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Occurrence of *Staphylococcus* spp. isolated from mastitic ewes and milking equipment

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Abstract: *This study aimed to analyze the occurrence of mastitis in dairy sheep herds with machine milking method . Totally 1893 samples of raw sheep´s milk , milk filters and swabs from udders' skin were collected during two milking seasons from 2022 until 2023 and identified by MALDI-TOF MS for detection of udder pathogens and risk of environmental contamination during milking process. The most prevalent mastitis agents detected from positive samples of raw milk and filters were non-Aureus Staphylococci (NAS; 46.8%) with S. aureus (15.7%). Other detected species were Enter. faecalis (11.7%) and E. coli (7%) isolated mainly from subclinical forms of mastitis. The statistically significant relationship between the occurrence of mastitis and contaminated environment caused by staphylococci was confirmed on a monitored farm.Therefore,the environmental hygiene and a proper milking program are two of the most important components of antimastitis measures. Both these practices significantly decrease bacterial spreading, transmission, and subsequent intramammary infection.*

Keywords: *ewes, milking, parlor, contamination*

I. INTRODUCTION

Inflammation of the mammary gland – mastitis remains one of the main health issues in ewes, significantly impacting the quantity and quality of milk production. The most common cause of mastitis in small ruminants is bacterial intramammary infections (IMI), which arise from the penetration of pathogens into the mammary gland, often through damage to its skin (e.g., during lamb suckling), with bacteria being spread during the milking process [1].

Failure to adhere to a proper milking hygiene program, combined with poor detection of mastitis, inadequate treatment, and improper milk transportation and processing, can lead to contamination with undesirable and health-threatening microflora [2]. Vršková et al. [3] emphasize the importance of the impact of microbiological contamination of raw sheep milk on the quality of dairy products. They highlight the need to meet stringent requirements and apply universally valid principles in primary milk production, with a specific approach to both traditional manual and mechanical sheep milking technologies. The risk of contamination persists if freshly milked milk is exposed to temperatures above 7°C during initial handling and storage.

Studies conducted on production farms have confirmed that the primary routes of contamination of raw sheep and cow milk with pathogenic bacteria are from the environment during its collection and processing, or the bacteria may already be present in the milk as a result of IMI [4,5].Therefore, the aim of the study was to monitor the occurrence of mastitis and the microbiological load of the milking environment over two milking seasons in a sheep farm using mechanical milking technology.

II. Material and Methods

Sheep Farm and Milking

Over a two-year period (2022 and 2023), the occurrence of mastitis and the microbiological contamination of produced milk were monitored on a sheep farm with 300 head of Improved Valachian, Tsigai, and their crossbreeds. The sheep were housed in two masonry barns with deep bedding during the winter, while a covered holding area, adjacent to the milking parlor, was also used for housing the sheep during the grazing season. During the grazing period (April – September), the sheep were gathered daily for milking in the parlor and were supplemented with hay and a grain feed additive.The milking parlor, a separate building, was equipped with an Alfa Laval Agri 2 x 14 milking system (Alfa Laval, Sweden) and a double-row fixation device. The parlor also included a milk cooling and storage room, as well as a staff room with hygienic facilities. Milk collection occurred once a day after morning milking, with the milk tanker being replaced after cleaning and disinfection.

Sheep Examination, Sample Collection, and Mastitis Diagnosis

In each year of the monitored period, the ewes from the observed flock were examined at the beginning of the grazing season (April), after the lambs were weaned during the first week of milking; in the middle of the season (July); and at the end of the season in September, a week before the conclusion of milking. A comprehensive examination focusing on the health of the ewes' mammary glands included clinical examination of the udder, supplemented with CMT tests as described by Vasil' [6], and the collection of individual sheep milk samples for microbiological analysis according to Tvarožková et al. [7].

All presumed *Staphylococcus* spp. after biochemical identification were verified using a matrix-assisted laser desorption/ionization (MALDI-TOF) biotyper (Bruker Daltonics, Leipzig, Germany) according to Ozbey et al. [8]. Standard *S. aureus* CCM 4750 (Czech Collection of Microorganisms, Brno, Czech Republic) was utilized to check for good quality.Based on a comprehensive assessment of the health status of the sheep's mammary glands, the individual forms were classified as subclinical and clinical intramammary infections (IMI).

Additionally, during the two-year observation period, samples were collected from the teat skin (n = 360) and milk filters (n = 42) as described by Vargová et al. [9]. These samples were taken six times over the two years, alongside milk samples at the beginning, middle, and end of the milking season. Swabs from the udder

skin were collected after the milking process (without teat disinfection), and swabs from the milk filters were taken during their replacement in the milking pipeline.

Data analysis

The Chi-square test was used to evaluate the relationship between environmental contaminations, occurrence of NAS, *S. aureus*, *E. coli* and *Ent. faecalis* on monitored surfaces (skin of udder and milk filters) and the incidence of mastitis caused by these pathogens in ewes from monitored farm.

III. Results and Discussion

Differences in climatic conditions, husbandry systems, management practices, and milking hygiene programs in sheep farming influence the distinct epidemiology, clinical manifestation of mastitis, and the quality of sheep milk. Under practical conditions in sheep farms, the prevalence of subclinical and clinical forms of mastitis varies, often reflecting the level of management and adherence to the established HACCP system [10].At the beginning (April), middle (June), and end (October) of the milking season, during the two-year monitoring period, 332 sheep (19.4%) tested positive for CMT with a score of 1-3. Of the positive CMT samples, 298 (17.4%) milk samples were confirmed through bacteriological analysis. Of a total of 1,709 milk samples, staphylococci were the most prevalent bacterial pathogens of the udder (Table 1).

Table 1. Prevalence of *Staphylococcus* spp. and other bacteria in milk samples over a two-year period

	No. of ewes	**Evalution of CMT**		**Infected milk samples**		**Staphylococcus sp.**		**Other bacteria**	
year	n	Negat. %	Posit. %	n	%	n	%	n	%
1.	863	77.8	22.2	171	19.8	108	12.5	63	7.3
2.	846	83.5	16.5	127	15.0	96	11.3	48	5.7
Total	**1709**	**80.6**	**19.4**	**298**	**17.4**	**204**	**10.9**	**111**	**6.5**

Note: CMT - California Mastitis Test, *Staphylococcus* sp. – *S. aureus* and NAS *(S. chromogenes, S. warneri, S. piscifermentans*).

Based on clinical examination of the udder, CMT assessment, and bacteriological analysis of the milk samples, the occurrence of mastitis was 16.1% on the monitored farm.The most common forms of IMI were subclinical mastitis (14.6%). NAS were isolated from milk as the causal udder pathogens of subclinical forms. The most common pathogen found in clinical mastitis was *Staphylococcus aureus* (1.6 %; Fig. 1).

By comparing the number of positive milk samples with those isolated from udder skin and milk filters, a statistically significant dependence ($P < 0.05$) was confirmed for NAS (*non-aureus staphylococci*) on both farms. The test criterion G ranged from 0.08 to 4.99, with a critical χ^2 value of 3.103. This led to the conclusion that the null hypothesis of dependence in characteristics was confirmed. This hypothesis indicates that the positivity of samples obtained from milk and the environment was not random.

Furthermore, on Farm 1, a test value of $\chi^2 = 3.26$ for *S. aureus* confirmed the link between mammary gland contamination and environmental transmission. In addition to NAS, Farm 2 demonstrated an association between the transmission of *E. coli*, *Enterococcus faecium*, and streptococci (Table 2).

The transmission dependency of staphylococci between the environment and milk contamination is also supported by Czech studies [11,12]. In the first study by Vyleťelová et al. [11], among 1,729 milk samples from dairy cows, sheep, goats, and milk tanks, the most common bacterial pathogens on Czech farms were *S. aureus* and coagulase-negative staphylococci (CNS; n = 634; 36.7%). Similarly, a study by Czech authors Bogdanovičová et al. [12], conducted on 50 dairy cattle farms, monitored bacterial pathogens in milk and

milking parlor environments. Of 261 samples of raw milk and filtration equipment, *S. aureus* was detected in 58 samples, 37 (14.2%) of which were isolated from raw milk.

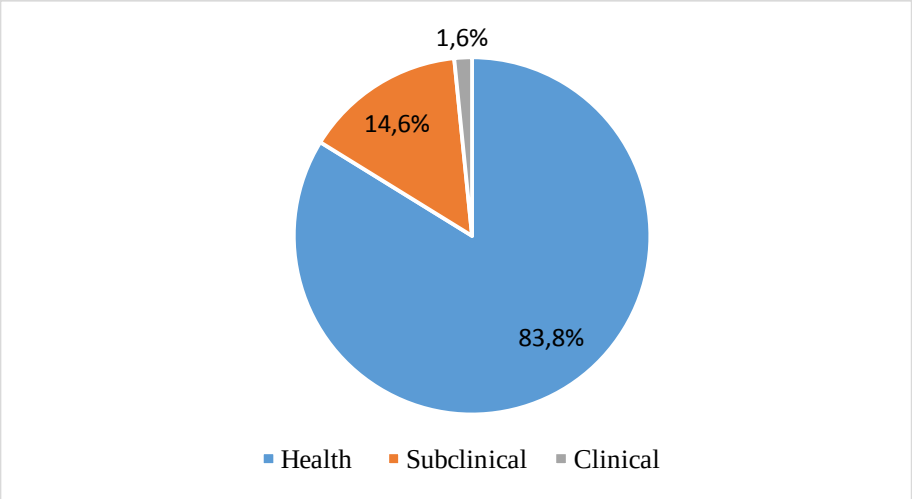

Figure 1. Occurrence of mastitis in sheep farm during a two-year monitoring period

Table2. The comparison of the dependence of positive bacterial isolates from mastitc milk with isolates from the environment (udder skin and milk filters)

Isolated Bacteria	Milk (n)	%	Skin of udder (n)	%	Filters (n)	%	Testing value χ^2	*p* Value
Staphylococci								
NAS	139	46.6	38	21.1	11	45.8	4.65	0.017*
S. aureus	65	21.8	16	8.9	4	16.7	3.26	0.036*
Other bacteria								
Str. spp.	36	12.1	9	5.0	2	8.3	1.93	0.264
E. coli	25	8.4	32	17.8	0	0	0.83	0.373
Ent. faecalis	31	10.4	19	10.6	1	4.2	1.87	0.229

Note: NAS - non aureus staphylococci; *Chi-squared test at a significance level $\alpha = 0.05$; critical value $\chi^2 = 3.103$; a positive testing value (G) regarding the statistical dependence of bacteria isolated from milk and the environment was confirmed when $G > \chi^2$; the dependence was not statistically significant when the testing value was $G < \chi^2$.

IV. Conclusion

The study highlights the dominant presence of staphylococci isolated from milk and environmental samples on a sheep farm utilizing machine milking technology. The most prevalent staphylococci were NAS (*S. chromogenes*, *S. haemolyticus*, *S. warneri*, *S. xylosus*) and *S. aureus*. A statistically significant relationship was confirmed between the occurrence of mastitis and environmental contamination caused by *S. aureus* and NAS.

NAS poses a risk of ewe contamination from the environment, manifesting as subclinical inflammatory changes in the mammary gland. These changes often go unnoticed by farmers but can significantly impact the further development of intramammary infections (IMI) and the quality of produced milk.Therefore the hygienic milking program and clean environment are most important components of a procedures to control mastitis.

Both these practices significantly decrease bacterial spreading, transmission, and subsequent intramammary infection.

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