Chapter

Occurrence of Mastitis in Dairy Herds and the Detection of Virulence Factors in Staphylococci

František Zigo, Zuzana Farkašová, Ibrahim Farag Mohammed Rehan and Ahmed Sayed-Ahmed

Abstract

Mastitis is still a major challenge that affects milk quality. The study is aimed to examine the health of the mammary gland and identify the udder pathogens and virulence factors that caused mastitis in 960 dairy cows and 940 ewes, respectively. We found that Staphylococci and streptococci are the most common causes of mastitis in those dairy animals. Coagulase-negative staphylococci (CoNS), along with the main udder pathogens such as *S. aureus, S. uberis, and S. agalactiae*, are a major concern for dairy animals. The majority of the virulence factors (production of hemolysis, gelatinase, biofilm, ability to hydrolyze DNA, and antibiotic resistance) were found in *S. chromogens, S. warneri, and S. xylosus* isolates from clinical and chronic cases of mastitis. S. aureus and CoNS strains tested by disk diffusion showed 77.0 and 44.2% resistance to one or more antimicrobial classes in mastitic milk samples from dairy cows and ewes, respectively. The presence of a methicillin-resistant gene *mecA* poses serious complications for treatment and indicates a health risk to milk consumers due to the resistance to β-lactam-antibiotics in two isolates of *S. aureus* and two species of CoNS isolated from cows' mastitic milk samples.

Keywords: dairy cows, ewes, mastitis, coagulase-negative staphylococci, biofilm, antibiotics, methicillin resistance gene

1. Introduction

Milk and milk products are important global dietary products, consumed by more than 6 billion people worldwide. In 2019, the recorded milk consumption was 852 million tons, distinguishing the dairy industry as a very profitable market. The milk obtained is a traditional raw material for the production of a range of dairy products, which are unique in their composition, but EU rules emphasize that such products must come from healthy animals, which significantly limits their production and quality [1].

Despite the increasing level of zoohygienic provision of dairy farming, inflammation of the mammary gland-mastitis is still one of the main health problems.

This disease is associated with pain and adversely affects animal health, welfare, milk quality, and the economics of milk production. Direct and indirect losses, caused by mastitis lead to economic losses. For direct losses, we can include treatment costs, discarded milk, labor time, fatalities, and the associated costs with repeated cases of mastitis. Regarding indirect losses, we can include increased culling, decreased milk production, decreased milk quality, loss of premiums, preterm drying-off, animal welfare aspects, and other associated health problems [2, 3]. According to a study by Hogeveen et al. [4], the losses for the global dairy industry are estimated at 16–26 billion euros per year, based on a global population of 271 million dairy cows, with a cost of €61–97 per animal for farmers. In the United States, economically bovine mastitis costs around $2 billion every year. It has also been identified as one of the most economically relevant diseases in Ireland by Animal Health Ireland [5]. In the Netherlands, van Soest et al. [6] estimated the total cost of mastitis in 108 dairy farms, and found that the average total cost of mastitis is €240/lactating cow per year. In addition, failure costs contributed €120/lactating cow per year and preventive costs also contributed another €120/lactating cow per year.

Due to their polyethological origin, infections of the mammary gland are most often caused by a complex of interactions among the host, environment, and infectious agents that result in bovine mastitis, one of the most frequent diseases of dairy cows and ewes (**Figure 1**). Mastitis has a significant impact on global dairy production, reducing both the quality and quantity of milk produced. In comparison with most other animal diseases, mastitis differs by the fact that several diverse kinds of bacteria can cause the infection. These pathogens are capable of invading the udder, multiplying there, and producing harmful, inflammation-causing compounds [7].

Up to this date, more than 137 different organisms have been recognized as causative agents of ruminant intramammary infection (IMI). They include bacteria,

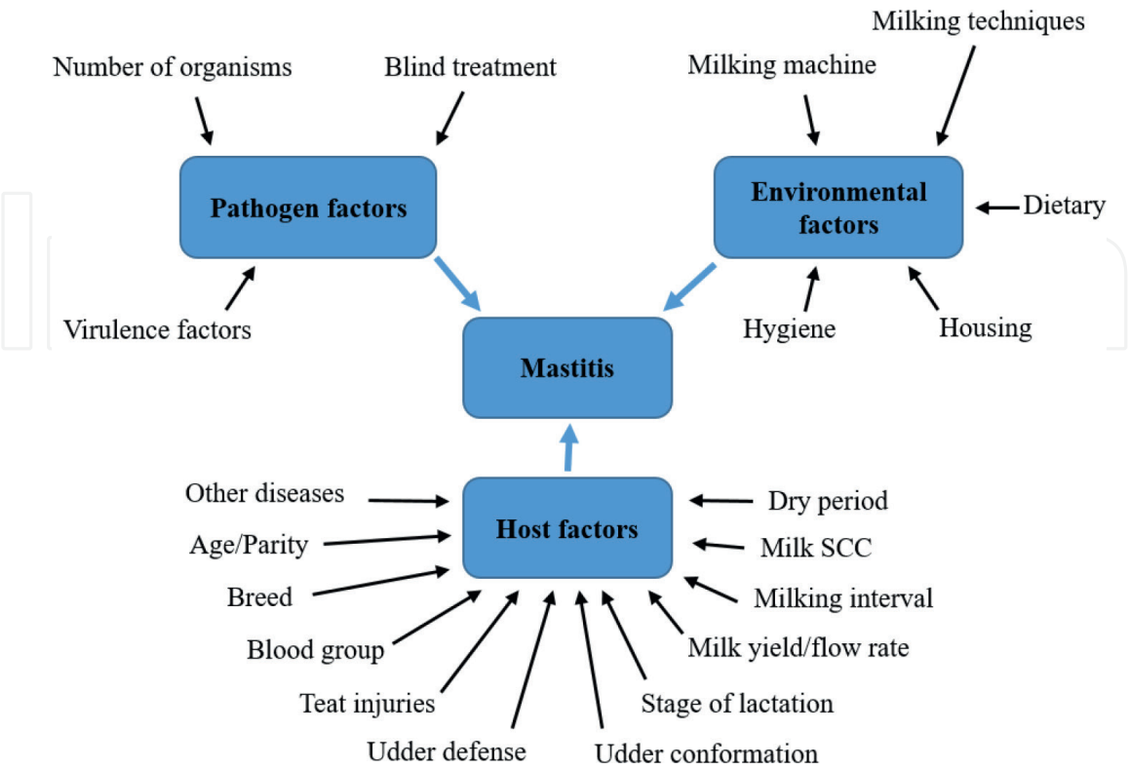

Figure 1.
Factors promoting mastitis. Source: Zigo et al. [3].

viruses, mycoplasma, yeasts, and algae, but bacteria have been identified as the principal causative agent of mastitis (95% of all IMI). In general, each mastitis case is believed to be caused by one primary pathogen, as in milk samples from the affected udder usually only one bacterial species has been identified. However, it is not rare to detect simultaneous infections by two different pathogen species, and even three pathogens have been found in a small proportion of samples [8, 9].

Major and minor pathogens are two main categories used to classify the microorganisms that cause mastitis. *Staphylococcus aureus, Streptococcus agalactiae*, or *Streptococcus dysgalactiale* are the most prevalent major pathogens or contagious udder pathogens, and when they can survive, these areas serve as their primary reservoirs in addition to the mammary gland (MG), the rumen, and the genital regions. As a result, the infection can spread from infected to uninfected quarters or halves [7]. Other pathogens that can cause intramammary infection in ruminants include *coliforms, enterococci*, *Streptococcus* spp., *Pseudomonas aeruginosa*, *Mannheimia hemolytica, Corynebacteria,* CoNS, and fungi, though their prevalence varies depending on the environment [10–12]. The most significant udder pathogens in this group are *Streptococcus uberis* and *E. coli*, which each have a number of pathogenic strains for both people and animals. Both pathogens can be present in the environment and the surroundings of the animals [7].

According to Slovak studies [7, 9, 13], *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, and *Staphylococcus xylosus* are the most common pathogens from CoNS causing mastitis, followed by *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Escherichia coli,* and Enterococci. Of the 42 monitored dairy farms, CoNS and *S. aureus* accounted for 36% and 12% of all positive mastitic cases, respectively.

Namely, *S. aureus* and CoNS have been among the most common microorganisms causing mastitis in dairy cows and health disorders among consumers of milk and dairy products in recent years. According to the World Health Organization, 420,000 lives are lost due to food poisoning; and *Staphylococcus* spp. is characterized as an important agent that can cause foodborne diseases. Poisoning occurs due to the ingestion of preformed enterotoxins in food. Symptoms include vomiting, diarrhea, and cramps; and an outbreak could lead to a public health problem [12–14].

The MG's inflammatory process manifests as symptoms and modifications in the milk and udder tissue. The IMI can be categorized as either persistent (chronic) mastitis or subclinical mastitis. Subclinical forms, which do not exhibit overt indications of inflammation but instead have elevated somatic cell counts (SCC) in the presence of the causative agents, are typically a serious silent issue and are the most common illnesses to result in significant financial loss for owners. Since they cannot be detected without a lab or field test, the subclinical types of IMI frequently become incurable in later stages [9].

Staphylococci can induce different types of intramammary infections depending on the quantity and pathogenicity of the strains, as well as the degree of the udder tissue's response to damage or infection. The interaction between dairy animals' innate resistance and adaptive immunity, as well as the virulence of Staphylococcal strains, determines the course of the clinical inflammation of MG caused by Staphylococci, which is characterized by local visible inflammatory changes in milk and udder tissue, either with or without systemic clinical signs [15]. In general, if there are enough *S. aureus* penetrates into the teat, one of two clinical forms of IMI may develop. Peracute Staphylococcal mastitis occurs infrequently and primarily affects cows and ewes in early lactation with compromised immune systems. The illness is very severe, and is manifested by a high fever, depression, and inappetence. The animals may become comatose and die within 24

hours after the onset of symptoms. The reluctance of infected animals to move is related to grossly swollen infected quarters, which is extremely painful. Blood-stained secretion with serous fluid from the infected part of the udder is usually observed. In surviving animals, blue gangrenous patches may be observed on the infected udder tissue that progress to black, exuding sores [16]. Although early treatment with effective ATB can save an animal suffering from peracute *S. aureus,* the affected quarter is almost always lost [14].

The more common form of *S. aureus* infection is less severe but chronic. The animals with chronic mastitis may not appear affected, and the infected part of the udder does not cause pain. No abnormalities may be observed in the milk. The main complications associated with the treatment of *S. aureus* infection include the fact that many strains can cause this disease and increasing number of them are becoming resistant to an increasing range of antibiotics available for veterinary use. One of the frequent causes of growing resistance is the normal practice on farms of drying dairy cows universally with antibiotics in addition to treating clinical cases of IMI.

According to the study by Ferroni et al. [17] management practices are associated with increased antibiotic consumption, especially in intensive dairy production. The authors analyzed 101 beef and dairy cattle farms in central Italy and compared the overall average antibiotic consumption during one year. The total course of administered ATBs was 3 times higher in the case of dairy cows than in beef farms. Their increased number was mainly related to the treatment of lactating and drying cows with ATBs (**Figure 2**).

The studies Vasil' et al. [18] and Holko et al. [19] confirm the increased resistance of mainly udder pathogens (*S. aureus, S. uberis*, *and S. agalactiae)* as well as CoNS to those ATBs, that are part of intramammary applicators used for dry treating.

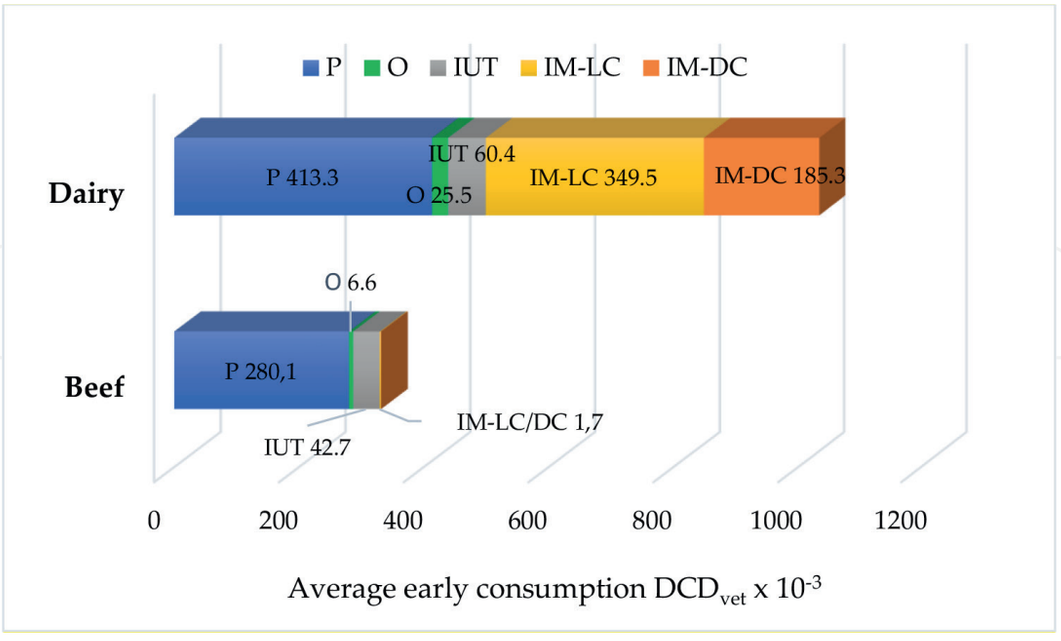

Figure 2.
Comparison of average early ATBs consumption between dairy and beef cattle.
Note: P = parenteral application, O = oral application, IUT = intrauterine application, IM-LC = intramammary treatment of lactating cows, IM-DC = intramammary application for drying). The overall average antibiotic consumption was expressed in defined course doses (DCD$_{vet}$)/year and is presented per livestock specialization and by administration route. The total courses administered were higher in farms with intensive dairy production (1034.1 × 10^{-3} DCD$_{vet}$/year) than in beef farms (330.7 × 10^{-3} DCD$_{vet}$/year).

Lately, CoNS have become a concern among dairy producers, as their potential as mastitis-pathogens has been observed; and they have already been found in majority of other pathogens. Their predominant isolation can be explained by the fact that CoNS are pathogens adapted to survive in cows or ewes and may be in the mammary gland of sick or healthy animals; while some species are also more resistant to antibiotics than *S. aureus* [13]. Among all the CoNS found in dairy animals, *S. haemolyticus*, *S. chromogenes, S. epidermidis, S. warneri, S. cohnii, S. simulans, S. hominis, S.capitis, and S. xylosus* are the most prevalent species [20, 21].

Following a decline in the incidence of mastitis brought on by the infectious bacteria, the causative CoNS became more prevalent and more resistant to typical ATBs and disinfectants used in dairy farm conditions (**Table 1**). When compared to *S. aureus*, the CoNS often exhibits less virulence and pathogenicity. Their primary pathogenicity factors are biofilm formation and ATB resistance, which enable them to survive the use of medicines and disinfectants during therapy. In a study by Nascimento et al. [20], the most popular antimicrobials used in veterinary practice were tested in vitro against CoNS isolated from mastitic cows. High resistance to the ATBs used to treat cows during lactation was found in tested strains of *S. epidermidis*, *S. saprophyticus*, *S. hominis*, and *S. aerletae*. Also, they could also make some of the Staphylococcal enterotoxins.

Particularly, Staphylococci bacteria that are multiresistant to multiple ATBs pose a severe threat to the public's health [16]. Recent research also suggests that the presence of methicillin-resistant Staphylococci (MRS), which have been found in raw milk and dairyproducts, such as cheese, is indicated by multiresistant Staphylococci, particularly to β-lactam ATBs. The public's health is threatened, according to WHO, by

Number of resistant CoNS	Antimicrobial groups	Percentage of resistant strains	Source
16/37	Oxacillin + chloramphenicol	5.4%	Khazandi et al. [22]
	Oxacillin + novobiocin	27.0%	
	Oxacillin + tetracycline	5.4%	
	Oxacillin + cefoxitin	5.4%	
3/8	Ampicillin + clindamycin + oxacillin erytromycin + gentamycin + penicillin + sulfonamide + trimethoprim/ sulfamethoxazole + tetracycline	37.5%	Dorneles et al. [23]
2/8	Ampicillin + penicillin + trimethoprim/sulfamethoxazole + trimethoprim/sulfamethoxazole	25.0%	
1/8	Ampicillin + gentamycin + oxacillin + penicillin + sulfonamide + trimethoprim/sulfamethoxazole + tetracycline	12.5%	
2/8	Ampicillin + gentamycin + oxacillin + penicillin + trimethoprim/sulfamethoxazole + tetracycline	25.0%	
18/170	Same group of antibiotics (β-lactam or MLS compounds)	10.58%	Sampimon et al. [24]
18/170	Diferent groups	10.58%	

Note: Number of resistant coagulase-negative staphylococci (CoNS); Antimicrobial groups they have resistance to; Percentage of resistant strains for each drug or group.

Table 1.
Resistance of CoNS to two or more antimicrobials.

the MRS strains' opportunistic capacity to induce mastitis. They might spread zoonotic diseases while acting as a gene repository for dairy cows' antimicrobial resistance. Of the MRS of concern, *Staphylococcus aureus* (MRSA) is the species most widely reported, however, in a number of studies CoNS were also identified as MRS isolates [23, 25, 26].

In addition to the increased antibiotic resistance of Staphylococci, the authors, Vasil et al. [18] and Haveri et al. [27] confirmed biofilm formation and lysines in mastitic milk samples and considered them as important virulence factors involved in the development of CM. Previous research has linked Staphylococci and their virulence factors to the pathogenesis and clinical manifestations of mastitis. They stressed the importance of thorough knowledge of their virulence factors, structures, and products. It is crucial to understand how these microorganisms facilitate adhesion and colonization of the mammary gland epithelium, which allows them to survive, successfully establish themselves, and persist in the host tissue. Therefore, the study was aimed at the occurrence and determination of contagious and environmental udder pathogens in dairy cows' and ewes' herds. Particularly in isolated Staphylococci, the presence of selected virulence factors such as hemolysis, gelatinase, biofilm, hydrolyzed DNA, and resistance to antibiotics with the detection of methicillin resistance gene-*mecA* and their effect on the severity of mastitis were determined.

2. Materials and methods

2.1 Monitored dairy farms

The practical part of the study was carried out in four different cows' and four sheep herds located in east Slovakia with conventional (nonorganic) farming. The selection of dairy farms for the study was based on criteria such as herd size, breed representation, and milk yield per lactation. Up to 70% of farms located in the east of Slovakia are in the range of 150–300 cattle and 200–400 ewes. Due to the study carried out in Slovakia, dairy farms were selected where there are national breeds of cattle and sheep. The practical part of the study on selected dairy farms with the clinical examination and collection of milk samples were approved by the Ethics Committee at the University of Veterinary Medicine and Pharmacy in Košice no. EKVP 2022/05.

From dairy cows, each herd size ranged from 150 to 300 Slovak spotted cattle bred between 1st and 4th lactation. The dairy cows under investigation on each of the four farms were housed in a system of free housing on straw litter with *ad libitum* access to water. According to international guidelines, a total mixed feed made up of silage, hay, and concentrate was given to them [28]. The rations met the nutritional requirements of cows weighing 650 kg, with an average milk yield of 20–30 kg per day. In the first phase of lactation, the mean average dry matter intake per cow per day was 23.6 kg +/- 3.7 kg. All cows were milked twice daily in parallel (Boumatic, USA) or fishing (DeLaval, Sveden) parlor. From all monitored dairy farms, 270 cows from the first, 215 cows from the second, 250 cows from the third and 225 cows from the fourth herd were investigated.

The four sheep farms were in herd sizes ranging from 200 to 400 animals and consisted of Improved Valachian, Tsigai, and Lacaune breeds. In April, the ewes were on pasture during the day and received concentrates in amounts of 200 g per day during milking. After their lambs were weaned in early April, the ewes were milked twice a day on each farm. In the first two herds, machine milking was performed using a two-line milk parlor 2×14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) and in two other herds, the sheep were milked in two-line milk parlor 2× 16 Alfa Laval Agri

(Alfa Laval, Sweden). From all the monitored sheep farms, during the first month of pasture (April), 220 ewes from the first, 250 ewes from the second, 270 ewes from the third, and 200 ewes from the fourth herd were investigated.

2.2 Dairy animals selection and udder health examination

The dairy cows from four monitored farms were selected on the basis of the formation of production groups according to the stage of lactation (early lactation 14–100 days of lactation) and the phase of nutrition, which were compiled by the zootechnicians. The selected dairy cows of the same performance class (early lactation) were housed in individual husbandry groups, which included 45–90 animals on each farm.

Ewes from four herds were included in the study two months after lambing between the 1st and 3rd lactation with a stay on pasture and milked twice a day. Complex examination of health status of udder in ewes from four monitored farms was carried out at the start of the milking season (April). On the basis of a clinical assessment, each dairy cow and sheep had a thorough inspection that included sensory evaluation and udder palpation. The California mastitis test (CMT) (Indirect Diagnostic Test, Krause, Denmark) was used to evaluate the milk from the fore-stripping of each udder quarter or halve – Raw milk samples from cows and ewes with positive test results were collected [19]. CMT scores were 0, +, ++ and +++ for "negative", "weak positive", "positive" or "strong positive", respectively [29].

Following that, of the 960 cows that were investigated, 689 had a negative CMT score, and 271 cows had a CMT score that indicated trace or positive symptoms based on clinical manifestations (score of 1–3), were chosen for aseptic collection of 12 mL mixed quarter milk samples by discarding first squirts with the cleaning of the teat end with 70% alcohol for laboratory analyses of bacterial pathogens, according to Holko et al. [19]. From 940 examined ewes, 756 animals had negative CMT scores and 184 animals with CMT score trace or 1–3 were taken with 12 mL mixed halves milk samples for laboratory analyses. All milk samples from cows and ewes were cooled to 4°C and immediately transported to the laboratory and were analyzed on the following day.

According to the National Mastitis Council [30], each instance of mastitis in positive animals was given a grade that was divided into subclinical, clinical, and chronic forms. A high SCC was found utilizing a CMT evaluation and a positive bacteriological result to identify subclinical mastitis (SM), which was distinguished from clinical mastitis by the absence of obvious symptoms in the udder or alterations in the milk. Clinical mastitis (CM), which can be seen in the milk or in the udder, is divided into three stages: mild mastitis, which is identified by visible changes in secretion; moderate mastitis, which also exhibits localized MG inflammation; and severe mastitis, which also exhibits general symptoms like loss of appetite, difficulty standing, fever, or low body temperature. Based on repeated therapy, a history of clinical evaluation of the MG with a positive CMT score, and the development of udder pathogens, chronic mastitis, or persistent mastitis was identified.

2.3 Bacteriological culture and evaluation of growth on plates

In the laboratory, 0.2 mL of milk was inoculated from each sample onto a blood agar plate (Oxoid LTD, Hamshire, UK) and incubated aerobically at 37°C for 24 hours. The primocultivated colony from blood agar and identification of *Staphylococcus* spp. were sub-cultured onto different selective bacteriological media (No. 110, Baird-Parker agar, Brilliance UTI Clarity Agar, Oxoid, Hampshire, UK) and incubated

at 37°C for next 24 hours. Cell morphology, Gram staining, the type of hemolysis, and the activities of catalase (3% H_2O_2, Merck, Darmstadt, Germany) were used to identify colonies, esculin hydrolysis and cytochrome oxidase C (Bactident Oxidase, Merck). The clumping factor test discovered potential *Staphylococcus aureus* (DiaMondiaL Staph Plus Kit, Germany). According to research by Vasil' et al. [18] and Holko et al. [19], esculin-positive streptococci were grown on modified Rambach agar to identify *Streptococcus uberis* or *Enterococcus* sp.. Lancefield serotyping (DiaMondiaL Strept Kit, Germany) was used to describe esculin-negative streptococci, and the MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) was utilized to identify all gram-negative species. The presence of one or more colony-forming units (CFU) of the major udder pathogens, such as *Staphylococcus aureus, Streptococcus dysgalactiae, or Streptococcus agalactiae*, was considered positive. The sample would be deemed positive if the growth of a significant udder pathogen was discovered in conjunction with other environmental species. Other pathogens were categorized as requiring at least three CFUs to be present. If infectious pathogens did not develop and three or more pathogens were isolated from a single milk sample, the grown samples were deemed contaminated.

2.4 Detection of virulence factors in Staphylococci

Confirmed Staphylococci based on MALDI-TOF analysis were exposed to deoxyribonuclease (DNase test) and to produce extracellular proteolytic enzymes (Gelatin hydrolysis test) according to Hiko [31]. The formation of biofilm was determined by a phenotypic method by growth on Congo Red agar (CRA) according to Vasil' et al. [13].

Additionally, it was established that Staphylococci can generate hemolysins, based on Moraveji et al. [32]. After 24 and 48 hours of incubation at 37°C, the lysis zone of each Staphylococcal isolate on plates of blood agar base supplemented with 5% sheep blood was used to phenotypically define the different types of hemolysis.

The susceptibility of Staphylococci isolated from cows' (n = 136) and sheep's (n = 86) infected milk was tested *in vitro* against 14 antimicrobial agents. The susceptibility tests of isolates were carried out on Mueller Hinton agar using a standard disk diffusion procedure [33]. In the current study, antibiotic discs containing penicillin (PEN; 10 μg), ampicillin (AMP; 10 μg), amoxicillin (AMC; 10 μg), amoxicillin+clavulanic acid (AXC; 20/10 μg), ceftiofur (CEF; μg), oxacillin (OXA; 1 μg), cefoxitin (CFX; 30 μg), ciprofloxacin (CPR; 5μg). The diameters determined were classified as susceptible, moderate, or resistant based on CLSI breakpoints, and the zone of inhibition was measured in millimeters [34]. Reference strains of *S. aureus* CCM 4750 and *S. chromogenes* CCM 3386 from the Czech Collection of Microorganisms in Brno, Czech Republic, served as the controls in the assays. The study's chosen antimicrobials represent the range of medications used in veterinary care on Slovak dairy cows.

2.5 Detection of the mecA gene from Isolated Staphylococci

Phenotypical positive Staphylococci (45 and 26 isolates from cows' and sheeps' mastitic milk samples) based on their antimicrobial resistance to β-lactams antimicrobials were subjected to PCR to test for methicillin resistance. Total genomic DNA was isolated according to Hein et al. [35]. Using a BioSpec spectrophotometer, the purity of the DNA recovered from the tested Staphylococci was evaluated (Shimadzu, Japan). According to Poulsen et al. [36], acquired DNA was used in PCR reactions

to detect the *mecA* gene using primers MecA1 and MecA2 (Amplia s.r.o., Bratislava, Slovakia). Sanger sequencing was used to confirm the identity of the PCR products (527 bp), in accordance with the guidelines provided by GATC Biotech (AG, Cologne, Germany). The BLAST tool was used to compare the DNA sequences acquired from the isolates to those found in the GenBank-EMBL (the European Molecular Biology Laboratory) database (NCBI software package). As a reference strain for PCR, *S. aureus* CCM 4750 (Czech Collection of Microorganisms, Brno, Czech Republic) was used in this investigation..

2.6 Statistical analysis

Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) was used to process the study's data, and SPSS version 20 and Excel were used to analyze it (IBM Corp., Armonk, USA). According to specific microbial species and mastitis types, the findings of grown udder pathogens from mastitic cows and ewes were processed and converted to percentages. The percentage of resistant isolates from milk samples that tested positive for *S. aureus* and CoNS for each type of antibiotic was also used to express the antimicrobial resistance results. According to the production of virulence factors, Staphylococcal isolates from clinical, subclinical, or chronic mastitis were compared using the chi-squared test. The significance level was set at 0.05, the critical value $\chi 2$ was 2.206 for cows and 1.824 for ewes, and the testing value was G. Within each species, statistical independence between isolates with and without virulence factors was verified when $G > \chi 2$, although the independence was not statistically significant when assessing $G > \chi 2$.

3. Results

A thorough analysis of 960 dairy cows from four farms during the early lactation phase (14–100 days of lactation) revealed that 271 animals (28.2%) and 689 cows (71.8%), respectively, had CMT scores of trace or 1–3 for one or more quarters. 756 (80.4%) of the 940 ewes evaluated for udder health during the first month of the grazing season showed negative CMT results. One-hundred eighty-four ewes (19.6%) had positive CMT with a score trace of 1–3. Of the mixed milk samples taken from each examined cow and sheep based on the anamnesis and positive CMT score, bacterial agents causing a mastitis were identified in 230 (84.8%) and 155 (84.2%), respectively (**Figure 3**). For the presence of udder pathogens, 41 (15.1%) and 29 (15.7%) samples from examined cows and ewes with a positive CMT score were identified as negative or contaminated.

Based on the clinical examination of the MG, assessment of CMT, and laboratory diagnosis of milk samples, the occurrence of CM in the monitored cows' and sheep's dairy farms was 9.1% and 4.5%, respectively. The most common form of IMI in monitored cows and ewes was subclinical mastitis, with an incidence of 11.3% and 10.2%, respectively. The occurrence of chronic mastitis was 3.6% and 1.8% in monitored dairy cows and ewes, respectively. Of the cows' and ewes' positive samples, 136 and 86 cases (59.1% and 55.4% of the infected samples) contained the most commonly isolated Staphylococci, respectively (**Table 2**).

The CoNS represented the most commonly detected bacteria (42.6% and 39.9% of positive findings in cows and ewes), causing mainly subclinical mastitis. The *S. aureus* was the second most common pathogen (16.5% and 18.2% of positive findings in cows

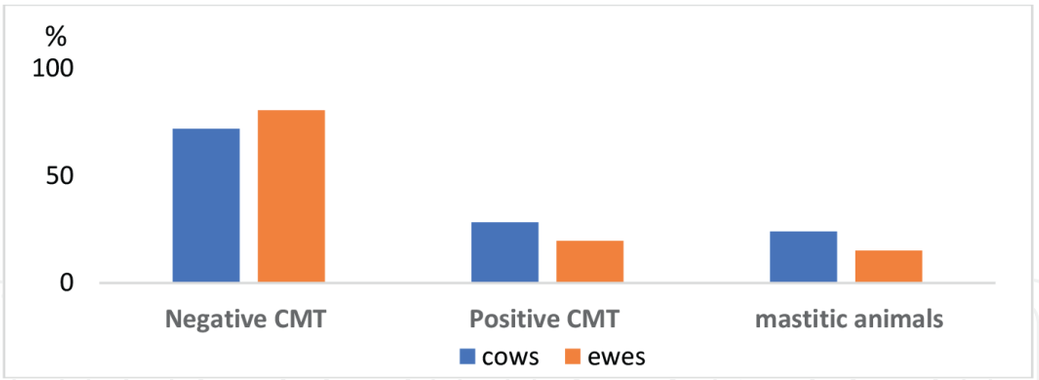

Figure 3.
Evaluation of CMT in monitored dairy herds.

and ewes, respectively), primarily causing clinical or chronic mastitis, followed by *E. coli*, streptococci, and enterococci (**Table 2**).

Tables 3 and **4** summarize, in descending frequency, the isolated strains of *Staphylococcus* spp., and indicate their role in the type of mastitis and the occurrence of selected virulence factors. Isolated *S. aureus* from clinical, chronic, or subclinical cases of mastitis has the highest ability to report virulence factors compared to CoNS and showed hemolysis in blood plates, production of gelatinase, biofilm, and the ability to hydrolyze DNA. The mecA gene was detected in two isolates of *S. aureus* from cows' clinical mastitis. Eight species of CoNS were isolated from mastitic cows, with the following recorded: *S. chromogenes* (22.4%), *S. warneri* (20.4%), *S. xylosus* (18.4%), *S. epidermidis* (9.1%), *S. haemolyticus* (7.1%), *S. hyicus* (10.2%), *S. capitis* (4.4%), and *S. piscifermentans* (4.4%) with testing value χ^2 = 2.206 for statistical significance. From mastitic ewes were isolated six species of CoNS with the following recorded: *S. warneri* (23.7%), *S. chromogenes* (18.6%), *S. xylosus* (18.6%), *S. haemolyticus* (15.2%), *S. caprae* (13.6%) and *S. epidermidis* (10.2%) with testing value χ^2 = 1.808 for statistical significance. From all the cows' and ewes' mastitic samples caused

Pathogens	**Cows**	**Ewes**	**Clinical[1] n/%**		**Subclinical n/%**		**Chronic n/%**	
	n/%	**n/%**	**cows**	**ewes**	**cows**	**ewes**	**cows**	**ewes**
CoNS	**98**/*42.6*	**59**/*39.9*	37/*16*	9/*6.1*	53/23.0	44/*29.7*	8/*3.5*	6/*4.1*
S. aureus	**38**/*16.5*	**27**/*18.2*	18/*7.8*	11/*7.4*	9/*3.9*	9/*6.1*	11/*4.7*	6/*4.1*
Escherichia coli	**26**/*11.2*	**18**/*12.2*	7/*3.0*	7/*4.7*	17/*7.4*	10/*6.7*	2/*0.9*	1/*0.7*
Str. uberis	**21**/*9.1*	**0**/0	9/*3.9*	0/0	5/*2.2*	0/0	7/*3.0*	0/0
Str. agalactiae	**8**/*3.4*	**4**/*2.7*	3/*1.3*	4/*2.7*	2/*0.9*	0/0	3/*1.3*	0/0
Streptococcus spp.	**10**/*4.3*	**9**/*6.0*	4/*1.7*	1/*0.7*	6/*2.6*	6/*4.1*	0/*0*	2/*1.4*
Enterococcus spp.	**14**/*6.1*	**24**/*16.2*	3/*1.3*	5/*3.4*	11/*4.8*	17/*1.5*	0/*0*	2/1.4
Mixed infection	**15**/*6.5*	7/*4.7*	6/*2.6*	5/*3.4*	5/*2.2*	10/*6.7*	4/*1.7*	0/0
Total	**230**/*100*	**155**/*100*	**87**/*37.8*	**42**/*27.1*	**108**/*46.9*	**96**/*62.0*	**35**/*15.2*	**17**/*11.0*

Clinical IMI[1] - clinical mastitis represented in mild, moderate, or severe forms of intramammary infection; n – number of mastitic animals. Modified from Zigo et al [9].

Table 2.
Pathogens isolated from milk samples of four monitored dairy cows and four sheep herds.

Staphylococcus *spp.*/number	IMI[1]/number	Hemolysins[2]	DNAse[3]	Gelatinase	Biofilm	*mecA* gene	Testing value
S. aureus (38)	clinical (22)	6α/4δ/1β	14	17	9	2	5.447*
	chronic (8)	3α/2δ/2β	8	7	7	0	
	subclinical (8)	3α/1β	6	7	5	0	
Coagulase-negative Staphylococci with significant production of virulence factors							
S. chromogenes (22)	clinical (11)	4β/3δ	3	4	4	0	3.204*
	chronic (4)	3β	1	1	2	1	
	subclinical (7)	2β/2δ	1	1	2	0	
S. warneri (20)	clinical (9)	4δ/2β	2	2	4	1	2.688*
	chronic (3)	3β	0	0	1	0	
	subclinical (8)	3β/1δ	2	0	2	0	
S. xylosus (18)	clinical (7)	2δ/2β	2	0	3	0	2.255*
	chronic (1)	0	0	0	0	0	
	subclinical (10)	4β/1δ	0	0	2	0	
Coagulase-negative Staphylococci without significant production of virulence factors							
S. epidermidis (9)	clinical (2)	1δ	0	0	1	0	1.012
	subclinical (7)	2δ	0	0	2	0	
S. haemolyticus (7)	clinical (4)	2β/1δ	1	0	2	0	0.742
	subclinical (3)	0	0	0	0	0	
S. capitis (6)	clinical (2)	2δ	0	0	0	0	0.401
	subclinical (4)	0	0	0	0	0	
S. piscifermentans (6)	clinical (2)	1β	0	0	1	0	0.851
	subclinical (4)	2δ	0	0	0	0	
S. hyicus (10)	clinical (0)	0	0	0	0	0	0.332
	subclinical (10)	1δ	0	0	1	0	

*Legend: IMI[1]: the number of isolates and their impact on the type of mastitis; hemolysins[2]: the production of hemolysin type α, β or δ; DNAse[3]: the capability of Staphylococci to hydrolyze DNA; *Chi-squared test significance level α= 0.05; critical value χ^2 = 2.206. In isolated Staphylococci, Testing value (G) and statistical independence of virulence factors were validated when G > χ^2; the independence was not statistically significant when G < χ^2. Modified from Zigo et al. [9].*

Table 3.
*The role of **S. aureus** and CoNS in the form of mastitis from infected cows and their virulence factors.*

by CoNS, 48 and 26 (48.9% and 44.1%) cases involved the production of hemolysins, 12 and 11 (12.2% and 18.6%) the hydrolysis of DNA, 8 and 12 (8.1% and 20.3%) the production of gelatinase, as well as 27 and 14 (27.5% and 23.7%) involved biofilm production.

In **Table 3**, the significance level of α = 0.05 was confirmed in the isolated Staphylococci *S. aureus, S. chromogenes, S. warner*, and *S. xylosus* from CM and chronic cows' mastitis, which, when compared to less virulent strains, has the highest representation of virulence factors (production of hemolysins, gelatinase, the ability to hydrolyze DNA, and biofilm). In addition, the *mecA* gene was confirmed in one chronic case of mastitis in *S. chromogenes* and one CM case in *S. warneri*. In isolated

Staphylococcus *spp.*/number	**IMI[1]/number**	**Hemolysins[2]**	**DNAse[3]**	**Gelatinase**	**Biofilm**	***mecA* gene**	**Testing value**
S. aureus (27)	clinical (11)	4α/2δ/2β	6	9	4	0	3.288*
	chronic (6)	2α/1β	3	6	4	0	
	subclinical (10)	4α/2β	4	8	3	0	
Coagulase-negative Staphylococci with significant production of virulence factors							
S. warneri (14)	clinical (3)	1α/1β	1	2	2	0	2.305*
	chronic (3)	2β	1	1	1	0	
	subclinical (8)	2α/2β/	3	4	3	0	
S. chromogenes (11)	clinical (2)	1β	0	0	1	0	1.824*
	chronic (2)	1β	1	1	1	0	
	subclinical (7)	3β/1δ	3	2	2	0	
Coagulase-negative Staphylococci without significant production of virulence factors							
S. xylosus (11)	clinical (1)	1β	1	0	0	0	1.140
	subclinical (10)	4α/2β	2	2	2	0	
S. haemolyticus (9)	clinical (2)	1β	0	0	0	0	0.435
	chronic (1)	0	0	0	1	0	
	subclinical (6)	3β	0	0	0	0	
S. caprae (8)	clinical (1)	1β	0	0	0	0	0.341
	subclinical (6)	0	0	0	0	0	
S. epidermidis (6)	subclinical (7)	0	0	0	1	0	0.215

*Legend: IMI[1] - number of isolates and their influence on type of mastitis; hemolysins[2] - production of hemolysin type α, β or δ; DNAse[3] - ability of Staphylococci to hydrolyze DNA; *Chi-squared test significance level α = 0.05; critical value χ^2 = 1.808; Testing value (G) and statistical independence of virulence factors in isolated Staphylococci was confirmed when $G > \chi^2$; the independence was not statistically significant when the testing value was $G < \chi^2$.*

Table 4.
*The role of **S. aureus** and NAS in the form of mastitis from infected ewes and their virulence factors.*

Staphylococci from mastitic ewes as demostrated in **Table 4**, the significance level in *S. aureus, S. warneri*, *S. chromogenes,* and *S. xylosus* was confirmed. The presence of the *mecA* gene has not been confirmed in tested *S. aureus* and CoNS.

In 136 and 86 isolates of Staphylococci from mastitic cows' and ewes' milk samples, *in vitro* resistance to 14 antimicrobials was tested by the standard disk diffusion method (**Table 5**). Generally, low resistance was shown to tetracycline, amoxicillin reinforced with clavulanic acid, rifaximin, and cephalexin. Of the tested Staphylococci, 95 and 38 isolates (70.0% and 44.2%) from mastitic cows and ewes showed resistance to one or more antimicrobials. To one antimicrobial, 50 and 22 isolates (36.7% and 25.6%) from mastitic cows and ewes were resistant. Mastitic cows and ewes produced 55 and 16 (39.7% and 18.6%) resistant Staphylococci isolates, respectively. Multidrug resistance to three or more antimicrobial classes was recorded in 16 and 4 isolates (11.7% and 4.7%) from cows' and ewes' samples. Tested Staphylococci showed multiresistance to a combination of antimicrobial classes, such as aminoglycosides, *β*-lactams, macrolides, and cephalosporins.

Number groups of antimicrobials	Phenotypic resistance profile	Cows (n = 136)		Ewes (n = 86)	
		No. of isolates	% of isolates	No. of isolates	% of isolates
0		41	30.1	48	55.9
1	PEN	7	5.1	4	4.7
1	STR	7	5.1	2	2.3
1	NMC	8	5.9	2	2.3
1	AMX	7	5.1	4	4.7
1	NVB	6	4.4	2	2.3
1	AMP	6	4.4	3	3.5
1	LNC	4	2.9	2	2.3
1	OXA	5	3.7	3	3.5
2	NMC, STR	8	5.9	2	2.3
2	OXA, NVB	0	0	2	2.3
2	OXA, TET	4	2.9	0	0
2	CPR, NVB	2	1.5	0	0
2	LNC, NVB	2	1.5	4	4.7
3	PEN, AMX, OXA	4	2.9	3	3.5
3	PEN, LNC, NVB	2	1.5	0	0
3	AMP, OXA, NMC	3	2.2	3	3.5
3	CPR, NMC, STR	4	2.9	0	0
3*	NVB, LNC, STR	4	2.9	2	2.3
4*	RFX, CPR, STR, TET	2	1.5	0	0
4*	CPR, LNC, NMC, NVB	3	2.2	1	1.2
4*	NVB, CPR, NMC, STR	2	1.5	0	0
4*	AMP, CEP, FOX, PEN	3	2.2	1	1.2
5*	OXA, AMP, LNC, NMC, STR	2	1.5	0	0
Total multidrug resistant isolates		16	11.8	4	4.7
Total antimicrobials resistant isolates		95	70.0	38	44.2

*Legend: *MDR: multidrug resistant isolates to three or more antimicrobial classes; AMX: amoxicillin, AMC: amoxicillin+clavulanat acid; AMP: ampicillin; CEP: cephalexin; CPR: ciprofloxacin; FOX: cefoxitin; LNC: lincomycin; NMC: neomycin; NVB:novobiocin; OXA: oxacillin; PEN:penicillin; RFX - rifaximin; STR:streptomycin; TET:tetracycline. Modified from Zigo et al. [9].*

Table 5.
Phenotypic resistance profile in isolates of ***Staphylococcus*** *spp. from mastitic cows and ewes.*

The 45 and 22 isolates (33.1% and 25.6% of all isolated Staphylococci) from mastitic cows and ewes in which phenotypic resistance was confirmed to β-lactam antimicrobials were tested by PCR for methicillin resistance with the detection of

the *mecA* gene. From positive cows' milk samples, four isolates of Staphylococci - two of *S. aureus*, one of each of *S. chromogenes* and *S. warneri*, and one of each - were shown to contain the *mecA* gene and to be resistant to both cefoxitin and oxacillin. The outcomes of our research indicated that these isolates were methicillin-resistant Staphylococci (MRS).

4. Discussion

Milk and milk products are important global dietary products, consumed by more than 6 billion people worldwide. The recorded milk consumption in 2019 was 852 million tons, distinguishing the dairy industry as a very profitable market [1]. However, an infection of the mammary gland caused mainly by bacteria, mastitis, is still a major problem affecting animal welfare, productivity, and the economy; especially in dairy production, which can lead to losses for the dairy industry [37]. The incidence of mastitis is, of course, highly dependent on the lactation stage and health status of dairy animals [29, 38].

During the first 100 days of lactation, we observed the prevalence and etiology of mastitis in four dairy farms with cows and ewes. The majority of cows on the farms and the ones who produce the most milk are those that are in this early lactation stage (14–100 days after calving). The dairy cow produces an amount of milk during the first 100 days of lactation that accounts for 42–45% of the total milk. Aside from hormonal changes, decreased feed intake (which is in contrast to increased milk production), increased lipomobilization with a negative energy balance, and changes in body condition score, cows are also subject to stress factors as a result of this heavy milk production burden [38].

All of the aforementioned risk factors have an impact on both the non-specific and specific immune systems, specifically the MG, via which pathogenic microorganisms from the environment can enter the body more easily. An elevated SCC is one sign of the start of intramammary infection [39]. The qualitative test used in practice to detect mastitis is CMT, which reflects changes in milk consistency and SCC. Based on anamnesis, evaluation of CMT and clinical examination 689 (71.7%) of the 960 examined dairy cows were negative while 271 cows (28.2) showed positive, with scores from 1 to 3, or trace CMT. 230 (84.9%) of 271 cows showing high SCC were positive for the isolation of udder pathogens. This constitutes a significant risk for individual and herd health due to the high risk of spreading the infection to the environment. On monitored sheep's farms during the first month of pasture season, 756 sheep (80.4%) a negative CMT and 184 animals (19.6%) had increased SCC on the basis of CMT score (**Figure 1**). Laboratory examination revealed that 136 samples (14.5%) were positive for the presence of an udder pathogen.

The development of infection often starts when pathogens enter the duct system, travel via the teat canal, interact with the mammary tissue, multiply, and spread throughout the functioning parts of the udder, such as the milk cisterns. The degree to which the udder tissue reacts to injury or infection largely determines how mastitis manifests [7]. The most clinical cases are manifested by increased body temperature, inappetence, redness, swelling, and/or painful udder and/or abnormal milk. In the subclinical forms that were most often confirmed in our study, there were no apparent clinical signs, but an increase in SCC was observed in milk. Of the 230 and 136 infected cows and ewes, 46.9% and 62.0% had subclinical, 37.8% and 27.1% had clinical, and 15.2% and 11.0% had chronic mastitis (**Table 2**).

The major economic and health issues caused by CM, according to Singha et al. [11], include decreased milk output, poorer milk quality, higher expenses for treatment, involuntary culling, early cow rejection, increased risk of antibiotic resistance, and decreased animal welfare. Therefore, in high-yield dairy cows, CM prevalence should be at its lowest level. Our results indicate that the prevalence of CM in monitored cows' dairy farms was 9.1% which is in contrast with the studies of Silva et al. [40] and Rahman et al. [10], who reported the prevalence of CM from 2.3% to 4.1% in lactating cows.

The incidence of mastitis in sheep farms is extremely variable. Fthenakis [41] found the occurrence of mastitis in sheep is between 4 and 50%. In our study, the incidence of mastitis at the beginning of the pasture season was 16.4% in monitored sheep herds, with the most frequently occurring subclinical form (11.5%). The occurrence of CM was 4.9%, which is considered an acceptable value. On the contrary, studies from British slaughterhouses reported a very high prevalence of CM, ranging from 13–50%. This suggests that CM, or chronic mastitis, is a major cause of the culling of ewes in the UK [42].

According to Wenz et al. [43] and our investigation, gram-positive bacteria (*Staphylococcus* spp. or *Streptococcus* spp.) are frequently the cause of CM in dairy ruminants. However, depending on the farm layout and cleanliness level, a significant number of cows and ewes with coliform mastitis develop bacteremia, and 20% of udder infections are brought on by gram-negative pathogens. This is in line with our findings, which showed that SM and CM brought on by *E. coli* accounted for 11.2% and 12.2%, respectively, of infections from all infected cows and ewes.

Pyörälä and Taponen [12] point to a much-increased risk of CM caused by *S. aureus* and CoNS in a Finnish investigation on the detection and etiology of mastitis, which was also confirmed in all monitored dairy herds. CoNS (42.6% and 39.9% of the 230 and 155 infected cows and sheep samples, respectively) and *S. aureus* (16.5% and 18.2%), which were found in 136 and 86 cases, respectively (59.1% and 55.5%), were the most frequently found. In the milk samples from mastitic cows and ewes, the isolates of *S. aureus* and CoNS of the CM were responsible for 7.8% and 7.4%, and 16.0% and 6.1%, respectively. However, due to ongoing IMI, *S. aureus*, *S. chromogenes*, *S. warneri*, and *S. xylosus* frequently caused chronic mastitis. According to the findings of our investigation, studies by Holko et al. [19] and Idriss et al. [25] found a similar incidence of clinical and chronic mastitis caused by *S. aureus* and certain CoNS in the investigated dairy farms. More than half of all clinical and chronic IMI were caused by Staphylococci occurring more frequently than other udder pathogens (**Table 2**).

Chronic IMI rather than new infections are assumed as suggested by Persson et al. [44]. It has been reported that cows and ewes showing IMI in early lactation stage were also positive during the previous lactation or when dried off. These can originate a persistent subclinical infection into a chronic mastitis in animals that turn immunocompromised after calving or lambing.

Our findings are consistent with Holko et al. investigation's [19], which found a significant incidence of Staphylococci (CoNS and *S. aureus*) identified from tainted milk samples from 42 dairy farms in western Slovakia. The most often found bacteria was the CoNS, which made up 35.9% of positive samples. In contrast to our findings, the authors also confirmed high resistance to aminoglycosides and β-lactam antimicrobials without the presence of methicillin resistance genes. The dominant CoNS strains identified from mastitis in dairy ruminants in recent years, according to many reports, are *S. haemolyticus*, *S. chromogenes*, *S. warneri*, and *S. xylosus* [45–47]. CoNS has been mainly isolated from CM in addition to subclinical forms of IMI [45],

which was validated in our investigation. CoNS-induced CM mastitis was associated with increased SCC, biofilm formation ability, and resistance to aminoglycosides and *β*-lactam antimicrobials, particularly penicillin, amoxicillin, and oxacillin.

The increasing prevalence of Staphylococcal infection in dairy ruminants is also influenced by the bacteria's level of pathogenicity and the production of certain virulence factors, which play a critical role in chronic and clinical mastitis cases [48, 49]. These contribute to the infection and include enterotoxins, different enzymes, and cell-associated factors. S. *aureus, S. chromogenes, S. warneri, S. xylosus*, and *S. haemolyticus* all produced hemolysins, hydrolyzed DNAse, and produced gelatinase from the various virulence factors. The isolated Staphylococci *S. aureus, S. chromogenes,* and *S. warneri* from mastitic cows and ewes had the most numerous representations of virulence factors, that may be contributing to the infection ability of isolated strains resulting in the increasing incidence of CM and persistent cases in comparison to strains with no virulence factors (**Tables 3** and **4**).

As biofilms promote Staphylococcal strains to adhere to both biotic and abiotic surfaces, they are regarded as having significant pathogenicity [48]. Bacteria generally produce a biofilm in order to protect themselves from fluctuations in environmental conditions. Substantial hygiene problems and economic losses are associated with biofilm formation in the dairy industry, as it can cause food spoilage and equipment impairment. The quality, quantity, and safety of food products are affected by the persistence of some foodborne pathogens on food contact surfaces and biofilms; and this problem has been reported more frequently [50]. Staphylococci are able to avoid immune defenses by creating biofilms that adhere to the MG epithelium, which leads to recurring or persistent infections [51]. Our findings indicated that seven species of NAS isolated from CM and chronic mastitis, as well as *S. aureus*, were mostly responsible for the biofilm-forming ability. The CoNS that produced chronic mastitis and CM showed the generation of hemolysins, the tendency to hydrolyze DNA, and resistance to antimicrobials as additional significant virulence factors in addition to *S. aureus*.

The relationship between hemolysins and biofilm formation, according to Perez et al. [49], can lessen the body's immunological response and response to antibiotic treatment while increasing Staphylococci interactions with bovine mammary epithelial cells. Our findings supported the idea that bacteria expressing these virulence characteristics had a high level of antibiotic resistance. In their study of Staphylococci isolated from mastitis milk in cows, Melchior et al. [51] indicated that the most frequent virulence factors in isolates recovered from CM were biofilm production and antibiotic resistance. Repeat episodes of mastitis following ineffective treatment showed increased biofilm production in CM strains. It is challenging to treat IMI brought on by *S. aureus* or CoNS even with intramammary antibiotics, therefore adequate care should be given to infections brought on by bacteria that produce biofilms.

The resistance to one or more antimicrobials in our study was detected in 95 and 38 isolates (77.0% and 44.2%) of Staphylococci isolated from infected cows and ewes, respectively. Multiresistant isolates for three or more groups of antimicrobial classes represented 16 and 4 isolates (11.8% and 4.7%). Multirresistance of staphylococci to a wide range of antibiotics such as β-lactams, macrolides, and cephalosporing (**Table 5**) was observed in our analysis. Methicillin resistance staphylococci were confirmed in 45 (33.1%) and 22 (25.6%) isolates from cows and ewes. By PCR the presence of the *mecA* gene was confirmed in two isolates of *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri*, only from mastitic cows. Oxacillin and cefoxitin resistance was present in all *mecA*-positive Staphylococci (n = 4; 2.9%), and these strains were categorized as MRS. When the entire genome was sequenced for a research by Khazandi et al. [22],

they discovered the presence of a *mecA* homolog in four oxacillin-resistant *S. sciuri* isolates. The homolog was not found using cefoxitin susceptibility testing or traditional *mecA* PCR. However, in our study, MRS was also phenotypically confirmed, so we do not assume the presence of a false positive *mecA* homolog.

The *S. aureus* and CoNS (n = 634; 36.7%) were the most frequently isolated bacteria from all tested samples in the study by Vyletelová et al. [52], which examined 1729 bulk milk and individual milk samples from ruminants in the Czech Republic. The species were also tested for the presence of the *mecA* gene using the PCR method and for antimicrobial susceptibility using the disc diffusion method. The most prevalent resistant strain was S. aureus (51%), followed by *S. epidermidis* (34.7%), and *S. chromogenes* (12.2%). A tital of 13 isolates of Staphylococci with *β*-lactam antibiotic resistance were found to have the mecA gene, which was primarily found in cow's milk. In a related investigation, Bogdanoviová et al. [53] tracked the prevalence and antibiotic resistance of *S. aureus* at 50 dairy farms in the Czech Republic. The authors found *S. aureus* positive in 58 samples from 261 raw milk and filtered milk samples, with 37 (14.2%) isolated from raw milk and 21 (8.1%) isolated from filtered milk. The majority of isolates from raw milk (17.8%) were found to be resistant to *β*-lactam antibiotics (amoxicillin and oxacillin), followed by isolates that were tetracycline- and macrolide-resistant. Methicillin-resistant *S. aureus* (MRSA) with the *mecA* gene present was found in two isolates from filtered milk and four isolates from raw milk samples using the PCR technique. We can affirm that IMI caused by Staphylococci, primarily *S. aureus*, with enhanced resistance to *β*-lactam antimicrobials is still a significant problem in Czech and Slovak dairy cow farms based on the findings of our study and the previous two investigations [52, 53]. The occurrence of MRS with the presence of the *mecA* gene is also worrying, which is in the range of 3–6% of isolates strains. In the monitored sheep, we did not record the presence of the *mecA* gene, which is probably a consequence of the higher culling of infected ewes with clinical and chronic mastitis and the renewal of herds with young sheep.

Among the resistant Staphylococci, *S. aureus* was identified by the WHO as the primary udder pathogen with the highest pathogenicity and most media attention. However, numerous other Staphylococci species have also been linked to methicillin resistance [54, 55]. In our work, we found the *mecA* gene to be present in two *S. aureus* isolates and one *S. chromogenes* and *S. warneri* strain. The CoNS is believed to be a reservoir for many resistance genes, which lead to greater resistance to antibiotics, according to Vinodkumar et al. [56]. The spread of resistance isolates may be caused, in part, by the presence of antimicrobials and their metabolites in the environment. This unfavorable effect of the heavy use of antimicrobials, along with delayed breakdown in the udder and drying out in cows (without antibiogram prior to application), maybe a contributing factor to rising resistance and MRS in veterinary medicine.

The MRS are usually resistant to *β*-lactam antimicrobials, and infections caused by these pathogens result in failed or frequent therapies, elevated SCC, and substandard milk quality. Studies from Norway revealed that MRSA has only ever been correlated to one case of cow mastitis when it comes to MRSA becoming the cause of the disease [23].

This contrasts with our findings and the current modeling in Belgium, where Bardiau et al. [56] revealed a comparable prevalence of MRSA in 4.4% of milk samples from clinical cases of mastitis and Vanderhaeghen et al. [57] identified MRSA in 9.3% of milk samples from farms relating with *S. aureus* mastitis, in contrast to our findings. Although our findings showed that the tested Staphylococci were more resistant to *β*-lactam antimicrobials than in previous studies, we can conclude that the occurrence of MRS in the monitored farms was roughly the same.

5. Conclusion

In dairy cows and ewes, Staphylococci and Streptococci were shown to be the most common causes of mastitis. Because of their virulence features, their prevalence poses a major risk to subsequent milk consumption. More than half of the mastitic cases from the cows and ewes under investigation were brought on by Staphylococci, particularly CoNS. Additionally, compared to other, less virulent CoNS strains, some strains of CoNS (*S. warneri, S. chromogenes*, and *S. xylosus*) with *S. aureus* isolated from clinical and chronic mastitis showed a high degree of pathogenicity in the synthesis of additional virulence factors. Resistance to aminoglycoside and *β*-lactam antimicrobials was frequently found in the tested Staphylococci, possibly because these are the antimicrobials most commonly used in dairy ruminant drying and mastitis treatment. Detection MRS by the presence of the *mecA* gene was confirmed in two isolates (2.9%) (one *S. aureus* and one isolate each of *S. chromogenes* and *S. warneri*) from mastitic cows. We can state that *S. aureus* still comes on top in the number of chronic or severe mastitis cases, as well as the number of virulence factors, but some CoNS species could have the same aggressive potential based on their production of gelatinase, hemolysis, biofilm, hydrolyzed DNA, and multidrug resistance.

According to the "*Farm to Fork*" strategy, the European Union intends to minimize the use of ATBs in cattle production by 50% by 2030 due to the frequent resistance of udder infections that cause mastitis and the occurrence of MRS in veterinary practice. Future use of antimicrobials during treatment in veterinary medicine and the dairy industry is still feasible, but only if it can be justified primarily in light of the findings of targeted diagnostics, which reveal each dairy animal's individual udder's physiological state through anamnestic data, clinical examination, SCC, and sample culture with an antibiogram. Designing effective prophylaxis and treatment guidelines to minimize the detrimental effects on milk yield and culling hazards in dairy animals requires knowledge of the virulence of both *S. aureus* and CoNS species associated with mastitis; particularly when combined with resistance patterns and the presence of MRS isolates.

Acknowledgements

The study was supported by Slovak grant **KEGA 009UVLF-4/2021**: *Innovation and implementation of new knowledge of scientific research and breeding practice to improve the teaching of foreign students in the subject of animal husbandry* and international **Visegrad Fund no. 22010056**: *Factors determining the occurrence of bovine mastitis in dairy herds situated in marginal regions.* The project is co-financed by the governments of Hungary, Poland, Czechia, and Slovakia through Visegrad Grants from the International Visegrad Fund.

Potential conflict of interest

The authors declare no conflict of interest.

DOI: http://dx.doi.org/10.5772/intechopen.108256

Author details

František Zigo[1*], Zuzana Farkašová[1], Ibrahim Farag Mohammed Rehan[2,3] and Ahmed Sayed-Ahmed[4]

1 Department of Nutrition and Animal Husbandry, University of Veterinary Medicine and Pharmacy, Košice, Slovakia

2 Faculty of Veterinary Medicine, Department of Husbandry and Development of Animal Health, Menoufia University, Menoufia, Egypt

3 Faculty of Pharmacy, Department of Pathobiochemistry, Meijo University, Aichi, Japan

4 Faculty of Veterinary Medicine, Department of Anatomy and Embryology, Menoufia University, Menoufia, Egypt

*Address all correspondence to: frantisek.zigo@uvlf.sk

References

[1] FAO. Dairy market review, [Internet]. 2020. Available from: https://www.fao.org/3/cb2322en/CB2322EN.pdf

[2] Augère-Granier ML. The EU dairy sector: Main features, challenges and prospects. Briefing EU. [Internet]. 2018. Available from: https://www.europarl.europa.eu/thinktank/en/document/EPRS_BRI(2018)63034

[3] Zigo F, Vasil M, Ondrašovičová S, Výrostková J, Bujok J, Pecka-Kielb E. Maintaining optimal mammary gland health and prevention of mastitis. Frontier in Veterinary Science. 2021;**8**:607311

[4] Hogeveen H, Huijps K, Lam TJGM. Economic aspects of mastitis: New developments. New Zealand Veterinary Journal. 2011;**59**:16-23

[5] O'Kennedy R, Murphy C. Immunoassays: Development, Applications and Future Trends. 1st ed. New York, USA: Jenny Stanford Publishing; 2017

[6] van Soest FJS, Santman-Berends IMGA, Lam TJGM, Hogeveen H. Failure and preventive costs of mastitis on Dutch dairy farms. Journal of Dairy Science. 2016;**99**:8365-8374

[7] Cobirka M, Tančin V, Slama P. Epidemiology and classification of mastitis. Animals. 2020;**10**:1-17

[8] Dufour S, Labrie J, Jacques M. The mastitis pathogens culture collection. Microbiological Resources Announcement. 2019;**8**(15):e00133-e00119

[9] Zigo F, Farkašová Z, Výrostková J, Regecová I, Ondrašovičová S, Vargová M, et al. Dairy cows' udder pathogens and occurrence of virulence factors in Staphylococci. Animals. 2022;**4**:1-18

[10] Rahman M, Bhuiyan M, Kamal M, Shamsuddin M. Prevalence and risk factors of mastitis in dairy cows. Veterinary. 2010;**26**:54-60

[11] Singha S, Koop G, Persson Y, Hossain D, Scanlon L, Derks M, et al. Incidence, etiology, and risk factors of clinical mastitis in dairy cows under semi-tropical circumstances in Chattogram, Bangladesh. Animals. 2021;**11**:1-17

[12] Pyörälä S, Taponen S. Coagulase-negative Staphylococci - Emerging mastitis pathogens. Veterinary Microbiology. 2009;**134**:3-8

[13] Vasiľ M, Farkasova Z, Elecko J, Illek J, Zigo F. Comparison of biofilm formation by Staphylococcus aureus and Staphylococcus epidermidis strains isolated from sheep milk using three diagnostic methods. Polish Journal of Veterinary Sciences. 2017;**20**:795-801

[14] El-Diasty M, Talaat H, Atwa S, Elbaz E, Eissa M. Occurrence of Coagulase-negative Staphylococcal mastitis in dairy cows. Mansoura Veterinary Medical Journal. 2019;**23**:35-39

[15] Monistero V, Graber HU, Pollera C, Cremonesi P, et al. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: Genotypes, detection of genes encoding different toxins and other virulence genes. Toxins (Basel). 2018;**10**(6):1-22

[16] Condas LAZ, De Buck J, Nobrega DB, Domonique AC, Roy JP, Greg PK, et al.

Distribution of non-aureus Staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. Journal of Dairy Science. 2017;**100**:5613-5627

[17] Ferroni L, Lovito C, Scoccia E, Dalmonte G, Sargenti M, Pezzotti G, et al. Antibiotic consumption on dairy and beef cattle farms of central Italy based on paper registers. Antibiotics. 2020;**9**:1-23

[18] Vasiľ M, Elečko J, Zigo F, Farkašová Z. Occurrence of some pathogenity factors in coagulase negative Staphylococci isolated from mastitis milk in dairy cows. Potravinarstvo Slovak Journal of Food Science. 2012;**6**:60-63

[19] Holko I, Tančin V, Vršková M, Tvarožková K. Prevalence and antimicrobial susceptibility of udder pathogens isolated from dairy cows in Slovakia. The Journal of Dairy Research. 2019;**86**:436-439

[20] Nascimento JS, Fugundes PC, Brito AV, Dos Santos KR, Bastos MC. Production of bacteriocins by coagulase-negative Staphylococci involved in bovine mastitis. Veterinary Microbiology. 2005;**106**:61-71

[21] De Buck J, Ha V, Naushad S, Nobrega DB, Luby C, Middleton JR, et al. Non-aureus Staphylococci and bovine udder health: Current understanding and knowledge gaps. Frontiers in Veterinary Science. 2021;**8**:1-16

[22] Khazandi M, Al-Farha AAB, Coombs GW, O'Dea M, Pang S, Trott DJ, etal.Genomiccharacterizationofcoagulase-negative Staphylococci including methicillin-resistant Staphylococcus sciuri causing bovine mastitis. Veterinary Microbiology. 2018;**219**:17-22

[23] Fergestad ME, Visscher A, De L'Abee-Lund T, Ngassam TC, Mainil G, Thiry JD, et al. Antimicrobial resistance and virulence characteristics in 3 collections of Staphylococci from bovine milk samples. Journal of Dairy Science. 2021;**104**:10250-10267

[24] Sampimon OC, Lam TJGM, Mevius DJ, Schukken YH, Zadoks RN. Antimicrobial susceptibility of coagulase-negative Staphylococci isolated from bovine milk samples. Veterinary Microbiology. 2011;**50**:173-179

[25] Idriss SE, Foltys V, Tančin V, Kirchnerová K, Zaujec K. Mastitis pathogens in milk of dairy cows in Slovakia. Slovak Journal of Animal Science. 2013;**46**:115-119

[26] Dorneles EMS, Fonseca MDAM, Abreu JAP, Lage AP, Brito MAVP, Pereira CR, et al. Genetic diversity and antimicrobial resistance in *Staphylococcus aureus* and coagulase-negative Staphylococci isolates from bovine mastitis in Minas Gerais, Brazil. Microbiologyopen. 2019;**8**:1-7

[27] Haveri M, Roslöf A, Pyörälä S. Virulence genes of bovine Staphylococcus aureus from persistent and nonpersistent intramammary infections with different clinical characteristics. Journal of Applied Microbiology. 2007;**103**:993-1000

[28] NRC. Nutrient Requirements of Dairy Cattle. 7th ed. Washington, USA: The National Academies Press; 2001. p. 381

[29] Tančin V, Mikláš Š, Čobirka M, Uhrinčať M, Mačuhová L. Factors affecting raw milk quality of dairy cows under practical conditions. Slovak Journal of Food Science. 2020;**14**:744-749

[30] NMC. National Mastitis Council Recommended Mastitis Control Program. Verona, WI: Natl. Mastitis

Counc. Ann. Mtg. Proc., Reno, NV. Natl. Mastitis Counc., Inc; 2001. p. 408

[31] Hiko A. DNase-cross-Coagulase test and antimi-crobial resistance test on Staphylococcus along beef abattoir line in Addis Ababa Ethiopia. Ethiopia Veterinary Journal. 2019;**23**:90-110

[32] Moraveji Z, Tabatabaei M, Shirzad AH, Khoshbakht R. Characterization of hemolysins of Staphylococ-cus strains isolated from human and bovine, southern Iran. Iranian Journal of Veterinary Research. 2014;**15**:326-330

[33] CLSI document M2-A13. Performance standards for antimicrobial disk susceptibility tests, Thirteenth informa-tional supplement. Clinical and Laboratory Standards Institute, Wayne, PA, USA. 2018. p. 92

[34] CLSI document M100–S31. Performance standards for antimicrobial susceptibility testing; Thirty–first informa-tional supplement. Clinical and Laboratory Standards Institute, Wayne, PA, USA. 2021. p. 350

[35] Hein I, Jorgensen HJ, Loncarevic S, Wagner M. Quantification of Staphylococcus aureus in unpasteurised bovine and caprine milk by real-time PCR. Research in Microbiology. 2005;**156**:554-563

[36] Poulsen AB, Skov R, Pallesen LV. Detection of methicillin resistance in coagulase-negative Staphylococci and in Staphylococci directly from simulated blood cultures using the EVIGENE MRSA Detection Kit. The Journal of Antimicrobial Chemotherapy. 2003;**51**:419-421

[37] Abera A. Prevalence and associated risk factors of bovine mastitis in lactating cows of small holder dairy farms in Ethiopia. Journal of Veterinary Medicine and Surgery. 2020;**4**(2):1-7

[38] Nitz J, Wente N, Zhang Y, Klocke D, Seeth M, Krömker V. Dry period or early lactation-time of onset and associated risk factors for intramammary infections in dairy cows. Pathogens. 2021;**10**:1-19

[39] Tančin V. Somatic cell counts in milk of dairy cows under practical conditions. Slovak Journal of Animal Science. 2013;**46**:31-34

[40] Silva AC, Laven R, Benites NR. Risk factors associated with mastitis in smallholder dairy farms in Southeast Brazil. Animals. 2021;**11**:1-10

[41] Fthenakis GC. Prevalence and aetiology of subclinical mastitis in ewes of Southern Greece. Small Ruminant Research. 1994;**13**(3):293-300

[42] Conington J, Cao G, Stott A, Bünger L. Breeding for resistance to mastitis in United Kingdom sheep, a review and economic appraisal. The Veterinary Record. 2008;**162**(12):369-376

[43] Wenz JR, Barrington GM, Garry FB. Bacteremia associated with naturally occuring acute coliform mastitis in dairy cows. Journal of the American Veterinary Medical Association. 2001;**219**:976-981

[44] Persson WK, Bengtsson B, Lindberg A, Nyman A, Ericsson UH. Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows- influence of breed and stage of lactation. Veterinary Microbiology. 2009;**134**:89-94

[45] Supre K, Haesebrouck F, Zadoks RN, Vaneechoutte M, Piepers S, De Vliegher S. Some coagu-lase-negative Staphylococcus species affect udder health more than others. Journal of Dairy Science. 2011;**94**:2329-2340

[46] Hosseinzadeh S, Saei HD. Staphylococcal species associated with bovine mastitis in the North West of Iran: Emerging of coagulase-negative Staphylococci. International Journal of Veterinary Science and Medicine. 2014;**2**:27-34

[47] Zigo F, Elecko J, Vasil M, Ondrasovicova S, Farkasova Z, Malova J, et al. The occurrence of mastitis and its effect on the milk malondialdehyde concentrations and blood enzymatic antioxidants in dairy cows. Veterinary Medicine. 2019;**64**:423-432

[48] Fredheim EGA, Klingenberg C, Rodhe H, Frankenberger S, Gaustad P, Flaegstad T, et al. Biofilm formation by Staphylococcus haemolyticus. Journal of Clinical Microbiology. 2009;**47**:1172-1180

[49] Pérez VKC, Da Costa GM, Sá Guimarães A, Heinemann MB, Lage AP, Dorneles EMS. Relationship between virulence factors and antimicrobial resistance in Staphylococcus aureus from bovine mastitis. Journal of Global Research. 2020;**22**:792-802

[50] Shemesh M, Ostrov I. Role of Bacillus species in biofilm persistence and emerging antibiofilm strategies in the dairy industry. Journal of the Science of Food and Agriculture. 2020;**100**:2327-2336

[51] Melchior MB, Van Osch MHJ, Lam TJGM, Vernooij JCM, Gaastra W, Fink-Gremmels J. Extended biofilm susceptibility assay for Staphylococcus aureus bovine mastitis isolates: Evidence for association between genetic makeup and biofilm susceptibility. Journal of Dairy Science. 2011;**94**:5926-5937

[52] Vyleťelova M, Vlkova H, Manga I. Occurrence and characteristics of methicillin resistant Staphylococcus aureus and methicillin resistant coagulase-negative Staphylococci in raw milk manufacturing. Czech Journal of Food Sciences. 2011;**29**:11-16

[53] Bogdanovičová K, Skočková A, Šťástková Z, Karpíšková R. Occurrence and antimicrobial resistance of Staphylococcus aureus in bulk tank milk and milk filters. Slovak Journal of Food Science. 2014;**8**:97-101

[54] Seixas R, Santos JP, Bexiga R, Vilela CL, Oliveira M. Short communication: Antimicrobial resistance and virulence characterization of methicillin-resistant Staphylococci isolates from bovine mastitis cases in Portugal. Journal of Dairy Science. 2014;**97**:340-344

[55] Vinodkumar K, Neetha N, Ashok S, Suchithra S, Justin D, Radhik S. Genotypic and phenotypic β-lactam resistance and presence of PVL gene in Staphylococci from dry bovine udder. PLoS One. 2017;**12**:1-19

[56] Bardiau MK, Yamazaki JN, Duprez B, Taminiau J, Mainil G, Ote I. Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. Letters in Applied Microbiology. 2013;**57**:181-186

[57] Vanderhaeghen W, Cerpentier T, Adriaensen C, Vicca J, Hermans K, Butaye P. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary Microbiology. 2010;**144**:166-171