their studies.

Probiotics tend to model natural resistance in many ways, including receptor antagonism, adapter protein binding and expression, expression of negative regulatory signaling molecules, endotoxin tolerance, and secretion of immunomodulatory substances (Li et.al., 2019; Villena and Kitazawa, 2020). As in our case, probiotics increased the concentration of immunoglobulins and enhanced the activity of macrophages and natural killer cells (Hussein, 2018; Ayala-Monter et al, 2019; Islam et.al., 2021).

Bifidobacteria have a direct effect on

the activation of the innate immune system of calves, which at an older age is reflected in a decrease in mortality and an increase in their productivity (Kargar et.al., 2021). The interaction of beneficial microflora and the intestinal mucosa occurs through the recognition of molecular mechanisms associated with the pathogen through pattern recognition receptors, of which toll-like receptors (TLRs) have the widest range of pathogen ligands (McKernan, 2020).

The immunological properties of probiotics are explained by the fact that bacteria are able to migrate into the circulatory and lymphatic systems with further reaching the site of alteration, promoting the biosynthesis of immunomodulatory and antibacterial substances. In addition, some strains of bacteria of the genus *Bacillus* initiate the synthesis of endogenous interferon in animals. These drugs have antiviral and antibacterial activity and increase the body's resistance.

Neutrophils are the most numerous group of blood leukocytes involved in the mechanisms providing local immunity. Pathogens entering the body cause an inflammatory reaction that attracts neutrophils from the peripheral blood into the tissues. At the site of inflammation, neutrophils destroy microorganisms through a number of mechanisms, mainly through phagocytosis, release of antimicrobial substances, and formation of neutrophil extracellular traps (Margraf et al., 2019; Poli and Zanoni, 2023). They also generate reactive oxygen species with cytopathic effects due to their high content of NADPH oxidase and myeloperoxidase (Hidalgo et al., 2022).

The use of probiotics led to an increase in the level of natural resistance of heifers due to an increase in the reactivity of blood neutrophils, as evidenced by an increase in the number of blood neutrophils capable of absorbing foreign material. As in our case, the use of probiotics increased natural resistance, immune status and optimized the microbiocenosis of the large intestine (Abenavoli et al., 2023; Gasmi et al., 2023). Probiotics containing lactic acid bacteria (*L. casei*, *L. rhamnosus*, *L. paracasei*) increased the absorption capacity of blood neutrophils and the phagocytic number (Garcia-Castillo et al., 2020).

Conclusions

It has been established that a probiotic containing *Lactobacillus* spp. and *Bacillus subtilis* is able to cause an increase in the functional activity of blood neutrophils and the immune function of beef heifers.

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Funkcionalna aktivnost neutrofila u krvi i imunološkog statusa junica pod utjecajem probiotika

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Ovaj članak tematizira učinak probiotika na organizam životinja i svrha mu je proučiti učinak probiotika pojedinih sojeva na funkcionalnu aktivnost neutrofila u krvi i na pojedine dijelove imunološkog sustava organizma goveda. U širokoj uporabi su različiti probiotici i oni omogućuju modernizaciju postojećih režima u stočarstvu. Zadatak pronalaska novih pristupa optimizaciji korisne flore organizma izuzetno je važan. Uporaba probiotika u stočarskoj proizvodnji je ekonomično rješenje i utvrđeno je da je uporaba probiotika *Lactobacillus* spp. i *Bacillus sb*. popraćena aktivacijom mehanizama prirodne otpornosti. Probiotici pomažu poboljšati i imunološki status, uglavnom zahvaljujući humoralnoj komponenti, a imaju i tendenciju optimizacije mikrobiocenoze debelog crijeva te imaju pozitivan učinak na povećanje produktivnosti životinja. Rezultati opsežnih studija stanja obrambenih sustava tijela goveda i njihovih promjena pod utjecajem lijeka proširuju i produbljuju postojeće podatke o učinku probiotika na homeostazu životinja.

Ključne riječi: *goveda, probiotici, neutrofili u krvi, fagocitoza, imunost*