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Mastitis prevention and control: Integration of microbiological and management approaches

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Abstract. Mastitis is a common cause of reduced dairy productivity in cows and poor milk quality. This paper considers a set of approaches based on microbiological studies and risk analysis aimed at reducing the number of cows with mastitis and improving milk quality. The study is aimed at examining microbial agents isolated from the milk of sick cows and means of ensuring udder health, and identifying the main dangers that arise due to errors in the implementation of mastitis prevention measures. The study included microbiological tests of milk, wipes and udder dipping using chromogenic media. For a qualitative risk assessment, a survey of consulting managers and veterinarians was conducted. The paper presents the results of microbiological tests of 115 samples of milk, 45 reusable napkins, and 111 samples of udder dips using conventional and chromogenic media. The most common microorganisms in milk samples were *Streptococcus spp.* 34.4% (*S. agalactiae* and *S. disgalactiae*), *Staphylococcus aureus* 31.1%, and *Escherichia coli* 28.9%. *Staphylococcus aureus* and *Streptococcus spp.* it is associated with infectious mastitis,

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and *Escherichia coli* – with sanitary mastitis. To reduce the incidence of mastitis, it is important to apply targeted measures aimed at various categories of pathogens. Udder wipes were contaminated with pathogens associated with the development of mastitis. The use of laundry detergent containing bactericidal components is crucial to minimise contamination of reusable udder wipes. Among the dips under study, 40.6% of the samples had a complete bactericidal effect, and 9.9% had no bactericidal effect. 3 factors of high risk of environmental mastitis and 6 factors of sanitary mastitis were identified. The results of the studies were tested on two dairy farms. Correction of high-risk risk factors led to an increase in the quality of milk in terms of somatic cells and the number of bacteria. The results obtained can be useful for improving the system of prevention and optimisation of mastitis treatment on dairy farms

Keywords: sanitary mastitis; infectious mastitis; mastitis risk; risk analysis; post-milking dipping; mastitis pathogens; milk quality improvement; *Streptococcus spp*.; *Staphylococcus spp*.

INTRODUCTION

Mastitis is the most common mammary gland pathology in cows in all countries, leading to a decrease in animal productivity and the overall economic efficiency of the industry. The development of this disease depends on various factors related to the infectious agent, animal health, the environment, and technological processes. Therefore, accurate and rapid identification of the main pathogens of the disease is important for diagnosis and selection of the optimal prevention and treatment regimen. Identifying the main hazards through risk analysis will help determine the shortcomings of preventive and technological measures on the farm.

Depending on the degree of manifestation of the infectious process, mastitis can be divided into clinical and subclinical. Clinical mastitis is accompanied by the development of pronounced signs of inflammation. Petersson-Wolfe *et al*. (2018) indicate that the pain that occurs, in this case, forms long-term consequences for the animal's health. Subclinical mastitis develops without any visible signs. Martins *et al*. (2020) note that chronic subclinical mastitis leads to a decrease in milk yield and a deterioration in milk quality.

The review by Cobirka *et al*. (2020) summarises the classification of mastitis depending on the causative agents and the degree of clinical manifestation. The researchers indicate that different infectious agents differ in their source of origin, transmission factors, and level of contagiousness. Depending on the pathogen, mastitis is divided into infectious, which has a high potential for transmission from animal to animal, and sanitary, which affects individual animals and has a low potential for transmission.

Milk testing results by Steinberg *et al*. (2022), using 16s RNA gene sequencing, show that the milk microbiota of healthy cows is diverse and variable, and undergoes changes during the development of mastitis. Identification of isolates from the milk of cows with mastitis by Nusrat *et al*. (2021) confirms that the causative agents of mastitis can be numerous genera of gram-positive and gram-negative microorganisms. According to Acharya *et al*. (2021), the genera of microorganisms identified as the main causative agents of mastitis changed during the study period.

Bacterial agents, due to their pathogenicity factors, have a destructive effect on the mammary gland. Thus, Egyedy & Ametaj (2022) note that the innate and acquired immunity of cows play an important role in protecting the mammary gland from mastitis pathogens. In this case, pathogens *Staphylococcus aureus* and *Streptococus uberis* may evade the immune response. A sanitary-associated pathogen *Escherichia coli* is able to multiply in milk without attachment to the epithelium. Authors Blum *et al*. (2020) studied in more detail the pathogenic effects of the sanitary pathogen *Escherichia coli* on the mammary gland. This microorganism, after entering the mammary gland, changes the metabolism in the epithelium and causes immune response processes, which leads to changes in the mammary gland and secretions. These changes may remain after bacterial cleansing of the udder.

Tomazi *et al*. (2018), after studying cases of mastitis in 20 Brazilian dairy farms, found that the risks of the disease are associated with the season of the year and the level of somatic cells in tanker milk. In their review, Girma and Tamir (2022) identified the main risk factors as breed, age, lactation stage, and nipple damage. They also noted the importance of monitoring sick livestock and implementing measures aimed at udder health. Zigo *et al*. (2021), in their systematic review, summarised all factors that may affect the level of occurrence of mastitis and the effectiveness of their treatment. The researchers note that the development of mastitis is a complex process that depends on the pathogen, the condition of the animal, and environmental factors. To effectively combat mastitis, it is necessary to clearly implement preventive measures. In the cross-sectional study with Bayesian network analysis, Horpiencharoen *et al*. (2019) identified clinically important risk factors influencing the occurrence of mastitis. They are associated with pathogens released from milk and animal care measures. However, the level of risk may vary depending on the specific situation. The importance of implementing mastitis management measures was also noted by Stevens *et al*. (2019). In addition, the process of evaluating management approaches increases the level of implementation of recommended measures, and leads to an improvement in the quality of

milk. Diagnostic methods include modern approaches to diagnose mastitis itself, advanced microbiological methods using chromogenic media, molecular genetic methods, and the use of nanotechnology.

Thus, Neculai-Valeanu & Ariton (2022) considered diagnostic tests that can be used for early detection of signs of the disease. The researchers note that the latest methods of non-invasive diagnosis of changes in the clinical condition of the breast can be applied to improve the health of the udder. El-Said & Kamel (2021) point to the possibility of using modern approaches to the diagnosis of mastitis using PCR, microbial gene sequencing, and nanotechnology. Microbiological methods have long been considered the gold standard for identifying mastitis pathogens. They do not lose their relevance even today. Granja *et al.* (2021) established the high sensitivity and specificity of a new, improved medium containing chromogenic components of CHROMagar™ Mastitis to detect the most common pathogens of udder infection.

The purpose of the study was to identify the factors that lead to the spread of mastitis pathogens on the farm, select optimal objects of microbiological analysis and methods for isolating and identifying microorganisms with subsequent risk analysis, and use the results obtained to improve animal welfare.

MATERIALS AND METHODS

The research was conducted in the research laboratory of the Department of veterinary and sanitary expertise and laboratory diagnostics of the Institute of Postgraduate Training of Heads and Specialists of Veterinary Medicine of Bila Tserkva NAU. Samples of milk from sick animals, napkins and udder dips were taken in the period May 2021-May 2022. The survey of consulting managers and veterinarians was conducted in June-July 2022. Visits to farms for experimental testing of the results obtained took place in the fall of 2022.

Milk sampling. Milk samples were taken during the cows' stay in the milking parlour. First, the udder skin was cleaned of dirt and debris with a disposable cloth soaked in alcohol to disinfect the skin and reduce the risk of contamination. A separate napkin and vessel were used for each nipple. The first jets of milk were milked and disposed of. Milk samples were taken separately from each udder lobe in a single-use individual sterile cup and immediately sent to the laboratory. 115 milk samples taken from 27 subclinically and 63 clinically sick animals were examined.

Microbiological studies of milk using non-selective and selective media. Milk samples were cultured on the blood agar (Conda) with the addition of 5% sheep red blood cells (Pharmstandard Biolek, Ukraine) and MacCONKEY Agar (Merc) at 37°C for 24-48 hours. Colonial characteristics and hemolysis were determined, gram-stained, and morphological characteristics of microorganisms were determined. Gram-positive cocci were tested with catalase and differentiated into positive and negative coagulase activity. Cocci catalase-positive colonies were then cultured in the Staph Api 20 kit. Coccoid catalase-negative colonies were studied using Hydrolysis of esculin, fermentation of hippurate and inulin, and growth in NaCl were determined using the CAMP test. For gram-negative bacteria, oxidase, indole formation, Voges Proskauer reaction, Lysine Decarboxylase test fermentation of glucose, lactose, mannitol, maltose, sucrose, urease, and citrate were used (Tarazona-Manrique *et al.,* 2019).

Microbiological studies using chromogenic media. 2 chromogenic media were used CHROMagar™ Orientation and CHROMagar™ Mastitis (CHROMagar, France). The growth pattern was determined visually.

CHROMagar™ Mastitis consists of 2 separate media for gram-positive bacteria (Mastitis GP) and gram-negative (Mastitis GN) bacteria. The growth of gram(-) bacteria is inhibited on Mastitis GP medium, and the growth of gram(+) bacteria is inhibited on Mastitis GN medium. CHROMagar™ Orientation is one-component, so it requires additional gram staining of smears taken from colonies. Differentiation of colonies occurs by analysing the colour in which they are coloured as a result of growth (Table 1).

For inoculation, five-fold serial dilutions were made, and native material and 0.1 ml of suspension were plated with dilution from 10^{-2} up to 10^{-5}. The cultures were cultivated for 24-48 hours at 37°C, after which the colonial morphology were analysed and their number was counted.

Table 1. *Colouration of microbial colonies grown on chromogenic media*

Microorganism	CHROMagar™ Orientation	CHROMagar™ Mastitis
Gram(-) bacterial		
Esherihia coli	dark pink to reddish	dark pink to reddish
Klebsiella spp., Enterobacter spp., Citrobacter spp., Serratia spp. (KECS group)	metallic blue (+/- reddish halo)	metallic blue (+/- reddish halo)
Proteus spp., Morganella spp.,Providencia spp.	brown halo	brown halo
Proteus vulgaris	blue with brown halo	
Pseudomonas spp.	Translucent (+/- natural pigmentation cream to green)	translucent (+/- natural pigmentation cream to green)
Acinetobacter spp.	cream	Variable

Table 1, Continued

Microorganism	CHROMagar™ Orientation	CHROMagar™ Mastitis
Stenotrophomonas spp.	colourless	Variable
Gram(+) bacterial		
*Streptococcus agalacti*ae	light blue	turquoise blue
Streptococcus uberis		metallic blue
Enterococcus spp.	turquoise blue	Variable
Staphylococcus aureus	golden, opaque, small	pink
Staphylococcus epidermidis	cream, pinpoint colonies	variable
Staphylococcus saprophyticus	pink, opaque, small	variable
Yeasts		
Candida albicans	cream, pinpoint colonies	cream, pinpoint colonies

Source: *compiled by the authors based on CHROMagar™ Mastitis Instructions For Use (n.d.) and CHROMagar™ Orientation Instructions for Use (n.d.)*

Sampling to determine the contaminating agent of napkins. A cut-off part of the napkin with sides of 10×10 cm was sent for the study. Sterile disposable gloves were worn and a sterile instrument was used before taking the sample. The cut material was placed in a sterile container. After washing according to the procedure performed on the farm, another area was re-cut off in the same napkin and placed in another sterile dish. After entering the laboratory, a piece of napkin was filled with 100 ml of sterile saline solution for 5 minutes. After exposure, the napkin was pressed against the wall of the test tube using sterile tweezers. To determine the contaminating agent, a bacterial suspension was inoculated in the media indicated above.

45 napkins were analysed. Of these, 29 were washed in a washing machine at a temperature of 60°C using domestic detergent, and another 16 – in a washing machine at a temperature of 60°C using a special detergent containing a bactericidal component.

Determination of the bactericidal activity of udder dipping. Museum strains were used to determine the antibacterial properties of dipping as control bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, as well as microorganisms isolated from the studied samples from farms. 111 dip samples were examined.

Microbial strains were transplanted from the storage medium to trypsin-soy agar (Conda) and cultivated for 24-48 hours at 37°C. A bacterial suspension with a bacterial cell concentration of 1.5×10^8 CFU/ml was prepared from the pure culture. To simulate a clean skin surface, the experiment used a mixing suspension consisting of a 1.5% solution of bovine albumin diluted in saline. A mixture of sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l was used to neutralise the active ingredients of the dips. To prepare mixture-1, 0.1 ml of bacterial suspension and 0.2 ml of mixing suspension were added to the test tube, mixed in an ice bath for 2 minutes at 0°C, and 9.7 ml of the test dip was added and kept for 60 seconds and 5 minutes at room temperature. After that, 1 ml of the resulting mixture-1 was transferred to a second tube containing 8 ml of neutralising suspension and 1 ml of water. The mixture-2 was kept in a water bath for 5 minutes at 20°C. After holding, 1 ml of mixture-2 was taken twice and placed in separate Petri dishes, and 15-20 ml of molten TCA was poured at a temperature of 45°C±1°C, and kept in a thermostat for 24-48 hours at 37°C.

Additionally, a decimal dilution was prepared, 0.5 ml of mixture-2 was added to 4.5 ml of neutralising agent, pipetted and sown with 1 ml of the resulting dilution, previously indicated by the method, on 2 agar plates. Additionally, a negative control was set, where sterile water was added instead of dip. The media were cultured during the day and the number of colonies formed was calculated, the criteria for evaluating bactericidal activity are shown in Table 2.

The bacterial load was determined by the equation:

$$N = \Sigma a / V(n1 + 0.1n2)d$$

where Σa – sum of colonies on cups selected for counting; V – volume of inoculate that was introduced into each cup, cm^3; $n1$ – number of cups of the first dilution selected for counting; $n2$ – number of cups of the second dilution selected for counting; d – degree of dilution (first) for which counting is performed.

Table 2. *Level of bactericidal action of the udder dipping depending on the growth of the microorganisms*

Level of bactericidal action of udder dipping	Growth characteristics
Full bactericidal effect	No growth
Incomplete bactericidal effect	1-99 CFU/ml
Subactericidal effect	100-299 CFU/ml

Source: *compiled by the authors*

Development of a matrix for assessing the risks associated with flaws at the milking stage. The survey of consulting managers was conducted by e-mail. 12 experts were interviewed. For the survey, 15 questions were formulated that correspond to the factors entered in the risk assessment matrix. A question was asked for each expert:

How do you assess the impact of *factor n* on the risk of mastitis?

Evaluation criteria: low impact, medium impact, significant impact.

The survey of doctors was conducted using paper questionnaires. 27 doctors took part in the survey. The factors described in the study were divided into two groups: 1 – violation of milking technology; 2 – laboratory tests conducted on the farm.

The question were formulated as follows:

How often have you detected violations of milking technology *n*?

Evaluation criteria: rarely, often, very often.

How often do you conduct research *x*?

Evaluation criteria: we never do it, sometimes we do it, and we do it all the time.

The survey questionnaire is available on request.

As a result, a matrix of qualitative risk assessment was developed. The vertical axis of the Matrix indicates the probability of detecting a factor on the farm, and the horizontal axis indicates the level of influence of the factor. The degree of danger of each factor can be determined according to the probability of manifestation and the level of exposure. The survey was anonymous, and the authors of the study did not receive or store the personal data of respondents.

Analysis of the causes of deterioration in milk quality. Two dairy farms with deteriorating milk quality in terms of somatic cells and bacterial load were selected for experimental testing of the effectiveness of corrective measures. During the first visit to the farm, the general sanitary condition of the farm, the quality of cow maintenance, and the system of grouping and keeping sick animals and cows of various technological groups were evaluated. Then the milking process was monitored. All lactating livestock were examined using a California test. The number of somatic cells was counted in milk from the affected animals and the infectious agent was identified. At each farm, flushes from milking cups, udder care products, and disinfectant samples were taken for microbiological studies.

Based on the results, farms were offered measures to reduce the impact of risk factors according to the risk analysis matrix. The effectiveness of the implemented measures was monitored based on weekly studies of tanker milk.

Statistical analysis. The analysis of experimental studies was carried out using the Microsoft Excel 2020 software suite. The results of the study were statistically calculated using a direct method for determining the standard deviation.

Experimental studies were conducted in accordance with modern methodological approaches and in compliance with the relevant requirements and standards, in particular, they meet the requirements of DSTU ISO/IEC 17025:2005 (2006). All manipulations were performed in accordance with the European Convention for the protection of vertebrates used for experimental and other scientific purposes (European Convention, 1986). The survey was conducted according to the International Compilation of Human Research Standards (2021).

RESULTS

Monoinfection was identified in 68 (75.6%) cows, and an association of 2 or more pathogens was found in 22 (24.4%) cows (Table 3).

Table 3. *Bacterial cultures found in the tested milk*

Pathogens isolated	Subclinical mastitis n=27	%	Clinical mastitis n=63	%	Total n=115	%
Staphylococcus aureus	4	14.8	9	14.3	13	14.4%
Staphylococcus epidermididis	2	7.4	4	6.3	6	6.7%
Staphylococus saprofiticus	1	3.7	3	4.8	4	4.4%
Streptococcus agalactiae	4	14.8	9	14.3	13	14.4%
Streptococcus disgalactiae	1	3.7	5	7.9	6	6.7%
Streptococus uberis	2	7.4	2	3.2	4	4.4%
Escherichia coli	5	18.5	11	17.5	16	17.8%
Klebsiella pneumoniae	0	0.0	6	9.5	6	6.7%
Staphylococcus aureus + Escherichia coli	1	3.7	6	9.5	7	7.8%
Staphylococcus aureus + Streptococcus agalactiae	2	7.4	0	0.0	2	2.2%

Table 3, Continued

Pathogens isolated	Subclinical mastitis n=27	%	Clinical mastitis n=63	%	Total n=115	%
Staphylococcus saprofiticus + Streptococcus disgalactiae	2	7.4	1	1.6	3	3.3%
Staphylococcus epidermididis + Staphylococcus aureus		0.0	2	3.2	2	2.2%
Staphylococcus aureus + Streptococcus disgalactiae	2	7.4	4	6.3	6	6.7%
Staphylococcus epidermididis + Escherichia coli + Streptococcus agalactiae	1	3.7	1	1.6	2	2.2%
Total	27	100.0	63	100.0	90	100.0

Source: *compiled by the authors*

Staphylococcus aureus was identified in 33.3% of cows with clinical and 30.1% with subclinical mastitis. *Staphylococcus epidermididis* was isolated in 11.1% of both animal groups. *Staphylococus saprofiticus* was detected in 11.1% of subclinically sick animals and 6.4% of clinically sick animals. *Streptococcus agalactiae* and *Streptococcus disgalactiae* more often isolated in subclinically sick animals – 25.9% and 18.5%, respectively, while the detection rate in clinically sick animals was 15.9%. *Streptococus uberis* was isolated from 7.4% of subclinically sick and 3.2% of clinically sick animals. *Escherichia coli* were present in 28.6% of clinically and 25.9% of subclinically sick animals. *Klebsiella pneumoniae* was isolated only as a monoculture in 6.6% of clinically sick animals.

To keep reusable udder treatment wipes clean, they need to be washed systematically. The level of contamination of napkins with microorganisms after washing with domestic detergent differed from that for washing with a special detergent containing bactericidal components (Table 4).

Table 4. *Number of contaminated wipes with each pathogen before and after washing*

Pathogen	Before washing with domestic detergent n=29	After washing with domestic detergent n=29	Before washing with antibacterial detergent n=16	After washing with antibacterial detergent n=16
Staphylococcus aureus	26	18	15	3
Staphylococcus coagulase negative	29	27	16	8
Streptococcus spp.	16	9	1	0
Escherichia coli	29	12	8	2
Klebsiella spp.	9	3	0	0
Pseudomonas spp.	11	6	6	1
Bacillus spp.	26	14	14	6
Proteus spp.	24	16	12	3
Candida spp.	25	16	9	2

Source: *compiled by the authors*

After washing with domestic detergent, the number of napkins that are contaminated with *Staphylococcus aureus* decreased by 31.6%, *coagulase negative Staphylococcus* by 6.9%, *Streptococcus spp.* by 43.7%, *Escherichia spp*. by 58.6%, *Klebsiella spp.* by 66.7%, *Pseudomonas spp.* by 45.5%, *Bacillus spp.* by 46.2%, *Proteus spp.* by 33.3%, and *Candida spp.* by 36%. After washing with a detergent containing bactericidal components, the number of napkins that are contaminated *Staphylococcus aureus* decreased by 80%, *coagulase negative Staphylococcus* by 50%, *Streptococcus spp.* by 100%, *Escherichia spp.* by 75%, *Klebsiella spp.* by 100%, *Pseudomonas spp.*by 83.3%, *Bacillus spp.* by 57.1%, *Proteus spp.* by 75%, and *Candida spp.* by 78.6%. None of the washing methods ensured the sterility of the napkins. Washing with a detergent containing bactericidal components led to a reduction in the level of contamination with various pathogens from 50 to 100% (by an average of 74.8%). Washing with domestic detergent resulted in a reduction in contamination from 6.9 to 66.7% (an average of 40.8%).

According to the results of dip studies, it was found that 40.6% of the studied samples had a complete bactericidal effect, 49.5% – incomplete bactericidal effect, and 9.9% had no bactericidal effect actions. 6.3% of all dips under study were contaminated (Table 5).

Table 5. *Bactericidal activity of dips depending on the active substance*

Active ingredient	Full bactericidal effect	Incomplete bactericidal effect	Subactericidal effect/of them contaminated
Iodine n=45	22	20	3/1
Lactic acid n=17	7	9	1/1
Chlorhexidine n=49	16	26	7/5
Total	45	55	11/7

Source: *compiled by the authors*

Dips which include the active substance iodine showed a complete bactericidal effect in 48.9% of cases, there was no bactericidal effect in 6.7% of the studied samples. Dips with lactic acid showed a complete bactericidal effect in 41.2% of cases, and 5.9% had no effect. However, the smallest number of samples were tested with this substance. The most frequent absence of bactericidal effect was found in dips with chlorhexedine – 14.3% of samples. 32.7% of the samples showed a complete bactericidal effect, which is the lowest indicator among all the samples under study. Of the 11 dips with no bactericidal action, 7 (63.6%) were contaminated. *Bacillus spp*. bacteria were identified in iodine-containing dips, and 3 – in chlorhexidine-containing dips. *Aspergillus spp*. fungi contaminated one dip with lactic acid and 2 dips with chlorhexidine.

According to the results of a survey of specialists, 16 risk factors that arise as a result of a violation of milking technology were identified, and the level of danger associated with them was determined. 3 factors had a high risk of developing mastitis caused by sanitary pathogens, medium – 9 factors, and low – 4 factors (Fig. 1). For pathogens of infectious mastitis, the number of factors for each risk group is 6, 5, and 5, respectively (Fig. 2).

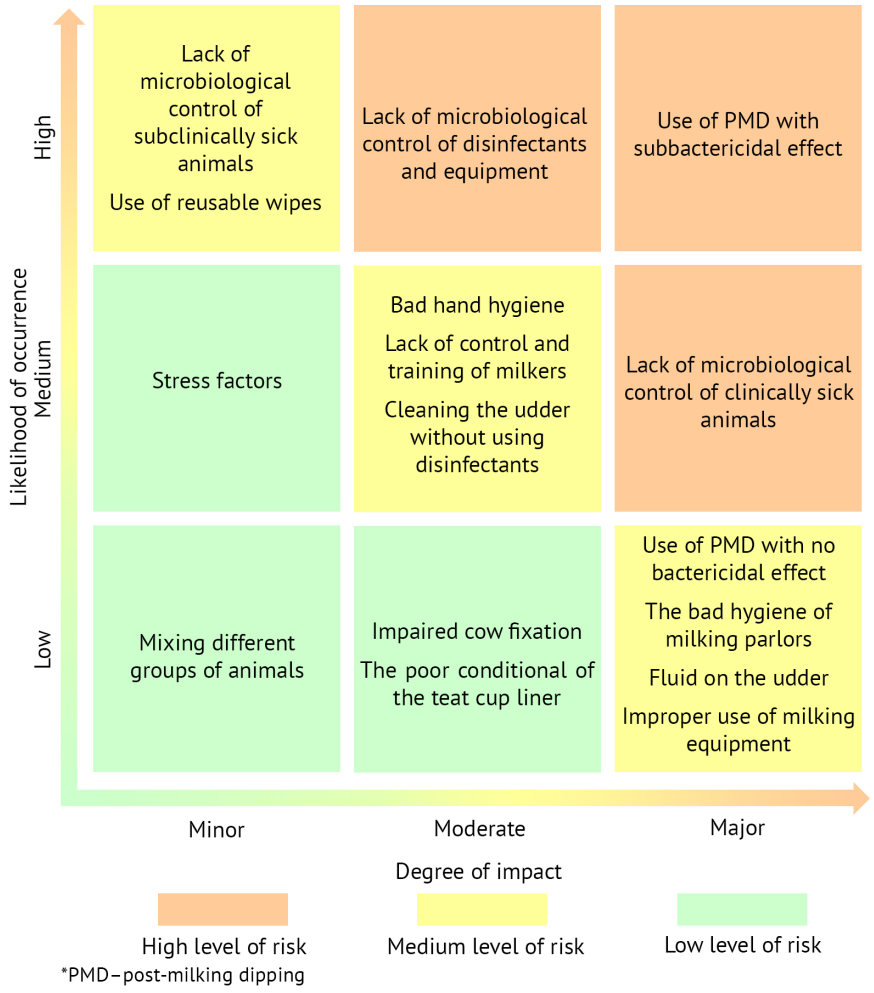

Figure 1. *Matrix of the risk level of sanitary mastitis*

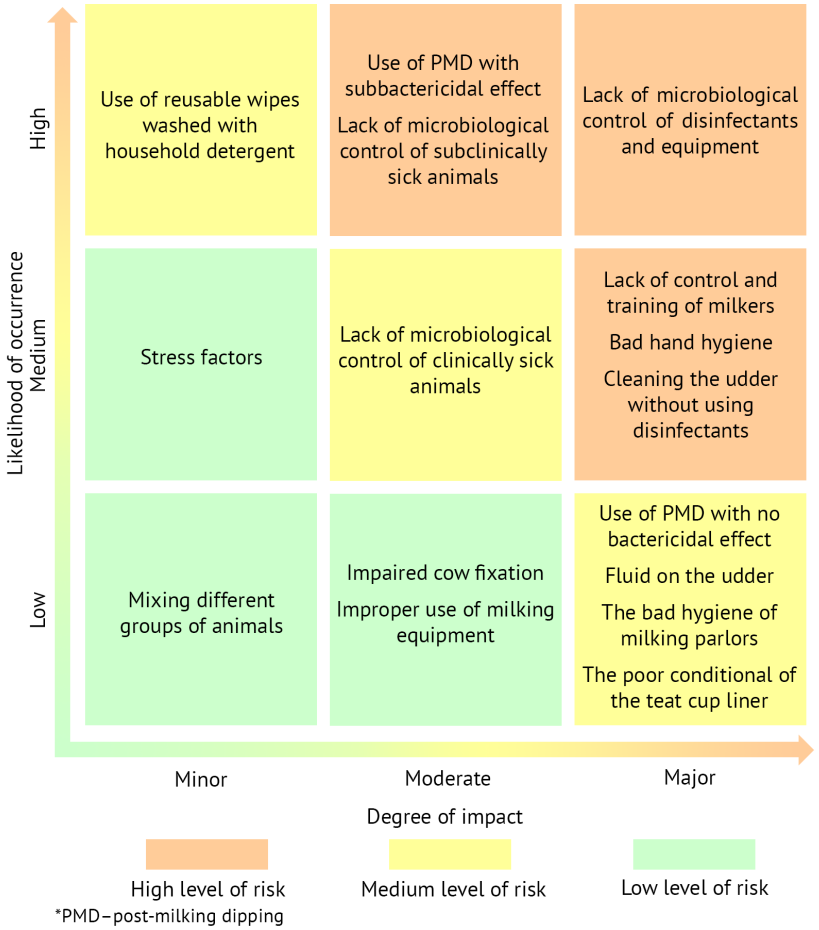

Figure 2. *Matrix of the risk level of infectious mastitis*

The developed risk matrix was tested to improve milk quality on two farms. Chromogenic media were used to accelerate the results of microbiological analysis (Fig. 3). The use of chromogenic media allowed identifying most of the pathogens of mastitis already in the first culture. This accelerated the identification of the main group of microorganisms that cause intramammary infection at each farm, and reduced the time required to select the correct corrective measures.

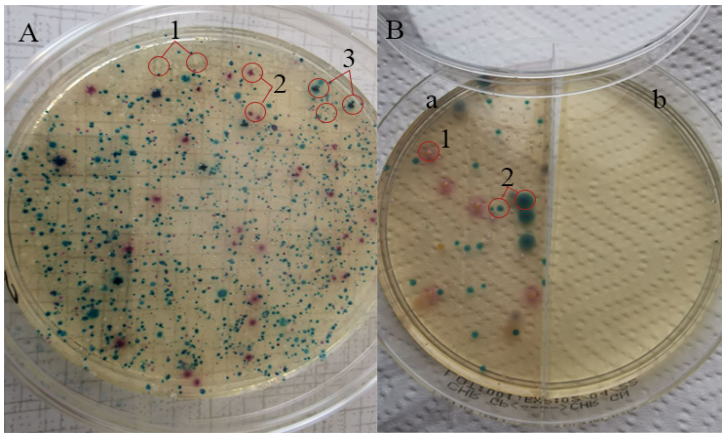

Figure 3. *Bacterial growth on chromogenic media*
A) CHROMagar™ Orientation 1. Staphylococcus epidermidis – cream, pinpoint colonies, 2. Staphylococcus saprophyticus – pink, opaque, small, 3. Streptococcus agalactiae – light blue B) a. Mastitis (GP) – 1. Staphylococcus aureus – pink 2. Streptococcus agalactiae – turquoise blue b. Mastitis (GN) – no growth

At the time of the study, 10 clinically sick animals and 17 subclinically sick animals were identified on the first farm. There are 7 clinically sick and 12 subclinically sick animals on the second farm (Table 6).

Table 6. *Data on livestock and milk quality at the beginning of the study*

	Number of livestock	Cows with mastitis	Bacterial load, thous. CFU/cm³	Somatic cells thous./cm³
Farm 1	970	27 (2.8%)	102	210
Farm 2	430	19 (4.5%)	145	275

Source: *compiled by the authors*

On Farm 1, the number of somatic cells in the mammary secretion ranged from 26.5 to 456.1×10⁴/cm³ (average 111.7±125.6×10⁴/cm³), on Farm 2 – from 32.2 to 567.6×10⁴/cm³ (average 111.7±125.6×10⁴/cm³). Animals from Farm 1 were found to have *Streptococcus agalactiae*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Esherihia coli*, and *Staphylococcus aureus*. In animals from Farm 2 – *Streptococcus disgalactiae*, *Staphylococcus epidermidis*, *Esherihia coli*, and *Staphylococcus aureus* (Table 7).

Table 7. *Results of milk tests from subclinically sick animals*

	Farm 1 n=30			Farm 2 n=17		
	Mean±SD, CFU×10⁴/cm³	Min, CFU×10⁴/cm³	Max, CFU×10⁴/cm³	Mean±SD, CFU×10⁴/cm³	Min, CFU×10⁴/cm³	Max, CFU×10⁴/cm³
Streptococcus agalactia	14.9±18.9	0.13	60	-	-	-
Streptococcus dysgalactiae	-	-	-	14.3±11	7	27
Staphylococcus saprophyticus	1.4±0.7	0.6	1.8	-	-	-
Staphylococcus epidermidis	1.3±1.5	0.16	4.5	14.2±18.2	1.3	27
E. coli	1±1.1	0.15	3	2.9±2.7	0.7	6.8
Staphylococcus aureus	0.4±0.19	0.39	0.56	6.5±7.1	1.1.	19

Source: *compiled by the authors*

It was established that both farms had good sanitary condition of the premises and balanced feeding. Factors leading to an increased risk of mastitis were identified at the milking stage (Table 8). These conclusions were also supported by the results of laboratory studies, the dominant microflora was pathogens associated with the development of subclinical mastitis.

Table 8. *Results of risk analysis on experimental farms*

Risk level	Farm 1	Farm 2
High level of risk	Lack of microbiological control of disinfectants and equipment Use of PMD with subbactericidal action Lack of a system for monitoring milkers' knowledge Lack of microbiological control of subclinically sick animals	Lack of microbiological control of disinfectants and equipment Udder cleaning without using disinfectants
Average level of risk	Poor hand hygiene of staff Use of reusable wipes washed with domestic detergent	Use of reusable wipes washed with domestic detergent
Results of analysis of post-milking dipping	Udder dipping had an incomplete bactericidal effect	Udder dipping had a full bactericidal effect
Results of equipment analysis	Bacteria of the genera *Str. agalactiae, Staph. aegeis*, *Esherihia coli* and groups *KECS* on three milk glasses	Bacteria of the genera *Str. dysgalactiae*, *Staph. aegeis*, *Esherihia coli* and groups *KECS* on five milk glasses

Source: *compiled by the authors*

Based on the results, it was recommended to immediately apply measures to correct factors that create a high risk of mastitis.

At Farm 1, the udder dipping with lactic acid showed an incomplete bactericidal effect, it was recommended to replace it with another one with iodine. The use of udder dips based on chlorhexidine and the use of disposable wipes was introduced. The staff was instructed on the use of pre- and post-milking dipping, and the patterns of spread of bacterial pathogens of infections and their role in the occurrence of mastitis was explained. Relevant educational and visualisation materials were provided.

At Farm 2, the use of pre-milking dipping based on hydrogen peroxide, additional treatment of the udder before milking with a disinfectant based on peracetic acid and hydrogen peroxide by aerosol method, and wiping the udder dry with disposable paper towels were introduced. In both farms, it is recommended to replace all rubber due to its condition (the presence of microcracks, loss of elasticity). When re-examined, the dipping had a complete bactericidal effect, and the new rubber on the milk cups was not contaminated. The number of mastitis cases on Farm 1 decreased to 18 of them 7 are subclinical, on Farm 2 – the number decreased to 12 of which 5 are subclinical (Table 9)

Table 9. *Data on livestock and milk quality at the end of the study*

	Number of livestock	Mastitis	Bacterial load, thous. CFU/cm³	Somatic cells, thous./cm³
Farm 1	970	18 (1.9%)	68	154
Farm 2	430	12 (2.8%)	102	145

Source: *compiled by the authors*

A week after the proposed interventions, the total number of bacteria and the number of somatic cells in tanker milk began to decrease (Fig. 4). Indicators of bacterial load decreased faster than indicators of somatic cells. However, at the time of the study, milk on two farms met the requirements for the "Extra" grade in terms of somatic cell indicators. While in terms of bacterial load, milk corresponded to the "Highest" grade.

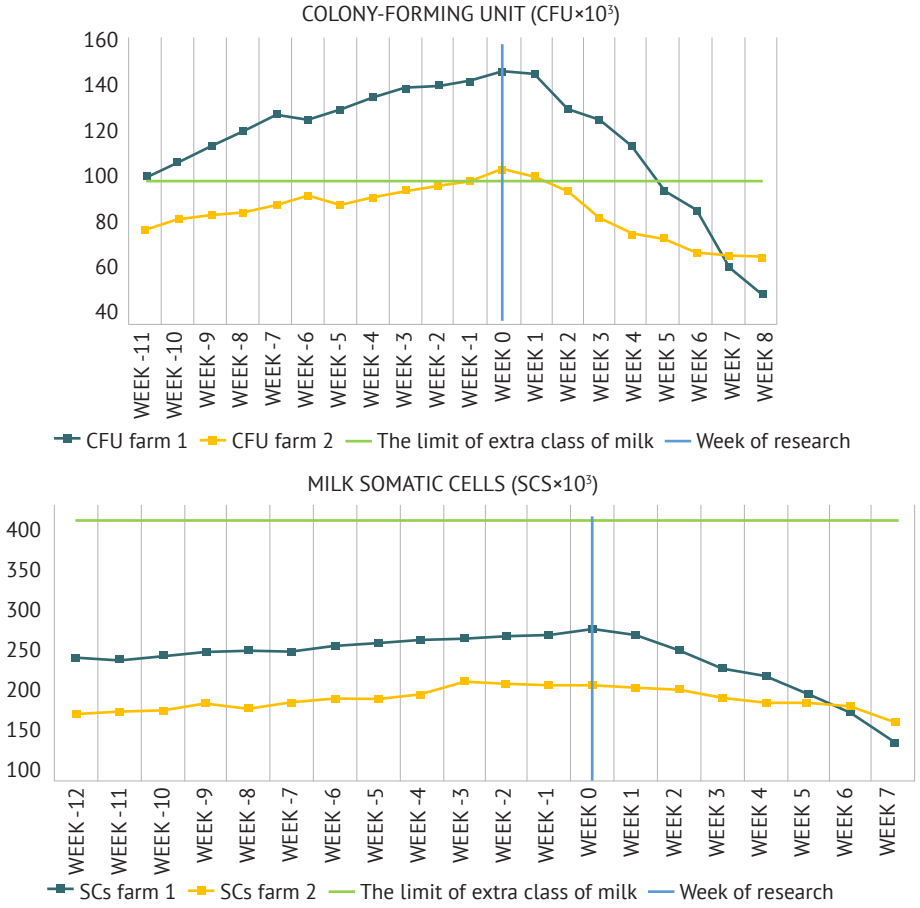

Figure 4. *Dynamics of changes in bacterial load indicators (CFU) and somatic cells (SCS)*

Recommendations for the two farms differed due to research results and specific factors that led to an increased risk of mastitis in each farm. Another important condition was the economic viability of farms to implement risk correction measures. As a result of the measures implemented on Farm 1, milk corresponded to the "Extra" grade two weeks after the implementation of the measures. More violations were detected on Farm 2, and milk quality improved in the fifth week after the recommendations were implemented.

In the study of milk from sick animals, pathogens associated with infectious mastitis *Staph. aureus*, *Str. Agalactiae*, and *Str. disgalactiae* were more often detected. On the visited dairy farms, this trend continued. But this may be conditioned by the choice of farms for research. Dips with incomplete bactericidal action were also used on one of the farms under study. This is consistent with previous data from studies of the bactericidal activity of udder dipping. Correction of the factors indicated in the risk analysis matrix contributed to an improvement in milk quality on farms where the dominant infectious agents were pathogens associated with the infectious mastitis.

DISCUSSION

The paper by Elias *et al.* (2020) described three infectious agents isolated from milk selected from Ukrainian farms. Among all pathogens isolated from cow's milk, *E. Coli* was identified in 23.3%, *S. aureus* – in 13%, *Str. agalactiae* – in 11% of isolates. The data obtained differ from the results of the present study, which may be conditioned by the analysis of a larger number of samples and the percentage of samples from clinically and subclinically sick animals.

In a study by Kenyan scientists Mbindyo *et al.* (2020), the main causative agent of subclinical and clinical mastitis was coagulase-negative staphylococci. Bacteria *Streptococcus spp.* – the second most common pathogen that caused clinical infections more often. The third most common pathogen, *S. aureus*, was more often isolated from the milk of subclinically sick animals. *E. coli* was isolated quite rarely, more often from the milk of clinically sick animals. Study of milk microflora using MALDI-TOF MS performed by Nonnemann *et al.* (2019) found that the most common pathogen in the biomaterial was *Streptococcus spp., S. aureus* and *E. coli*. Pascu *et al.* (2022) also found that the most common causative agent of mastitis in Romania was *S. aureus* bacteria. At the same time, *Staphylococcus spp.* and *Streptococcus spp.* are more often isolated from cows with subclinical mastitis, and *E. coli* – from clinically sick cows. In this study, there were similar trends in the distribution of major pathogens at the birth level, although the frequency of isolation of specific pathogens differed.

Several different authors (Horpiencharoen *et al.*, 2019; Ndahetuye *et al.*, 2020; Tomazi *et al.*, 2018) conducted statistical studies of the influence of various factors on the risks of mastitis. They paid attention to environmental factors, farm conditions, technological processes, pathogens, and animals in order to identify statistically significant correlations with the level of mastitis occurrence. The present study analysed aspects directly related to violations of milking technology, and the risk assessment was based on qualitative indicators. This approach may be more understandable for practitioners and farm owners.

Mistakes in preparing the udder for milking are associated with improper use of disinfectants and wipes. Often, farms used reusable wipes washed with domestic laundry powder, or did not use disinfectants to treat the udder. These factors have a stronger impact on the risk of infectious mastitis. Instead of disinfectants, some farms use running water, which leads to the washing of bacteria from the entire surface of the udder to the opening of the milk canal and their entry into the middle of the mammary gland. Dips are also available on the market to treat the udder before milking. Rowe *et al.* (2018) have concluded that pre-milking udder dipping has little effect on the development of clinical mastitis. Therefore, there is no need to apply protocols using these drugs in routine practice. Gleeson *et al.* (2018) also came to similar conclusions, but they note that the use of pre-milking dipping may be useful for herds with a high number of subclinically sick livestock.

Another mistake identified by this study, which does not happen often and significantly increases the risk of mastitis, is poor hand hygiene of personnel. Ndahetuye *et al.* (2020) note that washing staff's hands without using disinfectants increases the risk of mastitis caused by non-golden staphylococci.

At the milking stage, violations were identified related to incorrect use of milking equipment (incorrect dressing of milk glasses, exceeding the milking time), fixing animals and creating stressful factors. Odorčić *et al.* (2019) drew attention to the importance of proper milking equipment settings to reduce the number of mastitis cases. While the animal is in the milking parlour, the risk of infection can be reduced by other sanitary and technological measures, so the impact on the occurrence of infectious mastitis, in this case, is average. Since the nipple remains open, pathogens from the environment can get to it, so the risk of developing sanitary mastitis is high. Melvin *et al.* (2019), using ultrasound, found that the nipple canal remains open for some time after milking. But they noted that more research is needed to identify links between the size of the nipple canal diameter and the risk of udder infection.

On some farms, insufficient attention was paid to the condition of the milking rubber. This factor has an average effect on the risks of sanitary mastitis and a very strong effect on infectious mastitis. After exceeding the service life, the rubber loses its elasticity, microcracks form on its surface, where milk residues and bacteria that form a biofilm accumulate. In this case,

the disinfectants used to clean the equipment will be less effective. Latorre Data *et al.* (2020) indicate that biofilm formation on dairy equipment plays a role in the epidemiology of mastitis caused by *S. aureus*.

To protect the nipple canal after milking, post-milking dipping with bactericidal components is used. According to Lopez and Hillerton (2018), modern udder treatments not only create a physical barrier in the form of a film around the nipple opening. They also have a positive effect on the general condition of the nipple, which reduces the risk of mastitis. According to the study, a large number of such drugs have a subbactericidal effect. This is not an obvious problem for the farm owner, so it will not be fixed in time. This factor significantly increases the risk of sanitary mastitis, and has an average effect on the risk of infectious mastitis. Some dips may not have a bactericidal effect at all, the probability of this is not high, but in this case, the risk of mastitis increases significantly. As noted by Hohmann *et al.* (2020) the use of post-milking dipping has a long-term bactericidal effect on coliform bacteria and *S. aureus,* which can get on the skin of the nipple of healthy animals and are the main causative agents of mastitis. The bactericidal activity of dipping was studied in those farms where a large number of mastitis or a tendency to their growth were detected. Therefore, these data should be considered in the context of risks that may arise on the farm, and not for the analysis of the quality of goods available on the market.

Training of new personnel takes place in all farms and is part of the technological process. However, farms often lack periodic monitoring of personnel knowledge. Personnel who are not subject to control measures are not able to independently assess their wrong actions. Weak skills of milkers significantly increase the risk of infectious mastitis, and have less impact on the risk of sanitary mastitis. Rarely, farms may have a poor sanitary condition of pre-milking facilities, but this has a significant impact on the occurrence of sanitary mastitis. Bhakat *et al.* (2020) draw attention to the fact that in order to implement modern mastitis control strategies, it is necessary to provide training for farm owners. Alanis *et al.* (2022) also note the importance of training milkers. The introduction of an online training system has increased staff awareness of milking equipment failures and udder health assessment. Both authors note the importance of providing infrastructure for training and educational materials.

Without conducting bacteriological analyses, it is impossible to determine the risks of mastitis and use the risk analysis matrix developed by the study. Farms often showed a lack of microbiological control of subclinically ill animals. This significantly increases the risk of infectious mastitis, but has less effect on the risk of sanitary mastitis. Very often farms lack microbiological control of equipment and disinfectants, which has a significant impact on the risk of both groups of mastitis. Microbiological studies of clinically ill animals are often carried out on farms. Data obtained by Firth *et al.* (2019) show that microbiological studies are conducted more frequently on farms that are not affected by mastitis.

Identification of the source of danger and the nature of the infectious agent is extremely important for effective control of the pathogen. As this study has shown, performing microbiological control is also necessary to determine the effectiveness of disinfectants.

CONCLUSIONS

The most frequently detected infection-associated pathogens in milk from cows with mastitis were *Staphylococcus aureus* 33.3%, *Escherichia coli* 28.9%, and *Streptococcus agalactiae* 18.8%. *Escherichia coli* was more often identified with monoinfection, *Staphylococcus aureus* – with polyinfections.

Reusable wipes that are used to wipe the udder were contaminated with microorganisms that can become pathogens of mastitis. Washing with a detergent containing a bactericidal component significantly reduced the level of bacterial load compared to domestic laundry powder.

Most of the 49.5% of the udder dipping samples showed incomplete bactericidal action, 40.6% had a complete bactericidal effect, and 9.9% had a subbactericidal effect. Udder dips containing the active ingredient iodine had the best results. In the absence of microbiological monitoring and a system for assessing the skills of personnel, the risk of mastitis significantly increased. Priority correction of these factors will lead to an improvement in milk quality and a reduction in the level of infectious mastitis.

Measures, according to the risk analysis matrix on farms with milking technology violations, contributed to improving the quality of milk within a few weeks after their introduction. Microbiological studies allowed identifying the causative agents of mastitis, establishing contamination of care products, and determining the effectiveness of disinfectants. These data are necessary to determine the risks associated with various infectious agents.

To increase the effectiveness of the proposed risk analysis matrix, more research is needed on the correlation between risk factors and the level of mastitis in the herd. Along with the microbiological methods used in the research, the analysis of the possibility of using molecular genetic methods is promising.

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CONFLICT OF INTEREST

None.

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Профілактика та контроль маститу: інтеграція мікробіологічних та управлінських підходів

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Анотація. Мастити – це часта причина зменшення продуктивності корів та погіршення якості молока. У цій роботі розглянутий комплекс підходів, що базуються на мікробіологічних дослідженнях та ризик-аналізі, направлених на зменшення кількості хворих на мастит корів та поліпшення якості молока. Дослідження спрямоване на вивчення мікробних агентів ізольованих з молока хворих корів та засобів забезпечення здоров'я вимені і встановлення основні небезпеки, що виникають через помилки в провадженні заходів профілактики маститів. В дослідженні були застосовані мікробіологічні дослідження молока, серветок для обробки вимені та дипінгів з використанням хромогенних середовищ. Для якісної оцінки ризику було проведене опитування менеджерів-консультантів та ветеринарних лікарів. У дослідженні представлено результати мікробіологічних досліджень 115 зразків молока, 45 багаторазових серветок та 111 зразків дипінгів із застосуванням звичайних та хромогенних середовищ. Найпоширенішими мікроорганізмами в зразках молока були *Streptococcus spp.* 34,4 % (*S. agalactiae* та *S. disgalactiae*), *Staphylococcus aureus* 31,1 %, та *Escherichia coli* 28,9 %. *Staphylococcus aureus* та *Streptococcus spp.* асоціюється з інфекційним маститом, а *Escherichia coli* – з санітарним маститом. Для зниження захворюваності на мастит важливо застосовувати цілеспрямовані заходи, направлені на різні категорії збудників. Серветки для обтирання вимені були контаміновані збудниками асоційованими з розвитком маститу. Використання прального порошку, що містить бактерицидні компоненти, має вирішальне значення для мінімізації забруднення багаторазових серветок для вимені. Серед досліджених занурень дипінгів 40,6 % зразків мали повний бактерицидний ефект, а 9,9 % не мали бактерицидного ефекту. Встановлено 3 чинники високого ступеня ризику виникнення екологічного маститу та 6 чинників виникнення санітарного маститу. Результати досліджень були апробовані на двох молочних фермах. Виправлення ризик-факторів високого рівня небезпеки привело до підвищення якості молока за показниками соматичних клітин та кількості бактерій. Отримані результати можуть бути корисні для удосконалення системи профілактики та оптимізації лікування маститів на молочних фермах

Ключові слова: санітарний мастит; інфекційний мастит; ризик маститу; аналіз ризику; дипінги після доїння; збудники маститу; поліпшення якості молока; *Streptococcus spp.*; *Staphylococcus spp*.