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AGRICULTURAL INSTITUTE – STARA ZAGORA

Scientific Department "Breeding and Technologies in Cattle Breeding"

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“The influence of probiotic "Zoovit" on the main quality indicators and technological properties of milk from Holstein cows”

ABSTRACT

of a dissertation for the acquisition of the educational and scientific degree "PhD"

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I thank my family for their patience, love and faith in me.

The dissertation consists of 142 pages, 18 tables and 40 figures. In the list of cited literature, 282 literary sources are indicated, of which 58 are in Cyrillic and 224 are in Latin.

The numbering of the sections, tables and figures in the Abstract does not correspond to those in the dissertation.

The defense of the dissertation will take place on …… 2023 at …… h in the Meeting Hall of the Agricultural Institute - Stara Zagora. The materials related to the defense of the dissertation are available at the Scientific Secretary of ZI–Stara Zagora.

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The reviews and opinions of the members of the Scientific Jury, as well as the Abstract, are published on the website of the Agricultural Institute - Stara Zagora:

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I. INTRODUCTION

Livestock is one of the fastest-growing agricultural sectors, contributing about 40 percent of the global value of agricultural production, providing livelihood and food security to almost 1.3 billion people. The growing production of foods of animal origin raises the issue of subtherapeutic use of antibiotics in food for animals and the development of antibiotic resistance in microbial populations associated with human and animal diseases.

The global spread of antimicrobial resistance calls for the development of effective antibiotic alternative therapies in livestock, where almost two-thirds of the global antibiotic supply is consumed. Probiotics should provide both prophylactic and therapeutic efficacy to control antibiotic-resistant bacteria and influence treatment outcomes.

In order to use probiotics effectively, it is important to establish their performance in the gut, as well as their secondary effects in animal products and the environment. One of the critical fields of research is the impact of probiotic therapies on the quality of dairy products under specific processing technologies. Another critical point is the lack of experimental studies on levels of antibiotic residues and resistant bacteria in the environment after switching to probiotic therapy. Research are aimed at filling this data gap, developing effective antibiotic alternative therapies, reducing AMR in the environment and preserving the health of consumers and animals.

I. AIM AND TASKS

The aim of the present work was to investigate the influence of probiotic "Zoovit", taken with the ration, on the physicochemical, microbiological and technological properties of cow's milk.

To achieve the set goals, we set the following tasks:

1. Study of the influence of probiotic "Zoovit" on the main physicochemical indicators of cow's milk in two seasons - winter and summer.

2. Study of the influence of probiotic "Zoovit" on the main microbiological parameters of cow's milk in two seasons - winter and summer.

3 Detection of *Lactobacillus delbrueckii subsp. bulgaricus* in the faecal mass of animals.

4. Study of the influence of probiotic "Zoovit" on the main technological indicators of cow's milk from cows with the addition of a probiotic to the ration.

5. Study of the influence of probiotic "Zoovit" on the quality of yogurt using the classical technology.

II. MATERIALS AND METHODS

1. Experimental animals

The research was conducted in compliance with the Bulgarian legislation on humane treatment and protection of animals. Dairy cows from a livestock facility, city of Plovdiv, 4000-0001, were included in the experiment. The number of animals during the research at the site was 198, of which 110 were dairy cows, 66 heifers and 22 calves. During the research animals were fed according to recipes in accordance with their age and physiological state. To establish the influence of probiotic "Zoovit" on the physicochemical, microbiological and biological qualities of milk, two separate groups of 30 dairy animals were formed - experimental /group 1/ and control /group 2/. The two groups of animals were fed daily with 15 kg of combined fodder, 20 kg of silage and 4 kg of alfalfa hay. 0.600 kg of probiotic (0.020 kg per cow) was added to the combined feed of the experimental group. The tests were carried out in two seasons - winter - in December, January and February and summer - in June, July, September and October. Three parallel samples of morning raw milk (1 liter each) milked from the probiotic-fed animals and three of the control group animals were examined daily.

Six fecal samples from cows fed with feed containing the probiotic "ZOOVIT", collected in May and June 2017, as well as three randomly selected control samples from cows not receiving the probiotic preparation, were analized.

2. Probiotic “Zoovit”

Probiotic preparation "Zoovit" produced by "LB lact BAS" EOOD. It contains four strains of lactic acid bacteria - *Lactubacillus delbruedki subsp. bulgaricus* (F12), *Streptococcus salivarius subsp. thermophilus* (9), *Lactobacillus acidodophilus* (17), *Lactobacillus lactis* (B) and one strain of *Propionibacterium.* The presence of five strains improves its effectiveness and widens the spectrum of action.

3. Methods of analysis

The samples were analyzed in the Research Laboratory for Milk and Dairy Products "LB Lact" – Plovdiv according to the following indicators: physicochemical, microbiological and technological.

The technological properties of milk were characterized based on 4 main indicators: biological activity, through a fermentation sample, (h); rennet (enzymatic) coagulation (t /min); coagulum syneresis (mL); acid formation (^{0}T).

3.1. Physicochemical indicators

3.1.1. Active and titratable acidity

Active acidity - pH was determined potentiometrically using a MS 2011 pH-meter (Microsyst, Plovdiv, Bulgaria) equipped with a Sensoret pH electrode (Garden Grove, CA, USA). The total titratable acidity according to BDS 1111-1980.

- Milk fat – by applying the Gerber method (ISO 2446:2008 (IDF 226:2008)).

- Dry matter - by drying the sample at a temperature of 100÷105 ºC, for 4 h /BDS 1109:1989/.

- Total protein - according to BDS EN ISO 8968-1:2014.

3.2. Microbiological indicators

3.2.1. Determination of the number of viable cells of lactic acid bacteria (cfu.cm^{-3}), according to ISO 7889:2005

10 g of the test sample was weighted and transfered to a sterile 200 cm^3 flask. 90 cm^3 of sterile diluent was added to the sample. Ten-fold falling dilutions were made from this initial dilution, transferring 1 cm^3 of the previous dilution to the next tube with a sterile pipette. The number of tenfold dilutions was determined depending on the expected titer of active cells. Then, 1 cm^3 cultures were made from the last dilutions in a sterile petri dish.

After the incubation time, the petri dishes were removed from the thermostat and the grown colonies were counted and the amount of active cells was calculated according to the following formula:

$$N = \frac{\sum C}{V \cdot (n_1 + 0{,}1 \cdot n_2) \cdot d}$$

where: N is the numerical value of the number of characteristic microorganisms in 1 g of the sample, cfu.cm^{-3}; V-seed volume, cm^3; ∑C is the numerical value of the sum of the colonies of all listed petri dishes; n_1 - number of petri dishes from the first dilution; n_2 - number of petri dishes of the second dilution; 0.1 coefficient; d is the dilution factor corresponding to the first of the listed dilutions.

When the results were reported from three consecutive dilutions, the concentration of active cells was calculated according to the following relationship:

$$N = \frac{\sum C}{V \cdot (n_1 + 0{,}1 \cdot n_2 + 0{,}01 \cdot n_3) \cdot d}$$

where n_3 is the number of petri dishes of the third dilution.

3.2.2. Morphological characteristics of lactic acid bacteria

The morphological characteristics of the lactic acid bacteria strains were determined by coloured microscopic preparations, microscoped using Olympus BX41 microscope.

3.2.3. Determination of total number of microorganisms according to BDS EN ISO 4833-1:2013

The total number of microorganisms was determined by the tenfold dilution method. The raw milk was homogenized, 1 cm^3 was measured from it and transferred to a test tube containing 9 cm^3 of physiological solution, followed by homogenization and 1 cm^3 was taken from this test tube and transferred to the next one. This was repeated until the required degree of dilution was reached. The last three dilutions were inoculated with 1 cm^3 of seed material. The sawn petri dishes were filled with 15 cm^3 of melted and cooled to 44÷47 °C SMA nutrient medium. Waited for the medium to harden and placed the petri dishes in a thermostat where it was thermostatitrated at 30 °C for 72±3 hours. After the time was up, the petri dishes were removed from the thermostat, the grown colonies were counted in two consecutive dilutions and the total microbial number was calculated according to the formula:

$$N = \frac{\sum C}{V \cdot (n_1 + 0{,}1 \cdot n_2) \cdot d}$$

3.2.4. Determination of total number of somatic cells according to BDS EN ISO 13366-1:2008

The raw milk sample intended for the determination of somatic cells was cooled immediately after its collection to a temperature of 2÷6 °C. The examination of the sample was carried out no later than 6 hours after its reception in the laboratory.

The number of somatic cells in 1 cm^3 was calculated by multiplying the number of bands counted by the work factor of the microscope.

3.2.5.Determination of *Escherichia coli* according to ISO 16649-2:2014

For this purpose, 10 g of the examined fecal mass sample was weighed and placed in 90 cm^3 of a sterile diluent. From this initial dilution, several successive tenfold dilutions were made depending on the expected insemination of the sample. The typical colonies that are colored blue-green were counted in two successive dilutions and the amount of active *Escherichia coli* cells were calculated according to the following relationship:

$$N = \frac{\sum C}{V \cdot (n_1 + 0{,}1 \cdot n_2) \cdot d}$$

3.2.6. Determination of *Staphylococcus aureus* according to BDS EN ISO 6888-1:1999/A2:2018

The number (N) of coagulase-positive staphylococci in 1g of the tested product was calculated by the following formula:

$$N = \frac{\sum a}{v.(n_1 + 0{,}1n_2).d}$$

where a - sum of the colonies of coagulase-positive staphylococci contained in the petri dishes from two successive dilutions;

where: v – volume of seed material; n_1 – number of petri dishes from the first of the dilutions listed; n_2 – number of petri dishes from the second of the dilutions listed;

d – dilution factor corresponding to the first of the listed dilutions.

3.2.7. *Listeria monocytogenes* according to BDS EN ISO 11290-1:2017

3.2.8. Coliforms according to ISO 4832:2006

3.2.9. *Enterobacteriaceae* according to ISO 21528-1,2:2017

3.2.10. Molds and yeasts according to BDS ISO 6611:2004.

3.2.11. *Salmonella spp.* ISO 6579-1:2017

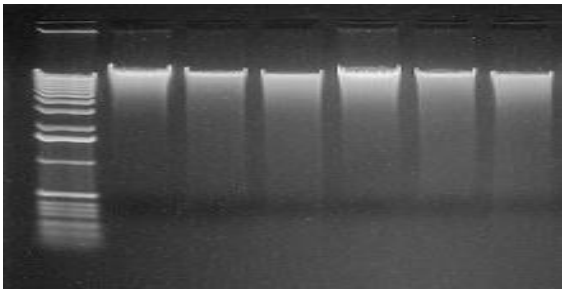
4. Determination of sulphite-reducing colonies in faecal mass according to ISO 15213:2003

5. Using the PCR analysis for detection of *Lactobacillus delbrueckii subsp. bulgaricus* in cow faeces

Six fecal samples from cows fed with feed containing the probiotic "ZOOVIT", collected in May and June 2017, as well as three randomly selected control samples from cows not receiving the probiotic preparation, were analyzed. One gram of faecal mass was suspended and homogenized very well in 9 cm^3 of sterile peptone water, after which tenfold falling dilutions were made. As from each dilution, a culture was made on MRS agar. The sawn petri dishes were incubated at 37±1°C for 48 hours.

A Gene JetTM Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltman, USA) was used to extract DNA from colonies from the various cow faecal samples. Initially, a lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA, 1.2 % Triton X-100) containing lysozyme 20 $mg.ml^{-1}$ was prepared. The samples were incubated for 30 min at 37 °C. Further isolation of DNA from faecal samples using this kit was performed according to the manufacturer's instructions..

The frequency of DNA in the extracts was determined by 0.7% agarose gel electrophoresis (Figure 1).



M 1 2 3 4 5 6

Figure 1. Determination of the frequency of the isolated DNA from the six samples of cow faeces

M - 100 bp molecular marker (Bioneer, Korea);

1-3 isolated DNA from faecal mass of control cows fed no probiotics;

4-6 isolated DNA from faecal mass of three cows that received probiotics.

Species-specific PCR analysis for the presence of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* in faecal samples.

To determine the presence of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* in cow faeces, a PCR analysis with species-specific primers was applied (Lick et al., 2000; Lick et al., 2001). The total PCR reaction in a volume of 25 µl of the Illustra™ puReTaq Ready-To-Go PCR Beads kit (GE Healthcare UK Limited, United Kingdom), contained: BSA, 200 µM concentration of each of the dNTP nucleotides (dATP, dCTP, dGTP, dTTP) , 2.5 units of PuReTaq DNA polymerase and reaction buffer (10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM MgCl2). Approximately 20÷30 ng of the purified DNA and 1 µM of each primer were added to the reaction mixture. The negative PCR control contained only sterile deionized water instead of DNA.

The nucleotide sequence of the primers used is presented in Table 1. The PCR reaction conditions for amplifying the PCR products are summarized in Table 2. The PCR products of approximately 678 and 715 kb in size were separated on a 1.2% agarose gel.

Ten randomly selected colonies from three cows from the control and three from the experimental group were used for PCR analysis to determine the presence of viable *L. delbrueckii subsp. bulgaricus* in faecal samples. Study was performed using DNA isolated from a single colony of a given lactic acid bacterium grown on MRS agar medium. For this purpose, the colony was dissolved in 50 µl TE buffer (10 mM

Tris-HCl and 1 mM EDTA, pH=8), to which 20 $mg.ml^{-1}$ lysozyme was previously added.

Table 1. Nucleotide sequence of species-specific primers, Fw-forward primer, Rv-reverse primer

Type	Nucleotide sequence	Size (base units)	Literature
L. delbrueckii	Fw-AAT TCC GTC AAC TCC TCA TC	715	Lick *et al*., 2000
	Rv-TGA TCC GCT GCT TCA TTT CA		
L.delbrueckii subsp. bulgaricus	Fw-CCT CAT CAA CCG GGG CT	678	Lick *et al*., 2000; Lick *et al*., 2001
	Rv-TGA TCC GCT GCT TCA TTT CA		

Samples were incubated at 37 °C for 30 min. DNA was precipitated by adding 3 times the sample volume of 100% ethanol and 1/10 of the volume of 3M sodium acetate. After centrifugation at 10000 x g, the sediment was washed twice with 70% ethanol and dissolved in 20 µl TE buffer. One µl of each sample was used as template in a PCR reaction under the same conditions described above.

Table 2. PCR reaction conditions for amplification of species-specific PCR products

Temperature regime	*L. delbrueckii*	*L. delbrueckii subsp. bulgaricus*
Initial denaturation	95°C-10 min	95°C-10 min
	10 cycles	
Denaturation	95°C-20 sek	95°C-20 sek
Hybridization	**55°C-20 sek**	**65°C-20 sek**
Extension	72°C-40 sek	72°C-40 sek
	35 cycles	
Denaturation	95°C-20 sek	95°C-20 sek
Hybridization	**50°C-30 sek**	**60°C-30 sek**
Extension	72°C-1 min	72°C-1 min
Ultimate extension	72°C-10 min	72°C-10 min

6. Organoleptic indicators

The organoleptic assessment of the yoghurt samples was carried out according to a ten-point hedonic scale. The main organoleptic indicators on which the organoleptic assessment is based were: appearance, color, type of coagulum, consistency, taste and aroma.

7. Mathematical-statistical analysis of the obtained data

A statistical analysis of the mean values of the triplicates was performed. Analysis of variables (oneway ANOVA) was performed with a significance level of $P \leq 0.05$ (Draper and Smith, 1998). Turkey's test for multiple comparisons was performed with a significance level of $P \leq 0.05$. Difference between values lower than 0.05 were considered statistically significant (Kenward, 1987). All statistical procedures were performed using Microsoft Excel 2010 and SigmaPlot 11.0 software.

III. RESULTS AND DISCUSSION

1. Study of the influence of the probiotic "Zoovit" on the physicochemical, microbiological and technological properties of milk

1.1. Studies on the influence of the probiotic "Zoovit" on basic physicochemical parameters of milk

The physicochemical parameters of raw milk limit its technological properties and quality. They depend on the type and quantity of the ration, stage of lactation and health status. The results of the conducted researches on the physicochemical parameters of the milk obtained from cows that received probiotics with the food rations during the winter and summer seasons are presented in figures and tables.

1.1.1. Research of the protein content of milk - winter and summer season

Figure 2 shows the results for the protein content of milk during the winter period. It can be seen from them that the values of the protein in the milk of the cows that received the probiotic during the period from 02.12. to 07.02. were higher and in the range from 3.34 to 3.52 %. As the intake of the probiotic continued, the influence of the probiotic on the amount of protein also increased, and was most significant 18 days after its intake by the animals.

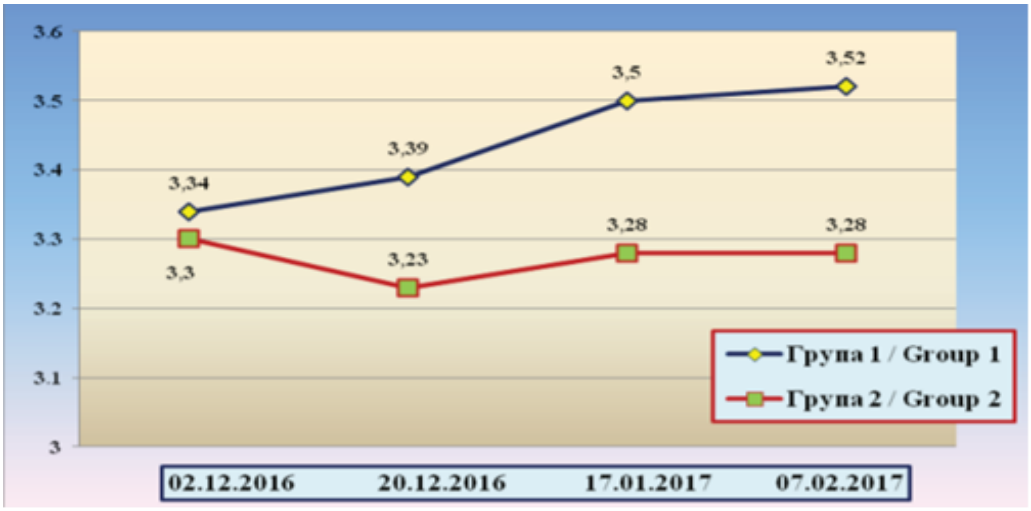


Figure 2. Protein content of milk - winter season

Figure 3 presents the results regarding the influence of probiotics on the protein content of milk during the summer period (June - October). It can be seen from them that the influence of probiotics during the studied period was less pronounced than that of the winter period - respectively 3.27 to 3.35 %. In contrast to the winter period, the influence of probiotics on the protein content in milk was markedly more active after 46 days of probiotic intake.

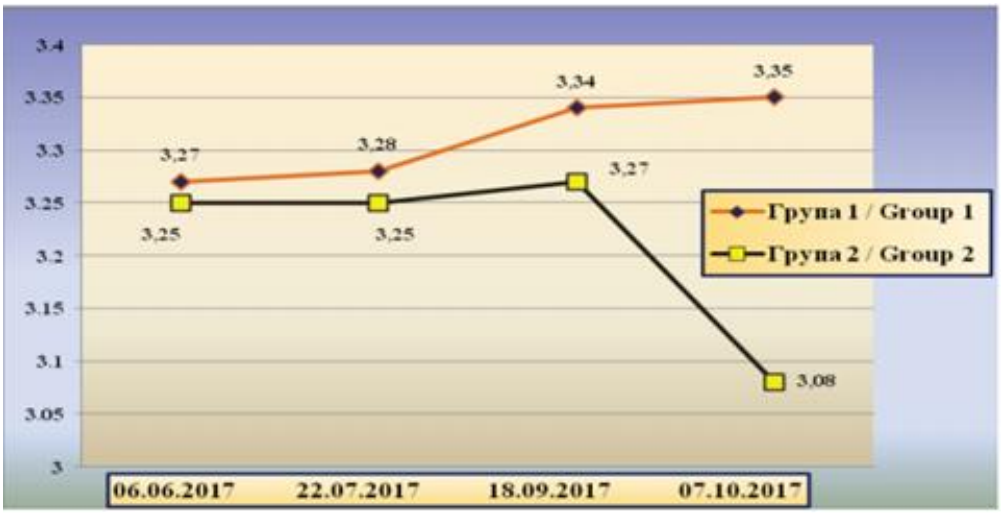


Figure 3. Protein content of milk - summer season

1.1.2. Study of the fat content in milk - winter and summer season

Figure 4 and 5 present the results of the research conducted on the influence of probiotics on the amount of fatty substances in milk during the summer and winter seasons. The data confirm the opinion of other authors that the fat content of milk depends mostly on diet and less on probiotics.

The percentage of fatty substances in the milk of the cows receiving the probiotic was slightly higher in both seasons, compared to those of the control group. In the summer period, the variation of this indicator was greater, with two peaks in the experimental group of animals, which we believe was due to the high temperatures, since the feeding was of the same type throughout the year.

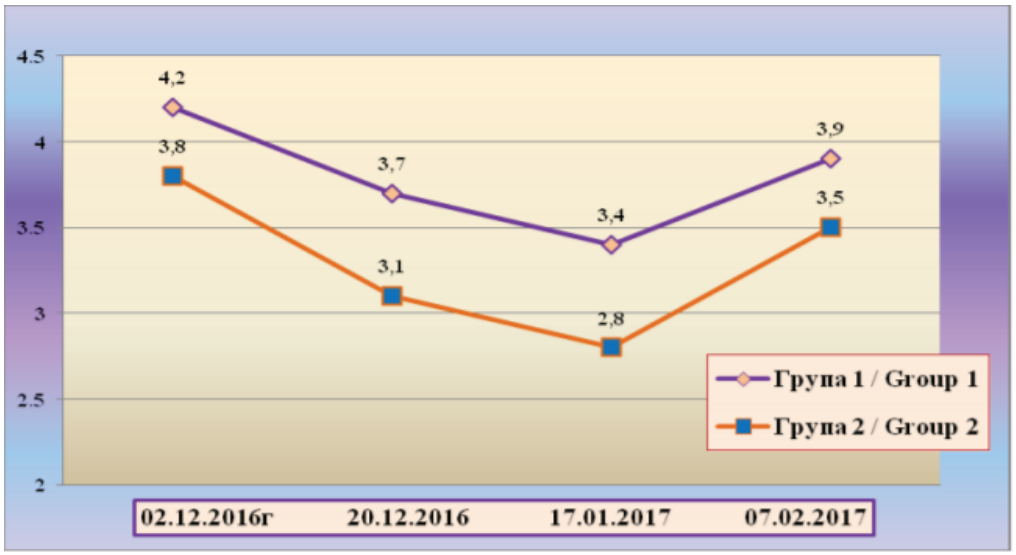


Figure 4. Fat content of milk - winter season

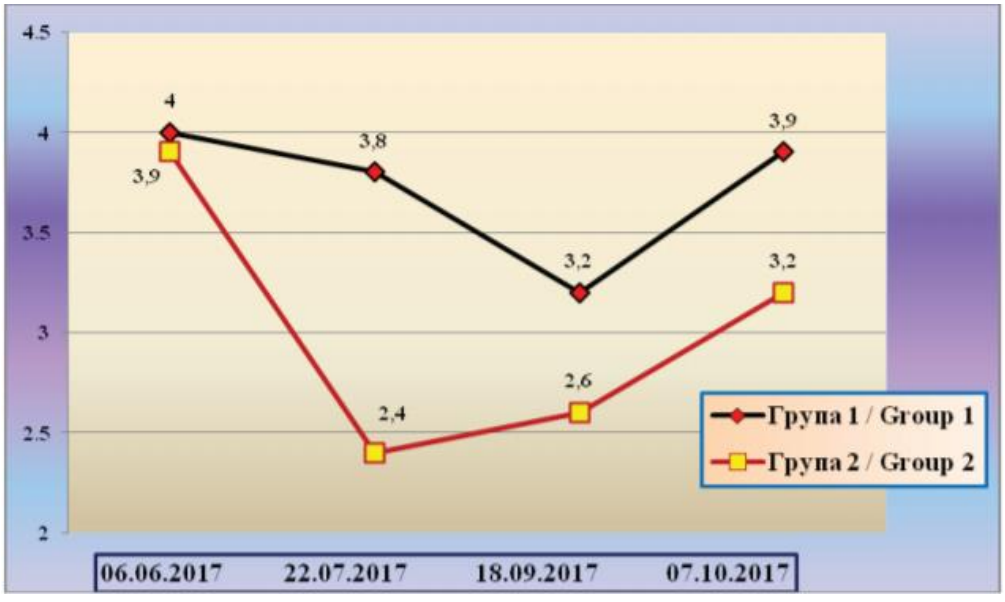


Figure 5. Fat content of milk - summer season

1.1.3. Study of dry matter and DNFR of milk - winter and summer season

Comparative studies of the milk of cows from the experimental and control groups (figure 6 and figure 7) show that during the winter period the difference in dry matter between them for the period 02.12.÷ 07.02. reached 1.96%, and the difference in dry matter during the summer period 06.06.÷07.10. (figure 9) was 1.26 % respectively.

This is probably due to the effect of the probiotic on the protein content and less on the fat content.

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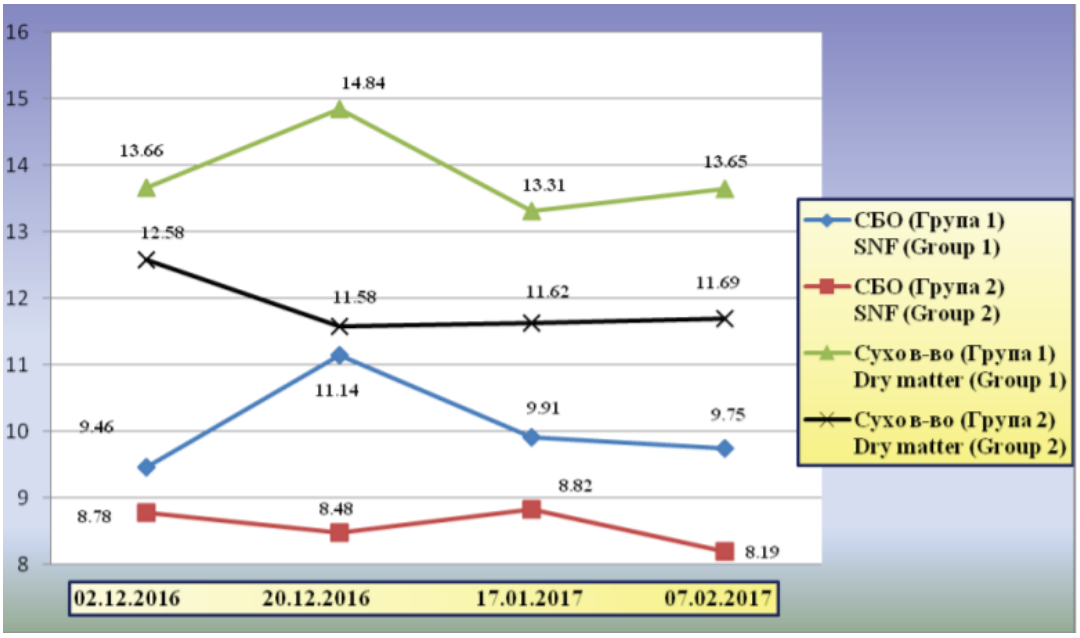


Figure 6. Dry matter and DNFR of milk - winter season

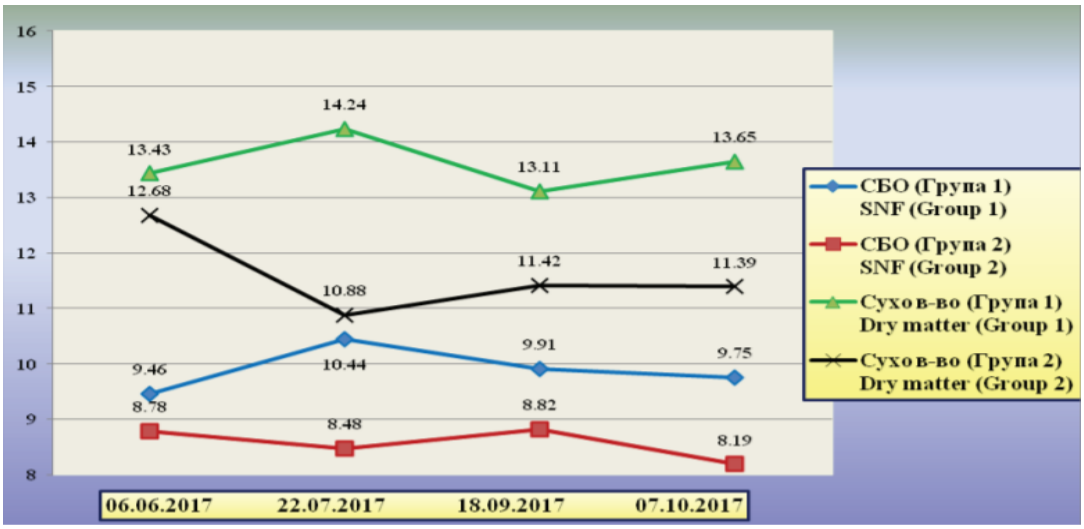


Figure 7. Dry matter and DNFR of milk – summer season

The obtained data for DNFR follows the trend for dry matter content. During the two seasons of the experiment, the milk of animals that received the probiotic "Zoovit" in their rations had a higher content of DNFR compared to those of the control group. Two peaks were observed - in August and December in the experimental group, while in the control group we had a drop in the content of DNFR at the end of the two experimental periods.

1.2. Researches on the influence of the probiotic "Zoovit" on basic microbiological traits of milk

1.2.1. Microbiological researches - summer season.

1.2.1.1. Amount of total microorganisms count in milk

Figure 8 shows the data on the total microorganisms count in the milk during the summer season in the two groups of animals – experimental and control. The amount of the total microorganisms count during the summer season in the milk from cows receiving the probiotic ration was from 8.7.105 to 1.3.105 cfu.mL^{-1}. In the milk from animals of the control group, the amount of the total microorganisms count was in the range of 2.7.106 to 2.5.106 cfu.mL^{-1}. The data show a tendency adding probiotic Zoovit to affect the total microorganisms count in milk during the indicated months.

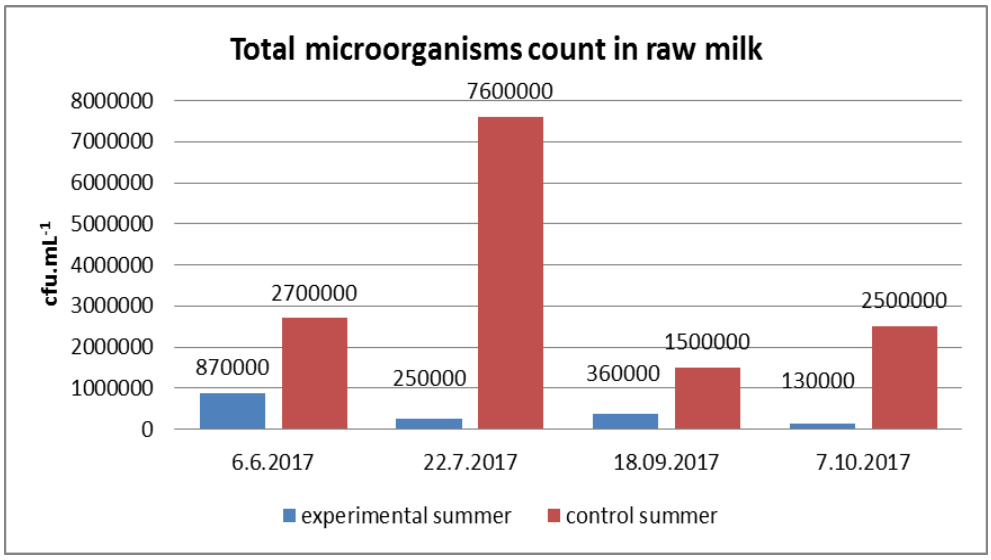


Figure 8. Total microorganisms count in milk - summer season

1.2.1.2. Total somatic cells count in milk

The effect of probiotics on the total somatic cells count is significant (figure 9). Their count in the studied period in the milk from experimental group ranged from 108 000 to 138 000 cells.mL^{-1}, compared to 200 000 to 202 000 cells.mL^{-1} of the control group for the same period. The effect of probiotics in the experiment to reduce somatic cells in milk was 46%.

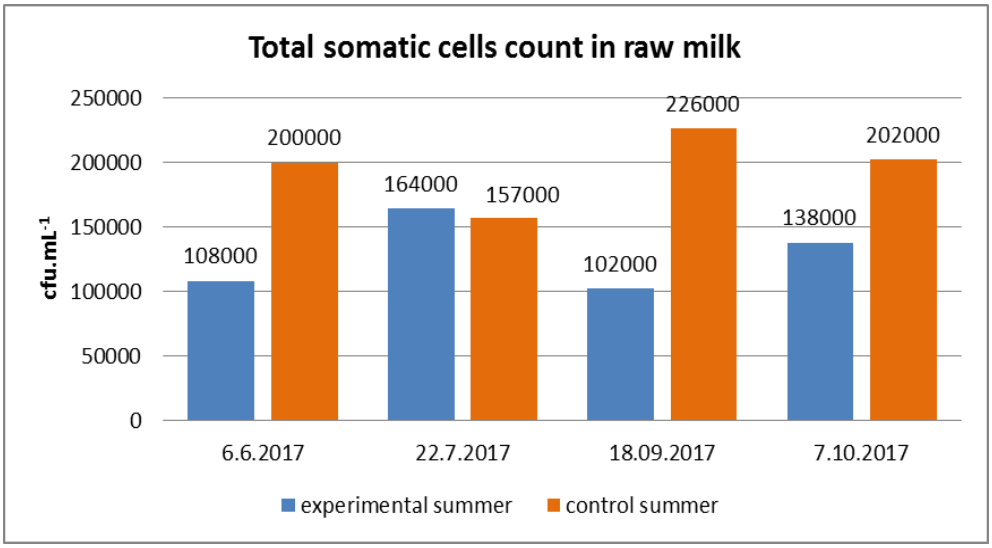


Figure 9. Total somatic cells count in milk - summer season

Salmonella and Listeria monocytogenes were not detected in the milk we examined in the experimental and control groups.

1.2.1.3. Total molds count in milk

The total count of molds in the milk of cows fed with probiotics suplemented ration was average from 36 to 77 cfu.mL^{-1}, and in the milk of the control group from 290 to 700 cfu.mL^{-1}. The data show that as a result of the influence of the probiotic, the amount of molds in the milk from experimental group was 6.2 times less compared to the control (figure 10).

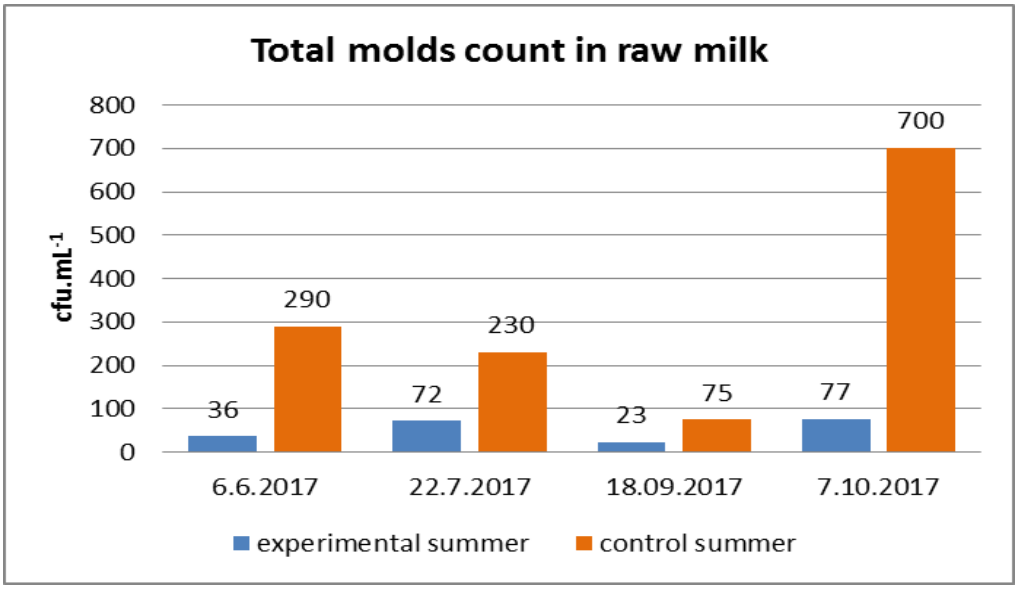


Figure 10. Total molds count in milk - summer season

1.2.1.4. Total of yeast count in milk

The positive effect of the probiotics on the yeast and enterobacteria content in the milk is shown on Figure 11. During the study period, the amount of yeast in the milk from cows receiving the probiotics was average 178 cfu.mL^{-1}, and the amount of yeast in the control group was an average of 7 655 cfu.mL^{-1}. The data showed that animal received probiotics produced milk with 43 times less yeast than the control group over the entire study period.

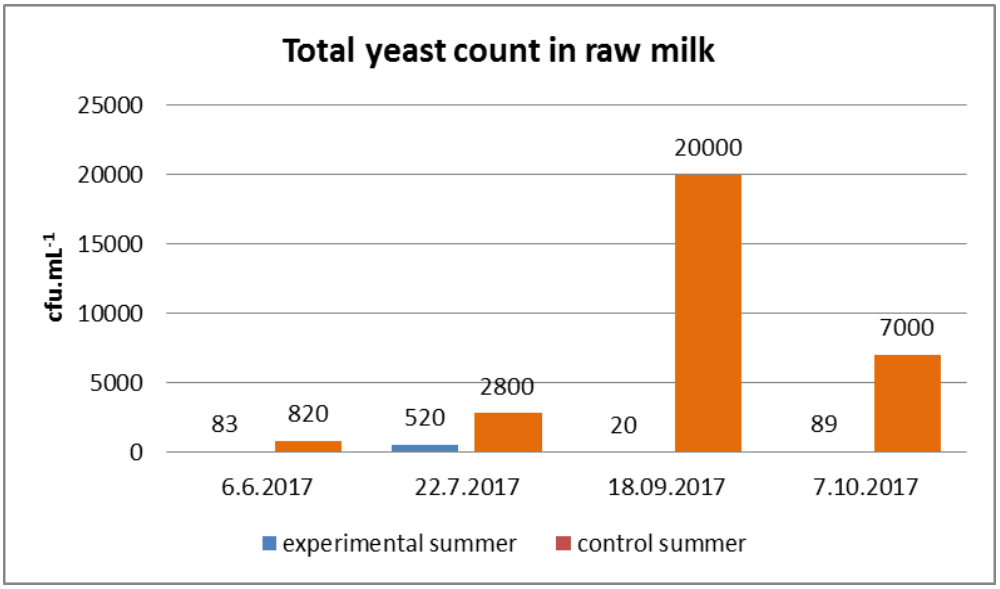


Figure 11. Total yeast count in milk - summer season

Probiotics supplements have been used to reduce intestinal carriage of antimicrobial-resistant *Enterobacteriaceae*. The milk from the experimental animals with probiotic feed rations averaged 1 940 cfu.mL^{-1} and the control group 37 500 cfu.mL^{-1}, or 19 times more *Enterobacteriaceae* in the milk from control group, the differences are significant (Figure 12). From Table 12 it is evident that these differences were more significant for the samples in September and October. During the same months, we indicated an increase in enterobacteria in the milk of cows from the control group.

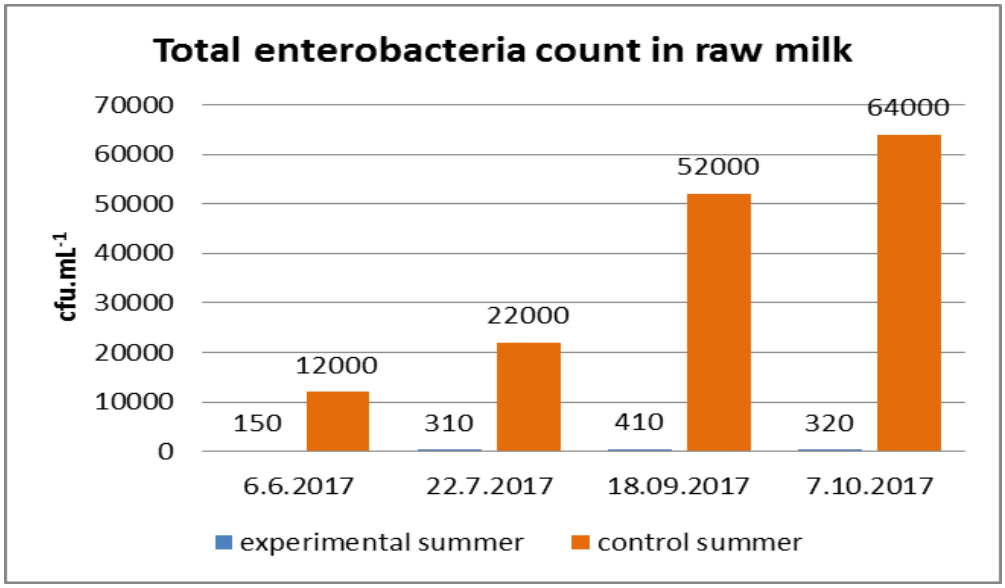


Figure 12. Total enterobacteria count in milk - summer season

1.2.2. Microbiological indicators - winter season

1.2.2.1. Total microorganisms count in milk - winter season

In the analyzes of milk during the winter period, a trend similar to the summer period was observed. The total microorganisms count in the winter season in the study period in milk from experimental group ranged from 3.7.105 to 3.3.105 cfu.mL^{-1}, and in the control, for the same period, it was within the limits of 4.9.106 to 2.2.106 cfu.mL^{-1}. It is clear from the data that the probiotic „Zoovit“ used by us reduced the total microorganisms count in the milk by 8.8 times (figure 13).

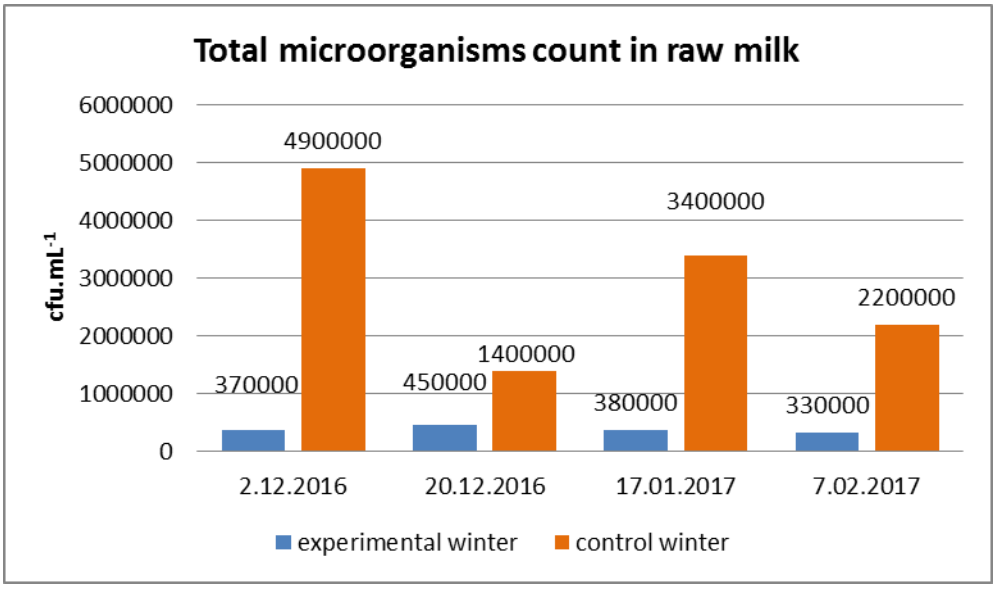


Figure 13. Total microorganisms count in milk - winter season

1.2.2.2. Total somatic cells count in milk - winter season

When analyzing the content of the total somatic cells count in the milk of cows that received probiotics with their rations, the same trend was established as in the summer period. The data showed a 7.7 times lower somatic cells count in the milk of animals receiving the probiotic compared to the control group, at a high level of probability (Figure 14).

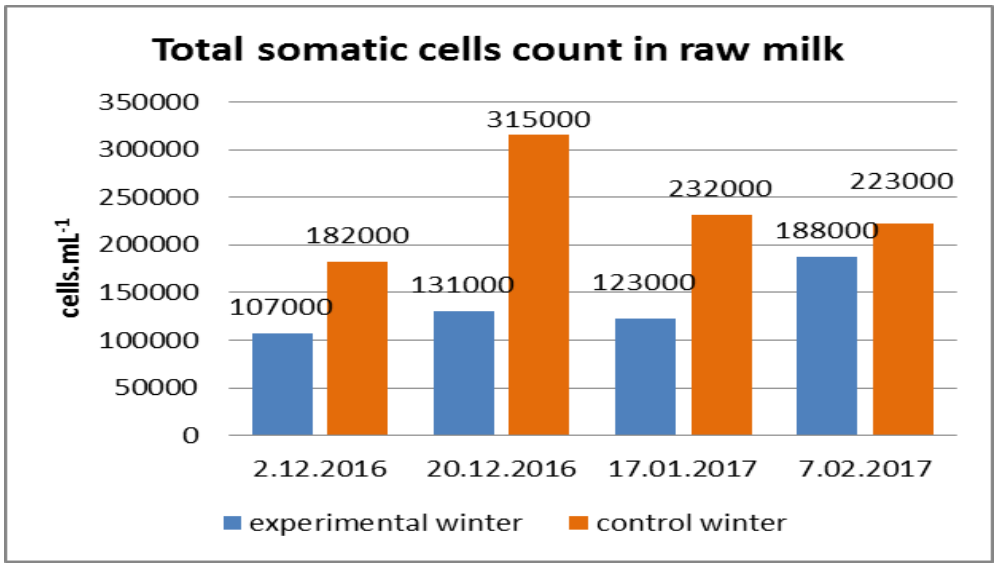


Figure 14. Total somatic cells count in milk - winter season

During the winter season, the presence of *Salmonella* and *Listeria monocytogenes* was not detected in the milk of animals from both groups.

1.2.2.3. Total molds count in milk - winter season

The total molds count in the milk from animals supplemented with probiotics had average values from 15 to 38 cfu.mL^{-1} and in the control group milk had 180 to 670 cfu.mL^{-1} (figure 15). This shows that as a result of the influence of the probiotics, the total molds count in the milk was 12 times less compared to the control. In addition to improving the health status of animals, probiotic „Zoovit“ also helps to obtain quality and safe milk.

1.2.2.4. Total yeast count in milk - winter season

Analysis of the yeast also reveals a positive influence of the probiotics. Figure 16 shows the data on the yeast count in the milk of cows from the experimental and control groups. In the studied period, the amount of yeast in the milk of cows receiving the probiotics was average 867.5 cfu.mL^{-1} and the amount of yeast in the control group was average 23660 cfu/ mL^{-1}. As with molds, the milk of the cows in the experimental group contained significantly less yeast (27 times) compared to the control group for the same period.

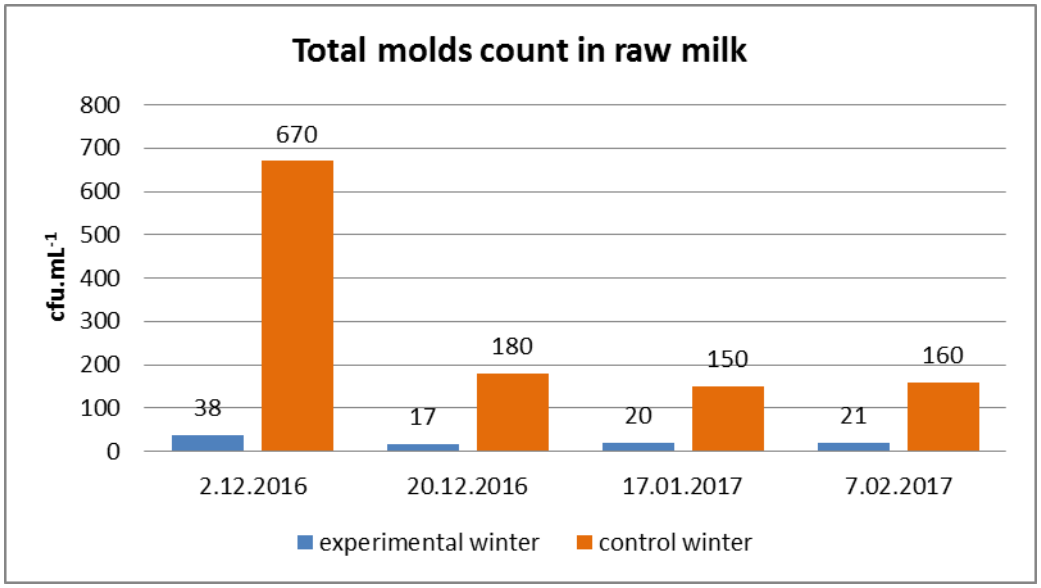


Figure 15. Total molds count in milk - winter season

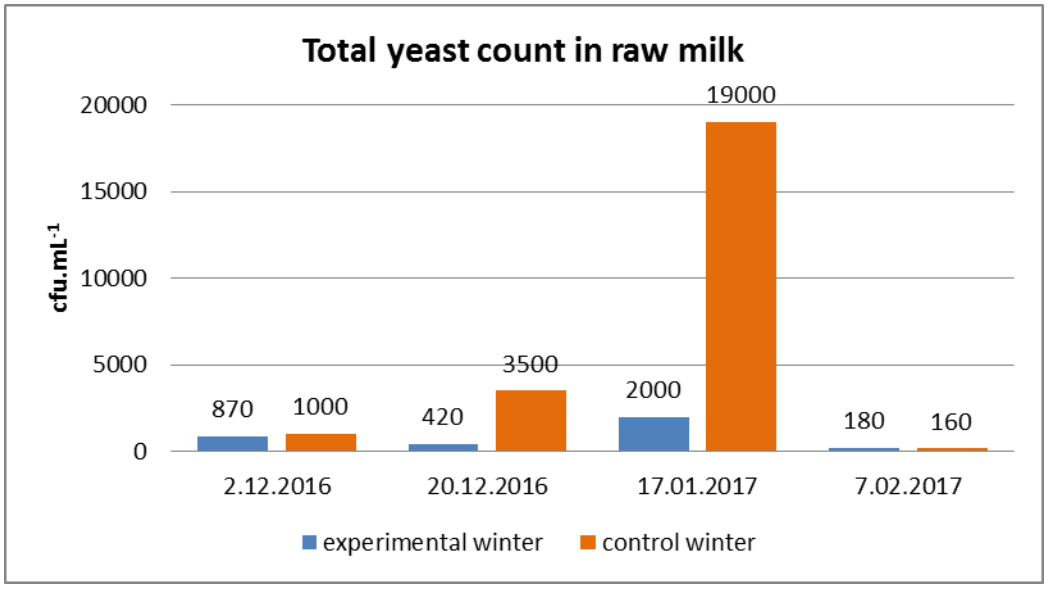


Figure 16. Total yeast count in milk - winter season

1.2.2.5. Total enterobacteria count in milk - winter season

Figure 17 shows the data regarding the amount of enterobacteria in milk during the winter season. As in the summer, a significant influence of probiotics was found. The milk from the experimental group fed with ration supplemented with probiotics had average 197.5 cfu.mL^{-1} *Enterobacteria* and the control group 2960 cfu.mL^{-1}, or 15 times more *Enterobacteria* in the milk. This is important for obtaining quality raw materials and dairy products.

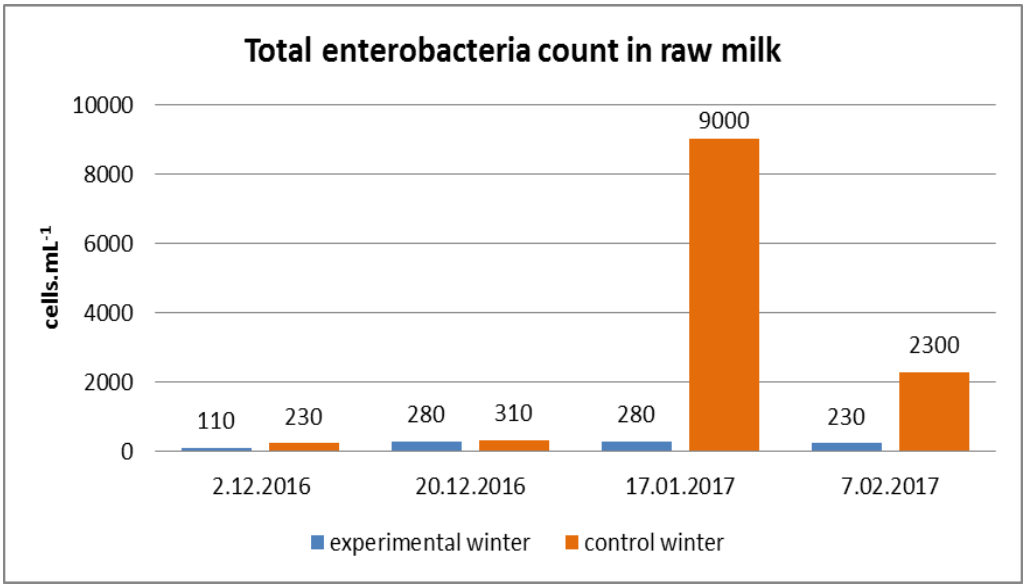


Figure 17. Total *Enterobacteria* count in milk - winter season

The influence of probiotic "Zoovit" on the total count of yeast and *Enterobacteria* was stronger in the winter period compared to the summer.

The effect of probiotics on yeast, which was about 2.5 times higher in the winter season, is explained by the richer possibilities of yeast contamination of the animals' winter ration. It is obvious that during the winter season when the animals were maintained in the cowshed and fed with fermented forages, which significantly increased the content of *Enterobacteria*. This also explains the large difference in the effect of probiotics on this group of microorganisms, which was 11.65 times higher in the winter season.

The obtained results regarding the influence of the probiotic "Zoovit" on the microbiological indicators of milk give us reason to consider that probiotics, taken with the rations of animals, had an impact on the following microbiological indicators of milk: total microorganisms, somatic cells, molds, yeasts , *Enterobacteria* count.

1.3.2. Effect of probiotics on *Escherichia coli*, Coliforms, *Staphylococcus aureus* and *Clostridium sp.* in the faecal mass of animals

The results of these studies are presented in table 3.

Table 3. Effect of probiotics on the content of microorganisms

Microorganism	Control	With probiotics
Escherichia coli	$1,5.10^5$ cfu/g	$5,5.10^3$ cfu/g
Koliforms	$3,5.10^5$ cfu/g	$2,1.10^4$ cfu/g
Staphylococcus aureus	Absence	Absence
Clostridium sp.	Absence	Absence

From the data presented, it is clear that *Staphylococcus aureus* and *Clostridium sp.* were not detected in the faecal mass, for both groups of animals.

In the case of *Escherichia coli* and Coliforms, a significant reduction of these microorganisms was found in the faecal masses of animals fed by supplemented ration with probiotics. As with *Escherichia coli*, a two-log reduction in active cells was observed in animals receiving the probiotic compared to the control group. For Coliforms, the reduction in the probiotics suplemented cows was one log unit compared to the control group. Expressed as a percentage the effectiveness of the probiotic regarding the reduction of the examined microorganisms in the faecal mass, a 3.67% reduction in the content of *Escherichia coli* in the fecal mass of the animals and a 6.0% reduction in the Coliform content was observed.

1.3.3. Detection of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* by PCR using total DNA from cow faecal samples

Microbiological characteristics

There are very few studies on the impact of probiotics on the fecal microbiome. A decrease in the amount of pathogenic bacteria in the faecal masses is an indicator of the health status of the animals and the number of pathogenic microflora in the rumen. This indicator is also important for the protection of the environment and the health of farm workers.

The content of MKB in cow faeces isolated on MRS in the control ranged from 7.17÷7.32 log cfu.g^{-1}, while in the samples with probiotics it ranged between 7.36÷7.54 log cfu.g^{-1}. The increased number of ICDs in the faeces of cows receiving probiotics compared to control cows.

Presence of DNA from *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* by PCR using total DNA from cow faecal samples.

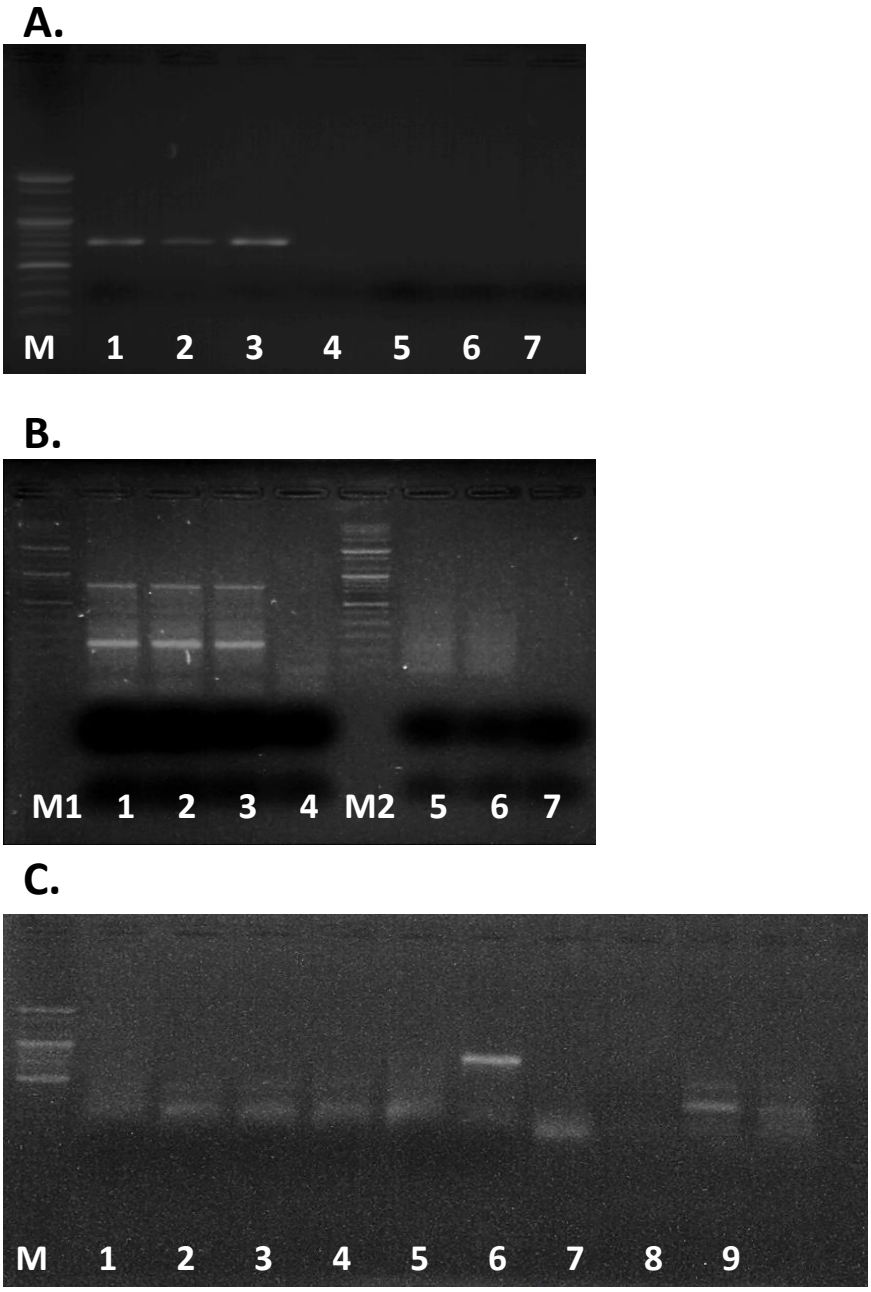
When conducting the PCR reactions, the presence of specific fragments of 715 bp and 678 bp, characteristic of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* (Figure 1A, and 1B;) from the faecal mass of cows receiving the probiotic "ZOOVIT" was established. In the DNA of the control group, fragments with a size of about 150 bp, characteristic of other types of lactic acid bacteria, were found. This shows that with the intake of probiotics in the gastrointestinal tract of the animals, the relative proportion of *L. delbrueckii subsp. Bulgaricus* go higher.

PCR analysis with a single colony of lactic acid bacteria grown on selective medium culture.

In the animals receiving the probiotics, the presence of colony number 6 with a colonial characteristic typical of *L. delbrueckii subsp. Bulgaricus* was found. Molecular genetic analysis of this colony revealed a fragment of the desired size of

678 nucleotides, when the analysis was performed with species-specific primers for *L. delbrueckii subsp. bulgaricus* (figure 18).

Figure 18. Detection of L. delbrueckii and L. delbrueckii subsp. bulgaricus by PCR using total DNA from cow faecal samples.



In the control group of animals, no colonies with the typical colonial characteristic of *Lactobacillus bulgaricus* were found. In these animals, Colonies 9 and 10 each gave small fragments on specific multiplication, most likely caused by other species of lactic acid bacteria contained in the faecal mass. This result coincides

with the data obtained for the presence of *L. delbrueckii subsp. bulgaricus* in the faecal mass of the animals that received probiotics (figure 2 B). When colonies from the control samples were used for analysis, the smaller fragment of 150 bp was not amplified, which proves the specificity of the analysis.

It was found that the total amount of lactic acid bacteria increased by 7.36-7.54 log cfu/g^{-1} in the animals receiving the probiotics "Zoovit", compared to the control group of animals, in which the total concentration of lactic acid bacteria was 7.17-7.32 log cfu. g^{-1}.

When conducting molecular genetic identification by means of the PCR method using total DNA from fecal mass of cows, the presence of *L. delbrueckii subsp. Bulgaricus* in the experimental and fragments of about 150 bp, characteristic of other types of lactic acid bacteria in the control group, were found, indicating the absence of *L. delbrueckii* and *L. delbrueckii subsp. Bulgaricus* at second group.

In cows receiving the probiotic supplement "Zoovit" the presence of colonies with a colonial characteristic typical of *L. delbrueckii subsp. Bulgaricus* were found. The molecular genetic analysis of this colony by the PCR method revealed a fragment of the desired size of 678 nucleotides when the analysis was performed with species-specific primers for *L. delbrueckii subsp. bulgaricus*. In the control group of animals, no colonies with the typical colonial characteristic of *Lactobacillus bulgaricus* were found.

When examining the fecal mass for the presence of *Staphylococcus aureus* and *Clostridium sp.* the absence of the indicated microorganisms was found, in both groups of animals.

In the cows receiving the probiotics, a decrease in the concentration of active cells of *Escherichia coli* by two logarithmic units and for coliforms by one logarithmic unit in the faecal mass was found, compared to the control group of animals. Expressed as a percentage of the effectiveness of the probiotics intake, regarding the reduction of the examined microorganisms in the faecal mass, a 3.67% reduction in the content of *Escherichia coli* in the faecal mass of the animals and a 6.0% reduction in the content of coliforms was observed.

1.4. Main technological properties of milk from cows with the addition of probiotic "Zoovit" to the feed rations

1.4.1. During the winter season

1.4.1.1. Biological activity (fermentation ability) of milk - winter season

During the first days of December, no difference was found in the activity time of the samples from the experimental and control groups /figure 19/.

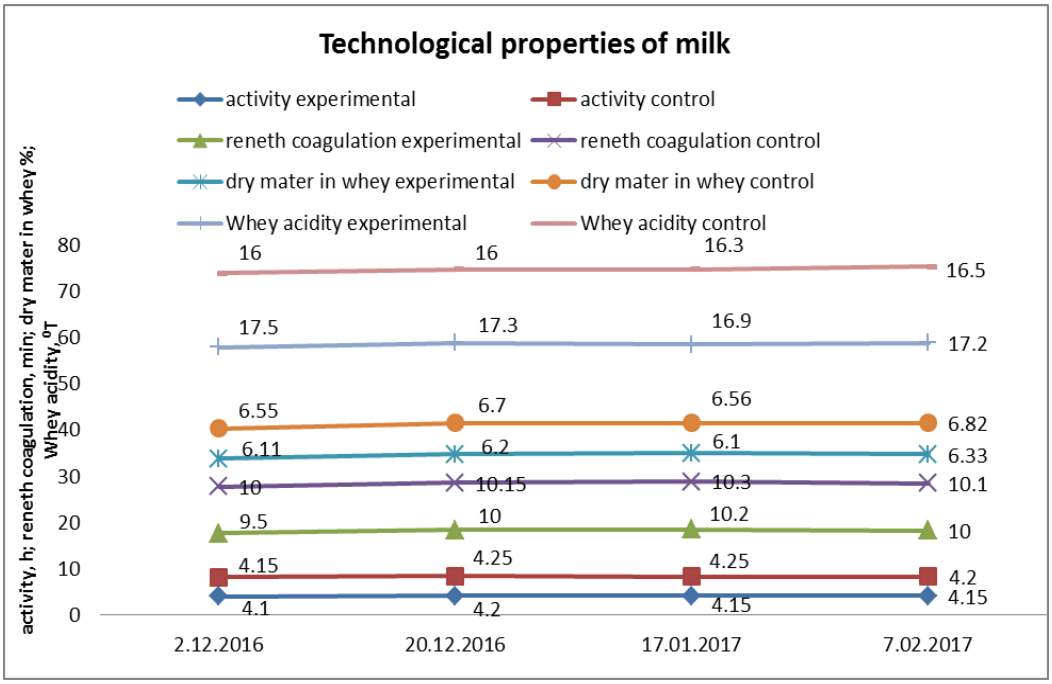


Figure 19. Technological properties of milk - winter season

Milk coagulation time was 4 hours for both samples. In the next period, the activity was increased for the milk from the experimental group, and coagulation time was reduced to 3.00 h, and for the control - 3.30 h respectively. In January, the activity was significantly accelerated and the time of the experimental batch for the fermentation process was 2.45 h, and for the control - 3.15 h.

From the obtained results, it can be definitely considered that the influence of probiotics on the studied period - December - February became more active as the time of its reception increases. This, in turn, is confirmed by the correspondence of the influence of probiotics on the total microorganism count in the milk samples.

1.4.1.2. Cuting properties of coagulum - winter season

The results presented in table 4 show that the coagulum of milk from cows that received the probiotic "Zoovit" had a more accelerated cutting properties compared to the control group, as a result of which the time of release of the required amount of water per unit of time was shorter, and this has implications for the conditions under which the cheese from the coagulum is subjected to pressing for the respective type of cheese.

The addition of probiotic to the feed rations of the cows also affects the cutting properties of the rennet coagulum during its processing. The results show that the control group had a slower cuting compared to the experiment. The acceleration of coagulum cutting properties in the milk with probiotic was 7.8% compared to the control /$p < 0.001$/.

Table 4. Basic biological and technological properties of milk obtained from cows fed with rations containing probiotics - winter season (December, January, February)*

experiment №	Date	Qty. milk,L sample / Control	Activity, (fermentation sample) h						Reneth (enzymatic) coagulation,min					
			Experimental			Control			Experimental			Control		
			X	S	C%	X	S	C%	X	S	C%	X	S	C%
1	02.12.16	3/3	4,10*	0,100	2,44	4,15*	0,100	2,41	9,5*	0,100	1,05	10,0*	0,100	1
2	20.12.16	3/3	4,20***	0,064	1,53	4,25***	0,064	1,51	10,0***	0,064	0,64	10,15***	0,064	0,63
3	17.01.17	3/3	4,15	0,162	3,90	4,25	0,162	3,80	10,2*	0,162	1,58	10,3*	0,162	1,57
4	07.02.17	3/3	4,15	0,150	3,61	4,20	0,150	3,57	10,0**	0,150	1,50	10,1**	0,150	1,48

* Reliability of differences between experimental and control groups:

* $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

Table 5. Characteristics of the coagulum

Experiment №	Characteristics of the coagulum																	
	Cutting propertie of the coagulum /100 ml of milk (whey, ml)						DM of whey,%						Whey acidity, ^{0}T					
	Experimental			Control			Experimental			Control			Experimental			Control		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
1	63,08 ***	0,064	0,10	61,18 ***	0,064	0,11	6,11 ***	0,105	1,72	6,55 ***	0,105	1,60	17,5 ***	0,105	0,60	16,0 ***	0,195	1,22
2	62,50 ***	0,116	0,18	60,25 ***	0,116	0,19	6,2 ***	0,064	1,04	6,7 ***	0,064	0,96	17,3 ***	0,064	0,37	16,0 ***	0,099	0,62
3	62,36 **	0,106	0,17	60,46 **	0,106	0,18	6,1 ***	0,116	1,89	6,56 ***	0,116	1,76	16,9 **	0,116	0,68	16,3 **	0,152	0,93
4	62,10 ***	0,100	0,16	60,20 ***	0,100	0,17	6,33 ***	0,106	1,67	6,82 ***	0,106	1,55	17,2 ***	0,106	0,62	16,5 ***	0,558	3,38

* Reliability of differences between experimental and control groups:

* $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

The obtained results for the dry matter in the whey /table 5/ show a significant difference between the experimental and control samples. The dry matter of the whey in the 8 sample variants during the period 02.12 - 07.02. in the experimental batch it ranged from 6.10 to 6.33 %, and in the control batch - from 6.55 to 6.8. Therefore, 0.48% less dry matter loss was obtained in the whey of the experimental lot compared to the control. The dry matter of the whey in the period from 06.06.17 to 07.10.17 of the experimental lot ranged from 6.00÷6.20 %, and of the control 6.18÷6.39 5. The losses of dry matter at the experimental group were 0.21% less compared to those in the control group at a reliability level of $p < 0.001$.

1.4.2. During summer season

1.4.2.1. Biological activity (fermentation ability) of milk - summer season

The obtained results are presented in table 6. Some differences were found regarding the technological indicators of the summer season compared to the winter season. The activity of the fermentation sample was accelerated in the summer season compared to the winter season by 7.2 min, whey coagulation was accelerated by 2.30 min, syneresis by 2.87 min, whey dry matter losses were reduced by 0.11%.

Table 6. Basic biological and technological properties of milk obtained from cows fed with rations containing probiotics - summer season (month June, July, September, October)*

experiment №	Date	Qty. milk,L sample / Control	Activity, (fermentation sample) h						Reneth (enzymatic) coagulation,min					
			Experimental			Control			Experimental			Control		
			X	S	C%	X	S	C%	X	S	C%	X	S	C%
1	02.12.16	3/3	3,4***	0,195	5,74	4,0***	0,195	4,88	9,5***	0,1	1,05	11,0***	0,195	1,77
2	20.12.16	3/3	3,0***	0,099	3,29	3,3***	0,099	2,99	10,0***	0,064	0,64	10,3***	0,099	0,96
3	17.01.17	3/3	2,45***	0,152	6,18	3,0***	0,152	5,05	8,45***	0,195	2,31	10,32***	0,152	1,47
4	07.02.17	3/3	2,45***	0,147	6,01	3,15***	0,147	4,68	8,5***	0,099	1,16	9,4***	0,147	1,57

* Reliability of differences between experimental and control groups:

* $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

Table 7. Characteristics of the coagulum

Experiment №	Characteristics of the coagulum																	
	Cutting propertie of the coagulum /100 ml of milk (whey, ml)						DM of whey,%						Whey acidity, ^{0}T					
	Experimental			Control			Experimental			Control			Experimental			Control		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
1	65,2 ***	0,195	0,30	60,3 ***	0,195	0,32	6,2 *	0,195	3,15	6,32 *	0,195	3,09	18,2 ***	0,195	1,07	17,0 ***	0,195	1,15
2	66,35 ***	0,099	0,15	60,5 ***	0,099	0,16	6,11 ***	0,099	1,61	6,28 ***	0,099	1,57	18,6 ***	0,099	0,53	17,1 ***	0,099	0,58
3	64,5 ***	0,152	0,23	61,2 ***	0,152	0,25	6,0 ***	0,152	2,53	6,39 ***	0,152	2,37	18,7 ***	0,152	0,81	17,4 ***	0,152	0,87
4	65,45 ***	0,147	0,23	61,5 ***	0,147	0,24	6,0 ***	0,147	2,46	6,18 ***	0,147	2,38	18,4 ***	0,147	0,80	16,8 ***	0,147	0,88

* Reliability of differences between experimental and control groups:

* $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

1.4.2.2. Casein/fat ratio and ratio of total to soluble protein in milk

The influence of the studied technological parameters of milk on its properties, presented above, depend in turn on the ratio of casein/fat in milk and on the ratio of total to soluble protein. The results are shown in Tables 8 and 9. It is evident from them that during the studied seasons there was a difference in the ratio between the protein content, which in the winter season was average 6.14 in the experimental compared to 6.11 of the control, and in the summer 5.66 compared to 6.0 of the control respectively.

The differences in the ratio was mainly due to the increased amount of soluble protein, which had average values of 0.55% in the winter season and 0.59% in the summer season, as well as the higher content of total protein in the winter season compared to the summer.

In summary, the results of a study of the influence of the probiotic "Zoovit" on the technological properties of milk are as follows: the duration of the intake of the probiotic affected the activity of the fermentation process. During the period 02.12 ÷ 07.02., the intake of probiotics accelerated the fermentation process.

With the introduction of probiotic "Zoovit" in the feed rations of cows, an acceleration of the rennet coagulation time of milk was achieved by 11.76%, compared to the milk from control group without supplementation of probiotics. The probiotic "Zoovit" affected whey protein losses. Under the same conditions of milk pasteurization and cheese processing, the effect of reducing losses was 11.8% compared to the whey from control group 6.1÷6.2%. In the summer season, the activity of the fermentation process was accelerated compared to the winter season by 1.33%; rennet coagulation tome was accelerated by 1.07%; dry matter losses in the whey were reduced by 0.11%.

Table 8. Total protein/soluble protein ratio in milk obtained from cows fed with rations supplemented probiotics *

Summer season																		
Date	Experimental group									Control group								
	Total protein,%			Soluble protein,%			Ratio. TP/SP			Total protein,%			Soluble protein,%			Total protein,%		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
15.06.2017	3,31	0,147	4,45	0,57 ***	0,011	1,84	5,8 ***	0,147	2,54	3,28	0,147	4,49	0,55 ***	0,011	1,91	5,96 ***	0,105	1,76
15.07.2017	3,3 ***	0,081	2,45	0,62 ***	0,006	0,98	5,32 **	0,081	1,52	3,12 ***	0,081	2,59	0,54 ***	0,006	1,13	5,37 **	0,061	1,13
26.09.2017	3,35 *	0,115	3,43	0,59 ***	0,012	1,95	5,67 ***	0,115	2,03	3,28 *	0,115	3,51	0,53 ***	0,012	2,17	6,18 ***	0,115	1,86
15.10.2017	3,4 ***	0,110	3,24	0,59 ***	0,011	1,87	5,76 ***	0,110	1,91	3,05 ***	0,110	3,61	0,5 ***	0,011	2,2	6,1 ***	0,110	1,81
Winter season																		
Date	Experimental group									Control group								
	Total protein,%			Soluble protein,%			Ratio. TP/SP			Total protein,%			Soluble protein,%			Total protein,%		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
02.12.2016	3,41	0,105	3,08	0,58 ***	0,011	1,81	5,87 ***	0,105	1,79	3,3	0,105	3,18	0,55 ***	0,011	1,91	6,0 ***	0,105	1,75
19.12.2016	3,25	0,061	1,87	0,55	0,006	1,11	6,09 ***	0,061	1,00	3,25	0,061	1,87	0,55	0,006	1,11	5,9 ***	0,061	1,03
23.12.2016	3,32 ***	0,115	3,46	0,55 ***	0,012	2,09	6,14	0,115	1,87	3,15 ***	0,115	3,65	0,51* **	0,012	2,26	6,17	0,115	1,86
20.01.2017	3,28	0,110	3,36	0,55 ***	0,011	2,00	6,14	0,110	1,79	3,28	0,110	3,36	0,53 ***	0,011	2,08	6,18	0,110	1,78

* Reliability of differences between experimental and control groups: * $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

Table 9. Casein/fat ratio in milk obtained from cows fed with rations supplemented probiotics*

Summer season																		
Date	Experimental group									Control group								
	Casein, %			Fat ,%			Ratio. C/F			Casein, %			Fat ,%			Ratio. C/M		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
12.06.2017	2,67*	0,105	3,93	4,0	0,105	2,63	0,66***	0,011	1,59	2,61*	0,105	4,02	3,9	0,105	2,69	0,67***	0,011	1,57
22.07.2017	2,62	0,061	2,32	3,8***	0,061	1,60	0,68***	0,006	0,89	2,6	0,061	2,34	2,4***	0,061	2,53	1,08***	0,006	0,56
18.09.2017	2,62	0,115	4,39	3,2***	0,115	3,59	0,81***	0,012	1,42	2,58	0,115	4,46	2,6***	0,115	4,42	0,99***	0,012	1,16
07.10.2017	2,66***	0,110	4,14	3,9***	0,110	2,82	0,68***	0,011	1,62	2,46***	0,110	4,48	3,2***	0,110	3,44	0,77***	0,011	1,43
Winter season																		
Date .	Experimental group									Control group								
	Casein, %			Fat ,%			Ratio. C/F			Casein, %			Fat ,%			Ratio. C/M		
	X	S	C%	X	S	C%	X	S	C%	X	S	C%	X	S	C	X	S	C%
02.12.2016	2,67	0,105	3,93	4,2***	4,2	4,2	0,64***	0,011	1,64	2,64	0,105	3,98	3,8***	0,105	2,76	0,69***	0,011	1,52
20.12.2016	2,71***	0,061	2,24	3,7***	3,7	3,7	0,73***	0,006	0,83	2,58***	0,061	2,36	3,1***	0,061	1,96	0,83***	0,006	0,73
17.01.2017	2,8***	0,115	4,11	3,4***	3,4	3,4	0,82***	0,012	1,40	2,62***	0,115	4,39	2,8***	0,115	4,11	0,93***	0,012	1,24
07.02.2017	2,81***	0,110	3,92	3,9***	3,9	3,9	0,72***	0,011	1,53	2,62***	0,110	4,20	3,5***	0,110	3,15	0,74***	0,011	1,49

* Reliability of differences between experimental and control groups: * $p < 0.05$ ** $p < 0.01$ **** $p < 0.001$

2. Influence of the probiotic "Zoovit" on the quality of yogurt produced by the classical technology

2.1. Technological characteristics of yogurt

Technological characteristics of the yogurt, depending on the intake of the "Zoovit" probiotic, were made in the summer season, after 30 days of intake. During this period, the effects of probiotics on the physicochemical, microbiological and technological parameters of raw milk were evaluated (table 10). The data shows a positive influence of probiotics on total protein, fat content, DNFR and dry matter. No differences were found between the two groups in terms of density, acidity and pH.

Table 10. Technological characteristics of yogurt

Groups	Total protein, %	Fat, %	DNFR	DM, %	Acidity, ^{0}T	pH	Density, g.cm^{-3}
Experimental	**3.27**	**4.0**	**9.46**	**13.43**	**17**	**6.65**	**1.031**
Control	3.15	3.7	8.78	12.48	17	6.65	1.030

2.2. Microbiological characteristics of the product

From the data in Figure 20, it can be seen that the probiotics had a significant effect on the growth of the starter cultures and their ratio.

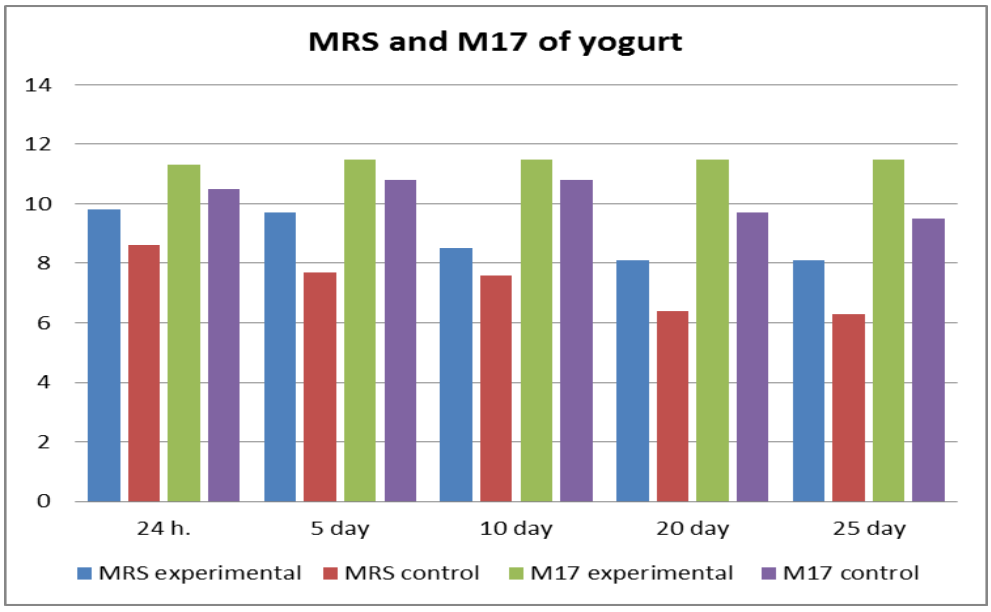


Figure 20. Microbiological studies of yogurt produced from raw milk obtained from animals with a probiotic included in the food rations

At the 24th hour, the amount of active cells of *Lb. bulgaricus* was 6.3.109 cfu. g^{-1} compared to 2.0.1011 cfu.g^{-1} of *Str. Thermophilus*.

2.3. Acidification during the process of fermentation and storage of yogurt

Table 11 shows the data on the acidity of yogurt obtained from milk from cows that received probiotics in the ration and from animals without supplementation.

Table 11. Acidity of yogurt obtained from milk with probiotic in the dietary rations of experimental animals and control group

Groups	Acidity, ^{0}T and pH									
	24 h		5 day		10 day		20 day		25 day	
	^{0}T	pH	^{0}T	pH	^{0}T	pH	^{0}T	pH	^{0}T	pH
Eperimental	85	4,8	101	4,7	110	4,6	115	4,55	118	4,45
Control	76	4,7	110	4,6	118	4,45	121	4,4	130	3,3

In the process of storing the milk, for the period of 24 hours to 25 days, in the experimental sample on the 10th day, the active cells of *Lb. bulgaricus* decreased by one degree and kept their activity at the same level - 108 cfu.g^{-1} until the 25th day. The active cells of *Str. Thermophilus* retained their content 3,2.10 11 cfu.g^{-1} until the 25th day. In milk without probiotics, active cells of *Lb. bulgaricus* and *Str. Thermophilus* were significantly lower and in the later periods of storage, a significant decrease in their amount was observed on the 20th and 25th day.

The acidity of the yogurt obtained from the milk of the experimental animals moved accordingly at the 24th hour – 85 0T, pH 4.8 and during the period from 5 to 25 days it increases from 101 0T pH 4.7 to 118 0T pH 4.45. For the control, the acidity at 24 hours was 76 0T pH 4.9 and during the period 5 to 25 days reached acidity of 110 0T pH 4.6 to 1300T pH 4.3, respectively (figures 21 and 22).

The influence of probiotics on the microflora is evident in the comparative data of the milk from experimental and control group. In the control, the amount of active microflora of *Lb. bulgaricus* in the storage process decreased by 1 to 2 degrees, and the amount of microflora of *Str. Thermophilus* decreased by one degree in the last storage period – 20÷25 days.

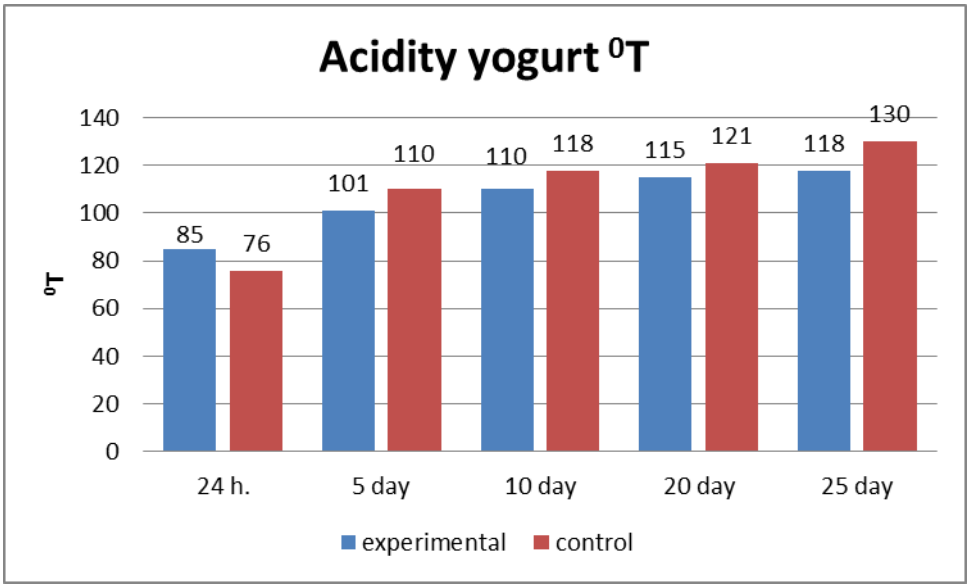


Figure 21. Titratable acidity of yogurt

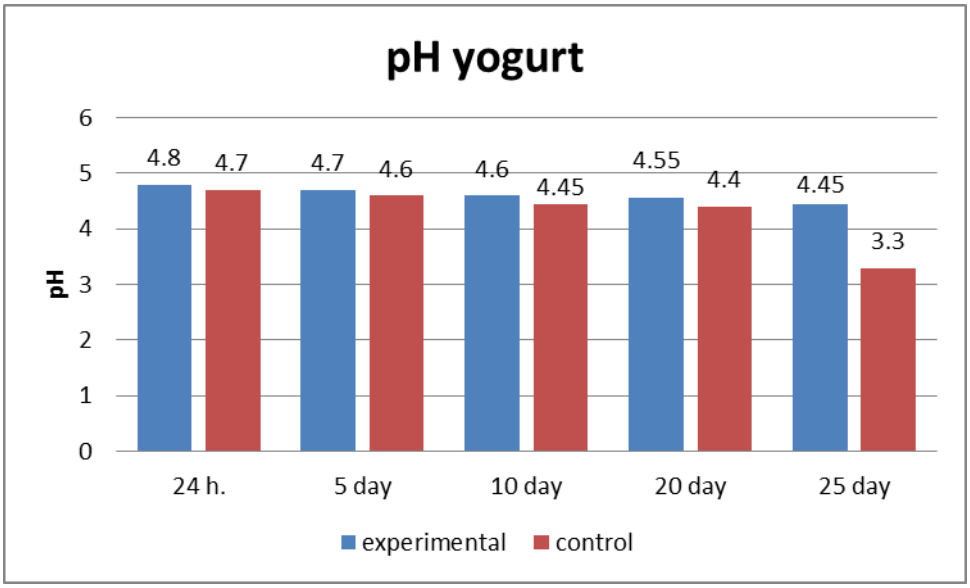


Figure 22. Active acidity of yogurt

2.4. Organoleptic analysis

The results of the organoleptic evaluation are presented in figure 23.

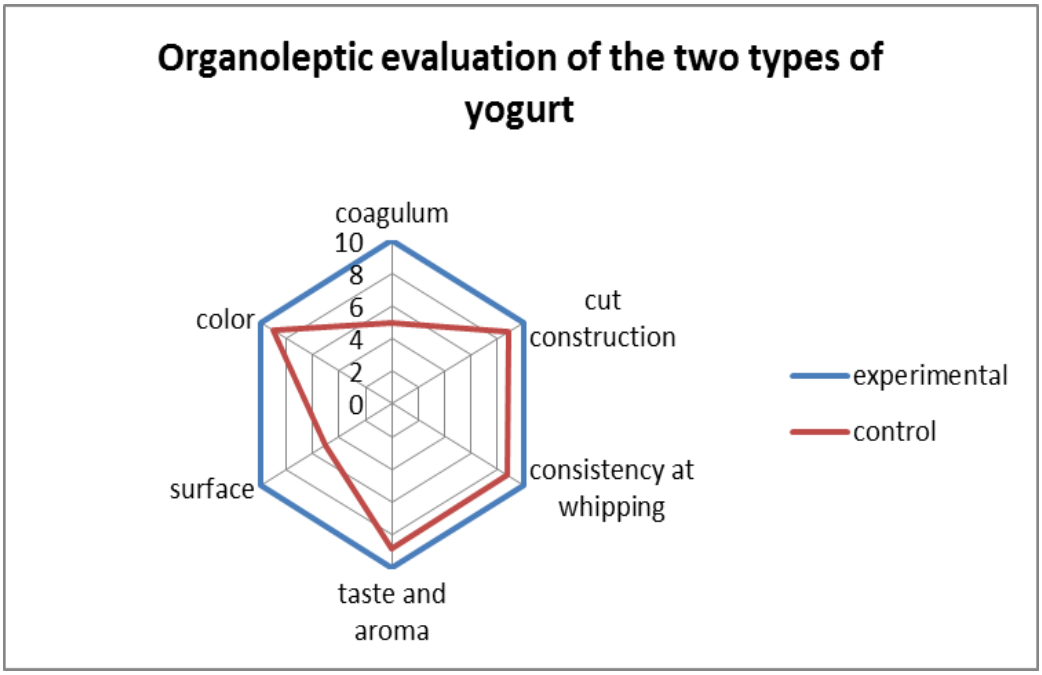


Figure 23. Organoleptic evaluation of the two types of yogurt

The data show that the yogurt obtained from raw milk produced from cows receiving the probiotic had a better organoleptic evaluation in all parameters compared to the yogurt from control group.

A loose coagulum with poor mechanical resistance, an uneven surface with cuted coagulum was found in the yogurt from control group. The taste was markedly sour and a faint aroma of diacetyl.

Yogurt obtained from animals receiving the probiotic supplement was characterized by a smooth surface without cuted coagulum, a dense and mechanically resistant coagulum. The milk had a pleasantly pronounced lactic acid taste and a very well-developed aroma of diacetyl, typical of traditional Bulgarian yogurt.

The yogurt from control group was of impaired quality in terms of structure stability, taste and aroma and damage with the growth of atypical microflora (molds and yeasts).

Probiotic "Zoovit" affected the development of bacteria in the starter cultures: it preserved the activity of the yogurt starter cultures during its storage from 24 hours to 25 days at a temperature of 4÷6 ^{0}C and stabilized its organoleptic properties for up to 35 days. Yogurt produced with milk obtained from cows receiving probiotics retained its quality indicators for up to 3 months, stored at a temperature of 4÷6 0C.

Table 12. Organoleptic analysis of yogurt stored for 3 months at temperature 5 ± 1 0C.

Parameters	*Sample № 1* – control (without probiotic supplementation)	*Sample № 2* – experimental (with probiotic supplementation)
Structure	dense, unstable	dense, denser than the control
Coagulum cut	more abundant in the control, presence of molds on the surface	without mold on the surface
Consistency	relatively more unstable consistency under mechanical impact	more stable compared to the control
Taste and aroma	yeasty flavor	clean, strongly lactic acid taste

3. Summarized discussion of the results of the dissertation

The research activity of the dissertation was carried out according to a methodical plan, covering a complex of tasks with scientific and applied importance for the introduction of probiotics in animal feeding.

A key moment in the research activity was the question of the quality of milk, its physicochemical and microbiological characteristics and technological properties, obtained from dairy cows that took probiotic "Zoovit" (fed) for 11 months, covering the winter and summer seasons.

The results of the analysis of the physicochemical parameters of the milk showed that the amount of the main components of the milk - protein and fat was affected by the probiotics, being significantly higher in the milk from cows that took the probiotic compared to the animals, under the same conditions, but without probiotics in the diet. In turn, it was confirmed by the higher percentage of dry mater of the milk, which in the winter season ranged from 13.31 to 14.84%, and in the summer season from 13.11 to 14.24%.

This effect of probiotics was observed in both seasons – summer and winter. More significant (higher) was the content of protein and fat in milk obtained in the winter season compared to the summer season.

This result shows that the climatic conditions have an influence on the synthesis of amino acids and fatty acids. Furthermore, we have reason to assume that the enzymatic processing of feed in the rumen of animals is favored by the enzymatic activity of the bacteria contained in the probiotics. In the favorable conditions of their

development at the animal's body temperature of 37.5÷39.5 0C, they create conditions for a more active synthesis of free amino acids in the formation of the protein chain and triglycerides of fatty acids.

The influence of probiotics on the microflora of milk also characterizes its effect on the quality of raw milk and the production obtained from it.

A significantly higher bactericidal effect of probiotic on the total microorganisms count contaminating raw milk during its production, milking and storage under farm conditions was found.

The bactericidal charge that raw milk obtained from animals that received a probiotics in the feed was due to the probiotic properties of the bacteria that are taken with the feed, at the favorable temperature for the development of the probiotic bacteria. They produced metabolic substances - lactic acid, bacteriocins, enzymes, immune substances, carry out enzymatic degradation of the nitrogenous components of proteins, etc.

Collectively, the metabolic products of the probiotic bacteria passed through the digestive system and colonized in the large intestine. Proof of this is the results of the PSR method for the detected DNA of *Lb. bulgaricus* and live active microflora in the animal's faecal mass.

Based on the research data on the main technological traits of milk - curd firmness ability and processing of curd, an important influence of probiotics on accelerating the coagulation process of milk and improving the density and elasticity of the coagulum has been established. This, in turn, reveals opportunities for improving the stability of the coagulum during its mechanized processing, its curd cuting properties - a prerequisite for reducing the loss of dry mater from the milk in the whey and achieving an economic effect in the production of dairy products. The overall results were due to an increase in the biological content of the milk and the acceleration of the fermentation process.

An essential role in the development of the fermentation process of dairy products is played by the biological content of enzymes in milk, which play an important role in the maturation of the products obtained from it and the formation of their organoleptic properties. In addition, the contributions of probiotics to the rheological properties - density and elasticity of the coagulum, are of essential importance for the introduction of appropriate technological approaches in the mechanization of cheese production processes. It is obvious that the milk of cows with better biological properties opens up opportunities to regulate the time of the fermentation process to obtain optimal quality of the coagulum.

Other conditions being equal (temperature, amount of enzyme, calcium ions, etc.), the time for enzymatic coagulation of milk was shorter in the summer season

compared to the winter season. This gives reason to assume that the main factor for the enzymatic processing of feed in the gastrointestinal tract of animals receiving probiotics is the climatic conditions - a question requiring targeted research, regarding the acceleration of the aggregation process of the paracasein micelles and, accordingly, their influence on the density and coagulum elasticity.

The significance of the obtained results of the study of the technological properties of milk is based on the possibility of finding rational guidelines and in the regulation of important technological factors - amount of rennet enzyme, coagulation time, optimal parameters of coagulum processing, regulation of the speed of cutting coagulum by physico-chemical parameters of the milk - dry matter, density, elasticity of the coagulum, level of cutting and others. By regulating the rate of coagulum cuting, the fermentation process can be successfully controlled - a matter of great importance for product quality.

Equally important is the curd cuting rate control approach to regulating dry matter losses, including protein and fat, in whey. Calculations of dry matter losses convincingly show the economic contribution of probiotics to increase the yield of cheese products.

The scientific information regarding the influence of the probiotic "Zoovit" on the physico-chemical, microbiological and technological properties of raw milk provokes interest in establishing its influence in the production of yogurt.

Yogurt is a basic sour-milk product in which the fermentation processes take place relatively more actively compared to other products. This characterizes it as a product with the highest demands regarding the quality of the source or with other words raw milk and its biological properties.

The milk from cows that receive the probiotic "Zoovit" has been proven to have better microbiological purity and biological properties, higher physico-chemical parameters - amount of protein and fat, dry matter and casein/fat ratio. All this favors the development of the starter culture and preserves its activity for a longer period of time. In addition, the positive influence of probiotics on the technological properties of milk determines the obtaining of a better density of the yogurt coagulum and lowers its cuting properties. The established technological effect has a serious impact on the organoleptic properties of the product and preservation of its fresh taste during storage.

The results of the quality indicators of the yogurt give reason to recommend the use of probiotic "Zoovit" in animal feedin and production of milk to obtain types of cheese and other dairy products, where economic results are also expected from the increase in the yield of the products.

V. CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS:

1. The influence of the probiotic "Zoovit", receaved with the feed of cows, on basic physicochemical parameters of milk was established.

1.1. The amount of proteins in milk increased by 0.18% in the winter season and 0.05% in the summer season, and the difference in dry matter for the period 02.12. ÷ 07.02. reaches 1.96%, and for the period 06.06. ÷ 07.10 is 1.26 % respectively.

1.2. The probiotic "Zoovit" has no significant effect on the amount of fat and acidity of milk.

2. The probiotic "Zoovit" taken with the feed rations of the cows has a specific bactericidal effect on the microbial content of the milk.

2.1. The probiotic "Zoovit" taken during the summer and winter season has a specific bactericidal effect: the total microorganisms count decreases during the summer season by 11.18%, winter season - 12.86%; the somatic cells count – 62.22% and 57.67%; molds – 16.06% and 8.25%; yeast – 2.93% and 6.37%; enterobacteria – 0.79% and 2.21% - respectively for summer and winte seasons.

3. It has been established that when the probiotic "Zoovit" is introduced into the feed rations of cows, the technological properties of the rennet coagulum change.

3.1. It was established that the introduction of the probiotic "Zoovit" into the rations of cows accelerates the coagulation process of milk, under the action of the rennet enzyme, by 11.76%.

3.2. The probiotic "Zoovit" affects whey protein losses. Under the same conditions of milk pasteurization and cheese processing, the effect of reducing whey protein losses was 11.8%.

3.3. In the summer season, the activity of the fermentation process is accelerated compared to the winter season by 1.33%; rennet coagulation is accelerated by 1.07%; whey dry matter losses were reduced by 0.11%.

4. The influence of the probiotic "Zoovit" on the development of the lactic acid microflora in the production of yogurt was established.

4.1. It was found that in yogurt the total count of lactic acid bacteria was 7.36 ÷ 7.54 log cfu.g^{-1}, obtained from cows receiving the probiotic "Zoovit", compared to the control group of cows - 7.17 ÷ 7.32 log cfu.g^{-1}, without probiotic.

5. When carrying out molecular genetic identification by means of the PCR method, by using total DNA from fecal mass of cows, the presence of L. delbrueckii subsp. Bulgaricus in animals receiving a probiotic was established.

5.1. In animals receiving the probiotic "Zoovit", the presence of colonies with a characteristic typical for L. delbrueckii subsp. bulgaricus, which was confirmed by the

PCR method was established. In the control group of animals, no colonies typical to Lactobacillus bulgaricus were found.

5.2. When examining faecal mass from cows for the presence of Staphylococcus aureus and Clostridium sp. was found absence of the bacterium, for both groups of animals.

5.3. In the cows receiving the probiotics, there was a decrease in the concentration of active Escherichia coli cells by two logarithmic units, and for coliforms - by one logarithmic unit in the faecal mass, compared to the control group of cows.

6. The probiotic "Zoovit" render an inhibitory effect on the metabolism of bacteria in yogurt.

6.1. The probiotics added to the cows' rations preserves the activity of the yogurt starter cultures during its storage from 24 hours to 35 days at a temperature of 4 ÷ 6 ^{0}C.

6.2. Yogurt produced from milk obtained from cows that received probiotics retains its organoleptic properties for up to 3 months, stored at a temperature of 4 ÷ 6 ^{0}C.

RECOMMENDATIONS:

1. The probiotic "Zoovit" taken during the summer and winter season has a specific bactericidal effect (reduces the total microorganisms count, somatic cells count, molds, yeasts and enterobacteria), therefore we recommend that, to be added to the ration of lactating cows.

2. **In order to improve** the technological properties of the coagulum, we recommend the addition of the probiotic “Zoovit”.

3. Based on the obtained results**, we recommend the use of probiotic “Zoovit” in the rations of cows to improve the quality of Bulgarian yogurt.**

VI. DISSERTATION CONTRIBUTIONS

1. Scientific contributions

1.1. A scientifically based method was developed to study the influence of the probiotic "Zoovit" taken by cows on the quality of milk and its technological properties. (for example, the probiotic "Zoovit").

1.2. The parameters of the influence of probiotics on basic physicochemical, microbiological and technological properties of milk from cows receiving probiotics were established.

2. Applied Contributions

2.1. The technological and economic importance of probiotics and its influence on the development of microflora, quality and technological properties of milk are characterized.

2.2. The importance of the introduction of probiotics in animal rations has been proven, by revealing possibilities for biological enrichment of milk and milk products and achieving an economic effect by reducing the loss of dry matter in the whey.

VII. PUBLICATIONS ON DISSERTATION

1. **Chavdarov, G. 2020.** Influence of probiotic "Zoovit" on basic physicochemical parameters of milk from cows of the Holstein-Friesian breed. Animal Sciences, LVII, 5, 25-33.

https://animalscience-bg.org/page/en/details.php?article_id=595