# RUMINANT MASTITIS: A 360° VIEW

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# RUMINANT MASTITIS: A 360° VIEW

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# Editorial: Ruminant mastitis: A 360° view

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Editorial on the Research Topic
Ruminant mastitis: A 360° view

In dairy ruminants, mastitis represents one of the most serious health issues which can contribute to the reduction of milk production, high veterinary care costs, increased use of antibiotics, and animal culling, all of which may account for severe economic losses. Mastitis consists of the inflammation of the udder and is mainly caused by contagious and/or environmental microorganisms leading to overt clinical or sub-clinical cases. Management of mastitis in dairy ruminants faces various challenges: the resilience of high-yielding animals, poor efficacy of therapies and prevention (antibiotic resistance, dubious efficacy of vaccines), quality of milk, reduced availability of food and water, climate change, and difficulties in early diagnosis. These issues could provoke the interest of researchers and practitioners to suggest more affordable and effective control measures.

This Research Topic is comprised of 20 articles. These include review articles, original research, and reports concerning the biology of mastitis pathogens, a comparison of several diagnostic tools, diverse prevention strategies, alternative treatments to antibiotics, and the prevalence of mastitis-causing agents.

The study of pathogens, pathogenesis, and host-pathogen interaction could be helpful in the definition of new prevention or treatment protocols for combating mastitis. Non-Aureus Staphylococci (NAS) involves several species and strains that have become the most frequently isolated bacteria in milk. Non-Aureus Staphylococci are associated with mastitis, with variable severity of cases, and some species are considered commensal in the mammary gland. These aspects prompted the scientific community to investigate the biology, pathogenicity, epidemiology, and antibiotic resistance of bacteria member of the NAS group (De Buck et al.). In emerging countries, different genetic strains of *Staphylococcus aureus* (SA) have been found in milk samples. Some of these strains are known to be adapted to dairy ruminant species but a highly diffuse one is a human-adapted strain. As all SA strains carry different antibiotic resistance genes, their isolation from milk poses important zoonotic concerns (Ndahetuye et al.). In China *Helcococcus ovis*, a mastitis-causing agent in sheep, was isolated on a dairy cow farm. Four genetic strains were identified, all susceptible to the majority of antibiotics. *Helcococcus*

*ovis* caused mild mastitis in a murine model when it was the only bacteria causing infection, but it caused severe mastitis when co-infecting the murine mammary gland with *Trueperella pyogenes* (Liu et al.). The role of co-infection in mastitis has been poorly investigated but it reveals interesting aspects for the management and treatment of mastitis. For example, the role of a viral infection such as the Bovine Leukemia Virus (BLV), can impair the defenses in the mammary gland predisposing it to secondary bacterial infections (da Souza Lima et al.). The knowledge of the pathogenesis of each agent could help in the management of this pathological condition for the udder. The mechanism by which SA infection impairs the milk protein synthesis by mammary epithelial cells has been investigated by Chen et al. demonstrating the involvement of mTORC1, STAT5, NF-kB, and two amino acid transporters (SLC1A3 and SLC7A5). Another aspect is the debated capability of some mastitis agents to develop biofilms that could explain their persistence in the udder, resistance to antibiotic treatments, and the presence of some chronic infections (Pedersen et al.). Moreover, in small ruminants such as high milk production goats, genetics may play a role in certain histological features that increase the permeability of skin to milk ("Weeping Teats" or WT), but limited information was available regarding an increased risk of mastitis on animals with WT. Gazzola et al. demonstrated a positive association between high SCC and bacteria in milk from goats suffering from WT, but these variables were not associated with the WT condition.

Dairy ruminant farming should try to develop early diagnostic tools and mastitis prevention strategies. Mastitis can occur as sub-clinical, mild clinical, or severe clinical pathological conditions. Depending on its appearance, the diagnosis could encounter difficulties. Somatic cell count (SCC) is the most widely used indicator of intra-mammary infection (IMI), but depending on the number of days in milk (DIM), cow breed, or other factors, the SCC value is sometimes inconclusive. The definition of the differential SCC (DSCC) has been proposed as a further indicator of IMI. The automated DSCC has become available recently with two instruments. Halasa and Kirkeby, and Alhussien et al. discussed insights about the use of the DSCC. They suggest that the collective use of SCC, DSCC, and evaluation of phagocyte activity is certainly helpful in the discrimination of different mastitis forms. They discussed the advantages and disadvantages of the two instruments, one for field investigations and the other for laboratory investigations. Another study proposed chromogenic media for the rapid identification of bacteria causing subclinical mastitis (SCM). Garcia et al. demonstrated the capability of this chromogenic medium to identify the main Gram-positive bacteria responsible for subclinical mastitis, with the exception of *S. aureus*.

Despite the improvement of the technologies for the management of dairy herds and milking, combined with the amelioration of veterinary interventions, IMI still remains an important issue in the dairy industry. For this reason, measures of prevention play a pivotal role in the reduction of the risk of mastitis. As documented by Zigo et al., given the multifactorial nature of the pathological condition of mastitis, the prevention strategies should relate to the control of SCC, correct diet, proper housing and management, milking systems, drying strategies, and eventually, immunization protocols. Research in the mastitis prevention field is very dynamic and new tools are often being proposed. Kabera et al. after a comparison between selective dry cow antimicrobial treatment (SD) and blanket dry cow treatment (BD), suggested applying SD in regard to antibiotic use reduction. Bedding choice can influence mammary gland health. Fréchette et al. highlight the risk of *K. pneumoniae* mastitis when using recycled manure solids bedding. The efficacy of immunizing dairy ruminants against mastitis is debated, but efficacious vaccines could represent a strong defense for combating IMI. Tassi et al. describe the development of an efficacious intramammary vaccine against *Mannheimia haemolytica*, based on the activation of innate immune responses. Effective vaccination against mastitis is not available and the causes of the failure of this type of prevention still need to be investigated. New control strategies such as immunomodulation may be helpful for the future fight against mastitis.

The treatment of mastitis is based mainly on antibiotics but the widespread increase of antibiotic-resistant bacteria poses several concerns about their use and stimulates the search for new therapeutic strategies. As stated by Ruegg, there are very few studies that investigate the real efficacy of antibiotic treatment. In order to develop efficacious therapeutic protocols for the treatment of mastitis, it is important to evaluate several aspects such as the pathogen involved, activity spectrum, antibiotic resistance, and animal factors (parity, stage of lactation, previous mammary infections). Non-steroidal anti-inflammatory drugs (NSAID) alone have been proposed by Krömker et al. as an alternative treatment to antibiotics, but only in case of mild or moderate clinical mastitis AMPK/NrF2/Nf-kB signaling pathway has been suggested as a potential target for the treatment of *S. aureus* bovine mastitis by Arbab et al.

Mastitis control and prevention are based on continuous and accurate monitoring of the bacterial species prevalence and evaluation of their antibiotic resistance. In our collection, we included recent data about the prevalence of mastitis pathogens in Northwest Pakistan (Ali et al.), Ontario, Canada (Acharya et al.), and the prevalence of *Klebsiella* spp. in China (Liu et al.). The distribution of mastitis pathogens around the world suggests the need for the application of differential strategy programs to control the disease in different countries.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Differential Somatic Cell Count: Value for Udder Health Management

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Intramammary infection (IMI) can cause mastitis, which is one of the costliest and most prevalent diseases in dairy cattle herds. Somatic cell count (SCC) is a well-established parameter to indicate IMI, and it represents the total count of immune cells in the milk. The differential somatic cell count (DSCC) has also long been suggested to indicate IMI, but no machine was available until recently to provide this parameter automatically. Two new machines have recently been introduced to measure the milk DSCC as an additional indicator of IMI. Here we provide insights about the DSCC measured by these two machines and the value it may provide for udder health management, based on the available literature. We also provide perspectives for future research to investigate potential value in using the DSCC to improve udder health.

**Keywords: management, cow, dairy, mastitis, differential somatic cell count**

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## INTRODUCTION

Mastitis is the inflammation of the mammary gland. It causes welfare and economic damage for the dairy industry worldwide (1). During the past decades, large efforts have been invested to monitor, prevent and control mastitis in dairy cattle herds (2). Several indicators have been proposed to monitor the occurrence of mastitis in dairy cows on a routine basis, including the widely used individual cow somatic cell count (SCC), which represents the total number of immune cells in the milk (3). Several studies have additionally suggested the use of differential somatic cell count (DSCC), which distinguishes between the different immune cells in the milk [see **Table 1**]. Until recently, the use of DSCC as an indicator of IMI was limited to research studies due to the unavailability of machinery to produce this parameter on a large scale. However, a high throughput machinery for regular Dairy Improvement Health testing (15) and an on-farm machine (16) have recently become available, allowing large scale measurement of DSCC. This warrants the investigation of the usefulness of this parameter for udder health management in dairy cattle herds. Missing information can thereafter be obtained in future research.

We here provide insights into the DSCC based on available literature and the potential value it may hold for udder health management. We also provide perspectives for future research to investigate the potential value of using the DSCC in dairy herds.

**TABLE 1 |** Proportion of polymorphonuclear cells (PMN) lymphocytes (Lym) and macrophages (Mac) observed in different studies, under different definitions of udder health statuses and using different methods of determination of these proportions.

| References | Definition | Method | PMN | Lym | Mac |
|---|---|---|---|---|---|
| (4) | Healthy: no pathogen and SCC <10,000 cells/ml | Cytology—Microscopy | 10 | 67 | 23 |
| | 1 day post-inoculation with *Staphylococcus aureus* | Cytology—Microscopy | 51 | 28 | 21 |
| | 4–8 days post-inoculation with *Staphylococcus aureus* | Cytology—Microscopy | 39 | 38 | 24 |
| | 9–14 days post-inoculation with *Staphylococcus aureus* | Cytology—Microscopy | 32 | 43 | 26 |
| | Healthy: no pathogen and SCC <10,000 cells/ml | Flow cytometry | 18 | 72 | 10 |
| | 1 day post-inoculation with *Staphylococcus aureus* | Flow cytometry | 61 | 18 | 20 |
| | 4–8 days post-inoculation with Staphylococcus aureus | Flow cytometry | 46 | 25 | 29 |
| | 9–14 days post-inoculation with *Staphylococcus aureus* | Flow cytometry | 31 | 34 | 34 |
| (5)[a] | Healthy: SCC <100,000 cells/ml | Flow cytometry | 28 | 11 | 13 |
| | Acute *E. coli* | Flow cytometry | 38 | 1 | 4 |
| | Acute *Staphylococcus aureus* | Flow cytometry | 36 | 5 | 11 |
| | Chronic *Staphylococcus aureus* | Flow cytometry | 42 | 37 | 17 |
| | Chronic non-aureus Staphylococci | Flow cytometry | 49 | 18 | 13 |
| | Chronic *Streptococcus dysgalactiae* | Flow cytometry | 73 | 8 | 5 |
| (6) | SCC <50,000 cells/ml | Flow cytometry | 31 | 26 | 43 |
| | SCC between 50,000 and 100,000 cells/ml | Flow cytometry | 51 | 14 | 35 |
| | SCC between 100,000 and 200,000 cells/ml | Flow cytometry | 59 | 12 | 29 |
| | SCC between 200,000 and 400,000 cells/ml | Flow cytometry | 64 | 11 | 25 |
| | SCC >400,000 cells/ml | Flow cytometry | 67 | 9 | 24 |
| (7) | SCC <6,250 cells/ml | Flow cytometry | 15 | 63 | 22 |
| | SCC between 6,250 and 25,000 cells/ml | Flow cytometry | 17 | 59 | 24 |
| | SCC between 25,000 and 100,000 cells/ml | Flow cytometry | 23 | 50 | 27 |
| | SCC >100,000 cells/ml | Flow cytometry | 60 | 19 | 21 |
| (8) | SCC <6,250 cells/ml | Cytology—Microscopy | 12 | 56 | 32 |
| | SCC between 6,250 and 25,000 cells/ml | Cytology—Microscopy | 12 | 49 | 38 |
| | SCC between 25,000 and 100,000 cells/ml | Cytology—Microscopy | 52 | 31 | 17 |
| | SCC >100,000 cells/ml | Cytology—Microscopy | 78 | 12 | 10 |
| (9) | Healthy: No pathogen and SCC <100,000 cells/ml | Cytology—Microscopy | 43 | 34 | 23 |
| | Culture positive and SCC <100,000 cells/ml | Cytology—Microscopy | 56 | 23 | 21 |
| | Culture negative and SCC >100,000 cells/ml | Cytology—Microscopy | 63 | 16 | 21 |
| | Culture positive and SCC >100,000 cells/ml | Cytology—Microscopy | 68 | 12 | 20 |
| (10)[a] | Healthy: no pathogen and SCC <200,000 cells/ml | Flow cytometry | 28 | 58 | 9 |
| | Moderate mastitis but SCC <100,000 cells/ml | Cytology—Microscopy | 47 | 20 | 33 |
| | Moderate mastitis and SCC between 100,000 and 400,000 cells/ml | Cytology—Microscopy | 59 | 10 | 31 |
| | Severe mastitis but SCC <100,000 cells/ml | Cytology—Microscopy | 59 | 18 | 23 |
| | Severe mastitis and SCC between 100,000 and 400,000 cells/ml | Cytology—Microscopy | 71 | 9 | 20 |
| | Severe mastitis and SCC >400,000 cells/ml | Cytology—Microscopy | 82 | 5 | 13 |
| (11) | Healthy: low SCC and no pathogen | Flow cytometry | 43 | 30 | 27 |
| (12) | Healthy: SCC <100,000 cells/ml | Flow cytometry | 32 | 16 | 52 |
| | SCC >800,000 cells/ml | Flow cytometry | 49 | 18 | 33 |
| (13) | Healthy: no pathogen and SCC <100,000 cells/ml | Flow cytometry | 20 | 8 | 72 |
| | Infused with endotoxin | Flow cytometry | 78 | 2 | 19 |
| (14) | Healthy: no pathogen and SCC <100,000 cells/ml | Cytology—Microscopy | 28 | 25 | 47 |

[a] *Leucocyte proportions do not necessarily add to 1 because of measuring other cells in the original studies. The numbers were obtained from (6).*

## FUNCTION AND DYNAMICS OF IMMUNE CELLS MEASURED IN THE MILK

The somatic cell count represent the immune cells in the milk, which are mainly lymphocytes, polymorphonuclear neutrophils (PMN) and macrophages (15). The task of the lymphocytes is to regulate the initiation and suppression of the immune response, while the macrophages ingest bacteria and cellular debris (15, 17). In addition, macrophages recognize invading pathogens and trigger an immune reaction, by the rapid recruitment of PMN (15, 18, 19). The task of PMN is to attack the invading pathogen and defend the

mammary glands at the start of an acute inflammatory reaction (15, 19).

As immune cells have different functions, their distributions in normal and mastitic milk differs (20). In milk samples obtained from healthy udders, macrophages and lymphocytes dominate the total cells, while PMNs dominate the cell count in milk obtained from infected mammary glands or milk with high SCC (see **Table 1**).

The proportions of these different leucocytes change following infection. Once the mammary glands are infected, a surge in PMN can be observed [Rivas et al. (4) in **Table 1**] triggered by the resident cells (lymphocytes, macrophages, and epithelial cells) (21). A few days later, a reduction in the proportion of PMN can be observed, while macrophages eliminate bacteria and debris (4). Sordillo et al. (17) indicated that the severity and duration of a mastitis case is highly related to the promptness of the leucocytes migratory response and the bactericidal activities of the immune cells at the site of infection. If the cells move fast from the blood to the mammary gland and clear the bacteria, cell recruitments ceases. Nevertheless, if the bacteria are capable of surviving the immune reactions, the inflammation continues and may become severe, leading to damage to the mammary gland tissue, which causes production losses (17). The reduction of the proportion of PMN following infection indicates the end of the acute stage, which may result in complete bacterial elimination, in which SCC returns to healthy levels (17). An acute stage maybe followed by a chronic stage, if bacterial elimination is not complete. During this stage, the immune system continues to respond to the presence of bacteria, which is characterized by elevated proportions of PMN [see Leitner et al. (5) in **Table 1**] and SCC (21). The SCC during the chronic stage may fluctuate, but it will generally remain elevated (21).

## THE RELATIONSHIP BETWEEN DSCC AND SCC

As indicated above, infection of mammary glands results in an increase in SCC and DSCC, when the number and relative proportion of PMN increase (**Table 1**). Thus, a high correlation between SCC and DSCC is expected, as also shown earlier (6, 22). However, studies have shown that elevations in PMN can be observed at low SCC levels, indicating that active bacterial eliminations without noticeable surges in SCC may take place [see (7–9) in **Table 1**]. This indicates that more information about the dynamics of udder health on cow level can be obtained by monitoring the DSCC.

Traditionally, the proportions of the leucocytes in milk are measured by either counting cells under a microscope following cell isolation and staining, or using a flow cytometer [see details about both procedures in Rives et al. (4)]. Microscopic examination is perhaps the standard method, as the margins of error for identification of cells are low when carried out by trained persons. Nevertheless, it is cumbersome and can therefore only be used as a tool for research. Rives et al. (4) compared both methods and concluded that flow cytometry is a valid diagnostic approach that provides results comparable to those obtained from manual cytology. Koess and Hamman (6) concluded that flow cytometry could be used reliably to differentiate milk leucocytes and to determine the percentages of the different cell types. These earlier studies paved the way for the development of technology and machinery that utilizes flow cytometry to produce the DSCC in milk.

To our knowledge, two instruments are currently able to measure DSCC in milk samples, automatically. The first machine is produced by FOSS Analytical A/S (Hillerød, Denmark), and is a laboratory based machine that allows high throughput measurement of FOSS DSCC (referred to as F-DSCC throughout the manuscript to distinguish it from the regular DSCC, where the proportions of the different leucocytes are reported) from milk samples using flow cytometry (15). The F-DSCC represent the proportion of PMN and lymphocytes compared to the total number of PMN, macrophages and lymphocytes (15). The second machine (namely QScout) is produced by Advanced Animal Diagnostics (USA), and can be used for on-farm diagnostics. This machine measures the absolute values and proportions of the leucocytes (neutrophils, macrophages, and lymphocytes) in the milk using fluorescent microscopy (16). We refer to the outcome of this machine throughout the paper as Milk Leucocytes differentials (MLD) as presented earlier (23). It is important to point out that limited data is available based in this machine in the literature, perhaps because this machine has only recently been introduced.

## FACTORS AFFECTING DSCC

SCC is affected by cow factors such as days in milk (DIM) and lactation number [e.g., Græsbøll et al. (24)]. Dosogne et al. (10) showed that lymphocytes decreased over DIM, while macrophages and PMN increased. On the other hand, Pilla et al. (9) found that only macrophages were significantly influenced by DIM, without finding a significant effect of lactation number or quarter position. However, the authors showed that the percentage of individual cell populations was significantly affected by the tested herd. Furthermore, the herd also affected the ratio of the different cell populations. This could be driven by the differences in the distributions of IMI causing pathogens and levels of IMI between the herds. It could also be attributed to genetic differences (25). Another study showed no significant effect of parity and quarter position on the ratio of the different populations of cells (11).

Kirkeby et al. (26) found a significant association between F-DSCC and both DIM and lactation number. F-DSCC generally decreased over the course of the lactation, and the relationship between F-DSCC and lactation number was found ambiguous (26). Schwarz et al. (27) found no significant effect of lactation number on F-DSCC, while in a more recent study Schwarz et al. (28) reported a significantly higher F-DSCC for cows with lactation number $>4$ compared to younger cows. In the latter study, no significant association between F-DSCC and DIM was found. In addition, the authors found no significant effect of milk weight (kg milk produced) on F-DSCC (28). In another recent study, the authors found that parity and DIM were significantly

associated with F-DSCC (29). In addition, the effect of cow was particularly high (29), indicating that a large part of the variability is attributed to differences between cows. Using the MLD, Lozado-Soto et al. (30) found significant effects of breed, lactation, sampling day, sampling time and quarter position on the MLD measurements. By relating these factors to the MLD measurements, the authors argued that our understanding of the somatic cell count recruitment could be improved, though more studies are needed to validate the findings (30).

These results show that the relationship between DSCC and the key herd- and cow factors is not yet fully resolved, pointing out the need for further studies.

## DSCC IN HEALTHY AND IMI QUARTERS

Only four studies investigated the level of F-DSCC in healthy (no pathogen growth) and IMI quarters (**Table 2**). In healthy quarters (no pathogen was isolated), the F-DSCC varied between 30 and 78%, while F-DSCC varied between 35 and 96% for IMI quarters.

The MLD machine provides absolute and proportions of the milk leucocytes. Thus, these outcomes can be directly compared to leucocytes absolute values and proportions of milk obtained from healthy quarters (e.g., see **Table 1**) to indicate subclinical mastitis.

## DSCC AS INDICATOR FOR IMI

The relationship between DSCC and IMI causative pathogens has been studied also. Leitner et al. (5) found that the distributions of leucocytes in quarters infected with *Streptococcus dysgalactiae* did not differ from that in quarters that had acute IMI caused by *Staphylococcus aureus* or *Escherichia coli*. Furthermore, the authors found no significant difference in PMN proportions between quarters that had chronic IMI caused by *Staphylococcus aureus* or non-aureus staphylococci (NAS) compared to healthy quarters. Wall et al. (31) studied the reaction of F-DSCC to IMI induced by lipopolysaccharide (LPS) or lipoteichoic acid (LTA), which resembles an *Escherichia coli* and *Staphylococcus aureus* IMI, respectively. The authors found a sharp increase in the F-DSCC a few hours after treatment with LPS or LTA from, 60 to 80%, indicating a shift in cell populations toward PMN. This indicates that the F-DSCC is capable of representing the change in DSCC following IMI.

Kirkeby et al. (26) investigated the association between F-DSCC and healthy quarters or quarters with IMI caused by major, minor and other IMI causing pathogens. The authors found a significant difference in F-DSCC between healthy and IMI quarters, also when SCC was already taken into account. However, this effect was influenced by a herd effect and the IMI causative pathogen group. Similarly, Schwarz et al. (27) studied the efficacy of F-DSCC to indicate IMI at dry off, and found that quarters with IMI had significantly higher F-DSCC than healthy quarters. Nevertheless, no significant difference in F-DSCC was observed between quarters that had IMI caused by major and minor IMI causing pathogens. In a subsequent study, Schwarz et al. (28) found that F-DSCC was significantly higher in quarters with IMI caused by major pathogens compared to healthy quarters and quarters with IMI caused by minor pathogens. In addition, the authors found that the F-DSCC response differed for different NAS pathogens (28). Another recent study investigated the association between F-DSCC and the IMI status of cows with SCC <50,000 cells/ml (32). For these cows, the authors found no significant association between F-DSCC and the IMI status.

Schwarz et al. (28) calculated the sensitivity and specificity of F-DSCC to indicate IMI. Using an F-DSCC cut-off value (threshold value to indicate IMI) of 60%, the sensitivity and specificity were estimated to 87.42 and 67.26%, respectively (28). Increasing the cut-off increased the sensitivity, but it reduced the specificity (28). The sensitivity could be further improved by combining the selection based on F-DSCC with SCC, as initially intended for the F-DSCC use (15), but this also

**TABLE 2 |** Values of differential somatic cell count in healthy and intramammary infected quarters calculated using the DSCC machine produced by FOSS analytical A/S (F-DSCC).

| References | Udder health status | Value of DSCC |
|---|---|---|
| (26) | Uninfected quarters—Herd 1 | Mean = 65% |
| | Infected with minor pathogen—Herd 1 | Mean = 68% |
| | Infected with major pathogen—Herd 1 | Mean = 71% |
| | Infected with other pathogen—Herd 1 | Mean = 73% |
| | Uninfected quarters—Herd 2 | Mean = 68% |
| | Infected with minor pathogen—Herd 2 | Mean = 73% |
| | Infected with major pathogen—Herd 2 | Mean = 74% |
| | Infected with other pathogen—Herd 2 | Mean = 74% |
| (27) | Uninfected quarters | Mean = 78%, std = 2%[a] |
| | Infected with minor pathogen | Mean = 88%, std = 2% |
| | Infected with major pathogen | Mean = 90%, std = 2% |
| (28) | Uninfected quarters | Mean = 30%, std = 3%[b] |
| | Infected with minor pathogen | Mean = 35%, std = 3.3% |
| | Infected with major pathogen | Mean = 68%, std = 3.5% |
| | Infected with other pathogen | Mean = 34%, std = 3.7% |
| (31)[c] | Uninfected quarters—Group A | 52–60% |
| | Infected quarters with lipopolysaccharides—Before infusion (healthy status) | 54–61% |
| | Infected quarters with lipopolysaccharides—after infusion (infected) | 69–94% |
| | Infected quarters with lipoteichoic acid—before infusion (healthy status) | 45–47% |
| | Infected quarters with lipoteichoic acid—after infusion (infected) | 54–96% |

[a] *Statistically different than infected quarters.*
[b] *Statistically different than infected quarters with major pathogens.*
[c] *Experimental study.*

reduced the specificity (28). Similar findings were reported by Schwarz et al. (27).

The sensitivity and specificity of the MLD using single or duplicate milk samples were measured by Godden et al. (23). The sensitivity varied between 25 and 86%, while the specificity varied between 32 and 95%, depending on the setting of the machine to optimize sensitivity or specificity. Nevertheless, the authors recommended the use of a cow sample (pooled milk sample from the four quarters of the udder) as it improved the sensitivity (23). Comparable values of the sensitivity and specificity were found by Lozado-Soto et al. (30).

Using a bioeconomic simulation model, Gussmann et al. (33) estimated the added value of using F-DSCC to indicate subclinical IMI when SCC is already known. In the simulated scenarios, the farmer selected cows based on F-DSCC and/or SCC for bacterial culturing and subsequent treatment and culling decisions in case of subclinical IMI. The authors found that using F-DSCC additionally to SCC for the selection would not affect the economic outcomes or the IMI status in the herd, but it would result in lower antibiotic usage.

## DSCC AND MILK PRODUCTION

One study investigated the association between milk production and different udder health groups based on the combination of F-DSCC and SCC (34). The authors classified cows based on their SCC and F-DSCC status into low F-DSCC and SCC (Group A), high F-DSCC and low SCC (Group B), high F-DSCC and SCC (Group C) and low F-DSCC and high SCC (Group D). They found that cows in groups B, C, and D produced 0.9–2.4%, 6–9.8%, and 17.5–38.5% less milk than cows in group A, respectively. The same approach was used by Bobbo et al. (35) to study the association between milk yield and composition and SCC combined with F-DSCC. The authors found that cows in group B had slightly higher milk production than those in group A, while cows in groups C and D had lower milk production than those in groups A and B. The picture was opposite for fat and protein production, perhaps due to a concentration effect as a result of the reduced milk production (35). In another recent study, Zecconi et al. (32) found no significant association between milk production and F-DSCC for cows with SCC $<$50,000 cells/ml.

## STRENGTHS AND LIMITATIONS OF DSCC FOR APPLICATION TO THE FIELD: PERSPECTIVES

As presented above and in **Table 1**, numerous studies have been conducted to investigate the ability of DSCC to indicate IMI. These studies focused on the theoretical understanding of the behavior of the different leucocytes in milk as a response to IMI, in order to establish the usefulness of differentiating these cells to indicate IMI. The work has shown evidence that differentiating milk leucocytes can indicate IMI.

The F-DSCC machine provides a measure of the DSCC in one value. It can be used for high throughput, and hence it provides the possibility to monitor cows regularly, which makes it quite practical and attractive for register-based applied research. The MLD machine on the other hand provides several measurements, which may give more insight into the reactions of the immune system toward infection. The MLD machine is not designed to provide high throughput analyses, which may limit its use as a tool to regularly monitor cows in dairy herds. However, it can be used on farm and hence measurements can be obtained for specific cows when desired.

The F-DSCC parameter is capable of indicating IMI during lactation and at dry off (26, 28) and may react differently between IMI with major and minor pathogens (26). Nevertheless, comparing values from four different studies (**Table 2**), F-DSCC may vary largely in both healthy and IMI quarters. This and the fact that several factors may affect F-DSCC, makes it difficult to set simple thresholds to discriminate healthy from IMI quarters using F-DSCC based on the currently available litterature. Therefore, further research is needed to establish IMI detection using F-DSCC both with and without SCC, and the optimal cut-off values should be determined, to facilitate the use of these parameters for udder health management. In addition, influential cow- and herd factors should also be considered when these cut-off values are proposed, as they may aid in providing cow- and herd-specific decision support to manage udder health. Gussmann et al. (33) used a threshold of 62% to identify cows with subclinical mastitis for treatment and/or culling decisions and showed that using F-DSCC combined with SCC (threshold of 200,000 cells/ml) can improve the selection of the cows and result in reduced antibiotics usage, while improving udder health. Further work investigating the effectiveness of using these parameters to select cows for dry cow therapy is needed. This may aid in further reduction of antibiotics usage in dairy cattle herds. In addition, the value of F-DSCC for breeding should be investigated thoroughly, as recent work showed that it may have the potential to improve udder health (25). Nevertheless, the authors recommended further work to quantify the weight that F-DSCC should receive in breeding programs to improve udder health (25).

The MLD machine provides proportions and absolute values of the milk leucocytes (16). This can provide insight into the immune system reactions toward IMI causing pathogens (30), and hence improve our understanding of how to tackle IMI caused by the different pathogens; e.g., when treatment is needed. Nevertheless, a better understanding of these relationships based on these measurements is necessary before recommendations can be made. In addition, production effects in relation to these measurements should be established, to allow conducting economic assessments based on these measurements. This will also facilitate their use from a practical standpoint. Thus, more work is needed to inform how the MLD measurements should be used to manage udder health and what value this may bring.

Finally, it is important that future research focuses on conducting field studies comparing management practices with and without using the DSCC, in order to validate the usefulness of this parameter under field conditions and allow translating research findings into the field. This would aid in the establishment of general guidelines for the use of DSCC to improve udder health in dairy cattle herds.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## REFERENCES

1. Halasa T, Huijps K, Østerås O, Hogeveen H. Economic effects of bovine mastitis and mastitis management: a review. *Vet. Q.* (2007) 29:18–31. doi: 10.1080/01652176200796952 24
2. Ruegg PL. A 100-year review: mastitis detection, management and prevention. *J. Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds2017-13023
3. Pyörälä S. Indicators of inflammation in the diagnosis of mastitis. *Vet. Res.* (2003) 34:565–78. doi: 10.1051/vetres:2003026
4. Rivas AL, Quimby FW, Blue J, Coksaygan O. Longitudenal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry and cytology. *J. Vet. Diagn. Invest.* (2001) 13:399–407. doi: 10.1177/104063870101300506
5. Leitner G, Shoshani E, Krifucks O, Chaffer M, Saran A. Milk leukocyte population patterns in bovine udder infection of different aetiology. *J. Vet. Med B.* (2000) 47:581–9. doi: 10.1046/j.1439-0450.200000388x
6. Koess C, Hamann J. Detection of mastitis in the bovine mammary gland by flow cytometry at early stages. *J. Dairy Res.* (2008) 75:225–32. doi: 10.1017/S0022029908003245
7. Schwarz D, Diesterbeck US, König S, Brügemann K, Schlez K, Zschöck M, Wolter W, Czerny CP. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *J. Dairy Sci.* (2011) 94:5033–44. doi: 10.3168/jds2011-4348
8. Schwarz D, Diesterbeck US, Konig S, Brugemann K, Schlez K, et al. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *J. Dairy Res.* (2011) 78:448–55. doi: 10.1017/S0022029911000574
9. Pilla R, Schwarz D, König S, Piccinini R. Microscopic differential cell counting to identify inflammatory rections in dairy cow quarter milk samples. *J. Dairy Sci.* (2012) 95:4410–20. doi: 10.3168/jds2012-5331
10. Dosogne H, Vangroenweghe F, Mehrzad J, Massart-Leen AM, Burvench C. Differential leukocyte count method for bovine low somatic cell count milk. *J. Dairy Sci.* (2003) 86:828–34. doi: 10.3168/jdsS0022-0302(03)73665-0
11. Pilla R, Malvisi M, Snel GGM, Schwarz D, König S, Czerny CP, Piccinini R. Differential cell count as an alternative method to diagnose dairy cow mastitis. *J. Dairy Sci.* (2013) 96:1653–60. doi: 10.3168/jds2012-6298
12. Wever P, Emanuelson U. Effects of systematic influences and intramammary infection on differential and total somatic cell counts in quarter milk samples from dairy cows. *Acta Vet. Scand.* (1989) 30:465–474.
13. Ostensson K. Total and differential leucocyte counts, N-acetyl-ß-Dglucosaminidase activity, and serum albumin in foremilk and residual milk during endotoxin-induced mastitis in cows. *Am. J. Vet. Res.* (1993) 54 231–8. doi: 10.1016/j.prevetmed.2020.105123
14. Merle R, Schröder A, Hamann J. Cell function in the bovine mammary gland: a preliminary study on interdependence of health and infected udder quarters. *J. Dairy Res.* (2007) 74:174–9. doi: 10.1017/S002202990600238X
15. Damm M, Holm C, Blaabjerg M, Bro MN, Schwarz D. Differential somatic cell count—a novel method for routine mastitis screening in the frame of dairy herd improvement testing programs. *J. Dairy Sci.* (2017) 100:4926–40. doi: 10.3168/jds2016-12409
16. Goncalves JL, Lyman RL, Hokett M, Rodriguez R, Santos MVD, Anderson KL. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. *J. Dairy Res.* (2017) 84:309–317. doi: 10.1017/S0022029917000267
17. Sordillo LM, Shafer-Weaver K, DeRosa D. Immunobiology of the mammary gland. *J. Dairy Sci.* (1997) 80:1851–65. doi: 10.3168/jdsS0022-0302(97)76121-6
18. Paape MJ, Mehrzad J, Zhao X, Detilleux J, Burvenich C. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *J. Mammary Gland Biol. Neoplasia.* (2002) 7:109–21. doi: 10.1023/A:1020343717817
19. Oviedo-Boyso J, Valdez-Alarcon JJ, Cajero-Juarez M, Ochoa- Zarzosa A, Lopez-Meza JE, Bravo-Patino A, Baizabal- Aguirre VM. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *J. Infect.* (2007) 54:399–409. doi: 10.1016/j.jinf.200606010
20. Nickerson SC. Immunological aspects of mammary involution. *J Dairy Sci.* (1989) 72:1665–78. doi: 10.3168/jdsS0022-0302(89)79278-X
21. Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky F, Gonzalez RN. Monitroing udder health and milk quality using somatic cell counts. *Vet. Res.* (2003) 34:579–96. doi: 10.1051/vetres:2003028
22. Pillai SR, Kunze E, Sordillo LM, Jayarao BM. Application of differential inflammatory cell count as a tool to monitor udder health. *J. Dairy Sci.* (2001) 84:1413–20. doi: 10.3168/jdsS0022-0302(01)70173-7
23. Godden AM, Royster E, Timmerman J, Rapnicki P, Green H. Evaluation of an automated milk leukocyt differential test and California mastitis test for detecting intramammary infection in early- and late-lactation quarter and cows. *J. Dairy Sci.* (2017) 100:6527–44. doi: 10.3168/jds2017-12548
24. Græsbøll K, Kirkeby C, Nielsen SS, Halasa T, Toft N, Christiansen LE. Models to estimate lactation curves of milk yield and somatic cell count in dairy cows at the herd level for the use in simulations and predictive models. *Front. Vet. Sci.* (2016) 3:115. doi: 10.3389/fvets201600115
25. Bobbo T, Penasa M, Cassandro M. Short communication: genetic aspects of milk differential somatic cell count in Holstein cows: a preliminary analysis. *J. Dairy Sci.* (2019) 102:4275–9. doi: 10.3168/jds2018-16092
26. Kirkeby C, Toft N, Schwarz D, Farre M, Nielsen SS, Zervens L, et al. Differential somatic cell count as an additional indicator for intramammary infections in dairy cows. *J. Dairy Sci.* (2019) 103:1759–75. doi: 10.3168/jds2019-16523
27. Schwarz D, Lipkens Z, Piepers S, De Vliegher S. Investigation of differential somatic cell count as a potential new supplementary indicator to somatic cell count for identification of intramammary infection in dairy cows at the end of the lactation period. *Prev. Vet. Med.* (2019) 172:104803. doi: 10.1016/j.prevetmed2019104803
28. Schwarz D, Santschi DE, Durocher J, Lefebvre DM. Evaluation of the new differential somatic cell count parameter as a rapid and inexpensive supplementary tool for udder health management through regular milk recording. *Prev. Vet. Med.* (2020) 181:105079. doi: 10.1016/j.prevetmed2020105079
29. Zecconi A, Zanini L, Cipolla M, Stefanon B. Factors affecting the patters of total amount and proportions of leukocytes in bovine milk. *Animals*. (2020) 10:992. doi: 10.3390/ani10060992
30. Lozado-Soto E, Maltecca C, Anderson K, Tiezzi F. Analysis of milk leukocyte differential measures for use in management practices for decreased mastitis incidence. *J. Dairy Sci.* (2020) 103:572–82. doi: 10.3168/jds2019-16355

31. Wall SK, Wellnitz O, Bruckmaier RM, Schwarz D. Differential somatic cell count in milk before, during and after lipopolysaccharide- and lipoteichoic-acid-induced mastitis in dairy cows. *J. Dairy Sci*. (2018) 101:5362–73. doi: 10.3168/jds2017-14152
32. Zecconi A, Dell'Orco F, Vairani D, Rizzi N, Cipolla M, Zanini L. Differential somatic cell count as a marker for changes of milk composition in cows with very low somatic cell count. *Animals*. (2020) 10:604. doi: 10.3390/ani10040604
33. Gussmann M, Kirkeby C, Schwarz D, Farre M, Halasa T. A simulation study to investigate the added value in using differential somatic cell count as an additional indicator for udder health management in dairy herds. *Prev. Vet. Med*. (2020) 182:105090. doi: 10.1016/j.prevetmed2020105090
34. Schwarz D, Kleinhans S, Reimann G, Stükler P, Reith F, et al. Investigation of dairy cow performance in different udder health groups defined based on a combination of somatic cell count and differential somatic cell count. *Prev. Vet. Med*. (2020) 183:105123. doi: 10.1016/j.prevetmed2020105123
35. Bobbo T, Penasa M, Cassandro M. Combining total and differential somatic cell count to better assess the association of udder health status with milk yield, composition and coagulation properties in cattle. *Ital. J. Anim. Sci*. (2020) 19:697–703. doi: 10.1080/1828051X20201784804

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frontiers in Veterinary Science

REVIEW
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# What Is Success? A Narrative Review of Research Evaluating Outcomes of Antibiotics Used for Treatment of Clinical Mastitis

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Treatment of clinical mastitis is the most common reason that antimicrobials are given to adult dairy cows and careful consideration of treatment protocols is necessary to ensure responsible antimicrobial stewardship. Clinical mastitis is caused by a variety of bacteria which stimulate an immune response that often results in spontaneous bacteriological clearance but can develop into long-term subclinical infections. Use of antimicrobial therapy is most beneficial for cases that are caused by pathogens that have a low rate of spontaneous cure but high rate of therapeutic cure. The purpose of this paper is to review studies that evaluated outcomes of antimicrobial therapy of clinical mastitis. Few studies reported differences in bacteriological cure among treatments and this outcome was rarely associated with clinical outcomes. Return to normal milk appearance was evaluated in most studies but demonstrated little variation and is not a reliable indicator of therapeutic success. Somatic cell count should be measured at quarter-level and will decline gradually after bacteriological clearance. Few researchers have evaluated important clinical outcomes such as post-treatment milk yield or culling. Few differences among approved antimicrobial therapies have been demonstrated and selection of antimicrobial therapy should consider the spectrum of activity relative to etiology.

**Keywords: dairy, antibiotics, bacteria, veterinary, mastitis**

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## INTRODUCTION

Mastitis is the most common bacterial disease of mature dairy cows (1–3) and is diagnosed based on recognition of an inflammatory response initiated after the immune system detects intramammary infection (IMI). Like most bacterial diseases, the magnitude of the inflammatory response is dependent on virulence of the pathogen and is regulated by the ability of the host to mount a rapid and effective immune response (4). A mild inflammatory response results in an influx of neutrophils into the gland without any visible changes in the gland (subclinical disease) whereas a larger inflammatory response results in observable localized or generalized signs (clinical mastitis). Subclinical mastitis is the most common outcome of IMI and is defined by enumeration of somatic cells in milk. When somatic cell count (SCC) of milk exceeds a healthy threshold (usually > 150,000 or 200,000 cells/mL) (5) the gland is considered to be subclinically infected. Milk of cows affected with subclinical mastitis has a normal appearance and can be co-mingled with milk from healthy cows and sold for consumption, so treatment of subclinical mastitis during lactation is rarely recommended (6, 7).

Clinical mastitis is diagnosed when the magnitude of the inflammatory response is sufficient to result in visible changes in the milk, the mammary gland or the cow. The incidence of clinical mastitis is estimated to range between about 17–40 cases per 100 cows per year (8–12). Clinical mastitis is often assessed based on severity of the presenting signs and only a small proportion of cows have acute systemic disease that requires immediate therapy (13–16). The appearance of clinical signs and the necessity of discarding abnormal milk results in strong motivation for farmers to effectively treat clinical cases (17). In the U.S., most cases of are treated with IMM products containing 1st or 3rd generation IMM cephalosporins without knowledge of etiology (8). Selection of antimicrobials was reported to be based on "historical effectiveness" (92% of farmers), "veterinary recommendation" (66%), "historical culture and sensitivity results," (33%) or individual cow culture before treatment (22%). The definition of "historical effectiveness" was not supplied and is illustrative of the ambiguities associated with evaluation of mastitis treatments. Oliveira et al. (18) reported treatment of 589 cases of clinical mastitis occurring on 51 Wisconsin dairy farms. Most cases were treated solely with IMM antibiotics but about 30% received either a second IMM antibiotic or were treated with both IMM and systemic antibiotics. In that study, farmers collected milk samples before treatment and later submitted them to a research laboratory. The culture results demonstrated that 32% of IMM antibiotics were administered to clinical cases that were bacteriologically negative before treatment and an additional 19% of IMM antibiotics were given to cases caused by *E. coli*. In these herds, symptomatic treatment of non-severe cases of clinical mastitis without determination of etiology resulted in over-prescription of antibiotics in almost 50% of cases. More recently, treatment data was reported for >26,000 cases of clinical mastitis occurring on 40 large Wisconsin dairy herds (2, 19). Based on review of computerized health records, the incidence of clinical mastitis on these farms was 34% and use of antimicrobials varied greatly. About 31% of cases were not treated using antimicrobials, while 53% received approved products containing IMM ceftiofur, 10% received IMM cephapirin, 3% each were treated with IMM hetacillin or pirlimycin and <1% received IMM amoxicillin. Systemic antibiotics were given to 14% of cases on 29 farms. The average duration of treatment using IMM antibiotics ranged from 3.3 to 5.7 d. There was no indication that efficacy varied among treatments.

Symptomatic treatment without knowledge of etiology results in unnecessary antimicrobial treatments (such as use of antimicrobials for treatment of culture negative cases) (18) and it is impossible to determine etiology without use of diagnostic tests (such as culturing or DNA based technologies). Knowledge of etiology is fundamental to prescribe an appropriate treatment and is necessary to properly evaluate outcomes. Pathogens vary in virulence and possess differing abilities to stimulate an immune response that may result in spontaneous bacteriological cure. Differences in bacterial cell wall structures result in differing susceptibilities to antimicrobials, and most antibiotics approved for treatment of mastitis have limited ability to inhibit or destroy Gram-negative bacteria. Exposure to mastitis pathogens varies among herds but overall, the distribution of etiologies on modern dairy farms is fairly consistent. When milk samples from quarters affected with clinical mastitis are properly collected and assessed, the results are typically distributed as no growth (15–30%), Gram-negative (20–30%), gram-positive (20–25%), and 10–15% other pathogens (*Prototheca* spp, *Serratia* spp., yeasts and others) (3, 10, 12, 18, 20, 21). While there are some exceptions, it is difficult to justify the use of antimicrobial therapy for treatment of non-severe cases of clinical mastitis that are culture negative (15, 16, 22–24) or Gram-negative (25–27) and inclusion of these cases in positively controlled efficacy studies may result in over-estimating the impact of antimicrobial therapy. Thus, pathogen-specific evaluation of therapeutic outcomes is strongly recommended.

Use of culture-based protocols to guide selective therapy have been shown to be cost effective and result in more judicious antimicrobial usage (16, 24, 28) but some researchers have created economic models suggesting that delayed therapy may have negative consequences for herds that have a significant proportion of clinical mastitis caused by Gram-positive organisms (29). Recommendations for treatment are frequently based on outcomes defined in clinical trials but relatively few clinical trials have been performed to generate evidence based protocols. On farms, treatment efficacy is often judged by the speed of return to normal appearance of milk, but this outcome has little variation and is not a good indicator of therapeutic success (30) so an understanding of the importance of other outcomes is needed. The purpose of this paper is to review clinical trials that were conducted to assess antimicrobial treatments of clinical mastitis and discuss the strengths and limitations of outcomes used to evaluate therapeutic success.

## CONSIDERATIONS WHEN EVALUATING MASTITIS TREATMENTS

### Spectrum of Activity

When evaluating outcomes of mastitis therapy, the spectrum of activity of the antimicrobial should be considered relative to the etiology of the cases enrolled in the study. In most countries, a variety of IMM antimicrobials are approved and veterinarians in some countries have access to systemically administered drugs that are able to penetrate mammary gland barriers (21). Most approved antimicrobials are relatively narrow spectrum (target Gram-positive organisms) but in some countries, broader spectrum products such as 3rd and 4th generation cephalosporins and some quinolones are approved. Dairy farmers in the U.S. have access to 7 approved IMM antimicrobial products but no systemic products are approved for treatment of clinical mastitis (limited extralabel usage of some products is allowed). One approved IMM product is classified as a lincosamide (pirlimycin) while 6 IMM products are classified as beta-lactams. The beta-lactams include 1st (cephapirin) and 3rd (ceftiofur) generation cephalosporins, aminopenicillins (amoxicillin and hetacillin), penicillin G, and a penicillinase-resistant penicillin (cloxacillin) (31). All approved IMM products are expected to have efficacy against most common Gram-positive mastitis pathogens and most are labeled

as efficacious against *Streptococci* and *Staphylococci*. While there is limited research to differentiate among products, based on the antimicrobial classes, it is unlikely that there are significant differences among approved IMM products in efficacy for treatment of common Gram-positive pathogens. Use of antimicrobials for treatment of non-severe mastitis caused by *E. coli* is generally not needed (26) because these cases have a high rate of spontaneous cure (25, 26), thus very few cases of clinical mastitis benefit from use of a broader spectrum antimicrobials.

## Intrinsic and Acquired Resistance

Before antimicrobials are approved for treatment of mastitis they are required to demonstrate efficacy for pathogens that are listed on the product label. Before antimicrobials are used to treat pathogens that are not listed on the label, the possibility of intrinsic resistance should be considered. Intrinsic resistance occurs when a bacterial genus or species lacks targets or possess defenses to render an antimicrobial ineffective. For example, Gram-negative bacteria are intrinsically resistant to pirlimycin because their cell walls lack a binding site. Most Gram-negative bacteria are intrinsically resistant to penicillin G, many *Klebsiella* spp. are intrinsically resistant to aminopencillins and intrinsic resistance to ampicillin and 1st and 2nd generation cephalosporins are common in *Enterobacter* spp. (32). Two IMM products (ceftiofur and hetacillin) have label claims that include efficacy for *E. coli,* but no products have efficacy claims for treatment of mastitis caused by *Klebsiella* spp. Some studies evaluating antimicrobial therapies of mastitis have included cases that are intrinsically resistant to the product (33, 34) and in these instances outcomes cannot be attributed to antimicrobial therapy. Knowledge of etiology is necessary to ensure that the spectrum of activity of an antimicrobial is appropriate for the case.

Acquired resistance refers to acquisition of resistance by a normally susceptible bacterial strain through some kind of genetic modification and is usually recognized by bi-modal distribution of minimum inhibitory concentration (MIC) values. Monitoring acquired resistance is useful to identify the potential of transmission of genetic determinants of resistance into environments and food systems. While there is little evidence that most mastitis pathogens found in N. American dairy herds have acquired widespread resistance to most IMM antimicrobials (35), a bimodal distribution of MIC values was observed for about one-third of *Klebsiella pneumoniae* included in a recent trial (25) and susceptibility of pathogens that are not included on product labels should be monitored on a regular basis.

## Accounting for Cow Factors

Therapeutic success is driven by both pathogen factors and cow characteristics (30, 36–38). Effective bacterial clearance depends on a robust immune response and factors such as parity, stage of lactation, and history of previous clinical or subclinical mastitis cases should be considered when evaluating efficacy of treatments administered for clinical mastitis.

# METHODS USED TO EVALUATE RESEARCH ABOUT CLINICAL MASTITIS TREATMENT

## Inclusion of Trials in This Review

Studies included in this review were retrieved by searching databases and web platforms using PubMed, Web of Science, and Google Scholar. Addition studies were added by reviewing bibliographies of relevant papers. Boolean search terms were used and included mastitis, bovine, clinical, randomized, non-inferiority and treatment. Only natural exposure clinical trials that utilized randomized or systematic allocation to evaluate antimicrobial treatments and were published since 2000 in English language journals were retrieved. Only studies from the last 20 years were included because there have been considerable changes in the distribution of pathogen from contagious organisms (such as *S. aureus* and *S. agalactiae*), to a more diverse mix of environmental organisms (5), use of antimicrobials is increasingly discouraged and management of clinical mastitis has gradually shifted to selective therapy of clinical cases using on-farm culture.

Studies that did not define the antimicrobial therapy or included only non-antimicrobial therapies were excluded. Studies evaluating homeopathic or herbal treatments were also excluded. While thorough, the search was not systematic and although no studies that met inclusion criteria were excluded, it is likely that some qualifying studies were missed.

Despite the global importance of bovine mastitis, relatively few clinical trials that evaluated antimicrobial treatments of clinical mastitis were identified (**Table 1**). While a systematic review or meta-analysis is the ideal method to summarize and compare studies, the wide diversity of study designs, variation in outcomes, differences in pathogens and treatment protocols included in mastitis trials creates a challenging situation relative to use of this method. The limited number of trials evaluating antimicrobial therapy used for treatment of bovine mastitis has been previously noted by authors of a systematic review who were unable to identify sufficient papers to establish networks to evaluate bacteriological cure and were unable to reach a conclusion about clinical efficacy of antimicrobials (59). Studies included in this review were conducted in the U.S. or Canada ($n = 9$), European Union ($n = 8$), New Zealand ($n = 6$), Brazil ($n = 2$) and Mexico ($n = 1$). The 26 studies included 65 study arms that included IMM therapy containing a single antimicrobial ($n = 28$), IMM therapy containing combination products [$n$ (60) $= 13$], combined IMM and systemic therapy ($n = 5$), systemic therapy only ($n = 11$), non-antibiotic treatments ($n = 2$) and no treatment (negative control; $n = 6$). Antibiotic classes included penicillins and extended spectrum penicillins, penicillin and aminoglycoside combinations, 1st−4th generation cephalosporins, lincosamides, a lincosamide combined with an aminoglycoside, tetracycline combined with several other classes, fluoroquinolones and macrolides. No studies were replicated, and a variety of outcomes were reported.

**TABLE 1 |** Description of 26 clinical mastitis treatment trials published since 2000 and meeting inclusion criteria for this review.

| Citation, Location & Cases (n) | Approximate distribution of pathogens[a] | Treatments evaluated |
|---|---|---|
| **NEGATIVELY CONTROLLED RANDOMIZED CLINICAL TRIALS** | | |
| Roberson et al. (39)<br>USA, 85 cases | 12% NG[b]<br>40% Gram negative<br>33% *Streptococcus* spp.<br>3% *S. aureus*<br>12% other | 1. Amoxicillin IMM for 1.5 d<br>2. Frequent Milk out only for 3 d @ 4 time per d<br>3. Amoxicillin IMM (1.5 d) + Frequent milk out<br>4. No antibiotic nor frequent milk out |
| Suojala et al. (27)<br>Finland, 132 cases | 100% *E. coli* | 1. Enrofloxacin – systemic for 2 d<br>2. Ketoprofen – oral for 1–3 d |
| Schukken et al. (40)<br>USA, 104 cases | 47% *E. coli*<br>39% *Klebsiella* spp.<br>14% *Enterbacter* spp. | 1. Ceftiofur IMM for 5 d<br>2. No treatment |
| Persson et al. (41)<br>Sweden, 56 cases | 100% *E. coli* | 1. Enrofloxacin – Systemic for 3 d<br>2. No treatment |
| Fuenzalida and Ruegg (22)<br>USA, 121 cases | 100% NG | 1. Ceftiofur IMM for 5 d<br>2. No treatment |
| Fuenzalida and Ruegg, (25)<br>USA, 168 cases | 47% *E. coli*<br>46% *Klebsiella pneumoniae*<br>8% other | 1. Ceftiofur IMM for 2 d<br>2. Ceftiofur IMM for 8 d<br>3. No Rx |
| **POSITIVELY CONTROLLED TRIALS - NOT TESTING A NON-INFERIORITY HYPOTHESIS** | | |
| Erskine et al. (34)<br>USA, 104 cases<br>(only severe cases) | 20% NG<br>54% Gram negative<br>13% *Streptococcus* spp.<br>0% *S. aureus*<br>13% other | 1. Ceftiofur SYS for 5 d + IMM pirlimycin for 3 d<br>2. IMM pirlimycin for 3 d<br>All cows received supportive fluids at initiation of treatment and anti-inflammatories |
| Wraight (42)<br>New Zealand, 416 cases | 12% NG<br>8% Gram negative<br>34% *Streptococcus* spp.<br>18% *S. aureus*<br>28% other | 3. Cefuroxime IMM for 1.5 d<br>4. Cloxacillin IMM 1 tube Q 48 h for 3 total Rx |
| McDougall (43)<br>New Zealand, 404 cases | 51% NG<br>2% Gram negative<br>30% *Streptococcus* spp.<br>5% *S. aureus*<br>12% other | 1. Lincomycin/Neomycin IMM for 1.5 d<br>2. Penicillin/streptomycin IMM for 1.5 d |
| Taponen et al. (44)<br>Finland, 117 cases | 100%<br>β-lactamase neg *S. aureus* | 1. Penicillin G IMM for 4 d<br>2. Penicillin G/neomycin IMM for 4 d<br>Both groups also received systemic PPG on day 1 |
| Serieys et al. (45)<br>France, 227 cases | 18% NG<br>28% Gram negative<br>18% *Streptococcus* spp.<br>13% *S. aureus*<br>23% other | 1. Penethemate systemic for 3 d<br>2. Cloxacillin/Ampicillin IMM for 3 d |
| Taponen et al. (46)<br>Finland, 166 cases | 100% *S. aureus* | 1. B-lactamase neg: Systemic Penicillin for 5 d & IMM Penicllin/neomycin for 4d<br>2. B-lactamase neg: Systemic Pencillin for 5 d<br>3. B-lactamase pos: Systemic Amoxicillin/Clavulanic acid for 5d & IMM Amoxicillin/clav.acid/prednisolone for 4 d<br>4. B-lactamase pos: Sys. Spiramycin for 5 d |
| Wenz et al. (33)<br>USA, 144 Cases | 0% NG<br>68% Gram negative<br>15% *Streptococcus* spp.<br>0% *S. aureus*<br>31% other | 1. Pirlimycin IMM for 2 d<br>2. Pirlimycin IMM for 2 d & systemic Ceftiofur for 3 d<br>3. Cephaparin IMM for 3 d (2x/d)<br>4. Cephaparin IMM for 3 d (2x/d) & systemic Ceftiofur for 3 d |
| Bradley and Green (47),<br>EU, 491 cases | 4% NG<br>25% Gram negative<br>36% *Strep sp.*<br>12% *S. aureus*<br>23% other | 1. Cefalexin/Kanamycin IMM for 2 d<br>2. Cefoperazone IMM for 2 d<br>3. Cefquinome IMM for 1.5 d |

*(Continued)*

**TABLE 1 |** Continued

| Citation, Location & Cases (n) | Approximate distribution of pathogens[a] | Treatments evaluated |
|---|---|---|
| Swinkels et al. (48) EU, 206 cases | 100% *S. aureus* | 1. Cefquinome IMM for 1.5 d<br>2. Cefquinome IMM for 5 d |
| Kalmus et al. (49) Estonia, 140 cases | 0% NG<br>0% Gram negative<br>71% *Streptococcus* spp.<br>6% *S. aureus*<br>23% other | 1. BenzylPenicillin systemic for 5 d<br>2. BenzylPenicillin IMM for 5 d |
| Truchetti et al. (50) Canada, 241 cases | 32% NG<br>9% Gram negative<br>20% *Streptococcus* spp.<br>26% *S. aureus*<br>13% other | 1. Ceftiofur IMM for 2 d<br>2. Ceftiofur IMM for 5 d |
| Cortinhas et al. (51) Brazil, 264 cases | 50% NG<br>10% Gram negative<br>22% *Streptococcus* spp.<br>8% *S. aureus*<br>10% other | 1. Ceftiofur IMM for 4d (moderate cases also received prednisolone)<br>2. Tetracycline/neomycin/bacitracin/prednisolone IMM for 4d |
| Viveros et al. (52) Mexico, 292 cases | 38% NG<br>18% Gram negative<br>13% *Streptococcus* spp.<br>9% *S. aureus*<br>22% other | 1. Enrofloxacin suspension IMM for 3 d<br>2. Enrofloxacin powder IMM for 3 d<br>3. Ceftiofur IMM for 3 d<br>4. Enrofloxacin systemic for 3 d |
| McDougall et al. (53) New Zealand, 304 cases | 23% NG<br>4% Gram negative<br>55% *Streptococcus* spp.<br>5% *S. aureus*<br>13% other | 1. Amoxicillin/clavulanic acid IMM for 1.5 d<br>2. Amoxicillin/clavulanic acid IMM for 2.5 d |
| **POSITIVELY CONTROLLED TRIALS – TESTING A NON-INFERIORITY OR "EQUIVALENCY" HYPOTHESIS** | | |
| McDougall et al. (54) NZ, 1,561 cases | 23% NG<br>1% Gram negative<br>38% *Streptococcus* spp.<br>17% *S. aureus*<br>18% other | 1. Penicillin G IMM for 1-1.5 d<br>2. Cefuroxime IMM for 1-1.5 d<br>3. Pencillin/streptomycin IMM for 1-1.5 d |
| McDougall et al. (21) New Zealand, 662 cases | 0% NG<br>0% Gram negative<br>79% *Streptococcus* spp.<br>6% *S. aureus*<br>15% other | 1. Penethemate systemic for 3 d<br>2. Tylosin systemic for 3 d |
| Schukken et al. (55) USA, 296 cases | 28% NG<br>25% Gram –<br>23% *Streptococcus* spp..<br>3% *S. aureus*<br>21% other | 1. Cephaparin IMM for 1 d<br>2. Ceftiofur IMM for 5 d |
| Vasquez et al. (56) USA, 588 cases | 36% NG<br>22% Gram negative<br>22% *Streptococcus* spp.<br>8% *S. aureus*<br>12% other | 1. Ceftiofur IMM for 5 d<br>2. Hetacillin IMM for 3 d |
| Bryan et al. (57) New Zealand, 458 Cases | 0% NG<br>3% Gram negative<br>58% *Streptococcus* spp.<br>27% *S. aureus*<br>12% other | 1. Penicillin/cloxacillin IMM for 3 d<br>2. Tetracycline/oleandromycin/neomysin/prednisolone IMM for 3 d |
| Tomazi et al. (58) Brazil, 346 cases | 30% NG<br>12% Gram negative<br>18% *Streptococcus* spp.<br>10% *S. aureus*<br>30% other | 1. Cephaparin/prednisolone IMM for 2 d<br>2. Tetracycline/neomycin/bacitracin/prednisolone IMM for 2 d |

*[a]Overall enrollment estimated from overall etiologies reported in results, subsets of data were often used to calculate various outcomes; [b]No growth on culture.*

## Study Designs Included in This Review

Mastitis efficacy trials are challenging to perform and use different study designs. Some trials are performed using experimental challenge where cows are purposefully infected using a well-described bacterial strain (61, 62). Experimental challenge studies are useful for answering narrow research questions, but natural exposure trials are preferred for evaluating treatments and no challenge studies were included. Natural exposure trials randomly allocate cows to treatments and compare outcomes to either a non-treated control group or a positive control group. Non-treated ("negatively-controlled") control groups are considered the gold standard for determining efficacy and are able to determine if treatments improve ("are superior to") outcomes as compared to non-treated cows (or cows treated with a placebo). The null hypothesis in a superiority trial states that treatments are equally effective while the alternative hypothesis states that they differ. The inclusion of non-treated animals allows the determination of spontaneous cure so the additional benefit of treatment can be determined. Welfare concerns about not treating cows affected with mastitis have limited the number of negatively controlled trials that have been performed and only 6 of the 26 trials included in this review were negatively controlled. Only one of the negatively controlled studies included Gram-positive pathogens (39).

Positively controlled clinical trials are frequently used to assess mastitis treatments (**Table 1**) and can be designed to demonstrate superiority or non-inferiority. Of positively-controlled studies included in this review ($n = 20$), 6 were specifically designed to test non-inferiority, 1 stated that they were testing a superiority hypothesis, while the remainder ($n = 13$) compared outcomes among treatments but did not define the type of hypothesis that they were testing. The lack of a non-treated control group makes it impossible to separate treatment effects from spontaneous cures and superiority is rarely demonstrated. The null hypothesis in a non-inferiority trial states that the treatments differ while the alternative hypothesis states that they are non-inferior. Non-inferiority studies, include a pre-defined margin of non-inferiority for each outcome (usually 15%) and conclude that the new treatment is superior, non-inferior, inconclusive or inferior to the comparison treatment (55, 63). It is important to recognize that the inclusion of culture-negative cases in trials will skew the results toward positive outcomes (regardless of treatment) as the majority of these cases have achieved spontaneous bacteriological cure at the time of detection (22, 23). Mathematical realities dictate that inclusion of a large proportion of culture-negative and non-severe Gram-negative cases in non-inferiority trials will almost always result in a finding of non-inferior unless the "true-efficacy" of one of the products is very low. Of 6 non-interiority trials included in this review, 4 enrolled cases regardless of etiology (including culture-negative) and all 6 concluded that the "new treatment" was non-inferior (or inconclusive) to the comparator product. All of the non-inferiority trails included in this review evaluated commercially available products which infers that the drug approval process resulted in an acceptable level of efficacy for at least some outcomes. Outcomes of non-inferiority trials should always be evaluated relative to the distribution of pathogens enrolled in the study with emphasis on the number of enrolled cases that would likely be within the spectrum of activity of the products being compared.

# OUTCOMES EVALUATED TO DETERMINE EFFICACY

Clinical trials can include both microbiological and clinical outcomes but other than bacteriological cure (BC), there is little consistency in outcomes that are reported (**Table 2**). In the studies included in this review, BC was reported by 23 of 26 studies. Other outcomes include new intramammary infections (NIMI) (reported in 6 studies), clinical cure (24 studies), recurrence of another clinical case (4 studies), post-treatment SCC (14 studies), post-treatment milk yield (6 studies), culling (8 studies), and miscellaneous other outcomes (such as measures of inflammation or variations of BC). Publication bias does not seem to have influenced these trials as only half of the studies reported a significant difference among treatment groups for at least 1 outcome.

## Bacteriological Cure and New Intramammary Infection

The purpose of antibiotic treatment is to enhance clearance of bacterial pathogens and treatment efficacy is initially evaluated based on estimates of BC. This outcome is very relevant for approving new products but is rarely evaluated in a clinical setting. Bacteriological cure is assessed by comparison of recovery of bacteria from milk samples collected when the case is detected to recovery of the same isolate from milk samples collected at various intervals after treatment is completed. Sampling strategies and intervals used to define BC vary among studies (**Table 3**). Some researchers defined BC based on results of a single post-treatment milk sample, while other studies require the absence of the causative pathogen in multiple samples (usually collected at 7-d intervals). In the included studies, apparent BC ranged from about 27–95%, but it is important to recognize that comparisons among studies are not accurate due to differences in the distribution of pathogens and sampling periods. The overall means and ranges of BC were 69% (27–95%; $n = 35$), 68% (33–91%; $n = 13$) and 60% (38–87%; $n = 6$) for all IMM antimicrobial therapies, systemic or systemic and IMM therapies combined or no antimicrobial treatment, respectively.

Bacteriological cures result from the combined effect of the host immune response (spontaneous cure) and the effect of treatment (treatment cure) and the value of antimicrobial therapy is greatest for pathogens that have a low rate of spontaneous cure and high rate of treatment cure (such as IMI caused by *S. agalactiae*). Among mastitis pathogens, expected rates of spontaneous bacteriological cure vary widely. The greatest contrast is between expectations of spontaneous bacteriological cure of IMI caused by *S. aureus* (close to zero) and *Escherichia coli* (about 90%) (25, 26). Limited efficacy of antibiotic therapy is well-documented for IMI caused by *S. aureus* (38, 64) and some pathogens lack targets for antimicrobial therapy (e.g., yeast, *Prototheca bovis*, *Mycoplasma* spp. and others) and are considered to be intrinsically resistant to all approved

**TABLE 2 |** Outcomes evaluated in 26 clinical mastitis treatment trials.

| Study | Bact. cure | New IMI | Clinical cure | SCC | Recurrence | Milk yield | Culling |
|---|---|---|---|---|---|---|---|
| **NEGATIVELY CONTROLLED RANDOMIZED CLINICAL TRIALS** | | | | | | | |
| Roberson et al. (39) | NSD[a] | NSD | NSD | SIG[b] | Defined as CC NSD | SIG | |
| Suojala et al. (27) | NSD | | NSD at day 21 | No stats | | | NSD |
| Schukken et al. (40) | SIG | | SIG | NSD | | NSD | SIG |
| Persson et al. (41) | | | | SIG | | | |
| Fuenzalida and Ruegg (22) | | NSD | NSD | NSD | NSD | NSD | NSD |
| Fuenzalida and Ruegg (25) | SIG | | NSD | NSD | NSD | NSD | NSD |
| **POSITIVELY CONTROLLED TRIALS – NOT TESTING A NON-INFERIORITY HYPOTHESIS** | | | | | | | |
| Erskine et al. (34) | | | NSD | | | | |
| Wraight (42) | NSD | | NSD | | | | |
| McDougall (43) | NSD | | SIG | NSD | SIG | NSD | |
| Taponen et al. (44) | NSD | | NSD | | | | |
| Sérieys et al. (45) | NSD | | NSD | SIG | | | |
| Taponen et al. (46) | SIG | | NSD | | | | |
| Wenz et al. (33) | NSD | | | | NSD | | NSD |
| Bradley and Green (47) | NSD | NSD | | | | | |
| Swinkels et al. (48) | NSD | NSD | SIG | NSD | | | |
| Kalmus et al. (49) | NSD | | NSD | NSD | | | NSD |
| Truchetti et al. (50) | SIG | NSD | NSD | | | | |
| Cortinhas et al. (51) | NSD | NSD | NSD | | | | |
| Viveros et al. (52) | NSD | | SIG | SIG | | | |
| McDougall et al. (53) | NSD | | SIG | NSD | | | |
| **POSITIVELY CONTROLLED TRIALS – TESTING A NON-INFERIORITY OR "EQUIVALENCY" HYPOTHESIS** | | | | | | | |
| McDougall et al. (54) | NSD | | SIG | | Defined as CC | | |
| McDougall et al. (21) | NSD | | SIG | NSD | Defined as CC | NSD | NSD |
| Schukken et al. (55) | NI[c] | | NSD | | | | NI |
| Vasquez et al. (56) | NI | | NI | NI | | | NI |
| Bryan et al. (57) | NSD | | NSD | | Defined as CC | | |
| Tomazi et al. (58) | Inconclusive | | Inconclusive | NI | | | |

[a]*No significant difference;* [b]*statistically significant difference among treatments;* [c]*non-inferior.*

antimicrobials. It is important to reiterate, that even with highly efficacious drugs the benefit of antimicrobial therapy is only for the cases that do not achieve spontaneous bacteriological cure; thus, the marginal value of antibiotic therapy decreases for cases caused by *E. coli* or other pathogens with high rates of spontaneous cure.

Among the 23 trials that evaluated bacteriological cure, statistically significant differences among treatments were observed in only 4 trials while 16 reported no significant differences and 3 trials concluded the evaluated treatments were non-inferior (**Table 3**). Two of the trials that reported significant differences in BC enrolled only Gram-negative cases and compared IMM antimicrobial treatment to non-treated controls (25, 40). The distribution of pathogens in both studies included a considerable proportion of *Klebsiella* spp. which influenced overall BC of the combined groups. Fuenzalida and Ruegg (25) identified an interaction of pathogen by treatment group and reported BC of 18% (non-treated *K. pneumoniae*), 74% (treated *K. pneumoniae*), 97% (non-treated *E. coli*) and 99% (treated *E. coli*). Schukken et al. (40) reported significant differences in BC for cases caused by both *E. coli* and *Klebsiella* spp. but this study is unique in reporting exceptionally low spontaneous cure rates of *E. coli* cases, which the authors attributed to persistent infections. In spite of finding significant differences in BC, neither of these studies reported biologically important differences in important clinical outcomes. Taponen et al. (46) reported BC of clinical mastitis caused by *S. aureus* that were either β-lactamase negative or positive and were treated with either systemic penicillin & an IMM combination product containing penicillin & neomycin or received systemic penicillin alone. While a significant difference was identified among treatments, BC was dramatically decreased for β-lactamase positive organisms. Truchetti et al. (50) compared shorter (2d) vs. longer (5d) therapy using IMM ceftiofur and reported a significant difference in BC but no differences in any clinical outcomes. Over 30% of cases enrolled in this study were culture-negative and 26% were caused by *S. aureus* thus the impact of 3d difference in therapy was likely biologically irrelevant. In general, no clear relationship between BC and important clinical outcomes (such as new IMI, clinical cure, recurrence, SCC, milk

**TABLE 3 |** Bacteriological cure (BC) definitions and outcomes for 24 studies reporting this outcome.

| Study | Bacteriological cure definition | % BC[a] | Treatment effects |
|---|---|---|---|
| **NEGATIVELY CONTROLLED RANDOMIZED CLINICAL TRIALS** | | | |
| Roberson et al. (39) | Etiology absent 3 consecutive days at 7 & 36 d | 67.0%<br>45.0%<br>53.0%<br>55.0% | No |
| Suojala et al. (27) | No *E. coli* at d 2 or d 21 | 90.5%<br>86.8% | Not at d 21 |
| Schukken et al. (40) | Etiology absent at d 7 & 14; if samples missing failure was defined | 73.0%<br>38.0% | Yes |
| Persson et al. (41) | Etiology absent at d 3 & 28 | 88.5%<br>84.2% | No statistics performed |
| Fuenzalida and Ruegg (25) | Etiology absent at 7, 14, 21, 28 d samples | 70.3%<br>77.8%<br>51.2% | Yes |
| **POSITIVELY CONTROLLED TRIALS – NOT TESTING A NON-INFERIORITY HYPOTHESIS** | | | |
| Wraight (42) | Only assessed if CC achieved; Etiology absent from all 3 post-treatment samples | 75.0%<br>64.3% | No |
| McDougall (43) | Etiology absent in 21 d sample | 77.0%<br>77.0% | No |
| Taponen et al. (44) | Etiology absent in 26 d sample | 73.2%<br>78.7% | No |
| Sérieys et al. (45) | Etiology absent at both 17 & 22 d | 54.3%<br>45.9% | No |
| Taponen et al. (46) | Etiology absent in 21- 28 d samples | 56.1%<br>79.1%<br>33.3%<br>33.3% | Yes |
| Wenz et al. (33) | Culture negative 7 d after leaving hospital | 27.0%<br>45.0%<br>33.0%<br>52.0% | No |
| Bradley and Green (47) | Etiology absent in both 16 and 25 d | Not reported | No |
| Swinkels et al. (48) | Etiology absent in both 14 and 21 d samples | 72.0%<br>79.0% | No |
| Kalmus et al. (49) | PCR negative at d 21 & 28 | 54.1%<br>55.7% | No |
| Truchetti et al. (50) | Etiology negative at d 7, 14, & 21 | 32.0%<br>61.0% | Yes |
| Cortinhas et al. (51) | Etiology absent at 14 or 21 d | 79.0%<br>76.0% | No |
| Viveros et al. (52) | Etiology absent in 7, 14 & 21 d samples | 90.0%<br>95.1%<br>88.9%<br>83.3% | No |
| McDougall et al. (53) | Etiology absent in both 14 & 21 d | 81.2%<br>83.8% | No |
| **POSITIVELY CONTROLLED TRIALS – TESTING A NON-INFERIORITY OR "EQUIVALENCY" HYPOTHESIS** | | | |
| McDougall et al. (54) | Etiology absent at 21 d | 84.0%<br>81.0%<br>85.0% | yes |
| McDougall et al. (21) | Etiology absent in both 14 and 21 d samples | 73.3%<br>72.0% | No |
| Schukken et al. (55) | Etiology absent at d 10 and 17 | 61.0%<br>73.0% | No |
| Vasquez et al. (56) | Etiology absent at 14, 21 d samples | 72.0%<br>67.0% | No |

*(Continued)*

**TABLE 3 |** Continued

| Study | Bacteriological cure definition | % BC[a] | Treatment effects |
|---|---|---|---|
| Bryan et al. (57) | Only assessed if CC achieved; Etiology absent at d 9,16, & 23 | 57.2%<br>65.7% | No |
| Tomazi et al. (58) | Etiology absent in 14 and 21 d samples | 68.0%<br>73.0% | No |

[a]*Proportion of BC are listed in same order of treatments defined in* ***Table 1****.*

**TABLE 4 |** Statistical significance of other study outcomes categorized by significance of bacteriological cure in 23 clinical trials that evaluated bacteriological cure.

| Bact. cure | New IMI | | Clin. cure | | SCC | | Recur | | Milk yield | |
|---|---|---|---|---|---|---|---|---|---|---|
| Result | *N*[a] | Sig[b] | *N* | Sig | *N* | Sig | *N* | Sig | *N* | Sig |
| Sig. difference ($n = 4$ studies) | 1 | 0 | 4 | 1 | 3 | 0 | 1 | 0 | 2 | 0 |
| No sig. diff. or non-inferior ($n = 19$ studies) | 3 | 0 | 19 | 7 | 10 | 2 | 2 | 1 | 4 | 1 |

[a]*Number of studies evaluating the outcome;* [b]*number of studies reporting significant difference among treatment groups in that outcome.*

yield or culling) were apparent (**Table 4**). Thus, while achieving BC is the goal of antimicrobial therapy, the finding of differences in BC in research trials does not appear to be predictive of differences in clinical outcomes.

New Intramammary infections are typically defined based on recovery of a different pathogen in follow-up milk samples but in the study that enrolled culture negative cases (22), NIMI was defined as recovery of any pathogen in the 14 and 21 d follow-up samples. While this outcome was not significant in any trials, recovery of both the etiological agent and new pathogens from follow-up samples after treatment is usually greater at earlier sampling periods (22, 25) as compared to samples collected after 14 d and in some instances may reflect differences among pathogens in duration of time to achieve both spontaneous and treatment clearance.

# CLINICAL OUTCOMES

## Clinical Cure (CC)

Almost all studies ($n = 24$; **Table 5**) evaluated "clinical cure" but the definition of this outcome varies enormously. Most researchers (67%) defined CC based on observations that the milk and/or the mammary gland returned to normal appearance, but the day of observation varied from 2 to 28 days after treatment and some relied on single observations, while other researchers required multiple observations. Of studies that defined CC based on observation of clinical signs, the proportion achieving CC was least (CC<15%) for studies that performed observations very early and evaluated systemic therapies (27, 41). For CC estimated based on visual observations after day 3, values of CC ranged from 25% to 98% with a median value of 81%. Other definitions of CC included retreatment within a defined time period (4 studies), use of scoring systems (2 studies) or a combination of methods (2 studies). Of 7 studies that reported significant differences in CC among treatments, 4 defined CC based on retreatment, 2 used comparison of defined scoring systems and 1 evaluated CC within 4 days of treatment. While achieving CC is the practical goal on farms, this outcome is not useful to determine effectiveness of antimicrobial therapy. In most cases of clinical mastitis, inflammation is self-limiting and regardless of BC, and for the majority of cases, milk will return to normal appearance by day 7 (30). There is almost no evidence that selection of an antimicrobial has a significant impact on this outcome and CC should not be used to make decisions about treatment efficacy.

## Post-treatment SCC

Similar to CC, a variety of definitions and sampling periods were used to assess SCC responses in the 14 studies that included this outcome (**Table 6**). Seven studies each assessed SCC at the quarter or composite level and dilution of healthy milk from unaffected quarters likely influenced results of studies that used composite milk samples. Sampling periods ranged from 7 to 90 days after treatment, and all studies that assessed SCC at multiple periods reported a gradual decline in SCC as time passed. While some researchers compared linear scores, other researchers compared the proportion of samples that were defined as "healthy" based on a threshold or either 200,000 or 250,000 cells/mL. Of the 14 studies that included this outcome, only 3 reported significant differences in their measure of SCC. One researcher used the California Mastitis Test on quarter milk samples collected at day 36 post-treatment and reported significantly fewer quarters below "trace score" for quarters that did not receive IMM treatment but received frequent milking (39). Interestingly, no difference was seen in non-treated control quarters that were not frequently milked. In another study, fewer quarters that received IMM antibiotics (as compared to systemic) achieved SCC <250,000 cells/mL by 22 days post-treatment (45) and a 3rd study reported significantly lower SCC at days 7 and 14 but those effects were not significant by day 22 (52). When assessing

**TABLE 5 |** Clinical cure definition and outcomes for 24 studies reporting this outcome.

| Study | Clinical cure definition | % Clinical cure[a] | Sig. treatment or pathogen effects |
|---|---|---|---|
| **NEGATIVELY CONTROLLED RANDOMIZED CLINICAL TRIALS** | | | |
| Roberson et al. (39) | Normal milk for 3 d or 2 weeks without recur assessed on d 7 & 36 | 57% Rx1<br>25% Rx2<br>53% Rx3<br>64% Con | No treatment effect<br>Large pathogen effect |
| Suojala et al. (27) | Absence of signs and normal milk at d2 and/or d21 | Day 2: 8% Rx1 & 20% Con<br>Day 21: 47% Rx2 & 57% Con | Sig. Treatment effect at d2 but not d21 |
| Schukken et al. (40) | <50% of original case severity score at 7 & 14 d | 54% Rx1<br>46% Con | Sig. treatment & pathogen effects |
| Persson et al. (41) | Absence of clinical signs and normal milk on d 3 | 21% Rx1<br>11% Con | Not reported |
| Fuenzalida and Ruegg (22) | Return to normal milk for 2 consecutive d within first 10 d after treatment | 86% Rx1<br>92% Con | No treatment effects |
| Fuenzalida and Ruegg (25) | Return to normal milk for 2 consecutive d within first 10 d after treatment | 92% Rx1<br>98% Rx2<br>90% Con | No treatment or pathogen effects |
| **POSITIVELY CONTROLLED TRIALS - NOT TESTING A NON-INFERIORITY HYPOTHESIS** | | | |
| Erskine et al. (34) | Additional treatment required within 48 h of initial therapy | 84% Rx1<br>77%Rx2 | Sig. treatment effect only for coliform cases |
| Wraight (42) | Normal milk at end of milk withholding | 83% Rx1<br>81% Rx2 | No treatment effect<br>Significant effect of pathogen |
| McDougall (43) | Re-treatment within 21 d of enrollment | 16% Rx1<br>5% Rx2<br>(% retreated) | Significant treatment effect |
| Taponen et al. (44) | No systemic or local signs evident by 3–4 weeks post-treatment | 73% Rx1<br>79% Rx2 | No treatment effect |
| Sérieys et al. (45) | Return to normal milk & udder at 3, 8, 17, & 22 d | >80% for all groups at all periods | No treatment effect |
| Sérieys et al. (45) | No systemic or local signs evident by 3–4 weeks post-treatment | 75% Rx1<br>74% Rx2 | No treatment effect<br>CNS CC higher |
| Swinkels et al. (48) | Severity grade 0 at d 1.5, 5, 14, 21 | 60% Rx1<br>82% Rx2 | Significant treatment effect |
| Kalmus et al. (49) | Normal milk and gland by 21 to 28 d | 80% Rx1<br>75% Rx2 | No treatment effect |
| Truchetti et al. (50) | Normal milk 21 d after last treatment | 89% Rx1<br>89% Rx2 | No treatment effect |
| Cortinhas et al. (51) | Normal Milk & Glands on d 4, 14, and 21 d | 79% Rx1<br>74% Rx2 | No treatment effect<br>No pathogen effect |
| Viveros et al. (52) | Absence of signs 4 d after 1st treatment | 95% Rx1<br>96% Rx2<br>68% Rx3<br>58% Rx4 | Significant effect of Treatment |
| McDougall et al. (53) | Visually abnormal milk at 14 & 21 d | 82% Rx1<br>81% Rx2 | No treatment effect |
| **POSITIVELY CONTROLLED TRIALS – TESTING A NON-INFERIORITY OR "EQUIVALENCY" HYPOTHESIS** | | | |
| McDougall et al. (54) | No new treatment within 30 d | 86% Rx1<br>80% Rx2<br>84% Rx3 | Significant effect of treatment |
| McDougall et al. (21) | No re-treatment within 21 d | 72% Rx1<br>87% Rx2 | Significant effect of treatment<br>CC sig worse in *S. aureus*<br>CC sig better in no growth |
| Schukken et al. (55) | Normal milk and gland at 10 and 17 d | 62% Rx1<br>62% Rx2 | No significant Rx Effect<br>Significant pathogen effects |

*(Continued)*

**TABLE 5 |** Continued

| Study | Clinical cure definition | % Clinical cure[a] | Sig. treatment or pathogen effects |
|---|---|---|---|
| Vasquez et al. (56) | Normal milk and udder 2 to 5 d | 64% Rx1<br>68% Rx2 | No Treatment effect |
| Bryan et al. (57) | Return to normal milk and no further RX up to day 23 | 80% Rx1<br>80% Rx2 | No significant treatment or pathogen effects |
| Tomazi et al. (58) | Milk and gland normal 48 h after last treatment | 88% Rx1<br>94% Rx2 | No<br>No |

[a] *Proportions of clinical cure are listed in same order of treatments defined in* ***Table 1****.*

**TABLE 6 |** SCC definitions and outcomes for 14 studies reporting this outcome.

| Study | SCC outcome definition | Quarter or composite | Response valuation | Sig. treatment or pathogen effects |
|---|---|---|---|---|
| **NEGATIVELY CONTROLLED RANDOMIZED CLINICAL TRIALS** | | | | |
| Roberson et al. (39) | % quarters with CMT < trace at day 36 | Quarter | 54% Rx1<br>21% Rx2<br>43% Rx3<br>44% Con | SIG difference between Rx1 and Rx2 |
| Schukken et al. (40) | Linear SCS value at 1st & 2nd post-treatment test day | Composite | 5.5 Rx1<br>5.4 Con | No treatment effect |
| Persson et al. (41) | Comparison of median SCC at monthly post-treatment test days for 6 months (month 6 shown) | Composite | 58,000 cells/mL<br>123,000 cells/mL | No treatment effect |
| Fuenzalida and Ruegg (22) | Post-treatment SCC weekly until 90 DIM | Quarter | 5.5 Rx1<br>5.4 Con | No treatment effects |
| Fuenzalida and Ruegg (25) | Post-treatment SCC weekly until 90 DIM | Quarter | 6.3 Rx1<br>6.0 Rx2<br>6.1 Con | No treatment effects |
| **POSITIVELY CONTROLLED TRIALS - NOT TESTING A NON-INFERIORITY HYPOTHESIS** | | | | |
| McDougall (43) | Test day SCC values | Composite | Values not shown | No treatment effects |
| Sérieys et al. (45) | SCC at days 8, 17, & 22 post-treatment; Also compared % of quarter SCC >250,000 cells/mL (day 22 data for % < 250,000 shown) | Composite | 70% Rx1<br>57% Rx2 | SCC of quarters that received IMM were significantly greater |
| Swinkels et al. (48) | SCC collected between days 21–27 post-treatment; compared median values and % of quarters with SCC <200,000 cells/mL | Quarter | 24% Rx1<br>31% Rx2 | No treatment effects |
| Kalmus et al. (49) | SCC collected monthly for 3 months post-treatment; also reported % below 200,000 cells/mL (month 3 data shown) | Composite | 43% Rx1<br>51% Rx2 | No treatment effect |
| Viveros et al. (52) | SCC at days 7, 14 & 21 post-treatment; only assessed on clinically cured cows | Quarter | Values shown only in figure | Significant effect of treatment at days 7 & 14 but not day 21 |
| McDougall et al. (53) | Linear SCS at days 14 & 21 post-treatment (day 21 data shown) | Quarter | 6.4 Rx1<br>6.3 Rx2 | No treatment effect |
| **POSITIVELY CONTROLLED TRIALS – TESTING A NON-INFERIORITY OR "EQUIVALENCY" HYPOTHESIS** | | | | |
| McDougall et al. (21) | Linear SCS at months 1–3 post-treatment | Composite | 4.5 Rx1<br>4.4 Rx2 | No treatment effect |
| Vasquez et al. (56) | Linear SCS at 1st month post-treatment | Composite | 3.1 Rx1<br>3.4 Rx2 | No Treatment effect |
| Tomazi et al. (58) | Proportion of linear SCS < 4.0 at day 21 post-treatment | Quarter | 29% Rx1<br>28% Rx2 | No Treatment effect |

SCC responses, it is important to recognize that quarter-level measurements will more accurately reflect ongoing inflammation that may indicate persistent IMI. When BC is achieved SCC will gradually decline and the speed of return to a "healthy" level is influenced by etiology. Assessment of SCC responses should be performed at the quarter level and should continue for at least 21 days. Somatic cell count responses are a practical outcome that can be used as an indicator of treatment success on farms, but a gradual (rather than immediate) decline should be expected. When using composite milk samples, a lower threshold

(<150,000 cells/mL) may help prevent misclassification of ongoing subclinical infections that can result after failure to cure clinical cases.

### Recurrence, Milk Yield, and Retention Within the Herd

A few studies have evaluated other important clinical outcomes. Only 4 studies reported recurrence as a distinct outcome (22, 25, 33, 43) but several included recurrence in their definition of clinical cure (or "clinical failure") (53, 54). Like other outcomes, recurrence can be defined at either the quarter or cow level, but when used to assess treatment this outcome should always be assessed relative to the affected quarter. Recurrence ranged from about 5–30% and was strongly influenced by additional risk factors such as parity (older cows are at greater risk of recurrence), etiology (culture positive are at greater risk as compared to culture negative), and increased milk yield. While two studies reported significant differences in recurrence based on treatment, this outcome is influenced by many other factors and should be interpreted cautiously. Post-treatment milk yield is an obviously important outcome that requires a prolonged follow-up period and has been evaluated infrequently in mastitis trials (22, 25, 39, 40, 43, 54). Of the 6 studies evaluating this outcome, 4 included a non-treated control group and the only significant finding was one study that reported non-treated control animals had the greatest post-treatment milk yield (39). Similarly, a significant difference in retention (or culling) after treatment was reported in only 1 of 8 studies that evaluated this outcome (22, 25, 27, 33, 40, 49, 55, 65). Culling is a very difficult outcome to assess as it is influenced by many factors including non-blinded studies that allow farmers to remove cows without a withholding period if they have not received antimicrobial therapy. While all of these outcomes are relevant and useful for dairy farmers, there is insufficient evidence to suggest that they are influenced by choices made about mastitis treatment.

## CONCLUSIONS AND CLINICAL IMPLICATIONS

Over the last 20 years, very few mastitis trials have been conducted to differentiate among mastitis treatment protocols and the inclusion of multiple etiologies and culture negative cases in efficacy studies have resulted in little ability to differentiate among treatments. Few studies have been conducted that evaluated antimicrobial therapies approved to treat clinical mastitis in N. America. With rare exceptions, researchers have not reported significant differences in most microbiological or clinical outcomes and non-inferiority trials have not concluded that there are differences among products. There is no-evidence that IMM products approved for treatment of clinical mastitis caused by Gram-positive organisms vary in efficacy and other characteristics of approved products (dosing interval, withholding period, price etc.) can be used to make treatment decisions. When possible, etiology should be determined before treatment, the probability of spontaneous cure should be considered and the spectrum of antimicrobial activity of the approved product should be appropriate for the etiological agent. Research has demonstrated that cases of mastitis that are culture-negative at detection or are caused by *E. coli* rarely benefit from antimicrobial therapy and use of antimicrobials to treat these cases should be considered on an individual case basis. Associations between BC and clinical outcomes are very weak, and resolution of inflammation (duration of abnormal milk) is not a reliable indicator of therapeutic efficacy. Among potential indicators that can be used clinically, evaluation of continued decline in quarter-level SCC appears to be the most reliable indicator of success.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

## REFERENCES


1. USDA. Dairy 2014. In: *Health and Management Practices on U.S. Dairy Operations, 2014*. Fort Collins, CO: USDA-APHIS-VS-CEAH-NAHMS (2018).
2. Leite de Campos J, Steinberger A, Goldberg TS, Safdar N, Kates A, Shutske J, et al. Frequency of antimicrobial usage on treatment for bacterial diseases occurring in cows on large dairy farms. *J Dairy Sci.* (2019) 101(Supp. 2):135.
3. Olde Riekerink RGM, Barkema HW, Kelton DF, Scholl DT. Incidence rate of clinical mastitis on canadian dairy farms. *J Dairy Sci.* (2008) 91:1366–77. doi: 10.3168/jds.2007-0757
4. Sordillo LM, Shafer-Weaver K, DeRosa D. Immunobiology of the mammary gland. *J Dairy Sci.* (1997) 80:1851–65. doi: 10.3168/jds.S0022-0302(97)76121-6
5. Ruegg PL. A 100-Year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023
6. Swinkels JM, Rooijendijk JG, Zadoks RN, Hogeveen H. Use of partial budgeting to determine the economic benefits of antibiotic treatment of chronic subclinical mastitis caused by *Streptococcus uberis* or *Streptococcus dysgalactiae*. *J Dairy Res.* (2005) 72:75–85. doi: 10.1017/S0022029904000603
7. Steeneveld W, Swinkels J, Hogeveen H. Stochastic modelling to assess economic effects of treatment of chronic subclinical mastitis caused by *Streptococcus uberis*. *J Dairy Res.* (2007) 74:459–67. doi: 10.1017/S0022029907002828
8. USDA. Dairy 2014. In: *Milk Quality, Milking Procedures, and Mastitis in the United States, 2014*. Fort Collins, CO: USDA-APHIS-VS-CEAH-NAHMS. (2016).
9. Levison LJ, Miller-Chuson EK, Tucker AL, Bergeron R, Leslie KE, Barkema H, et al. Incidence rate of pathogen-specific clinical mastitis on conventiaonl and organic Canadian dairy farms. *J Dairy Sci.* (2016) 99:1341–50. doi: 10.3168/jds.2015-9809
10. Verbeke J, Piepers S, Supré K, De Vliegher S. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. *J Dairy Sci.* (2014) 97:6926–34. doi: 10.3168/jds.2014-8173
11. Kossaibati MA, Hovi M, Esslemont RJ. Incidence of clinical mastitis in dairy herds in England. *Vet Rec.* (1998) 143:649–53. doi: 10.1136/vr.143.24.649
12. Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L, et al. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *J Dairy Sci.* (2017) 100:4797–806. doi: 10.3168/jds.2016-12334
13. Wenz JR, Barrington GM, Garry FB, Dinsmore RP, Callan RJ. Use of systemic disease signs to assess disease severity in dairy cows

with acute coliform mastitis. *J Am Vet Med Assoc.* (2001) 218:567–72. doi: 10.2460/javma.2001.218.567
14. Ruegg PL. Making antibiotic treatment decisions for clinical mastitis. *Vet Clin North Am Food Anim Pract.* (2018) 34:413–25. doi: 10.1016/j.cvfa.2018.06.002
15. Lago A, Godden SM, Bey R, Ruegg PL, Leslie K. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. *J Dairy Sci.* (2011) 94:4441–56. doi: 10.3168/jds.2010-4046
16. Vasquez AK, Nydam DV, Capel MB, Eicker S, Virkler PD. Clinical outcome comparison of immediate blanket treatment versus a delayed pathogen-based treatment protocol for clinical mastitis in a New York dairy herd. *J Dairy Sci.* (2017) 100:2992–3003. doi: 10.3168/jds.2016-11614
17. Valeeva NI, Lam TJGM, Hogeveen H. Motivation of dairy farmers to improve mastitis management. *J Dairy Sci.* (2007) 90:4466–77. doi: 10.3168/jds.2007-0095
18. Oliveira L, Hulland C, Ruegg PL. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. *J Dairy Sci.* (2013) 96:7538–49. doi: 10.3168/jds.2012-6078
19. Ruegg PL. Understanding the economic impact of mastitis. The role of duration and drug selection. In: Third *Am Assoc Bov Pract Annual Recent Graduate Conference.* Columbus OH: Am. Assoc Bov Prac. (2020). p. 84–91.
20. Bradley AJ, Leach KA, Breen JE, Green LE, Green MJ. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet Rec.* (2007) 160:253–7. doi: 10.1136/vr.160.8.253
21. McDougall S, Agnew KE, Cursons R, Hou XX, Compton CR. Parenteral treatment of clinical mastitis with tylosin base or penethamate hydriodide in dairy cattle. *J Dairy Sci.* (2007) 90:779–89. doi: 10.3168/jds.S0022-0302(07)71562-X
22. Fuenzalida MJ, Ruegg PL. Negatively controlled, randomized clinical trial to evaluate use of intramammary ceftiofur for treatment of nonsevere culture-negative clinical mastitis. *J Dairy Sci.* (2019) 102:3321–38. doi: 10.3168/jds.2018-15497
23. Latosinski GS, Amzalak MJ, Pantoja JCF. Efficacy of ketoprofen for treatment of spontaneous, culture-negative, mild cases of clinical mastitis: a randomized, controlled superiority trial. *J Dairy Sci.* (2020) 103:2624–35. doi: 10.3168/jds.2019-17504
24. Lago A, Godden SM, Bey R, Ruegg PL, Leslie K. The selective treatment of clinical mastitis based on on-farm culture results: II. Effects on lactation performance, including clinical mastitis recurrence, somatic cell count, milk production, and cow survival. *J Dairy Sci.* (2011) 94:4457–67. doi: 10.3168/jds.2010-4047
25. Fuenzalida MJ, Ruegg PL. Negatively controlled, randomized clinical trial to evaluate intramammary treatment of nonsevere, gram-negative clinical mastitis. *J Dairy Sci.* (2019) 102:5438–57. doi: 10.3168/jds.2018-16156
26. Suojala L, Kaartinen L, Pyorala S. Treatment for bovine *Escherichia coli* mastitis - an evidence-based approach. *J Vet Pharmacol Ther.* (2013) 36:521–31. doi: 10.1111/jvp.12057
27. Suojala L, Simojoki H, Mustonen K, Kaartinen L, Pyorala S. Efficacy of enrofloxacin in the treatment of naturally occurring acute clinical *Escherichia coli* mastitis. *J Dairy Sci.* (2010) 93:1960–9. doi: 10.3168/jds.2009-2462
28. Pinzon-Sanchez C, Cabrera VE, Ruegg PL. Decision tree analysis of treatment strategies for mild and moderate cases of clinical mastitis occurring in early lactation. *J Dairy Sci.* (2011) 94:1873–92. doi: 10.3168/jds.2010-3930
29. Down PM, Bradley AJ, Breen JE, Green MJ. Factors affecting the cost-effectiveness of on-farm culture prior to the treatment of clinical mastitis in dairy cows. *Prev Vet Med.* (2017) 145:91–9. doi: 10.1016/j.prevetmed.2017.07.006
30. Pinzon-Sanchez C, Ruegg PL. Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *J Dairy Sci.* (2011) 94:3397–410. doi: 10.3168/jds.2010-3925
31. Wagner S, Erskine R. Antimicrobial drug use in mastitis. In: Giguere S, Prescott JF, Dowling PM, editors. *Antimicrobial Therapy in Veterinary Medicine*. 5th ed. Ames IA: Wiley Blackwell (2013). p. 519-28. doi: 10.1002/9781118675014.ch30
32. Boerlin P, White DG. Antimicrobial resistance and its epidemiology. In: Giguere S, Prescott JF, Dowling PM, editors. *Antimicrobial Therapy in Veterinary Medicine*. 5th ed. Ames, IA: Wiley Blackwell (2013). p. 21-40. doi: 10.1002/9781118675014.ch3
33. Wenz JR, Garry FB, Lombard JE, Eilia R, Prentice D, Dinsmore RP. Efficacy of parenteral ceftiofur for treatment of systemically mild clinical mastitis in dairy cattle. *J Dairy Sci.* (2005) 88:3496–9. doi: 10.3168/jds.S0022-0302(05)73034-4
34. Erskine RJ, Bartlett PC, VanLente JL, Phipps CR. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. *J Dairy Sci.* (2002) 85:2571–5. doi: 10.3168/jds.S0022-0302(02)74340-3
35. Oliver SP, Murinda SE, Jayarao BM. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne Pathog Dis.* (2011) 8:337–55. doi: 10.1089/fpd.2010.0730
36. Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A, Duchateau L. Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet Res.* (2003) 34:521–64. doi: 10.1051/vetres:2003023
37. Berry DP, Meaney WJ. Cow factors affecting the risk of clinical mastitis. *Irish J Ag and Food Research.* (2005) 44:147–56. Available online at: http://hdl.handle.net/11019/463
38. Barkema HW, Schukken YH, Zadoks RN. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci.* (2006) 89:1877–95. doi: 10.3168/jds.S0022-0302(06)72256-1
39. Roberson JR, Warnick LD, Moore G. Mild to moderate clinical mastitis: efficacy of intramammary amoxicillin, frequent milk-out, a combined intramammary amoxicillin, and frequent milk-out treatment versus no treatment. *J Dairy Sci.* (2004) 87:583–92. doi: 10.3168/jds.S0022-0302(04)73200-2
40. Schukken YH, Bennett GJ, Zurakowski MJ, Sharkey HL, Rauch BJ, Thomas MJ, et al. Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. *J Dairy Sci.* (2011) 94:6203–15. doi: 10.3168/jds.2011-4290
41. Persson Y, Katholm J, Landin H, Mork MJ. Efficacy of enrofloxacin for the treatment of acute clinical mastitis caused by *Escherichia coli* in dairy cows. *Vet Rec.* (2015) 176:673. doi: 10.1136/vr.102667
42. Wraight MD. A comparative efficacy trial between cefuroxime and cloxacillin as intramammary treatments for clinical mastitis in lactating cows on commercial dairy farms. *New Zeal Vet J.* (2003) 51:26–32. doi: 10.1080/00480169.2003.36326
43. McDougall S. Intramammary treatment of clinical mastitis of dairy cows with a combination of lincomycin and neomycin, or penicillin and dihydrostreptomycin. *New Zeal Vet J.* (2003) 51:111–6. doi: 10.1080/00480169.2003.36349
44. Taponen S, Dredge K, Henriksson B, Pyyhtia AM, Suojala L, Junni R, et al. Efficacy of intramammary treatment with procaine penicillin G vs. procaine penicillin G plus neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria - a double blind field study. *J Vet Pharmacol Therap.* (2003) 26:193–8. doi: 10.1046/j.1365-2885.2003.00473.x
45. Sérieys F, Raguet Y, Goby L, Schmidt H, Friton G. Comparative efficacy of local and systemic antibiotic treatment in lactating cows with clinical mastitis. *J Dairy Sci.* (2005) 88:93–9. doi: 10.3168/jds.S0022-0302(05)72666-7
46. Taponen S, Jantunen A, Pyorala E, Pyorala S. Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused by penicillin-susceptible or penicillin-resistant Staphylococcus aureus. *Acta Vet Scand.* (2003) 44:53–62. doi: 10.1186/1751-0147-44-53
47. Bradley AJ, Green MJ. Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparations. *J Dairy Sci.* (2009) 92:1941–53. doi: 10.3168/jds.2008-1497
48. Swinkels JM, Cox P, Schukken YH, Lam TJGM. Efficacy of extended cefquinome treatment of clinical Staphylococcus aureus mastitis. *J Dairy Sci.* (2013) 96:4983–92. doi: 10.3168/jds.2012-6197
49. Kalmus P, Simojoki H, Orro T, Taponen S, Mustonen K, Holopainen J, et al. Efficacy of 5-day parenteral versus intramammary benzylpenicillin for treatment of clinical mastitis caused by gram-positive bacteria susceptible to penicillin *in vitro*. *J Dairy Sci.* (2014) 97:2155–64. doi: 10.3168/jds.2013-7338
50. Truchetti G, Bouchard E, Descôteaux L, Scholl D, Roy JP. Efficacy of extended intramammary ceftiofur therapy against mild to moderate clinical mastitis in Holstein dairy cows: a randomized clinical trial. *Can J Vet Res.* (2014) 78:31–7.

51. Cortinhas CS, Tomazi T, Zoni MSF, Moro E, dos Santos MV. Randomized clinical trial comparing ceftiofur hydrochloride with a positive control protocol for intramammary treatment of nonsevere clinical mastitis in dairy cows. *J Dairy Sci.* (2016) 99:5619–28. doi: 10.3168/jds.2016-10891
52. Viveros M, Lopez-Ordaz R, Gutierrez L, Miranda-Calderon JE, Sumano H. Efficacy assessment of an intramammary treatment with a new recrystallized enrofloxacin vs ceftiofur and parenteral enrofloxacin in dairy cows with nonsevere clinical mastitis. *J Vet Pharmacol Ther.* (2018) 41:e1–9. doi: 10.1111/jvp.12441
53. McDougall S, Clausen L, Hintukainen J, Hunnam J. Randomized, controlled, superiority study of extended duration of therapy with an intramammary antibiotic for treatment of clinical mastitis. *J Dairy Sci.* (2019) 102:4376–86. doi: 10.3168/jds.2018-15141
54. McDougall S, Arthur DG, Bryan MA, Vermunt JJ, Weir AM. Clinical and bacteriological response to treatment of clinical mastitis with one of three intramammary antibiotics. *N Z Vet J.* (2007) 55:161–70. doi: 10.1080/00480169.2007.36762
55. Schukken YH, Zurakowski MJ, Rauch BJ, Gross B, Tikofsky LL, Welcome FL. Noninferiority trial comparing a first-generation cephalosporin with a third-generation cephalosporin in the treatment of nonsevere clinical mastitis in dairy cows. *J Dairy Sci.* (2013) 96:6763–74. doi: 10.3168/jds.2013-6713
56. Vasquez AK, Nydam DV, Capel MB, Ceglowski B, Rauch BJ, Thomas MJ, et al. Randomized noninferiority trial comparing 2 commercial intramammary antibiotics for the treatment of nonsevere clinical mastitis in dairy cows. *J Dairy Sci.* (2016) 99:8267–81. doi: 10.3168/jds.2016-11258
57. Bryan MA, Hea SY, Mannering SA, Booker R. Demonstration of non-inferiority of a novel combination intramammary antimicrobial in the treatment of clinical mastitis. *New Zeal Vet J.* (2016) 64:337–42. doi: 10.1080/00480169.2016.1210044
58. Tomazi T, Lopes TAF, Masson V, Swinkels JM, Santos MV. Randomized noninferiority field trial evaluating cephapirin sodium for treatment of nonsevere clinical mastitis. *J Dairy Sci.* (2018) 101:7334–47. doi: 10.3168/jds.2017-14002
59. Winder CB, Sargeant JM, Hu D, Wang C, Kelton DF, Godkin MA, et al. Comparative efficacy of antimicrobials for treatment of clinical mastitis in lactating dairy cattle: a systematic review and network meta-analysis. *Anim Health Res Rev.* (2019) 20:229–46. doi: 10.1017/S1466252319000318
60. Ruegg PL. Mastitis, detection, diagnosis and management. *J Dairy Sci.* (2017).
61. Hillerton JE, Kliem KE. Effective treatment of *Streptococcus uberis* clinical mastitis to minimize the use of antibiotics. *J Dairy Sci.* (2002) 85:1009–14. doi: 10.3168/jds.S0022-0302(02)74161-1
62. Oliver SP, Almeida RA, Gillespie BE, Headrick SJ, Dowlen HH, Johnson DL, et al. Extended ceftiofur therapy for treatment of experimentally-induced *Streptococcus uberis* mastitis in lactating dairy cattle. *J Dairy Sci.* (2004) 87:3322–9. doi: 10.3168/jds.S0022-0302(04)73468-2
63. Piaggio G, Elbourne DR, Altman DG, Pocock SJ, Evans SJW, Grp C. Reporting of noninferiority and equivalence randomized trials - an extension of the CONSORT Statement. *JAMA-J Am Med Assoc.* (2006) 295:1152–60. doi: 10.1001/jama.295.10.1152
64. Philpot WN. Role of therapy in mastitis control. *J Dairy Sci.* (1969) 52:708–13. doi: 10.3168/jds.S0022-0302(69)86633-6
65. Andreani NA, Carraro L, Zhang L, Vos M, Cardazzo B. Transposon mutagenesis in *Pseudomonas fluorescens* reveals genes involved in blue pigment production and antioxidant protection. *Food Microbiol.* (2019) 82:497–503. doi: 10.1016/j.fm.2019.03.028


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REVIEW
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# Maintaining Optimal Mammary Gland Health and Prevention of Mastitis

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In dairy industry, quality of produced milk must be more important than quantity without a high somatic cells count (SCC) or pathogens causing mastitis of dairy cows and consumer diseases. Preserving the good health of dairy cows is a daily challenge for all involved in primary milk production. Despite the increasing level of technological support and veterinary measures, inflammation of the mammary gland–mastitis, is still one of the main health problems and reasons for economic losses faced by cow farmers. The mammary gland of high-yielding dairy cows requires making the right decisions and enforcing the proper measures aimed at minimizing external and internal factors that increase the risk of intramammary infection. Due to the polyfactorial nature of mastitis related to its reduction, the effectiveness of commonly used antimastitis methods tends to be limited and therefore it is necessary to find the areas of risk in udder health programs and monitoring systems. Only by implementing of complete udder health programs should be accompanied by research efforts to further development these complete udder health control. The present review analyses the current knowledge dealing with damping and prevention of mastitis include SCC control, proper nutrition, housing and management, milking and drying as practiced in dairy farming conditions. This information may help to improve the health of the mammary gland and the welfare of the dairy cows as well as the production of safe milk for consumers.

**Keywords: dairy cows, mastitis, somatic cells count, nutrition, bedding, dry period, treatment**

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## INTRODUCTION

Ruminant milk is a traditional raw material for the production of a range of dairy products that are unique in their composition. Many of them can be classified as functional foods in different geographical and social localities. However, European Union regulations oblige producers to obtain milk only form healthy animals in order to increase consumers safety, what however may limit milk production and consumption (1–4).

A number of factors influence the health status of ruminants in large dairy herds. Both single factors and their combination create the conditions in which the virulence of pathogens, especially bacteria, break the host's immunity. Various organ diseases may be induced among others by inadequate housing hygiene, poor nutrition, and mistreatment and when many of the animals are affected they may be defined as so-called production diseases (5).

In publications on dairy farming, mastitis, laminitis, and metritis are distinguished as the main three production diseases. The US Animal Welfare Council concluded that production diseases are currently considered the most serious problem in dairy farming, causing, in addition to deterioration of dairy animals health and welfare, huge economic losses (6).

Despite the increasing quality of zootechnical control and better hygiene of milk production, mastitis remains the most serious and demanding disease of dairy cows with significant negative economic impact. The negative economic consequences of clinical or subclinical mastitis include a decrease in milk production and lower price for milk with high SCC, increased rate of culling, and higher cost of veterinary treatment, which can climb up to 185 EUR/cow (7). A survey conducted by Turk et al. (8) showed that 23% of cows leaving the herds too early are culled because of udder health problems.

Equally important negative impacts are related to the poor technological quality of raw milk used in the dairy industry, and the presence of mastitis pathogens and their toxins in milk and dairy products (9, 10). Based on over 70 years of systematic studies on mastitis in ruminants, a general thesis is accepted that the disease is polyethiological and multifactorial, and therefore it requires a comprehensive approach to reduce its incidence (11–13).

Methods of disease prevention and control must be based primarily on the results of targeted diagnostics, including history data, to reveal the clinical status of the udder, and confirm the extent of anatomical and pathophysiological changes in the mammary gland (MG) (14, 15).

An overview analyzes antimastitis measures aimed at damping and prevention of mastitis include SCC control, proper nutrition, housing and management, milking and drying as practiced in dairy farming conditions to improve the health and welfare of the cows.

## Causes of Mastitis

According to Holko et al. (16), the causes of mastitis can be fundamentally divided into two groups. In the first group, inflammations of the MG and milk ducts are caused by microbial. In the second group are applied incorrect technological procedures during milking, metabolic disorders, udder injuries and various stress factors in the development of mastitis.

Acceptance of the mutual relationship between the infectious agent and the dairy cow organism is of fundamental importance in influencing all factors of the external environment, while the susceptibility of the dairy cow to mastitis is also given by factors such as: age, order of lactation, its stage, milk yield, anatomical dispositions, but mainly by immunological condition and reactivity of the mammary gland. Due to the multitude of internal and external causes leading to mastitis, it should be considered a multifactorial disease [(17), **Figure 1**].

To date, over 137 different organisms have been identified as being causative agents of bovine mastitis, including bacteria, viruses, mycoplasma, yeasts and algae but bacteria remain the principle causative agents (95% of all IMI) of such complex (18, 19). Generally, every mastitis case is considered to be caused by one primary pathogen, because usually only one bacterial species is identified in milk samples from diseased glands. Nevertheless, simultaneous infections by two different pathogen species are not rare, and three pathogens may be found in a small proportion of cases.

The bacteria causing the most common forms of mastitis may be considered within two groups. Contagious pathogens (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae* or *Streptococcus dysgalactiale*) (20). These organisms can survive and grow within the MG so that transmission of infection from infected to uninfected quarters and from cow to cow is most likely to occur during milking. Environmental pathogens thrive in the environment especially where cows' feces are involved. Of this group, *E. coli* is the most important with multiple strains of varying pathogenicity for animals and humans. Others include *Streptococcus uberis,* coagulase-negative staphylococci (CNS), *Corynebacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Proteus* spp., *Pasteurella* spp., *Listeria* spp., *Leptospira* spp., *Yersinia* spp., *Enterobacter* spp., *Brucella* spp. and *Mycobacterium* spp. (21–25).

Pathogens can invade into MG in various ways. The most common way is penetration through teat orifice and teat duct as well as through microlesions and damaged skin of the udder (26, 27). With the exception of a few pathogens that can invade via the bloodstream (e.g., *Mycobacterium avium* ssp. *Paratuberculosis* or *Brucella abortus* from other organs. The entero-mammary pathway has been invoked to explain the transfer of gut lumen bacteria to the MG. It is speculated that bacteria taken up from the gut lumen by leucocytes such as dendritic cells or macrophages would be carried to the MG by phagocytes migrating to the MG by the haematogenous route, then making their way to the MG lumen to be finally shed in milk. It has been shown that translocation of bacteria from the gut lumen to milk in mononuclear leucocytes may occur in lactating mice for a short period after delivery (28).

The manifestations of the inflammatory process vary widely, as they depend on the degree of reaction of the udder tissue to injury or infection (29). The clinical manifestations of MG inflammation as well as its further course depend on the interplay between the innate resistance and adaptive immunity of the dairy cow and the type, concentration, and virulence of udder pathogens. If the MG has been infected with a large number of pathogens or more germs that are virulent and the host's defense systems have not been sufficient to control the infection, an clinical or chronic form of mastitis will develop (22, 30).

Clinical form of mastitis is characterized by a sudden onset, alterations in milk composition and appearance, decreased milk production, and the presence of the cardinal signs of inflammation in infected mammary quarters. In contrast, a lower concentration of pathogens with lower virulence leads to subclinical infections without visible symptoms in the udder or milk, but the milk production decreases and the SCC increases. According to Sharma et al. (21), subclinical mastitis is considered the most economically important type of mastitis because of its higher prevalence and long term devastating effects as compared to clinical mastitis. The symptoms of subclinical IMI may only be recognized from evidence of an elevated SCC. In some cows it may persist throughout lactation without presenting clinical signs, in others give rise

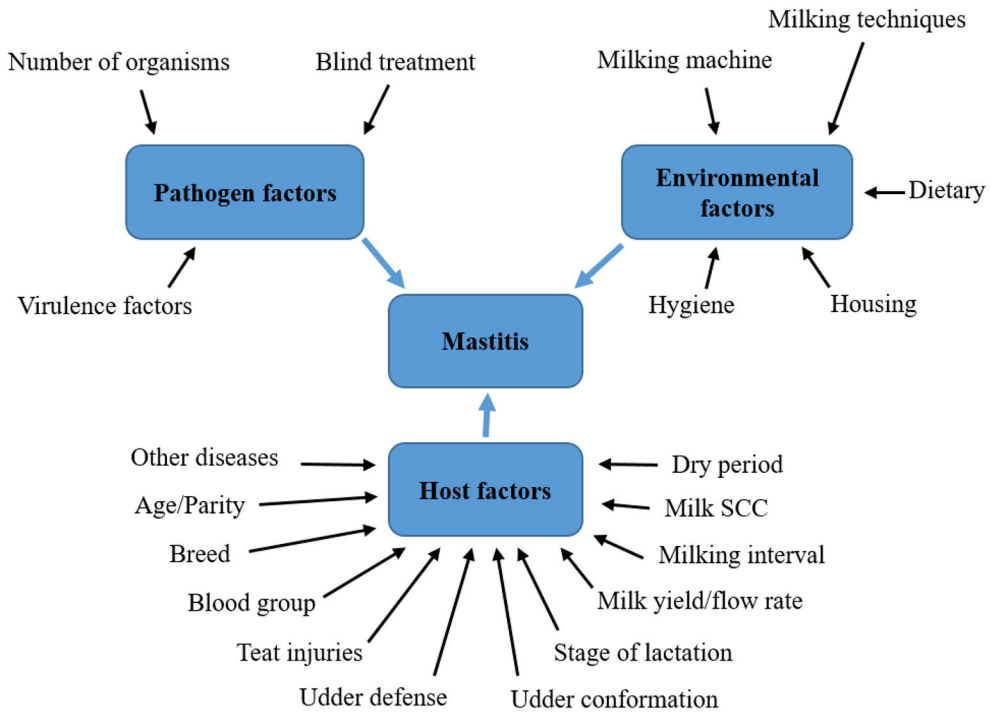


**FIGURE 1 |** Factors influencing the development of mastitis in dairy cows. Soure: Adapted from Abebe et al. (17).

to repeated episodes of (probably mild) clinical symptoms. In these circumstances, cows would be defined as cases of chronic mastitis. Whether subject to repeat clinical episodes or not, cow with chronic mastitis (especially with *S. aureus*) continue to shed organisms so present a risk of cross-infection at the time of milking.

In recent years, one of the most common microorganisms causing mastitis in dairy cows is *S. aureus* and coagulase negative staphylococci (CNS) (18). Bacteria *S. aureus* is a natural inhabitant of the skin of cows and humans, where it does no harm unless the cow s teat or the milker's and is cracked, when it can cause the wound to turn septic. If the organism is able to penetrate the teat in sufficient numbers the disease taken one of two clinical forms of IMI. Peracute staphylococcal mastitis can occur rarely, but especially in early lactation when the immune defenses of the cow are depressed. The cow becomes very ill with a high fever, depression, inappetence and may become comatose and die within 24 h of the onset of symptoms. The infected quarter is grossly swollen and extremely painful, which makes the cow very reluctant to move. The secretion from the infected quarter is usually a lood-stained, serous fluid. If the cow survives, blue gangrenous patches may appear on the quarter and proceed to blackened, oozing sores. Although the cow with peracute *S. aureus.* infection can be saved by an effective antibiotic, if caught in time, the quarter is almost invariably lost (20).

The more common form of *S. aureus* infection is less severe but chronic. The affected cow may not appear ill and the affected quarter may not be paiful. The foremilk may or may not show abnormalities (31). However, as with *Strep. agalactiae,* chronically infected quarters are sources of cross infection and become progressively less productive as scar tissue replaces secretory tissue. Treatment of *S. aureus* infection is complicated by the fact there are many strains and more and more of them are becoming resistant to more and more of the antibiotics within the veterinary armory (19, 32).

Coagulase-negative staphylococci are considered to be minor pathogens in dairy mastitis however, there is increasing work by authors to emphasize their role in the development of MG inflammation (23, 33–35). The increase of their occurrence in dairy farms occurs after the reduction of the occurrence of the main pathogens; the CNS that are present are characterized by increased resistance to commonly used antibiotics and disinfectants (34). Compared to *S. aureus*, CNS usually have a lower proportion of virulence factors but their essential factor of pathogenicity is the production of a biofilm and thus resist the applied disinfection and sanitation procedures. In addition, in their study, Nascimento et al. (36) confirmed that the CNS (*S. epidemidis, S. saprophyticus, S. hominis and S. aerletae*) which were isolated from cow mastitis, were resistant to the antibiotics used and were able to produce some of the staphylococcal enterotoxins. Haveri et al. (37) and Vasil et al. (38) consider the ability to produce staphylococcal enterotoxins to be an important virulence factor which is responsible for the development of, in particular, clinical forms of mastitis. Previous studies indicate that CNS with some virulence factors and multiple resistance, are very important in pathogenesis of mastitis in dairy cows.

## Immunocompetence in the Mammary Gland

Immunocompetence in the mammary gland (MG) is a complex of non-immune anatomical factors, and a plethora of immune-mediated defense mechanisms that include innate and adaptive immune responses. Immunocompetence can vary during lactation, showing depression in the peripartum period due to the hormonal and metabolic stress of calving and milk production. Decreased immunity after calving with the negligence in an application of milking hygiene program, housing hygiene, nutrition and breeding work increases transmission of pathogenic microorganisms and the contamination of the mammary quarters (39). In most cases, mastitis-causing microorganisms enter the MG tissue and milk through the teat duct, from where they are transmitted and spread to remaining structures of the glandular tissue [(21), **Figure 2**].

The teat orifice and teat duct are therefore considered as one of the most important physical barriers of the defense system against the penetration of microorganisms into the mammary gland. The teat duct epithelium produces keratin that physically traps bacteria and blocks their migration to the mammary cistern. Keratin also has antimicrobial activity due to some bacteriostatic fatty acids (lauric, myristic, palmitoleic, and linoleic), as well as fibrous proteins that bind and damage the microorganism cell wall. Damage of keratin, perhaps as a result of incorrect intramammary therapy infusion or by faulty machine milking, has been reported to increase susceptibility of the teat canal to bacterial invasion and colonization (20, 40, 41).

One of the important barriers that affects the transfer of pathogens from the environment and may govern mastitis susceptibility is commensal microbiota residing in the udder. Bacteriocins produced by certain non-aureus Staphylococci and Corynebacterium species colonizing the teat apices and teat canals may inhibit growth of major mastitis pathogens. According to Bronzo et al. (39) commensal microbiota of MG can change immune responses through direct and indirect mechanisms, such as through bacterial secretion of antimicrobial compounds or through influencing the expression of genes hosts' immune responses.

Within complex ecosystems, ecosystem diversity can increase resiliency against an influx of external species by supporting favorable interactivity. The complexity of microbe to microbe communications concerning the functional properties of the mammary ecosystem are difficult to understand. It is essential to identify those bacterial species in the milk microbiota that contribute to mammary homeostasis and mastitis pathogen susceptibility (42).

The MG displays both innate and adaptive (or acquired) immune mechanisms that defend the tissue against pathogens. The innate immune system (IIS) is the first line of defense against pathogens after penetration through physical barrier of the teat canal and before the adaptive immune system comes into play, and it evolves into a highly effective host defense. Innate host-defenses depend on germline-encoded receptors that recognize conserved structures expressed by a wide range of microbes, and early induced cellular and soluble defenses (43).

A wide variety of innate immune components have been identified in colostrum and milk, including cellular defense components (e.g., leukocytes, neutrophils, macrophages) components contributing to humoral defense (e.g., complement system, immune-modulating factors, cytokines, lactoferrin, transferrin, lysozyme, and components of the lactoperoxidase/myeloperoxidase systems, oligosaccharides, gangliosides, reactive oxygen species, acute phase proteins), ribonucleases, and a wide range of antimicrobial peptides and proteins. These components of IIS respond quickly to microbes during early stages of infection and are tightly integrated with the adaptive immune system (44).

The adaptive immune system uses a diverse repertoire of antigen specific receptors expressed by clonally expanded B and T-lymphocytes to regulate or eliminate the signal elicited by recognition events. Additionally, the induced adaptive immune response has the capacity to establish antigen specific memory for a rapid and augmented response upon subsequent exposure to the same antigen (45).

Generally, the microorganisms located on the surface of the teat penetrate the udder during or after milking through the teat canal. Especially, after milking, the teat canal is open for 1–2 h or for the entire period between milking if damaged. This condition facilitates the entry of microorganisms from the environment, in particular from the dirty bedding. Microorganisms, after penetration of the MG, attack and colonize tissues. Some of the microorganisms spread to the higher parts of the glandular tissue of the MG when the cow moves after the milk has mixed in the milk cistern (22).

As mentioned, both innate and adaptive immune response are coordinating and operating together in very complicated pathways to provide the optimal defense against infections. After contacting the bacteria with leukocytes in the milk or the lining gland epithelium accompanied by exerting various virulence mechanisms and liberating toxins, irritation or even damage to MG epithelium and, thereby, activation of the IIS occur through the transcriptional activation of key response genes (41).

Inflammatory products from damaged epithelium induce locally located leukocytes and healthy MG epithelium to release several chemoattractants for the migration and recruitment of both bone marrow and circulating immune cells into the MG environment, mainly neutrophils. Proinflammatory cytokines are the main effectors to initiate the inflammatory responses at both local and systemic levels (46). They act in collaboration with transforming growth factors and several chemotactic factors to potently trigger circulation-into-MG migration of neutrophils via induction of vascular endothelial adhesion molecules expression. These processes lead to the recruitment of further leukocytes from blood, their passage to milk and an infiltration of udder tissue. The migration of immune cells during IMI plus desquamation of MG epithelia results in an increase of SCC accompanied with decreased milk production according to the severity of the process (42).

If the udder cannot be cleared from the invading microorganisms, persistently activated leukocytes may injure the intralobar ducts and the alveoli. Damage to the alveolar epithelial cells increases the permeability of the capillaries

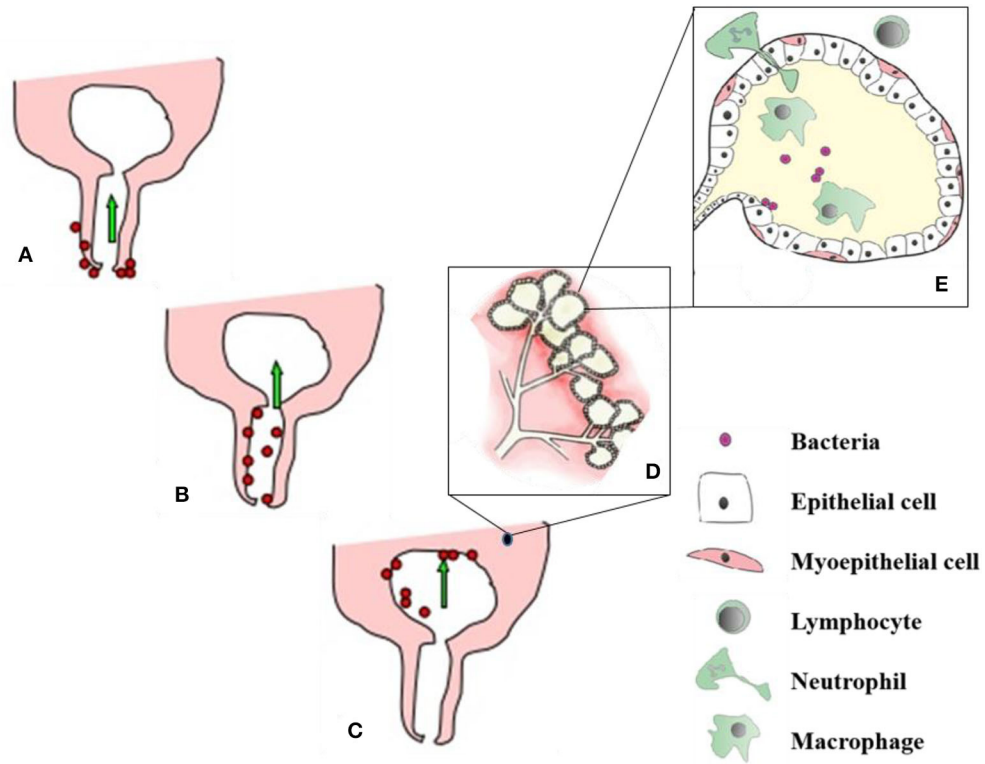


**FIGURE 2 |** Process of udder infection. **(A)** – Organisms invade the udder through teat canal, **(B,C)** - Migrate up the teat canal and colonize all parts where the milk gets and finally setup the infection in mammary gland, **(D)** - alveoli and secretory mammary epithelial cells. After getting bacterial infection, cellular defense mechanism become active and phagocytic cells (from blood) effort to engulf and kill the bacteria, phagocytosis by products and release of bacterial toxins damage to the secretory mammary epithelial cells **(E)**. Soure: Adapted from Sharma and Jeong (21).

leading to further increase in the number of white blood cells in the infected tissues and to the influx of the minerals and clotting factors from the blood (14).

The interaction between the pathogenic microorganisms and the host's immune system leads to the coagulation and retention of milk, which results in the closure of the ducts. The activity of secretory cells is suppressed, the alveoli reduce in size, then secretory cells are destroyed and replaced by connective tissue (22, 29).

Trinidad et al. (26) using histological analysis of mammary tissue samples from primipary cows showed that percentages of alveolar epithelium and lumen in quarters infected with *Staph. aureus* were lower than those in uninfected quarters. Quarters infected with *Staph. aureus* also showed a greater percentage of interalveolar stroma than did uninfected quarters. Additionally, quarters infected with *Staph. aureus* exhibited significantly greater infiltration of leukocytes (mainly lymphocytes and neutrophils) compared with uninfected tissues.

## Damping and Control of Mastitis

A comprehensive approach is necessary to determine appropriate control and prevention measures in dairy farming for the production of high quality milk while maintaining udder health. It should be borne in mind that mastitis cannot be completely eliminated from the herd but only kept at the lowest possible incidence. Improving MG health at farm level is based on the application of two basic principles:

1. shortening the duration of an existing intramammary infection
2. reducing the incidence of new intramammary disease.

Since mastitis is a multifactorial disease, a successful breeder should have certain characteristics to apply these two basic principles; He must fully understand the complexities of the disease, know the principles of prevention and control, be motivated and determined, be able to motivate his employees and lastly, be able to put (comprehensive) knowledge into practice. Improving the health of the mammary gland and the production of quality milk can only be achieved through the application of broad-spectrum mastitis prevention and control programs (47).

Antimastitis measures must consider all aspects of both the external and the internal environments based on daily husbandry practices that affect the health of the dairy cow and its milk production. Required aspects include:

- the benefit of reducing the incidence of mastitis in the herd must be more than the cost of treating and controlling it,
- the choice of measures to control mastitis must be applicable throughout the herd,

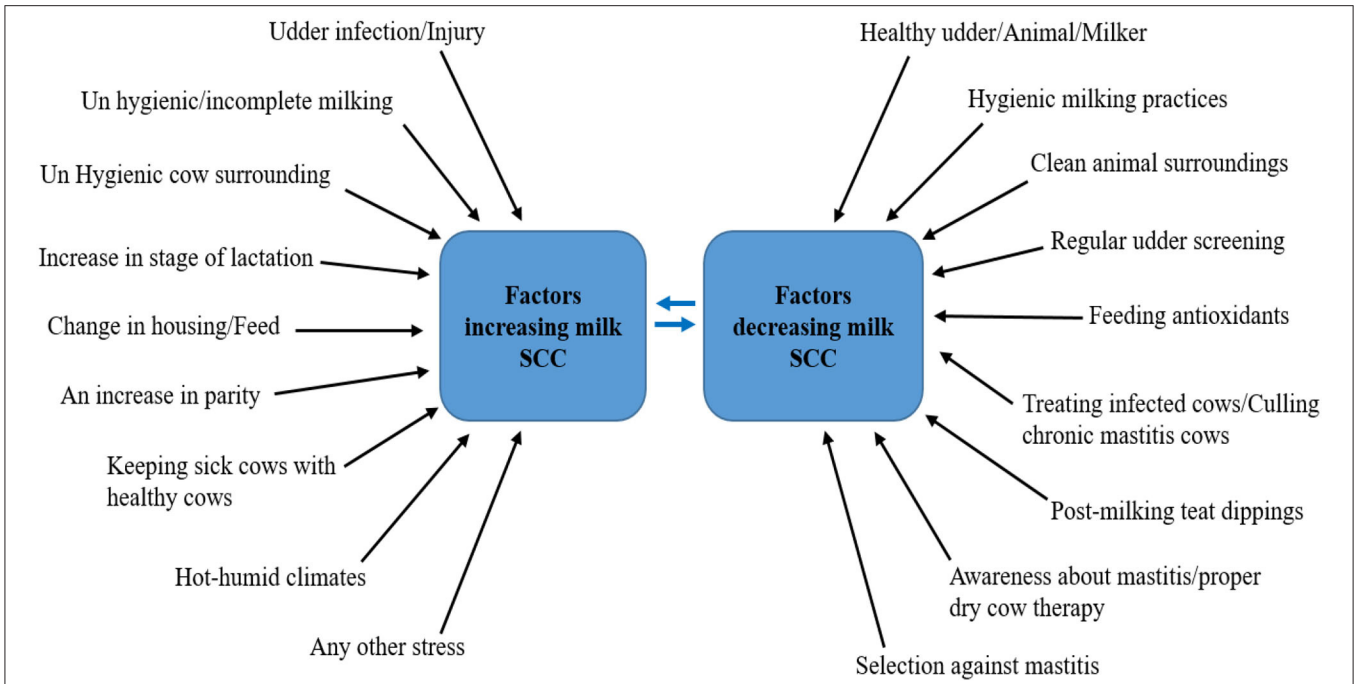


**FIGURE 3 |** Factors and management practices affecting the release of milk SCC. Source: Adapted from Alhussien and Dang (49).

- the measures in place must be effective against all mastitis pathogens (25, 48).

## Somatic Cells Count Monitoring and Reduction

There are many factors and management practices that affect the release of milk SCC and can cause a decrease or increase in their levels [(28), **Figure 3**]. Researchers over the years have found associations between various management practices adopted on dairy farms and herd SCC (14, 15, 50, 51).

Low SCC milk production is an important tool for a good dairy economy. Somatic cells are predominantly white blood cells that are produced by the immune system to prevent inflammation of the mammary gland (mastitis). Inflammation of the mammary gland can have a clinical course (when changes in the udder and milk are visible), and subclinical as well when there are no visible symptoms, however, increased SCC in milk results to a decrease in milk production [(52), **Table 1**]. The increase in the number of cells in milk due to the inflammatory response can be enormous. From a base level of only 100.000 ($10^5$) per mL, i.e., a cell count of 100, it may increase to as many as 100.000.000 ($10^8$) per ml (a cell count of 100.000) in just a few hours, and many quarters rapidly reach a cell count of 10 billion ($10^9$).

The system of mastitis control in a specific herd can be implemented through the control of SCC in a pool sample based on monthly reports from the performance control. If the pool somatic cells rise above 400 x $10^3$ in 1 mL within 2 months, then there is a problem with the classification of milk in the breeding, thus, requires solutions to the causes of the unfavorable condition (2).

**TABLE 1 |** Estimated milk losses due to increased SCC.

| SCC/ml | Loss of milk (%) | Losses of milk production per dairy cow/year (kg) |
|---|---|---|
| 100 000 | 3 | 180 |
| 200 000 | 6 | 360 |
| 300 000 | 7 | 450 |
| 400 000 | 8 | 550 |
| 500 000 | 9 | 590 |
| 600 000 | 10 | 635 |
| 700 000 | 10.5 | 680 |
| 800 000 | 11 | 725 |
| 900 000 | 11.5 | 750 |
| 1 000 000 | 12 | 770 |
| 1 600 000 | 12 | 770 |

*Source: Tongel and Mihina (52).*

The fastest way is to sort out cows with increased SCC. Such dairy cows should be milked last in special cans so that milk with elevated SCC (often contaminated with microorganisms) is not mixed with the remaining milk in the cooling tank. However, this is a short-term quick solution to reduce SCC in the pool sample. Often, breeders in the created group of dairy cows called "millionaires" (>1,000 x $10^3$ SCC), classify the chronically ill or incurable dairy cows in which no effect on SCC reduction or elimination of the mastitis-causing pathogen was observed even after multiple treatments. Additionally, dairy cows that

repeatedly fail to respond to treatment are considered high-risk vectors for the transference of resistant strains to other dairy cows in the established group and the herd as well (21).

The breeder should consider their exclusion based on a good record and frequency of chronic and incurable mastitis caused by contagious pathogens in individual dairy cows in selected groups. The culling of dairy cows with recurrent mastitis and ineffective treatment represents a very effective way to reduce SCC as well as bacterial pressure in a herd, especially in contagious infections (53).

The second way is prevention, particularly the control of mastitis animals and their subsequent treatment. The distinction between new (mainly subacute and acute forms) and long-term inflammation is possible mainly based on daily diagnosis and evidence (54). Cows with severe infections will likely need veterinary intervention and require immediate and aggressive treatment with fluids, systemic and intramammary antibiotics, anti-inflammatories and calcium. But severe cases only occur 15% of the time; the other 85% are the mild and moderate cases where milk cultures are most informative (55).

In practical conditions, antimastitis protocols include sampling from suspected dairy cows for the purpose of rapid cultivation and differentiation of G– and G + bacteria in a thermobox directly on the farm. Zootechnics are in charge of the anamnesis and determination of the degree of severity of mastitis according to the clinical signs of inflammation (milk, udder, cow). After culturing the samples for 12 or 24 h, the result of the bacteriological examination is read and, based on the obtained result and the previous anamnesis, the attending veterinarian applies antibiotics [(56), **Figure 4**].

For the attending veterinarian, according to Tančin et al. (14) it is important to know if the cow has $G^-$ and $G^+$ bacteria in the udder. In the second case, antibiotic treatment is started after re-culturing the sample taken 24 h after the last application of antimicrobials. Based on the etiological agents, there are 1st, 2nd, and 3rd line antibiotics listed in the antimastitis protocols. The first-line antibiotics are novobiocin, penicillin, dihydro-streptomycin and neomycin. Second-line antimicrobial drugs are amoxycillin-based preparations. Third-line antibiotic is lincomycin-based preparation. When choosing intramammary formulations, it is necessary to start from the long-term monitoring of the pathogen sensitivity in a given herd, and individually evaluate the occurrence of new cases. The main task of the veterinarian in this scheme is to evaluate the effectiveness of individual drugs as well as to adjust the antimastitis protocol, e.g., in the case of some pathogens, to omit the first-line antibiotics, etc. The main advantages of this system are an increase in the effectiveness of antibiotic therapy and a reduction in the occurrence of resistant strains in breeding (55).

Only early diagnosis of mastitis with the cultivation of bacterial mastitis agents with the selection of a suitable antibiotic to which their highest sensitivity has been recorded increases the effect of the treatment with a positive impact on SCC reduction and restoration of the required milk production. This method is only long-term, but also more economical and efficient. Noteworthy, by reducing the SCC from 600 000 to 300 000 for a herd of 100 dairy cows at 34.0 €/100 kg of milk (purchase price of milk in Slovakia), the farmer can obtain up to 6 290 €/year by reducing the loss of milk production in the same herd conditions and feed [(52), **Table 1**].

## Nutrition

Having a healthy herd with proper nutrition is the first step to becoming a successful breeder. A balanced diet plays an important role in udder resistance to infection because certain nutrients affect various mammary resistance mechanisms, namely: (1) leukocyte function, (2) antibody transport and (3) mammary tissue integrity (57, 58).

Cattle breeders with correctly compiled and mixed feed ration, which meets all the requirements imposed during the individual stages of lactation (phase nutrition) can achieve increased resistance of dairy cows to mastitis-causing pathogens. When composing and mixing the feed ration, it is necessary to use feeds that are safe from fungi and mycotoxins. Contaminated feeds adversely affect the immune system, weakening it, hence, making it unable to defend itself against pathogens entering the udder. Similarly, a similar effect on immunity is caused by an overall impoverished feed ration for energy, nitrogenous substances and other essential nutrients necessary for the proper functioning of the body.

Not only can dietary nutrients have a direct impact on immune function and susceptibility to mastitis, but they can indirectly increase cow susceptibility to mastitis through their impact on peripartum metabolic diseases. All essential nutrients can induce one or more metabolic diseases when either deficient or in excess in the transition diet. Hypocalcemia (milk fever) has been shown to slow the closure of the teat sphincter. Cows with milk fever are 8.1 times more likely to have mastitis and nine times more likely to have a coliform mastitis event as a result. Mastitis was also associated with ketosis and retained placenta. Cows with fatty infiltration of the liver have been shown to be slower in clearing E. coli from their mammary gland. Consequently, this translates that a feed ration with the necessary amounts of vitamins, minerals and other immunostimulatory additives improves the body's defenses against pathogenic bacteria (59, 60).

Recently, when compiling feed rations, breeders use various feed additives in the form of humic acids (uptake of mycotoxins, optimization of rumen pH, stabilization of symbiotic microflora and increased utilization of nutrients) (61, 62) or mineral-vitamin supplements (vitamins E, C, and A and essential trace elements; zinc - Zn, copper - Cu, and selenium - Se) with significant antioxidant and immunostimulatory effects to reduce the risk of mastitis in the postpartum period (63–66).

### *Vitamin E and Selenium*

Vitamin E and selenium (Se) compounds are among the most effective antioxidant nutrients, although often deficient in compound feed during the dry period and peripartum period. Selenium is a vital component of the antioxidant enzyme glutathione peroxidase, which is essential for the protection of cells and body tissues. The beneficial effects of Se can be attributed to the decreased damage to cells by oxygen radicals and

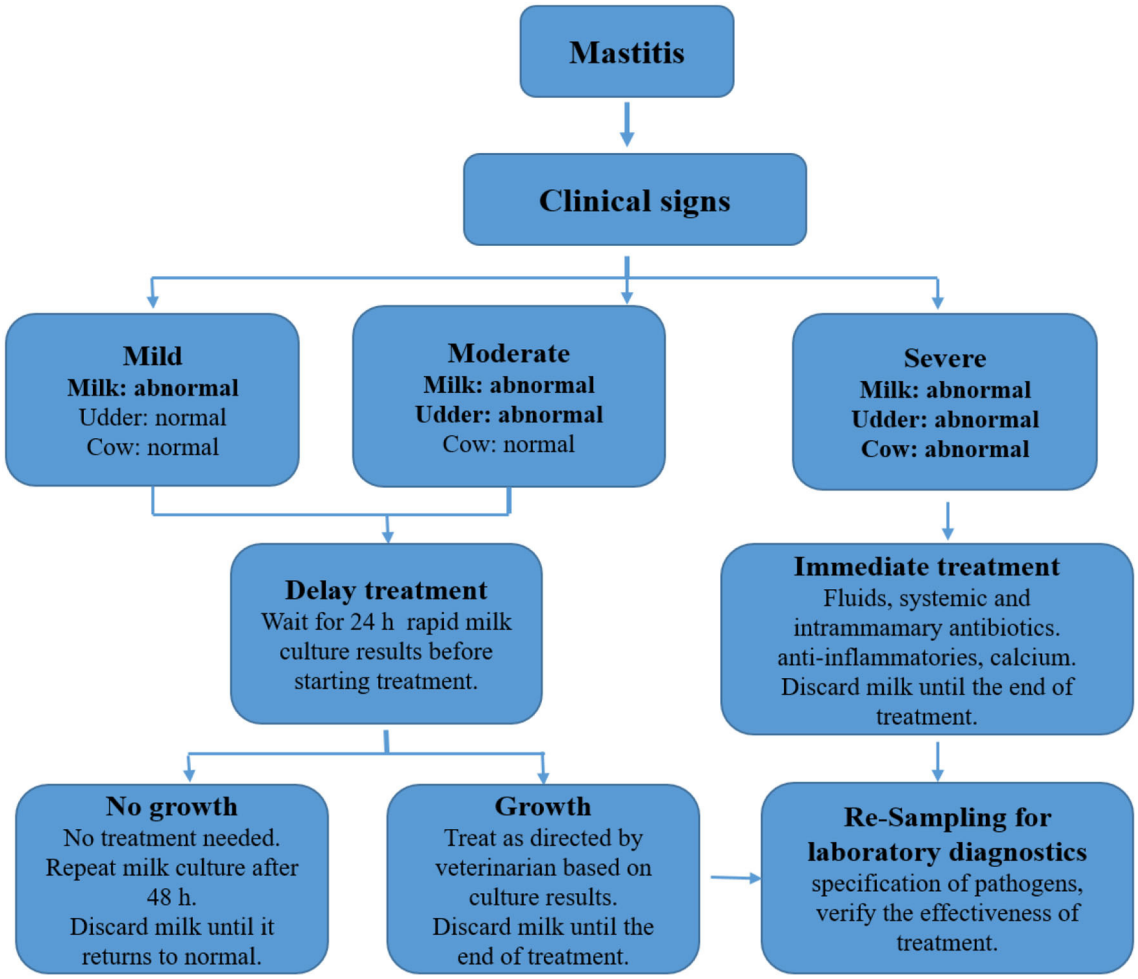


**FIGURE 4 |** Determination of severity and treatment of mastitis according to clinical symptoms. Source: Adapted from Bargren (56).

peroxidases with an increased efficiency of the enzymes that are involved in intracellular killing mechanisms (67).

The proportion of Se in grains or in forage depends on the presence of this element in the soil. The Se concentration in the soil varies greatly even over relatively small areas. Because of this, Se supplement is recommended to maintain the minimum consumption level and to ensure effective immune response. According to the National Research Council (68), the Se recommendations for dairy cattle vary from 0.1 to 0.3 mg/kg DM, regardless of the age and the physiological state of the animal, but there is no reference to the form in which Se supplement should be used, i.e., organic or inorganic. The most used Se inorganic forms are sodium selenite and sodium selenate. In feeds in general, and in yeasts, Se is found in the organic form (69).

Erskine (57) and Hogan et al. (70) documented that Se deficiencies in compound feed can have serious consequences on MG health. The same authors confirmed the benefits of dietary supplementation with organic as well as anorganic Se sources for the control of bovine mastitis. Neutrophil killing of *S. aureus* and other environmental pathogens (e.g., *E. coli*) is greatly enhanced for dairy cows receiving an Se supplement compared with cows that were deficient in Se. Erskine (57) also showed that the supplemented cows experienced clinical mastitis of lesser severity and shorter duration than those of unsupplemented cows. Similarly, Sordillo et al. (71) reported a decrease of the phagocytic ability of blood and milk neutrophil to kill pathogens in dairy cows with a Se deficiency. The opposite situation was reported for neutrophils cows having a higher status of selenium.

According to Slavik et al. (65), Se-organic dietary sources (Se enriched yeast) are more effective than sodium selenite for increasing the concentration of Se in blood, colostrum and milk. On the other hand, other studies confirm that there are no differences between the supplementation of organic and inorganic forms of selenium. Oltramari et al. (72) in his study demonstrated that dairy cows supplemented with organic Se and in organic Se during 124 days showed a reduced incidence of subclinical positive mastitis and strongly positive mastitis as decreased SCC compared to control group feed with 0.278 $mg.kg^{-1}$ DM of Se during experimental phase. Similar results as in the previous study were verified in study

by Viero et al. (73) using doses of sodium selenite and selenium yeast.

From our previous studies suggest that the supplementation of diets for dairy cows, is not important form of Se but its length of supplementation. Ideally, when elevated concentrations of Se (0.3–0.5 mg.kg$^{-1}$ of DM) are added to the feed throughout the dry period and first stage of lactation. Due to the increased immunostimulatory effect of Se is good to add vitamin E to the feed ration (67, 69).

Vitamin E, which is similar to Se in its biological properties. Is an important component of all cell membranes and provides stability and prevents the debilitating peroxidation of membrane lipids. Vitamin E also plays a regulatory role in the biosynthesis of various inflammatory mediators that are necessary for the integrity of integument and wound healing and has shown increased neutrophil bactericidal activity (71).

This essential antioxidant is found in high quantities in fresh, green food stuffs; however, the concentration of vitamin E decreases as age of plants and length of storage increase, and vitamin E is often destroyed in silages. Therefore, deficiencies are common in unpastured cows and in cows during seasons when pasture is not available (74).

Research, as well as practical results, show that Se in particular, together with vitamin E, have a synergistic effect, reducing the prevalence of clinical mastitis, new IMI at calving, and SCC, as well as reducing the severity and duration of clinical mastitis to a greater degree than the supplementation of either micronutrient alone (69, 70, 75).

Selenium and vitamin E are part of the protection of individual antioxidant levels of cellular structures against the amount of free radicals formed, maintaining low tissue concentrations of reactive oxygen species, which are beneficial for the body in many cases. Moreover, they are also used in the etiopathogenesis of diseases and pathological processes (74, 76).

Deficient intake of Se and vitamin E in feed rations and a long-term decrease in plasma concentrations (**Table 2**) of these two important nutrients, which are part of the body's antioxidant system, lead to increased lipid peroxidation and damage to cell membranes (67).

According to Hogan et al. (70) vitamin E and Se deficiency in cows leads to increased formation and accumulation of peroxide radicals in tissues and lipid structures, resulting in placental retention, MG swelling and an increased incidence of mastitis. In dairy cows with a low intake of vitamin E at a dose of 20 IU/kg of dry matter (DM) and 0.1 mg Se/kg of DM during the dry period, incidence of mastitis was increased by 57% compared to the group of cows fed 50 IU/kg of DM vitamin E and 0.3 mg Se/kg of DM.

Eulogio et al. (77) demonstrated that the incorporation of Se and vitamin E in commercial diets of grazing first lactation cows increases milk production and percentage of crude protein, solids non-fat and lactose content and decreases SCC. This data confirms earlier findings that Se and vitamin E supplementation are related to mammary health gland. The performance and economic feasibility of the use of Se plus vitamin E allowed us to obtain a profit margin of $ 0.21 per animal per day in this study.

**TABLE 2 |** Overview of Se and vitamin E deficiency syndromes in ruminants.

| Species | Syndrome | Affected system, resp. organ |
|---|---|---|
| Cattle | Nutritional myodystrophy of calves | Skeletal muscle, myocardium |
| | Retained placenta | Placental connection with the uterus |
| | Ovarian cysts | Ovaries |
| | Decreased production, mastitis | Udder, mammary gland |
| | Immune system disorders | Decrease Th lymphocyte production and phagocytic activity |
| | Anemia | Erythrocytes |
| Sheep/goat | Nutritional myodystrophy | Skeletal muscle, myocardium |
| | Infertility | Loss of uterine tone |
| | Decreased production, mastitis | Udder, mammary gland |
| | Immune system disorders | Decrease Th lymphocyte production and phagocytic activity |

*Source: Adapted from Zigo et al. (67).*

It seems that Se and vitamin E should not only be supplemented and determined in the feed ration but also monitored in the blood plasma of animals. Breeders often rely on the feed intake, but even increased supplementation of vitamin E and Se in the form of concentrates and premixes may not correlate with their current blood concentrations, as was demonstrated in our previous study in cows during the dry and postpartal period. Significant decrease in plasma concentrations of Se and vitamin E below the recommended physiological range during the dry period cannot be compensated by an increased supplementation in feed ration. Rapid rise in plasma concentrations of Se and vitamin E is best ensured by parenteral administration, while long-term stabilization may be achieved by feeding ratios with an increased content of these antioxidants throughout the dry period (67, 69).

In dairy cows, a minimum daily intake of vitamin E from a feed ration of 500–600 IU/head and Se of 0.1–0.3 mg/kg DM is recommended to maintain optimal health. In dry cows and at the initial phase of lactation, daily vitamin E supplementation of 1,000–2,000 IU/head should be provided and the feed ration should contain 0.3–0.4 mg Se/kg DM to achieve a positive effect on the health of the MG and reproduction (69).

### *Humic Acids*

In addition to the supplementation of mineral and vitamin supplements, humic acids have been added to feed ration in recent years to increase the body's defenses and eliminate adverse conditions that could lead to the occurrence of various diseases and ailments. Humic acids are natural organic substances that are formed by the chemical and biological decomposition of organic matter of plant origin and synthetic activity of microorganisms. It alongside fulvic acids and humin are among humic substances that are part of humus. They are based on lignin collectively with other components of plant biomass (sugars, fats, proteins, waxes and resins) (78).

The increased use of humic acids in animal nutrition is further exacerbated by the fact that from 28th January 2022, the legislation will be applied in all Member States of the European Community prohibiting the preventive and mass administration of antibiotics for all groups of farmed animals. In practice, this means continued administration of antibiotics to sick animals, however, only individually, with a clinical examination performed before their administration, respecting the withdrawal period for animal products after their administration. Oral administration of humic acids is one of the approved real alternatives to antimicrobials and zinc oxide (79).

Due to increasing milk production to its maximum level and the associated risk of intramammary infections with subsequent antibiotic treatment, the addition of humic acids to the feed ration is increasingly used, especially during the drying period and the first half of lactation. Rich nutrition with nuclear feeds (especially in the first 100 days of lactation) with a high content of protein, energy and at the same time, low fiber content, which in the case of ruminants is unnatural, negatively affects their health, reproductive and economic indicators (80). Addition of humic acids in a daily dose of 100 g per dairy cow for 60–70 days leads to decreased SCC, reduction in the incidence of subclinical mastitis as well as an increase in both protein levels and fat by 0.2–0.5% and 0.3–0.5%, respectively (81).

Furthermore, the supplementation of humates to the feed stimulates the immune system and the growth of symbiotic rumen microflora. Their mechanism of stimulation of the immune system is related to the ability of humates to bind sugars in the body to complexes. A large number of these complexes allow the body to synthesize glycoproteins that bind to NK and T cells as a modulator and communication link between cells. Thus, they regulate the immune system and prevent the imbalance of T and NK cells (61).

Humates added to feed ration stimulate the growth of symbiotic microorganisms depending on the species while suppressing pathogenic microorganisms. Species that have been inhibited by natural humic agents include *Candida albicans, Enterobacter cloacae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Streptococcus epidermidis*, and *Streptococcus pyogenes* (62).

## Herd Environment and Management

The cleanliness of the environment in which the animals are located is important for the improvement of the health of the udder and elimination of mastitis. The main influences on the hazards and risks associated with aspects of housing and its management are illustrated in [(82), **Figure 5**]. The aim of daily care and maintenance of the stalls is to have clean, dry and satisfied dairy cows when entering the milking parlor. The occurrence of environmental mastitis (environmental mastitis) is related to the level of housing hygiene. From this viewpoint, achieving the lowest possible pollution of the body, especially the udder, deserves high priority in the breeding of high-yielding dairy cows (83).

Among the main actions that help to reduce environmental pathogens is the regular replacement of bedding (straw, sawdust) and the removal of manure. The bed must always be dry and clean to prevent the formation of a breeding ground for bacteria (fecal enterococci and streptococci), which causes environmental mastitis. It is good to use lime or special commercial products available on the market to disinfect and absorb excess moisture from the lying boxes (22, 84).

Due to the frequent lack of straw, recycled manure solids (RMS) have been used as a substitute bedding material in recent years to create sufficient comfort for dairy cows. RMS consists of dry matter and a nutrient-rich fraction obtained by mechanical or gravitational separation of slurry manure removed from dairy cow housing systems. To ensure its hygienic quality and optimum pH, RMS is often combined with straw and other components such as limestone or zeolite (85, 86).

In our previous study, improved composition of bedding per one cubicle consisted of ground limestone (100 kg), water (80 L), recycled manure solids (15 kg), and straw (25 kg) influenced the level of hygiene on indicator microorganisms in comparison to conventional straw bedding. Samples of lying boxes with improved bedding showed reduced total viable count, coliform bacteria, fecal coliform bacteria and fecal streptococci in one-day-fresh improved composition bedding as well as the first 2 months after it was laid. In addition to reducing the number of microorganisms, by using the improved composition bedding for a period of 3 months, the effect of reduced infection pressure from the environment was demonstrated, which resulted in an increased number of healthy quarters with negative CMT score and a reduced incidence of subclinical mastitis in dairy cows (87).

More so, regular cleaning and disinfection of the milking parlor and waiting room should be observed. Usually, the cleanliness of the housing of heifers and dried cows is neglected, making room for pathogens to enter the MG. In herd management, it is important to separate mastitis or otherwise sick dairy cows from healthy animals until they are completely cured or eliminated. Additionally, it is likewise ideal to create a group for primipary cows to prevent the transmission of infection from cows (22).

## Lactating Cows and Milking

During this period, the dairy cows are inspected based on a once a month performance check, giving us a detailed picture of SCC, while highlighting the level of California mastitis test (CMT) needed on the farm (88). At the end of the colostrum period on the 4–6th day, it is necessary to examine each dairy cow by CMT, that is, upon which only healthy animals are moved to the production group. In case of a positive CMT result, it is necessary to proceed with a possible treatment according to **Figure 4**. An important outcome is also the early culture of positive samples from cows with subclinical mastitis based on the positive CMT (89).

This form of mastitis is the most prevalent type of intramammary infection, but it cannot be detected by looking at the MG or the milk because both appear normal. The majority of infections are caused by the staphylococci and streptococci. If antibiotic therapy is to make a significant contribution toward reducing the herd level of mastitis as well as the bulk tank SCC, it is necessary to treat subclinical infections as well as the clinical cases. It is not unusual to have 15–40 subclinical cases

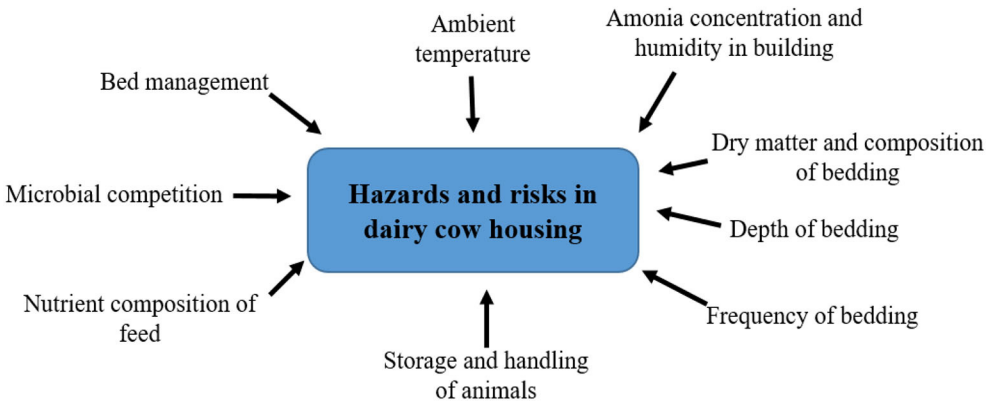


**FIGURE 5 |** Factors affecting hazards and risks associated with bedding materials in dairy cow housing. Source: Adapted from Bradley et al. (82).

for every clinical case caused by contagious pathogens. Generally, antibiotic intramammary therapy of subclinical mastitis during lactation is indicated only when *Strep. agalactiae* or *S. aureus* are present, or the producer is in danger of losing his milk market due to a high bulk tank SCC (58).

Blowey and Edmondson (1) observed that the treatment of cows subclinically infected with *Strep. agalactiae* is usually successful and results in increased production and a dramatic decrease in bulk tank SCC. In contrast, it is not considered cost-effective to treat cows that are chronically infected with *S. aureus* because cure rates during lactation are rather poor.

A proper milking hygiene program that meets all biological and hygienic requirements of the dairy cow significantly influences the maintenance of good udder health. The purpose of milk hygiene is, of course, not just the control of mastitis in the cows, but to guarantee that the milk sent for sale is fit for human consumption. This requires attention to hygiene at all stages of the milking process and storage in bulk tanks. The standard measure of the milk hygiene is the total bacteria count (TBC). This is measured routinely by the dairies collecting milk off farm. Within the EU, herd counts of TBC >100 000 per ml are penalized to the extent that action is called for; TBC >50 000 may incur a reduction in the milk price. A marker of good practice would be TBC that were consistently <15 000/mL (20).

The most common bad practices and mistakes in milking hygiene program are: spraying water on the udder when the cows enter the parlor (**Figure 6**), emptying the teat cistern (first sprays of milk) on the ground, weak stimulation and insufficient udder toilet (ineffective predipping) before milking, soiled and unwashed milking clusters of the milking machine, attachment of the milking cups to a dirty or insufficiently wiped udder, incorrect attachment of milking machines, failure to disinfect the teats after each milking, too short or ineffective postdipping, etc. (48).

Milkers must follow a well-defined workflow that includes conditions associated with milking on a particular business; these are divided into the following steps: (1). washing and drying of teats, (2). make the first sprays from all the quarters in a container with a black bottom and perform a sensory assessment of the quality of the milk, (3). application of pre-dip (preparation before milking), (4). drying of teats, (5). put on the milking equipment,

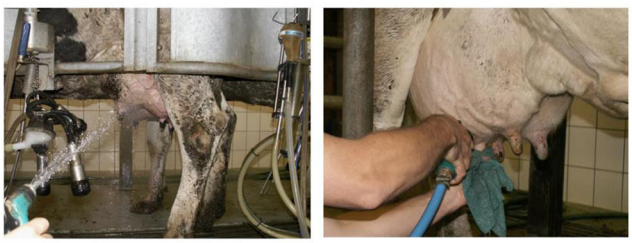

**FIGURE 6 |** Incorrect and correct udder washing. Note: Direct spraying with a strong stream of water on the udder is unacceptable. Slightly flowing hot water is possible to use only on contaminated teats of the udder. Source: Tančin (64).

(6). no milking on dry, (7). post-dip application (preparation after milking), (8). rinsing and regular technical maintenance of milking equipment, (9). after milking, feed the cows to keep them upright until the teat close (∼20–30 min.) (40).

Other important factors in milk production are milking efficiency and regular maintenance of the milking equipment. Mistakes and undesired effects of machine milking that increase the risk of colonization of the teat duct and new IMI include: incomplete milking of the udder, incomplete empty milking, large pressure fluctuations in vacuum regulator, too fast or too slow pulsation, slow flow of milk from the milk claw distributor causing "flooding of teats," removing the milking clusters before switching off the vacuum, etc. It is also necessary to keep in mind the service life of the individual components of the milking equipment as well as the service and setting the functional parameters of the milking equipment. Any underestimation or delay in regular inspections of the milking equipment to "save money" later draws much more money out of the cash register (48).

## Drying Cows

A very important part of the further milk production after the previous lactation is the management in the dry period. This is a period when ideal conditions are created for the regeneration

of MG tissue after previous production, on many aspects–physiological, morphological and immunological (90, 91).

Cows are naturally protected against intramammary infections during the dry period by formation of a keratin plug in the teat canal. However, time of teat canal closure varies among cows. In a study conducted by Williamson et al. (92), 50% of teat canals were classified as closed by 7 days after dry off, 45% closed over the following 50–60 days of the dry period, and 5% had not closed by 90 days after dry off. Teats which do not form a plug-like keratin seal are thought to be most susceptible to infection.

However, a quarter that becomes infected during the dry period, or that remains infected from the previous lactation, will produce 30–40% less milk (54). One of the most important aspects in this period is the length of the drying interval of the cows. Cows should be dried no later than 60 days before planned calving, except for dairy cows with a high daily intake (more than 25 L), where the dry period can be reduced by 10 days. The risk of mastitis is greatest at the beginning and end of this period; this is the reason, it is necessary to pay extreme attention to pregnant animals with proper hygiene procedures and principles of application of intramammary preparations (93).

Dairy cows ready for drying must be examined by CMT, if they are positive, they must be treated and only then dried with an effective intramammary long-term effect antibiotic. Treatment of all quarters of all cows at drying off (blanket dry cow therapy) is one of the most important components of a comprehensive plan of mastitis control. This is because dry cow therapy both cures existing infections, caused mainly by contagious pathogens, and prevents the development of new infections, caused mainly by environmental pathogens (5, 94).

For drying cows, a "Combo" application therapy for the administration of preparations based on antibiotics and keratin seal is effective. After the last udder milking, it is necessary to administer intramammary antibiotic injections for drying and injections with teat sealant providing an external physical barrier for the teat orifice during critical times in the dry period. Thereafter, it is recommended to soak the teats in a post-dip. When a cow is prepared in this way, the onset of a new infection during the dry period would be prevented (27).

Godden et al. (95) demonstrated a significant reduction in the incidence of clinical mastitis during the first stage of lactation in cows that had been treated with intramammary anantibiotic with teat seal when compared to cows treated only teat seal. Their results confirmed that intramammary infusion with teat sealant as an adjunct to long-term effect antibiotic at dry off had a significant effect on reducing the risk for acquiring a new IMI between 1 and 3 days in milking (DIM; treatment = 22.8%, control = 29.1%), 6–8 DIM (treatment = 20.6%, control = 25.9%) and 60 DIM (treatment = 5.9%, control = 8.0%) (**Figure 7**).

Legislative and consumer pressure is to reduce antibiotic use in primary milk production. One way to achieve that is to selectively use antibiotics in dry cows. Selective use of antibiotics can be a useful tool, but only in individual quarters of the udder or in whole udders with low SCC and without the presence of pathogens. In farming conditions selective application of antibiotics to dry cows is done on the basis of a history, clinical examination of the udder and evaluation of the SCC from milk utility monitoring records (96).

After considering all aspects, breeders classify dairy cows into three categories. The first category includes dairy cows in which no problems with MG health were observed during lactation, the SCC in the sample taken before drying did not exceed 200 x $10^3$ in 1 mL and no clinical or subclinical form of mastitis was currently diagnosed. These dairy cows are ideal for drying without the application of antibiotics using a teat sealant (it definitely closes the teat canal and prevents pathogens from the entering MG) or by immersing the teats in a protective solution. The second group consists of dairy cows that have an SCC higher than 200 x $10^3$ in 1 mL, in which mastitis was recorded during lactation, but currently the MG is free of clinical signs of inflammation. In this case, a long-acting intramammary antibiotic is applied to each quarter in conjunction with an internal teat sealant or a protective solution. The antibiotic for dry period should be chosen based on the overall susceptibility of the bacteria to the antibiotics used (antibiogram) on the farm as well as on the last examination of milk samples from individual dairy cows (56, 93).

The third group includes dairy cows with current clinical or chronic mastitis as well as dairy cows with non-milked quarters to which an antibiotic preparation may be applied. In these dairy cows, the infected quarters must first be treated during lactation with adequate intramammary antibiotics and proper milking. Subsequently, the cow is dried according to the above scheme for the second group with an intramammary antibiotic preparation and teat sealant (97, 98).

Intramammary treatment with blanket drying-off therapy of all udder quarters with long acting antibiotics at the beginning of the dry period, has been recommended for a long time because it proved to reduce efficiently the level of infection at the herd level. The products used are mainly effective against Gram positive bacteria, yet there is no increase in the prevalence of infections by Gram negative bacteria associated to their use. After the cessation of treatment and before calving, the cow is at risk of developing a new infection when it is no longer under antibiotic protection. The antibiotic concentrations more than 3 weeks during drying period has the potential to interfere durably with any protective intramammary microbiota, and microbiota disruption by antibacterial products is known to favor dysbiosis, which may increase the susceptibility to infections. At this point, sterile udder and created colostrum that are an ideal breeding ground for bacteria, is beginning to form (42).

For these purposes, probiotics have been used to protect the udder and support the protective microflora. According to the concept of mastitis as a manifestation of dysbiosis, i.e., an imbalance of the intramammary microbiota, the use of probiotics to re-equilibrate the microbiota appears as a possible corrective measure. Oral supplementation of probiotics for the treatment of IMI have not been effectively in polygastric animals such as ruminants, especially since the enteromammary pathway is poorly operative in these species. This is probably why probiotics for the bovine MG have been administered through the teat canal (99).

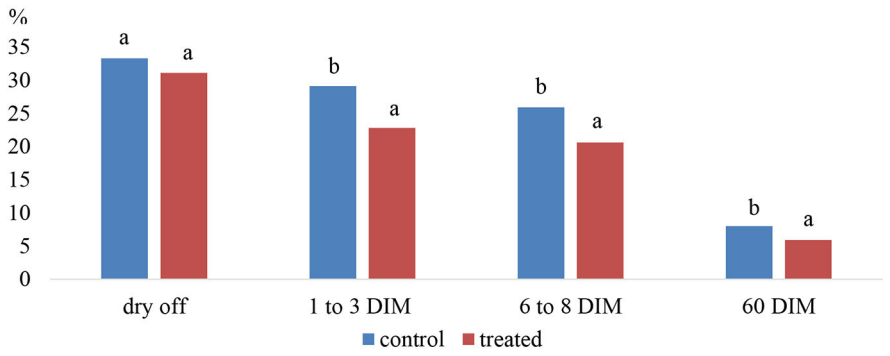


**FIGURE 7 |** Prevalence of IMI in control (antibiotic only) and treated (seal plus antibiotic) quarters at dry off, 1–3 days in milking (DIM), 6–8 DIM and 60 DIM. Note: [a,b]% of all quarters with IMI between columns are significantly different ($P < 0.05$). Source: Adapted from Godden et al. (95).

Several Lactobacillus species as *L. lactis, L. acidophilus, L. casei* or *L. perolens* has been used as an alternative non-antibiotic treatment of mastitis. Intramammary inoculation of a probiotic mixture to cure mastitis has been found to be efficient only in minor pathogens as *Corynebacterium bovis* and coagulase-negative staphylococci. Intrammary infusion of $10^6$ cfu *L. perolens* or other probiotic mixture has been found ineffective to major pathogens such as *S. aureus*, streptococci and *E. coli*. Application of probiotics through teat canal causes an inflammatory response from the MG, and this is probably why they have been used for therapy rather than for prevention of IMI. Although quite a few studies reported some protective effect by minor pathogens, others have found the converse or no effect, and several recent reviews conclude that a protective effect would be of low magnitude in any case (100, 101).

## Vaccination Procedures Against Mastitis Pathogens

Immunoprophylaxis of mastitis involves means and methods for targeted enhancement of specific immunity to an infectious agent. The possibility of vaccinating cows against specific pathogens causing intramammary infections is a relatively new specific tool for suppressing, controlling and preventing MG inflammation, but this method also has its drawbacks (102).

Most vaccines are based on achieving high levels of specific antibodies in the blood of dairy cows that pass into milk. The first drawback is that the transfer of antibodies from the blood to the milk does not take place by diffusion. It occurs by active transport independent of the concentration of antibodies in the blood and is dependent on the physiological state of the MG (103).

Another drawback is the low level of complement in the milk and the low performance of neutrophils. Their lack of performance is due to the fact that they only express a small percentage of immunoglobulin receptors on their surface. The fact that they consume a high amount of oxygen limits their function due to the fact that the oxygen concentration in milk is a 100 times lower than in blood. In addition, phagocytic cells need energy for the process of phagocytosis, which they can draw from glucose, but this is also present in low concentrations in milk. Another problem is that a large part of phagocytic cells also absorbs harmless fat droplets, which depletes their number (104).

Monovalent (*Staphylococcus aureus*) or polyvalent (streptococci/staphylococci) vaccines can be used in a mastitis control programs. Available vaccines can shorten the duration of infection and limit the circulation of some contagious microorganisms causing MG inflammation, mainly *Staphylococcus aureus*, throughout the herd. In the USA for example, four preparations are used which, although do not reduce the frequency of MG infections, they alleviate its manifestations (105).

Toušová et al. (106) recommends the use of a polyvalent vaccine in dairy herds with recurrent mastitis and to reduce clinical signs caused by coliform bacteria, *S. aureus* and coagulase negative staphylococci. The author also reports a significant reduction in SCC in a pooled milk sample during lactation in vaccinated dairy cows throughout lactation. At the same time, it was found that dairy cows treated according to the vaccination schedule in three doses showed a reduction in the incidence of mastitis up to 43.5%, in contrast to untreated dairy cows, when the incidence was 65%.

According to Doležal et al. (107) most vaccination schedules consist of three doses. The first dose is given when the dairy cows are dried, the second a month later and the third 2 weeks after birth. The efficiency of immunoprophylaxis in dairy cows at second and higher lactations is reported to be in the range of 10 to 20%, both by reducing clinical forms of mastitis and also by reducing the number of subclinical and latent mastitis. In order to achieve the maximum effectiveness of immunoprophylaxis, the author recommends starting vaccination in heifers before the first mating. The first dose is given at 6 months of age and the next one in 14 days. This procedure is repeated every 6 months until calving. Then, revaccinations occur at calving and 6 months after calving. The author further states in his study that after five administered doses, the primipary cows in the calving period has a significant reduction in the incidence of mastitis.

Although preventive use of the vaccine in production herds is an economic burden associated with higher costs of purchase and self-administration, its positive benefits associated with better

milk monetization due to reduced SCC with lower mastitis is one way to improve profitability of milk production, healthy cows and reduces the number of weaned dairy cows (105).

## CONCLUSION

Taking into account all measures affecting the reduction of mastitis, effective prevention programs can be developed. One of the most proven antimastitis programs is based on strict adherence to ten steps, which include: (1). setting a mammary gland health target, (2). ensuring clean and dry housing of animals, (3). adherence to the order of dairy cows in the milking parlor (calved cows, production groups, end of lactation and treated), (4). correctly chosen milking procedure, (5). care of milking equipment, (6). initiation of early and adequate treatment of clinical cases of mastitis, (7). keeping records of treated cows with evaluation and updating of antibiotics used, (8). effective management and selective use of antibiotics in the drying off cows, (9). culling of the chronically ill resp. incurable dairy cows, (10). periodic assessment of the antimastitis program.

These 10 measures in the antimastitis program provide a comprehensive system that allows breeders to control the most important actions that are directly related to the origin and spread of mastitis in the herd. Only respect for current scientific knowledge in a logical context and the complex in the daily application of proven prevention and control practices in production farms can positively affect the overall production, quality and nutritional value of milk with a positive impact on consumer health.

## AUTHOR CONTRIBUTIONS

FZ proposed and wrote the manuscript. JB and EP-K provided critical review the manuscript. MV provided helpful comments on the article. SO editing the manuscript. JV and FZ corrected manuscript according to the reviewers' recommendations. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. Blowey R, Edmondson P. *Mastitis Control in Dairy Herds*. 2nd Edition,sss CAB International (2010). p. 272. ISBN 978-1-84593-550-4. Available online at: https://www.lactoscan.com/editor/ufo/manuals/SCC/Mastitis_Control_in_Dairy_Herds_2nd(veterinary-student.blogfa.com).pdf (accessed October 2, 2020).
2. Zajac P, Tomaska M, Murarova A, Capla J, Curlej J. Quality and safety of raw cow's milk in Slovakia in 2011. *Slovak J Food Sci*. (2012) 6:64–73. doi: 10.5219/189
3. Tvarožková K, Tančin V, Holko I, Uhrinčat M, Mačuhová L. MASTITIS IN EWES: somatic cell counts, pathogens and antibiotic resistance. *J Microbiol Biotechnol Food Sci*. (2019) 9:661–70. doi: 10.15414/jmbfs.2019/20.9.3.661-670
4. Tvarožková K, Tančin V, Uhrinčat M, Hleba L, Mačuhová L. Mastitis pathogens and somatic cell count in ewes milk. *Slovak J Food Sci*. (2020) 14:164–9. doi: 10.5219/1338
5. Tiwari JG, Babra C, Tiwari HK, Williams V, Wet SD, Gibson J, et al. Trends in therapeutic and prevention strategies for management of bovine mastitis: an overview. *J Vaccines Vaccin*. (2013) 4:176. doi: 10.4172/2157-7560.1000176
6. Fourichon C, Seegers H, Malher X. Effect of disease on reproduction in the dairy cow: a meta-analysis. *Theriogenology*. (2000) 53:1729–59. doi: 10.1016/S0093-691X(00)00311-3
7. Dubuc J, Duffield TF, Leslie KE, Walton JS, Leblanc SJ. Effects of postpartum uterine diseases on milk production and culling in dairy cows. *J Dairy Sci*. (2011) 94:1339–46. doi: 10.3168/jds.2010-3758
8. Turk R, Koledić M, Macešić N, Benic M, Dobranic V, Duricic D, et al. Mastitis in dairy cows. *Mljekarstvo*. (2017) 67:91–101. doi: 10.15567/mljekarstvo.2017.0201
9. Patil MP, Nagvekar AS, Ingole SD, Bharucha SV, Palve VT. Somatic cell count and alkaline phosphatase activity in milk for evaluation of mastitis in Buffalo. *Vet World*. (2015) 8:363–6. doi: 10.14202/vetworld.2015.363-366
10. Tančin V, Baranovič Š, Uhrinčat M, Mačuhová L, Vršková M, Oravcová M. Somatic cell counts in raw ewes' milk in dairy practice: Frequency of distribution and possible effect on milk yield and composition. *Mljekarstvo*. (2017) 67:253–60. doi: 10.15567/mljekarstvo.2017.0402
11. Legates JE, Grinnells CD. Genetic relationships in resistance to mastitis in dairy cattle. *J Dairy Sci*. (1952) 35:829–33. doi: 10.3168/jds.S0022-0302(52)93763-6
12. Oliver SP, Mitchell BA. Susceptibility of bovine mammary gland to infections during the dry period. *J Dairy Sci*. (1983) 66:1162–6. doi: 10.3168/jds.S0022-0302(83)81913-4
13. Ruegg PL. A 100-year review: mastitis detection, manage-ment, and prevention. *J Dairy Sci*. (2017) 100:10381–97. doi: 10.3168/jds.2017-13023
14. Tančin V, Ipema AH, Hogewerf P. Interaction of somatic cell count and quarter milk flow patterns. *J Dairy Sci*. (2007) 90:2223–8. doi: 10.3168/jds.2006-666
15. Uhrinčaťová M, Tančin V, Tvarožková K, Mačuhová L, Vršková M, Ptáček M, et al. The electrical conductivity of sheep's milk and the possibility of mastitis detection. *Potravinarstvo Slovak J Food Sci*. (2019) 13:562–5. doi: 10.5219/1074
16. Holko I, Tančin V, Vršková M, Tvarožková K. Prevalence and antimicrobial susceptibility of udder pathogens isolated from dairy cows in Slovakia. *J Dairy Res*. (2019) 86:436–9. doi: 10.1017/S0022029919000694
17. Abebe R, Hatiya H, Abera M, Megersa B, Asmare K. Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Vet Res*. (2016) 12:270. doi: 10.1186/s12917-016-0905-3
18. Zigo F, Elečko J, Farkašová Z, Zigová M, Vasil M, Ondrašovičová S, et al. Preventive methods in reduction of mastitis pathogens in dairy cows. *J Microbiol Biotechnol Food Sci*. (2019) 9:121–6. doi: 10.15414/jmbfs.2019.9.1.121-126
19. Nazmul HM, Istiaq A, Shaminur RM, Rafiul IM, Anwar A, Zonaed SA, et al. Microbiome dynamics and genomic determinants of bovine mastitis. *Genomics*. (2020) 112:5188–203. doi: 10.1016/j.ygeno.2020.09.039
20. Webster J. *Understanding the Dairy Cows*. 3rd ed. Oxford, UK: Wiley Blackwell (2020). p. 258.
21. Sharma N, Jeong DK. Stem cell research: a novel boulevard towards improved bovine mastitis management. *Int J Biol Sci*. (2013) 9:818–29. doi: 10.7150/ijbs.6901

22. Brouček J, Mihina Š, Uhrinčať M, Lendelová J, Hanus A. Impact of gestation and lactation stage on the dairy cow response following removal to unfamiliar housing and milking system. *Ital J Anim Sci.* (2015) 14:233–7. doi: 10.4081/ijas.2015.3410
23. Pyörälä S, Taponen S. Coagulase-negative staphylococci - emerging mastitis pathogens. *Vet Microbiol*. (2009) 134:3–8. doi: 10.1016/j.vetmic.2008.09.015
24. Garcia AB, Shalloo L. Invited review: the economic impact and control of paratuberculosis in cattle. *J Dairy Sci*. (2015) 98:5019–39. doi: 10.3168/jds.2014-9241
25. Fox LK, Gay JM. Contagious mastitis. The Veterinary clinics of North America. *Food Anim*. (1993) 9:475–87. doi: 10.1016/S0749-0720(15)30615-0
26. Trinidad P, Nickerson SC, Adkinson RW. Histopathology of staphylococcal mastitis in unbred dairy heifers. *J Dairy Sci*. (1990) 73:639–47. doi: 10.3168/jds.S0022-0302(90)78715-2
27. Sol J, Sampimon OC, Snoep JJ, Schukken YH. Factors associated with bacteriological cure after dry cow treatment of subclinical staphylococcal mastitis with antibiotics. *J Dairy Sci.* (1994) 77:75–9. doi: 10.3168/jds.S0022-0302(94)76930-7
28. Rainard P. Mammary microbiota of dairy ruminants: fact or fiction? *Vet Res.* (2017) 48:25. doi: 10.1186/s13567-017-0429-2
29. Tančin V, Apolen D, Botto L, Brestenský V, Brouček J, Dano J, et al. *Livestock Farming in Marginal Areas*. LuŽianky, Slovakia: CVŽV Nitra (2013). p. 70.
30. Mbindyo CM, Gitao GC, Mulei CM. Prevalence, etiology, and risk factors of mastitis in dairy cattle in Embu and Kajiado Counties, Kenya. *Vet Med Int*. (2020) 2020:8831172. doi: 10.1155/2020/8831172
31. Sharma N, Singh NK, Bhadwal MS. Relationship of somatic cell count and mastitis: an overview. *Asian Aust J Ani Sci.* (2011) 24:429–38. doi: 10.5713/ajas.2011.10233
32. Fox LK. Prevalence, incidence and risk factors of heifer mastitis. *Vet Microbiol.* (2009) 134:82–8. doi: 10.1016/j.vetmic.2008.09.005
33. Otto M. Virunce factors of the coagulase-negative staphylococci. *Front Biosci*. (2004) 9:841–63. doi: 10.2741/1295
34. Moniri R, Dastehgoli K, Akramian A. Increasing resistant CNS in bovine clinical mastitis. *Pakistan J Biol Sci*. (2007) 10:2465–9. doi: 10.3923/pjbs.2007.2465.2469
35. Melchior MB, Fink-Gremmels J, Gaastra W. Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J Vet Med B Infect Dis Vet Public Health*. (2006) 53:326–32. doi: 10.1111/j.1439-0450.2006.00962.x
36. Nascimento JS, Fugundes PC, Brito AV, Dos Santos KR, Bastos MC. Production of bacteriocins by coagulase-negative staphylococci involved in bovine mastitis. *Vet Microbiol*. (2005) 106:61–71. doi: 10.1016/j.vetmic.2004.10.014
37. Haveri M, Roslöf A, Pyörälä S. Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. *J Appl Microbiol*. (2007) 103:993–1000. doi: 10.1111/j.1365-2672.2007.03356.x
38. Vasil M, Elečko J, Zigo F, Farkašová Z. Occurrence of some pathogenity factors in coagulase negative staphylococci isolated from mastitis milk in dairy cows. *Potravinarstvo*. (2012) 6:60–3. doi: 10.5219/186
39. Bronzo V, Lopreiato V, Riva F, Amadori M, Curone G, Addis MF, et al. The role of innate immune response and microbiome in resilience of dairy cattle to disease: the mastitis model. *Animals*. (2020) 10:1397. doi: 10.3390/ani10081397
40. Tančin V, Uhrinčať M, Mačuhová L, Bruckmaier RM. Effect of pre-stimulation on milk flow patternand distribution of milk constituents at a quarter level. *Czech J Anim Sci.* (2007) 52:117–21. doi: 10.17221/2234-CJAS
41. Ezzat Alnakip M, Quintela-Baluja M, Böhme K, Fernández-No I, Caamaño-Antelo S, Calo-Mata P, et al. The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. *J Vet Med.* (2014) 2014:659801. doi: 10.1155/2014/659801
42. Rainard P, Fromageau A, Cunha P, Gilbert FB. *Staphylococcus aureus* lipoteichoic acid triggers inflammation in the lactating bovine mammary gland. *Vet Res*. (2008) 39:52. doi: 10.1051/vetres:2008034
43. Thompson-Crispi K, Atalla H, Miglior F, Mallard BA. Bovine mastitis: frontiers in immunogenetics. *Front Immunol*. (2014) 5:493. doi: 10.3389/fimmu.2014.00493
44. Stelwagen K, Carpenter E, Haigh B, Hodgkinson A, Wheeler TT. Immune components of bovine colostrum and milk. *J Anim Sci*. (2009) 87:3–9. doi: 10.2527/jas.2008-1377
45. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science*. (2010) 327:291–5. doi: 10.1126/science.1183021
46. Bougarn S, Cunha P, Gilbert FB, Harmache A, Foucras G, Rainard P. Staphylococcal-associated molecular patterns enhance expression of immune defense genes induced by IL-17 in mammary epithelial cells. *Cytokine*. (2011) 56:749–59. doi: 10.1016/j.cyto.2011.09.020
47. *NMC: National Mastitis Council 56th Annual Meeting: Proceedings, January 28-31*, St. Pete's Beach, Florida (2017). p. 190.
48. Hovinen M, Pyörälä S. Invited review: udder health of dairy cows in automatic milking. *J Dairy Sci.* (2011) 94:547–62. doi: 10.3168/jds.2010-3556
49. Alhussien MN, Dang AK. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. *Vet World*. (2018) 11:562–77. doi: 10.14202/vetworld.2018.562-577
50. Idriss SH, Tančin V, Foltýs V, Kirchnerová K, Tančinová D, Vršková M. Relationship between mastitis causative pathogens and somatic cell count in milk of dairy cows. *Potravinárstvo*. (2013) 7:207–12. doi: 10.5219/304
51. Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez NR. Monitoring udder health and milk quality using somatic cell counts. *Vet Res*. (2003) 34:579–96. doi: 10.1051/vetres:2003028
52. Tongel P, Mihina Š. *Mastitis - a milk thief. Institute of Scientific and Technical Information for Agriculture, Nitra*. (2005). Available online at: http://www.agroporadenstvo.sk/zv/hd/mlieko/mastitida.htm
53. Reksen O, Solverod L, Branscum AJ, Osterås O. Relationships between milk culture results and treatment for clinical mastitis or culling in Norwegian dairy cattle. *J Dairy Sci.* (2006) 89:2928–37. doi: 10.3168/jds.S0022-0302(06)72565-6
54. Nickerson SC, Ryman VE. *Antibiotic Therapy in Mastitis Control for Lactating and Dry Cows*. University of Georgia, UGA Cooperative Extension Bulletin 1516, (2019). p. 11. Available online at: https://secure.caes.uga.edu/extension/publications/files/pdf/B%201516_1.PDF (accessed January 28, 2021).
55. Zadoks R, Fitzpatrick J. Changing trends in mastitis. *Ir Vet J*. (2009) 6:59–70. doi: 10.1186/2046-0481-62-S4-S59
56. Bargren C. *Culture Milk to Save Money*. River Valley Veterinary Clinic, Newsletter (2017). p. 1–2. Available online at: https://rivervalleyvet.net/wp-content/uploads/2018/08/july-17-milk-cultures.pdf (accessed December 30, 2020).
57. Erskine RJ. Nutrition and mastitis. *Vet Clin North Am Food Anim Pract*. (1993) 9:551. doi: 10.1016/S0749-0720(15)30621-6
58. Nickerson SC, Kautz FM, Nace EL. *Managing Mastitis in Dairy Heifers to Improve Overall Herd Health*. Bulletin 1416, UGA Cooperative Extension. (2013). Available online at: https://secure.caes.uga.edu/extension/publications/files/pdf/B%201416_2.PDF (accessed December 28, 2020).
59. Eastridge ML. Major advances in applied dairy cattle nutrition. *J Dairy Sci.* (2006) 89:1311–23. doi: 10.3168/jds.S0022-0302(06)72199-3
60. Cobirka M, Tancin V, Slama P. Epidemiology and classification of mastitis. *Animals*. (2020) 10:2212. doi: 10.3390/ani10122212
61. Islam KMS, Schumacher A, Gropp JM. Humic acid substances in animal agriculture. *Pak J Nutr*. (2005) 4:126–34. doi: 10.3923/pjn.2005.126.134
62. Galip N, Polat U, Biricik H. Effects of supplemental humic acid on ruminal fermentation and blood variables in rans. *Ital J Anim Sci.* (2010) 9:390–3. doi: 10.4081/ijas.2010.e74
63. Dias RS, López S, Montanholi, Smith B, Haas LS, Miller SP, France J. A meta-analysis of the effects of dietary copper, molybdenum, and sulfur on plasma and liver copper, weight gain, and feed conversion in growing-finishing cattle. *J Anim Sci*. (2013) 91:5714–23. doi: 10.2527/jas.2013-6195
64. Dang AK, Prasad S, De K, Pal S, Mukherjee J, Sandeep IVR, et al. Effect of supplementation of vitamin E, copper and zinc on the in vitro phagocytic activity and lymphocyte proliferation index of peripartum Sahiwal (*Bos indicus*) cows. *J Anim Physiol Anim Nutr*. (2013) 97:315–32. doi: 10.1111/j.1439-0396.2011.01272.x
65. Slavik P, Illek J, Brix M, Hlavicova J, Rajmon R, Jilek F. Influence of organic versus inorganic dietary selenium supplementation on the concentration of

selenium in colostrum, milk and blood of beef cows. *Acta Vet Scand.* (2008) 50:1–6. doi: 10.1186/1751-0147-50-43
66. Mehdi Y, Dufrasne I. Selenium in cattle: a review. *Molecules*. (2016). 21:545. doi: 10.3390/molecules21040545
67. Zigo F, Farkašová Z, Elečko J, Lapin M, Chripková M, Czerski A. Effect of parenteral administration of Selenium and vitamin E on health status of mammary gland and on selected antioxidant indexes in blood of dairy cows. *Pol J Vet Sci.* (2014) 17:217–23. doi: 10.2478/pjvs-2014-0031
68. National Research Council, *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. Washington, DC, USA: National Academy Press (2001). Available online at: http://www.nmconline.org/wp-content/uploads/2016/08/RECOMMENDED-MASTITIS-CONTROL-PROGRAM-International.pdf (accessed November 2, 2020).
69. Vasil M, Zigo F, Elečko J, Zigová M, Farkašová Z. Effect of peroral supplementation with selenium and vitamin E during late pregnancy on udder health and milk quality in dairy cows. *Potravinarstvo Slovak J Food Sci*. (2017) 11:535–8. doi: 10.5219/766
70. Hogan JS, Weiss WP, Smith KL. Role of vitamin E and selenium in host defense against mastitis. *J Dairy Sci.* (1993) 76:2795–803. doi: 10.3168/jds.S0022-0302(93)77618-3
71. Sordillo LM, Shafer-Weaver K, DeRosa D. Immunobiology of the mammary gland. *J Dairy Sci*. (1997) 80:1851–65. doi: 10.3168/jds.S0022-0302(97)76121-6
72. Oltramari CE, Pinheiro MG, de Miranda MS, Arcaro JRP, Castelani L, Toledo LM, et al. Selenium sources in the diet of dairy cows and their effects on milk production and quality, on udder health and on physiological indicators of heat stress. *Ital J Anim Sci*. (2014) 13:1. doi: 10.4081/ijas.2014.2921
73. Viero V, Fischer V, Machado SC, Zanela MB, Ribeiro MER, Barbosa RS, et al. Efeitoda suplementação com diferentes níveisde selênio orgânico e inorgânico na pro-dução e na composição do leite e nosangue de vacas em lactação. *Arq Bras Med Vet Zoo*. (2010) 62:382–39. doi: 10.1590/S0102-09352010000200019
74. Bouwstra RJ, Nielen M, Stegeman JA, Dobbelaar P, Newbold JR, Jansen EHJM. Vitamin E supplementation during the dry period in dairy cattle. Part I: adverse effect on incidence of mastitis postpartum in a double-blind randomized field trial. *Dairy Sci.* (2010) 93:5684–95. doi: 10.3168/jds.2010-3159
75. Hoque MN, Das ZC, Rahman ANM.A, Hoque MM. Effect of administration of vitamin E, selenium and antimicrobial therapy on incidence of mastitis, productive and reproductive performances in dairy cows. *Int J Vet Sci Med*. (2016) 4:63–70. doi: 10.1016/j.ijvsm.2016.11.001
76. Pilarczyk B, Jankowiak D, Tonza MA, Pilarczyk R, Sablik P, Drozd R, et al. Selenium concentration and glutathione peroxidase (GSH-Px) activity in serum of cows at different stages of lactation. *Biol Trace Elem Res.* (2012) 147:91–6. doi: 10.1007/s12011-011-9271-y
77. Eulogio GLJ, Hugo CV, Antonio CN, Alejandro CI, Juan MQ. Effects of the selenium and vitamin E in the production, physicochemical composition and somatic cell count in milk of Ayrshire cows. *J Anim Vet Adv*. (2012) 11:687–91. doi: 10.3923/javaa.2012.687.691
78. Mudroňová D, Karaffová V, Pešulová T, Koščová J, Maruščaková I, Bartkovský M, et al. The effect of humic substances on gut microbiota and immune response of broilers. *Food Agric Immunol.* (2020) 31:137–49. doi: 10.1080/09540105.2019.1707780
79. Semjon B, Marcinčáková D, Koréneková B, Bartkovský M, Nagy J, Turek P, et al. Multiple factorial analysis of physicochemical and organoleptic properties of breast and thigh meat of broilers fed a diet supplemented with humic substances. *Poult Sci.* (2020) 99:1750–60. doi: 10.1016/j.psj.2019.11.012
80. Potúčková M, Kourimská L. Effect of humates diet of dairy cows on the raw milk main components. *Potravinarstvo Slovak J Food Sci.* (2017) 11:558–63. doi: 10.5219/748
81. Zigo F, Vasil M, Farkašová Z, Ondrašovičová S, Zigová M, Malová J, et al. Impact of humic acid as an organic additive on the milk parameters and occurrence of mastitis in dairy cows. *Potravinarstvo Slovak J Food Sci.* (2020) 14:358–64. doi: 10.5219/1340
82. Bradley AJ, Leach KA, Green MJ, Gibbons J, Ohnstad IC, Black DH, et al. The impact of dairy cows' bedding material and its microbial content on the quality and safety of milk - a cross sectional study of UK farms. *Int J Food Microbiol.* (2018) 269:36–45. doi: 10.1016/j.ijfoodmicro.2017.12.022
83. Haley DB, de Passille AM, Rushen J. Assessing cow comfort: effect of two types and two tie stall designs on the behaviour of lactating dairy cows. *Appl Anim Behav Sci.* (2001) 71:105–17. doi: 10.1016/S0168-1591(00)00175-1
84. Leach KA, Archer SC, Breen JE, Green MJ, Ohnstad IC, Tuer S, et al. Recycling manure as cow bedding: potential benefits and risks for UK dairy farms. *Vet J.* (2015) 206:123–30. doi: 10.1016/j.tvjl.2015.08.013
85. Popescu S, Borda C, Diugan EA, Niculae M, Stefan R, Sandru CD. The effect of the housing system on the welfare quality of dairy cows. *Ital J Anim Sci.* (2014) 13:2940. doi: 10.4081/ijas.2014.2940
86. Fournel S, Godbout S, Ruel P, Fortin A, Duquette-Lozeau K, Létourneau V, et al. Production of recycled manure solids for use as bedding in Canadian dairy farms: II. Composting methods. *J Dairy Sci.* (2019) 102:1847–65. doi: 10.3168/jds.2018-14967
87. Zigo F, Sasáková N, Gregová G, Výrostková J, Ondrašovičová S. Effects of using an alternative bedding composition on the levels of indicator microorganisms and mammary health in dairy farm conditions. *Agriculture.* (2020) 10:250. doi: 10.3390/agriculture10060245
88. Jackson PGG, Cockroft PD. *Clinical Examination of Farm Animals*. Oxford, UK: Blackwell Science Ltd., Wiley-Blackwell (2002). p. 303.
89. Viguier C, Arora S, Gilmartin N, Welbeck K, Okennedy R. Mastitis detection: current trends and future perspectives. *Trends Biotechnol.* (2009) 27:486–93. doi: 10.1016/j.tibtech.2009.05.004
90. Berry EA, Hogeveen H, Hillerton JE. Decision tree analysis to evaluate dry cow strategies. *J Dairy Res.* (2004) 71:409–18. doi: 10.1017/S0022029904000433
91. Berry EA, Hillerton JE. The effect of an intramammary teat seal on new intramammary infections. *J Dairy Sci.* (2002) 85:2512–20. doi: 10.3168/jds.S0022-0302(02)74334-8
92. Williamson JH, Woolford MW, Day AM. The prophylactic effect of a dry-cow antibiotic against *Streptococcus uberis*. *N Z Vet J*. (1995) 43:228. doi: 10.1080/00480169.1995.35898
93. Kaczorek-Łukowska E, Małaczewska J, Wójcik R, Naumowicz K, Blank A, Siwicki AK. *Streptococci* as the new dominant aetiological factors of mastitis in dairy cows in north-eastern Poland: analysis of the results obtained in 2013–2019. *Ir Vet J.* (2021) 74:2–6. doi: 10.1186/s13620-020-00181-z
94. White RE, Abdulla Z, Toni F, Lemarchand F, Miciletta M, De P.rado A, et al. Effect of introducing OrbeSeal into a dry cow management programme and the impact on farm economics. *In: Hogeveen* H, Lam TJG, editors. *Udder Health and Communication*. Wageningen: Wageningen Academic Publishers (2011). Available online at: https://link.springer.com/chapter/10.3920%2F978-90-8686-742-4_73 (accessed October 14, 2020).
95. Godden S, Rapnicki P, Stewart S, Fetrow J, Johnson A, Bey R, et al. Effectiveness of an internal teat seal in the prevention of new intramammary infections during the dry and early-lactation periods in dairy cows when used with a dry cow intramammary antibiotic. *J of Dairy Sci.* (2003) 86:3899–911. doi: 10.3168/jds.S0022-0302(03)73998-8
96. Crispie F, Flynn J, Ross PR, Hill C, Meaney JW. Dry cow therapy with a non-antibiotic intramammary teat seal - a review. *Ir Vet J.* (2004) 57:412–8. doi: 10.1186/2046-0481-57-7-412
97. Hogeveen H. *Mastitis in Dairy Production.* Wageningen: Wageningen Academic Publishers (2005). p. 744. doi: 10.3920/978-90-8686-550-5
98. Hassan AA, Salem AZM, Elghandour MMY, Hafsa ASH, Reddy PRK, Atia SES, et al. Humic substances isolated from clay soil may improve the ruminal fermentation, milk yield, and fatty acid profile: a novel approach in dairy cows. *Animal Feed Sci Technol.* (2020) 268:114601. doi: 10.1016/j.anifeedsci.2020.114601
99. Reyher KK, Haine D, Dohoo IR, Revie CW. Examining the effect of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens - a systematic review and meta-analysis. *J Dairy Sci*. (2012) 95:6483–502. doi: 10.3168/jds.2012-5594
100. Aguilar C, Vanegas C, Klotz B. Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. *J Dairy* Res. (2010) 78:136–43. doi: 10.1017/S0022029910000877

101. Porcherie A, Gilbert FB, Germon P, Cunha P, Trotereau A, Rossignol C, et al. IL-17A is an important effector of the immune response of the mammary gland to Escherichia coli infection. *J Immunol*. (2016) 96:803–12. doi: 10.4049/jimmunol.1500705
102. Boerhout E, Vrieling M, Benedictus L, Daemen I, Ravesloot L, Rutten V, et al. Immunization routes in cattle impact the levels and neutralizing capacity of antibodies induced against *S. aureus* immune evasion proteins. *Vet Res*. (2015) 46:115. doi: 10.1186/s13567-015-0243-7
103. Ticháček A, Ponížil A, Pechová A, Pavlata L, Olejník P, Kopunecz P, et al. Poradenství jako nástroj bezpečnosti v prvovýrobě mléka/Consulting as a safety tool in primary milk production (In Czech). In: Grafotic Š, editor. *Šumperk Agritec*. 1st ed. (2007). p. 89.
104. Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO, van Neerven RJJ. Effects of bovine immunoglobulins on immune function, allergy, and infection. *Front Nutr*. (2018) 5:52. doi: 10.3389/fnut.2018.00052
105. Hofírek B. Nemoci skotu/Diseases of cattle (In Czech). Brno: Noviko (2009). p. 1149.
106. Toušová R, Stádník L, Ducháček J, Baráková M. Vliv vakcinace prvotelek proti mastitidě na její výskyt, počet somatických buněk a obsah pevných sloŽek v mléce/Influence of vaccination against mastitis on its incidence, somatic cells amount and content of solid components in milk of primiparous dairy cows. *Výzkum v chovu skotu - Cattle Res*. (2011) 53:84–90. Available online at: https://www.researchgate.net/publication/280573113_Vliv_vakcinace_prvotelek_proti_mastitide_na_jeji_vyskyt_pocet_somatickych_bunek_a_obsah_pevnych_slozek_v_mlece (accessed December 30, 2020).
107. Doležal O, Kopunecz P. *Management dojení, jeho optimalizace a hodnocení kvality dodávek mléka/Milking Management, Its Optimization and Evaluation of Milk Supply Quality*. Praha: Institut vzdělávání v zemědělství, (2010). p. 20.

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REVIEW
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# Non-*aureus* Staphylococci and Bovine Udder Health: Current Understanding and Knowledge Gaps

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Despite considerable efforts to control bovine mastitis and explain its causes, it remains the most costly and common disease of dairy cattle worldwide. The role and impact of non-*aureus* staphylococci (NAS) in udder health are not entirely understood. These Gram-positive bacteria have become the most frequently isolated group of bacteria in milk samples of dairy cows and are associated with (mild) clinical and subclinical mastitis. Different species and strains of NAS differ in their epidemiology, pathogenicity, virulence, ecology and host adaptation, and antimicrobial resistance profiles. They have distinct relationships with the microbiome composition of the udder and may also have protective effects against other mastitis pathogens. Some appear to persist on the skin and in the teat canal and udder, while others seem to be transient residents of the udder from the environment. Analyzing genotypic and phenotypic differences in individual species may also hold clues to why some appear more successful than others in colonizing the udder. Understanding species-level interactions within the microbiome and its interactions with host genetics will clarify the role of NAS in bovine mastitis and udder health.

**Keywords: mastitis, bovine, *Staphylococcus*, mammary gland, udder, bacterial infection**

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## INTRODUCTION

Staphylococci can be subdivided into two groups, coagulase-positive and coagulase-negative, based on their ability to clot rabbit plasma, a key diagnostic step in clinical microbiology laboratories. Staphylococcal coagulase is an extracellular protein encoded by the *coa* gene. *Staphylococcus* coagulase-associated clotting involves formation of a coagulase-prothrombin complex that recognizes fibrinogen as a substrate and directly converts it into fibrin. Coagulase secretion is a key virulence strategy in pathogenesis and persistence of staphylococcal diseases (1) and has often been used to distinguish *S. aureus* from other staphylococci (2). In the context of bovine mastitis, staphylococci were historically classified into two groups: one that included *S. aureus,* considered more pathogenic and thus a "major pathogen," and a second including other staphylococci that were lumped together as "minor pathogens" and termed the coagulase-negative staphylococci (CNS).

Another classification scheme adopted in more recent mastitis literature involves grouping all staphylococci other than *S. aureus* into a single category, non-*aureus* staphylococci (NAS). Some coagulase-positive and coagulase-variable mastitis pathogens (e.g., *Staphylococcus hyicus* and *Staphylococcus agnetis*) were also often included in the coagulase-negative category. In addition, coagulase-negative variants of *S. aureus* are known to exist, some of which can have similar pathogenicity to their coagulase-positive variants (3). Some *S. aureus* isolates of bovine origin react negatively to the standard coagulase test and are PCR-negative for the *coa* gene (4, 5). Additionally, the von Willebrand factor-binding protein exhibits coagulating ability, resulting in *S. aureus* producing two proteins that coagulate plasma (6). In a study analyzing the distribution of virulence factor genes among isolates belonging to 25 NAS species of bovine origin, the corresponding gene for the von Willebrand factor-binding protein, *vWbp* was detected in *S. agnetis, S. hyicus,* and *S. chromogenes* (7). Because the term coagulase-negative staphylococci, based on the ability of proteins to cause coagulation as a diagnostic test, may result in ambiguity in the context of mastitis, non-*aureus* staphylococci (NAS) provides a better term to classify pathogens associated with bovine mastitis by providing a clear dichotomy between *S. aureus* and the other staphylococcal species. Furthermore, NAS are often considered pathogens of lesser importance in dairy production (so-called minor pathogens), especially compared to *S. aureus*, some streptococci and some coliforms (8). However, in most studies they have been the most frequently isolated bacteria from udder quarters with subclinical mastitis (SCM) (9) and their ability to cause clinical mastitis (CM) cannot be understated. Approximately 20% of milk samples collected on Canadian dairy farms were NAS-positive and the prevalence of NAS in quarters with a somatic cell count (SCC) $<$ 200,000 cells/mL, oftentimes regarded as healthy udder quarters, was ∼43% (10, 11), suggesting at least some can be considered commensals (12). In a Canada-wide clinical mastitis (CM) study (13), NAS were isolated from 10.7% of culture-positive samples, whereas in a CM study from Wisconsin (14), 6.1% of isolates were NAS. In two Belgian studies 5 and 12% quarters with CM, respectively, were NAS-positive (15, 16). Other studies in the US and Belgium also concluded that NAS are the principal cause of IMI on modern dairy farms (17, 18). Prevalence of IMI with NAS is especially high in virgin and first lactation heifers (18–24). In addition, it has been argued that modern mastitis control programs, which focus on major udder pathogens (and are apparently less effective against minor pathogens such as NAS), may have contributed to marked increases in prevalence of IMI due to NAS (23, 25). On dairy farms implementing modern mastitis control practices, the prevalence of major pathogen IMI has decreased resulting in a lower bulk tank SCC. NAS IMI have become relatively more important and are considered the leading cause of SCM (23).

NAS do not seem to be the main cause of mastitis in herds with significant milk quality problems (**Table 1**); yet, in herds with low bulk tank SCC, NAS IMIs contribute to a substantial proportion of the bulk tank SCC (8). However, a recent longitudinal study demonstrated that NAS IMI early in lactation results in only a small but significant increase of SCC (24), and other studies demonstrated that when compared to non-infected quarters, NAS-infected quarters did not generally have reduced milk production (26, 27). While their effect on milk yield at the whole cow level has no negative impact (28), NAS-infected heifers out-produced non-infected counterparts, presumably due to a lower incidence of CM (29, 30). One study reported a positive correlation of *S. caprae* with milk yield in goats, further suggesting that NAS IMI may have a positive effect in early lactation on milk yield (31, 32) yet cows with SCM produced milk of poorer quality (2). Elucidating factors to better understand the role of NAS in IMI (**Table 1**) may lead to more effective prevention and control measures of SCM.

## SPECIES DISTRIBUTION AND DIVERSITY

Staphylococci have been isolated from many animal species. Very few of these NAS species (e.g., *S. hyicus, S. pseudointermedius, S. arlettae, S. felis, S. equorum, S. delphini,* and *S. caprae*) demonstrate a level of host specificity (32–37). NAS are very prevalent in bovine IMI, especially in dairy heifers (38, 39). In fact, 53 different species are recognized in the genus *Staphylococcus*, 23 of which have been isolated from a Canadian collection of $>$ 5,000 bovine milk samples (9). Twenty-five species were identified from 300 samples in another study (40), whereas in a smaller study only 10 species were found from 105 NAS isolates (41).

In order to understand the variety of NAS species isolated from milk, it is important to clearly determine phylogenetic relationships among species (**Figure 1**). Previously, this relationship was determined through construction of a phylogenetic tree based on 16S rDNA sequences of 42 NAS isolates (42). More recently, the genomes of over 400 bovine isolates were sequenced and several methods were applied to understand evolution and relationships between species (e.g., based on core protein set, entire genomes, SNPs). As a result, 5 main clades were identified, each with a varying number of species (43). Construction of a phylogenetic tree based on whole genome sequencing provided a highly reliable classification of bovine NAS species. Earlier studies using single gene sequencing revealed contradicting phylogenies when compared to each other, failing to show true evolutionary histories and speciation of *Staphylococcus*. By dividing bovine NAS species into 5 distinct clades, shared biological properties among related species such as virulence and host specificity can be better characterized. These properties will provide the basis for studies on the role and significance of individual and related NAS species for udder health, as there is also diversity within species isolated from different body sites on the same animal (44).

The diversity of NAS species begs the question of why so many *Staphylococcus* species can be isolated from bovine milk samples (**Table 1**). It is unclear if all NAS species fill the same niche and are therefore interchangeable; whether they are all unique in their interactions with the udder, or whether synergisms exist among species or strains. There are reasons to believe that a bacterial species only evolves to adapt to a certain niche

**TABLE 1 |** Knowledge gaps in understanding the role of NAS on udder health.

| Section | Knowledge gaps |
|---|---|
| Species distribution and diversity | • Interactions between individual NAS (e.g., synergistic) in the udder<br>• Interactions between individual NAS and the udder<br>• Acquired genes giving the ability to colonize and persist in udders and on teat apices |
| Dominant NAS species | • Factors that underlie success of certain NAS as colonizers and the most prevalent species such as *S. chromogenes* |
| Impact of NAS on inflammation | • Potential strain differences and factors of NAS species that provoke inflammation |
| Virulence and host association | • Association between virulence genes and disease severity<br>• The role of capsular genes in NAS virulence<br>• Correlation between capsular genotype/phenotype and biofilm formation<br>• Biofilm production and its association with pathogenicity of *S. chromogenes* and other NAS species<br>• Elucidating the role of specific virulence factors (e.g., β-hemolysins) for *S. chromogenes* and other NAS species<br>• Tracking evolutionary history of NAS species in the context of virulence genes |
| Antimicrobial resistance | • Clarifying if NAS species represent a reservoir of AMR genes for major mastitis pathogens<br>• Possibility of new resistance mechanisms in NAS species<br>• Characterization of intrinsic AMR mechanisms<br>• Correlation between co-resistance profiles of NAS species and its effect on udder health |
| Niche adaptation and host association | • Classification of NAS species as commensal microbiota or opportunistic or obligate pathogens |
| Interactions within the udder microbiome | • Causes of NAS being disruptors of the udder microbiome<br>• Role of bacteriocins produced by NAS species in modulating the udder microbiome<br>• Clarifying if NAS species IMI increase susceptibility to major pathogens or, on the contrary, prevent them from infecting the udder<br>• Characterizing the host genetic component and its relationship to NAS colonization |
| Understanding how mastitis control measures influence NAS incidence and prevalence | • Further evaluation of the associations among mastitis control measures and incidence and prevalence of mastitis caused by different NAS species |

and that every mutation in every gene needs to provide an advantage to be maintained. Following that reasoning, every NAS species, each with roughly 2.4 Mb genomes and many thousands genetic polymorphisms, must have vastly different behaviors. Besides, each species has a large pan genome, suggesting large strain differences within species. Analysis of NAS by Pulsed Field Gel Electrophoresis (PFGE) has demonstrated that diversity exists within species with respect to persistence and SCC in corresponding milk samples (45), and between isolates of the same species isolated from different body sites (44). This diversity suggests important differences in virulence and host adaptation genes, differences in gene expression between species, and differences in interactions with other microbes. Importantly, the identification methods and study designs in previous literature may have influenced these results. Two phenotypic tests (API Staph ID 32 and Staph-Zym) were shown to be inaccurate in species identification from bovine milk samples, and genotypic methods were shown to have higher type ability and accuracy in the identification of bovine NAS (46, 47). API Staph has been shown to have moderate to low performance in goat NAS identification as well (48). Moreover, biases in biochemical testing developed for human NAS should be considered in the context of characterizing species diversity and prevalence of bovine NAS as genotypic methods are considered more accurate than biochemical galleries (47). Additional large-scale longitudinal studies are needed to provide insight into how both strain and evolutionary differences affect prevalence and distribution of NAS species causing IMI, and the resulting impact on udder health. MALDI-TOF is an accurate technique in this regard, able to correctly identify almost all NAS isolates at the species-level, as long as the library is updated with relevant field isolates and strains from new species (49–51).

The ability of different NAS species to persist and colonize different niches may be due to acquired genes which confer selective advantages in their respective environment. For example, several factors, such as surface proteins, biofilm resistance genes, and phenol-soluble modulin peptides, increased the ability of *S. epidermidis* to persist in blood isolates obtained from newborn humans (52). Recently, the molecular relationship was determined between *S. agnetis* isolates from cattle and chickens. The chicken isolates were closely related to cattle isolates and clustered together, indicating a common ancestor and possibly a single jump from cattle to chickens (53). However, no unique virulence genes were identified in a hypervirulent chicken isolate, resulting in the speculation of small alterations in virulence associated factors.

## *STAPHYLOCOCCUS CHROMOGENES*: THE DOMINANT NAS SPECIES

In a Canada-wide study, 50% of NAS isolates were *S. chromogenes* (**Table 2**). This NAS species had the highest prevalence in IMI of any bacteria in milk samples of cattle with SCM (and either a low or high SCC) (9, 38). *Staphylococcus chromogenes* was also the most prevalent species in a US study (55) and Belgian studies (56). In Canada, *S. chromogenes* also has the highest (of any NAS) prevalence in CM (as well in high or low SCC quarters). Of all NAS species, *S. chromogenes* (followed by *S. epidermidis*, and *S. simulans*)-positive milk samples had the

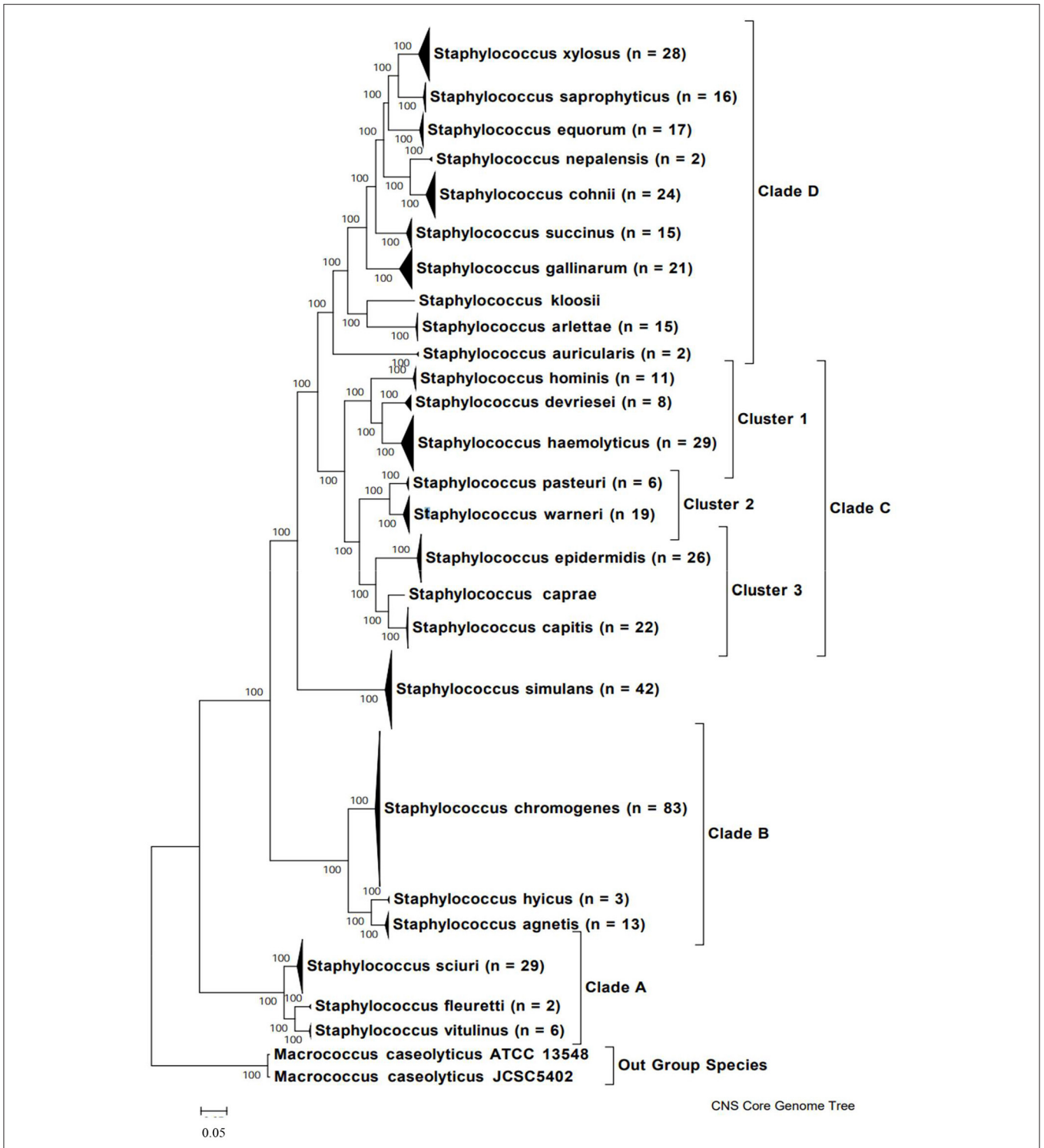


**FIGURE 1 |** Phylogenetic tree of NAS species based on whole genome sequences, indicating major clades. Copied from Naushad et al. (43).

highest SCC (23, 60–62). *S. chromogenes* IMI is associated with higher SCC and is considered an important species in quarters with a high SCC, persistent cases and CM (38, 45, 61, 63). It was also reported that *S. chromogenes* is responsible for significantly increased SCC in cows with persistent SCM (64), as well as for greater inflammatory responses and more pronounced clinical signs (65). NAS also play a major role in small ruminant mastitis. One study found that SCC increase was three times higher in

**TABLE 2 |** Overview of the top 3 most frequently isolated non-*aureus* staphylococci species in various countries from cows having subclinical or clinical mastitis.

| Country | Top 3 species | Prevalence (%) | Reference(s) |
|---|---|---|---|
| Canada | *S. chromogenes* | 49 | (9) |
| | *S. simulans* | 17 | |
| | *S. xylosus* | 12 | |
| Belgium | *S. equorum* | 34 | (40) |
| | *S. haemolyticus* | 13 | |
| | *S. epidermidis* | 9 | |
| Finland | *S. chromogenes* | 49 | (54) |
| | *S. simulans* | 23 | |
| | *S. warneri* | 5 | |
| The Netherlands | *S. chromogenes* | 30 | (23) |
| | *S. epidermidis* | 13 | |
| | *S. capitis* | 11 | |
| United States of America | *S. chromogenes* | 48 | (55) |
| | *S. haemolyticus* | 18 | |
| | *S. simulans* | 7 | |
| Belgium | *S. chromogenes* | 41 | (56) |
| | *S. sciuri* | 13 | |
| | *S. cohnii* | 11 | |
| China | *S. arlettae* | 12 | (57) |
| | *S. sciuri* | 12 | |
| | *S. xylosus* | 12 | |
| Poland | *S. warneri* | 37 | (58) |
| | *S. chromogenes* | 33 | |
| | *S. xylosus* | 23 | |
| Argentina | *S. chromogenes* | 47 | (59) |
| | *S. haemolyticus* | 32 | |
| | *S. warneri* | 7 | |
| Belgium | *S. chromogenes* | 10 | (16) |
| | *S. haemolyticus* | 9 | |
| | *S. equorum* | 7 | |
| Belgium | *S. chromogenes* | 29 | (27) |
| | *S. xylosus* | 9 | |
| | *S. vitulinus* | 9 | |

*Quarter milk samples were examined in each study, apart from one which used bulk tank samples (40). Samples were considered NAS-positive according to the National Mastitis Council guidelines, in addition to genotypic characterization of species.*

small ruminant NAS than in bovine NAS IMI, and another reported an elicited immune response in goats after inoculation with *S. chromogenes* (66–68). This suggests that either the host immune response or differences in NAS must be taken into account when discussing bacterial virulence and commensalism.

Interestingly, *S. chromogenes* is most frequently isolated from milk and skin (69), but not from other environmental sources, suggesting that it is likely host-adapted (49, 62, 70–72). Literature suggests that this species is largely isolated from samples of bovine origin, although it can be isolated from the milk of other dairy ruminants including goats and dairy buffalo (73). According to Taponen et al. (54), 55% of *S. chromogenes* persisted throughout lactation, while Fry et al. (45) showed persistence based on PFGE. Another study reported the average duration of IMI caused by *S. chromogenes* to be ∼40 days longer than that of other species (28). Additionally, 45% IMI caused by *S. chromogenes* was shown to persist over at least two sampling days, compared to only 9.8% of other species persisting for that long (28). The average duration of *S. chromogenes* IMI was reported to be 150 d in another study (63). Moreover, infection by one *S. chromogenes* genotype, followed by recovery, then re-infection with a different *S. chromogenes* genotype may be misclassified as a chronic *S. chromogenes* IMI in the absence of strain-typing data. Although PFGE-based strain-typing of the first and last IMI isolates in a series from the same quarter (45) indicated persistence, the duration of *S. chromogenes* IMI and all other NAS species may therefore be overestimated by studies that have not included a strain-typing method.

## OTHER PREVALENT NAS SPECIES IN DIFFERENT GEOGRAPHICAL REGIONS

Following *S. chromogenes*, the most frequently identified NAS are *S. simulans*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis*. While there are some regional differences in overall prevalence (**Table 2**), these species are consistently isolated from the udder and milk samples. In contrast, the other NAS species together represent <10% of the NAS isolates. While it might be concluded that regional and environmental differences affect the prevalence and distribution of individual NAS species (**Table 2**), it is also reasonable to conclude that species distribution is most likely impacted by herd management (69). Hence, regional differences are perhaps more impacted by the nature of the studied herds than geography. These findings suggest that additional studies are needed to better characterize these influences.

Interestingly, some *Staphylococcus* species are infrequently isolated from milk, e.g., *S. rostri* was isolated in one study from feces (16, 74). NAS species which can be isolated from other sites on the cow, but not from milk or the exterior of the udder might provide an opportunity to help clarify which genes allow NAS to either infect or colonize the udder.

## ASSOCIATION OF IMI AND UDDER INFLAMMATION FOR DIFFERENT NAS SPECIES

Host-pathogen interactions for many mastitis pathogens has not been well-established because of the complex interactions *in vivo*, and much of the evidence derived from the interactions of *S. aureus* with its host. It is of great interest to determine whether all NAS species provoke inflammation and increases in SCC. Most studies, evaluating the associations between mammary inflammation (e.g., SCC) and presence of NAS in milk samples have been observational. Some conflicting information on effect of NAS IMI on udder health (8, 63, 69) and the impact on milk yield (26, 29) exists within literature. Large scale studies using 16S, *rpoB* sequencing (9) and MALDI (50, 75) are targeting these questions. Interestingly, when comparing the prevalence of individual NAS species between milk samples with low SCC (<200,000 cells/mL) or high SCC (≥200,000 cells/mL), all species had higher prevalence in the latter, suggesting that NAS provoke some inflammatory response (38). In addition to an increase in SCC, NAS IMI was shown to have elicited host immune

responses, an important consideration is the role of NAS in modulating these responses, as they may offer cross-protection against other mastitis pathogens (64, 65, 76, 77).

Few studies have evaluated NAS species and their influence on udder inflammation in experimental intramammary challenge trials (76, 77). Based on these studies, it was demonstrated that intramammary challenge with *S. chromogenes* stimulated an inflammatory response and that a strain previously isolated from an IMI was more inflammatory than a teat apex strain. Furthermore, while *S. fleurettii* could be isolated from milk of experimentally infected udder quarters and was associated with an increase in SCC, the strain was cleared from milk within 12 h (76). Hence, more data are needed to truly understand the relationship between IMI and udder inflammation in the context of NAS.

## VIRULENCE AND HOST ASSOCIATION

In one study, the virulence potential of each *Staphylococcus* species and the profile of all Virulence Factors (VFs) were determined by defining a species-specific VF gene set from each species and analyzing variation within them (7). Virulence genes may explain why some species are more successful at colonizing and surviving within the udder, and products of such genes are considered VFs (7). The phylogenetic distribution, sharing and evolution of VFs can reveal how these different species evolved (7). Accordingly, if some NAS are commensals, a question of interest would be whether they individually became commensals or if they evolved from a common commensal ancestor. If the latter is true, they would have become more aggressive in claiming niches by accumulating VFs, leading to their evolution into a different species (7).

The distribution of 191 VFs and their possible associations with pathogenesis in 25 NAS species were determined along with the relationship between VFs and udder health (high SCC and signs of CM) (7). The overall number of VFs was not associated with disease severity. This confirmed data from another study in which virulence gene profile or accumulation of virulence genes did not predict the type of mastitis (SCM or CM) or the severity of inflammation (78). In one study, more severe disease outcomes were correlated with increasing numbers of toxin and host immune evasion genes (7). Although the effects of individual VFs have been analyzed (**Table 3**), these findings suggest that development of disease and interactions of VFs with the host are complex and determined by interplay of genes rather than just presence of specific virulence genes. Interactions of VFs expressed by these genes with the host could also depend on the specific staphylococcal species. Some NAS strains associated with mastitis had varying proportions of virulence genes, and biofilm formation genes were only detected in a small percentage of examined species (58). The contribution of virulence genes on disease outcomes or development can also be affected by intrinsic factors (within the udder) or extrinsic factors (in the cow's environment) that influence gene expression. The latter is likely influenced by factors such as herd management, climatic conditions, and geographic location. One study using NAS isolates from a single Chinese herd reported lower prevalence of exotoxin and biofilm-associated genes compared to previous studies (57). These findings suggest the need for additional studies on presence or absence of these genes, and further gene expression studies to resolve which are associated with disease severity. The lack of expression studies prevents us from understanding associations between specific NAS species and NAS IMI, as well as which genetic elements are responsible for differences in prevalence and distribution among NAS species. Additionally, molecular characterization resistance and virulence factors have also been conducted for small ruminant NAS (66). While there may be opportunities to learn from these studies, an important consideration is the potentially different host-pathogen interactions between cattle and small ruminants.

Analyses of the distribution of 191 VFs in 441 genomes of 25 NAS species by t-Distributed Stochastic Neighbor Embedding (T-SNE), a method to visualize high-dimensional datasets, demonstrated that all species studied can be defined as separate and homogenous bacteria (7) because of clear clustering by species (**Figure 2**). Virulence potential was also associated with the different phylogenetic clades. These findings suggest that virulence potential developed gradually during evolution into distinct species. This is in contrast to the possibility that some species acquired several virulence factors relatively suddenly, turning them into somewhat more virulent pathogens or more adapted commensals.

As discussed above, it is unclear what mechanisms enable *S. chromogenes* to be the most prevalent organism in bovine mastitis (and successful in causing persistent IMI and SCM). In-depth studies on genomes of 440 NAS isolates determined that closely related *S. chromogenes*, *S. agnetis,* and *S. hyicus* had the highest virulence potential (i.e., number of virulence genes), largely due to exotoxin, host evasion and capsular genes, of all NAS (82). However, *S. chromogenes* (∼50% of NAS isolates) did not differ greatly in VF profile from the closely related species *S. agnetis* (<0.5% of NAS isolates) and *S. hyicus* (<0.1% of NAS isolates) (9). The lack of clear differences in detected virulence genes between *S. chromogenes* and the other Clade B NAS, despite the large differences in species distribution, suggest that an unknown mechanism is at play which makes *S. chromogenes* the most frequently isolated species in NAS IMI.

Interestingly, in the T-SNE plot (**Figure 2**), *S. chromogenes* is the only species split into 2 populations with respect to virulence genes, with a minority of the strains clustering with other members of the clade B, while the majority of the *S. chromogenes* strains have a distinct profile. An important caveat is that more *S. chromogenes* isolates were included in this study than other species, but it is tempting to speculate that the larger population of *S. chromogenes* might represent a pathotype that has adapted to the udder. Additional evidence for this was presented in a study demonstrating that *S. chromogenes* isolated from a chronic IMI had greater ability to adhere to bovine mammary epithelial cells compared to a strain isolated from the teat apex (83). Another study compared a *S. fleurettii* strain isolated from sawdust bedding and a *S. chromogenes* strain from a persistent IMI; the latter strain persisted longer after experimental inoculation into the udder (76). If true, the finding needs to be confirmed with

**TABLE 3 |** Summary of virulence factors and their related genes that were detected in several NAS species, as well as the relationship between these genes and pathogenesis in the context of NAS IMI.

| Virulence factors (related genes) | Associations of virulence factors with pathogenesis of NAS | Reference(s) |
|---|---|---|
| Methicillin-resistance and biofilm-related genes (*mecA, eno*) | • Isolates from clinical mastitis cases had a significantly higher presence of methicillin-resistant (*mecA*) genes (21 out of 43 isolates)<br>• All 43 isolates tested positive for the presence of the biofilm-related gene, *eno* | (16) |
| Intracellular adhesin (*icaA/B/C*) | • In human-associated NAS, it is a genetic determinant for biofilm formation<br>• Presence of *icaA* was associated with greater biofilm formation in bovine NAS species. Almost half the isolates tested positive for this gene | (7, 79, 80) |
| Iron-regulated surface determinant (*isdA/B/C/I*) | • *IsdI* the most frequently distributed gene among NAS species in a Canadian study<br>• Every NAS isolate contained at least one gene related to iron uptake and metabolism<br>• Staphylococci require iron to replicate and persist in infections | (7) |
| Hemolysin (*hla/b/d)* | • Hemolysins lysed erythrocytes of cattle, sheep, and goats<br>• β-hemolysin (*hlb*) was the most frequent gene in NAS isolates in a Canadian study<br>• In Iran, bovine NAS isolates primarily produced δ-hemolysin (*hld*) | (7, 81) |
| Phenol-soluble modulins (PSMβ1/2/3/4) | • Lysis of red and white blood cells, linked to biofilm formation and stimulation of inflammatory responses<br>• β-type PSMs were associated with bovine NAS isolates in a Canadian study | (7) |

a larger number of strains, as this might hold clues about why *S. chromogenes* has become the dominant NAS species isolated from milk of dairy cattle.

No clear difference was present between the two *S. chromogenes* populations with respect to severity of mastitis (**Figure 2B**). The subsequent sections will analyze how virulence factors may explain why *S. chromogenes* is the only species that diverges into two distinct populations. It is also important to note that other reasons may include differences in AMR profiles, host adaptation, interactions with host genetics and interactions with the microbiome.

In some *S. chromogenes* isolates capsular genes from the larger VF-based cluster are missing (7), which seems to be one factor that causes the population split in this species. In *S. aureus,* expression of these genes results in formation of a polysaccharide capsule that helps resist phagocytic cell uptake, thus playing a role in evasion of the host immune response (84). However, there is conflicting evidence on the associations between capsule genes and overall virulence of *Staphylococcus* species. In one study, presence of these genes and formation of polysaccharide capsules enhanced *S. aureus* virulence in a murine model, but decreased virulence of *S. aureus* when causing IMI (7). Based on a strong association between the amount of biofilm formed and the capsular genotype and phenotype, these factors may be important to virulence of *S. aureus* and its ability to persist in chronic IMIs. In a Canadian study, biofilm formation had no effect on disease severity (79). However, it has been suggested that biofilms increase the ability of NAS to persist in the mammary environment (79, 80). When analyzing *S. aureus* isolates *in vitro*, isolates which harbored genes coding for capsule type 5 (*cap5)* formed more biofilm and produced a thinner capsular polysaccharide layer than those with genes coding for capsule type 8 (*cap8)* (85). *S. chromogenes* isolates had *cap5* but *cap8* was not present (78). Additional *in vivo* testing is needed to better characterize the associations between pathogenicity and biofilm production in *S. chromogenes.*

Conversely, the absence of these capsular genes increased both intracellular survival rates as well as invasion rates of *S. aureus* (86). Persistence of this pathogen in an infected host has been linked to the loss of capsular polysaccharide 5 and 8 (CP5/8). This was confirmed in a murine model where isogenic acapsulated mutants persisted for a longer period of time and in higher numbers when compared to their capsulated counterparts (87). In clinical studies, human patients with chronic osteomyelitis had a higher proportion of non-typeable (NT) *S. aureus*, compared to those with acute osteomyelitis (87). NT strains are non-reactive with antibodies to CP types 1, 2, 5, or 8 (87), and these isolates from chronically infected hosts were shown to have conserved their acapsulated phenotype over successive passages on artificial media without reverting back to encapsulation (87). Isolates from cows with SCM revealed that the proportion of non-typeable (NT) *S. aureus* strains was 86% (88). These findings reveal that ability to persist in chronic infections is strongly associated with NT strains (i.e., acapsulated pathogens). With a majority of *S. chromogenes* isolates lacking capsule genes, it may be of further interest to study the relationship between acapsulation and the persistence of *S. chromogenes* in IMI.

Other previously identified VFs associated with pathogenicity of *S. aureus* have also been detected in NAS. β-hemolysin (*hlb*) was the most frequent and predominant gene detected in *S. chromogenes* isolates and other species of clade B (7). The *hlb* gene was detected in all isolates in clade D3, while only a few of the isolates in one clade E species carried this gene (7). Strains of *S. aureus* isolated from bovine CM produced predominantly β-hemolysin, in combination with other hemolysins (89). This was confirmed in a study which found that 97% of *S. aureus* isolates from Europe and the US either produced or were PCR-positive for β-hemolysin (90). It was also determined that 45–90 CFU of a β-hemolytic *S. aureus* strain could result in CM (89). These findings suggest that β-hemolysins may play an important role in the pathogenesis of mastitis caused by some strains of *S. aureus* but that it is not the sole virulence factor that influences disease severity. With almost all clade B isolates expressing the *hlb* gene

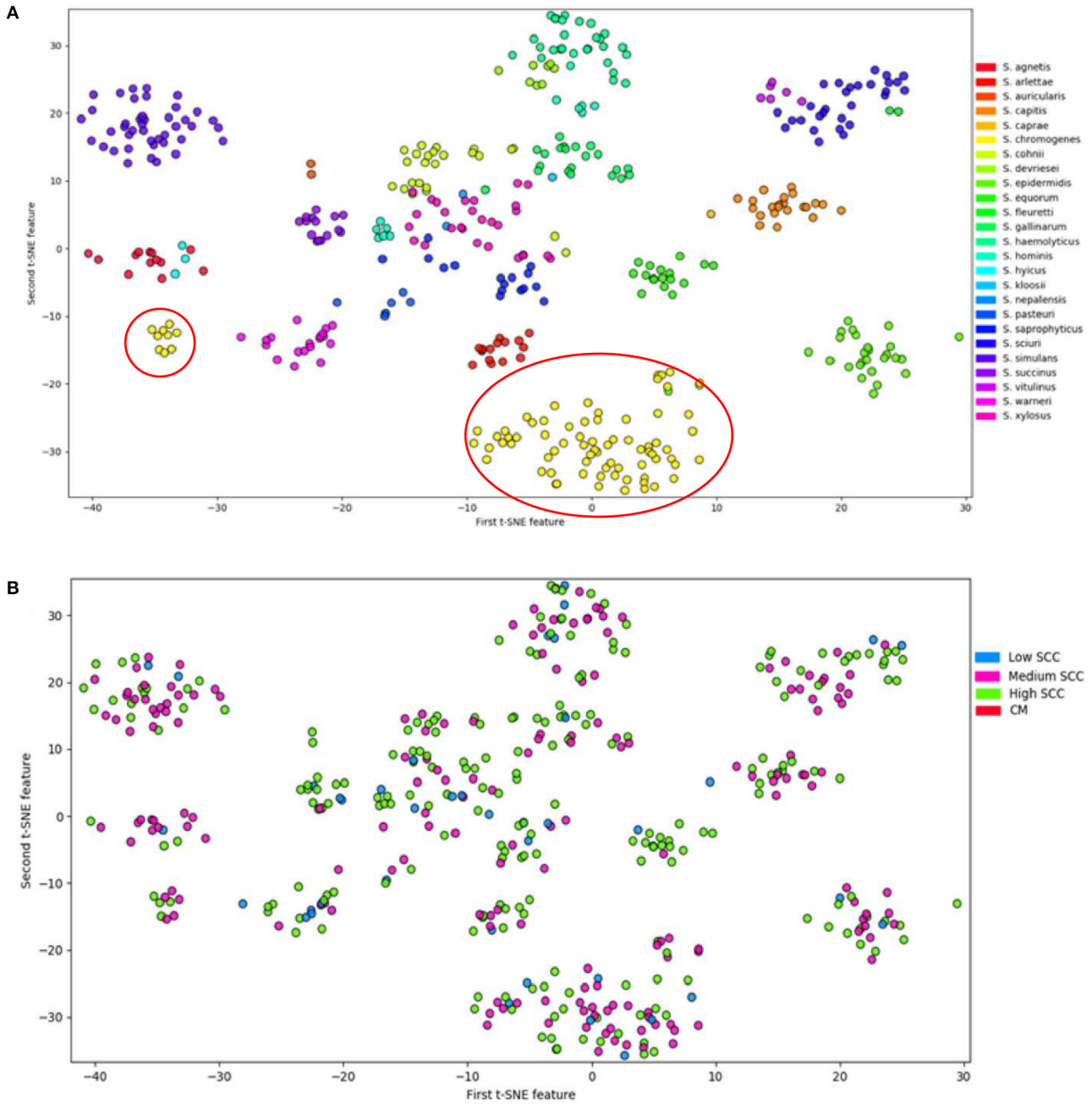


**FIGURE 2 |** Visualization of 25 NAS species by t-Distributed Stochastic Neighbor Embedding (T-SNE) based on virulence gene content, demonstrating that each of the NAS species is a discrete microorganism; the red circles indicate the distinctive split between *S. chromogenes* populations **(A)**. Labeling of severity of mastitis of the NAS isolates separated based on virulence genes **(B)**. Modified from (7).

(7), it may be of interest to elucidate its role in the pathogenesis of these species.

Adenosine synthase A was another *S. aureus* virulence gene detected in *S. chromogenes*. Adenosine synthase A is an immune evasion factor for *S. aureus* responsible for increasing the overall abundance of extracellular adenosine, which may be the most potent immuno-suppressive signaling molecule. This factor is necessary for staphylococcal survival within neutrophils, allowing *S. aureus* to escape bactericidal activity of leukocytes and other host immune responses (91, 92).

In the same study analyzing VF genes, all NAS species contained at least one gene from the iron-responsive surface determinant (*isd*) operon (7). Staphylococci require iron to replicate and sustain infections, and it was shown that the *isdI*

gene, the most frequently distributed *isd* gene among all NAS species in this study, is necessary for *S. aureus* pathogenesis (7). Similarly, most NAS species contained β-type phenol-soluble modulins (PSMs), which have been considered major determinants of *S. aureus* virulence (7). Phenol-soluble modulins have multiple roles in staphylococcal pathogenesis, causing lysis in red and white blood cells, contributing to biofilm development and stimulation of inflammatory responses (93) (**Table 3**). The numerous studies above have studied the roles of these VFs in the pathogenesis of *S. aureus*. Perhaps elucidating their role in pathogenesis of *S. chromogenes* in future studies may explain its dominance in bovine mastitis and persistence in the udder (**Table 3**).

A correlation was observed between the average SCC of milk samples from which specific NAS species were isolated and the number of exoenzyme, host evasion and iron uptake genes these species carried (7, 9). These virulence genes might hold the key to why certain NAS species provoke somewhat more inflammation than others. Absence of these virulence genes may result in NAS species becoming more host adapted or even commensal. This is somewhat illustrated by *S. chromogenes*, which is considered a host-adapted NAS, and has moderate numbers of exoenzyme, host evasion and iron uptake genes. Furthermore, interesting associations were found between virulence genes identified in NAS, with striking differences in the strength of these associations between isolates that caused low SCC and CM isolates (7).

## ANTIMICROBIAL RESISTANCE

The high prevalence of *S. chromogenes* relative to other NAS species is likely multifactorial. Antimicrobial resistance (AMR) might be one explanation for the predominance of a single species of *Staphylococcus* associated with the udder, but based on available data this does not seem to be the case. *S. chromogenes* have relatively low phenotypic and genotypic prevalence of AMR when compared to other NAS species isolated from the udder (82). Another study demonstrated that *S. epidermidis* had increased resistance rates against penicillin when compared to *S. chromogenes* (94). Additionally, researchers reported presence of β-lactamase in *S. chromogenes* is relatively low when compared to either *S. haemolyticus* or *S. epidermidis* (61).

In Canada, higher numbers of AMR genes, with a strong correlation between AMR genotype and phenotype, were identified in NAS rather than *S. aureus* originating from the same dairy herds (10, 82). This is in agreement with previous reports where *S. aureus* isolated from SCM and CM cases were less resistant than NAS against commonly used antimicrobials (60, 75, 95, 96). In addition, studies have demonstrated that NAS could serve as reservoirs for AMR genes for major mastitis pathogens including *S. aureus* (97, 98).

A study (60) investigated the association between AMR and antimicrobial use in NAS. An association was present when penicillins, third-generation cephalosporins or macrolides were administered systemically, but not when antimicrobials were administered via the intrauterine and intramammary route (99). It was hypothesized that antimicrobials administered systemically for conditions other than mastitis, if partitioning to the udder, could cause prolonged bacterial exposure to sub-therapeutic antimicrobial concentrations in the udder. Similarly, one study suggested that increasing systemic administration resulted in a decrease of antimicrobial susceptibility of NAS to β-lactams, as opposed to intramammary treatment of SCM. Systemic administration was expressed as antimicrobial treatment incidence, with units of the number of defined daily doses animal used per 1,000 cow-days (100). In addition, there is a higher likelihood of NAS being present in the udder compared *S. aureus*, which would therefore result in an increased window of exposure of NAS to antimicrobials used in dairy herds.

Methicillin-resistant NAS were an important reservoir of AMR and virulence genes in a Belgian study (98). Most cases saw an association between presence of AMR genes and phenotypic resistance, and only a few cases had a negative correlation between presence of AMR genes and resistance. This study also identified some isolates which did not carry any of the investigated AMR genes yet still displayed a non-wild type (epidemiologically resistant) phenotype (98). *Staphylococcus sciuri* appeared resistant to fusidic acid but this phenotype was not correlated to any of the known fusidic acid resistance genes (98) (**Table 4**). In a Swiss study (98) *in vitro* phenotypic resistance to several antimicrobials such as erythromycin, clindamycin and streptomycin was not explained by the presence of any tested genes (102), suggesting development of new resistance mechanisms. Previous studies have also characterized associations between resistance determinants and AMR in NAS. These include β-lactam resistance being associated with *blaZ* and *mecA* genes, and chloramphenicol resistance having a correlation with the FexA transporter. Daptomycin resistance was explained by the presence of the *mprF* gene, whereas *tetK* and *tetL* genes were associated with tetracycline resistance (82). Even though bovine NAS isolates may acquire resistance to these antimicrobials, it has been suggested through phenotypic AMR patterns that intrinsic mechanisms of AMR may be present for a subset of NAS species as well (82). It is worth noting that many of these antimicrobials are not labeled for use in lactating dairy cows. For example in North America and Europe chloramphenicol is illegal for use in food-producing animals, suggesting the absence of selective pressures.

A study in Portugal characterized the AMR profile of methicillin-resistant staphylococci (MRS) isolates from bovine SCM and CM cases, identifying 9.3% of isolates as being MRS and associated with the *mecA* virulence gene (101). Despite the low percentage of MRS detected, the majority of isolates still had a multi-resistance profile (101) (**Table 4**). This study, in addition to a Swedish one (61), revealed that AMR and virulence gene profiles are species dependent. The Swedish study revealed that the prevalence of β-lactamase varied among NAS species and was more common in isolates originating from SCM cases than from CM cases (61). β-lactamase is the most common resistance mechanism in staphylococci, and while the prevalence was high in *S. epidermis* and *S. haemolyticus*, there was little to no detection in *S. chromogenes* and *S. simulans* (61). In this study, *S. chromogenes* and *S. epidermis* were the most commonly

**TABLE 4 |** Summary of antimicrobial resistance profiles and prevalence of single gene resistance determinants of frequently isolated NAS species across several studies.

| Species | Antimicrobial resistance profile[a] | Resistance determinants (% prevalence of determinant) | | References |
|---|---|---|---|---|
| | | Acquired | Intrinsic | |
| *S. lentus* | PEN, KAN, STR, TET, CHL, TMP, FUS | *tetK (23), tetM (10), tetL (14), fexA (32)* | – | |
| *S. sciuri* | PEN, FOX, GEN, KAN, STR, ERY, CLI, SYN, TET, CHL, TMP, FUS | *tetK (5), tetL (25), fexA (5)* | – | (98) |
| *S. epidermidis* | PEN, FOX, GEN, KAN, STR, ERY, CLI, TET, CHL, TMP, FUS | *tetK (45), tetL (9), fexA (27)* | – | |
| *S. epidermidis* | NA, ERY, KAN, GEN, TOB | – | – | |
| *S. simulans* | NA, ERY, STR, CLI | – | – | (101) |
| *S. haemolyticus* | NA, SXT | – | – | |
| *S. chromogenes* | NA, TET | – | – | |
| *S. chromogenes* | PEN, OXA, STR, TET, ERY | *blaZ (72), mecA (2)* | – | |
| *S. epidermidis* | PEN, OXA STR, TET, ERY | *blaZ (48), mecA (4)* | – | (94) |
| *S. xylosus* | PEN, OXA, STR, ERY | *blaZ (87)* | – | |
| *S. chromogenes* | PEN, OXA, TET, STR, ERY, CLI, CHL, KANA, GEN, TRM | – | – | |
| *S. xylosus* | PEN, OXA, TET, ERY, CLI, CHL, GEN | – | – | (102) |
| *S. sciuri* | OXA, TET, STR, CLI, KAN, GEN, TRM | – | – | |
| *S. chromogenes* | CHL, TET, CLI, PNV, PIR, ERY, AMP, PEN | *blaZ (10), tet38 (100), tetK (2), tetL (3),* | *NorA (100), Sav1866 (100),* | |
| *S. simulans* | CHL, TET, PIR, ERY, PEN, MDR | *tetK (3), tetL (3), tetM (3)* | *norA* (100), *Sav1886* (100). | (82) |
| *S. xylosus* | CHL, TET, CLI, PIR, ERY, AMP, PEN, MDR | *msrA (14), tetK (19)* | *norA (100), norB (100), sav1886 (100)* | |
| *S. arlettae* | – | – | $bla_{ARL}$ | (103) |

*Isolates from bovine milk diagnosed with clinical and subclinical mastitis were used in all studies with the exception of the first study which used nasal swab samples collected from veal calves.*

[a]*Multi-drug resistant profiles are not included. NA, nalidixic acid; ERY, erythromycin; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; STR, streptomycin; SXT, sulphamethoxazole-trimethoprim; TET, tetracycline'; PEN, penicillin; CLI, clindamycin; CHL, chloramphenicol; TRM, trimethoprim; FUS, fusidic acid; FOX; OXA, oxacillin; PIR, pirlimycin.*

isolated species in SCM cases (61). In a Dutch study, 70% of *S. epidermis* isolates and 18% of *S. chromogenes* isolates were resistant to penicillin (94) (**Table 4**), suggesting that a high prevalence of penicillin resistance in SCM was associated with the high prevalence of *S. epidermis* (61). These findings confirm the existence of inter-species variation in AMR profiles, emphasizing the need to continue monitoring co-resistance profiles among NAS populations associated with bovine mastitis cases. Coupled with the possible development of resistance mechanisms not associated with previously characterized virulence genes, additional studies analyzing AMR in NAS are needed alongside the characterization of bovine NAS specific clinical antimicrobial susceptibility breakpoints, as this presents a challenge in treating bovine mastitis cases.

## NICHE ADAPTATION AND HOST ASSOCIATION

NAS prevalence and distribution is impacted by many environmental and management factors such as geographic region, climate, water sources, access to pasture, barn type, bedding and host factors (parity, quarter location, antibiotic use). In this context, it is useful to determine the natural habitat of different NAS species. This defines whether they should be considered as environmental or host-adapted pathogens. This also relates to their commensal nature and their level of host adaptation to the skin, teat canal and/or udder.

Host adaptation relates to colonization and persistence of isolates as well as the level of inflammation caused. Adaptation can be quite specific, demonstrated by the fact that species and frequency of isolation of NAS differs between teat canal and milk samples (104). Some studies find the most predominant NAS species, *S. chromogenes* and *S. xylosus*, to be equally ubiquitous in CM, SCM, skin, and environment (61, 75). These two species are also more frequently associated with persistent IMI and SCM compared to other NAS species (55). Other studies report differences in distribution and in genotypes between milk, udder and environment (60, 105). In contrast, molecular epidemiology studies demonstrate that *S. haemolyticus*, *S. fleurettii,* and *S. equorum* are predominantly environmental species (55, 105).

It was clearly demonstrated that some NAS species are more associated with IMI than with environmental (e.g., parlor-associated) niches (105). Interestingly, *S. chromogenes* is almost uniquely associated with IMI and not found in the environment of the dairy cow. Unpublished data from Walpole et al. comparing isolates identified in milk vs. body sites, failed to detect *S. chromogenes* a single time on other body sites of dairy

cattle, whereas it was by far the most frequently isolated species from IMI. In contrast, Adkins et al. (44) isolated *S. chromogenes* from pre-partum mammary secretions, milk, the inguinal region skin, teat skin muzzle, and perineum of peripartum dairy heifers. Hence, these data demonstrate likely adaption to niches on the cow which seems to underpin its success as an IMI organism. Similar data have been reported for *S. aureus*, another host-adapted udder pathogen (106).

A recent longitudinal study, identified 4 udder-adapted NAS species, 2 of which were considered persistent and some demonstrated characteristics of contagious pathogens (*S. chromogenes* and *S. simulans*) (55). Contagious transmission routes for *S. chromogenes* and *S. simulans* seem plausible from this study, possibly in addition to environmental transmission patterns.

## INTERACTIONS WITHIN THE MICROBIOME

Knowledge is emerging that an udder microbiome exists that is distributed over the milk, milk ducts, cistern, teat canal, teat apex and teat skin, where staphylococci seem to play an important role (12). Previous literature used the NMC procedure to define NAS-positive samples (**Figure 2**), importantly at the time of publication, the National Mastitis Council (NMC) did not publish guidelines for classifying quarters as infected or not in the context of clinical diagnosis for IMI (107). Another consideration is that culture-independent genotypic methods have only been implemented during the past decade for use in IMI diagnosis (108). The bovine milk microbiome in both culture-based and DNA-based methods has proven to be more complex than expected, with the role of different species in milk samples—either as pathogens, commensals, or contaminants—being essential in assessing the analyses (108). Currently, the best definition of IMI is offered Dohoo et al. (107), but even these criteria can be open to interpretation. *S. chromogenes* is one of the organisms most negatively influencing the microbiome of the udder based on the observation that it has the most negative connections with other members of the milk microbiota. These negative connections presumably reduce diversity and therefore microbiome stability (109). A similar negative effect was observed for *S. xylosus*. In general, staphylococci are negatively correlated with Shannon and Simpson indices of diversity (109). Conflicting evidence exists on whether or not they are disruptors of the normal milk microbiome (12, 109). The negative interactions might be due to indirect mechanisms that involve the host, such as the induction of immune responses, or may be due to other genera in the microbiome that are overshadowed by NAS.

Direct mechanisms, including the production of antimicrobial factors such as bacteriocins, may also result in negative correlation between *S. chromogenes* and other members of the milk microbiota. NAS produce many of these bacteriocins with capacity to inhibit the growth of mostly Gram-positive bacteria but also some with potential to inhibit Gram-negatives (110). A Belgian study found that 38 of 254 NAS isolates displayed bacteriocin-like activity, and that 7 of these strains displayed activity against at least one major pathogen associated with bovine mastitis (111). Interestingly, the bacteriocin produced by an inhibitory *S. chromogenes* strain used in this study (nukacin L217) inhibited the growth of all mastitis-causing pathogens tested (111). This bacteriocin may hold clues to the success of *S. chromogenes* as an NAS species in IMI and its possibly negative associations with major mastitis pathogens such as *S. aureus*, as antibacterial production is often advantageous for strain colonization in a certain niche (111). These findings are mostly based on *in vitro* studies. It remains unclear if these bacteriocins play an actual role in modulating the microbiota inside the udder or on the skin, as in the Belgian study bacteriocin production was abundant on growth agar medium but did not grow in broth (111). Other species apart from *S. chromogenes* also inhibit the growth of major mastitis pathogens. In a recent study, cytoplasmic bacteriocins from *S. epidermidis* selectively inhibited growth of *S. aureus,* including methicillin-resistant strains (112). These studies suggest the need for additional *in vivo* studies to determine how bacteriocins influence NAS species-level interactions in the milk microbiome.

Previous studies have clearly established that co-infections with other NAS and pathogens occur (113–115), yet there exists conflicting evidence as to whether NAS increase susceptibility to major pathogens such as *S. aureus* or prevent it from colonizing the udder. Because major pathogens are generally considered more virulent and damaging to the udder than minor mastitis pathogens such as NAS, it would be of interest to clarify what impact NAS has on major pathogens. Several studies detailed analyses which concluded that NAS colonization protected quarters against IMI by major pathogens (88, 116, 117), whereas another reported that the presence of NAS was a risk factor for acquiring *S. aureus* IMI (118). Interestingly, certain strains of *S. chromogenes* can inhibit the *in vitro* growth of all *S. aureus, S. dysgalactiae,* and *S. uberis* strains. The intensity of inhibition varied amongst target species, with only 2 out of 10 *S. chromogenes* isolates showing consistent inhibitory activity (117). A systematic review of the current literature revealed that strong protective effects were observed in studies that had higher underlying risks, as well as in challenge studies which introduced major pathogens into the udder through the teat end (113). Studies that used larger doses of challenge organisms and those with more stringent diagnostic criteria for pathogen IMI reported reduced protective effects. Larger scale studies are needed to resolve the existing conflicting evidence and better characterize the association between NAS and major pathogens.

Interestingly, there also seems to be a host genetic component to whether NAS are part of the milk microbiome. Two main variants of the bovine antigen presenting major histocompatibility complex protein Bola DrB3.2 strongly defined what organisms are "accepted" to form the milk microbiome (119). Each of the genetic variants seems to promote the presence of different NAS species: *S. equorum*, *S. gallinarum*, *S. sciuri,* and *S. haemolyticus* were enriched in microbiota of one of the variants, whereas *S. chromogenes* was enriched within microbiota of the second variant. These findings spark hypotheses related to the predominance of *S. chromogenes* and the dichotomy between "environmental" and "host adapted" NAS.

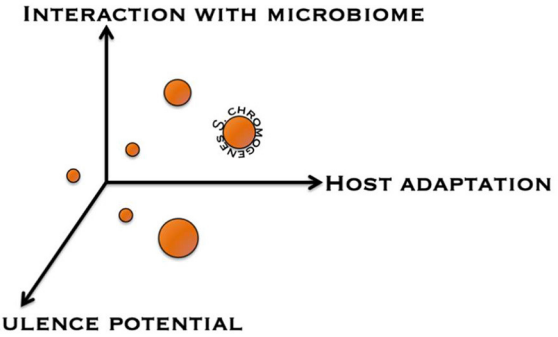


**FIGURE 3 |** Conceptual discrimination of different non-*aureus* staphylococci based on several factors. Displayed are the interaction with the udder microbiome, host adaptation, and virulence potential.

## CONCLUSIONS

To help improve our understanding as to whether NAS species are commensals, opportunistic pathogens, or obligate (minor) pathogens with respect to the udder, a framework was conceptualized to categorize NAS based on different discriminating factors (**Figure 3**). A first factor is the nature of the interaction the NAS species has with the udder, ranging from a commensal interaction to a pathogenic interaction. A second factor is the strength and specialization behind this interaction, from environmental organism to obligate symbiont. A third factor is the impact of the NAS species on the milk microbiome and on major mastitis pathogens. NAS make up a significant fraction of the milk microbiome (109) and they also seem to contribute to many of the predicted interactions between milk microbiome members. This categorization might help in defining which NAS dairy producers should consider more important than others when designing control programs. Additional factors could include antimicrobial resistance and compatibility with host immune genetics and response.

Although many recent studies have focused on NAS at the species level, many questions remain (**Table 1**). The true nature of each NAS species has yet to be identified, either as commensals or pathogens, or as environmental or contagious pathogens. The effects of these interactions between NAS with the rest of the milk microbiome as well as its associations with host genetics and the immune response need to be elucidated. Interactions in the milk microbiome may influence factors such as AMR or virulence in NAS species, leading to their success as colonizers of the udder. Further investigations into the role of NAS as an AMR reservoir for major and minor pathogens are needed. In addition, more data is needed to clarify if NAS truly prevent other mastitis pathogens from colonizing or infecting the udder.

It will also be worthwhile to elucidate the reason for dominance of *S. chromogenes* with the NAS in many parts of the world. This is particularly important as it is unclear if *S. chromogenes* should be considered beneficial or harmful. Given *S. chromogenes'* dominance as NAS and IMI commensal or pathogen in general and its potentially positive or negative impacts, it seems that new strategies to support or eliminate *S. chromogenes* from the bovine udder would go a long way in reducing the prevalence and impact of mastitis in dairy herds. It should be determined which other NAS species have the same impact as *S. chromogenes*, in addition to which species require less or no attention, as NAS may represent a natural mechanism to reduce IMI with (other) mastitis pathogens, which could be implemented as an intervention method. It is also important to focus on strain differences related to interactions of NAS with the udder, as they may override differences at the species level.

Finally, for herds that have successfully controlled other mastitis pathogens, controlling cases of NAS CM and SCM may be an important step in further lowering bulk milk SCC. While much has been reported to help define the ecology and epidemiology of these bacteria, a clear understanding of how existing mastitis control practices can be applied or where new control measures are needed to mitigate IMI is needed.

## AUTHOR CONTRIBUTIONS

JD, VH, SN, DN, CL, JM, SD, and HB contributed to writing and editing the review. All authors contributed to the article and approved the submitted version.

## REFERENCES

1. Bonar E, Miedzobrodzki J, Władyka B. *The Staphylococcal Coagulases*. London: Elsevier Inc. (2018). doi: 10.1016/B978-0-12-813547-1.00007-8
2. Leitner G, Krifucks O, Merin U, Lavi Y, Silanikove N. Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk. *Int Dairy J.* (2006) 16:648–54. doi: 10.1016/j.idairyj.2005.10.020
3. Fox LK, Besser TE, Jackson SM. Evaluation of a coagulase-negative variant of *Staphylococcus aureus* as a cause of intramammary infections in a herd of dairy cattle. *J Am Vet Med Assoc.* (1996) 209:1143–6.
4. Akineden Ö, Hassan AA, Schneider E, Usleber E. A coagulase-negative variant of *Staphylococcus aureus* from bovine mastitis milk. *J Dairy Res.* (2011) 78:38–42. doi: 10.1017/S0022029910000774
5. Sundareshan S, Isloor S, Babu Y, Sunagar R, Sheela P, Tiwari J, et al. Isolation and molecular identification of rare coagulase-negative *Staphylococcus aureus* variants isolated from bovine milk samples. *Ind J Comparat Microbiol Immunol Infect Dis.* (2017) 38:66–73. doi: 10.5958/0974-0147.2017.00011.3
6. Bjerketorp J, Jacobsson K, Frykberg L. The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. *FEMS Microbiol Lett.* (2006) 234:309–14. doi: 10.1111/j.1574-6968.2004.tb09549.x
7. Naushad S, Naqvi SA, Nobrega D, Luby C, Kastelic JP, Barkema HW, et al. Comprehensive virulence gene profiling of bovine non-*aureus* staphylococci based on whole-genome sequencing data. *mSystems.* (2019) 4:1–23. doi: 10.1128/mSystems.00098-18
8. Schukken YH, González RN, Tikofsky LL, Schulte HF, Santisteban CG, Welcome FL, et al. CNS mastitis: nothing to worry about? *Vet Microbiol.* (2009) 134:9–14. doi: 10.1016/j.vetmic.2008.09.014
9. Condas LAZ, De Buck J, Nobrega DB, Carson DA, Naushad S, De Vliegher S, et al. Prevalence of non-*aureus staphylococci* species causing intramammary infections in Canadian dairy herds. *J Dairy Sci.* (2017) 100:5592–612. doi: 10.3168/jds.2016-12478

10. Reyher KK, Dufour S, Barkema HW, Des Coteaux L, Devries TJ, Dohoo IR, et al. The National Cohort of Dairy Farms–a data collection platform for mastitis research in Canada. *J Dairy Sci.* (2011) 94:1616–26. doi: 10.3168/jds.2010-3180
11. Dufour S, Dohoo IR, Barkema HW, Descoteaux L, Devries TJ, Reyher KK, et al. Epidemiology of coagulase-negative *Staphylococci* intramammary infection in dairy cattle and the effect of bacteriological culture misclassification. *J Dairy Sci.* (2012) 95:3110–24. doi: 10.3168/jds.2011-5164
12. Porcellato D, Meisal R, Bombelli A, Narvhus JA. A core microbiota dominates a rich microbial diversity in the bovine udder and may indicate presence of dysbiosis. *Sci Rep.* (2020) 10:21608. doi: 10.1038/s41598-020-77054-6
13. Olde Riekerink RG, Barkema HW, Kelton DF, Scholl DT. Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci.* (2008) 91:1366–77. doi: 10.3168/jds.2007-0757
14. Oliveira L, Ruegg PL. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. *J Dairy Sci.* (2014) 97:5426–36. doi: 10.3168/jds.2013-7756
15. Verbeke J, Piepers S, Supré K, De Vliegher S. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. *J Dairy Sci.* (2014) 97:6926–34. doi: 10.3168/jds.2014-8173
16. Wuytack De Visscher A, Piepers S, Boyen F, Haesebrouck F, De Vliegher S. Distribution of non-*aureus* staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential. *J Dairy Sci.* (2020) 103:10658–75. doi: 10.3168/jds.2020-18265
17. Timms LL, Schultz LH. Dynamics and significance of coagulase-negative *Staphylococcal* intramammary infections. *J Dairy Sci.* (1987) 70:2648–57. doi: 10.3168/jds.S0022-0302(87)80335-1
18. Piepers S, De Meulemeester L, de Kruif A, Opsomer G, Barkema HW, De Vliegher S. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J Dairy Res.* (2007) 74:478–83. doi: 10.1017/S0022029907002841
19. Fox LK, Chester ST, Hallberg JW, Nickerson SC, Pankey JW, Weaver LD. Survey of intramammary infections in dairy heifers at breeding age and first parturition. *J Dairy Sci.* (1995) 78:1619–28. doi: 10.3168/jds.S0022-0302(95)76786-8
20. Adkins PRF, Dufour S, Spain JN, Calcutt MJ, Reilly TJ, Stewart GC, et al. Cross-sectional study to identify staphylococcal species isolated from teat and inguinal skin of different-aged dairy heifers. *J Dairy Sci.* (2018) 101:3213–25. doi: 10.3168/jds.2017-13974
21. Nickerson SC, Owens WE, Boddie RL. Mastitis in dairy heifers: initial studies on prevalence and control. *J Dairy Sci.* (1995) 78:1607–18. doi: 10.3168/jds.S0022-0302(95)76785-6
22. Oliver SP, Jayarao BM. Coagulase-negative *Staphylococcal* intramammary infections in cows and heifers during the non-lactating and periparturient periods. *Zentralbl Veterinarmed B.* (1997) 44:355–63. doi: 10.1111/j.1439-0450.1997.tb00986.x
23. Sampimon OC, Barkema HW, Berends IM, Sol J, Lam TJ. Prevalence and herd-level risk factors for intramammary infection with coagulase-negative *Staphylococci* in Dutch dairy herds. *Vet Microbiol.* (2009) 134:37–44. doi: 10.1016/j.vetmic.2008.09.010
24. Valckenier D, Piepers S, De Visscher A, Bruckmaier RM, De Vliegher S. Effect of intramammary infection with non-*aureus* staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. *J Dairy Sci.* (2019) 102:6442–53. doi: 10.3168/jds.2018-15913
25. Tenhagen BA, Köster G, Wallmann J, Heuwieser W. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J Dairy Sci.* (2006) 89:2542–51. doi: 10.3168/jds.S0022-0302(06)72330-X
26. Tomazi T, Gonçalves J, Barreiro J, Arcari M, Dos Santos M. Bovine subclinical intramammary infection caused by coagulase-negative staphylococci increases somatic cell count but has no effect on milk yield or composition. *J Dairy Sci.* (2015) 98:3071–8. doi: 10.3168/jds.2014-8466
27. Valckenier D, Piepers S, De Visscher A, De Vliegher S. The effect of intramammary infection in early lactation with non-*aureus* staphylococci in general and *Staphylococcus* chromogenes specifically on quarter milk somatic cell count and quarter milk yield. *J Dairy Sci.* (2020) 103:768–82. doi: 10.3168/jds.2019-16818
28. Valckenier SPD, Schukken YH, De Visscher A, Boyen F, Haesebrouck F, De Vliegher S. Longitudinal study on the effects of intramammary infection with non-*aureus* staphylococci on udder health and milk production in dairy heifers. *J Dairy Sci.* (2020) 104:899–914. doi: 10.3168/jds.2020-18685
29. Piepers S, Opsomer G, Barkema HW, de Kruif A, De Vliegher S. Heifers infected with coagulase-negative staphylococci in early lactation have fewer cases of clinical mastitis and higher milk production in their first lactation than noninfected heifers. *J Dairy Sci.* (2010) 93:2014–24. doi: 10.3168/jds.2009-2897
30. Piepers S, Schukken YH, Passchyn P, De Vliegher S. The effect of intramammary infection with coagulase-negative staphylococci in early lactating heifers on milk yield throughout first lactation revisited. *J Dairy Sci.* (2013) 96:5095–105. doi: 10.3168/jds.2013-6644
31. Piepers S, De Vliegher S, de Kruif A, Opsomer G, Barkema HW. Impact of intramammary infections in dairy heifers on future udder health, milk production, and culling. *Vet Microbiol.* (2009) 134:113–20. doi: 10.1016/j.vetmic.2008.09.017
32. Koop G, De Vliegher S, De Visscher A, Supré K, Haesebrouck F, Nielen M, et al. Differences between coagulase-negative *Staphylococcus* species in persistence and in effect on somatic cell count and milk yield in dairy goats. *J Dairy Sci.* (2012) 95:5075–84. doi: 10.3168/jds.2012-5615
33. Devriese L, Hajek V, Oeding P, Meyer S, Schleifer K. *Staphylococcus hyicus* (sompolinsky. 1953) comb. nov, and *Staphylococcus hyicus* subsp. chromogenes subsp. nov. *Int J System Evolution Microbiol.* (1978) 28:482–90. doi: 10.1099/00207713-28-4-482
34. Bannoehr J, Guardabassi L. *Staphylococcus* pseudintermedius in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. *Vet Dermatol.* (2012) 23:253–66.e51–2. doi: 10.1111/j.1365-3164.2012.01046.x
35. Schleifer K, Kilpper-Bälz R, Devriese L. *Staphylococcus arlettae* sp. nov *S. equorum* sp. nov, and *S. k1oosii* sp. nov.: three new coagulase-negative, novobiocin-resistant species from animals. *Syste Appl Microbiol.* (1984) 5:501–9. doi: 10.1016/S0723-2020(84)80007-7
36. Worthing K, Pang S, Trott DJ, Abraham S, Coombs GW, Jordan D, et al. Characterisation of *Staphylococcus felis* isolated from cats using whole genome sequencing. *Vet Microbiol.* (2018) 222:98–104. doi: 10.1016/j.vetmic.2018.07.002
37. Varaldo PE, Kilpper-Bälz R, Biavasco F, Satta G, Schleifer KH. *Staphylococcus delphini* sp. nov, a coagulase-positive species isolated from dolphins. *Int J Syste Evolution Microbiol.* (1988) 38:436–9. doi: 10.1099/00207713-38-4-436
38. Condas LAZ, De Buck J, Nobrega DB, Carson DA, Roy JP, Keefe GP, et al. Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, clinical mastitis. *J Dairy Sci.* (2017) 100:5613–27. doi: 10.3168/jds.2016-12479
39. De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW. Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. *J Dairy Sci.* (2012) 95:1025–40. doi: 10.3168/jds.2010-4074
40. De Visscher Piepers S, Haesebrouck F, Supré K, De Vliegher S. Coagulase-negative *Staphylococcus* species in bulk milk: prevalence, distribution, and associated subgroup-and species-specific risk factors. *J Dairy Sci.* (2017) 100:629–42. doi: 10.3168/jds.2016-11476
41. Mahmmod YS, Klaas IC, Svennesen L, Pedersen K, Ingmer H. Communications of *Staphylococcus aureus* and non-*aureus Staphylococcus* species from bovine intramammary infections and teat apex colonization. *J Dairy Sci.* (2018) 101:7322–33. doi: 10.3168/jds.2017-14311
42. Casaes Nunes RS, Pires de Souza C, Pereira KS, Del Aguila EM, Flosi Paschoalin VM. Identification and molecular phylogeny of coagulase-negative staphylococci isolates from Minas Frescal cheese in southeastern Brazil: Superantigenic toxin production and antibiotic resistance. *J Dairy Sci.* (2016) 99:2641–53. doi: 10.3168/jds.2015-9693
43. Naushad S, Barkema HW, Luby C, Condas LA, Nobrega DB, Carson DA, De Buck J. Comprehensive phylogenetic analysis of bovine non-*aureus* staphylococci species based on whole-genome sequencing. *Front Microbiol.* (2016) 7:1990. doi: 10.3389/fmicb.2016.01990
44. Adkins PRF, Dufour S, Spain JN, Calcutt MJ, Reilly TJ, Stewart GC et al. Molecular characterization of non-*aureus Staphylococcus* spp. from heifer

intramammary infections and body sites. *J Dairy Sci.* (2018) 101:5388–403. doi: 10.3168/jds.2017-13910
45. Fry PR, Middleton JR, Dufour S, Perry J, Scholl D, Dohoo I. Association of coagulase-negative *Staphylococcal* species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. *J Dairy Sci.* (2014) 97:4876–85. doi: 10.3168/jds.2013-7657
46. Sampimon OC, Zadoks RN, De Vliegher S, Supré K, Haesebrouck F, Barkema HW, et al. Performance of API Staph ID 32 and Staph-Zym for identification of coagulase-negative staphylococci isolated from bovine milk samples. *Vet Microbiol.* (2009) 136:300–5. doi: 10.1016/j.vetmic.2008.11.004
47. Zadoks RN, Watts JL. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. *Vet Microbiol.* (2009) 134:20–8. doi: 10.1016/j.vetmic.2008.09.012
48. Koop G, De Visscher A, Collar CA, Bacon DA, Maga EA, Murray JD, et al. Short communication: Identification of coagulase-negative staphylococcus species from goat milk with the API Staph identification test and with transfer RNA-intergenic spacer PCR combined with capillary electrophoresis. *J Dairy Sci.* (2012) 95:7200–5. doi: 10.3168/jds.2012-5747
49. Tomazi T, Goncalves JL, Barreiro JR, de Campos Braga PA, Prada e Silva LF, Eberlin MN, et al. Identification of coagulase-negative staphylococci from bovine intramammary infection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol.* (2014) 52:1658–63. doi: 10.1128/JCM.03032-13
50. Cameron M, Barkema HW, De Buck J, De Vliegher S, Chaffer M, Lewis J, et al. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. *J Dairy Sci.* (2017) 100:2137–47. doi: 10.3168/jds.2016-12020
51. Cameron M, Perry J, Middleton JR, Chaffer M, Lewis J, Keefe GP. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. *J Dairy Sci.* (2018) 101:590–5. doi: 10.3168/jds.2017-13226
52. Klingenberg C, Rønnestad A, Anderson A, Abrahamsen T, Zorman J, Villaruz A, et al. Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness. *Clin Microbiol Infect.* (2007) 13:1100–11. doi: 10.1111/j.1469-0691.2007.01818.x
53. Shwani Adkins PRF, Ekesi NS, Alrubaye A, Calcutt MJ, Middleton JR, Rhoads DD. Whole-genome comparisons of *Staphylococcus* agnetis isolates from cattle and chickens. *Appl Environ Microbiol.* (2020) 86:12. doi: 10.1128/AEM.00484-20
54. Taponen S, Koort J, Bjorkroth J, Saloniemi H, Pyorala S. Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. *J Dairy Sci.* (2007) 90:3301–7. doi: 10.3168/jds.2006-860
55. Jenkins SN, Okello E, Rossitto PV, Lehenbauer TW, Champagne J, Penedo MCT, et al. Molecular epidemiology of coagulase-negative *Staphylococcus* species isolated at different lactation stages from dairy cattle in the United States. *PeerJ.* (2019) 7:e6749. doi: 10.7717/peerj.6749
56. De Visscher Piepers S, Haesebrouck F, De Vliegher S. Intramammary infection with coagulase-negative staphylococci at parturition: species-specific prevalence, risk factors, and effect on udder health. *J Dairy Sci.* (2016) 99:6457–69. doi: 10.3168/jds.2015-10458
57. Xu J, Tan X, Zhang X, Xia X, Sun H. The diversities of *Staphylococcal* species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microb Pathog.* (2015) 88:29–38. doi: 10.1016/j.micpath.2015.08.004
58. Malinowski E, Kłossowska A, Zastempowska E. Virulence factors in coagulase-negative staphylococci isolated from cows with subclinical mastitis. *Bull Vet Inst Pulawy.* (2011) 55:681–4.
59. Raspanti CG, Bonetto CC, Vissio C, Pellegrino MS, Reinoso EB, Dieser SA, et al. Prevalence and antibiotic susceptibility of coagulase-negative *Staphylococcus* species from bovine subclinical mastitis in dairy herds in the central region of Argentina. *Rev Argent Microbiol.* (2016) 48:50–6. doi: 10.1016/j.ram.2015.12.001
60. Taponen S, Pyörälä S. Coagulase-negative staphylococci as cause of bovine mastitis — Not so different from *Staphylococcus aureus*? *Vet Microbiol.* (2009) 134:29–36. doi: 10.1016/j.vetmic.2008.09.011
61. Persson Waller K, Aspán A, Nyman A, Persson Y, Grönlund Andersson U. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Vet Microbiol.* (2011) 152:112–6. doi: 10.1016/j.vetmic.2011.04.006
62. Thorberg BM, Danielsson-Tham ML, Emanuelson U, Persson Waller K. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J Dairy Sci.* (2009) 92:4962–70. doi: 10.3168/jds.2009-2184
63. Supré K, Haesebrouck F, Zadoks RN, Vaneechoutte M, Piepers S, De Vliegher S. Some coagulase-negative *Staphylococcus* species affect udder health more than others. *J Dairy Sci.* (2011) 94:2329–40. doi: 10.3168/jds.2010-3741
64. Simojoki H, Orro T, Taponen S, Pyorala S. Host response in bovine mastitis experimentally induced with *Staphylococcus* chromogenes. *Vet Microbiol.* (2009) 134:95–9. doi: 10.1016/j.vetmic.2008.09.003
65. Simojoki H, Salomäki T, Taponen S, Iivanainen A, Pyörälä S. Innate immune response in experimentally induced bovine intramammary infection with *Staphylococcus* simulans and S epidermidis. *Vet Res.* (2011) 42:1–10. doi: 10.1186/1297-9716-42-49
66. Martins KB, Faccioli PY, Bonesso MF, Fernandes S, Oliveira AA, Dantas A, et al. Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *J Dairy Sci.* (2017) 100:2184–95. doi: 10.3168/jds.2016-11583
67. Leitner G, Merin U, Krifucks O, Blum S, Rivas AL, Silanikove N. Effects of intra-mammary bacterial infection with coagulase negative staphylococci and stage of lactation on shedding of epithelial cells and infiltration of leukocytes into milk: comparison among cows, goats and sheep. *Vet Immunol Immunopathol.* (2012) 147:202–10. doi: 10.1016/j.vetimm.2012.04.019
68. Lasagno M, Ortiz M, Vissio C, Yaciuk R, Bonetto C, Pellegrino M, et al. Pathogenesis and inflammatory response in experimental caprine mastitis due to *Staphylococcus* chromogenes. *Microb Pathog.* (2018) 116:146–52. doi: 10.1016/j.micpath.2018.01.031
69. Vanderhaeghen W, Piepers S, Leroy F, Van Coillie E, Haesebrouck F, De Vliegher S. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. *Vet J.* (2015) 203:44–51. doi: 10.1016/j.tvjl.2014.11.001
70. Piessens V, Van Coillie E, Verbist B, Supre K, Braem G, Van Nuffel A, et al. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. *J Dairy Sci.* (2011) 94:2933–44. doi: 10.3168/jds.2010-3956
71. Quirk T, Fox LK, Hancock DD, Capper J, Wenz J, Park J. Intramammary infections and teat canal colonization with coagulase-negative staphylococci after postmilking teat disinfection. species-specific responses. *J Dairy Sci.* (2012) 95:1906–12. doi: 10.3168/jds.2011-4898
72. Pate M, Zdovc I, Avbersek J, Ocepek M, Pengov A, Podpecan O. Coagulase-negative staphylococci from non-mastitic bovine mammary gland: characterization of *Staphylococcus* chromogenes and *Staphylococcus* haemolyticus by antibiotic susceptibility testing and pulsed-field gel electrophoresis. *J Dairy Res.* (2012) 79:129–34. doi: 10.1017/S0022029911000811
73. Gosselin VB, Lovstad J, Dufour S, Adkins PRF, Middleton JR. Use of MALDI-TOF to characterize *Staphylococcal* intramammary infections in dairy goats. *J Dairy Sci.* (2018) 101:6262–70. doi: 10.3168/jds.2017-14224
74. Wuytack De Visscher A, Piepers S, Boyen F, Haesebrouck F, De Vliegher S. Non-*aureus* staphylococci in fecal samples of dairy cows: first report and phenotypic and genotypic characterization. *J Dairy Sci.* (2019) 102:9345–59. doi: 10.3168/jds.2019-16662
75. Wald R, Hess C, Urbantke V, Wittek T, Baumgartner M. Characterization of *Staphylococcus* species isolated from bovine quarter milk samples. *Animals.* (2019) 9:200. doi: 10.3390/ani9050200
76. Piccart K, Verbeke J, De Visscher A, Piepers S, Haesebrouck F, De Vliegher S. Local host response following an intramammary challenge with *Staphylococcus fleurettii* and different strains of *Staphylococcus* chromogenes in dairy heifers. *Vet Res.* (2016) 47:56. doi: 10.1186/s13567-016-0338-9
77. Verbeke J, Piccart K, Piepers S, Van Poucke M, Peelman L, De Visscher A, et al. Somatic cell count and milk neutrophil viability of dairy heifers with specific CXCR1 genotypes following experimental intramammary infection

with *Staphylococcus* chromogenes originating from milk. *Vet J.* (2015) 204:322–6. doi: 10.1016/j.tvjl.2015.04.010
78. Åvall-Jääskeläinen S, Taponen S, Kant R, Paulin L, Blom J, Palva A, et al. Comparative genome analysis of 24 bovine-associated *Staphylococcus* isolates with special focus on the putative virulence genes. *PeerJ*. (2018) 6:e4560. doi: 10.7717/peerj.4560
79. Tremblay YD, Lamarche D, Chever P, Haine D, Messier S, Jacques M. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *J Dairy Sci.* (2013) 96:234–46. doi: 10.3168/jds.2012-5795
80. Piessens V, De Vliegher S, Verbist B, Braem G, Van Nuffel A, De Vuyst L, et al. Characterization of coagulase-negative *Staphylococcus* species from cows' milk and environment based on bap, icaA, and mecA genes and phenotypic susceptibility to antimicrobials and teat dips. *J Dairy Sci.* (2012) 95:7027–38. doi: 10.3168/jds.2012-5400
81. Moraveji Z, Tabatabaei M, Shirzad Aski H, Khoshbakht R. Characterization of hemolysins of *Staphylococcus* strains isolated from human and bovine, Southern Iran. *Iran J Vet Res.* (2014) 15:326–30. doi: 10.1016/j.micpath.2013.10.003
82. Nobrega DB, Naushad S, Naqvi SA, Condas LAZ, Saini V, Kastelic JP, et al. Prevalence, genetic basis of antimicrobial resistance in non-*aureus Staphylococci* isolated from Canadian dairy herds. *Front Microbiol.* (2018) 9:256. doi: 10.3389/fmicb.2018.00256
83. Souza FN, Piepers S, Della Libera AMMP, Heinemann MB, Cerqueira MOP, De Vliegher MS. Interaction between bovine-associated coagulase-negative staphylococci species and strains and bovine mammary epithelial cells reflects differences in ecology and epidemiological behavior. *J Dairy Sci.* (2016) 99:2867–74. doi: 10.3168/jds.2015-10230
84. Kuipers Stapels DAC, Weerwind LT, Ko YP, Ruyken M, Lee JC, van Kessel KPM, et al. The *Staphylococcus aureus* polysaccharide capsule and Efb-dependent fibrinogen shield act in concert to protect against phagocytosis. *Microbiology.* (2016) 162:1185–94. doi: 10.1099/mic.0.000293
85. Salimena P, Lange CC, Camussone C, Signorini M, Calvinho LF, Brito MA, et al. Genotypic and phenotypic detection of capsular polysaccharide and biofilm formation in *Staphylococcus aureus* isolated from bovine milk collected from Brazilian dairy farms. *Vet Res Commun.* (2016) 40:97–106. doi: 10.1007/s11259-016-9658-5
86. Bardiau M, Detilleux J, Farnir F, Mainil JG, Ote I. Associations between properties linked with persistence in a collection of *Staphylococcus aureus* isolates from bovine mastitis. *Vet Microbiol.* (2014) 169:74–9. doi: 10.1016/j.vetmic.2013.12.010
87. Tuchscherr L, Löffler B, Buzzola FR, Sordelli DO. *Staphylococcus aureus* adaptation to the host and persistence: role of loss of capsular polysaccharide expression. *Future Microbiol.* (2010) 5:1823–32. doi: 10.2217/fmb.10.147
88. Lam TJ, Schukken YH, van Vliet JH, Grommers FJ, Tielen MJ, Brand A. Effect of natural infection with minor pathogens on susceptibility to natural infection with major pathogens in the bovine mammary gland. *Am J Vet Res.* (1997) 58:17–22.
89. Naidu TG, Newbould FH. Significance of beta-hemolytic *Staph. aureus* as a pathogen to the bovine mammary gland. *Zentralbl Veterinarmed B.* (1975) 22:308–17. doi: 10.1111/j.1439-0450.1975.tb00592.x
90. Larsen HD, Aarestrup FM, Jensen NE. Geographical variation in the presence of genes encoding superantigenic exotoxins beta-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe USA. *Vet Microbiol.* (2002) 85:61–7. doi: 10.1016/S0378-1135(01)00478-3
91. Kim HK, Thammavongsa V, Schneewind O, Missiakas D. Recurrent infections and immune evasion strategies of *Staphylococcus aureus*. *Curr Opin Microbiol.* (2012) 15:92–9. doi: 10.1016/j.mib.2011.10.012
92. Thammavongsa V, Kern JW, Missiakas DM, Schneewind O. *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J Exp Med.* (2009) 206:2417–27. doi: 10.1084/jem.20090097
93. Peschel A, Otto M. Phenol-soluble modulins and *Staphylococcal* infection. *Nat Rev Microbiol.* (2013) 11:667–73. doi: 10.1038/nrmicro3110
94. Sampimon OC. *Coagulase-Negative Staphylococci Mastitis in Dutch Dairy Herds*. Utrecht: Utrecht University (2009).
95. Kot B, Piechota M, Wolska KM, Frankowska A, Zdunek E, Binek T, et al. Phenotypic and genotypic antimicrobial resistance of staphylococci from bovine milk. *Pol J Vet Sci.* (2012) 15:677–83. doi: 10.2478/v10181-012-0105-4
96. de Jong Garch FE, Simjee S, Moyaert H, Rose M, Youala M, Siegwart E. Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. *Vet Microbiol.* (2018) 213:73–81. doi: 10.1016/j.vetmic.2017.11.021
97. Fišarová L, Pantuček R, Botka T, Doškar J. Variability of resistance plasmids in coagulase-negative staphylococci and their importance as a reservoir of antimicrobial resistance. *Res Microbiol.* (2019) 170:105–11. doi: 10.1016/j.resmic.2018.11.004
98. Argudín MA, Vanderhaeghen W, Butaye P. Diversity of antimicrobial resistance and virulence genes in methicillin-resistant non-*Staphylococcus* aureus staphylococci from veal calves. *Res Vet Sci.* (2015) 99:10–16. doi: 10.1016/j.rvsc.2015.01.004
99. Nobrega DB, De Buck J, Barkema HW. Antimicrobial resistance in non-aureus staphylococci isolated from milk is associated with systemic but not intramammary administration of antimicrobials in dairy cattle. *J Dairy Sci.* (2018) 101:7425–36. doi: 10.3168/jds.2018-14540
100. Stevens M, Piepers S, Supré K, De Vliegher S. Antimicrobial consumption on dairy herds and its association with antimicrobial inhibition zone diameters of non-*aureus* staphylococci and *Staphylococcus aureus* isolated from subclinical mastitis. *J Dairy Sci.* (2018) 101:3311–22. doi: 10.3168/jds.2017-13365
101. 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. *J Dairy Sci.* (2014) 97:340–4. doi: 10.3168/jds.2013-7130
102. Frey Y, Rodriguez JP, Thomann A, Schwendener S, Perreten V. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *J Dairy Sci.* (2013) 96:2247–57. doi: 10.3168/jds.2012-6091
103. Andreis SN, Perreten V, Schwendener S. Novel β-lactamase $bla_{ARL}$ in *Staphyloccocus arlettae*. *mSphere*. (2017) 2:e00117-17. doi: 10.1128/mSphere.00117-17
104. Traversari J, van den Borne BHP, Dolder C, Thomann A, Perreten V, Bodmer M. Non-*aureus Staphylococci* species in the teat canal and milk in four commercial swiss dairy herds. *Front Vet Sci.* (2019) 6:186. doi: 10.3389/fvets.2019.00186
105. De Visscher Supré K, Haesebrouck F, Zadoks RN, Piessens V, Van Coillie E, Piepers S, et al. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. *Vet Microbiol.* (2014) 172:466–74. doi: 10.1016/j.vetmic.2014.06.011
106. Roberson JR, Fox LK, Hancock DD, Gay JM, Besser TE. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. *J Dairy Sci.* (1994) 77:3354–64. doi: 10.3168/jds.S0022-0302(94)77277-5
107. Dohoo R, Smith J, Andersen S, Kelton DF, Godden S, Conference MRW. Diagnosing intramammary infections: evaluation of definitions based on a single milk sample. *J Dairy Sci.* (2011) 94:250–61. doi: 10.3168/jds.2010-3559
108. Taponen S, McGuinness D, Hiitiö H, Simojoki H, Zadoks R, Pyörälä S. Bovine milk microbiome: a more complex issue than expected. *Vet Res.* (2019) 50:44. doi: 10.1186/s13567-019-0662-y
109. Derakhshani H, Plaizier JC, De Buck J, Barkema HW, Khafipour E. Composition of the teat canal and intramammary microbiota of dairy cows subjected to antimicrobial dry cow therapy and internal teat sealant. *J Dairy Sci.* (2018) 101:10191–205. doi: 10.3168/jds.2018-14858
110. Carson DA, Barkema HW, Naushad S, De Buck J. Bacteriocins of non-*aureus Staphylococci* isolated from bovine milk. *Appl Environ Microbiol.* (2017) 83:e01015-17. doi: 10.1128/AEM.01015-17
111. Braem G, Stijlemans B, Van Haken W, De Vliegher S, De Vuyst L, Leroy F. Antibacterial activities of coagulase-negative staphylococci from bovine teat apex skin and their inhibitory effect on mastitis-related pathogens. *J Appl Microbiol.* (2014) 116:1084–93. doi: 10.1111/jam.12447
112. Jang T, Yang M, Kim HJ, Park JK. Novel cytoplasmic bacteriocin compounds derived from *Staphylococcus* epidermidis selectively kill *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus* (MRSA). *Pathogens*. (2020). 9:87. doi: 10.3390/pathogens9020087
113. Reyher KK, Haine D, Dohoo IR, Revie CW. Examining the effect of intramammary infections with minor mastitis pathogens on the

acquisition of new intramammary infections with major mastitis pathogens–a systematic review and meta-analysis. *J Dairy Sci.* (2012) 95:6483–502. doi: 10.3168/jds.2012-5594

114. Dolder C, van den Borne BHP, Traversari J, Thomann A, Perreten V, Bodmer M. Quarter- and cow-level risk factors for intramammary infection with coagulase-negative staphylococci species in Swiss dairy cows. *J Dairy Sci.* (2017) 100:5653. doi: 10.3168/jds.2016-11639
115. Derakhshani H, Plaizier JC, De Buck J, Barkema HW, Khafipour E. Composition and co-occurrence patterns of the microbiota of different niches of the bovine mammary gland: potential associations with mastitis susceptibility, udder inflammation, teat-end hyperkeratosis. *Anim Microbiome.* (2020) 2:11. doi: 10.1186/s42523-020-00028-6
116. Piepers S, Peeters K, Opsomer G, Barkema HW, Frankena K, De Vliegher S. Pathogen group specific risk factors at herd, heifer and quarter levels for intramammary infections in early lactating dairy heifers. *Prev Vet Med.* (2011) 99:91–101. doi: 10.1016/j.prevetmed.2011.02.010
117. De Vliegher S, Opsomer G, Vanrolleghem A, Devriese LA, Sampimon OC, Sol J, et al. *In vitro* growth inhibition of major mastitis pathogens by *Staphylococcus* chromogenes originating from teat apices of dairy heifers. *Vet Microbiol.* (2004) 101:215–21. doi: 10.1016/j.vetmic.2004.03.020
118. Reyher KK, Dohoo IR, Scholl DT, Keefe GP. Evaluation of minor pathogen intramammary infection, susceptibility parameters, and somatic cell counts on the development of new intramammary infections with major mastitis pathogens. *J Dairy Sci.* (2012) 95:3766–80. doi: 10.3168/jds.2011-5148
119. Derakhshani H, Plaizier JC, De Buck J, Barkema HW, Khafipour E. Association of bovine major histocompatibility complex (BoLA) gene polymorphism with colostrum and milk microbiota of dairy cows during the first week of lactation. *Microbiome.* (2018) 6:203. doi: 10.1186/s40168-018-0586-1

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# Evaluation of Chromogenic Culture Media for Rapid Identification of Gram-Positive Bacteria Causing Mastitis

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The present study aimed to evaluate the diagnostic performance specificity (Sp), sensitivity (Se), positive predictive value (PPV), negative predictive value (NPV), and accuracy (Acc) of two chromogenic culture media for rapid identification of Gram-positive bacteria causing subclinical mastitis (SCM) in dairy cows. For this, the performance of chromogenic culture media Gram-positive (GP) and *Staphylococcus* (Staph) (CHROMagar $^{TM}$, Paris—France) was evaluated in milk samples collected from: (1) lactating cows with SCM ($n = 504$), and (2) cows in the post-partum period (PP) ($7 \pm 3$ days post-partum; $n = 536$). Rapid identification of Gram-positive bacteria in chromogenic media was performed by visual inspection of colony colors after 24 h of incubation at 37°C. Bacterial identification by MALDI-TOF mass spectrometry was considered the reference methodology for calculating: Acc, Se, Sp, PPV, NPV, and Cohen's *Kappa* coefficient of agreement (*k*). The chromogenic media GP showed high Acc for *Strep. agalactiae/dysgalactiae* identification in both samples of SCM (Se: 89.1%; Sp: 96.3% and Acc: 95.6%) and of cows in PP (Se: 100%; Sp: 99.0% and Acc: 99.1%). Similar results were observed for *Strep. uberis/Enterococcus* spp. identification (Se: 90.5%; Sp: 92.5% and Acc: 92.3%) in SCM samples and Se: 100%; Sp: 99.6% and Acc: 99.6% in samples of PP cows using the GP media. However, the GP chromogenic media showed low Se (25.0% in SCM samples and 50.0% in samples of cows in PP) for *Staph. aureus* identification, despite Sp and Acc were high (Sp: 98.3% and Acc: 95.4% in SCM and Sp samples: 99.4% and Acc: 98.9% in PP cow samples). Staph culture media showed high Acc for *Staph. aureus* identification (Se: 80.0%; Sp: 98.8% and Acc: 98.0% in SCM samples and Se: 66.7%; Sp: 100% and Acc: 99.6% in PP cow samples), although the low prevalence of *Staph. epidermidis* and *Staph. saprophyticus* limit inferences about the performance of identifying these pathogens in Staph media. In conclusion, despite the limitation of the GP media for identification of *Staph. aureus*, GP, and Staph chromogenic media obtained satisfactory diagnostic performance results for the rapid identification of the main Gram-positive pathogens associated with SCM.

**Keywords: chromogenic media, microorganism identification, mastitis, milk quality, on-farm culture**

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## INTRODUCTION

Mastitis is a disease that causes major losses in dairy production (1). The most prevalent form of the disease is subclinical mastitis (SCM) (2), which leads to decreased milk production, increased somatic cell count (SCC), and increased risk of clinical mastitis (CM) during lactation (3–5).

The occurrence of SCM in the dairy herd leads to direct as well as indirect economic losses, such as the decrease in milk production, which reduces the profitability of farms (6, 7). These losses are further worsened when SCM is caused by contagious agents, which can be transmitted between cows in the same herd (8), and, in this sense, controlling the spread of contagious SCM is essential for attaining higher profitability in dairy farms. In this situation, rapid identification of subclinical mastitis-causing pathogens is fundamental for rapid control measures.

The use of microbiological culture associated with biochemical tests is the standard method for identifying mastitis-causing agents. However, laboratory microbiological culture is not widely used in dairy herds across different countries (9). The main challenges for using laboratory microbiological culture are the logistical limitations and cost of shipping samples, along with the time of analysis to obtain the results, which can vary from 3 to 5 days (10). Alternatively, the on-farm microbiological culture has been used to rapidly identify clinical mastitis-causing pathogens, which allows greater agility in decision making for treatment, and, alternatively, it could also be used to choose preventive or control measures in the herd, such as segregation or culling (9). In similar ways, rapid and correct identification of SCM causing agents could assist in guiding measures to control mastitis pathogens in the herd, strategic treatments for intramammary infection (IMI), when treatment is viable, or selective dry cow therapy (10).

Chromogenic culture media are alternatives for rapid microbiological identification, as they make it possible to presumptively differentiate bacterial species and/or groups according to colony color, reducing the need for biochemical tests (11). These culture media outperform other conventional microbiological rapid methods (e.g., Minnesota Easy System Triplets; Mastitis SSGN Quad Culture Plates) concerning specificity (Sp), sensitivity (Se), and accuracy (Acc) (12), in addition to identifying contamination in the samples more efficiently (13), which minimizes the occurrence of false positives in the identification of agents.

The use of chromogenic media for detecting mastitis-causing pathogens has been described in Europe (13); North America (14) and South America (15). According to Ganda (2016), chromogenic media presented 100% Se and 99.8% Sp for the identification of *Staph. aureus* in CM milk samples. Therefore, the use of chromogenic culture media on the dairy farm routine could be an alternative for the rapid identification of SCM. However, no previous study evaluated the effectiveness of chromogenic media in milk samples from cows with SCM, and for the monitoring of cows in post-partum (PP); which is a period of the high risk of new IMI and the manifestation of IMI not cured during the dry period can manifest (16).

Thus, the present study hypothesizes that the chromogenic media Mastitis GP and Staph. (CHROMagar ™, Paris—France), which are selective for Gram-positive (GP) and *Staphylococcus* spp. (Staph), respectively, present high Acc, Se, and Sp for rapid identification of the main SCM causing agents during lactation and in the PP period compared to standard laboratory microbiological identification methods such as mass spectrometry.

## MATERIALS AND METHODS

The present study was carried out under the ethical principles of animal experimentation and followed the rules established by the National Council for the Control of Animal Experimentation (CONCEA). The experimental protocol adopted was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo under registration No. 4579250719.

### Herd Selection and Sample Collection

The milk samples were obtained from six dairy herds located in the states of São Paulo ($n = 5$) and Minas Gerais ($n = 1$), Brazil. Dairy herds were selected based on non-probabilistic convenience sampling (availability to participate in the study and proximity to the host institution, Milk Quality Research Laboratory—Qualileite, Pirassununga, SP). Herds had an average of 125 (± 60) lactating cows housed in compost-bedded pack barns, with average milk production of 27.5 (± 2.5) liters/cow/day. Also, all herds performed individual monthly SCC analysis of all lactating cows. Before the beginning of the study, milking personnel and/or those responsible for the udder health of each herd were trained for procedures of sampling collecting collection, and clinical mastitis diagnosis.

Five hundred and four lactating cows with SCM (SCC> 200,000 cells/mL) had composite milk samples (pool of the four mammary quarters) collected for microbiological identification of mastitis-causing pathogens. Additionally, 541 cows in the PP period (7 ± 3 days after calving) had composite milk samples ($n = 226$) or individual samples ($n = 315$) of mammary quarter collected for microbiological culture. In our study, cows with CM, or cows that present concomitant diseases other than mastitis and/or treated with antimicrobials (systemic or intramammary) in < 14 days of sample collection, were excluded.

The procedures of milk sample collection for microbiological analysis were performed as described by NMC (17). Briefly, prior to milk collection, teat end was cleaned and disinfected with 70% iodized alcohol (70% alcohol + 2% iodine). The first milk strips were discarded and the milk was collected directly in a sterile tube, previously identified. The collection of milk samples from cows in PP was carried out by previously trained farm workers. Samples were stored at −20°C on the farm for a maximum period of 30 days until the microbiological culture analysis was carried out.

## Chromogenic Culture Media

Two selective chromogenic culture media, GP and Staph (CHROMagar ™, Paris, France), for identification of Gram-positive bacteria and *Staphylococcus* spp., respectively, were evaluated. The results of presumptive bacterial identification were visually performed based on the interpretation of the colony color characteristics, according to the manufacturer's recommendation.

The GP culture media identification results were interpreted according to the following colony colors: (a) turquoise blue—*Streptococcus agalactiae/dysgalactiae*; (b) dark blue/metallic blue—*Streptococcus uberis/Enterococcus* spp.; (c) pink/mauve—*Staphylococcus aureus* (**Figure 1**). Additionally, the identification results of Staph culture media were interpreted according to the following colony colorations: (a) pink—*Staph. aureus*; (b) colorless/pinky colony—*Staphylococcus epidermidis*; (c) turquoise blue—*Staphylococcus saprophyticus*. In both culture media, colonies showing other colors not described in the manufacturer's recommendations were considered as "other microorganisms."

## Evaluation of Chromogenic Culture Media for Mastitis-Causing Agents Identification

For the microbiological identification of mastitis-causing pathogens, 0.01 mL of milk samples were inoculated, using a calibrated platinum loop, simultaneously in three culture media: a) blood agar supplemented with 5% bovine blood; b) GP chromogenic media and c) Staph chromogenic media. After inoculation, the plates were incubated at 37°C for 24 h, and then they were subjected to visual inspection to evaluate the microbiological growth. Samples that presented $\geq$ 3 microorganisms with different characteristics in composite milk samples, or individual mammary quarter, were considered contaminated (18). Besides, samples with the isolation of two microorganisms with different colony morphologies were considered "mixed culture." In chromogenic media, colony colors were analyzed for microbiological identification, according to the manufacturer's recommendations (**Figure 1**). The evaluation of the bacterial colonies on the chromogenic culture media was carried out with a white background, for better differentiation of colony color. The microbial growth was recorded by a digital camera. In the GP chromogenic media, samples were considered positive for *Strep. uberis/Enterococcus* spp. or *Strep. agalactiae/dysgalactiae* when they presented a growth of 3 or more colonies with the color pattern defined for the species. For *Staph. aureus*, the growth of only 1 colony was considered the threshold for the identification of the agent. In the Staph culture media, the growth of $\geq$ 1 colony was sufficient for the identification of *Staph. aureus*, *Staph. epidermidis*, and *Staph. saprophyticus*. After the visual inspection of morphological characteristics, all the isolates that showed positive growth in blood agar and the chromogenic culture media were submitted to microbiological identification by Matrix Associated Laser Desorption-Ionization—Time of Flight (MALDI-TOF MS), as previously described (19). Throughout the experimental period, the evaluation of microbial growth in the culture media was carried out by the same evaluator, who had no prior knowledge of the results of identification by MALDI-TOF of the isolates.

## Diagnostic Performance

The visual identification of mastitis-causing agents by GP and Staph chromogenic media was compared to the gold standard method (MALDI-TOF MS) to estimate performance indicators. Samples that showed contamination in one of the two results (blood agar and/or chromogenic media) were excluded in the final analysis.

The performance indicators of Acc, Se, Sp, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of the chromogenic media were calculated based on the following results: True Positive (TP; when there was microbial growth and the result of visual microbiological identification of the chromogenic media was corresponding to that of the gold standard method), True Negative (TN; when there was no growth of any microorganism in the chromogenic culture media and blood agar), False Positive (FP; when there was a growth of any microorganism whose result was different between the microbiological identification of the gold standard method and the visual microbiological identification of the chromogenic media), False Negative (FN; when there was no growth of microorganisms in the chromogenic media and there was a growth of any microorganism in the gold standard method) (12).

The SENSPAC option of PROC FREQ; SAS version 9.4 (SAS INC., North Carolina, USA) was used to calculate Se, Sp, PPV, and NPV. Acc was also calculated by PROC FREQ, considering the TP and TN results. The results of Acc, Se, Sp, PPV, and NPV for the microbiological identification of chromogenic culture media were classified as low (results <60%), intermediate (between 60 and 80%), and high (> 80%) (20).

The results of Cohen's Kappa coefficient of agreement (k) were calculated by PROC FREQ of SAS version 9.4 (SAS INC., North Carolina, USA). Almost perfect agreement was considered when results of the coefficient between 0.81 and 1.00; substantial agreement (results from 0.61 to 0.80); moderate agreement (results from 0.41 to 0.60); fair agreement (results between 0.21 and 0.40), slight agreement (results between 0.00 and 0.20), and values $\leq$ 0.00 poor agreement (21).

# RESULTS

A total of 504 composite milk samples from SCM cases were evaluated during the 02/01/2019–03/15/2019 periods. A total of 51 different species of microorganisms (that includes bacteria, yeast and algae) were isolated from the three culture media (GP; S and blood agar), considering that the microorganisms isolated in each media are not necessarily the same for the same sample. No contamination was observed in SCM samples evaluated.

A total of 541 milk samples (226 composite and 315 from mammary quarter) from post-partum dairy cows were evaluated during the 04/12/2019–07/25/2019 periods. A total of 36 different species of microorganisms (bacteria, yeast, and algae) were isolated considering the growth results for the three culture media evaluated. In 0.9% milk samples ($n = 5$; four

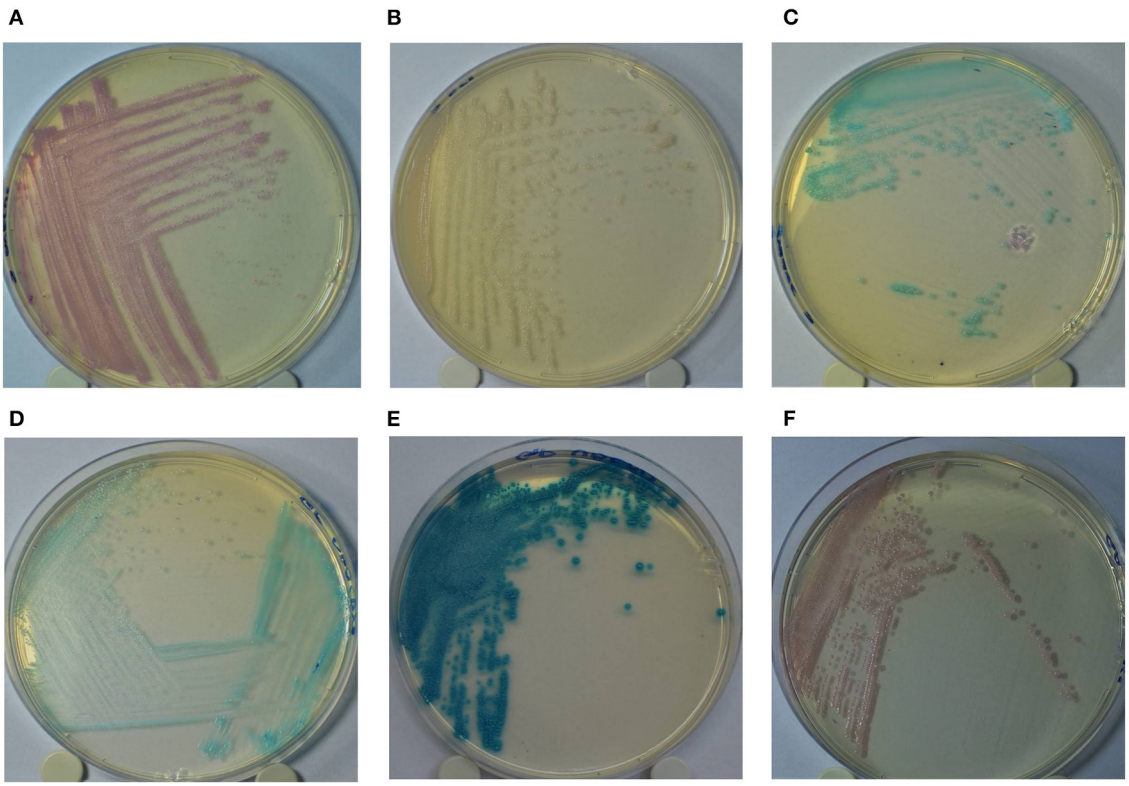


**FIGURE 1 |** Colony color pattern of *Staphylococcus aureus* (**A**: pink); *Staphylococcus epidermidis* (**B**: pinkish/colorless); *Staphylococcus saprophyticus* (**C**: turquoise blue) grown in Staphylococcus chromogenic culture media **(A–C)**; and *Streptococcus agalactiae/Streptococcus dysgalactiae* (**D**: turquoise blue); *Streptococcus uberis/Enterococcus* spp. (**E**: dark/metallic blue) and *Staphylococcus aureus* (**F**: pink) grown in Mastitis GP culture media **(D–F)**.

composite and one of the mammary quarter) contamination was observed, and these milk samples were not evaluated in diagnostic performance analysis.

## Frequency of Mastitis Pathogens Identification

Based on blood agar growth, 61.3% (309/504) of the SCM milk samples and 30.5% (165/541) of PP milk samples had microbiological growth. Of these, mixed culture was observed in 3.8% (19/504) SCM and in 2.4% (13/541) PP milk samples. Gram-positive bacteria had higher isolation frequency (57.1%; 288/504 in SCM and 28.3%; 153/541 in PP) and non-aureus *Staphylococcus* (NAS) was the microorganism group that had the largest isolation frequency (25%; 126/504 in SCM and 22.6% 122/541 in PP). *Staph. chromogenes* was the most frequently isolated pathogen in both SCM (20.6%; 104/504) and PP (15.5%; 84/541; **Table 1**) milk samples.

In GP culture media, 49.4% (249/504) of SCM and 13.3% (72/541) of PP milk samples had microbiological growth. Mixed culture was observed in 5.8% (29/504) of SCM and 1.5% (8/541) of PP milk samples. Also, 4.8% (24/504) of SCM and 0.6% (3/541) of PP milk samples had microbiological isolation in GP chromogenic media and had no growth in blood agar. *Staph. chromogenes* were the most frequent pathogen isolated in GP media (16.5%; 83/504 in SCM and 7.2%; 39/541 in PP milk samples; **Table 1**).

In Staph chromogenic media, 32.74% (165/504) of SCM and 5.5% (30/541) of PP milk samples had microbiological isolation. Of these, mixed culture was observed in 2.4% (12/504) of SCM and 0.6% (3/541) of PP milk samples. Also, 5.6% (28/504) of SCM and 0.7% (4/541) of PP milk samples had no growth in blood agar and had microbiological isolate in Staph chromogenic media. *Staph. chromogenes* was the most isolated pathogen (19.4%; 98/504 in SCM and 2.0%; 11/541 in PP milk samples), followed by *Staph. aureus* (4.0%; 20/504 in SCM and 0.9%; 5/541 in PP). *Strep. uberis* was isolated in Staph media in 0.4% (2/504) of SCM milk samples. Colonies of *Strep. uberis* identified in the Staph media and the respective milk sample were re-inoculated in Staph media plates and no microbiological isolation was observed.

## Diagnostic Performance of Chromogenic Culture Media

Accuracy of GP chromogenic media identification varied according to the pathogen group from 92.3% (*Strep. uberis*/*Enterococcus* spp.) to 95.6% (*Strep. agalactiae*/*dysgalactiae*) for SCM milk samples, and from 98.9% (*Staph. aureus*) to 99.3% (*Strep. uberis*/*Enterococcus* spp.) for PP milk samples. Additionally, Se of GP media varied according

**TABLE 1 |** Frequency of mastitis-causing agents isolated from milk samples of lactating cows with subclinical mastitis and in post-partum period, identified using two chromogenic culture media (Gram-positive and Staphylococcus) and by MALDI-TOF MS.

| | SCM[a] | | | | | | PP[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **BA[c]** | **%** | **GP[d]** | **%** | **Staph[e]** | **%** | **BA** | **%** | **GP** | **%** | **Staph** | **%** |
| Total samples | 504 | 100 | 504 | 100 | 504 | 100 | 541 | 100 | 541 | 100 | 541 | 100 |
| Negative culture | 195 | 38.6 | 255 | 50.6 | 339 | 67.3 | 371 | 68.6 | 464 | 85.8 | 506 | 93.5 |
| Positive culture | 309 | 61.3 | 249 | 49.4 | 165 | 32.7 | 165 | 30.5 | 72 | 13.3 | 30 | 5.5 |
| Single morphology colonies | 290 | 57.5 | 220 | 43.7 | 153 | 30.4 | 152 | 28.1 | 64 | 11.8 | 27 | 5.0 |
| Mixed morphology[f] | 19 | 3.8 | 29 | 5.8 | 12 | 2.4 | 13 | 2.4 | 8 | 1.5 | 3 | 0.6 |
| Contamination[g] | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0.9 | 5 | 0.9 | 5 | 0.9 |
| Gram-positive bacteria | 288 | 57.1 | 249 | 49.4 | 165 | 32.7 | 153 | 28.3 | 70 | 12.9 | 29 | 5.4 |
| *Strep.*[h] *agalactiae* | 25 | 5 | 34 | 6.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Strep. dysgalactiae* | 21 | 4.2 | 16 | 3.2 | 0 | 0 | 7 | 1.3 | 5 | 0.9 | 0 | 0 |
| *Strep. uberis* | 61 | 12.1 | 69 | 13.7 | 2 | 0.4 | 4 | 0.7 | 4 | 0.7 | 0 | 0 |
| *Enterococcus* spp. | 2 | 0.4 | 4 | 0.8 | 0 | 0 | 2 | 0.4 | 2 | 0.4 | 0 | 0 |
| *Staph.*[i] *aureus* | 20 | 4 | 20 | 4 | 20 | 4 | 6 | 1.1 | 5 | 0.9 | 5 | 0.9 |
| NAS[j] | 126 | 25 | 102 | 20.2 | 124 | 24.6 | 122 | 22.6 | 48 | 8.9 | 19 | 3.5 |
| *Staph. chromogenes* | 104 | 20.6 | 83 | 16.5 | 98 | 19.4 | 84 | 15.5 | 39 | 7.2 | 11 | 2 |
| *Staph. epidermidis* | 1 | 0.2 | 0 | 0 | 2 | 0.4 | 4 | 0.7 | 2 | 0.4 | 1 | 0.2 |
| *Staph. saprophyticus* | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other NAS | 22 | 4.4 | 21 | 4.2 | 29 | 5.8 | 38 | 7 | 8 | 1.5 | 8 | 1.5 |
| Other Gram-positive | 48 | 9.5 | 23 | 4.6 | 26 | 5.2 | 17 | 3.1 | 6 | 1.1 | 6 | 1.1 |
| Gram-negative bacteria | 17 | 3.4 | 0 | 0 | 0 | 0 | 11 | 2 | 0 | 0 | 0 | 0 |
| Other pathogens | 5 | 1 | 2 | 0.4 | 0 | 0 | 1 | 0.2 | 1 | 0.2 | 0 | 0 |

*[a]subclinical mastitis samples; [b]post-partum samples; [c]blood agar; [d]mastitis GP (CHROMagar™. Paris—France); [e]Staphylococcus (CHROMagar™. Paris—France); [f]plates containing colonies with two different morphologies; [g]colonies with more than two different morphologies; [h]Streptococcus spp.; [i]Staphylococcus spp.; [j]Non-aureus Staphylococcus.*

to the group of pathogens from 25.0% (*Staph. aureus*) to 90.5% (*Strep. uberis*/*Enterococcus* spp.) in SCM milk samples, and from 50% (*Staph. aureus*) to 100% (*Strep. agalactiae*/*dysgalactiae*) for PP milk samples. Gram-positive culture media Sp for SCM ranged from 92.5% (*Strep. uberis*/*Enterococcus* spp.) to 98.7% (*Staph. aureus*), and in PP milk samples ranged from 99.1% (*Strep. agalactiae*/*dysgalactiae*) to 99.6% (*Strep. uberis*/*Enterococcus* spp.).

Positive predictive value results of GP media identification varied according to the pathogen group from 38.5% (*Staph. aureus*) to 70.4% (*Strep. agalactiae*/*dysgalactiae*) in SCM milk samples and 50.0% (*Staph. aureus*) to 71.4% (*Strep. uberis*/*Enterococcus* spp.) in PP milk samples. Also, NPV results range from 97.0% (*Staph. aureus*) to 98.9% (*Strep. agalactiae*/*dysgalactiae*) in SCM milk samples, and range from 99.4% (*Staph. aureus*) to 100% (*Strep. agalactiae*/*dysgalactiae*) in PP milk samples.

Kappa coefficient of agreement values varied according to the pathogen group from 0.27 (*Staph. aureus*) to 0.76 (*Strep. agalactiae*/*dysgalactiae*) in SCM milk samples and range from 0.45 (*Staph. aureus*) to 0.72 (*Strep. uberis*/*Enterococcus* spp.) in PP milk samples. Indicators of diagnostic performance for negative results of SCM in GP chromogenic media were: Acc = 82.1%, Se = 88.7%, Sp = 77.3%, PPV = 74.1%, NPV = 90.4% and $k$ = 0.60. Similarly, for PP negative milk samples, Acc was 83.4%, Se = 99.2%, Sp = 44.5%, PPV = 81.5%, NPV = 95.8%, and $k$ = 0.50.

Considering the low isolation frequency of *Staph. epidermidis* ($n$ = 1 in SCM and $n$ = 4 in PP milk samples) and *Staph. saprophyticus* ($n$ = 1 in SCM and $n$ = 0 in PP milk samples), it was not possible to evaluate the diagnostic performance of Staph chromogenic media for microbiological identification of these pathogens. However, *Staph. aureus* identification by Staph media in SCM milk samples had Acc = 98.0%, Se = 80.0%; Sp = 98.8%; PPV = 72.7% and NPV = 99.2%. In addition, for *Staph. aureus* identification in PP milk samples, Staph media had results of Acc = 99.6%; Se = 66.7%; Sp = 100%; PPV = 100%, NPV = 99.6%, and $k$ was 0.71 for SCM and 0.80 for PP milk samples (**Table 2**). Indicators of diagnostic performance for negative results of SCM in Staph chromogenic media were: Acc = 86.3%, Se = 91.4%, Sp = 77.0%, PPV = 87.9%; NPV = 83%, and $k$ = 0.53 (**Table 3**). Similarly, for PP milk samples that had negative results the Acc was 80.4%, Se was 99.0%, Sp was 20.5%, PPV was 80.0%, NPV was 86.7%, and $k$ was 0.29.

## DISCUSSION

Chromogenic culture media may be used as alternatives for rapid microbiological identification, considering the potential to differentiate species and/or groups of microorganisms (11). This study evaluated the ability of GP and Staph chromogenic culture media for rapid identification of mastitis-causing pathogens in milk from SCM and cows in PP.

In chromogenic media GP, it was observed high Se and Sp for identification of *Strep. uberis*/*Enterococcus* spp. and *Strep. agalactiae*/*dysgalactiae* groups, both in SCM and PP samples. Se and Sp values obtained for SCM samples (Se = 89.1%; Sp = 96.3% for *Strep. agalactiae*/*dysgalactiae* and Se = 90.5%; Sp = 92.5% for *Strep. uberis*) were similar to those obtained for the identification of *Streptococcus* spp. from the Minnesota Tri-Plate triplet (92.6 and 89.5%, respectively) (9); and also, for the results obtained by the Accumast chromogenic media (Se: 90% and Sp: 93%, respectively) (14). However, direct comparisons between our results and previous studies are limited because Ganda et al. (14) and MacCarron et al. (9) did not differ between *Strep. uberis* and *Strep. agalactiae*/*dysgalactiae*, grouping them into *Streptococcus* spp. This differentiation capacity observed in the chromogenic media GP enables farms to specifically identify the group and/or pathogen species and to adopt specific prophylactic control measures according to the species of *Streptococcus* spp. isolated, since these agents have distinct pathogenicity and transmission profile. The high Se and SP in PP samples (Se = 100%; Sp = 99% for *Strep. agalactiae*/*dysgalactiae* and Se = 71.4%; Sp = 99.6% for *Strep. uberis*) indicate that it is possible to obtain identification results reliable for these pathogens from PP samples.

The positive predictive values were classified as low/moderate for the *Strep. agalactiae*/*dysgalactiae* and *Strep. uberis*/*Enterococcus* spp. groups, both in SCM samples (PPV = 70.7% and PPV = 63.3; respectively) and for PP samples (PPV = 58.3 and PPV = 71.4; respectively). These results were inferior to those obtained by Ganda et al. (14) (Accumast) and MacCarron et al. (9) (Minnesota plate) but they were close to those reported by both the Minnesota Easy Culture System Bi-Plate and Tri-Plate (20). However, if we take into account the isolates that were grown in the GP media that were not isolated on blood agar, the diagnostic performance would be higher, especially in the samples of SCM (PPV = 89.7% for *Strep. agalactiae*/*dysgalactiae* and PPV = 80% for *Strep. uberis*/*Enterococcus* spp.) which would match the results obtained by Accumast and Minnesota plate.

The NPV result of the groups of *Strep. agalactiae*/*dysgalactiae* and *Strep. uberis*/*Enterococcus* spp. was considered high for both SCM samples (NPV = 98.9% and NPV = 98.5%; respectively) and for PP samples (NPV = 100% and NPV = 99.6). The results obtained were superior to those of the Minnesota Easy Culture System biplane for *Streptococcus* spp., and similar to those obtained by the Minnesota Easy Culture System Tri-Plate (20) and by the Accumast method (14). The high NPV values indicate the assertiveness of negative results in GP media when there is no isolation of agents from the *Strep. agalactiae*/*dysgalactiae* and *Strep. uberis*/*Enterococcus* spp. groups.

The Se values of the chromogenic media GP for presumptive identification of *Staph. aureus* was lower concerning the other groups of pathogens for samples of SCM (Se = 25%) and PP samples (Se = 50%). This low Se can be attributed to an inconsistency in the color pattern generated for the diversity of *Staph. aureus* isolates that were evaluated in this study. Different shades of pink were observed, not necessarily consistent with the characteristic color associated with *Staph. aureus* identification according to the manufacturer's recommendations, which caused misidentification and high numbers of FN results. Due to the high amount of FN results, both in SCM samples (FN = 75%; 15/20) and in PP samples (FN = 50% 3/6), PPV was also compromised (SCM = 38.5% and PP = 50%, respectively). Similar results for Se and PPV were obtained for *Staph. aureus* in

**TABLE 2 |** Indicators of diagnostic performance of the Gram-positive chromogenic media in milk samples from cows with SCM ($n = 504$) and cows in PP ($n = 536$).

| Indicator | | Gram-positive chromogenic media[a] | | |
|---|---|---|---|---|
| | **Negative** | ***Strep. uberis*/*Ent.*[b]** | ***Strep. aga*/dys[c]** | ***Staph. aureus*[d]** |
| N[e] | | | | |
| SCM[f] | 249 | 63 | 46 | 20 |
| PP[g] | 433 | 6 | 7 | 6 |
| Acc%[h] | | | | |
| SCM | 82.1 (78.8–85.5) | 92.3 (89.9–94.6) | 95.6 (93.9–97.4) | 95.4 (93.6–97.3) |
| PP | 98 (96.8–99.2) | 99.3 (98.5–100) | 99.1 (98.3–99.9) | 98.9 (98–99.8) |
| Se%[i] | | | | |
| SCM | 88.7 (84.5–93) | 90.5 (83.2–97.7) | 89.1 (80.1–98.1) | 25 (6–44) |
| PP | 99.2 (98.3–100) | 71.4 (38–100) | 100 (100–100) | 50 (10–90) |
| Sp%[j] | | | | |
| SCM | 77.3 (72.5–82.1) | 92.5 (90–95) | 96.3 (94.6–98) | 98.3 (97.2–99.5) |
| PP | 44.5 (36.7–52.3) | 99.6 (99.1–100) | 99 (98.2–99.9) | 99.4 (98.8–100) |
| PPV%[k] | | | | |
| SCM | 74.1 (68.7–79.5) | 63.3 (53.4–73.3) | 70.7 (59–82.4) | 38.5 (12–64.9) |
| PP | 81.4 (77.9–85) | 71.4 (38–100) | 58.3 (30.4–86.2) | 50 (10–90) |
| NPV%[l] | | | | |
| SCM | 90.4 (86.7–94) | 98.5 (97.4–99.7) | 98.9 (97.9–99.9) | 96.9 (95.4–98.5) |
| PP | 95.8 (91.2–100) | 99.6 (91.1–100) | 100 (100–100) | 99.4 (98.8–100) |
| *k* (IC 95%)[m] | | | | |
| SCM | 0.60 (0.53–0.67) | 0.71 (0.61–0.79) | 0.76 (0.61–0.79) | 0.30 (0.09–0.5) |
| PP | 0.50 (0.42–0.59) | 0.72 (0.42–1) | 0.69 (0.46–0.93) | 0.49 (0.14–0.84) |
| *k*—*P* Value[n] | | | | |
| SCM | < 0.0001 | < 0.0001 | 0.01 | 0.04 |
| PP | < 0.0001 | 0.56 | 0.01 | 1 |

[a]*gram-positive selective chromogenic media- Mastitis GP (CHROMagar™. Paris—France);* [b]*Streptococcus uberis/Enterococcus spp.;* [c]*Streptococcus agalactiae/dysgalactiae;* [d]*Staphylococcus aureus;* [e]*isolation frequency;* [f]*subclinical mastitis samples;* [g]*post-partum samples;* [h]*accuracy;* [i]*sensitivity;* [j]*specificity;* [k]*positive predictive value;* [l]*negative predictive value;* [m]*Cohen's Kappa concordance test;* [n]*Kappa Coefficient P-value.*

SCM samples by the rapid microbiological identification method VetoRapid kit (22) and CM by the SSGN and SSGNC quadrates (12). Contrary to our results for SCM samples, 3M Petrifilm system (9), and, in CM Accumast (14) and Minnesota Easy Culture System II (20), presented high Se and PPV results for *Staph. aureus* identification. In our study, SCM composite milk samples were used, which may result in a lower bacterial count necessary for the isolation of microorganisms in comparison to CM samples. Thus, it is possible to have a lower Se in the isolation of microorganisms in composite samples concerning CM samples per mammary quarter (23).

For the chromogenic media Staph, the results of Se and Sp for *Staph. aureus* was high in SCM samples (Se = 80%; Sp = 98.8%) and moderate to high for PP samples (Se = 66.7%; Sp = 100%; respectively). The PPV result was intermediate for SCM samples (PPV = 72.7) and the NPV result was high (NPV = 99.2). However, PPV would be higher (PPV = 90.9%) if we included samples with the isolation of *Staph. aureus* in Staph culture media (correctly identified by the chromogenic media), but without growth on blood agar were considered TP. These results are similar to those obtained by Accumast (14) and Minnesota Easy Culture System II (20). The ability to rapidly identify *Staph. aureus* infected cows are essential for the control of SCM in problem-herds, considering the potential of contagious transmission and low cure risk (24). The results of *Staph. aureus* identification by chromogenic media Staph can assist in decision making regarding the segregation and/or culling of *Staph. aureus* infected cows. Besides, the Staph chromogenic media makes it possible to differentiate between two other species of *Staphylococcus* spp. (*Staph. saprophyticus* and *Staph. epidermidis*), which has not been described in Minnesota Easy Culture System II and Accumast. However, due to the low isolation frequency of *Staph. saprophyticus* and *Staph. epidermidis*, it was not possible to determine the diagnostic performance in these species.

The agreement between the GP chromogenic media and the gold standard method, as measured by the *k* coefficient, was substantial for the *Strep. agalactiae/dysgalactiae* ($k = 0.76$) and *Strep uberis/Enterococcus* spp. ($k = 0.70$) groups for SCM samples. In PP samples, we also observed substantial agreement for the groups of pathogens mentioned above ($k = 0.72$ for *Strep. uberis/Enterococcus* spp. and $k = 0.69$ for *Strep. agalactiae/dysgalactiae*). The agreement observed in the present

**TABLE 3 |** Indicators of diagnostic performance in milk samples from cows with SCM ($n = 504$) and cows in PP ($n = 536$).

| Indicator | | *Staphylococcus* chromogenic media[a] | | |
|---|---|---|---|---|
| | **Negative** | ***Staph. aureus***[b] | ***Staph. epi.***[c] | ***Staph. sapro.***[d] |
| N[e] | | | | |
| SCM[f] | 338 | 20 | 1 | 1 |
| PP[g] | 493 | 6 | 4 | 0 |
| Acc%[h] | | | | |
| SCM | 86.3 (83.3–89.3) | 98 (96.8–99.2) | 98.6 (97.6–99.6) | 96.2 (94.6–97.9) |
| PP | 80.4 (77–83.8) | 99.6 (99.1–100) | 99.3 (98.5–100) | 98.9 (9 8–99.8) |
| Se%[i] | | | | |
| SCM | 91.4 (88.4–94.5) | 80 (62.5–97.5) | 0 (.) | 0 (.) |
| PP | 99 (98.1–100) | 66.7 (29–100) | 25 (0–67.4) | . |
| Sp%[j] | | | | |
| SCM | 77 (70.8–83.2) | 98.8 (97.8–99.8) | 98.8 (.) | 96.4 (.) |
| PP | 20.5 (13.5–27.5) | 100 (100–100) | 99.8 (99.4–100) | 98.9 (.) |
| PPV%[k] | | | | |
| SCM | 87.9 (84.4–91.4) | 72.7 (54.1–91.3) | 0 (.) | 0 (.) |
| PP | 80 (76.6–83.5) | 100 (100–100) | 50 (0–100) | 0 (.) |
| NPV%[l] | | | | |
| SCM | 83 (77.3–88.8) | 99.2 (98.4–100) | 99.8 (.) | 99.8 (.) |
| PP | 86.7 (74.5–98.8) | 99.6 (99.1–100) | 99.4 (98.8–100) | 100 (.) |
| *k* (IC 95%)[m] | | | | |
| SCM | 0.53 (0.44–0.61) | 0.73 (0.56–0.89) | (−0.003) [(−0.008)−0.002] | (−0.004) [(−0.010)−0.003] |
| PP | 0.29 (0.19–0.39) | 0.80 (0.52–1.00) | 0.33 [(−0.16)−0.82] | 0 |
| *k*−*P* Value[n] | | | | |
| SCM | < 0.0001 | 0.52 | 0.17 | 0.006 |
| PP | < 0.0001 | 0.15 | 0.31 | . |

[a]*selective chromogenic media to Staphylococcus spp.—Staph. (CHROMagar™. Paris—França);* [b]*Staphylococcus aureus;* [c]*Staphylococcus epidermidis;* [d]*Staphylococcus saprophyticus;* [e]*isolation frequency;* [f]*subclinical mastitis samples;* [g]*post-partum samples;* [h]*accuracy;* [i]*sensitivity;* [j]*specificity;* [k]*positive predictive value;* [l]*negative predictive value;* [m]*Cohen's Kappa concordance test.;* [n]*Kappa Coefficient P-value.*

study was higher than that obtained for *Streptococcus* spp. by the bi-plate ($k < 0.6$) and similar to the tri-plate ($k$ between 0.73 and 0.85) Minnesota Easy Culture System (20), however, it was lower than that obtained by Accumast ($k = 0.91$) (14). The ability to discern between *Strep. uberis/Enterococcus* spp. and *Strep. agalactiae/dysgalactiae* groups can help control SCM, making possible, for example, the segregation and strategic treatment of cows presumptively positive for *Strep. agalactiae*, which is the only SCM causing agent which treatment during lactation is indicated (25).

In SCM samples, the $k$ result obtained indicates that GP chromogenic media had a fair agreement for the rapid identification of *Staph. aureus* (k = 0.30), which corroborates the unsatisfactory results of Se (25.0%), and PPV (38.5%). For PP samples, the agreement was moderate ($k = 0.49$). The agreement results were lower than those obtained by Accumast ($k = 0.93$) (14) and by the Minnesota Easy Culture System Bi-Plate and Tri-Plate ($k = 0.62$ and k = 49; respectively) (20) but were similar to those obtained for the CHROMagar Mastitis chromogenic tri-plate ($k = 0.33$) (13). The low $k$ coefficient for *Staph. aureus* identification indicates a limitation of the GP media.

Staph chromogenic media had a substantial agreement for the rapid identification of *Staph. aureus* for both SCM samples ($k = 0.73$) and PP samples ($k = 0.79$). Our results were slightly lower than the almost perfect agreement obtained by Accumast ($k = 0.93$) (14) in CM samples, which may be associated with a higher concentration of pathogens present in CM samples compared to SCM samples. However, the $k$ coefficient of the Staph media was higher than that obtained by the Minnesota Easy Culture System Bi-Plate and Tri-Plate (both $k < 0.7$) (20), CHROMagar Mastitis, Hardy Diagnostics Mastitis Tri-Plate, and VetoRapid chromogenic triplets ($k = 0.33$ and $k = 0.40$, respectively) (13), indicating high agreement between the reference method and the presumptive identification of *Staph. aureus* by Staph culture media.

In the case of samples with no growth in the GP chromogenic media, high Se (88.7%) and moderate Sp (77.3%) results were obtained in SCM samples, indicating the assertiveness of the media for negative results. In the PP samples, GP chromogenic media obtained high Se for negative results (99.2%) but presented low Sp (44.5%). The low Sp may be associated with the high amount of FP ($n = 86$). The results obtained for the GP chromogenic media were superior to those described by Ferreira

et al. (12) for the Accumast, Minnesota Easy System, SSGN, and SSGNC media, which may be partially associated with the selective capacity of the media.

The Se of samples without growth in the Staph chromogenic media was also high in SCM (Se = 91.4%), while the Sp was moderate (Sp = 77%). As observed in the GP chromogenic media, for PP samples, Sp was low (Sp = 20.5%) and Se was high (Se = 99%). The results indicate assertiveness in the diagnosis of negative samples for microbiological isolation, but little Sp in this identification, which again may be partially associated with the occurrence of high numbers of FP results, especially in PP samples ($n = 101$). Also, two samples of SCM had isolation of *Strep. uberis*, a pathogen that should have growth inhibited in the Staph chromogenic media, which is selective for *Staphylococcus* spp. However, in both cases, when these colonies were transferred to a new plate of the Staph chromogenic media, there was no microbial isolation, and the same occurred when a new inoculation of the milk sample was performed.

It should be noted that our study has some limitations. Visual inspection of the colonies and the presumptive identification of the pathogens in chromogenic media were performed by only one researcher and at the laboratory conditions, which makes it difficult to extrapolate the result to farm conditions. However, to minimize bias, the reader was blinded to the results of identification on the gold standard.

The main limitation of this study was the use of MALDI-TOF MS as the only reference method for comparing the results of bacterial identification in two chromogenic media. The identification of isolates is based on the manufacturer's spectral database of the specific species or strains (26). Since the Biotyper library is mainly based on human pathogens strains, the identification of some mastitis isolates is not possible. However, this limitation can be solved by adding mass spectra of strains from mastitis-causing pathogens in the local library (27). On the other hand, isolates correctly identified by chromogenic media (agreement between visual identification on the chromogenic media and by MALDI-TOF MS) were considered FP, when no bacterial growth was observed in blood agar. For example, a total of 31 isolates had correct identification in the GP media (29 from SCM and 2 from PP) and six isolates in the Staph media ($n = 6$ samples from SCM), which were considered FP results. These results may suggest that the selectivity of the chromogenic media could reduce the competition between microorganisms, thus allowing the growth of non-isolated microorganisms in blood agar (11).

In general, regardless of the type of sample (SCM or PP), with the only exception of *Staph. aureus* in GP, both chromogenic culture media tested showed satisfactory performances for the rapid identification of the pathogens they were designed to identify. The GP chromogenic media was efficient for the identification of the *Strep. agalactiae/dysgalactiae* and *Strep uberis/Enterococcus* spp. groups, enabling the differentiation between *Strep. uberis* and *Strep. agalactiae/dysgalactiae* without the use of additional biochemical tests (e.g., Christie, Atkins, Munch-Petersen test). The ability of GP chromogenic culture media to differentiate between the groups *Strep. uberis*/*Enterococcus* spp. from *Strep. agalactiae*/*dysgalactiae* would be helpful for immediate SCM control, such as the segregation of suspected positive cows for *Strep. agalactiae*. Additionally, the identification of *Strep. agalactiae*/*dysgalactiae* positive cows using the GP media is an indication of the need to screen the whole herd for identification of *Strep. agalactiae* cows based on laboratory microbiological identification.

In this study, GP media presented limitations in the identification of *Staph. aureus*, which is possibly related to the inconsistency of the color pattern associated with the identification of this pathogen. The performance of the Staph chromogenic media was generally satisfactory for *Staph. aureus*, the main pathogen to which the media proposed to identify, what indicates the potential of the Staph media to be used in on-farm culture systems for specific identification of *Staph. aureus*. The identification of *Staph. aureus* positive cows would be helpful for control measures, such as the segregation and/or culling of infected cows.

Finally, the tested chromogenic media obtained diagnostic performance similar to other methods of rapid microbiological identification used in on-farm culture systems, with the advantage of allowing between *Strep. uberis* and *Strep. agalactiae/dysgalactiae* in GP media, as well as it possible to differentiate between *Staph. aureus*; *Staph. epidermidis* and *Staph. saprophyticus,* when using Staph media.

## CONCLUSION

The results of diagnostic performance of GP and Staph chromogenic media were satisfactory in identifying the main pathogens associated with mastitis in milk samples from cows in PP and SCM samples during lactation, except for *Staph. aureus* identification using GP media. The possibility of rapid microbiological identification (from 18 to 24 h), the high values obtained for the diagnostic performance in most groups of pathogens, and the ability to differentiate between some species of *Staphylococcus* spp. and *Streptococcus* spp. genus suggests that GP and Staph media are adequate alternatives for using rapid identification of Gram-positive pathogens causing SCM.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by National Council for the Control of Animal Experimentation (CONCEA) Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

BGa and MS took part in designing the experiment. BGa, CF, GF, BGr, and MS performed the experiment, data analysis, and wrote the paper. All authors participated in reading, provided a critical review, and approved the final manuscript.

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## REFERENCES

1. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023
2. Pumipuntu N, Kulpeanprasit S, Santajit S, Tunyong W, Kong-ngoen T, Hinthong W, et al. Screening method for *Staphylococcus aureus* identification in subclinical bovine mastitis from dairy farms. *Vet World.* (2017) 10:721–6. doi: 10.14202/vetworld.2017.721-726
3. Reksen O, Sølverød L, Branscum AJ, Østerås O. Relationships between milk culture results and treatment for clinical mastitis or culling in Norwegian dairy cattle. *J Dairy Sci.* (2006) 89:2928–37. doi: 10.3168/jds.S0022-0302(06)72565-6
4. Van den Borne BHP, Vernooij JCM, Lupindu AM, van Schaik G, Frankena K, Lam TJGM, et al. Relationship between somatic cell count status and subsequent clinical mastitis in Dutch dairy cows. *Prev Vet Med.* (2011) 102:265–73. doi: 10.1016/j.prevetmed.2011.07.013
5. van den Borne BHP, van Schaik G, Lam TJGM, Nielen M, Frankena K. Intramammary antimicrobial treatment of subclinical mastitis and cow performance later in lactation. *J Dairy Sci.* (2019) 102:4441–51. doi: 10.3168/jds.2019-16254
6. Gonçalves JL, Cue RI, Lima Netto EP, Gameiro AH, dos Santos M V. Herd-level associations between somatic cell counts and economic performance indicators in Brazilian dairy herds. *J Dairy Sci.* (2020) 104:1855–63. doi: 10.3168/jds.2019-17834
7. Hogeveen H, Huijps K, Lam TJGM. Economic aspects of mastitis: new developments. *N Z Vet J.* (2011) 59:16–23. doi: 10.1080/00480169.2011.547165
8. Cervinkova D, Vlkova H, Borodacova I, Makovcova J, Babak V, Lorencova A, et al. Prevalence of mastitis pathogens in milk from clinically healthy cows. *Vet Med.* (2013) 58:567–75. doi: 10.17221/7138-VETMED
9. McCarron JLL, Keefe GPP, McKenna SLL, Dohoo IRR, Poole DEE. Evaluation of the University of Minnesota Tri-plate and 3M Petrifilm for the isolation of *Staphylococcus aureus* and *Streptococcus species* from clinically mastitic milk samples. *J Dairy Sci.* (2009) 92:5326–33. doi: 10.3168/jds.2009-2333
10. Lago A, Godden SMM, Bey R, Ruegg PLL, Leslie K. The selective treatment of clinical mastitis based on on-farm culture results: II. Effects on lactation performance, including clinical mastitis recurrence, somatic cell count, milk production, and cow survival. *J Dairy Sci.* (2011) 94:4457–67. doi: 10.3920/978-90-8686-742-4_47
11. Perry JD. A decade of development of chromogenic culture media for clinical microbiology in an era of molecular diagnostics. *Clin Microbiol Rev.* (2017) 30:449–79. doi: 10.1128/CMR.00097-16
12. Ferreira JC, Gomes MS, Bonsaglia ECR, Canisso IF, Garrett EF, Stewart JL, et al. Comparative analysis of four commercial on-farm culture methods to identify bacteria associated with clinical mastitis in dairy cattle. *PLoS ONE.* (2018) 13:1–15. doi: 10.1371/journal.pone.0194211
13. Griffioen K, Velthuis AGJ, Lagerwerf LA, Heuvelink AE, Lam TJGM. Agreement between four commercial diagnostic tests and routine bacteriological culture of milk to determine the udder infection status of dairy cows. *Prev Vet Med.* (2018) 157:162–73. doi: 10.1016/j.prevetmed.2018.07.003
14. Ganda EK, Bisinotto RS, Decter DH, Bicalho RC. Evaluation of an on-farm culture system (Accumast) for fast identification of milk pathogens associated with clinical mastitis in dairy cows. *PLoS ONE.* (2016) 11:1–16. doi: 10.1371/journal.pone.0155314
15. Srednik ME, Crespi E, Testorelli MF, Puigdevall T, Pereyra AMD, Rumi MV, et al. First isolation of a methicillin-resistant *Staphylococcus aureus* from bovine mastitis in Argentina. *Vet Anim Sci.* (2019) 7:100043. doi: 10.1016/j.vas.2018.11.004
16. Morrill KM, Polo J, Lago A, Campbell J, Quigley J, Tyler H. Estimate of serum immunoglobulin G concentration using refractometry with or without caprylic acid fractionation. *J Dairy Sci.* (2013) 96:4535–41. doi: 10.3168/jds.2012-5843
17. Adkins PRF, Middleton JR, Fox LK, Pighetti G, Petersson-Wolfe C, (U.S.) NMC. *National Mastitis Council (NMC). Laboratory Handbook on Bovine Mastitis*. New Prague, MN: National Mastitis Council Inc. (2017).
18. Parker KI, Compton CWR, Anniss FM, Heuer C, McDougall S. Quarter-level analysis of subclinical and clinical mastitis in primiparous heifers following the use of a teat sealant or an injectable antibiotic, or both, precalving. *J Dairy Sci.* (2008) 91:169–81. doi: 10.3168/jds.2007-0212
19. Barcelos MM, Martins L, Grenfell RC, Juliano L, Anderson KL, dos Santos MV, et al. Comparison of standard and on-plate extraction protocols for identification of mastitis-causing bacteria by MALDI-TOF MS. *Brazilian J Microbiol.* (2019) 50:849–57. doi: 10.1007/s42770-019-00110-5
20. Royster E, Godden S, Goulart D, Dahlke A, Rapnicki P, Timmerman J. Evaluation of the minnesota easy culture system ii bi-plate and tri-plate for identification of common mastitis pathogens in milk. *J Dairy Sci.* (2014) 97:3648–59. doi: 10.3168/jds.2013-7748
21. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics.* (1977) 33:159. doi: 10.2307/2529310
22. Viora L, Graham EM, Mellor DJ, Reynolds K, Simoes PBA, Geraghty TE. Evaluation of a culture-based pathogen identification kit for bacterial causes of bovine mastitis. *Vet Rec.* (2014) 175:89. doi: 10.1136/vr.102499
23. Reyher KK, Dohoo IR. Diagnosing intramammary infections: evaluation of composite milk samples to detect intramammary infections. *J Dairy Sci.* (2011) 94:3387–96. doi: 10.3168/jds.2010-3907
24. Misra N, Pu X, Holt DN, Mcguire MA, Tinker JK. Immunoproteomics to identify *Staphylococcus aureus* antigens expressed in bovine milk during mastitis. *J Dairy Sci.* (2018) 101:6296–309. doi: 10.3168/jds.2017-14040
25. Saini V, McClure JT, Léger D, Keefe GP, Scholl DT, Morck DW, et al. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. *J Dairy Sci.* (2012) 95:4319–32. doi: 10.3168/jds.2012-5373
26. Singhal N, Kumar M, Kanaujia PK, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front Microbiol.* (2015) 6:791. doi: 10.3389/fmicb.2015.00791

27. Nonnemann B, Lyhs U, Svennesen L, Kristensen KA, Klaas IC, Pedersen K. Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. *J Dairy Sci.* (2019) 102:2515–24. doi: 10.3168/jds.2018-15424

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REVIEW
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# Biofilm Research in Bovine Mastitis

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Bovine mastitis is one of the most important diseases in the dairy industry and has detrimental impact on the economy and welfare of the animals. Further, treatment failure results in increased antibiotic use in the dairy industry, as some of these mastitis cases for unknown reasons are not resolved despite standard antibiotic treatment. Chronic biofilm infections are notoriously known to be difficult to eradicate with antibiotics and biofilm formation could be a possible explanation for mastitis cases that are not resolved by standard treatment. This paper reviews the current literature on biofilm in bovine mastitis research to evaluate the status and methods used in the literature. Focus of the current research has been on isolates from milk samples and investigation of their biofilm forming properties *in vitro*. However, *in vitro* observations of biofilm formation are not easily comparable with the *in vivo* situation inside the udder. Only two papers investigate the location and distribution of bacterial biofilms inside udders of dairy cows with mastitis. Based on the current knowledge, the role of biofilm in bovine mastitis is still unclear and more *in vivo* investigations are needed to uncover the actual role of biofilm formation in the pathogenesis of bovine mastitis.

**Keywords: biofilm, bovine mastitis, chronic infections, antimicrobial resistance, antimicrobial therapy**

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## INTRODUCTION

Bovine mastitis is an important disease in the dairy industry with severe consequences for the welfare of dairy cows and the economy of the industry (1). Antibiotic treatment of bovine mastitis account for the highest antibiotic use in the dairy industry (2).

Bovine mastitis is defined as inflammation of the mammary gland and is most commonly caused by bacterial infection (3). Bovine mastitis occurs in two different clinical manifestations; subclinical and clinical mastitis, and ranges from mild, moderate to severe cases. Subclinical mastitis can be diagnosed by tests, e.g., the somatic cell count in milk, however, no clinical signs are apparent (4). Clinical mastitis manifests with visible changes to the milk in the form of clots or flakes and clinical signs of infection and inflammation, such as fever, redness, pain, and swelling of udder and lymph nodes (4). Some cases of bovine mastitis resolve themselves and most cases resolve after standard antibiotic treatment (2), however, some cases can progress to a detrimental point where the cow is culled, and in severe cases, spontaneous death may even occur (1, 4).

The most common infectious agents of bovine mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae* (2), *Escherichia coli* (5), and *Streptococcus uberis* (6). *S. aureus* is a common and challenging mastitis pathogen, as *S. aureus* has a high persistence rate (7, 8) and a low bacteriological cure rate in clinical mastitis cases (9). During bovine mastitis, bacteria potentially upregulate expression of virulence factors that can lead to higher resistance to phagocytosis (10)

and upregulation of genes that destruct host tissue and the ability of the host cells to capture iron, e.g., lactoferrin (11). The pathogens are adapted to infection of the tissue in the mammary gland by a broad variety of virulence factors, e.g., the propensity to invade and escape host cell defenses by hemolysins (12), adhesion to host cells and production of leukotoxins to destroy monocytes and polymorphonuclear cells (12). Further, some of the pathogens are low shedders (13) and some form biofilm (12). All resulting in pathogens capable of causing long-lasting infections.

Bovine mastitis is normally treated with antibiotics, however, in some cases, the antibiotics are not resolving the disease and the infection becomes chronic. Continued antibiotic treatment in these cases where antibiotics do not eradicate the microbial agents increases the risk of developing antibiotic resistance, which is one of the greatest threats to human and animal health (14).

Chronic and recurrent cases of bovine mastitis share similar characteristics with chronic biofilm infections observed in humans and other animals. Biofilm is defined as "a coherent cluster of bacterial cells imbedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense" (15), however the role of matrix is unclear *in vivo* (16). Biofilm is suggested to be the default mode of growth for bacteria (17). Antibiotic treatment of biofilm infections is often unsuccessful and thus the infections are difficult to eradicate (18, 19). Being part of a biofilm can provide protection for the bacteria against threats from the environment, including antibiotics and host defenses (20).

The role of biofilm in human infections has been an expanding research field since bacterial aggregates were observed in 1977 in the lungs of patients with cystic fibrosis (21), and since 1982 where the first report on a medical biofilm causing recurrent infection (bacteremia) was described (22). However, in veterinary medicine, few reports exist on biofilms' direct role in infections, as most literature focuses on *in vitro* characteristics of pathogenic bacteria/biofilms and not their role *in vivo*.

In human medicine, biofilms are known to contribute to a wide variety of infections and diseases including wound infections, implant related infections, lung infections, osteomyelitis, chronic otitis media, urinary tract infections, chronic sinusitis, dental plaque, endocarditis, etc. (23). *Pseudomonas aeruginosa* biofilm infection in cystic fibrosis patients is one of the most well-studied biofilm infections to date, and intense research has revealed both pathogenetic, diagnostic, and therapeutic breakthroughs, and has increased the life expectancy of these patients dramatically (24–26). Biofilms are found in the majority of human chronic wounds and are considered to play a consistent role in the pathogenesis of impaired wound healing (27–29). Major biofilm pathogens in chronic wounds are *S. aureus*, *P. aeruginosa*, and *Enterobacteriaceae* (27, 30, 31). Implant related infections are often also driven by biofilms that cause low grade, difficult to detect infections with delayed onset (32, 33). Biofilm infections are thus important and are estimated to account for 550,000 deaths and 17 million infections yearly in the USA (34).

Understanding the role of biofilm in bovine mastitis will potentially unlock new treatment options and avoid unnecessary antibiotic treatment. If thereby being able to cure these chronic and recurrent bovine mastitis cases, the economy of the dairy industry, as well as animal welfare will improve and use of antibiotics will decrease.

In this paper, we review the literature on the development of bovine mastitis biofilm research with focus on the last two decades. In addition, we review the methods applied in published research and propose new methods for future research of biofilms' role in bovine mastitis.

## DEVELOPMENT OF RESEARCH OF BOVINE MASTITIS

The first studies investigating biofilm forming abilities of bovine mastitis pathogens emerged in the early 1990s. In 1993, "slime production" (exopolysaccharide matrix) was observed in bovine coagulase-negative staphylococci (CNS) strains. This slime production was observed *in vitro* by using the tube method together with Congo Red Agar plates and suggested that the slime-production functioned as a virulence factor (35). Later, strains of *S. aureus* isolated from bovine mastitis cases were found to bind to milk fat globules. This suggested that the bacteria were in a biofilm mode of growth *in vitro* (36). During the first decade of 2000, most papers concentrated on investigating the *in vitro* biofilm forming abilities of *S. aureus* and *Staphylococcus epidermidis* isolates from bovine mastitis cases (37–39), the genes that were associated with biofilm formation (39–41), the susceptibility to antimicrobial agents (42), and potential treatment options against biofilm infections (43–45).

After 2010, the research of biofilm in bovine mastitis accelerated, and during the last decade, over 170 studies have been published. The focus of the research is still the *in vitro* biofilm forming abilities of bovine mastitis pathogens but also investigations of antibiotic resistance, molecular investigations of biofilm related genes, and the search for potential treatments and vaccines; the majority of these paper have focused on *S. aureus.* In only two *in vivo* studies, bacterial biofilms have been directly identified in bovine udders with mastitis (46, 47).

## BIOFILM METHODS APPLIED TO THE RESEARCH OF BIOFILM IN BOVINE MASTITIS

### Methods Used for Investigation of Biofilm Forming Abilities of Bovine Mastitis Pathogens

The biofilm forming abilities of bovine mastitis pathogens *in vitro* have been investigated by multiple traditional biofilm methods. Most studies have focused on bacterial isolates from milk samples of bovine mastitis cases and the main focus has been on *S. aureus*, a well-known *in vitro* biofilm producer (48) and one of the most common pathogens in chronic bovine mastitis (49). The majority of the studies, i.e., more than 140 papers, have been using microtiter plates with crystal violet staining for

quantification of the bacterial biomass. When using this biofilm assay, the bacteria are grown in polystyrene microtiter plates. The wells are emptied and washed at different time points, whereby the remaining biofilm biomass can be stained and quantified with crystal violet (50, 51). The crystal violet stain is used to quantify the total biomass in these system, as the stain binds to negatively charged molecules, which means to both the bacteria and exopolysaccharides (50). *S. aureus* is the most common species investigated using the microtiter assay in biofilm and bovine mastitis research to investigate its ability to form biofilm *in vitro*. Multiple studies found that majorities of *S. aureus* isolates from bovine mastitis cases can form biofilm *in vitro* by this assay (52–54). Applying the same method, 20–30% of *S. agalactiae* mastitis isolates also showed biofilm forming abilities *in vitro* when cultivated in different atmospheric conditions and growth media (55–57). This assay was also used to investigate the biofilm forming abilities of 53 mastitis isolates of *Klebsiella* spp. and 17 *Pseudomonas aeruginosa* mastitis isolates, all isolates were able to form biofilm (58, 59).

Although not as common as the crystal violet assay, several studies use the Congo Red Agar (CRA) test. The CRA method was developed by Freeman et al. (60) in 1989 for "detecting the production of slime by coagulase-negative staphylococci." The "slime-forming" strains are black and the strains not capable of forming slime appear red on the agar (60). The CRA test is a qualitative method to estimate whether staphylococci isolates are able to produce biofilm *in vitro* and is often followed by a quantitative assay—such as the tube method or the microtiter assay. Half of *S. aureus* isolates from dairy cows with subclinical mastitis were able to produce biofilm by the CRA method (61, 62).

In the standard tube method, bacteria are cultivated in culture tubes, washed and then stained with crystal violet, safranine, or other stains. Biofilm production is observed by color on the sides and bottom of the tube (63). When the biofilm forming ability of *S. aureus* isolates from bovine mastitis cases was investigated by the tube method using safranine stain, 25–70% of the isolates were able to form biofilm (61, 64, 65).

Using yet another staining method, ∼85% of CNS isolates from mastitis milk samples were able to form biofilm when their biofilm forming ability was investigated by the microtiter assay and stained using the LIVE/DEAD technique with subsequent confocal laser scanning microscopy (CLSM) to study the composition of the matrix (66). Confocal laser scanning microscopy is widely used in the visualization of medical biofilm, as some of the advantages of this technique are the possibility to visualize 3D and spatial structures of biofilms (51). Furthermore, it is possible to quantify volume and other parameters of the biofilm and to apply different fluorescent probes (51).

Quantitative and qualitative assays for investigating the biofilm forming abilities of bovine mastitis pathogens *in vitro* are inexpensive, fairly simple and fast. In the last years, microscope techniques have become more accessible and would facilitate more detailed investigations of the biofilm phenotype and interactions between antimicrobial compounds and biofilms.

## Investigations of Antimicrobial Compounds Against Biofilm Forming Mastitis Pathogens

Different antimicrobial compounds and antibiotics have been tested on bovine mastitis isolates' ability to form biofilm. The biofilm forming ability of *E. coli* in the presence of different antibiotics was investigated using CLSM and revealed increased adhesion of the isolates (67) and a greater biofilm formation of *E. coli* bovine mastitis isolates in the presence of enrofloxacin (68). When grown as biofilms, *S. aureus* bovine mastitis isolates are highly resistant to antimicrobial agents (42). The antibacterial use of the traditional medicinal plant *Plectranthus ornatus* (spur flowers) used in Brazil for treatment of skin infections was investigated for its anti-biofilm properties by using the plant as a herbal soap on gloves contaminated with *S. aureus* from dairy cows with bovine mastitis. There was no microbial growth after the gloves were submerged in the herbal soap and when the biofilm inhibitory concentration by microtiter plates and crystal violet staining was investigated, the plant was able to inhibit biofilm formation (69). Anti-biofilm agents against *S. aureus* have also been investigated *in vivo*. Ethanolic extracts from the leaves of *Rhodomyrtus tomentosa* (*rose myrtle*) were investigated as a possible antimicrobial agent against biofilm producing *S. aureus* in combination with the antibiotic pirlimycin. When extracts were used alone, there was no significant reduction in the bacterial load in a murine mastitis model. In combination with the antibiotic, a significant antibacterial effect was observed, but there was no significant difference between the antibiotic used alone compared with the combination of antibiotics and extract (70). The possible inhibitory effect of the Argentinian medicinal plant *Minthostachys verticillata* was tested on *Escherichia coli*, *Bacillus pumilus*, and *Enterococcus faecium* isolated from mastitis milk. The essential oil of the plant had inhibitory effect on the production of biofilm of all isolates in 96 well-microtiter plates (71). The naturally occurring signaling molecule of bacteria, cyclic dinucleotide 3′,5′-cyclic diguanylic acid (c-di-GMP), has been investigated to inhibit biofilm formation of *S. aureus*, and a decrease in the colonization of the pathogen in the mammary glands was shown in a murine mastitis model (44). The alternative drug, 1-hydroxyanthraquinone, was found to have a significant inhibitory action against *Staphylococcus xylosus in vitro* as well as a reduction in inflammation in the mammary glands of murine models (72).

## Biofilm-Associated Genes in Bovine Mastitis Pathogens

The molecular identification of pathogens is another direction in the research of biofilm in bovine mastitis and several studies have investigated different biofilm-associated genes of bovine mastitis isolates. The intercellular gene cluster adhesion operon (*ica*) is one of the genes that has been investigated for its role in biofilm formation and has been found in 40% of *S. aureus* isolates from bovine mastitis by analyzing their biofilm forming abilities within the microtiter assay and then sequencing the isolates (73). However, whether the isolates carrying the *ica* genes actually produce biofilm *in vitro*, depends on the biofilm assay. Some

studies found that even if the isolates carried the *ica* genes, not all of the isolates produced biofilm in the microtiter plate (74) and that some isolates would form black colonies (indicating slime-formation) when grown on CRA plates but not necessarily form biofilm in the microtiter assay (41). Biofilm-associated proteins (bap) has been researched by several bovine mastitis studies and *S. aureus* isolates have been investigated for the presence of *bap* genes and their biofilm forming ability (40). A study found that over 90% of isolates carried *icaADBC* genes and of these 25% carried the *bap* genes. When the isolates were positive for both *icaADBC* and *bap*, they were strong biofilm producers *in vitro*, however, when only positive for *icaADBC*, they produced less biofilm. The role of *bap* was investigated by constructing a mutant only positive for *bap* and found that the mutant had the same biofilm forming capacity as the wild type (40). However, in other studies, the *bap* gene was not found at all in *S. aureus* isolates from bovine mastitis cases (39, 74).

# OTHER TOPICS ADDRESSED IN THE RESEARCH OF BIOFILM IN BOVINE MASTITIS

## Multispecies Biofilm

The research of bovine mastitis and biofilm often focuses on one specific pathogen and its ability to form biofilm *in vitro*. When only single species are investigated, there is a risk of overseeing keystone species (75) or possible interactions between commensals and pathogens or amongst pathogens, which might be important in the understanding of biofilms' role in bovine mastitis. However, the majority of studies investigating the role of biofilm in bovine mastitis focuses solely on *S. aureus*. In the environment, there is often more than one bacterial strain present and multispecies biofilms are commonly observed (76). Bovine mastitis infections can have multiple bacterial agents (9) and it is also important to consider the possible role of commensal bacteria in udders. Lactic acid bacteria (LAB) are commonly isolated from the teat canals and milk of dairy cows (77). Wallis et al. investigated the effect of growing two probiotic LAB strains together with a challenge between biofilm of probiotic LAB and *S. aureus* biofilm. They observed that when two LAB strains were co-cultured with *S. aureus*, it resulted in no growth of *S. aureus*, suggesting the beneficial use of probiotic bacteria against pathogenic biofilms in bovine mastitis (78). The presence of specific bacteria can either promote or decrease growth of other bacteria (79, 80) and the competition between these bacteria can cause damage to the surrounding environment or tissue (81). Immune responses toward bacteria and biofilm may similarly cause collateral damage to the surrounding tissue (82). Therefore, it is important to consider possible interactions between other bacteria and bovine mastitis pathogens as well as between pathogens and the immune response.

## Potential Vaccines Against Biofilm Forming Mastitis Pathogens

Two mastitis vaccine candidates against *S. uberis* have shown a significant reduction in the mortality of mice infected with the pathogen (83). Different candidates for a *S. aureus* vaccine is currently being investigated; in one study, live-attenuated small-colony variants have shown promising results compared to inactivated bacteria in murine models (84). However, in another study, a formalin-killed whole-cell vaccine candidate of *S. aureus* biofilm showed a significant reduction in the colonization of *S. aureus* in the udder in vaccinated mice compared to mice vaccinated with a vaccine candidate from planktonic *S. aureus* (85). A killed bacterin vaccine candidate against *S. aureus* was tested in primiparous gestating cows. There were no observations of any prevention of intramammary infection by *S. aureus* but a reduced multiplication of *S. aureus* in the mammary glands was observed (86). *S. aureus*' protein A has also been investigated as a possible vaccine target and a vaccine candidate has shown a significant reduction in bacterial load of the mammary glands of pregnant mice. However, the immunized mice were not protected when they subsequently were infected with biofilm producing encapsulated *S. aureus* (87). Currently, two mastitis vaccines are available on the market against *S. aureus* and *S. uberis* from the company HIPRA (Amer, Spain).

## *In vivo* Investigations of Biofilm in Bovine Mastitis

Most of the so-called *in vivo* investigations of biofilm in bovine mastitis have used experimental models (mice and sheep), and the majority of these studies focused on anti-biofilm treatment or vaccines against biofilm udder infections (**Table 1**) (44, 69, 70, 72, 83–85). Only a few studies investigated and confirmed biofilm *in vivo* within udder tissue of dairy cows with bovine mastitis. Two studies directly detected biofilm inside udders of dairy cows with mastitis. Clustering of *S. aureus* bacteria in udders of dairy cows with bovine mastitis were observed by microscopy to be located in the lumen of the alveoli and lactiferous ducts of the udders of experimentally infected dairy cows (47). In another study, the presence of biofilm was investigated directly in the udders of dairy cows by collecting swabs from the udders of slaughtered dairy cows with *S. aureus* infection. Swabs were obtained from the teat cistern, gland cistern, and parenchyma and were subsequently stained using immunofluorescence staining of polysaccharide intercellular adhesions (PIA), which is a component of the *S. aureus* biofilm matrix. The samples were investigated by fluorescence microscopy and PIA was found in 71 out of 184 swabs (46).

## The Bovine Mammary Immune Response to Biofilm Infection

The response to infections is crucial for the survival of mammals. The response mechanisms to bacterial and viral infections are widely investigated, however much less is known about the immune response toward biofilm. As per definition, host immune responses are tolerated by biofilms, and no specific anti-biofilm immune responses have been identified (82).

The protection against infectious agents in the bovine mammary gland has been recently reviewed by Sordillo (93). As for biofilm infections in general, the mammary gland response toward bacterial biofilms is not fully understood yet, and as

**TABLE 1 |** Studies investigating biofilm forming bovine mastitis pathogens in animal models or the effect of possible antibacterial agents and vaccines in animal models.

| References | Year | Sample type | Focus of study | Pathogen | Experimental animal |
|---|---|---|---|---|---|
| Cucarella et al. (88) | 2001 | Bovine subclinical mastitis and human isolates | Molecular basis of biofilm | *S. aureus* | Mice |
| Brouillette et al. (44) | 2005 | Clinical bovine mastitis isolates | Antibacterial treatment | *S. aureus* | Mice |
| Gogoi-Tiwari et al. (85) | 2015 | Bovine mastitis isolates | Vaccine | *S. aureus* | Mice |
| Collado et al. (83) | 2016 | Clinical bovine mastitis isolates | Vaccine | *S. uberis* | Mice |
| Gogoi-Tiwari et al. (87) | 2016 | Bovine mastitis isolates | Vaccine | *S. aureus* | Mice |
| Mordmuang et al. (70) | 2019 | Bovine mastitis isolates | Antibacterial treatment | *S. aureus* | Mice |
| Montironi et al. (89) | 2019 | Subclinical bovine mastitis isolates, milk samples | Investigation of phenotype, genotype and virulence | *Enterococcus faecium* | Mice |
| Côté-Gravel et al. (84) | 2019 | Bovine mastitis isolates | Vaccine | *S. aureus* | Mice |
| Marbach et al. (90) | 2019 | Subclinical bovine mastitis isolates, milk samples | Interactions between host and bacteria | *S. aureus* | Mice |
| Wang et al. (72) | 2020 | Isolates (Purchased strains) | Antibacterial treatment | *S. xylosus* | Mice |
| Prenafeta et al. (86) | 2010 | Ruminant mastitis isolates | Vaccine | *S. aureus* | Heifers, cows |
| Savijoki et al. (91) | 2014 | Bovine mastitis isolates | Genomics and proteomics | *S. epidermidis* | Cows |
| Seroussi et al. (92) | 2018 | Bovine mastitis isolates | Antibacterial treatment | *E. coli*, *S. aureus* | Cows |
| Cucarella et al. (40) | 2004 | Bovine subclinical mastitis isolates | Molecular basis of biofilm | *S. aureus* | Sheep |

described above very few *in vivo* investigations of mammary biofilm infections exist. Some studies have investigated the response of mammary cells to biofilm-producing strains of known mastitis-causing pathogens *in vitro*. The ability of *S. aureus* biofilm forming strains to adhere and invade the mammary cells is especially investigated. A study found that *S. aureus* biofilm showed lower invasion ability into mammary epithelial cells compared to planktonic *S. aureus* cultures and that the biofilm culture induced less cellular activation than the planktonic cultures. Both planktonic culture and *S. aureus* biofilm culture induced expression of interleukin 6 by mammary alveolar cells, which could be an anti-inflammatory response (94). This corresponds well to human research of biofilm infections and immune response, where biofilms do not trigger any specific immune responses (82) and downregulates specific virulence genes when the cell density is low to "fly under the radar" so the immune system does not detect the bacteria. Whenever the cell density is high enough, the bacteria can upregulate the virulence factors (95). However, other *in vitro* studies found no difference in the ability to invade host cells by non-biofilm producing mastitis strains compared to biofilm producing mastitis strains (96, 97). The role of the Bap protein expressed by *S. aureus* has been investigated in a lactating mouse model, where the surface protein Bap, involved in biofilm matrix, adhered to epithelial cells and bound to host receptor Gp96. The bacteria expressing the surface protein Bap did not invade the cells and had increased persistence in the mammary glands of the lactating mice, indicating that the protein promotes adhesion to the cells and limits invasion of the host cells (98).

The main question still not resolved is how the biofilms go undetected and survive the immune response (99) and more research is needed to answer that question both for mastitis and for all other biofilm infections. The current research is based on *in vitro* experiments and as discussed earlier in this review, more *in vivo* research is needed to fully understand the role of biofilm in mastitis.

## DISCUSSION

The role of biofilm in the pathogenesis of bovine mastitis infection is still unclear. To the authors' best knowledge, only two papers investigate and detect the presence of biofilms inside udders from dairy cows with mastitis (46, 47). Plenty of *in vitro* studies investigate the biofilm forming abilities of mastitis pathogens isolated from milk samples. Similar to swabs and wound fluid samples, analyzing milk samples is a great, easy and quick way to investigate and culture the bacteria present in the samples and to determine, e.g., the genetic composition and antibiotic susceptibility. However, disadvantages are the risk of contamination from the environment and that it is only possible to detect bacteria present in or released into the milk, however bacteria embedded in the tissue, encapsulated bacteria, low-shedding bacteria, and potential biofilms might not be detectable in milk samples (100). Even if bacteria are isolated from milk samples and are able to form biofilm *in vitro*, this does not provide any information on the bacteria's phenotype *in vivo* in the infected udder. *In vitro* biofilms of *P. aeruginosa* have a markedly different genetic expression profile than *in vivo* biofilms during human infections (101). This is due to, e.g., the environment in the host tissue, interactions with the immune system, and antibiotic treatment that are impossible to fully mimic *in vitro*. Further, major physical differences exist between *in vitro*

and *in vivo* biofilms; for example, *in vitro* biofilms normally form large mushroom-shaped structures, which are never observed *in vivo*, where the biofilms are markedly smaller in size (16).

Hence to find the actual role of biofilm in bovine mastitis, the approach needs to change from *in vitro* to *in vivo* investigations of biofilms in infected udders. When diagnosing biofilm infections in human medicine, the gold standard is to directly visualize the biofilms and concurrent immune response in the tissue. This can be done with, e.g., CLSM or scanning electron microscopy (15, 18, 102). Sample collection for biofilm diagnosis naturally varies for different diseases/tissue, e.g., from cystic fibrosis patients, expectorated sputum samples, bronchoalveolar lavage, or biopsies from removed lung tissues during lung transplantation can be collected (18, 102), and from chronic wounds, biopsies or debrided tissue can be investigated (103). Especially in wound infections, the spatial distribution of different bacterial biofilms within the tissue can be observed using microscopic examination; this method has further found the pathogen *P. aeruginosa* to be underestimated when performing culture of standard wound swabs (104, 105). A good technique to detect bacterial biofilms in tissue is peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH), with probes that hybridize to bacterial ribosomal RNA, which can subsequently be detected using CLSM. This is a sensitive method that is well-established in the research of biofilm infections in humans (102, 104, 106–108). This method would be applicable to udder tissue samples as well.

If biofilms are found present in mastitis udders, e.g., by use of the methods just described, the next question is whether the biofilms are part of the pathogenesis of bovine mastitis? Therefore, the immune response to the biofilms is also important to investigate. The cytological cure of mastitis is delayed compared to the bacteriological cure, meaning that when the infection appears cleared, the inflammation can continue in the udder (9). The cytological cure rate can be as low as around 20% and therefore it should be considered that chronic mastitis cases could be due to long-lasting inflammation, potentially driven by biofilms, after the apparent bacterial cure (9). The treatment of especially *S. aureus* mastitis cases is difficult, and therefore the connection between these cases and *S. aureus* biofilm presence and many virulence factors that are upregulated during mastitis infections should be further investigated (10, 11). We propose that udder cell and tissue models could potentially be applied to investigate how biofilms affect bovine udder tissue, however, studies of natural or experimentally induced mastitis will provide more information as a competent immune system would respond to the infection.

Collecting udder tissue biopsies from live dairy cows with mastitis for microscopy is difficult, if not impossible. However, biopsies can easily be obtained after euthanasia and by applying relevant staining and microscopy techniques, a more accurate view of biofilms' potential location and distribution as well as the related host immune response during mastitis can be revealed. Only a few papers provide information on biofilms' presence in udders from dairy cows with mastitis (46, 47) and more research is needed to elucidate biofilms' role in mastitis pathogenesis. Therefore, the collection of biopsies from euthanized animals might not have any direct clinical relevance, as the animals would be dead, but has important scientific relevance to better understand the disease and relate this to findings in milk samples. If biofilms play a role in bovine mastitis, diagnostic methods to detect biofilm in milk samples could be a possible way to easily diagnose the biofilms. However, for now, no such biofilm marker, specific biofilm product, or specific biofilm immune response have been identified that would be usable for quick and simple biofilm diagnostics neither in human or veterinary medicine. This is naturally the topic and aim of many human research groups' intense work, as biofilm infections play an important part of many human infections, and whenever found this would hopefully also be applicable to milk samples from bovine mastitis. By understanding the bacteria and biofilms including their interactions with the host immune system during mastitis infections, potentially new possible diagnostic methods could be developed as well as new optimized treatment options.

## CONCLUSIONS

Bovine mastitis is one of the most important diseases in the dairy industry and a better understanding of the role of biofilm in the disease is of high importance to achieve more successful treatments. Chronic biofilm infections are recognized as serious and difficult-to-treat diseases in human medicine. The majority of the research on biofilm and bovine mastitis has so far focused on *in vitro* studies; however, to uncover the presence of biofilm in udders of dairy cows suffering from mastitis, direct methods need to be applied. Some of the methods used in the diagnosis and research of biofilms in human infections could be applied to the research of biofilm in bovine mastitis. There is a need for *in vivo* research where the location and distribution of biofilms are investigated directly in the udder of dairy cows with mastitis and where these findings are related to findings in milk samples. The continuous unsuccessful antibiotic treatment of potential biofilm mastitis infections can increase the risk of antibiotic resistance, which is one of the biggest threats to human and animal health. The role of biofilm infections in bovine mastitis therefore seems a key to unlock the required knowledge to develop new diagnostic methods and treat the persistent and chronic cases of bovine mastitis.

## METHODS

This review has included studies that examine biofilm in relation to mastitis in dairy cows. We have included studies investigating biofilm abilities, molecular properties, treatment options, prevention and interactions of bovine mastitis related pathogens. Studies published since 2000 were included. Reviews and manuscripts in other languages than English have not been included in this review.

The literature search was carried out using the database Pubmed on October 1st 2020 with the search words "bovine mastitis + biofilm." Over 170 papers investigated the role of

biofilm in bovine mastitis by *in vitro* methods and 16 papers used *in vivo* methods.

## AUTHOR CONTRIBUTIONS

RP: original draft preparation, methodology, and writing. EJ: writing, editing, and supervision. VK, TB, KD-P, and RB: editing and supervision. All authors have read the manuscript and agreed to the published version.

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## REFERENCES


1. Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R. Mastitis detection: current trends and future perspectives. *Trends Biotechnol.* (2009) 27:486–93. doi: 10.1016/j.tibtech.2009.05.004
2. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023
3. Bradley A. Bovine mastitis: an evolving disease. *Vet J.* (2002) 164:116–28. doi: 10.1053/tvjl.2002.0724
4. Adkins PRF, Middleton JR. Methods for diagnosing mastitis. *Vet Clin North Am Food Anim Pract.* (2018) 34:479–91. doi: 10.1016/j.cvfa.2018.07.003
5. Suojala L, Kaartinen L, Pyörälä S. Treatment for bovine *Escherichia coli* mastitis - an evidence-based approach. *J Vet Pharmacol Ther.* (2013) 36:521–31. doi: 10.1111/jvp.12057
6. Oliver SP, Almeida RA, Gillespie BE, Headrick SJ, Dowlen HH, Johnson DL, et al. Extended ceftiofur therapy for treatment of experimentally-induced *Streptococcus uberis* mastitis in lactating dairy cattle. *J Dairy Sci.* (2004) 87:3322–9. doi: 10.3168/jds.S0022-0302(04)73468-2
7. Wente N, Grieger AS, Klocke D, Paduch J-H, Zhang Y, Leimbach S, et al. Recurrent mastitis-persistent or new infections? *Vet Microbiol.* (2020) 244:108682. doi: 10.1016/j.vetmic.2020.108682
8. Lundberg Å, Nyman A-K, Aspán A, Börjesson S, Unnerstad HE, Waller KP. Udder infections with *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at calving in dairy herds with suboptimal udder health. *J Dairy Sci.* (2016) 99:2102–17. doi: 10.3168/jds.2015-9487
9. Schmenger A, Krömker V. Characterization, cure rates and associated risks of clinical mastitis in Northern Germany. *Vet Sci.* (2020) 7:170. doi: 10.3390/vetsci7040170
10. Zecconi A, Cesaris L, Liandris E, Daprà V, Piccinini R. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. *Microb Pathog.* (2006) 40:177–83. doi: 10.1016/j.micpath.2006.01.001
11. Oogai Y, Matsuo M, Hashimoto M, Kato F, Sugai M, Komatsuzawa H. Expression of virulence factors by *Staphylococcus aureus* grown in serum. *Appl Environ Microbiol.* (2011) 77:8097–105. doi: 10.1128/AEM.05316-11
12. Pérez VKC, Custódio DAC, Silva EMM, de Oliveira J, Guimarães AS, Brito MAVP, et al. Virulence factors and antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Brazil. *Braz J Microbiol.* (2020) 51:2111–22. doi: 10.1007/s42770-020-00363-5
13. Hamel J, Zhang Y, Wente N, Krömker V. Non-*S. aureus* staphylococci (NAS) in milk samples: Infection or contamination? *Vet Microbiol.* (2020) 242:108594. doi: 10.1016/j.vetmic.2020.108594
14. Dhingra S, Rahman NAA, Peile E, Rahman M, Sartelli M, Hassali MA, et al. Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. *Front Public Health.* (2020) 8:535668. doi: 10.3389/fpubh.2020.535668
15. Burmølle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homøe P, et al. Biofilms in chronic infections - A matter of opportunity - Monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol.* (2010) 59:324–36. doi: 10.1111/j.1574-695X.2010.00714.x
16. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, et al. The *in vivo* biofilm. *Trends Microbiol.* (2013) 21:466–74. doi: 10.1016/j.tim.2013.06.002
17. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol.* (2016) 14:563–75. doi: 10.1038/nrmicro.2016.94
18. Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, et al. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clin Microbiol Infect.* (2015) 21:S1–S25. doi: 10.1016/j.cmi.2014.10.024
19. Høiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PØ, et al. The clinical impact of bacterial biofilms. *Int J Oral Sci.* (2011) 3:55–65. doi: 10.4248/IJOS11026
20. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol.* (2004) 2:95–108. doi: 10.1038/nrmicro821
21. Hoiby N. *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. A survey. *Acta Pathol Microbiol Scand Suppl.* (1977) 58:1–96.
22. Marrie TJ, Nelligan J, Costerton JW. A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. *Circulation.* (1982) 66:1339–41. doi: 10.1161/01.CIR.66.6.1339
23. Lebeaux D, Ghigo J-M. Management of biofilm-associated infections: what can we expect from recent research on biofilm lifestyles? *Med Sci (Paris).* (2012) 28:727–39. doi: 10.1051/medsci/2012288015
24. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. *BMC Pulm Med.* (2016) 16:174. doi: 10.1186/s12890-016-0339-5
25. Bjarnsholt T. The role of bacterial biofilms in chronic infections. *APMIS Suppl.* (2013) 136:1–51. doi: 10.1111/apm.12099
26. Stephenson AL, Tom M, Berthiaume Y, Singer LG, Aaron SD, Whitmore GA, et al. A contemporary survival analysis of individuals with cystic fibrosis: a cohort study. *Eur Respir J.* (2015) 45:670–9. doi: 10.1183/09031936.00119714
27. Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, et al. Why chronic wounds will not heal: a novel hypothesis. *Wound repair.* (2008) 16:2–10. doi: 10.1111/j.1524-475X.2007.00283.x
28. Malone M, Bjarnsholt T, McBain AJ, James GA, Stoodley P, Leaper D, et al. The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *J Wound Care.* (2017) 26:20–5. doi: 10.12968/jowc.2017.26.1.20
29. James GA, Swogger E, Wolcott R, Pulcini E deLancey, Secor P, Sestrich J, et al. Biofilms in chronic wounds. *Wound repair.* (2008) 16:37–44. doi: 10.1111/j.1524-475X.2007.00321.x
30. Gjødsbøl K, Christensen JJ, Karlsmark T, Jørgensen B, Klein BM, Krogfelt KA. Multiple bacterial species reside in chronic wounds: a longitudinal study. *Int Wound J.* (2006) 3:225–31. doi: 10.1111/j.1742-481X.2006.00159.x
31. Wolcott RD, Hanson JD, Rees EJ, Koenig LD, Phillips CD, Wolcott RA, et al. Analysis of the chronic wound microbiota of 2,963 patients by 16S rDNA pyrosequencing. *Wound Repair.* (2016) 24:163–74. doi: 10.1111/wrr.12370
32. Trampuz A, Zimmerli W. Diagnosis and treatment of implant-associated septic arthritis and osteomyelitis. *Curr Infect Dis Rep.* (2008) 10:394–403. doi: 10.1007/s11908-008-0064-1
33. Jakobsen TH, Eickhardt SR, Gheorghe AG, Stenqvist C, Sønderholm M, Stavnsberg C, et al. Implants induce a new niche for microbiomes. *APMIS.* (2018) 126:685–92. doi: 10.1111/apm.12862

34. Wolcott RD, Rhoads DD, Bennett ME, Wolcott BM, Gogokhia L, Costerton JW, et al. Chronic wounds and the medical biofilm paradigm. *J Wound Care.* (2010) 19:45–6, 48–50, 52–53. doi: 10.12968/jowc.2010.19.2.46966
35. Baselga R, Albizu I, De La Cruz M, Del Cacho E, Barberan M, Amorena B. Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. *Infect Immun.* (1993) 61:4857–62. doi: 10.1128/IAI.61.11.4857-4862.1993
36. Ali-Vehmas T, Westphalen P, Myllys V, Sandholm M. Binding of *Staphylococcus aureus* to milk fat globules increases resistance to penicillin-G. *J Dairy Res.* (1997) 64:253–60. doi: 10.1017/S0022029996002026
37. Fox LK, Zadoks RN, Gaskins CT. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. *Vet Microbiol.* (2005) 107:295–9. doi: 10.1016/j.vetmic.2005.02.005
38. Oliveira M, Nunes SF, Carneiro C, Bexiga R, Bernardo F, Vilela CL. Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol.* (2007) 124:187–91. doi: 10.1016/j.vetmic.2007.04.016
39. Melchior MB, van Osch MHJ, Graat RM, van Duijkeren E, Mevius DJ, Nielen M, et al. Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. *Vet Microbiol.* (2009) 137:83–9. doi: 10.1016/j.vetmic.2008.12.004
40. Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzón M, Peris C, et al. Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect Immun.* (2004) 72:2177–85. doi: 10.1128/IAI.72.4.2177-2185.2004
41. Vasudevan P, Nair MKM, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Vet Microbiol.* (2003) 92:179–85. doi: 10.1016/S0378-1135(02)00360-7
42. Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: a role in recurrent mastitis infections? *Vet J.* (2006) 171:398–407. doi: 10.1016/j.tvjl.2005.01.006
43. Arrizubieta MJ, Toledo-Arana A, Amorena B, Penadés JR, Lasa I. Calcium inhibits bap-dependent multicellular behavior in *Staphylococcus aureus*. *J Bacteriol.* (2004) 186:7490–8. doi: 10.1128/JB.186.22.7490-7498.2004
44. Brouillette E, Hyodo M, Hayakawa Y, Karaolis DKR, Malouin F. 3',5'-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. *Antimicrob Agents Chemother.* (2005) 49:3109–13. doi: 10.1128/AAC.49.8.3109-3113.2005
45. Karaolis DKR, Rashid MH, Chythanya R, Luo W, Hyodo M, Hayakawa Y. c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. *Antimicrob Agents Chemother.* (2005) 49:1029–38. doi: 10.1128/AAC.49.3.1029-1038.2005
46. Schönborn S, Krömker V. Detection of the biofilm component polysaccharide intercellular adhesin in Staphylococcus aureus infected cow udders. *Vet Microbiol.* (2016) 196:126–8. doi: 10.1016/j.vetmic.2016.10.023
47. Hensen SM, Pavičić MJAMP, Lohuis JACM, De Hoog JAM, Poutrel B. Location of *Staphylococcus aureus* within the experimentally infected Bovine Udder and the expression of capsular polysaccharide type 5 *in situ*. *J Dairy Sci.* (2000) 83:1966–75. doi: 10.3168/jds.S0022-0302(00)75073-9
48. Moormeier DE, Bayles KW. *Staphylococcus aureus* biofilm: a complex developmental organism. *Mol Microbiol.* (2017) 104:365–76. doi: 10.1111/mmi.13634
49. Rossi BF, Bonsaglia ECR, Castilho IG, Dantas STA, Salina A, Langoni H, et al. Genotyping of long term persistent *Staphylococcus aureus* in bovine subclinical mastitis. *Microb Pathog.* (2019) 132:45–50. doi: 10.1016/j.micpath.2019.04.031
50. Stiefel P, Rosenberg U, Schneider J, Mauerhofer S, Maniura-Weber K, Ren Q. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Appl Microbiol Biotechnol.* (2016) 100:4135–45. doi: 10.1007/s00253-016-7396-9
51. Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, et al. Critical review on biofilm methods. *Crit Rev Microbiol.* (2017) 43:313–51. doi: 10.1080/1040841X.2016.1208146
52. Darwish SF, Asfour HAE. Investigation of biofilm forming ability in *Staphylococci* causing bovine mastitis using phenotypic and genotypic assays. *Sci World J.* (2013) 2013:378492. doi: 10.1155/2013/378492
53. Notcovich S, DeNicolo G, Flint SH, Williamson NB, Gedye K, Grinberg A, et al. Biofilm-forming potential of *Staphylococcus aureus* isolated from clinical mastitis cases in New Zealand. *Vet Sci.* (2018) 5:8. doi: 10.3390/vetsci5010008
54. Salimena APS, Lange CC, Camussone C, Signorini M, Calvinho LF, Brito MAVP, et al. Genotypic and phenotypic detection of capsular polysaccharide and biofilm formation in *Staphylococcus aureus* isolated from bovine milk collected from Brazilian dairy farms. *Vet Res Commun.* (2016) 40:97–106. doi: 10.1007/s11259-016-9658-5
55. Bonsaglia ECR, Latosinski GS, Rossi RS, Rossi BF, Possebon FS, Pantoja JCF, et al. Biofilm production under different atmospheres and growth media by *Streptococcus agalactiae* isolated from milk of cows with subclinical mastitis. *Arch Microbiol.* (2020) 202:209–12. doi: 10.1007/s00203-019-01727-8
56. Pang M, Sun L, He T, Bao H, Zhang L, Zhou Y, et al. Molecular and virulence characterization of highly prevalent *Streptococcus agalactiae* circulated in bovine dairy herds. *Vet Res.* (2017) 48:65. doi: 10.1186/s13567-017-0461-2
57. Varhimo E, Varmanen P, Fallarero A, Skogman M, Pyörälä S, Iivanainen A, et al. Alpha- and β-casein components of host milk induce biofilm formation in the mastitis bacterium *Streptococcus uberis*. *Vet Microbiol.* (2011) 149:381–9. doi: 10.1016/j.vetmic.2010.11.010
58. Sela S, Hammer-Muntz O, Krifucks O, Pinto R, Weisblit L, Leitner G. Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* strains isolated from mastitis outbreaks in dairy herds. *J Dairy Res.* (2007) 74:425–9. doi: 10.1017/S0022029907002610
59. Massé J, Dufour S, Archambault M. Characterization of Klebsiella isolates obtained from clinical mastitis cases in dairy cattle. *J Dairy Sci.* (2020) 103:3392–400. doi: 10.3168/jds.2019-17324
60. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol.* (1989) 42:872–4. doi: 10.1136/jcp.42.8.872
61. Dhanawade NB, Kalorey DR, Srinivasan R, Barbuddhe SB, Kurkure N V. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Vet Res Commun.* (2010) 34:81–9. doi: 10.1007/s11259-009-9326-0
62. Castelani L, Pilon LE, Martins T, Pozzi CR, Arcaro JRP. Investigation of biofilm production and icaA and icaD genes in Staphylococcus aureus isolated from heifers and cows with mastitis. *Anim Sci J.* (2015) 86:340–4. doi: 10.1111/asj.12284
63. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun.* (1982) 37:318–26. doi: 10.1128/IAI.37.1.318-326.1982
64. Aslantaş Ö, Demir C. Investigation of the antibiotic resistance and biofilm-forming ability of *Staphylococcus aureus* from subclinical bovine mastitis cases. *J Dairy Sci.* (2016) 99:8607–13. doi: 10.3168/jds.2016-11310
65. Shah MS, Qureshi S, Kashoo Z, Farooq S, Wani SA, Hussain MI, et al. Methicillin resistance genes and *in vitro* biofilm formation among *Staphylococcus aureus* isolates from bovine mastitis in India. *Comp Immunol Microbiol Infect Dis.* (2019) 64:117–24. doi: 10.1016/j.cimid.2019.02.009
66. Tremblay YDN, Lamarche D, Chever P, Haine D, Messier S, Jacques M. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *J Dairy Sci.* (2013) 96:234–46. doi: 10.3168/jds.2012-5795
67. Silva VO, Soares LO, Silva Júnior A, Mantovani HC, Chang Y-F, Moreira MAS. Biofilm formation on biotic and abiotic surfaces in the presence of antimicrobials by Escherichia coli Isolates from cases of bovine mastitis. *Appl Environ Microbiol.* (2014) 80:6136–45. doi: 10.1128/AEM.01953-14
68. Costa M, Freitas I De. Increased production of biofilms by *Escherichia coli* in the presence of enrofloxacin. *Vet Microbiol.* (2012) 160:488–90. doi: 10.1016/j.vetmic.2012.05.036
69. Nascimento FR, Albuquerque KRS, Oliveira MR, Pizziolo VR, Brasileiro BG, Diaz G, et al. Antibiotic activity of *Plectranthus ornatus* Codd., a Traditional Medicinal Plant. *An Acad Bras Cienc.* (2017) 89:2461–9. doi: 10.1590/0001-3765201720170068
70. Mordmuang A, Brouillette E, Voravuthikunchai SP, Malouin F. Evaluation of a *Rhodomyrtus tomentosa* ethanolic extract for its therapeutic potential on *Staphylococcus aureus* infections using *in vitro* and *in vivo* models of mastitis. *Vet Res.* (2019) 50:49. doi: 10.1186/s13567-019-0664-9
71. Cerioli MF, Moliva MV, Cariddi LN, Reinoso EB. Effect of the essential oil of *Minthostachys verticillata* (Griseb.) epling and limonene on biofilm

production in pathogens causing bovine mastitis. *Front Vet Sci.* (2018) 5:146. doi: 10.3389/fvets.2018.00146
72. Wang J, Qu Q, Liu X, Cui W, Yu F, Chen X, Xing X, Zhou Y, Yang Y, Bello-Onaghise G, et al. 1-Hydroxyanthraquinone exhibited antibacterial activity by regulating glutamine synthetase of Staphylococcus xylosus as a virulence factor. *Biomed Pharmacother*. (2020) 123:109779. doi: 10.1016/j.biopha.2019.109779
73. Rumi MV, Huguet MJ, Bentancor AB, Gentilini ER. The icaA gene in staphylococci from bovine mastitis. *J Infect Dev Ctries.* (2013) 7:556–560. doi: 10.3855/jidc.2670
74. Szweda P, Schielmann M, Milewski S, Frankowska A, Jakubczak A. Biofilm production and presence of ica and bap genes in *Staphylococcus aureus* strains isolated from cows with mastitis in the eastern Poland. *Polish J Microbiol.* (2012) 61:65–9. doi: 10.33073/pjm-2012-009
75. Tan CH, Lee KWK, Burmølle M, Kjelleberg S, Rice SA. All together now: experimental multispecies biofilm model systems. *Environ Microbiol.* (2017) 19:42–53. doi: 10.1111/1462-2920.13594
76. Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ. Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* (2014) 22:84–91. doi: 10.1016/j.tim.2013.12.004
77. Frola ID, Pellegrino MS, Espeche MC, Giraudo JA, Nader-Macias ME, Bogni CI. Effects of intramammary inoculation of *Lactobacillus perolens* CRL1724 in lactating cows' udders. *J Dairy Res.* (2012) 79:84–92. doi: 10.1017/S0022029911000835
78. Wallis JK, Krömker V, Paduch J-H. Biofilm challenge: lactic acid bacteria isolated from bovine udders versus staphylococci. *Foods.* (2019) 8:79 doi: 10.3390/foods8020079
79. Ren X, Wang L, Chen W. Oxytropis glabra DC. Inhibits Biofilm Formation of *Staphylococcus epidermidis* by Down-regulating ica operon expression. *Curr Microbiol.* (2020) 77:1167–73. doi: 10.1007/s00284-019-01847-w
80. Wolcott R, Costerton JW, Raoult D, Cutler SJ. The polymicrobial nature of biofilm infection. *Clin Microbiol Infect.* (2013) 19:107–12. doi: 10.1111/j.1469-0691.2012.04001.x
81. Brown SP, Fredrik Inglis R, Taddei F. Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. *Evol Appl.* (2009) 2:32–9. doi: 10.1111/j.1752-4571.2008.00059.x
82. Jensen PØ, Givskov M, Bjarnsholt T, Moser C. The immune system vs. *Pseudomonas aeruginosa* biofilms. *FEMS Immunol Med Microbiol.* (2010) 59:292–305. doi: 10.1111/j.1574-695X.2010.00706.x
83. Collado R, Prenafeta A, González-González L, Pérez-Pons JA, Sitjà M. Probing vaccine antigens against bovine mastitis caused by *Streptococcus uberis*. *Vaccine.* (2016) 34:3848–54. doi: 10.1016/j.vaccine.2016.05.044
84. Côté-Gravel J, Brouillette E, Malouin F. Vaccination with a live-attenuated small-colony variant improves the humoral and cell-mediated responses against Staphylococcus aureus. *PLoS One.* (2019) 14:e0227109. doi: 10.1371/journal.pone.0227109
85. Gogoi-Tiwari J, Williams V, Waryah CB, Eto KY, Tau M, Costantino P, et al. Comparative studies of the immunogenicity and protective potential of biofilm vs planktonic *Staphylococcus aureus* vaccine against bovine mastitis using non-invasive mouse mastitis as a model system. *Biofouling.* (2015) 31:543–54. doi: 10.1080/08927014.2015.1074681
86. Prenafeta A, March R, Foix A, Casals I, Costa L. Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. *Vet Immunol Immunopathol.* (2010) 134:208–17. doi: 10.1016/j.vetimm.2009.09.020
87. Gogoi-Tiwari J, Williams V, Waryah CB, Mathavan S, Tiwari HK, Costantino P, et al. Intramammary immunization of pregnant mice with staphylococcal protein a reduces the post-challenge mammary gland bacterial load but not pathology. *PLoS One.* (2016) 11:e0148383. doi: 10.1371/journal.pone.0148383
88. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol.* (2001) 183:2888–96. doi: 10.1128/JB.183.9.2888-2896.2001
89. Montironi ID, Moliva M V, Campra NA, Raviolo JM, Bagnis G, Cariddi LN. Characterization of an *Enterococcus faecium* strain in a murine mastitis model. *J Appl Microbiol.* (2019) 128:1289–300. doi: 10.1111/jam.14554
90. Marbach H, Mayer K, Vogl C, Lee JYH, Monk IR, Sordelli DO, et al. Within-host evolution of bovine Staphylococcus aureus selects for a SigB-deficient pathotype characterized by reduced virulence but enhanced proteolytic activity and biofilm formation. *Sci Rep.* (2019) 9:13479. doi: 10.1038/s41598-019-49981-6
91. Savijoki K, Iivanainen A, Siljamäki P, Laine PK, Paulin L, Karonen T, et al. Genomics and proteomics provide new insight into the commensal and pathogenic lifestyles of bovine- and human-associated *Staphylococcus epidermidis* strains. *J Proteome Res.* (2014) 13:3748–62. doi: 10.1021/pr500322d
92. Seroussi E, Blum SE, Krifucks O, Lavon Y, Leitner G. Application of pancreatic phospholipase A2 for treatment of bovine mastitis. *PLoS One.* (2018) 13:e0203132. doi: 10.1371/journal.pone.0203132
93. Sordillo LM. Mammary gland immunobiology and resistance to mastitis. *Vet Clin North Am Food Anim Pract.* (2018) 34:507–23. doi: 10.1016/j.cvfa.2018.07.005
94. Bohl LP, Isaac P, Breser ML, Orellano MS, Correa SG, Tolosa de Talamoni NG, et al. Interaction between bovine mammary epithelial cells and planktonic or biofilm *Staphylococcus aureus*: the bacterial lifestyle determines its internalization ability and the pathogen recognition. *Microb Pathog.* (2021) 152:104604. doi: 10.1016/j.micpath.2020.104604
95. Bjarnsholt T, Givskov M. The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*. *Anal Bioanal Chem.* (2007) 387:409–14. doi: 10.1007/s00216-006-0774-x
96. Zaatout N, Ayachi A, Kecha M. Interaction of primary mammary bovine epithelial cells with biofilm-forming staphylococci associated with subclinical bovine mastitis. *Iran J Vet Res.* (2019) 20:27–32.
97. Oliveira M, Bexiga R, Nunes SF, Vilela CL. Invasive potential of biofilm-forming Staphylococci bovine subclinical mastitis isolates. *J Vet Sci.* (2011) 12:95–7. doi: 10.4142/jvs.2011.12.1.95
98. Valle J, Latasa C, Gil C, Toledo-Arana A, Solano C, Penadés JR, et al. Bap, a biofilm matrix protein of Staphylococcus aureus prevents cellular internalization through binding to GP96 host receptor. *PLoS Pathog.* (2012) 8:e1002843. doi: 10.1371/journal.ppat.1002843
99. Mulcahy LR, Isabella VM, Lewis K. *Pseudomonas aeruginosa* biofilms in disease. *Microb Ecol.* (2014) 68:1–12. doi: 10.1007/s00248-013-0297-x
100. Krömker V, Friedrich J, Klocke C. Ausscheidung und Nachweis von Staphylococcus aureus über Milch aus infizierten Milchdrüsenvierteln. [Excretion and detection of Staphylococcus aureus via milk from infected mammary gland quarters]. *Tierärztl Prax.* (2008) 389-392. doi: 10.1055/s-0038-1623865
101. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Møller K, et al. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A.* (2018) 115:E5125–E34. doi: 10.1073/pnas.1717525115
102. Høiby N, Bjarnsholt T, Moser C, Jensen PØ, Kolpen M, Qvist T, et al. Diagnosis of biofilm infections in cystic fibrosis patients. *APMIS.* (2017) 125:339–43. doi: 10.1111/apm.12689
103. Thomsen TR, Aasholm MS, Rudkjøbing VB, Saunders AM, Bjarnsholt T, Givskov M, et al. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. *Wound repair.* (2010) 18:38–49. doi: 10.1111/j.1524-475X.2009.00561.x
104. Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, et al. Distribution, organization, and ecology of bacteria in chronic wounds. *J Clin Microbiol.* (2008) 46:2717–22. doi: 10.1128/JCM.00501-08
105. Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, et al. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. *J Clin Microbiol.* (2009) 47:4084–9. doi: 10.1128/JCM.01395-09
106. Fazli M, Bjarnsholt T, Høiby N, Givskov M, Tolker-Nielsen T. PNA-based fluorescence *in situ* hybridization for identification of

bacteria in clinical samples. *Methods Mol Biol.* (2014) 1211:261–71. doi: 10.1007/978-1-4939-1459-3_21

107. Rudkjøbing VB, Thomsen TR, Xu Y, Melton-Kreft R, Ahmed A, Eickhardt S, et al. Comparing culture and molecular methods for the identification of microorganisms involved in necrotizing soft tissue infections. *BMC Infect Dis.* (2016) 16:652. doi: 10.1186/s12879-016-1976-2
108. Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent *in situ* hybridization (PNA FISH). *Microbiology.* (2009) 155:2603–11. doi: 10.1099/mic.0.028712-0

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## NOMENCLATURE

CNS, Coagulase-negative staphylococci
CRA, Congo Red Agar
CLSM, confocal laser scanning microscopy
LAB, lactic acid bacteria
PIA, polysaccharide interstellar adhesions
PNA-FISH, peptide nucleic acid fluorescence *in situ* hybridization.

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# Non-inferiority Trial Investigating the Efficacy of Non-steroidal Anti-inflammatory Drugs and Antimicrobial Treatment of Mild to Moderate Clinical Mastitis in Dairy Cows With Long-lasting Udder Diseases

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To reduce ineffective antimicrobial usage in the treatment of non-severe clinical mastitis (CM) in cows with long-lasting udder diseases, non-antibiotic therapy with a non-steroidal anti-inflammatory drug (NSAID) was conducted and evaluated in a non-blinded, positively controlled, non-inferiority trial. Therefore, three-time systemic ketoprofen treatment at intervals of 24 h was evaluated in comparison with the reference treatment of solely antibiotic therapy in a field study on nine free-stall dairy farms located in Northern Germany. Cows with previous CM cases in current lactation and/or with long-lasting high somatic cell counts in preceding dairy herd improvement test days were randomly allocated to one of the two treatment groups in cases of mild to moderate CM. Quarter foremilk samples of the affected quarters were taken for cyto-bacteriological investigation before treatment as well as ∼14 and 21 d after termination of therapy. Both treatment groups were compared regarding the bacteriological cure (BC) as the primary outcome. Clinical cure (CC) and no CM relapse within 60 d after the end of treatment (no R60) were chosen as secondary outcomes. The study resulted in the following outcomes: *Streptococcus uberis* was most frequently identified in microbiological culture from pre-treatment samples, followed by *Staphylococcus aureus* and *Escherichia coli* and other coliforms. No significant differences between the NSAID treatment and the reference treatment were detected regarding CC and CM recurrence (no R60). Although the sole ketoprofen therapy resulted in a numerically lower likelihood of BC, there were no significant differences to the reference treatment. Considering the selection criteria in this study, the results indicate that in mild to moderate CM cases exclusive

treatment with ketoprofen may constitute an alternative to antimicrobial intramammary therapy, providing an opportunity for reduction of antibiotic usage. However, non-inferiority evaluations were inconclusive. Further investigations with a larger sample size are required to confirm the results and to make a distinct statement on non-inferiority.

**Keywords: bovine, mastitis treatment, NSAID, chronic, cure, reduction of antibiotic usage**

## INTRODUCTION

Due to the increasing development of antibiotic resistance, antimicrobial usage in livestock farming is a critically discussed subject and a matter of public concern. With maximum cure rates in mind, antibiotic overtreatment was propagated for clinical mastitis (CM) over a long period of time (1). It was reported that over 95% of CM was treated with antibiotics in the U.S. (2). Recent available data from Germany suggest that in the field, three out of four CM cases are treated immediately with antibiotics (1). Nevertheless, modern therapeutic strategies also indicate that not every case of mastitis requires antibiotic therapy, and using evidence-based decision criteria, cure rates similar to antibiotic therapy can be achieved with extensive antibiotic savings (3–5).

The goal of antibiotic treatment is to eliminate the causative pathogens from the infected udder quarter and thus achieve a bacteriological cure (BC) (6–9). For chronic disease cases, according to Trevisi et al., antibiotic treatments do not lead to improved animal health and are not appropriate in terms of cost-benefit analysis (10).

The influence of cow-related factors on the BC risk of CM cases treated with antibiotics has been a topic in many studies. It was shown that probability of BC decreases with increasing numbers of previous CM cases in the current lactation (9, 11) as well as high cow somatic cell counts (SCC) prior to CM (9, 11–14). As a result, decreasing the likelihood of BC leads to decreasing efficacy and benefit of antibiotic treatment. Prolonged udder disease is present in cows that have recurrent CM cases or episodes that are interrupted by symptom-free periods with elevated cow SCC - i.e., subclinical mastitis (15, 16). Consideration of the CM history in the current lactation and persistent elevation in cow SCC allows a determination of cows with a low probability of BC following antibiotic treatment. Especially in chronic mastitis cases involving *Staphylococcus (S.) aureus*, BC rates after antibiotic therapy seldom exceed self-cure rates (17).

Rather, it is important to consider whether it is reasonable to define a bacteriological cure as the goal in the treatment of CM. Recent work has shown that 20–30% of all mastitis cases are recurrent mastitis (18, 19). Thus, a large proportion of all CM may be attributed to animals with longer lasting udder infections with clinical flare ups. Infections with *Streptococcus (Sc.) uberis* in particular resolve well, and subsequent infections are largely caused by other strains of the pathogen (19). In conclusion, chronically diseased animals may cure bacteriologically between infections, but a cow with a compromised udder parenchyma will remain chronically ill, showing persistent elevated quarter SCC and will very likely develop clinical signs in turn. Even if a BC can be achieved for some pathogens, it is often not long-lasting. Thus, the value of antibiotics in treatments of such chronic disease cases must be reconsidered.

If possible, those cows should be removed from the herd (20) or treated symptomatically in the case of CM to avoid useless application of antibiotics (21). From a farmer's point of view, such cows, especially the high-yielding animals, are still profitable as long as they show no clinical symptoms and the milk is saleable. Therefore, treatment should focus on decreasing the symptoms of inflammation. In actual practice, however, in the case of chronic mastitis with recurrent clinical flare ups, farmers tend to prolong antibiotic treatment (1, 5).

Non-steroidal anti-inflammatory drugs (NSAIDs) based on ketoprofen are approved in many countries for the adjunctive treatment of clinical mastitis. By preventing the function of the key enzyme cyclooxygenase, ketoprofen inhibits the synthesis of prostaglandin. By NSAID treatment, affected animals benefit from pain relief, which can prevent milk starvation due to insufficient feed intake. Moreover, it has shown positive effects on BC and cows regain physiological milk secretion earlier (22, 23). However, farmers underestimate the positive impact and, against recommendations, tend to omit NSAIDs (5). Demonstrating treatment success with sole NSAID medication in cases of chronically diseased cows could convince skeptical farmers to abandon antibiotic therapy for those animals.

The aim of this study was to evaluate non-inferiority of ketoprofen against antibiotic treatment of mild to moderate CM in cows with long-lasting udder diseases.

## MATERIALS AND METHODS

### Study Design

This was a randomized non-inferiority study, comparing the outcomes of the test treatment group (ketoprofen treatment) with the outcomes of the reference treatment group (antibiotic treatment). This study is similar to a study we have conducted previously that assessed non-inferiority of an enzyme therapy to AB treatment (24). For better understanding and readability, the study design is described again. The idea of a non-inferiority study is to prove equality of the two treatments by defining an equivalence margin, which specifies a range of values for which the margins between differences in clinical outcome are sufficiently close to be considered equivalent ($\Delta$) (7, 25, 26). The null hypothesis was in our study that a 3-d treatment with ketoprofen is inferior compared to an antibiotic treatment. The alternative hypothesis implied that the 3-d treatment with ketoprofen is non-inferior compared to the antibiotic treatment

by more than the equivalence margin of 15% (−Δ) (7, 25):

$$H_0 : [P_{outcome}(\text{ketoprofen}) - P_{outcome}(\text{antibiotictreatment})] \leq -\Delta$$
$$H_A : [P_{outcome}(\text{ketoprofen}) - P_{outcome}(\text{antibiotictreatment})] > -\Delta$$

Whereby, $P_{outcome}$ is the probability of outcome variables for the ketoprofen and antibiotic treatment. To establish non-inferiority of a test treatment to a reference treatment, the null hypothesis ($H_0$) must be rejected in order to have the alternative hypothesis accepted ($H_A$). The evaluations of possible study results, applying for this study, were described by Schukken et al. (7).

## Ethical Approval

This study was conducted in accordance with the guidelines on good clinical practice (27). The clinical trial registry number is TVO-2016-V-78. The study complies with the Consolidated Standards of Reporting Trials (CONSORT Checklist).

## Sample Size Determination

Based on former studies of our group and on investigations of Schukken et al. (28), the margin of non-inferiority (Δ) was determined as 0.15 for this study. Furthermore, other scientific working groups previously adopted this value for the non-inferiority margin in CM studies (8, 29). The confidence interval (CI; 95%) approach was used to calculate the required sample size based on the BC rate. In this model, treatments are assumed to achieve similar cure and recurrence rates and we want to assure using the 95% level that the difference is not higher than 15% regarding the margin of non-inferiority (Δ) and the null effect. The sample size was calculated assuming that the antibiotic cure risk was ∼50%, and a statistical significance of 5% and power of 80% were chosen. The calculations were performed with the use of StudySize 2.0 (Creostat HB, www.creostat.com) and it resulted in an estimated sample size per group of 137 cases.

Using the estimation of the recurrences due to the higher required sample size, we calculated that if a further 5% of CM cases dropped out of the study post admission, around 145 cases were needed per treatment group. Therefore, a total of 290 cows with CM had to be included.

## Farms and Cows

Inclusion criteria for farms were that farms were motivated to reduce antimicrobials in the treatment of chronic mastitis, participated in the German Dairy Herd Improvement program (DHI), and farm staff were experienced in aseptic sampling in accordance with the guidelines of the German Veterinary Association (30).

The study was conducted on nine free-stall dairy farms located in Northern Germany from October 2014 to September 2018. Herd sizes were between ∼160 and 900 lactating Holstein-Friesian dairy cows. The milk production ranged from 9,500 and 11,800 kg/cow/year with bulk milk somatic cell counts between 138,000 and 226,000 cells/ml. None of the farms produced organic milk. All farms used modern milking systems and common hygiene management methods were implemented in daily milking routines (milkers wore gloves, one tissue per cow to clean the teats before milking, teat disinfection after milking). All herds were milked twice a day. A rotary milking parlor was installed on two farms, whereas seven farms owned a herringbone/side by side parlor. All farms fed their cows total mixed rations.

Only cows that met the criteria for chronic, longer lasting udder disease were included in the trial. Every cow had to be registered with a unique ear tag to clearly identify every animal, as stipulated in Germany. Definition criteria were fulfilled in the case of at least three consecutively high cow SCC (> 400,000 somatic cells/ml) in the previous three monthly DHI samplings and/or at least two CM cases in the current lactation. Cows included in the study had shown a period of normal milk secretion before CM occurred. Lactating Holstein-Friesian dairy cows of all parities with CM signs in one or more quarters were eligible for inclusion. Mastitis severity score was defined according to the International Dairy Federation guidelines (31). A CM case was classified as mild (grade 1) if there was only change in the appearance of milk (color, viscosity, consistency; i.e., flaky sediments, watery appearance, discoloration). A moderate CM (grade 2) additionally showed local clinical signs of inflammation of the udder parenchyma (i.e., swelling, heat, pain, redness). In the case of general clinical signs (fever, lack of appetite) the CM was defined as a severe mastitis (grade 3). Only cases of mild to moderate CM were included in the study and only cows free of significant udder, teat, or teat orifice lesions or another additional disease at the same time were used.

## Treatment and Randomization

If a case of CM occurred in an animal that met the definition of a chronically udder-diseased cow, classification of the severity score and the treatment was performed by instructed farm staff. Two different treatment regimens were investigated in the study: animals of the first group, the AB group, received local antimicrobial treatment according to the label of the respective products used on the farms ($\beta$-lactam antibiotics); animals of the second group, the NSAID group, received systemic treatment with ketoprofen (three applications at 24 h intervals with 3 mg of ketoprofen per kg bodyweight Kelaprofen® (Veyx-Pharma GmbH, Schwarzenborn, Germany). Cows were randomly allocated to one treatment group based on the last number of their respective barn number (even/odd) and therapy applied following strict asepsis by trained farm personnel. Cows with CM in more than one quarter were also included in the study and all affected quarters received the same therapy. Animals from both treatment groups were not separated for the trial, but were kept under the same conditions on the farms.

## Study Procedure

Farms received a monthly list containing eligible cows based on the monthly DHI results and the farm records of cow CM history of the current lactation. Farm staff were instructed to record clinical data and to fill in treatment protocols in accordance with the study procedure. A cow with a mild or moderate CM case in one or more quarters was identified by the milking personnel and checked for inclusion criteria using the list of eligible cows. If a cow was included in the study, a milk sample

was taken in accordance with the guidelines of aseptic milk sampling (30). Following the aforementioned randomization, the animals received the appropriate treatment depending on the assigned treatment group. Each cow was included in the study with only one CM case. Treatment was performed according to the label of the respective product. At day 5 after the end of treatment of a case, the clinical score of the affected quarter was assessed by the milkers. In the case of a deterioration of the clinical appearance, the case was recorded as treatment failure and farmers treated their cow additionally. CM cases without clinical symptoms on day 5 were assessed as clinically cured. These cured quarters were observed from days 6 to 60 after the end of treatment for recurrent CM cases and a quarter foremilk sample was taken in case of return of clinical signs. After treatment, pre-milk samples were collected from all clinically cured quarters on day 14 (±2) and day 21 (±2) after the end of treatment by a veterinarian of the study personnel. All samples were refrigerated and were picked up weekly during farm visits by a veterinarian of our working group. During these regular farm visits, we exchanged information with the herd personnel to resolve inaccuracies and ensure data quality. Any deviations from the study protocol were noted and investigated for eligibility to include in the study. Commonly used cow-level data including lactation number, affected quarter location, cow SCC of the three most recent DHI recordings prior to CM, days in milk (DIM) at CM occurrence, and concurrent diseases and treatments for a period of 30 d after enrolment were recorded.

## Blinding

It was not possible to blind either the study personnel or the farmers/herdspersons to product administration by virtue of the differences in treatment regimens. The laboratory personnel performing cyto-microbiological diagnostic examinations were unaware of the treatment given to the quarters being sampled. But due to the study design it was not possible to blind either the farm staff or the farmer as these people implemented the treatment depending on the treatment group.

## Laboratory Procedure

All milk samples were collected aseptically and were stored below 8°C until analysis. Ly20, containing boric acid as the preserving agent, was used in test tubes (30). The samples were sent to the microbiological laboratory at the University of Applied Sciences and Arts Hannover (Germany). Microbiological examinations were performed in accordance with the guidelines of the German Veterinary Association (30), which are similar to National Mastitis Council recommendations (31). From each milk sample, 10 µl was plated onto one quadrant of an esculin blood agar plate (Oxoid, Germany) and incubated for at least 48 h at 37°C under aerobic conditions. By the assessment of Gram staining, morphology of the colonies and cells, hemolysis patterns, esculin hydrolysis, and activity of catalase (3% $H_2O_2$; Merck, Germany), an initial evaluation of the grown colonies was performed. Subsequently several biochemical tests were done to determine the growing microorganisms. The clumping factor test (DiaMondiaL Staph Plus Kit, Sekisui Virotech, Germany) instead of the coagulase test was used to differentiate presumptive *Staphylococcus* (*S.*) *aureus* from non-*aureus* staphylococci (N*a*S). Different esculin-negative streptococci were distinguished by the serological tests for Lancefield Group B [*Streptococcus (Sc.) agalactiae*], C (*Sc. dysgalactiae*), and G (DiaMondiaL Streptococcal Extraction Kit Sekisui Virotech, Germany). To differentiate between *Sc. uberis* and *Enterococcus* spp. the modified Rambach agar according to Watts et al. (32) was used. Gram-positive, beta-hemolytic, catalase-negative irregular rods with a V- or Y-shaped configuration were identified as *Trueperella (T.) pyogenes*. Coryneform bacteria form small colonies on esculin blood agar. They are Gram-positive and catalase-positive. Both, *T. pyogenes* and coryneform bacteria are asporogenic. *Bacillus* spp. form large colonies on esculin blood agar. *Bacillus* spp. are Gram-positive, catalase-positive rods and can form endospores. Coliform bacteria are Gram-negative, catalase-negative, and cytochrome oxidase-negative (Bactident oxidase, Merck, Germany) rod-shaped bacteria, which can metabolize glucose fermentatively (OF basal medium with the addition of D (+)-glucose monohydrate, Merck, Germany). On Chromocult Coliform Agar (Merck, Germany), *Escherichia* (*E.*) *coli* forms blue colonies under aerobic incubation at 37°C for 24 h, other coliforms form pink-red colonies. *Klebsiella* spp. are immobile during the performance of the OF test. Pseudomonads were identified as Gram-negative, catalase-positive, cytochrome oxidase-positive rod-shaped bacteria that break down glucose oxidatively. Yeasts, moulds, and *Prototheca* spp. were differentiated microscopically after subculturing on YGC agar (Merck, Germany). Environment-associated, mastitis-causing microorganisms (*Sc. uberis*, *E. coli*, N*a*S, *Klebsiella* spp., coliform bacteria, yeasts, *Pseudomonas* spp., and *Prototheca* spp.) were recorded as a microbiologically positive result if ≥5 cfu/0.01 ml were cultured to reduce bias due to contamination. If two pathogens were cultured, the case was included in the study and both microorganisms were documented. A milk sample was considered as contaminated when more than two pathogens were identified, except in cases where also *S. aureus*, *Sc. agalactiae*, *Sc. dysgalactiae,* and *T. pyogenes* were cultured. Then only the growth of these pathogens was recorded and the cases were classified as contaminated if the samples contained more than two of these pathogens. Somascope Smart (Delta Instruments, The Netherlands) was used to determine the SCC by flow cytometry.

## Outcome Variables

Primary outcome was BC and secondary outcomes were CC and no CM recurrence within 60 d after the end of treatment (no R60). Quarter somatic cell count was additionally determined to identify quarters with cytological cure (CYC). CC was defined as absence of clinical symptoms in milk, this meant without flaky sediments, watery appearance, or discoloration and on udder quarter, this meant without swelling, heat, redness, or pain at day 5. CM cases of cows, which received additional or different treatment due to deterioration of clinical symptoms within the 5 days or after the end of initial therapy or were removed from the herd due to udder disease were assessed as failure of CC.

Quarters with clinically cured cases were observed for the time frame of days 6–60 after the end of treatment and defined as

recurrent quarters when one or more CM cases were detected. A quarter showed no R60 if it was free of CM within the observed time frame.

BC was defined as the absence of the pathogen cultured pre-treatment in both post-treatment samples at days 14 and 21. If a bacterial species other than the pathogen cultured pre-treatment was isolated in the post-treatment samples, the case was still defined as bacteriologically cured. If one post-treatment sample was contaminated, the outcome of the other post-treatment sample was used to determine the BC. If two pathogens were isolated in the pre-treatment sample the case was enrolled as mixed infection and applied as bacteriologically cured if neither of the two pathogens were cultured in both of the post-treatment samples. When a clinically cured quarter suffered from a CM recurrence within days 6–21 after the end of treatment, available post-treatment samples and the recurrence sample were used to determine BC.

CYC was defined as a quarter SCC with <200,000 cells/ml in both post-treatment samples at days 14 and 21. If one post-treatment sample was missing, the CYC of the other post-treatment sample was used to determine the outcome. When a clinically cured quarter suffered from a CM recurrence within days 6–21 after the end of treatment, the CM case was assessed as failure of CYC. Quarters with CM cases experiencing no CC were also included in the analysis as failure of BC and CYC to take the principle of "intention-to-treat" into account (8, 26).

## Statistical Analysis

The data were collected and analyzed using Excel, Office 2010 (Microsoft Corporation) and SPSS (IBM SPSS 26.0.0.0, Armonk, USA). The statistical unit was the CM case of an udder quarter. For every CM case, CC or no CC, R60 or no R60, BC or no BC, and CYC or no CYC (encoded as 1 or 0, respectively) were determined according to the aforementioned definitions, constituting the binary dichotomous-dependent variables. Outcomes were analyzed using generalized linear mixed models including lactation number, DIM, and pathogen (grouped) cultured pre-treatment as important covariates. As clustering was present in the design (i.e., gland within cow, and cow within herd) the analysis was corrected using random effects, but had no relevant influence. The treatment group was the main variable of interest. Statistical significance was assumed at $\alpha = 0.05$.

The linear predictor was calculated as

$$\text{Logit(outcome)} = \text{intercept} + \text{treatment} + \text{lactationnumber} + \text{DIM} + \text{pathogen} + \text{herd*cow*gland (random)}.$$

BC, CC, no R60, or CYC are the outcomes and lactation number is the lactation number of the included cow grouped as 1, 2, and over 2. DIM is days in milk of the cow at CM occurrence grouped as 0–100, 101–200, and over 200. Pathogens cultured pre-treatment were grouped into *Enterobacteriaceae*, streptococci, staphylococci, other pathogens, contaminated samples, mixed infections, and no growth.

For BC, CC, and no R60, the model was used to calculate least square means of the various treatment groups. Thereby, the differences between treatments were estimated. Confidence intervals of the therapy differences were calculated utilizing the least square means and standard deviation (8).

# RESULTS

## Descriptive Results

A total of 296 CM cases were enrolled in the study. In 17 CM cases, the dataset was incomplete because not all samples were taken (forgotten by the milker) and/or examined (leaked during transport). Antibiotic treatment was applied in 144 CM cases (AB group), whereas 135 cases received ketoprofen (NSAID group) (**Table 1**). No further treatment had to be initiated in any case due to worsening of the mastitis severity score. No adverse events of treatment were observed. The median of lactation number for all CM cases amounted to 3 (minimum 1; maximum 11) and of milk yield last DHI before CM occurrence, 31.5 kg (minimum 9.6 kg; maximum 58.0 kg). In 135 CM cases the front quarters and in 144 cases the rear quarters suffered from CM. In 178 cases, mastitis severity was classified as mild and as moderate in 101 cases. A proportion of 15.8% of the CM cases occurred in cows in their first 100 DIM, 43.3% in 101–200 DIM, and 40.9% in over 200 DIM, respectively.

The results of bacteriological culture are presented in **Table 2**. The pathogen most cultured from the pre-treatment sample was *Sc. uberis* (16.8%), followed by *S. aureus* (15.4%), and coliforms (11.1%). No microbiological growth was found in 62 cases (22.2%), 15 quarters showed mixed infections (5.4%), and 28 samples were contaminated (10.0%). In 53.3% of the mixed infections, *Sc. uberis* was one of the cultured pathogens and in 33.3% N*a*S was one of the isolated microorganisms.

The treatment groups were similar in terms of the lactation number, DIM, mastitis score, and pathogen distribution ($P > 0.05$). For good measure, herd as random effect, DIM, lactation number, and pathogen cultured pre-treatment were included in the generalized linear mixed models to take these factors into account.

## Bacteriological Cure

Bacteriological cure was determined for 189 CM cases. The remaining cases were excluded because no microorganisms were cultured (62 cases) or pre-treatment samples were contaminated

**TABLE 1 |** Number of cows per herd assigned to either the reference group with solely antibiotic treatment (AB) or the test treatment group with solely systemic ketoprofen treatment (NSAID).

| Participation (from–until) | Farm | Cows per herd (size) | AB | NSAID |
|---|---|---|---|---|
| Oct 2014–Sep 2018 | A | 270 | 21 | 15 |
| Oct 2015–Mar 2017 | B | 160 | 6 | 1 |
| Sep 2017–Mar 2018 | C | 740 | 4 | 5 |
| Oct 2014–Sep 2018 | D | 180 | 9 | 11 |
| Oct 2014–Sep 2016 | E | 850 | 24 | 20 |
| Oct 2014–Mar 2017 | F | 900 | 26 | 43 |
| Oct 2016–Sep 2017 | G | 250 | 4 | 2 |
| Oct 2014–Sep 2017 | H | 780 | 28 | 22 |
| Oct 2014–Sep 2018 | I | 550 | 22 | 16 |
| **Total** | **9** | **4,680** | **144** | **135** |

**TABLE 2 |** Bacteriological culture results ($n$= 79 CM) of pre-treatment samples of the reference group with solely antibiotic treatment (AB) and the test treatment group with solely systemic ketoprofen treatment (NSAID).

| Microorganism | AB ($n$ = 144) | NSAID ($n$ = 135) |
|---|---|---|
| ***Enterobacteriaceae*** | **13** | **18** |
| Coliforms (other than *E. coli* and *Klebsiella* spp.) | 9 | 5 |
| *E. coli* | 4 | 13 |
| **Streptococci** | **32** | **30** |
| *Sc. Uberis* | 23 | 24 |
| *Sc. Dysgalactiae* | 5 | 3 |
| Other streptococci | 4 | 3 |
| **Staphylococci** | **18** | **30** |
| *S. aureus* | 14 | 29 |
| NaS | 4 | 1 |
| **Other pathogens** | **22** | **11** |
| Coryneforms | 5 | 3 |
| *Pseudomonas* spp. | 5 | 2 |
| *Prototheca* spp. | 4 | 2 |
| Enterococci | 3 | 1 |
| *T. pyogenes* | 2 | 3 |
| Yeasts | 3 | 0 |
| No growth | 31 | 31 |
| Mixed infections | 10 | 5 |
| Contaminated | 18 | 10 |
| **Total** | **144** | **135** |

**TABLE 3 |** Final mixed logistic regression model results for the outcome variable bacteriological cure.

| Variable | Coefficient | | OR | 95% CI | $P$-value[a] |
|---|---|---|---|---|---|
| | X | SE | | | |
| Intercept | 0.616 | 0.453 | 1.852 | 0.758–4.523 | 0.175 |
| **Treatment** | | | | | |
| AB | 0.099 | 0.336 | 1.104 | 0.568–2.143 | 0.769 |
| NSAID (reference) | 0 | | | | |
| **Lactation number of the cow at the day of clinical mastitis occurrence** | | | | | |
| 1 | 1.228 | 0.554 | 3.414 | 1.145–10.181 | **0.028** |
| 2 | 0.170 | 0.372 | 1.185 | 0.569–2.468 | 0.648 |
| >2 (reference) | 0 | | | | |
| **Days in milk at the day of clinical mastitis occurrence** | | | | | |
| 0–100 | −1.319 | 0.497 | 0.267 | 0.100–0.713 | **0.009** |
| 101–200 | −0.151 | 0.373 | 0.860 | 0.412–1.796 | 0.686 |
| >200 (reference) | 0 | | | | |
| **Pathogen cultured from the pre-treatment milk sample** | | | | | |
| Mix | −0.818 | 0.676 | 0.441 | 0.116–1.676 | 0.228 |
| Other | −0.149 | 0.548 | 0.862 | 0.292–2.542 | 0.786 |
| Staphylococci | −2.222 | 0.555 | 0.108 | 0.036–0.324 | **0.000** |
| Streptococci | −0.656 | 0.477 | 0.519 | 0.202–1.331 | 0.171 |
| *Enterobacteriaceae* (reference) | 0 | | | | |

[a] *Significance set at $P < 0.05$. Bold value indicates significant value.*
*Two different treatment regimens were investigated: NSAID, solely ketoprofen comprising three treatments at an interval of 24 h; AB, antibiotic treatment as usual on the farm according to the label of the respective product.*

(28 cases). The overall BC rate was 44.4% (84/189). The probability of BC in the AB group was 48.4% (46/95) and in the NSAID group 40.4% (38/94).

Results of the generalized linear mixed model showed the least square means of 48.1% for the AB group and 45.6% for the NSAID group. The model demonstrated that no significant differences in BC of the reference treatment AB to the test treatment NSAID were found ($P = 0.769$) (**Table 3**). Animals with CM within 1 to 100 DIM showed a significantly higher probability of BC than cows suffering from CM > 100 DIM ($P = 0.028$). Cows with staphylococcal infections had a significantly lower BC rate than animals with other pathogens ($P = 0.028$). The point estimate of the calculated differences in BC from the logistic regression and the associated 95% CI is shown in **Figure 1**. Non-inferiority was inconclusive but very close to non-inferior for NSAID treatment in comparison to the solely antibiotic treatment.

## Clinical Cure

The overall CC rate was 50.5% (141/279). The probability of CC in the AB group was 51.4% (74/144) and in the NSAID group 49.6% (67/135).

Results of the generalized linear mixed model showed least square means of 57.9% for the AB group and 57.4% for the NSAID group. Again, no significant differences in CC of the reference treatment AB to the test treatment NSAID ($P = 0.57$) were present (**Table 4**). Cows suffering from CM with streptococcal infections showed a significantly lower probability of CC than cows affected with other pathogens ($P = 0.004$). The point estimate of the calculated differences in CC from the logistic regression and the associated 95% CI is shown in **Figure 1**. Non-inferiority was inconclusive but very close to non-inferior in comparison to the reference treatment.

## No Recurrence After 60 d

Only CM cases of cows that reached a CC and were still in milk 60 d after the end of treatment were included in this analysis (11). Of the 141 clinically cured quarters, only two cases were excluded because the cows had been sold within the considered timeframe. Consequently, 139 CM cases were included in the analysis. The overall no R60 rate was 54.7% (76/139). The probability of achieving no CM recurrence 60 d after the end of treatment in the AB group was 58.3% (42/72) and in the NSAID group 50.7% (34/67).

Results of the generalized linear mixed model showed numerically different least square means of 63.3% for the AB group and 64.9% for the NSAID group. However, no significant differences in no R60 of the reference treatment AB to the test treatment NSAID ($P = 0.556$) were found (**Table 5**). Cows in their second lactation ($P = 0.009$) showed a significantly higher probability of no R60 than cows in the third or higher lactation ($P = 0.030$). Furthermore, animals with CM at the beginning of lactation (<100 DIM) had a significantly lower likelihood of no R60 compared to cows suffering from mastitis later in lactation

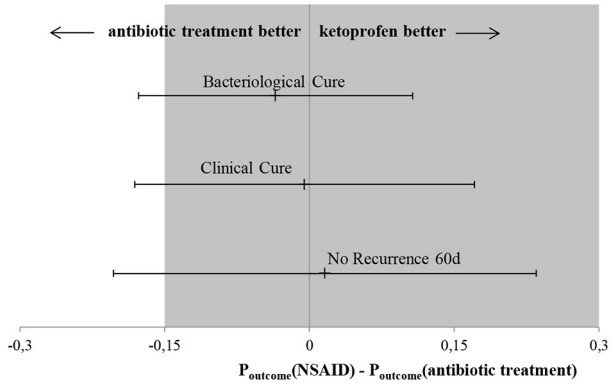


**FIGURE 1 |** Main results of this non-inferiority trial. Black point presents point-estimate of difference in outcome variables between the test treatments (NSAID) and the reference treatment (AB) with the associated 95% CI indicated by the arrowheads. Dark field represents area of non-inferiority. Bacteriological cure: difference in BC between test treatment (NSAID) in comparison to the reference treatment (AB), the CI spans both 0 and −Δ, non-inferiority is inconclusive and there are no significant differences between the two treatments. Clinical cure: difference in CC between test treatment (NSAID) in comparison to reference treatment (AB), the CI spans both 0 and non-inferiority margin (−Δ), non-inferiority is inconclusive and there are no significant differences between the two treatments. No recurrence 60 d: difference in no R60 between test treatment (NSAID) in comparison to reference treatment (AB), the CI spans both 0 and −Δ, non-inferiority is inconclusive and there are no significant differences between the two treatments.

**TABLE 4 |** Mixed logistic regression model results for the outcome variable clinical cure.

| Variable | Coefficient | | OR | 95% CI | *P*-value[a] |
|---|---|---|---|---|---|
| | X | SE | | | |
| Intercept | −0.261 | 0.466 | 1.298 | 0.519–3.246 | 0.576 |
| **Treatment** | | | | | |
| AB | 0.021 | 0.278 | 1.021 | 0.590–1.766 | 0.569 |
| NSAID (reference) | 0 | | | | |
| **Lactation number of the cow at the day of clinical mastitis occurrence** | | | | | |
| 1 | 0.166 | 0.402 | 1.181 | 0.535–2.604 | 0.957 |
| 2 | −0.286 | 0.319 | 0.751 | 0.400–1.409 | 0.371 |
| >2 (reference) | 0 | | | | |
| **Days in milk at the day of clinical mastitis occurrence** | | | | | |
| 0–100 | 0.348 | 0.395 | 1.417 | 0.651–3.082 | 0.378 |
| 101–200 | 0.547 | 0.310 | 1.728 | 0.939–3.182 | 0.079 |
| >200 (reference) | 0 | | | | |
| **Pathogen cultured from the pre-treatment milk sample** | | | | | |
| Mix | −0.161 | 0.637 | 0.851 | 0.243–2.984 | 0.800 |
| Other | −0.015 | 0.475 | 0.985 | 0.387–2.510 | 0.975 |
| Staphylococci | −0.549 | 0.437 | 0.578 | 0.244–1.366 | 0.211 |
| Streptococci | −1.145 | 0.398 | 0.318 | 0.146–0.697 | **0.004** |
| *Enterobacteriaceae* | 0.176 | 0.500 | 1.192 | 0.445–3.191 | 0.725 |
| Contaminated | 0.148 | 0.521 | 1.160 | 0.416–3.233 | 0.776 |
| No growth (reference) | 0 | | | | |

[a] *Significance set at $P < 0.05$. Bold value indicates significant value.*
*Two different treatment regimens were investigated: NSAID, solely ketoprofen comprising three treatments at an interval of 24 h; AB, local antibiotic treatment as usual on the farm according to the label of the respective product.*

($P = 0.014$). CM cases caused by staphylococci and streptococci showed a significantly lower probability of no R60 than CM cases where another or no pathogen was isolated ($P = 0.022$; $P = 0.038$ resp.). The point estimate of the calculated differences in no R60 from the logistic regression and the associated 95% CI is shown in **Figure 1**. Non-inferiority was inconclusive for the NSAID treatment in comparison to the AB treatment.

## Cytological Cure

The overall CYC was 3.9% (11/279). The probability of CYC in the AB group was 4.2% (6/144) and in the NSAID group 3.7% (5/135).

Including the important covariates of the aforementioned generalized linear mixed model, there were no significant differences between the investigated treatment groups for the outcome variable CYC ($P = 0.872$; data not shown).

# DISCUSSION

The aim of the present study was to evaluate the efficacy of an NSAID treatment in comparison to a reference therapy with solely local antibiotic treatment in the case of non-severe CM in cows with a long-lasting udder disease. In the case of CM, farmers assessed mastitis severity. Cows with severe cases were excluded from the trial and were treated systemically with antibiotics, according to the farms' treatment protocols. These animals were at risk of developing bacteremia, so irrespective of a previous onset of chronic mastitis, parenteral antibiotic treatment is recommended (33, 34).

The primary outcome in this trial was BC. Although BC risk of the reference group with antibiotic treatment was numerically higher with 48.1% compared to the NSAID group with 45.6%, no significant differences were confirmed by statistical analysis. As the CI spans the non-inferiority margin (−Δ), non-inferiority was inconclusive but very close to non-inferior for NSAID treatment in comparison to the solely antibiotic treatment (**Figure 1**). An inconclusive result could possibly occur due to a wide range of the CI. However, the confidence interval only slightly exceeded delta. With a larger number of cases, the non-inferiority could possibly be confirmed, as the span of the CI would become smaller. The non-inferiority margin of 15% was chosen according to previous CM trials (7, 29, 35, 36). Sample size was calculated to give the study sufficient power and to show a difference between test and reference therapy if there was a real difference of at least 15% according to Schukken et al. (7). The NSAID treatment in our study showed a numerically almost identical BC risk and no significant differences to the reference treatment; non-inferiority was inconclusive due to the lack of power. The overall BC risk was low in this study with 44.4%, as was the BC risk for CM cases treated with antibiotics (48.1%). This study exclusively included CM cases of cows with long-lasting udder diseases. Therefore,

**TABLE 5 |** Final mixed logistic regression model results for the outcome variable no recurrence 60 d.

| Variable | Coefficient | | OR | 95% CI | *P*-value[a] |
|---|---|---|---|---|---|
| | X | SE | | | |
| Intercept | 1.759 | 1.323 | 2.461 | 0.721–8.399 | 0.149 |
| **Treatment** | | | | | |
| AB | −0.475 | 0.804 | 0.932 | 0.372–2.338 | 0.881 |
| NSAID (reference) | 0 | | | | |
| **Lactation number of the cow at the day of clinical mastitis occurrence** | | | | | |
| 1 | 1.843 | 0.809 | 2.939 | 0.883–9.790 | 0.079 |
| 2 | 2.137 | 0.791 | 3.346 | 1.126–9.947 | **0.030** |
| >2 (reference) | 0 | | | | |
| **Days in milk at the day of clinical mastitis occurrence** | | | | | |
| 0–100 | −2.815 | 1.050 | 0.207 | 0.059–0.721 | **0.014** |
| 101–200 | −1.939 | 0.941 | 0.734 | 0.291–1.854 | 0.510 |
| >200 (reference) | 0 | | | | |
| **Pathogen cultured from the pre-treatment milk sample** | | | | | |
| Mix | −1.002 | 1.744 | 0.273 | 0.041–1.838 | 0.180 |
| Other | 0.628 | 0.980 | 4.628 | 0.873–24.542 | 0.072 |
| Staphylococci | −2.028 | 1.008 | 0.184 | 0.044–0.780 | **0.022** |
| Streptococci | −0.510 | 0.884 | 0.246 | 0.065–0.926 | **0.038** |
| *Enterobacteriaceae* | −1.888 | 1.767 | 1.410 | 0.387–5.135 | 0.600 |
| Contaminated | | | 0.649 | 0.179–2.344 | 0.506 |
| No growth (reference) | 0 | | | | |

[a] *Significance set at P < 0.05. Bold value indicates significant value.*

*Two different treatment regimens were investigated: NSAID, solely ketoprofen comprising three treatments at an interval of 24 h; AB, antibiotic treatment as usual on the farm according to the label of the respective product.*

low likelihood of BC was expected, as studies had shown before (7, 12, 17, 37). In comparison, focusing on all occurring cases in a dairy herd, studies demonstrated BC risks of ∼70% (7, 8, 18). The high differences in BC rates support the selection criteria used in this study to choose cows suffering from CM with a low likelihood of BC. Nevertheless, a tendency for the efficacy of antibiotic treatment against mastitis pathogens was shown.

A CC of the affected quarters was a secondary outcome in this study. Clinical cure risk was almost identical in both study groups (LSM; mixed model) and no statistically significant difference was found between the treatment groups. In the AB group, CC risk was slightly better with 0.5%. Again, due to the large calculated confidence interval of 17.6%, the statement regarding non-inferiority must also be inconclusive here. Other studies found a slightly higher likelihood of CC of ∼60% for CM cases treated with antibiotics compared with our results, despite different, changing definitions of CC (7, 8). It is possible that CC risk worsens with increasing chronicity of mastitis.

The other secondary outcome variable was no R60. The probability of achieving no CM recurrence 60 d after the end of treatment was almost numerically identical for animals of the AB group (63.3%) and animals of the NSAID group (64.9%). Statistical analysis showed no significant differences between these two treatments. Non-inferiority was inconclusive because the CI also had a wide range (18.0%) and spanned −Δ and 0. Recurrences were observed only for clinically cured cases. Hence, the amount of evaluable cases was lower as in the models for the other outcomes and therefore CI increased. The no R60 risk of NSAID (64.9%) was numerically better than the rate of the AB treatment (63.3%). Comparing this result with those of previous trials which are also exclusively dealing with cows suffering from longer-lasting udder disease, no recurrence risks were similar (24). Studies performed without comparable selection criteria for enrolled CM cases described higher no recurrence rates of 80% within 60 d (13). CM is a disease with recurrent character (38). Cha et al. (39) showed that a cow with two CM cases in current lactation had a higher risk of contracting a third case. Thus, there was strong evidence that animals in this study were more likely to develop recurrent CM.

The evaluations of non-inferiority resulted in inconclusive findings for the targeted outcomes. A larger sample size of CM cases is required to confirm the detected results of the study and to make a clear statement on non-inferiority.

The specification of the non-inferiority margin is often controversial (40). As the control group received antibiotic treatment, primary outcome was BC. A non-inferiority margin of 0.15 was chosen because it had been used in antibiotic comparative studies and also in one of our studies when comparing antibiotic with an enzymatic mastitis therapy (7, 24, 25, 29, 36). Due to the very low BC rate to be expected when dealing with chronically udder-diseased cows, a wider margin might have been better suited for this trial. Based on the available literature on chronic mastitis, a large delta, as chosen in comparative studies with negative controls or placebo groups, did not seem appropriate to our study design. In addition, the choice of the primary outcome can be controversial, as BC is the actual goal of antibiotic mastitis therapy, but it is of little importance in the field. Ultimately, the acceptance in terms of cure rate reduction is a practical question.

The NSAID group received systemic treatment with ketoprofen (three applications at 24 h intervals with 3 mg of ketoprofen per kg bodyweight Kelaprofen®, Veyx-Pharma GmbH, Schwarzenborn, Germany). The prescribed withdrawal period on milk is 0 days, which ensures that there are no residues in the milk as a result of the usage. This is potentially the greatest advantage for farmers of this alternative treatment, as the milk can be sold again as soon as the cow is free of clinical signs of disease (although other NSAIDs might have a different prescribed withdrawal period on milk). This will also have a positive impact on farmers' costs due to the reduction in milk loss. Another advantage is that the risk from iatrogenic infection due to improper use of udder injectors is thus avoided. For cattle, according to the standard operating procedure of Kelaprofen®, the maximum treatment duration of 3 days should not be exceeded in order to avoid any unwanted side effects on the animals' gastrointestinal tract. The biggest challenge for farms might be the documentation of chronically udder-diseased cows and the implementation of the alternative treatment for these animals into existing treatment protocols and the daily procedure in cases of clinical mastitis. Avoiding useless antibiotic treatment complies with public demands and offers a sustainable

treatment strategy in a broader perspective, but it can be challenging to convince farmers that these cows will not benefit from antibiotic treatment (5). The sharpened farmers' awareness of chronically udder-diseased cows in the herd might contribute to a targeted culling scheme and therefore might have a positive effect on the udder health at a herd level. Our intention was to reflect the situation in daily practice on dairy farms. Information about the causative pathogen was not available at the time of CM occurrence. Therefore, and because power calculations were made on overall therapy level, evaluations of treatment efficacy at a pathogen level gave no reliable results due to lack of power. Moreover, farmers were allowed to use their routine mastitis treatment procedure (AB) for CM cases of the reference group. That resulted in a wide range of used antibiotic products with different durations of treatment and withholding times. However, there were no indications of the various antimicrobial therapies influencing the study outcomes.

No completely untreated control group was included in our investigation. Mastitis is a painful condition for the cow. Therefore, for reasons of animal welfare a treatment is indicated. Also, so far there is no evidence-based information on the further course of CM in untreated animals. The participating farms were all economically oriented and the animals in the trial were in the regular production cycle. Thus, the formation of an untreated experimental group could not have been justified to the voluntarily participating farmers. Thus, we did not know whether the selection criteria chosen were correct to identify animals with a low probability of BC in lactation. As an additional outcome of the study, these selection criteria turned out to be well-adapted for this purpose. It is possible that stricter inclusion criteria (>3 clinical cases prior to the case under study and/or higher cow SCC cell count thresholds) would provide even clearer results.

Since the SCC significantly determines the value of the milk and thus influences the payment amount to the farmers, treatment is also intended to reduce the SCC of the affected quarter. In this study cows with mastitis history and persistent high cow SCC's were chosen and a low likelihood of BC was expected and proved. Antimicrobial treatment can solely target a BC and therefore a decreasing SCC can only be expected as a consequence of a reached BC (21). CYC rates in this study turned out quite low with 3.9% overall and with no significant differences between the treatment groups. Compared to a recently published study of Ziesch et al. (24), showing an overall CYC of 9.9%, this percentage is even lower. The authors suggested that a cow fulfilling the used selection criteria had a very low probability to recover from a physiological SCC in the affected udder quarter. In addition, the low CYC rate, accompanied by a CC rate of ∼57%, was interpreted as an indication that the observed CM cases may turn subclinical with the remaining elevated quarter and therefore cow SCCs.

The actual very low BC rates achieved in this study demonstrate that an antibiotic treatment of cows with longer-lasting mastitis history can hardly be justified. Nevertheless, the milk of these cows is still saleable as long as they show no clinical symptoms. Therefore, farmers are particularly interested in a CC, a low recurrence rate, and a short time of discarding milk (41). With respect to the outcomes, the NSAID treatment seemed to achieve similar results in comparison to the reference group treated with antibiotics without having a withdrawal period for milk, which may decrease time of discarding milk, and a reduced risk of antibiotic residues. The results of this study will further encourage farmers and veterinarians to consider the impact of NSAID treatment, avoiding useless application of antibiotics in cases of chronically diseased cows.

## CONCLUSION

A randomized, multi-herd, non-inferiority study was conducted evaluating the efficacy of the test treatment ketoprofen in comparison to antibiotic treatment (AB; reference) of mild to moderate cases in cows with chronic mastitis. The test treatment showed no significant differences to the reference treatment with respect to the outcome variables BC, CC, no R60, and CYC. Solely NSAID therapy showed a numerically lower probability of BC and CC without significant differences to the reference treatment. NSAID treatment resulted in a numerically higher non-recurrence rate than the antibiotic treatment. The study findings indicate that solely using NSAID for treatment of mild to moderate CM in cows with long-lasting udder diseases may constitute an alternative therapy to reduce antibiotic usage. However, a greater sample size is needed to accomplish a reliable non-inferiority evaluation. Overall, the results for the different cure rates suggest that the used selection criteria of cows should be monitored in dairy herds. The quickest possible removal of such animals is recommended.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the animal welfare officer of the University of Veterinary Science Hannover; ethics committee and animal welfare officer of the University of Hanover.

## AUTHOR CONTRIBUTIONS

VK: conceptualization and formal analysis. VK and EM-dV: methodology. VK and DK: software and data curation. VK and AS: validation and writer-original design preparation. EM-dV, VK, AS, NW, and YZ: investigation. VK, SL, DK, NW, and YZ: resources. VK, AS, DK, and SL: writer review and editing. AS: visualization. VK and SL: supervision and project management. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. Falkenberg U, Krömker V, Heuwieser W, Fischer-Tenhagen C. Survey on routines in udder health management and therapy of mastitis on German Dairy Farms. *Milk Sci Int.* (2019) 72:11–5. doi: 10.25968/MSI.2019.2
2. Oliveira L, Ruegg PL. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. *J Dairy Sci.* (2014) 97:5426–36. doi: 10.3168/jds.2013-7756
3. Mansion-de Vries EM, Lücking J, Wente N, Zinke C, Hoedemaker M, Krömker V. Comparison of an evidence-based and a conventional mastitis therapy concept with regard to cure rates and antibiotic usage. *Milk Sci Int.* (2016) 69:27–32. doi: 10.25968/MSI.2016.6
4. Kock J, Wente N, Zhang Y, Paduch JH, Leimbach S, Klocke D, et al. Udder health effects of an evidence-based mastitis therapy concept in Northwestern Germany. *Milk Sci Int.* (2018) 71:14–20. doi: 10.25968/MSI.2018.4
5. Schmenger A, Leimbach S, Wente N, Zhang Y, Biggs AM, Krömker V. Implementation of a targeted mastitis therapy concept using an on-farm rapid test: antimicrobial consumption, cure rates and compliances. *Vet Rec.* (2020) 187:401. doi: 10.1136/vr.105674
6. Krömker V, Paduch JH, Klocke D, Friedrich J, Zinke C. Efficacy of extended intramammary therapy to treat moderate and severe clinical mastitis in lactating dairy cows. *Berl Munch Tierarztl Wochenschr.* (2010) 123:10–5.
7. Schukken YH, Zurakowski MJ, Rauch BJ, Gross B, Tikofsky LL, Welcome FL. Noninferiority trial comparing a first-generation cephalosporin with a third-generation cephalosporin in the treatment of nonsevere clinical mastitis in dairy cows. *J Dairy Sci.* (2013) 96:6763–74. doi: 10.3168/jds.2013-6713
8. Swinkels JM, Krömker V, Lam TJGM. Efficacy of standard vs. extended intramammary cefquinome treatment of clinical mastitis in cows with persistent high somatic cell counts. *J Dairy Res.* (2014) 81:424–33. doi: 10.1017/S0022029914000442
9. Ziesch M, Krömker V. Factors influencing bacteriological cure after antibiotic therapy of clinical mastitis. *Milk Sci Int.* (2016) 69:7–14. doi: 10.25968/MSI.2016.2
10. Trevisi E, Zecconi A, Cogrossi S, Razzuoli E, Grossi P, Amadori M. Strategies for reduced antibiotic usage in dairy cattle farms. *Res Vet Sci.* (2014) 96:229–33. doi: 10.1016/j.rvsc.2014.01.001
11. Pinzón-Sánchez C, Ruegg PL. Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *J Dairy Sci.* (2011) 94:3397–410. doi: 10.3168/jds.2010-3925
12. Sol J, Sampimon OC, Barkema HW, Schukken YH. Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *J Dairy Sci.* (2000) 83:278–84. doi: 10.3168/jds.S0022-0302(00)74875-2
13. Bradley AJ, Green MJ. Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparations. *J Dairy Sci.* (2009) 92:1941–53. doi: 10.3168/jds.2008-1497
14. Swinkels JM, Cox P, Schukken YH, Lam TJGM. Efficacy of extended cefquinome treatment of clinical *Staphylococcus aureus* mastitis. *J Dairy Sci.* (2013) 96:4983–92. doi: 10.3168/jds.2012-6197
15. GVA. *Guidelines for Combating Bovine Mastitis as a Stock Problem*. 5th ed. Gießen: German Veterinary Association (2012).
16. Grieger AS, Zoche-Golob V, Paduch JH, Hoedemaker M, Krömker V. Recurrent clinical mastitis in dairy cattle – importance and causes. *Tierarztl Prax Ausg G.* (2014) 42:156–62. doi: 10.1055/s-0038-1623218
17. Linder M, Paduch JH, Grieger AS, Mansion-de Vries E, Knorr N, Zinke C, et al. Cure rates of chronic subclinical *Staphylococcus aureus* mastitis in lactating dairy cows after antibiotic therapy. *Berl Munch Tierarztl Wochenschr.* (2013) 126:291–6.
18. Schmenger A, Krömker V. Characterization, cure rates and associated risks of clinical mastitis in Northern Germany. *Vet Sci.* (2020) 7:170. doi: 10.3390/vetsci7040170
19. Wente N, Grieger AS, Klocke D, Paduch JH, Zhang Y, Leimbach S, et al. Recurrent mastitis–persistent or new infections? *Vet Microbiol.* (2020) 244:108682. doi: 10.1016/j.vetmic.2020.108682
20. Krömker V, Friedrich J. Recommendations for diagnostic measures regarding mastitis control on herd level. *Prakt Tierarzt.* (2011) 92:516–24.
21. Degen, S, Paduch JH, Hoedemaker M, Krömker V. Factors affecting the probability of bacteriological cure of bovine mastitis. *Tierarztl Prax Ausg G.* (2015) 43:222–7. doi: 10.15653/TPG-141082
22. Krömker V, Paduch JH, Abograra I, Zinke C, Friedrich J. Effects of an additional nonsteroidal anti-inflammatory therapy with carprofen (Rimadyl Rind®) in cases of severe mastitis of high yielding cows. *Berl Munch Tierarztl Wochenschr.* (2011) 124:161−7.
23. McDougall S, Bryan MA, Tiddy RM. Effect of treatment with the nonsteroidal antiinflammatory meloxicam on milk production, somatic cell count, probability of re-treatment, and culling of dairy cows with mild clinical mastitis. *J Dairy Sci.* (2009) 92:4421–31. doi: 10.3168/jds.2009-2284
24. Ziesch M, Wente N, Zhang Y, Zaremba W, Engl S, Krömker V. Noninferiority trial investigating the efficacy of a nonantibiotic intramammary therapy in the treatment of mild-to-moderate clinical mastitis in dairy cows with longer lasting udder diseases. *J Vet Pharmacol Ther.* (2018) 41:11–21. doi: 10.1111/jvp.12415
25. Piaggio G, Elbourne DR, Altman DG, Pocock SJ, Evans SJW. Reporting of non-inferiority and equivalence randomized trials: an extension of the CONSORT statement. *JAMA.* (2009) 295:1152–60; 1842. doi: 10.1001/jama.295.10.1152
26. O'Connor AM, Sargeant JM, Gardner IA, Dickson JS, Torrence ME, Dewey CE, et al. The REFLECT statement: methods and processes of creating reporting guidelines for randomized controlled trials for livestock and food safety. *J Vet Intern Med.* (2010) 24:57–64. doi: 10.1111/j.1939-1676.2009.0441.x
27. EMEA. *VICH Topic GL9 (GCP): Guideline on Good Clinical Practices*. London: The European Agency for the Evaluation of Medicinal Products (2000).
28. Schukken YH, Bennett GJ, Zurakowski MJ, Sharkey HL, Rauch BJ, Thomas MJ, et al. Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. *J Dairy Sci.* (2011) 94:6203–15. doi: 10.3168/jds.2011-4290
29. Vasquez AK, Nydam DV, Capel MB, Ceglowski B, Rauch BJ, Thomas MJ, et al. Randomized noninferiority trial comparing 2 commercial intramammary antibiotics for the treatment of nonsevere clinical mastitis in dairy cows. *J Dairy Sci.* (2016) 99:8267–81. doi: 10.3168/jds.2016-11258
30. GVA *Guidelines for Aseptic Milk Sampling and Guidelines to Isolate and Identify Mastitis Pathogens*. 2nd ed. Gießen: German Veterinary Association (2009).
31. IDF. *Suggested Interpretation of Mastitis Terminology. Bulletin of the IDF 338*. (1999). Brussels: International Dairy Federation (1999).
32. Watts JL, Salmon SA, Yancey RJJ. Use of modified Rambach agar to differentiate *Streptococcus uberis* from other mastitis streptococci. *J Dairy Sci.* (1993) 76:1740–3. doi: 10.3168/jds.S0022-0302(93)77506-2
33. Wenz JR, Barrington GM, Garry FB, McSweeney KD, Dinsmore RP, Goodell G, et al. Bacteremia associated with naturally occurring acute coliform mastitis in dairy cows. *J Am Vet Med Assoc.* (2001) 219:976–81. doi: 10.2460/javma.2001.219.976
34. Erskine RJ, Wagner S, DeGraves FJ. Mastitis therapy and pharmacology. *Vet Clin North Am Food Anim Pract.* (2003) 19:109–38. doi: 10.1016/S0749-0720(02)00067-1
35. Schukken YH, Deluyker HA. Design of field trials for the evaluation of antibacterial products for therapy of bovine clinical mastitis. *J Vet Pharmacol Ther.* (1995) 18:274–83. doi: 10.1111/j.1365-2885.1995.tb00591.x
36. Deluyker HA, Chester ST, van Oye SN. A multilocation clinical trial in lactating dairy cows affected with clinical mastitis to compare the efficacy of treatment with intramammary infusions of a lincomycin/neomycin combination with an ampicillin/cloxacillin combination. *J Vet Pharmacol Ther.* (1999) 22:274–82. doi: 10.1046/j.1365-2885.1999.00205.x
37. Krömker V, Wente N, Zhang Y, Bolte J, Renner R, Schmenger A, et al. Comparison of a non-antibiotic treatment with an antibiotic treatment of chronic mastitis. *Milk Sci Int.* (2019) 72:34−8. doi: 10.25968/MSI.2019.6
38. Schukken YH, Bar D, Hertl J, Gröhn YT. Correlated time to event data: modeling repeated clinical mastitis data from dairy cattle in New York State. *Prev Vet Med.* (2010) 97:150–6. doi: 10.1016/j.prevetmed.2010.09.012
39. Cha E, Hertl J, Schukken Y, Tauer L, Welcome F, Gröhn Y. Evidence of no protection for a recurrent case of pathogen specific clinical mastitis from a previous case. *J Dairy Res.* (2016) 83:72–80. doi: 10.1017/S002202991500062X
40. Powers JH, Fleming TR. Non-inferiority trials: clinical understandings and misunderstandings. *Clin Invest.* (2013) 3:215–8. doi: 10.4155/cli.12.157

41. Ruegg PL. *The Application of Evidence Based Veterinary Medicine to Mastitis Therapy*. Santiago: World Buiatrics Congress (2010).

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# Intramammary Immunisation Provides Short Term Protection Against *Mannheimia haemolytica* Mastitis in Sheep

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Mastitis affects both dairy and meat/wool sheep industries with losses due to reductions in milk quality and quantity, increased treatment costs and restricted lamb growth. Effective vaccines would be important tools for mastitis control. However, the development of vaccines against mastitis has proved challenging due to the failure to target protective immunity to the mammary gland. In order to target responses to the mammary gland, this study tested whether local administration directly into the gland through the teat canal or in the udder skin confers protection against an intramammary infection. In this study, we tested a vaccine that confers protection against respiratory disease caused by *Mannheimia haemolytica* to determine if it also protects against intramammary infection by the same organism. No evidence of protection was observed in animals that received a subcutaneous immunisation in the udder skin, however, intramammary immunisation provided almost complete protection against an experimental challenge administered 7 days post immunisation but not if the challenge was delivered 14 days post immunisation. To investigate further the nature of this variation in response, the somatic cell count and concentration of cytokines Interleukin-1β, Interleukin-10 and Interleukin-17A was determined in milk over the course of each study. Intramammary immunisation induced an inflammatory response within the mammary gland, characterised by increases in SCC and in the production of cytokines IL-1β, IL-10, and IL-17A. This response was similar to that observed in un-vaccinated control animals post challenge. The SCC and cytokine levels had returned to levels comparable with un-vaccinated controls prior to challenge at both 7 and 14 days post immunisation. The transient nature of the protective effect is consistent with the priming of an innate antibacterial response within the mammary gland which provides protection against challenge at 7 days but is diminished by 14 days post-vaccination. Further studies are planned to determine the nature of the innate immune mechanisms associated with the protective effect described here to determine whether it may be exploited to improve ruminant udder health.

**Keywords: mastitis, ovine, *Mannheimia haemolytica*, intramammary immunisation, Interleukin-17A**

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## INTRODUCTION

Mastitis or inflammation of the mammary gland is most often caused by an intramammary infection (IMI) by a range of Gram-positive and negative species of bacteria. IMIs impact both dairy and meat/fibre sheep production systems worldwide through reduction in the quantity and quality of milk production. In suckling flocks the consequence of an IMI is a slowing of the growth rate of lambs with a corresponding increase in the time required to achieve target weights (1). Mastitis appears in a number of forms including a long lasting subclinical inflammatory form or a more acute clinical form which is a painful inflammatory condition with clear animal welfare implications and which may lead to the death or premature culling of animals from affected flocks (2). Accurate estimates of the economic losses attributed to ovine mastitis are not available, although it has been calculated in the UK alone that a reduction of just 10% in ovine mastitis cases would save £2.7 million per annum (3).

The most common bacterial species associated with ovine mastitis are *Staphylococcus aureus*, *Streptococcus uberis*, *Mannheimia haemolytica*, and several species of coagulase negative staphylococci (CNS) (4, 5). In meat production systems *M. haemolytica* is one of the most frequently identified causes of mastitis (5–8).

Several risk factors that predispose animals to mastitis have been identified. These include poor conformation of the udder, teat lesions, litter size (two or more lambs), poor body condition of the ewe and previous cases of mastitis (9). Suckling lambs have also been identified as a possible source of intramammary infections as young animals can carry the *M. haemolytica* bacteria in the upper respiratory tract (10).

As with cattle dairy, control of mastitis in sheep dairy systems relies on reducing the impact of bacterial contamination of the milking environment and rapid treatment of clinical cases. If implemented effectively such measures can substantially reduce the impact of mastitis in a flock (11). However, in more extensive sheep meat and fibre production systems the options available to the farmer are more limited. These include culling of old or previously affected ewes and breeding for an udder conformation that minimises the risk of teat damage and contamination of the teat apex (9, 12). The treatment of infected animals relies on antimicrobial drugs. Although the usage of antimicrobials in sheep farming does not appear to be as high as in other farmed animals (13) it may still represent a risk for the induction of antimicrobial resistance. Thus, there is a requirement to reduce the use of antimicrobials and the development of alternative mastitis prevention strategies including vaccines (14).

Despite many attempts at developing mastitis vaccines for dairy ruminant species, few are currently available and all target disease in dairy cattle. Notable examples include vaccines against *Escherichia coli* (15) and *S. aureus* (16). As these vaccines appear to reduce the clinical symptoms of mastitis but not the infection rate, current research is focused on vaccines that induce not only a strong humoral response but also a cellular response within the mammary gland (17). Recent studies suggest that a cellular response may be key in clearing an intramammary infection (17–19), specifically a Th17 type response (20, 21). The Th17 response targets extracellular bacteria by enhancing innate immune mechanisms such as phagocyte activity and the production of antimicrobial peptides by mammary epithelial cells (22). Also several studies suggest that delivery of the vaccine directly into the mammary gland as opposed to a systemic route, may enhance its efficacy (23–25).

Vaccines which protect sheep from the respiratory disease caused by *M. haemolytica* have been available for many years. Such vaccines consist of several serotypes of *M. haemolytica* grown in iron deficient medium. This induces expression of iron regulated proteins on the surface of the bacteria (26). These proteins are immunogenic and induce a protective response in immunised animals. The vaccine is generally administered to pregnant ewes in order to induce colostral antibodies which protect their lambs during the first few weeks of their life. Despite its routine use there are no studies reported which investigate the potential of these vaccines to protect against mastitis caused by *M. haemolytica*. Therefore, the aim of this study was to test whether a vaccine originally developed against the respiratory disease caused by *M. haemolytica* also provides protection against mastitis when administered in the mammary gland's subcutaneous tissue or directly infused into the mammary gland through the teat canal.

## MATERIALS AND METHODS

### Animals

Lactating Scottish Mules between 2 and 6 years old and ∼1 month into lactation were sourced from the Moredun Research Institute (MRI) flock. Prior to enrolling into each study, milk samples from each mammary half were screened for pre-existing intramammary infection by bacterial culture as described by Zadoks et al. (5). Animals were housed with their lambs in loose pens with straw bedding and had access to water and grass hay *ad libitum* and concentrate was fed twice daily. After weaning animals were hand milked once (study 1) or twice (studies 2 and 3) each day to maintain lactation. All studies were conducted under UK Government Home Office licence following approval of the MRI Animal Welfare and Ethical Review Body (AWERB) in accordance with the Animals (Scientific Procedures) Act 1986.

### Immunisation Regime

We conducted three studies to test whether local administration of the Ovipast plus vaccine (MSD animal health, Milton Keynes, UK) confers protection against an intramammary infection caused by *M. haemolytica.* The Ovipast plus vaccine includes several serotypes of *M. haemolytica* and *Bibersteinia threalhosi* grown in an iron deficient medium prior to formalin inactivation. Aluminium hydroxide is used as an adjuvant with this vaccine preparation. As part of standard flock management, this vaccine is used in the control of the systemic and respiratory disease caused by these bacterial species in ewes and lambs. All ewes are immunised between 4 and 6 weeks prior to parturition by subcutaneous injection in the lateral side of the upper neck, so are not immunologically naïve. In our studies, animals were boosted with the same vaccine 24 h after weaning by one of the two routes


**FIGURE 1 |** Overview of the study design. Lambs were weaned from their mothers 24 h prior to immunisation at D1. Each ewe was immunised subcutaneously in the udder skin (study 1) or directly into the mammary gland through the teat canal (studies 1, 2, and 3). Animals were challenged by infusion of the teat canal with *M. haemolytica* at D8 (study 1 and 2), or at D15 days (study 3). Animals were followed for 7 days after challenge.

detailed below. An overview of the time line of each study is described in **Figure 1**.

### Study 1

The effect of local immunisation with the Ovipast vaccine when delivered directly into the mammary gland through the teat canal or subcutaneously over the supramammary lymph node was tested in study 1 in 29 sheep. Both mammary halves were stripped of milk and the teat ends disinfected with cotton wool swabs soaked in 70% (vol/vol) ethanol. Two milliliters of vaccine were administrated subcutaneously each side of the mammary gland, ∼5 cm from the supramammary lymph node ($n = 10$) or directly into the mammary gland ($n = 10$) using a J-12 teat infusion cannula (Jorgens Laboratories, Loveland, USA). The control group ($n = 9$), did not receive the vaccine. One animal was removed from the control group due to an unrelated IMI detected prior to immunisation. The efficacy of the vaccine was tested after 7 days by infusion of the teat canal with *M. haemolytica*.

### Study 2

Study 2 was conducted to ensure reproducibility of the intramammary vaccination data observed in the first study. In study 2, group sizes were reduced to five animals based on power calculations using data from study 1 in accordance with the principles of the 3Rs (27). Two milliliters of vaccine were administrated directly into the mammary gland via a teat infusion cannula ($n = 5$). The control group ($n = 5$) did not receive the vaccine.

### Study 3

Study 3 was conducted to test the efficacy of intramammary vaccination 14 days after administration. In this study, a total of 10 animals were used in two groups of 5 animals. Two milliliters of vaccine were administrated directly into the mammary gland via a teat infusion cannula in the experimental group ($n = 5$) and the control group ($n = 5$) did not receive the vaccine.

## Intramammary Challenge With *Mannheimia haemolytica*

To test whether immunisation with the Ovipast vaccine confers protection against mastitis, all animals were challenged directly into the mammary gland via the teat canal with *M. haemolytica* FSL T1-008. FSL T1-008 was isolated from a case of sheep mastitis (5) and in our preliminary study it was shown to cause clinical mastitis in lactating ewes when infused into the mammary gland via the teat canal (data not shown). Animals were challenged in both mammary halves with ∼2,000 cfu. The challenge was delivered either 7 days (studies 1 and 2) or 14 days (study 3) after immunisation (**Figure 1**).

### Preparation and Administration of the Bacterial Challenge

The bacterial inoculum was prepared from stock cultures stored at −80°C. A stock culture was thawed, plated on 5% sheep blood agar (E&O Laboratories, Bonnybridge, UK) and incubated overnight at 37°C to check for viability and colony purity. Two to five colonies were inoculated into 45 ml of nutrient broth (Difco, Cambridge, UK) and incubated for 14 h at 37°C with 150 rpm shaking. Based on preliminary experiments the bacterial suspension was diluted in sterile phosphate buffered saline (PBS) to the target concentration of ∼1,000 cfu/ml. The actual inoculum concentration was checked by the viable count method. A series of 10-fold dilutions were prepared in sterile PBS and three 10 µl drops per dilution plated onto horse blood agar plates (E&O Laboratories) and incubated overnight at 37°C. Colonies were counted when they were in the range of 5–50 cfu per spot, and the bacterial concentration for each time point (cfu/ml) was calculated based on average colony counts for the appropriate dilution. Prior to challenge, sheep were milked, the teat ends disinfected and 2 ml of inoculum (target dose: 2,000 cfu/mammary half) infused into each mammary half. After infusion sheep were returned to their pen.

## Data and Sample Collection

Milk samples collected throughout the study were used for qualitative and quantitative bacteriology, measurement of the somatic cell count (SCC) and quantification of pro and anti-inflammatory cytokines levels. Milk samples for bacteriological analysis were collected using aseptic technique (28) and stored on ice until refrigeration at 4°C.

## Qualitative and Quantitative Bacteriology Analysis

For qualitative bacteriology, 10 µl of milk from each mammary half were plated on 5% horse or sheep blood agar (E&O Laboratories) and incubated at 37°C for 24 h. Identification of bacterial species was based on morphology and standard biochemical tests including Gram staining, catalase test, and esculin splitting. For quantitative analysis, milk samples were serially diluted 10-fold in sterile PBS. Triplicate 10 µl aliquots of each dilution were spotted on blood agar plates (E&O Laboratories), allowed to air dry and incubated overnight at 37°C. Colonies with morphology consistent with *M. haemolytica* were counted, if possible for the dilution showing between 5 and 50 cfu per spot. The bacterial concentration in milk (cfu/ml) was calculated based on average colony counts for appropriate dilutions.

## SCC and Preparation of Skimmed Milk for Cytokine Analysis

Approximately 35 ml of milk from each mammary half was collected in a 50 ml Falcon tube (Corning, Amsterdam, The Netherlands) and stored at 4°C until analysis. Five millilitres of milk was used for SCC no later than 48 h after collection using a DeLaval DCC Cell counter (DeLaval, Cardiff, UK). Samples were diluted up to 10-fold in PBS when the cellular concentration in the neat sample was beyond the detection limit. Cell count data is presented as cells/ml. The remaining 30 ml of milk were centrifuged at 3,500 × g at 4°C for 20 min. The fat layer was discarded and the supernatant was transferred to a new 50 ml Falcon tube. Centrifugation was repeated and the supernatant stored at −80°C.

## Measurement of Cytokines in Milk

The concentration of cytokines Interleukin-1β (IL-1β), Interleukin-10 (IL-10) and Interleukin-17A (IL-17A) were measured in skimmed milk samples by sandwich ELISA tests using the antibodies detailed in **Table 1**. Microtitre plates (Immunolon 2 HB, Thermo Electron Corporation, Langenselbold, Germany for IL-1β and IL-10 ELISAs and Elisa Medium Binding M129A, Greiner Bio-One, Kremsmünster, Austria for the IL-17A ELISA) were coated overnight at 4°C with 100 μl/well of the appropriate coating antibody in 0.5 M carbonate buffer (0.5 M $Na_2CO_3$, 0.5 M $NaHCO_3$, pH 9.6) at concentrations detailed in **Table 1**. Wells were washed with washing buffer (PBS, pH 7.4 and 0.05% vol/vol Tween 20) and non-specific binding sites blocked with 300 μl/well of PBS containing 3% (wt/vol) BSA and 0.05% (vol/vol) Tween 20 at room temperature for 1 h. Plates were washed and incubated for 1 h at room temperature with 100 μl/well of skimmed milk. Each sample was tested in duplicate. When necessary, samples were diluted with PBS supplemented with 0.05% (vol/vol) Tween 20 and 1% (wt/vol) BSA (reagent diluent). A standard curve of known cytokine concentrations was determined using dilutions of appropriate standards. Recombinant bovine standards were used for IL-1β (Bio-Rad-antibodies), and IL-17A (Kinghfisher Biotech, Saint Paul, USA). For IL-10 a recombinant ovine standard was provided by Sean Wattegedera (MRI, UK). ELISA plates were washed and 100 μl/well of detection antibody (**Table 1**) added, followed by incubation for 1 h at room temperature. For IL-10 and IL-17A, 100 μl/well of horseradish peroxidase (HRP)-streptavidin (Sigma-Aldrich) diluted 1:500 in reagent diluent were added, followed by incubation for 45 min at room temperature. IL-1β plates were incubated with 100 μl/well of HRP-conjugated polyclonal goat anti-rabbit immunoglobulins (Dako, Ely, UK) diluted 1:1,000 in reagent diluent for 1 h at room temperature. After incubation with HRP-streptavidin or HRP-conjugated antibody, plates were washed and incubated for 20 min at room temperature with 100 μl per well of o-Phenylenediamine dihydrochloride substrate (Sigma-Aldrich). The reaction was stopped with 25 μl per well of 2.5 M $H_2SO_4$ and optical density measured at 492 nm using a sunrise absorbance reader (Tecan, Theale, UK). Cytokine concentrations in skimmed milk samples were then calculated from the standard curve.

## Statistical Analysis

The effect of vaccination on bacterial counts, SCC and cytokine concentration was assessed using mixed models, which account for correlation between repeated observations from the same animal. The data from studies 1 and 2 were analysed together, as inoculation and challenge were administered at the same time in each experiment, with a random intercept included to account for potential differences between the two studies. Data from study 3 was analysed separately, as the time of challenge was different in this study. In all models, time, treatment and the interaction between time and treatment were considered as potential explanatory variables (in both zero and non-zero parts of zero-inflated models) and random intercepts were included for each animal. Model selection was carried out using likelihood ratio tests (LRTs) with final models fitted using restricted maximum likelihood (REML). The false discovery rate method was used to adjust for multiple comparisons across levels of several factors. All statistical analyses were conducted in R 4.0.2 (29). The models fitted to each response variable are explained in more detail below.

Bacterial counts were transformed (ln+1) and analysed using a zero-inflated linear mixed model (LMM) with random intercepts considered to account for differences between experiments 1 and 2 and differences between animals. A zero-inflated LMM was used to account for the fact that a large proportion (63%) of the non-missing values following challenge were zero. By fitting a zero-inflated model we fit models to the zero-generating process and the non-zero values separately. The models were fitted using the R package glmmTMB (30).

SCC data were transformed (ln) and analysed using generalised additive mixed models (GAMMs) fitted to the log of SCC. GAMMs fit smooth splines to capture potentially nonlinear relationships between the explanatory variables (time and treatment) and the response (SCC). GAMMs were fitted using the mgcv package in R (31). The fitted model included separate thin plate splines fitted to each treatment group and separate factor smooth splines with equivalent levels of smoothness fitted to each animal to account for variability in the trajectories of individual animals.

A linear mixed effects model was also fitted to the log-transformed SCC data to check for statistically significant differences specifically at time point 8 (immediately pre-challenge) in studies 1 and 2 and at time point 15 in study 3.

Cytokine concentrations were transformed (ln+1) and analysed using (zero-inflated linear) mixed models, as for bacterial counts. Given the clear non-linearity in data a GAMM was considered, however there were insufficient time points to fit such a model. Consequently, time was included as a categorical (factor) variable in the analysis and comparisons between treatment groups were made at each time point. To minimise the loss in power associated with multiple comparisons and to avoid issues arising from complete separation (all responses as zero or non-zero in some groups), the measurements taken before challenge were assigned to a "pre-challenge" category (rather

**TABLE 1 |** Details of antibodies used for cytokine ELISAs.

| Target | Antibody | Isotype, Origin | Conc. (μg/ml) | Source |
|---|---|---|---|---|
| Ovine IL-1β Coating | 1D4 | IgG1, mouse | 1 | Bio-Rad-Antibodies |
| Ovine IL-1β Detection | Polyclonal | n/a, rabbit | 2 | Bio-Rad-Antibodies |
| Bovine IL-10 Coating | CC318 | IgG2b, mouse | 4 | Bio-Rad-Antibodies |
| Bovine IL-10 Detection | Biotinylated CC320 | IgG1, mouse | 1 | Bio-Rad-Antibodies |
| Bovine IL-17A Coating | Polyclonal | n/a, rabbit | 2 | Kingfisher Biotech |
| Bovine IL-17A Detection | Biotinylated polyclonal | n/a, rabbit | 1 | Kingfisher Biotech |

than carrying out separate comparisons at the four pre-challenge timepoints). Consequently, there were five timepoints considered in each study (pre-challenge, day 9, 10, 12, and 14 for studies 1 and 2 and pre-challenge, day 16, 17, 19, and 21 for study 3).

For IL-1β and IL-17A a zero-inflated LMM was used, as for bacterial counts. For IL-10 data from studies 1 and 2 a zero-inflated generalised LMM with a Tweedie error distribution was used. The Tweedie distribution was chosen to better satisfy the assumptions regarding the distribution of residuals, as the non-zero values were typically small. A standard LMM resulted in confidence intervals for estimated IL-10 concentrations that included negative values (which was nor observed under a Tweedie distribution). In the case of the IL-10 data from experiment 3 a LMM with no zero-inflation component was fitted to only the data from day 16 onwards as the data prior to this was almost exclusively zero (creating model fitting issues due to complete separation) but from day 16 onwards there were few zeroes.

The output of the models used is shown in **Supplementary Tables 1–10**.

# RESULTS

## Clinical and Bacteriology Data

To test whether immunisation targeted to the mammary gland provided protection against mastitis caused by *M. haemolytica,* we immunised and challenged groups of sheep in a series of studies over a 2 year period. All unvaccinated control animals developed an IMI in one or both mammary halves following intramammary *M. haemolytica* challenge. In total, 15 of the 18 control mammary halves were bacteriologically positive 24 h post challenge (PC) in study 1 (**Figure 2A**) and all 10 control mammary halves were bacteriologically positive 24 h PC in studies 2 and 3 (**Figures 2B,C**). The number of the infected mammary halves in control animals showed a reduction from 48 h PC. By the end of the study, 5/18, 4/10, and 3/10 halves remained bacteria positive in the control groups of all three studies, respectively (**Figures 2A–C**).

In the first study we tested two vaccine delivery routes, subcutaneous in the udder skin and an intramammary administration directly into the mammary gland via the teat canal. No evidence of early protection against challenge was observed in animals that received the subcutaneous vaccination, with 16 of the 20 mammary halves infected at 24 h PC (**Figure 2A**). In contrast to the subcutaneous and control groups, all intramammary immunised animals appeared fully protected at 24 h PC. This group remained protected until the end of the study 1 week PC (**Figure 2A**). On repeating this in study 2, an additional five animals received the vaccine via the intramammary route. Of these, one of the 10 challenged halves developed an infection (**Figure 2B**). In total, of the 30 mammary halves in studies 1 and 2 that received the vaccine via the intramammary route only 1 developed an infection at 24 h PC compared to 25 of the 28 control halves. However, in the third study when the challenge was delivered 2 weeks post immunisation this impressive level of protection was not observed. On this occasion, 9 of the 10 mammary halves became infected (**Figure 2C**).

The quantitative analysis of bacteria in milk samples from control animals peaked at 24 h in all three studies (**Figures 2D–F**). Bacterial counts gradually decreased thereafter with several control animals clearing the infection by the end of the study (**Figures 2D–F**).

Of the animals immunised directly into the mammary gland through the teat canal in study 1, very few bacteria were recovered in milk samples after challenge (**Figure 2D**). The mean bacterial concentration across the intramammary immunised animals in study 2 reached a peak of $3.13 \times 10^3 \pm 8.27 \times 10^3$ cfu/ml 48 h PC (**Figure 2E**) $13.8 \times 10^3$ fold lower than the control values of $4.34 \times 10^8 \pm 4.04 \times 10^8$.

The bacterial concentration in milk samples from animals subcutaneously immunised in study 1 followed a similar pattern to those observed in un-vaccinated control animals. However, the peak bacterial concentration ($2.95 \times 10^7 \pm 4.37 \times 10^7$ cfu/ml) at 24 h PC was ∼14 fold lower than the peak concentration of $4.34 \times 10^8 \pm 4.04 \times 10^8$ observed in the unvaccinated control group at the same time point (**Figure 2D**).

The final fitted LMM for studies 1 and 2 included time after challenge and a quadratic term for time after challenge as fixed effects in the non-zero model and treatment and time after challenge as fixed effects in the zero model. Random intercepts for animal were included in both parts of the model and a random intercept for experiment was included in the non-zero part of the model. Both the zero and non-zero parts of the model indicate that subcutaneous vaccination did not significantly reduce the *S. uberis* bacterial concentration compared to the non-immunised controls (**Supplementary Table 1**). However, there was strong evidence that the intramammary vaccine reduced the number of animals shedding bacteria after challenge ($p < 0.001$).

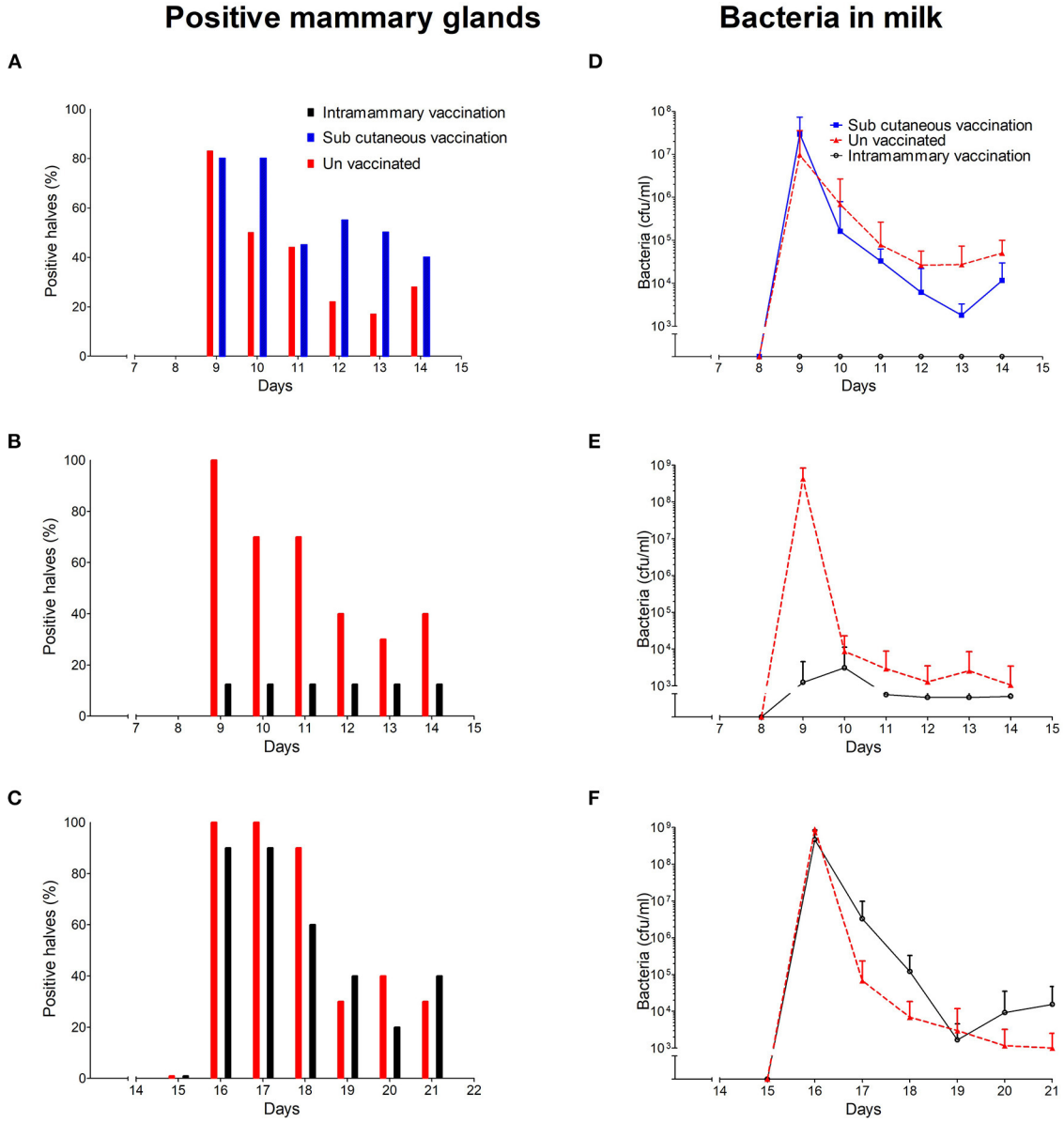


**FIGURE 2 |** The percentage of *M. haemolytica* positive mammary glands in study 1 **(A)**, study 2 **(B)**, and study 3 **(C)**. The concentration of *M. haemolytica* in milk from study 1 **(D)**, study 2 **(E)**, and study 3 **(F)**. Average bacterial concentrations with standard deviations are shown.

In contrast to animals in studies 1 and 2 which were immunised and challenged after 1 week, bacterial concentrations in milk samples from animals challenged 2 weeks post vaccination followed a similar pattern to that observed in the control animals (**Figure 2F**). In this group the maximal mean bacterial concentration of $9 \times 10^8 \pm 2 \times 10^8$ cfu/ml was observed 24 h PC, a concentration ∼2-fold higher than that observed in control animals ($4.1 \times 10^8 \pm 1.5 \times 10^8$). Thereafter, the average bacterial concentration in milk decreased. The statistical analysis showed no differences in the mean concentration between the vaccinated and the control group both in the count and zero inflated part of the model (**supplementary Table 2**).

## Somatic Cell Response to Immunisation and Challenge

The SCC increased in all three studies in response to intramammary immunisation compared with the unvaccinated controls. The increase was observed 24 h after immunisation in studies 1 and 2 and reached a peak of $9.49 \times 10^6 \pm 4.06 \times 10^6$ and $5.57 \times 10^6 \pm 2.85 \times 10^6$ cells/ml, respectively at 72 h (**Figures 3A,B**). This represented an increase of 1.4 and 3.8-fold compared with pre-immunisation levels. The SCC remained statistically significantly higher in intramammary immunised animals compared with control and subcutaneously immunised animals until day 7 ($p < 0.001$, **Supplementary Figure 1**). Prior

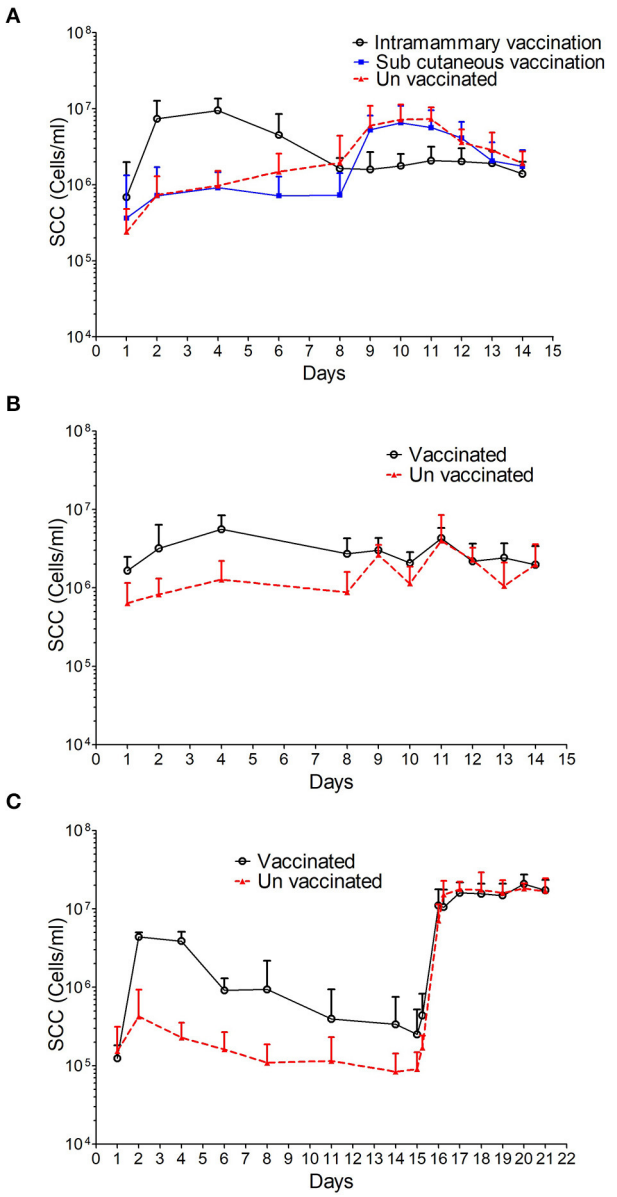


**FIGURE 3 |** SCC data from sheep immunised and challenged with *M. haemolytica*. The average SCC in cells/ml with standard deviation is shown for study 1 **(A)** where three groups are represented: i, intramammary immunisation; ii, sub-cutaneous immunisation and iii, unvaccinated controls; study 2 **(B)** where two groups are represented: i, intramammary immunisation and ii, un-vaccinated controls and study 3 **(C)** where 2 groups are represented: i, intramammary immunisation and ii, unvaccinated controls.

to challenge at day 8 no statistically significant differences were observed between immunised and control groups ($p = 0.050$).

In study 3 the SCC increased at 24 h post-immunisation in the intramammary group with a peak concentration of $4.39 \times 10^6 \pm 6.38 \times 10^5$ cells/ml, which was 35-fold higher than the pre-immunisation levels. The SCC decreased thereafter and by day 8 was comparable to that observed in the unvaccinated control group prior to challenge ($p = 0.184$).

No differences in SCC were observed in animals that received the subcutaneous immunisation in study 1 compared to the control animals (**Supplementary Figure 1**).

The SCC increased in all control animals in response to intramammary challenge (**Figures 3A–C**). At 24 h post-challenge the mean SSC corresponded to $6.01 \times 10^6 \pm 4.88 \times 10^6$ and $2.63 \times 10^6 \pm 9.02 \times 10^5$ cells/ml in studies 1 and 2, respectively, representing a 2-fold increase when compared with pre-challenge levels. In study 3, the SCC changed from $9.06 \times 10^4 \pm 5.83 \times 10^4$ cells/ml to $7.1 \times 10^6 \pm 4.25 \times 10^6$ cells/ml representing an 80-fold increase when compared with pre-challenge levels and remained at this level until the end of the study. In studies 1 and 2, a 3 and 1.5-fold decrease, respectively in the mean SCC was observed in control animals at 6 days PC. The SCC in animals subcutaneously immunised in study 1 followed a pattern similar to that observed in control animals with a 7-fold increase in count from $7.34 \times 10^5 \pm 6.94 \times 10^5$ to $5.28 \times 10^6 \pm 2.86 \times 10^6$ cells/ml 24 h PC.

In studies 1 and 2, no increase in SCC was observed following challenge in those animals that received an intramammary immunisation. In contrast, the SCC in animals immunised in study 3 followed a pattern similar to that observed in control animals with a 40-fold increase in cell counts from $2.5 \times 10^5 \pm 2.71 \times 10^5$ to $1.11 \times 10^7 \pm 6.75 \times 10^6$ cells/ml at 24 h PC (**Figures 3A–C**). Overall, despite the variability in the SCC after challenge no statistically significant differences were found between vaccinated animals and control animals after the challenge (**Supplementary Figure 1**).

Cytokine responses in milk following immunisation and intramammary challenge

In order to understand the specific mechanisms behind the protective immune response observed when animals were challenged 7 days post immunisation we measured the concentration of some pro and anti-inflammatory cytokines in milk samples from studies 1 to 3. Changes in IL-1β, IL-10, and IL-17A concentrations are shown in **Figure 4** and actual values at key time points are shown in **Table 2**. The output of the models used is reported in **Supplementary Tables 5–10**.

## Interleukin-1β

During the pre-challenge period low levels of IL-1β were detected in milk samples from control and vaccinated animals in all three studies (**Table 2**). In studies 1 and 3, the concentration of IL-1β increased 14 and 7-fold, respectively at 24 h post intramammary immunisation when compared to pre immunisation levels (**Figures 4A,D,G**). No increase in IL1β was detected in animals immunised via the intramammary route in study 2. Similarly, no increase in IL-1β was observed in animals which received the subcutaneous immunisation in study 1.

Over the course of all three studies, pre challenge concentrations of IL-1β in immunised animals were not statistically different from control animals (**Supplementary Tables 5,6**). Following challenge, IL-1β concentrations increased in control animals from all three studies (**Figures 4A,D,G** and **Table 2**). Statistically significant lower concentrations of IL-1β were observed in intramammary immunised animals when compared to controls 24 h after

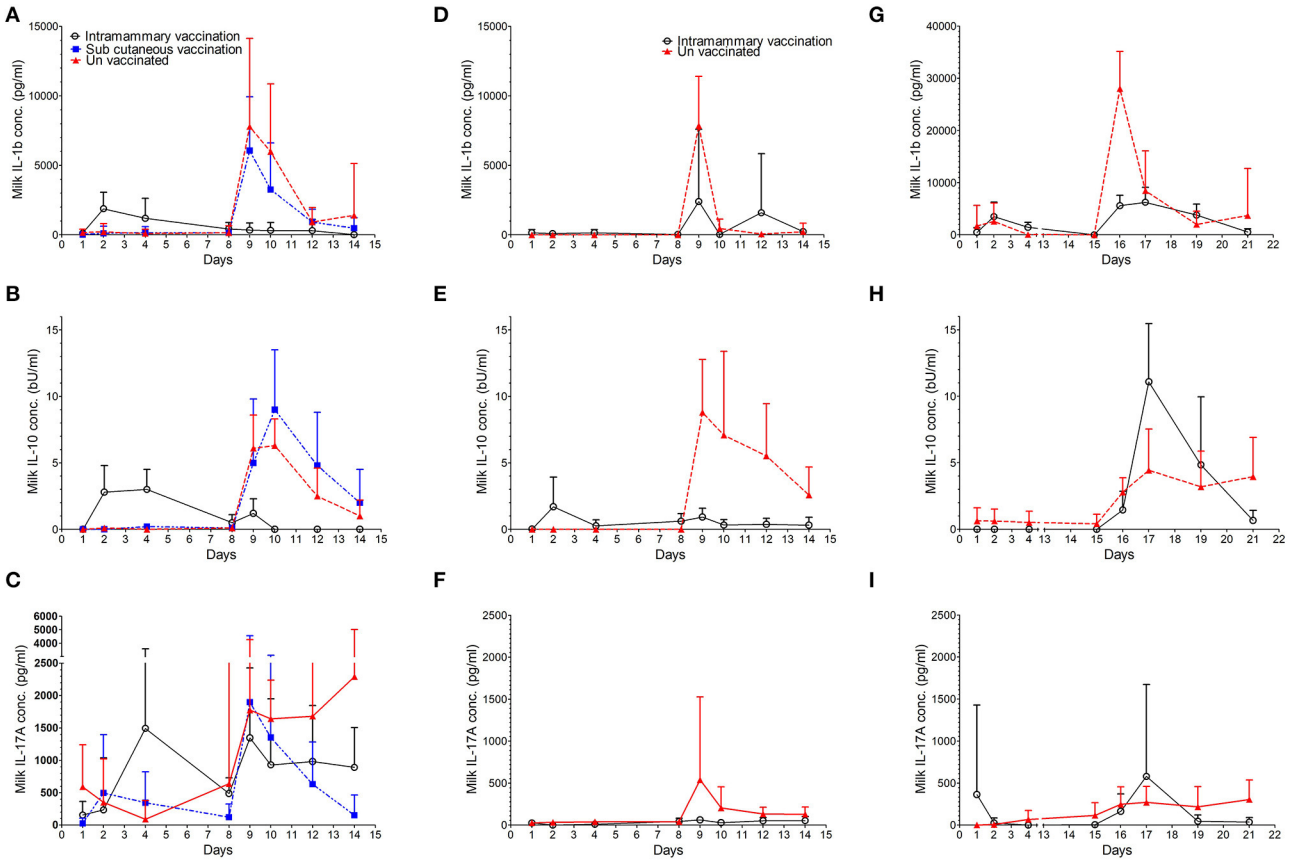


**FIGURE 4 |** Concentration of cytokines IL-1β, IL-10, and IL-17A in milk. The average concentration with standard deviation is shown for IL-1β in study 1 **(A)**, study 2 **(D)**, and study 3 **(G)**. The average concentration with standard deviation is shown for IL-10 in study 1 **(B)**, study 2 **(E)**, and study 3 **(H)**. The average concentration with standard deviation is shown for IL-17A in study 1 **(C)**, study 2 **(F)**, and study 3 **(I)**.

challenge ($p < 0.001$ in studies 1 and 2 and $p = 0.024$ in study 3). In study 3, a further increase of IL-1β was observed in control animals at day 6 when compared to animals that received the intramammary vaccination ($p = 0.034$).

## Interleukin-10

During the pre-challenge period low levels of IL-10 were detected in milk samples from control and vaccinated animals in all three studies (**Supplementary Tables 7**, **8**). IL-10 concentrations increased from 24 h post intramammary immunisation in studies 1 and 2 (**Figures 4B,E,H** and **Table 2**) peaking at 72 h post immunisation in animals intramammary vaccinated (**Table 2**). In these experiments no increase of IL-10 was observed following subcutaneous immunisation or in control animals. No increase of IL-10 following vaccination was observed in experiment 3 in either the vaccinated or the control animals.

In experiments 1 and 2 this resulted in a higher level of IL-10 in animals that received the intramammary immunisation compared with the control animals ($p < 0.001$). The concentration of IL-10 in study 3 could be assessed only post challenge due to model fitting issues stemming from a high proportion of zero responses pre-challenge.

Following challenge, an increase in IL-10 was observed in control animals from all three studies. This resulted in statistically significant lower levels of IL-10 in intramammary immunised animals compared to controls at 24 h ($p < 0.001$), 48 h ($p < 0.001$), and 72 h ($p = 0.008$) PC (**Figures 4D–F**) in studies 1 and 2. In study 3, however, an increased IL-10 concentration compared to the controls was observed in intramammary immunised animals 48 h PC ($p = 0.046$). This was reversed 6 days post-challenge when control animals had a statistically significantly higher concentration of this cytokine compared to the intramammary immunised animals ($p = 0.046$).

## Interleukin-17A

During the pre-challenge period low levels of IL-17A were detected in milk samples from control and vaccinated animals in all three studies (**Supplementary Tables 9**, **10**). IL-17A concentration increased in study 1, 24 h following intramammary immunisation. Levels peaked at 72 h representing a 10-fold

**TABLE 2 |** Concentration of milk cytokines above the baseline pre-vaccination, post-vaccination peak, and post-challenge peak.

| | Pre-vaccination conc. (Day1) | Post-vaccination peak conc. and peak time (Day) | Post-challenge peak conc. and peak time (Day) |
|---|---|---|---|
| **Interleukin-1β (pg/ml)** | | | |
| Experiment 1 | | | |
| Intramammary vaccination | NA | 1882 ± 1117 **(2)** | NA |
| Subcutaneous vaccination | NA | NA | 6076 ± 3749 **(9)** |
| Controls | NA | NA | 7788 ± 6139 **(9)** |
| Experiment 2 | | | |
| Intramammary vaccination | NA | NA | 2401 ± 5204 **(9)** |
| Controls | NA | NA | 7861 ± 3547 **(9)** |
| Experiment 3 | | | |
| Intramammary vaccination | 486 ± 875 | 3501 ± 2760 **(2)** | 6245 ± 2858 **(17)** |
| Controls | 1715 ± 3927 | 2589 ± 3504 **(2)** | 28058 ± 7088 **(16)** |
| **Interleukin-10 (bu/ml)** | | | |
| Experiment 1 | | | |
| Intramammary vaccination | NA | 3.19 ± 1.31 **(4)** | 1.23 ± 1.26 **(9)** |
| Subcutaneous vaccination | NA | NA | 8.67 ± 4.29 **(10)** |
| Controls | NA | NA | 6.27 ± 1.93 **(10)** |
| Experiment 2 | | | |
| Intramammary vaccination | NA | 1.70 ± 2.23 **(2)** | 0.93 ± 0.65 **(9)** |
| Controls | NA | NA | 8.79 ± 3.98 **(9)** |
| Experiment 3 | | | |
| Intramammary vaccination | NA | NA | 11.09 ± 4.38 **(17)** |
| Controls | NA | NA | 4.44 ± 3.09 **(17)** |
| **Interleukin-17A (pg/ml)** | | | |
| Experiment 1 | | | |
| Intramammary vaccination | NA | 1317 ± 2067 **(4)** | 1359 ± 1086 **(9)** |
| Subcutaneous vaccination | NA | 507 ± 790 **(2)** | 1913 ± 2326 **(9)** |
| Controls | 629 ± 679 | NA | 1725 ± 2240 **(14)** |
| Experiment 2 | | | |
| Intramammary vaccination | NA | NA | NA |
| Controls | NA | NA | 540 ± 989 **(9)** |
| Experiment 3 | | | |
| Intramammary vaccination | 364 ± 1066 | NA | 580 ± 1094 **(17)** |
| Controls | NA | NA | 305 ± 231 **(21)** |

*For each experimental group the average concentration, standard deviation and peak day is shown.*

increase compared with pre-immunisation levels (**Table 2**). In animals which received a subcutaneous immunisation, an 18-fold increase in IL-17A was observed 24 h post immunisation (**Figure 4C**; **Table 2**). No increase of IL-17A levels was observed in intramammary vaccinated animals in studies 2 and 3. No increase of IL-17A was observed during the pre-challenge period in control animals in the three experiments.

Despite this variability, there were no statistically significant differences in the pre challenge levels of IL-17A between the vaccinated and the control animals in the three experiments (**Supplementary Tables 9, 10**). Following challenge, IL-17A levels increased in the control animals from all three studies. The increase was detected 24 h PC which coincided with the maximal levels in study 2 (**Figure 4F** and **Table 2**). In studies 1 and 3 IL-17A stayed at elevated levels until the end of the study (**Figures 4C,I**). This resulted in a statistically significant higher concentration compared to the intramammary immunised animals. This difference was detected in studies 1 and 2, 24 h PC ($p = 0.008$), 48 h PC ($p = 0.015$), 96 h PC ($p = 0.018$), and 144 h PC ($p = 0.008$). In study 3, however, the increase of IL-17A in control animals compared to the intramammary immunised ($p < 0.001$) animals was observed only 6 days PC ($p = 0.036$). No differences in the concentration of the animals vaccinated subcutaneously was observed in comparison to the control animals (**Supplementary Table 9**).

## DISCUSSION

The aim of this study was to test whether local administration of a vaccine, originally developed to protect sheep against respiratory disease caused by *M. haemolytica*, would also protect against an IMI caused by the same bacterial species. We initially tested two different administration routes; subcutaneous in an area located close to the supramammary draining lymph nodes and intramammary, which consisted of infusion of the vaccine directly into the mammary gland through the teat orifice. The results clearly demonstrated that intramammary immunisation protected against subsequent bacterial challenge with *M. haemolytica*. In two independent studies, animals which received the vaccine through the intramammary route did not develop intramammary infections following challenge. Over these studies, only 2 of the 24 mammary halves that were immunised tested positive for bacteria 1 week post challenged. In comparison, 28 out of the 30 control mammary halves developed intramammary infections. However, the protective effect observed at 7 days was not observed when animals were challenged 14 days after intramammary immunisation. In this study, immunisation failed to reduce infection, bacterial concentration in the milk or impact the SCC when compared with controls. Similarly, no protection against challenge was observed in animals immunised through the subcutaneous route close to the supramammary draining lymph nodes although a five-fold reduction in bacterial load was observed compared to the control animals, this was not statistically different to that observed in unvaccinated control animals.

In our intramammary challenge model, infected glands showed clinical signs of mastitis such as redness, pain on palpation and the presence of clots and discoloration of the milk within 24 h. This is in accordance with the experimental models previously described in the literature (32, 33). Bacteria could be recovered from the milk by 12 h PC and reached a peak 24 h PC. The bacterial concentration decreased thereafter and many animals progressively cleared the infection. Bacterial clearance appears to be related to an increase in SCC and is likely to include neutrophils and macrophages which are known to play a bactericidal role (34). A similar infection pattern has been described for other Gram-negative mastitis pathogens in cattle such as *E. coli*, *Serratia marcescens*, and *Klebsiella pneumoniae* (35).

The delivery of vaccine antigens directly into the mammary gland through the teat has previously been investigated as a means of protecting against IMI in ruminant livestock. Both antibody and cellular immune responses to immunogens such as tuberculin (36) and ovalbumin (37) may be induced by delivery of antigens directly into the mammary gland. Importantly for vaccine development, the induction of immune memory through delivery of vaccine antigens directly into the mammary gland has also been demonstrated (36). These observations were further expanded in studies that applied the intramammary delivery route to important mastitis pathogens (19). Studies conducted mainly in cattle with either whole killed bacteria or bacterial antigens demonstrated that delivery directly into the mammary gland elicited an enhanced response compared to the same vaccine delivered through a subcutaneous or an intramuscular injection. For example, Finch et al. (23) demonstrated that intramammary immunisation with killed *S. uberis* during the dry period induces a protective response to homologous challenge. In the same study animals were immunised subcutaneously with the same vaccine preparation but with the addition of an adjuvant. The addition of an adjuvant reduced the number of bacteria in the challenged mammary glands whereas intramammary vaccinated animals did not. Protection did not appear to correlate with milk antibody titre as both subcutaneous and intramammary immunisations showed similar concentration of antibodies.

In contrast to studies carried out in dairy cattle where intramammary immunisation was administered during the dry period, we have focused on sheep during lactation. For these reasons care should be taken when making direct comparisons between studies. Nevertheless, the protective effect of intramammary immunisation described here is similar to related studies conducted in cattle. The protective effect of the intramammary immunisation protocol we tested appears to be limited to 7 days. It is possible that the duration of protection may be extended by employing multiple administrations of the vaccine, different adjuvants and combinations of intramammary and subcutaneous immunisation regimes. Also in the studies described here immunologically naïve animals were not available as pregnant ewes are immunised subcutaneously with Ovipast or similar vaccines, prior to parturition as part of routine flock management in order to protect against respiratory disease. Our immunisation strategy may therefore be considered a systemic prime followed by an intramammary boost.

It was surprising therefore that boosting the immune response through re-stimulation of previously primed sheep via intramammary administration of vaccine antigens was not mirrored by subcutaneous administration over the supramammary lymph nodes. It is possible that the limited effect on bacterial numbers following subcutaneous immunisation reflects a boosting of an acquired immune response. On the other hand an intramammary boost may have primed innate immune effectors cells such as neutrophils and macrophages as well as boosting the antibody responses. Future cellular and serological analyses will seek to confirm this.

In our study, intramammary immunisation induced an inflammatory response within the mammary gland, characterised by increases in SCC (**Figure 3**) and in the production of cytokines IL-1β, IL-10 and IL-17A (**Figure 4**). The SCC and cytokine levels appeared to have returned to levels comparable with un-immunised controls prior to challenge except for IL-10 in animals challenged 7 days after immunisation. This raises a number of questions regarding the immune mechanisms associated with protection at 7 days and why it is greatly reduced at 14 days post-immunisation. The protective effect may be due to boosting and targeting to the mammary gland of an adaptive immune response which includes immunoglobulin and cell mediated responses. However, the reduced levels of protection observed at 14 days when we would expect adaptive responses to remain effective, suggests that mechanisms other than adaptive immunity may be involved. Rainard et al. (38) found that infusion of inflammatory

compounds such as lipopolysaccharides (LPS) and ovalbumin into the mammary gland induced an inflammatory response that lasted no longer than 72 h. Our immunisation strategy may have induced similar innate responses within the mammary gland which remained effective at 7 days post-immunisation but substantially reduced at 14 days.

Intramammary immunisation with gram negative bacterial cell wall components such as LPS (39) also produced results on challenge similar to those we describe here. In addition to bacterial antigens that induce protective responses against respiratory infection, the Ovipast vaccine is likely to also include bacterial components that act as ligands for pathogen recognition receptors including the Toll-like receptors (TLR). TLR are expressed by mammary epithelial cells (40) which in *in vitro* and *in vivo* studies rapidly respond to bacterial antigens including LPS (41). Activation of the mammary epithelium results in the production of antibacterial peptides (42), as well as cytokines and chemokines which recruit macrophages and neutrophils into the mammary gland (43). The rapid and almost sterile protection we observed in study 1 indicates that the challenge was effectively eliminated upon delivery to the mammary gland. Whether this was mediated by such innate mechanisms or through a combination of innate and adaptive immune mechanisms will be determined in future studies using this model.

To begin the investigation of the nature of the immune response in the ovine mammary gland following immunisation and challenge we measured the concentration of IL-1β, IL-10, and IL-17A in milk samples over the course of each study. The pro inflammatory cytokine IL-1β closely mirrors the SCC reaching its peak 24 h PC and declining thereafter. This cytokine is produced in response to pathogens by both macrophages (44) and mammary epithelial cells (45) and plays a fundamental role in initiating the inflammatory response in the mammary gland (35). In this study, the early detection of this cytokine is consistent with such a role. The peak of IL-1β is followed by IL-10 which is mainly produced by macrophages and lymphocytes. IL-10 has an anti-inflammatory role which is crucial in down regulating inflammation in order to avoid excessive tissue damage. A similar pattern of pro- and anti-inflammatory cytokine production has been described for several cattle mastitis pathogens (35).

We also detected IL-17A in response to infection although the cellular source of this cytokine and its role in protection are not clear. The protection observed at 7 days following intramammary immunisation was not related to an increase in IL-17A in milk either after vaccination or in response to the challenge. This suggest that IL-17A may not be produced by Th17 lymphocytes as part of an adaptive immune response to challenge. It may however be produced by a range of innate immune cells which have central roles in the maintenance of mucosal immunity (46). Notwithstanding it source, IL-17 is considered important in the response to extra cellular pathogens as it has been shown to recruit and enhance the bactericidal activity of phagocytes (47). *In vitro*, IL-17 family cytokines up regulate the production of antimicrobial proteins from bovine mammary epithelial cells (22), which contributes to bacterial clearance. We have previously reported that induction of IL-17A in cattle infected with *S. uberis* appeared to be associated with neutrophil recruitment and clearance of bacterial infection (20). The detection of IL-17A in multiple hosts and in response to different pathogen species confirms its importance in the context of mastitis and suggests that its induction through innate and or adaptive immune stimulation may be important in protecting against IMI.

In conclusion, we have demonstrated that delivery of a vaccine directly into the mammary gland via the teat canal can induce a protective response against subsequent IMI. While the protective effect was limited in duration it provides us with a cost effective, small ruminant immunisation, and challenge model to study the induction, nature and manipulation of immunity within the ruminant mammary gland. Future studies will seek to determine the relative importance of innate and adaptive responses in the protection observed 7 days post-immunisation and whether these may ultimately be exploited to improve ruminant udder health.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Moredun Research Institute Animal Welfare and Ethical Review Body.

## AUTHOR CONTRIBUTIONS

RT and KB conceived and designed the experiments. RT, JF, MS, HT, and KB performed the experiments. DE performed the statistical analysis. RT, DE, and KB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.659803/full#supplementary-material

## REFERENCES


1. Grant C, Smith EM, Green LE. A longitudinal study of factors associated with acute and chronic mastitis and their impact on lamb growth rate in 10 suckler sheep flocks in Great Britain. *Prev Vet Med.* (2016) 127:27–36. doi: 10.1016/j.prevetmed.2016.03.002
2. Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X. Mastitis of dairy small ruminants. *Vet Res.* (2003) 34:689–716. doi: 10.1051/vetres:2003030
3. Conington J, Cao G, Stott A, Bünger L. Breeding for resistance to mastitis in United Kingdom sheep, a review and economic appraisal. *Vet Rec.* (2008) 162:369–76. doi: 10.1136/vr.162.12.369
4. Watkins GH, Burriel AR, Jones JET. A Field Investigation of subclinical mastitis in sheep in Southern England. *Brit Vet J.* (1991) 147:413–20. doi: 10.1016/0007-1935(91)90083-Y
5. Zadoks RN, Tassi R, Martin E, Holopainen J, McCallum S, Gibbons J, et al. Comparison of bacteriological culture and PCR for detection of bacteria in ovine milk-sheep are not small cows. *J Dairy Sci.* (2014) 97:6326–33. doi: 10.3168/jds.2014-8351
6. Arsenault J, Dubreuil P, Higgins R, Bélanger D. Risk factors and impacts of clinical and subclinical mastitis in commercial meat-producing sheep flocks in Quebec, Canada. *Prev Vet Med.* (2008) 87:373–93. doi: 10.1016/j.prevetmed.2008.05.006
7. Koop G, Rietman JF, Pieterse MC. *Staphylococcus aureus* mastitis in Texel sheep associated with suckling twins. *Vet Rec.* (2010) 167:868–69. doi: 10.1136/vr.c3375
8. Omaleki L, Browning GF, Allen JL, Barber SR. The role of Mannheimia species in ovine mastitis. *Vet Microbiol.* (2011) 153:67–72. doi: 10.1016/j.vetmic.2011.03.024
9. Cooper S, Huntley SJ, Crump R, Lovatt F, Green LE. A cross-sectional study of 329 farms in England to identify risk factors for ovine clinical mastitis. *Prev Vet Med.* (2016) 125:89–98. doi: 10.1016/j.prevetmed.2016.01.012
10. Fragkou IA, Gougoulis DA, Billinis C, et al. Transmission of *Mannheimia haemolytica* from the tonsils of lambs to the teat of ewes during sucking. *Vet Microbiol.* (2011) 148:66–74. doi: 10.1016/j.vetmic.2010.08.008
11. Gelasakis AI, Mavrogianni VS, Petridis IG, Vasileiou NG, Fthenakis GC. Mastitis in sheep–The last 10 years and the future of research. *Vet Microbiol.* (2015) 181:136–46. doi: 10.1016/j.vetmic.2015.07.009
12. Casu S, Sechi S, Salaris SL, Carta A. Phenotypic and genetic relationships between udder morphology and udder health in dairy ewes. *Small Rum Res.* (2010) 88:77–83. doi: 10.1016/j.smallrumres.2009.12.013
13. Davies P, Remnant JG, Green MJ, Gascoigne E, Gibbon N, Hyde R, et al. Quantitative analysis of antibiotic usage in British sheep flocks. *Vet Rec.* (2017) 181:511. doi: 10.1136/vr.104501
14. O'Neill J (chaired by). *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations.* (2016). Available online at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf
15. Wilson DJ, Grohn YT, Bennett GJ, González RN, Schukken YH, Spatz J. Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. *J Dairy Sci.* (2007) 90:4282–88. doi: 10.3168/jds.2007-0160
16. Pérez MM, Prenafeta A, Valle J, Penadés J, Rota C, Solano C, et al. Protection from *Staphylococcus aureus* mastitis associated with poly-N-acetyl beta-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. *Vaccine.* (2009) 27:2379–86. doi: 10.1016/j.vaccine.2009.02.005
17. Rainard P, Foucras G, Fitzgerald JR, Watts JL, Koop G, Middleton JR. Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. *Transbound Emerg Dis.* (2018) 65:149–65. doi: 10.1111/tbed.12698
18. Schukken YH, Günther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O, et al. Host-response patterns of intramammary infections in dairy cows. *Vet Immunol Immunopathol.* (2011) 144:270–89. doi: 10.1016/j.vetimm.2011.08.022
19. Bharathan M, Mullarky IK. Targeting mucosal immunity in the battle to develop a mastitis vaccine. *J Mammary Gland Biol Neoplasia.* (2011) 16:409–19. doi: 10.1007/s10911-011-9233-1
20. Tassi R, McNeilly TN, Fitzpatrick JL, Fontaine MC, Reddick D, Ramage C, et al. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of *Streptococcus uberis* in dairy cattle. *J Dairy Sci.* (2013) 96:5129–45. doi: 10.3168/jds.2013-6741
21. Roussel P, Cunha P, Porcherie A, Petzl W, Gilbert FB, Riollet C, et al. Investigating the contribution of IL-17A and IL-17F to the host response during Escherichia coli mastitis. *Vet Res.* (2015) 46:56. doi: 10.1186/s13567-015-0201-4
22. Bougarn S, Cunha P, Gilbert FB, Harmache A, Foucras G, Rainard P. Staphylococcal-associated molecular patterns enhance expression of immune defense genes induced by IL-17 in mammary epithelial cells. *Cytokine.* (2011) 56:749–59. doi: 10.1016/j.cyto.2011.09.020
23. Finch JM, Hill AW, Field TR, Leigh JA. Local vaccination with killed Streptococcus uberis protects the bovine mammary gland against experimental intramammary challenge with the homologous strain. *Infect Immun.* (1994) 62:3599–03. doi: 10.1128/IAI.62.9.3599-3603.1994
24. Smith JL, Hogan JS, Smith KL. Efficacy of intramammary immunization with an *Escherichia coli* J5 bacterin. *J Dairy Sci.* (1999) 82:2582–88. doi: 10.3168/jds.S0022-0302(99)75513-X
25. Pomeroy B, Gurjar A, Sipka A, Klaessig S, Salmon S, Quesnell R, et al. Intramammary immunization with ultraviolet-killed *Escherichia coli* shows partial protection against late gestation intramammary challenge with a homologous strain. *J Dairy Sci.* (2016) 99:9014–26. doi: 10.3168/jds.2016-11149
26. Donachie W. Vaccine development against pasteurella haemolytica infections in sheep. In: Donachie W, Lainson FA, Hodgson JC, editors. *Haemophilus, Actinobacillus, and Pasteurella*. Boston, MA: Springer (1995). p. 25–37.
27. Russell WMS, Burch RL. *The Principles of Humane Experimental Technique.* Universities Federation for Animal Welfare (1959, as reprinted 1992). Available online at: https://caat.jhsph.edu/principles/the-principles-of-humane-experimental-technique
28. National Mastitis Council. *Laboratory Handbook on Bovine Mastitis*. National Mastitis Council (1999).
29. R Core Team 2020. *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing. Available online at: https://www.R-project.org/
30. Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, et al. glmmTMB balances speed and flexibility among packages for zero-inflated Generalized Linear Mixed Modeling. *R J.* (2017) 9:378–400. doi: 10.32614/RJ-2017-066
31. Wood SN. *Generalized Additive Models: An Introduction with R, 2nd edn*. Boca Raton, FL: Chapman and Hall/CRC (2017).
32. el-Masannat ET, Jones JE, Scott MJ. The experimental production of mastitis in sheep by intramammary inoculation of Pasteurella haemolytica. *J Comp Pathol.* (1991) 105:455–65. doi: 10.1016/S0021-9975(08)80115-6
33. Mavrogianni VS, Fthenakis GC, Brooks H, Papaioannou N, Cripps PJ, Taitzoglou I, et al. The effects of inoculation of *Mannheimia haemolytica* into the teat of lactating ewes. *Vet Res.* (2005) 36:13–25. doi: 10.1051/vetres:2004048
34. Sordillo LM, Streicher KL. Mammary gland immunity and mastitis susceptibility. *J Mammary Gland Biol Neoplasia.* (2002) 7:135–46. doi: 10.1023/A:1020347818725
35. Bannerman DD. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. *J Anim Sci.* (2009) 87:10–25. doi: 10.2527/jas.2008-1187
36. Nonnecke BJ, Elsken LA, Kehrli ME. Local and systemic immune response in the cow after intramammary vaccination during lactation. *Vet Immunol Immunopathol.* (1986) 11:31–44. doi: 10.1016/0165-2427(86)90086-3
37. Fitzpatrick JL, Cripps PJ, Hill AW, Bland PW, Stokes CR. MHC class II expression in the bovine mammary gland. *Vet Immunol Immunopathol.* (1992) 32:13–23. doi: 10.1016/0165-2427(92)90065-X
38. Rainard P, Cunha P, Bougarn S, Fromageau A, Rossignol C, Gilbert FB, et al. T helper 17-associated cytokines are produced during antigen-specific inflammation in the mammary gland. *PLoS One.* (2013) 8:e63471. doi: 10.1371/journal.pone.0063471
39. Petzl W, Günther J, Pfister T, Sauter-Louis C, Goetze L, von Aulock S, et al. Lipopolysaccharide pretreatment of the udder protects against experimental *Escherichia coli* mastitis. *Innate Immun.* (2012) 18:467–77. doi: 10.1177/1753425911422407

40. Günther J, Petzl, W, Zerbe H, Schuberth HJ, Seyfert HM. TLR ligands, but not modulators of histone modifiers, can induce the complex immune response pattern of endotoxin tolerance in mammary epithelial cells. *Innate Immun.* (2017) 23:155–64. doi: 10.1177/1753425916681076
41. Günther J, Petzl W, Zerbe H, Schuberth HJ, Koczan D, Goetze L, et al. Lipopolysaccharide priming enhances expression of effectors of immune defence while decreasing expression of pro-inflammatory cytokines in mammary epithelia cells from cows. *BMC Genomics.* (2012) 13:17. doi: 10.1186/1471-2164-13-17
42. Riollet C, Rainard P, Poutrel B. Cells and cytokines in inflammatory secretions of bovine mammary gland. *Adv Exp Med Biol.* (2000) 480:247–58. doi: 10.1007/0-306-46832-8_30
43. Morgante M, Ranucci S, Pauselli M, Beghelli D, Mencaroni G. Total and differential cell count by direct microscopic method on ewe milk. *Zentralbl Veterinarmed A.* (1996) 43:451–58. doi: 10.1111/j.1439-0442.1996.tb00474.x
44. Politis I, McBride BW, Burton JH, Zhao X, Turner JD. Secretion of interleukin-1 by bovine milk macrophages. *Am J Vet Res.* (1991) 52:858–62.
45. Boudjellab N, Chan-Tang HS, Zhao X. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. *Comp Biochem Physiol A Mol Integr Physiol.* (2000) 127:191–9. doi: 10.1016/S1095-6433(00)00257-9
46. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol.* (2010) 10:479–89. doi: 10.1038/nri2800
47. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* (2008) 4:e1000159. doi: 10.1371/journal.ppat.1000159

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SYSTEMATIC REVIEW
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# Comparing Blanket vs. Selective Dry Cow Treatment Approaches for Elimination and Prevention of Intramammary Infections During the Dry Period: A Systematic Review and Meta-Analysis

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A systematic review and a series of meta-analyses were conducted to investigate the efficacy of selective dry cow antimicrobial treatment (SDCT) (in which only infected quarters/cows were treated with an antimicrobial) compared with blanket dry cow treatment (BDCT) (all quarters/all cows received an antimicrobial, regardless of their infection status). A full detailed protocol was published before initiating this review. Studies reporting on the (1) proportion of untreated quarters or cows when using SDCT, (2) intramammary infection (IMI) incidence risk over the dry period, (3) IMI elimination risk, (4) post-calving IMI prevalence, (5) early lactation clinical mastitis incidence, or (6) subsequent lactation milk yield and somatic cell counts were considered eligible. Thirteen articles representing 12 controlled trials, whether randomized or not, were available for analyses. SDCT reduced the use of antimicrobials at dry off by 66% (95% CI: 49–80). There was no difference in the elimination of existing IMI at dry off, between SDCT and BDCT. Meta-regression showed that the risk of IMI incidence during the dry period, IMI risk at calving, early lactation clinical mastitis risk, and early lactation milk yield and somatic cell counts did not differ between SDCT and BDCT as long as an internal teat sealant (65% bismuth subnitrate) was administered to untreated healthy quarters/cows at dry off. For trials not using internal teat sealants, SDCT resulted in higher risk than BDCT of acquiring a new IMI during the dry period and of harboring an IMI at calving. Lines of evidence strongly support that SDCT would reduce the use of antimicrobials at dry off, without any detrimental effect on udder health or milk production during the 1st months of the subsequent lactation, if, and only if, internal teat sealants are used for healthy, untreated quarters/cows.

**Keywords: dairy cows, dry period, selective antimicrobial treatment, intramammary infection, antimicrobial use**

# INTRODUCTION

Blanket dry cow therapy (BDCT), where all quarters of all cows are treated with a long-acting antimicrobial at dry off, was introduced many years ago (1) and is widely used by dairy farmers. This practice permits to increase the elimination of existing intramammary infections (IMI) at dry off and prevent the occurrence of new IMI during the dry period. In fact, persistent and new IMI during the dry period can result in the development of clinical mastitis (CM) early in the next lactation (2–4).

However, with changes in mastitis epidemiology and increasing public health concerns regarding the use of antimicrobials in food-producing animals, selective dry cow therapy (SDCT) is a potential alternative to BDCT to reduce antimicrobial usage in dairies (5–7). With the SDCT approach, antimicrobial treatment is reserved for cows or quarters suspected of having an IMI, while uninfected cows and quarters usually do not receive an antimicrobial treatment. In addition, internal teat sealants (ITS) have been shown to be a very effective non-antimicrobial alternative to prevent new IMI during the dry period (8–10). A teat sealant could be used to protect untreated cows or quarters when a selective antimicrobial treatment approach at dry off is applied. Thus, SDCT could prevent the use of antimicrobials for a prophylactic purpose and that it could possibly be without detrimental changes to udder health parameters (11).

A systematic review comparing blanket and selective dry cow therapy and describing the various advantages and potential negative impacts would be of great importance for decision-makers to engage in an effective and judicious use of antimicrobials at dry off. Recently, a systematic review (12) reported on the impact of selective vs. blanket dry cow therapy, but on only one outcome, prevalence of IMI at calving. In this latter study, reduction in the use of antimicrobials at dry off (the main reason for choosing SDCT) was not investigated, nor was the risk of CM, milk yield, or somatic cell counts (SCC) in the early next lactation. These outcomes are all very important for choosing the best dry cow treatment protocol. Moreover, IMI dynamics during the dry period (i.e., acquisition and elimination of IMI during the dry period) was not investigated in the study of Winder et al. (12). Nevertheless, studying IMI dynamics can provide better insights on the underlying biological processes, compared with studying prevalence at a single point in time (e.g., at calving).

## Objective

The objective of the current study was to investigate the efficacy of SDCT, compared with the treatment of all quarters of all cows, for (1) reducing the use of antimicrobials at dry off, (2) preventing IMI incidence during the dry period, (3) eliminating existing IMI at dry off, (4) reducing the prevalence of IMI at calving, and (5) preventing early lactation CM. Another objective was also to investigate whether milk yield and SCC in the early lactation would be affected. Our hypothesis was that SDCT protocols could be implemented without negative health or production effects and would result in a substantially lower usage of antimicrobials at dry off.

The population, intervention, comparator, outcome (PICO) questions answered by the current study were formulated as: in dairy cows (i.e., the population), is SDCT (i.e., the intervention) as efficient as BDCT (i.e., the comparator), (1) in preventing new IMI during the dry period, (2) in eliminating existing IMI at dry off, (3) in reducing IMI risk at calving, and (4) in preventing early lactation CM; and (5) what are the impacts of dry cow treatment approach on milk yield and SCC in the early lactation (i.e., the outcomes)?

**Abbreviations:** SDCT, selective dry cow therapy; BDCT, blanket dry cow therapy; IMI, intramammary infections; CM, clinical mastitis; ITS, internal teat sealants; SCC, somatic cell counts; DIM, days in milk; ROB, risk of bias.

# METHODS

This current systematic review was reported using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines (13). The detailed protocol for this review was published elsewhere prior to initiating the review (14). The complete protocol targeted three independent objectives: (1) choice of antimicrobial at drying off, (2) comparison of blanket vs. selective dry cow treatment, and (3) complementing an antimicrobial treatment with a teat sealant. However, the current manuscript reports only on the comparison of blanket and selective dry cow treatments. The other two objectives will be addressed in two future independent manuscripts.

The complete search strategy described in the published protocol was initially conducted on May 1st, 2018, and updated on June 16th, 2020, prior to finalizing the analyses and manuscript. The search strategies were all conducted on the same day for the three electronic sources of information (Medline, CAB Abstracts, and Web of Science) and for conference proceedings from the National Mastitis Council and the American Association of Bovine Practitioners. Modifications and precisions to the published protocol with their justifications are described in the following sections.

## Modifications and/or Precisions to the Published Protocol

### Eligibility Criteria

In the published protocol, we planned to include studies where the post-calving IMI status was determined within 14 days in milk (DIM), to ensure that the new IMI or elimination most likely occurred during the dry period (vs. in the early lactation). However, in some articles, cows were sampled twice after calving (for instance, 3–4 DIM and 5–18 DIM), and a parallel interpretation of the two milk samples was used to define IMI status. Hence, some studies relied on testing within an interval that extended slightly beyond 14 DIM but was mostly within 0–14 DIM. We decided to retain these studies (5, 6, 15). In the published protocol, we indicated CM incidence during the first 0–4 months after calving as a studied outcome. More precisely, we did use the CM data from studies with a shorter follow-up

period and otherwise extracted the data up to a maximum of 4 months in milk.

### Risk of Bias in Individual Studies

As it was planned in the protocol, we proposed to record different domains of risk of bias (ROB) by outcome's type. In fact, the ROB 2.0 makes it clear that the assessment is typically specific to a particular result, and consequently, the assessments of ROB need to be outcome-specific (16). However, all measured outcomes yielded the same evaluation within a given trial. Hence, for simplicity, we only reported the risk for a domain for all outcomes of a trial at once. As all the included studies were controlled trials (whether randomized or not), only the Cochrane Collaboration's tool for assessing ROB was used for assessing ROB in selected studies (Cochrane Handbook for Systematic Reviews of Interventions, version 5.1.0).

### Summary Measures

Daily mean milk production (kg/day) or mean ln SCC during the first 4 months was extracted directly from included trials or obtained from personal communications with the authors. Thus, raw mean difference (MD) was used as the effect size, for those two outcomes.

### Data Synthesis and Meta-Analysis

Meta-analyses were conducted in R version 4.0.0 [R Foundation for Statistical Computing Platform: ×86_64-w64-mingw32/×64 (64-bit)] using RStudio version 1.2.1335 (RStudio Inc., Boston, MA, USA) using the "meta" package version: 4.12-0 (2020-05-04). Studies were weighed using the inverse variance method based on the logit transformation. A random effects approach was used, as it was described in the published protocol (14) and the between-study variability was estimated using the method of restricted maximum likelihood (REML) (17) and the Knapp–Hartung adjustment for random effects model (18). Heterogeneity was assessed by the $I^2$ statistic. Effects of trial level characteristics were tested using a meta-regression model with one covariate and only if at least three trials were included in each category of the covariate.

### Confidence in Cumulative Estimates

The Grading of Recommendations Assessment, Development, and Evaluation (GRADE) approach involves rating, for each comparison made, the confidence in effect estimate based on an assessment of eight domains: number of trials, ROB, inconsistency, indirectness, imprecision, publication bias, number of individuals (in our case quarters or cows) followed, and a summary measure of association with its 95% CI. Then, an overall assessment is made regarding the level of confidence in the summary effect estimate observed. For rating the different domains of the GRADE, in the current review, we used the guidelines suggested by Dufour et al. (8).

Briefly, for the ROB domain, a trial was rated at low ROB, when at least four out of seven evaluated domains for an individual trial were rated at low risk with a maximum of one domain evaluated at high risk. When at least four domains were rated at low risk but with two domains evaluated at high risk, the trial was rated at moderate ROB. In other cases, the trial was rated at high ROB. For the inconsistency domain, we visually appraised, using forest plot, whether a uni-, bi-, or multimodal distribution of point estimates was observed across trials and rated these, respectively, as no serious, serious, and very serious limitations. Regarding the indirectness domain, we computed independently the proportion of trials for which the investigated population, intervention, and outcome matched those of interest, and an equal weight was given to these three subdomains. Comparisons with a score $\geq$66%, between 65 and 33%, and of $\leq$33% for that domain were then rated as no serious, serious, and very serious limitations, respectively.

For the imprecision domain, the difference between the natural logarithm of the higher and lower bounds of the summary relative risk (RR) was computed. Comparisons with confidence interval bounds, differences $\leq$1.1 on the logarithmic scale (equivalent to an RR interval of 1.0–3.0 points), between 1.1 and 1.6 (equivalent to an RR interval of 3.0–5.0 points), and $\geq$1.6 (equivalent to an RR interval of $\geq$5.0 points) were rated, respectively, as no serious, serious, and very serious limitations. For the imprecision domain for milk yield and SCC, in addition to the examination of upper and lower limits of the 95% confidence intervals, we considered the calculation of an optimal information size (19). When the optimal information size criterion was not met, the precision was rated as serious limitations. When the optimal information size criterion was met and the 95% CI length $<$2 (i.e., a mean difference of $-$1.00 to $+$1.00) for milk yield and ln SCC, we rated precision as no serious limitations. When the optimal information size criterion was met, and the 95% CI length $\geq$2 and $\leq$4 for milk yield and ln SCC, the precision was rated as serious limitations. When the optimal information size criterion was met, and the 95% CI length $\geq$4 for milk yield and ln SCC, the precision was rated as very serious limitations.

Finally, for the publication bias domains, we considered whether the number of trials allowed us to fully appraise funnel plot asymmetry. We also considered whether the outcomes studied would be associated with any commercial advantages.

## RESULTS

### Study Selection

Results of the different steps for searching and assessing eligibility of studies are presented in **Figure 1**. After removing duplicates and exclusion due to language restriction, a total of 991 records were identified from three databases: CAB Abstracts, Web of Science, and Medline. Of the 991 records, after reviewing the content of the abstracts and full texts, only 89 records met the inclusion criteria for at least one of the PICO questions on antimicrobial-based dry cow therapy approaches.

In addition, 43 records were identified through the search of the National Mastitis Council (NMC) and American Association of Bovine Practitioners (AABP) conference proceedings. Finally, after excluding proceeding papers for which an equivalent full article was available ($n$ = 27), 105 records combining 89 full articles and 16 conference papers were included.

The references cited in these 105 retained records and 78 non-primary studies were screened for any additional relevant

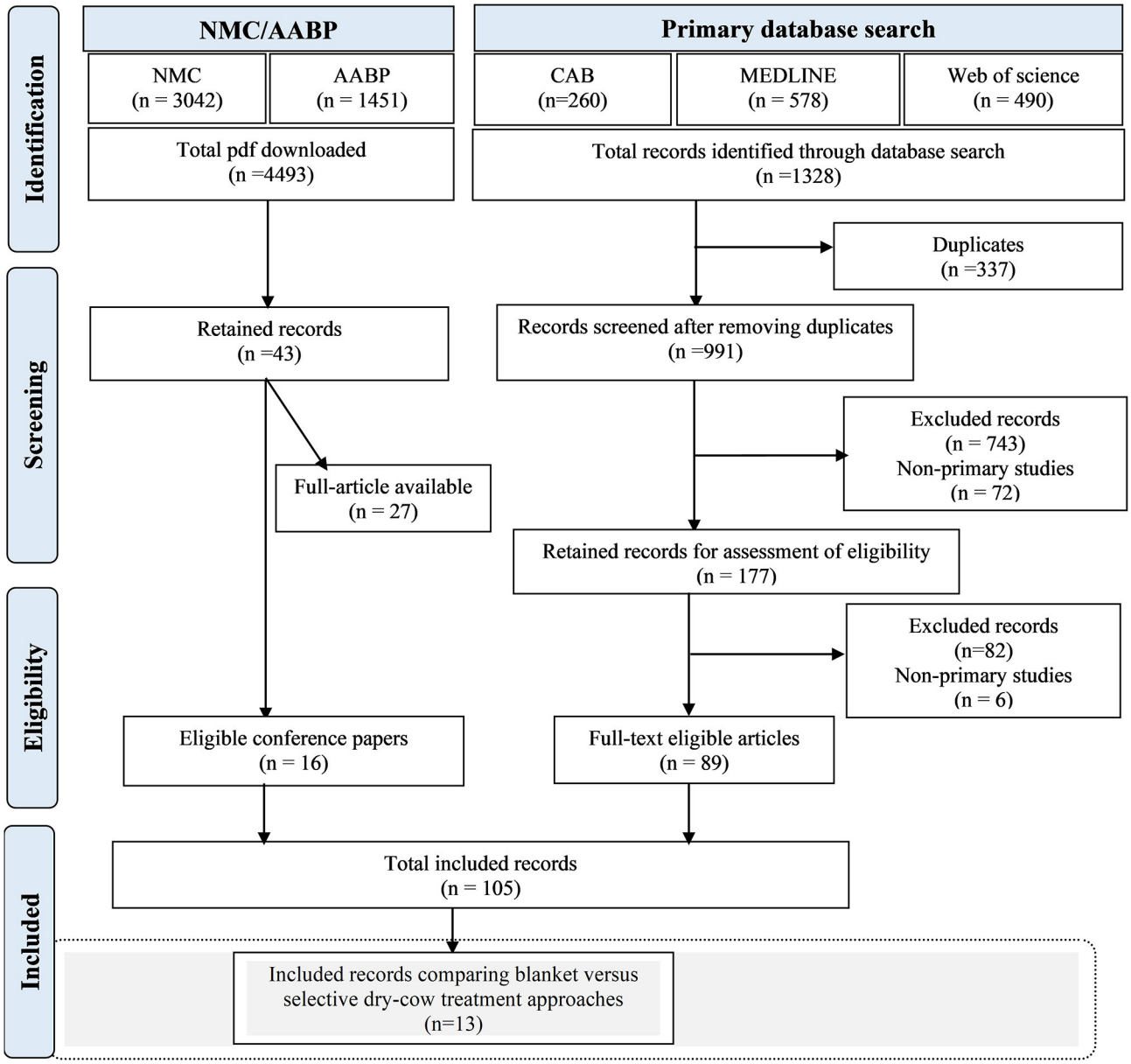


**FIGURE 1 |** Result of the different steps for searching and identifying relevant records for the systematic review and meta-analysis on antimicrobial-based dry cow therapy approaches. The search was conducted to answer three research objectives: (1) choice of antimicrobial at drying off; (2) comparison of blanket vs. selective dry cow treatment; and (3) complementing an antimicrobial treatment with a teat sealant. The gray box indicates results specific for objective (2), comparison of blanket vs. selective dry cow treatment, and the other two objectives will be presented in subsequent independent articles. Screening of references cited by the included articles was also conducted but did not lead to the addition of eligible articles specific to the comparison of selective and blanket dry cow therapies. This latter part of the search strategy will be presented for the other two objectives in the subsequent associated articles. NMC, National Mastitis Council; AABP, American Association of Bovine Practitioners.

study which was not initially retrieved through the databases or conference proceedings search, but no additional eligible records were identified from this process for the comparison of SDCT and BDCT.

Of the 105 records retained, 13 articles representing 12 trials reported on the comparison of SDCT and BDCT and, therefore, were included in this part of the systematic review. Other retained records will be discussed in two other manuscripts reporting on the choice of antimicrobial at drying off or on complementing an antimicrobial treatment with an ITS.

## Included Studies

Characteristics of the 13 included articles representing 12 trials are described in **Table 1**. Those 12 trials include six trials reported

**TABLE 1 |** Characteristics of 13 articles representing 12 trials included in the systematic review comparing selective dry cow therapy and blanket dry cow therapy for curing and preventing intramammary infections.

| References | Country | Study design | #[a] herds | # cows | # quarters | Inclusion criteria |
|---|---|---|---|---|---|---|
| Ward and Schultz (15) | USA | CT | 4 | 402 | 1,600 | No criteria |
| Roguinsky and Serieys (20) | France | CT | 1 | 40 | 159 | NR |
| Rindsig et al. (7) | USA | CT | 1 | 232 | 928 | NR |
| Browning et al. (21, 22) | Australia | CT | 12 | 1,044 | 4,176 | BTSCC 100,000–400,000 cells/ml; cow's expected dry period ≥2 months; and <4 infected quarters at dry off |
| Williamson et al. (23) | New Zealand | CT | 4 | 371 | NR | NR |
| Hassan et al. (24) | Australia | CT | 3 | 150 | 600 | NR |
| Cameron et al. (5, 25) | Canada | RCT | 16 | 603 | 2,287 | BTSCC < 250,000 cells/ml; cow's SCC < 200,000 cells/ml on the last three DHI tests; no CM on the same period; cow's expected dry period 30–90 days; cow had no antimicrobial treatment in the last 14 days; all quarters of the cow had CMT < 2 on the day prior to drying off. |
| Patel et al. (26) | USA | RCT | 1 | 56 | 224 | Four functional quarters; no antibiotic or anti-inflammatory medication during the 14-day period prior to dry off; clinically healthy; no signs of CM at enrollment or on the day of dry off; expected dry period 30–90 days |
| Rowe et al. (27, 28) | USA | RCT | 7 | 1,243 | 5,100 | Herd size sufficient to dry off ≥ 15 cows per week; BTSCC < 250,000 cells/ml; record CM, culling, and death events; cow's expected dry period 30–90 days; no antibiotic or anti-inflammatory treatment within 14 days; no CM; no lameness (>3/5) or poor body condition (<2/5) |
| Kabera et al. (6) | Canada | RCT | 9 | 569 | 2,142 | BTSCC < 250,000 cells/ml; no CM or antimicrobial treatment during 14 days prior to dry off; and cow's expected dry period 35–75 days |

*CT, controlled trial (no randomization reported); NR, not reported; BTSCC, herd mean 12-month bulk-tank somatic cell count; RCT, randomized controlled trial; SCC, somatic cell counts; DHI, dairy herd improvement; CM, clinical mastitis; CMT, California Mastitis Test.*
[a]*Number of units analyzed.*

in six articles (7, 15, 20, 23, 24, 26), two trials where each trial was reported in two articles for different outcomes (5, 21, 22, 25), two trials reported in two articles where each article reported on both trials (27, 28), and two trials reported in one article (6). Furthermore, the description of the SDCT group and of the reported outcomes are summarized in **Table 2**. Finally, the follow-up period after calving and the definitions of IMI at dry off and calving, of new IMI, and of elimination of IMI during the dry period used in each study are provided as (**Supplementary Table 1**).

Briefly, six included trials were randomized controlled trials and six did not clearly report a randomization process and were, therefore, considered simply as controlled trials. Seven trials reported using herd and/or cow level recruitment criteria, one trial did not set criteria to recruit cows and/or quarters, and four trials did not report on selection criteria. One trial set a selection criteria at the herd level only, while the other six trials set them both at cow and herd levels. The most common herd-level selection criteria was to have a bulk milk SCC below a predetermined threshold (ranging from 250,000 to 400,000 cells/ml). For cow-level criteria, having a standard expected dry period length was often used, as well as no recent treatment prior to dry off, and no CM at dry off. Among the six trials where breed was reported, three were conducted in crossbred and purebred (Holstein and Holstein–Jersey or Friesian and Friesian–Jersey), while the other three were conducted solely in purebred cows (Holstein/Friesian). Of the 12 trials, the selection approach was based at the quarter level for eight trials and at the cow level for the other four trials.

In all trials, measures of new IMI, of IMI elimination, and of prevalence of IMI were based on bacteriological culture of milk samples collected before drying off and after calving. Predry off samples were taken within 1 month before dry off, and days in milk at post-calving sampling ranged from 0 to 4 weeks. Of the 12 included trials, IMI incidence during the dry period was the most often reported outcome ($n = 11$), followed by elimination of IMI during the dry period ($n = 10$) and prevalence of IMI at calving ($n = 9$). Clinical mastitis in the subsequent lactation was reported in 10 trials. However, two of the 10 trials reporting on CM in the subsequent lactation were excluded from the meta-analysis, as the follow-up period was more than 4 months and it was not possible to have data specifically for the 0–120 DIM period.

Four trials reported daily mean milk production during the first 120 DIM of the subsequent lactation (6, 28), and one trial reported daily mean milk production during the first 180 DIM (25). Six trials reported on SCC during the subsequent lactation. One trial reported on an arithmetic mean scale for the 1st week and between 28 and 56 days after calving (7). One trial reported test-day ln SCC 0–180 DIM (25), two on mean milk somatic cell score for 0–120 DIM (6), and two on SCC geometric mean for 0–120 DIM (28). After contacting the authors, data could be obtained on the same scale (mean ln SCC) for five trials. Moreover, for the trial reporting on a period of 0–180 DIM, we were able to obtain data specifically for the 0–120 DIM

**TABLE 2 |** Treatment regimens and outcomes studied in 13 articles representing 12 trials included in a systematic review comparing selective dry cow therapy (SDCT) and blanket dry cow therapy (BDCT).

| References | SDCT description | | | | | | Outcomes measured | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Method for identifying units to treat | Level[a] | Threshold for treatment | Tx[b] if + | Tx[c] if – | % with no ATB[d] | New IMI[e] | Elimination of IMI[f] | IMI[g] | Others in next lactation |
| Ward and Schultz (15) | CM[h] | Q | ≥1 CM in last month | Neomycin sulfate | No Tx | 96.1 | Yes | Yes | Yes | CM |
| Roguinsky and Serieys (20) | CMT | C | ≥1 quarter with CMT ≥3 in last month | Cloxacillin or penicillin and streptomycin (half of the cows received each treatment) | No Tx | 68.2 | Yes | Yes | Yes | None |
| Rindsig et al. (7) | SCC, CMT, and CM | C | Cow SCC > 500,000 cells/ml or CMT ≥ 2 in any quarter or ≥1 CM | Penicillin and streptomycin | No Tx | 42.9 | Yes | Yes | Yes | SCC |
| Browning et al. (21, 22) | Lab-based milk culture | Q | NR | Benzathine cloxacillin | No Tx | 67.5 | Yes | Yes | Yes | CM |
| Williamson et al. (23) | Lab-based milk culture | Q | NR | Cephalonium | No Tx | NR | Yes | No | No | CM |
| Hassan et al. (24)* | N-acetyl-beta-D-glucosaminidase | Q | High NAGase on a sample taken 24 h before dry off | Benzathine cloxacillin | No Tx | 81.1 | No | No | Yes | CM |
| Cameron et al. (5, 25) | Aerobic count Petrifilm | C | ≥50 CFU/ml in composite milk | Ceftiofur hydrochloride and ITS | ITS | 45.6 | Yes | Yes | Yes | MY, CM, SCC |
| Patel et al. (26) | Minnesota Easy culture system | Q | ≥100 CFU/ml in quarter milk | Ceftiofur hydrochloride + ITS | ITS | 48.1 | Yes | Yes | Yes | CM |
| Kabera et al. (6) | Aerobic count Petrifilm | Q | ≥50 CFU/ml in quarter milk | Penicillin G procaine and novobiocin | ITS | 57.4 | Yes | Yes | No | MY, CM, SCC |
| | Aerobic count Petrifilm | Q | ≥50 CFU/ml in quarter milk | Penicillin G procaine and novobiocin + ITS | ITS | 58.6 | Yes | Yes | No | MY, CM, SCC |
| Rowe et al. (27, 28) | Minnesota Easy® 4Cast® plate | Q | ≥100 CFU/ml in quarter milk | Ceftiofur hydrochloride + ITS | ITS | 55.5 | Yes | Yes | Yes | MY, CM, SCC |
| | Algorithm (SCC + CM) | C | ≥2 CM during lactation or any DHIA test with SCC > 200,000 cells/ml during lactation | Ceftiofur hydrochloride + ITS | ITS | 55.2 | Yes | Yes | Yes | MY, CM, SCC |

*CMT, California mastitis test; SCC, somatic cell counts; NR, not reported; CFU/ml, colony forming units per milliliter; ITS, internal teat sealant (65% bismuth subnitrate); MY, milk yield; DHIA, Dairy Herd Improvement Association.*

[a] *Selection for treatment applied at the cow (C) or quarter level (Q).*

[b] *Treatment for infected cow/quarter.*

[c] *Treatment for uninfected cow/quarter.*

[d] *Percentage of antimicrobial use reduction.*

[e] *New intramammary infections during the dry period.*

[f] *Elimination of intramammary infections during dry period.*

[g] *Prevalence of intramammary infections at calving.*

[h] *Clinical mastitis history in current lactation.*

**This study had both a positive and a negative control group.*

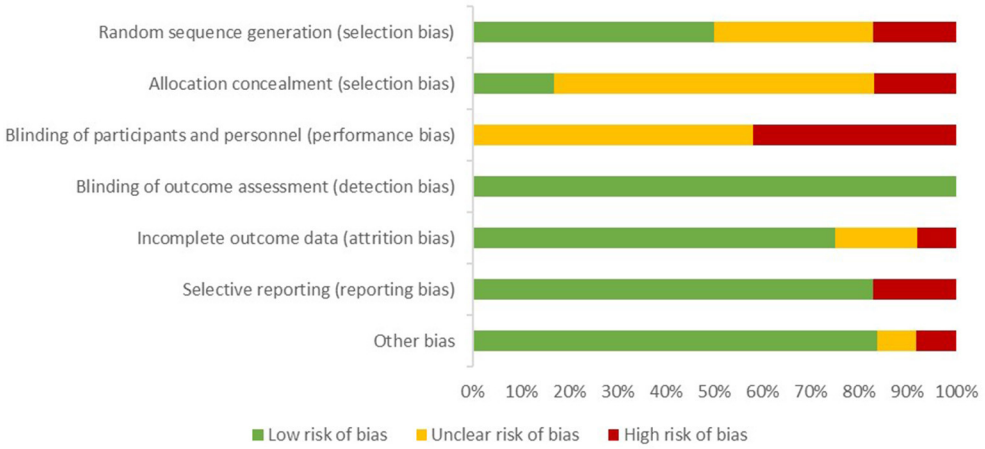


**FIGURE 2 |** Proportion of studies with a given risk of bias among 12 trials included in a systematic review comparing selective dry cow therapy and blanket dry cow therapy.

period. This latter trial was, therefore, included in the meta-analysis comparing mean milk yield and ln SCC between SDCT and BDCT.

## Risk of Bias Within Studies

The ROB for each individual study is reported as **Supplementary Figure 1**. A summary of the ROB assessment for the 12 trials included in the meta-analysis is presented in **Figure 2**. All trials had at least one potential source of bias rated as high or unclear. The ROB was evaluated for 13 articles reporting on 12 trials and the components with the smallest proportion of low risk trials were blinding of participants and personnel (0/12), then allocation concealment (2/12), and finally, random sequence generation (6/12). The method used to generate a random sequence was described for only six trials (5, 6, 25–28). Two trials had cows allocated alternately to two treatment groups and were consequently assessed as "high risk" (7, 15), and four other trials did not report on the randomization process in sufficient details for assessing potential bias (20–24). The allocation concealment was not described at all in eight trials. Consequently, they were classified as having an unclear risk regarding potential source of bias. It was appraised as "low risk" in two trials (6) and as "high risk" in two other trials where cows were allocated alternately (7, 15). Similarly, blinding of participants and personnel was not mentioned in seven trials. This latter component was evaluated at high risk in five other trials, as producers were not blinded to the treatment, and thus, we could not exclude an influence on the management of cows in different treatment groups. Bias due to blinding of outcome assessment (detection bias) was considered "low risk" in all trials relying mainly on laboratory analyses, which was considered to be an objective measurement.

## Meta-Analyses Comparing Selective and Blanket Dry Cow Therapies

A total of 12 trials reported on the effect of SDCT on IMI during the dry period and on udder health and milk production in the subsequent lactation, in comparison with BDCT. In addition to a positive control group (BDCT), one trial (24) included a second control group where cows did not receive any therapy at dry off. Data from this control group were not extracted, as our focus was the comparison between SDCT and BDCT.

The most important study characteristics suspected as potential sources of heterogeneity and tested in meta-regression were (1) method used to identify infected cows/quarters at dry off (milk culture vs. combination of SCC and/or history of clinical mastitis and/or California Mastitis Test and/or N-acetyl-beta-D-glucosaminidase), (2) whether the selective treatment was applied at the cow or quarter level, and (3) whether an ITS was applied for healthy cows/quarters. Meta-regression by the preceding variables was attempted if at least three trials were included in each category. For all the meta-analyses conducted, results by category of the covariate were presented rather than a general summary measure, whenever a variable tested in a meta-regression yielded a $p < 0.05$.

### Reduction of Antimicrobial Use at Dry Off

Eleven trials reported on the reduction of antimicrobial use; however, only 10 of them could be used to summarize reduction of usage of antimicrobial at dry off. In fact, one of the trials (5, 25) reported on the reduction in the use of antimicrobials in cows preselected [individual SCC <200,000 cells/ml and no CM on the last three dairy herd improvement (DHI) tests; (**Table 1**) before the randomization into selective and blanket treatment groups. Thus, it was not comparable with other trials, regarding the reduction of antimicrobial use.

Three trial characteristics [diagnostic test used to identify infected cows/quarters at dry off; whether the selective antimicrobial treatment was applied at cow or quarter level and whether an ITS was applied for untreated (healthy) cows/quarters or not] were tested. None of them could explain the observed heterogeneity ($I^2 = 97\%$). **Figure 3** presents the proportion of antimicrobial use reduction for each trial and a summary measure.

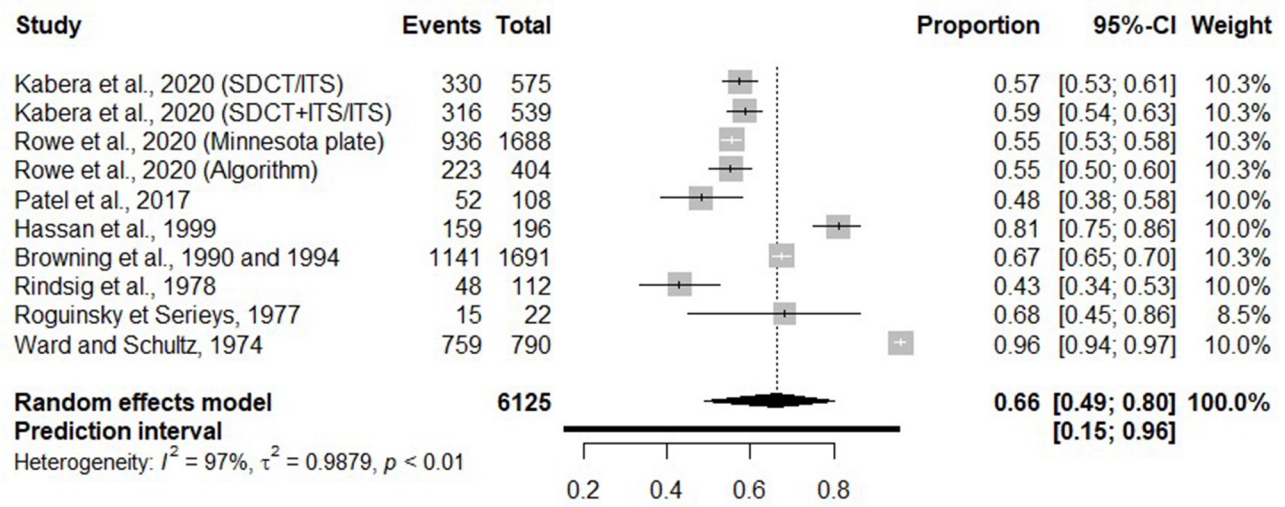


**FIGURE 3 |** Forest plots showing the proportion of antimicrobial use reduction.

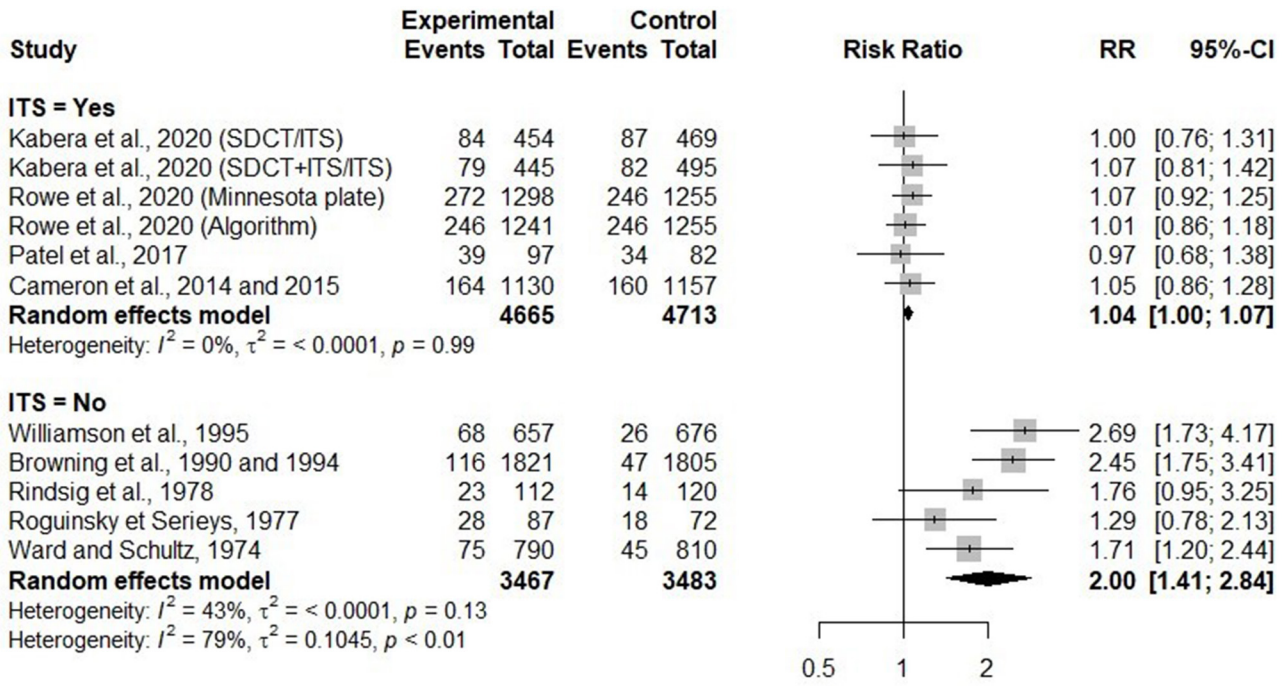


**FIGURE 4 |** Forest plots showing the effect of selective dry cow treatment compared with blanket dry cow therapy on risk of acquiring new IMI during the dry period, grouped by studies where untreated cows/quarters with antimicrobial received an internal teat sealant (ITS = Yes) and those where they did not receive an internal teat sealant (ITS = No).

## Effects of Selective Dry Cow Therapy on IMI Incidence Over the Dry Period

In 11 trials, IMI incidence risk during the dry period was investigated and reported at the quarter level. When comparing IMI incidence over the dry period in SDCT and BDCT, one trial characteristic [whether an ITS was applied for untreated (healthy) cows/quarters] was significantly associated with the estimate effect size ($p < 0.01$; $tau^2 = 0.00$). An ITS consisting of 65% bismuth subnitrate was used in the six trials where it was applied for untreated (healthy) cows/quarters. **Figure 4** presents the RR comparing risk of acquiring a new IMI over the dry period between selective and BDCT for

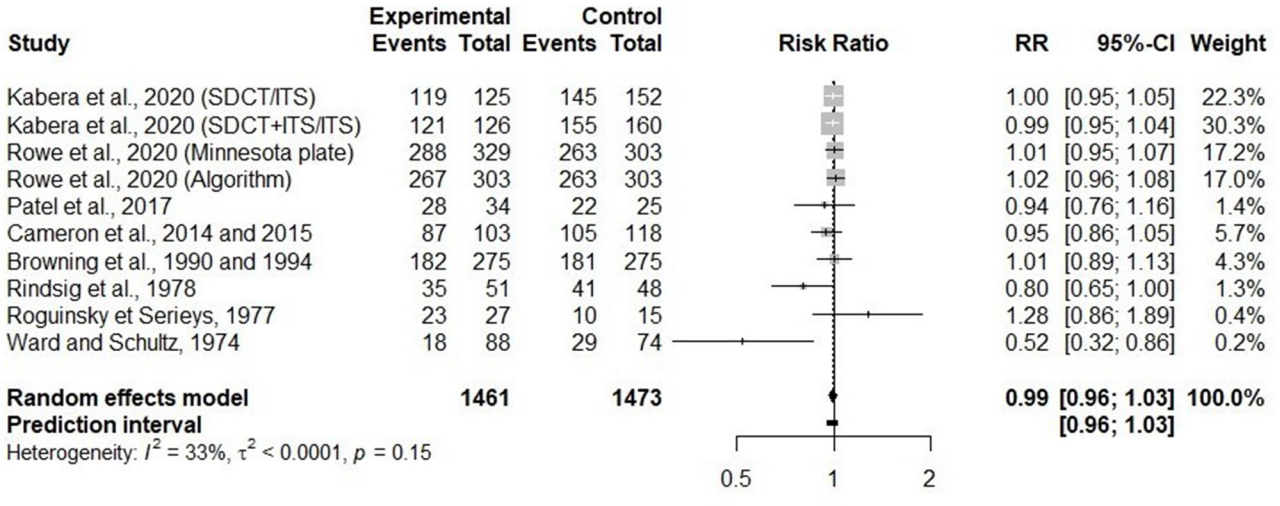


**FIGURE 5 |** Forest plots showing the effect of selective dry cow treatment compared with blanket dry cow therapy on risk of IMI elimination during the dry period.

each trial, as well as summaries of RR for trials using ITS or not.

For studies without ITS, the risk of new IMI during the dry period was significantly higher for selectively treated compared with blanket dry treated cows/quarters (RR = 2.00, 95% CI = 1.41, 2.84). Conversely, for studies where an ITS was used to protect healthy cows/quarters, the risk of new IMI during the dry period was not different for selectively treated compared with blanket dry treated cows/quarters (RR = 1.04, 95% CI = 1.00, 1.07).

### Effects of Selective Dry Cow Therapy on IMI Elimination Over the Dry Period

In 10 trials, elimination of IMI during the dry period was investigated. None of the variables evaluated in the meta-regressions were significantly associated with the risk of IMI elimination. **Figure 5** presents the RR comparing the risk of IMI elimination over the dry period between selective and BDCT for each trial, as well as a summary measure for all trials together. There was no difference (RR = 0.99, 95% CI = 0.96, 1.03) between SDCT and BDCT, regarding the elimination of IMI during the dry period.

### Effects of Selective Dry Cow Therapy on IMI Prevalence at Calving

In nine trials, IMI prevalence at calving was reported. Only one trial characteristic (whether an ITS was applied for healthy cows/quarters) was significant ($p < 0.01$, $tau^2 = 0.01$). **Figure 6** presents the RR comparing the risk of IMI at calving between SDCT and BDCT for each trial, as well as RR summaries for each category of ITS usage. For trials without ITS ($n = 5$), the risk of IMI at calving was significantly higher for selectively treated cows/quarters than blanket treated cows/quarters (RR = 1.57, 95% CI = 1.19, 2.06), but substantial heterogeneity was still present within this category ($I^2 = 60\%$). For trials using an ITS ($n = 4$), the risk of IMI at calving was not different for selectively and blanket treated cows/quarters (RR = 1.03, 95% CI = 0.97, 1.09). For this latter category, no heterogeneity was seen ($I^2 = 0\%$).

### Effects of Selective Dry Cow Therapy on Clinical Mastitis Incidence in the Early Lactation

Incidence risk of CM early in the following lactation was investigated in eight trials. Two of them reported CM incidence at the cow level and the other six at the quarter level. Before commingling these studies together, it would have been interesting to investigate in a meta-regression the impact of reporting CM, the outcome, at the cow vs. quarter level, but there were not enough trials where CM were reported at the cow level. Among the other potential predictors, only the method used to identify infected cows/quarters at dry off could be tested in a meta-regression and it was not significant. **Figure 7** presents the RR of CM incidence during the first 120 days of lactation between SDCT and BDCT for each trial, as well as a summary RR for all trials.

The risk of CM incidence during the first 4 months of lactation was not significantly different between selectively and blanket dry treated cows/quarters (RR = 1.03, 95% CI = 0.65–1.64). However, there was an important heterogeneity among trials ($I^2 = 83\%$). When we considered only the six trials where an ITS was used for healthy cows/quarters, the risk of CM was still not different between SDCT and BDCT (RR = 0.84, 95% CI = 0.65–1.08); however, the heterogeneity was reduced to an almost null value ($I^2 = 3\%$).

### Effects of Selective Dry Cow Therapy on Milk Yield in the Early Lactation

Only five trials reported on milk yield during the first 4 months of the subsequent lactation. **Figure 8** presents the mean difference of milk production during the first 4 months of lactation after a SDCT approach, in comparison with a BDCT, for each trial, as well as a summary measure for all trials. There was no difference in milk yield during the 1st months of the subsequent lactation

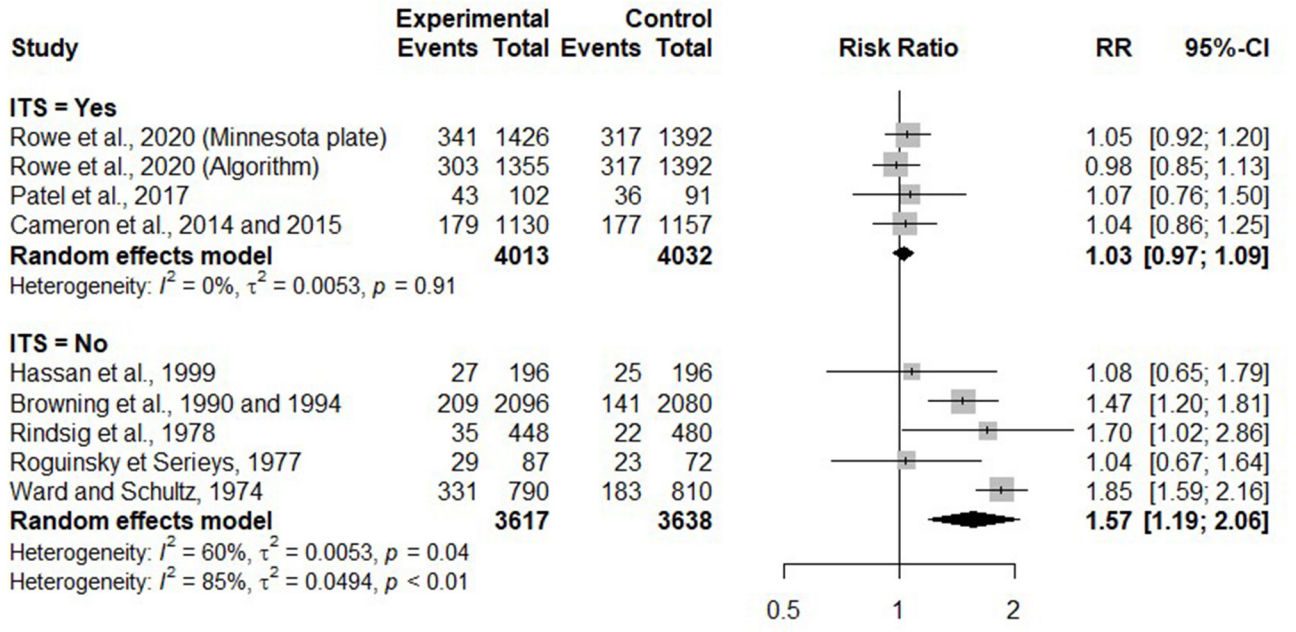


**FIGURE 6 |** Forest plots showing the effect of selective dry cow treatment compared with blanket dry cow therapy on risk of IMI prevalence at calving.

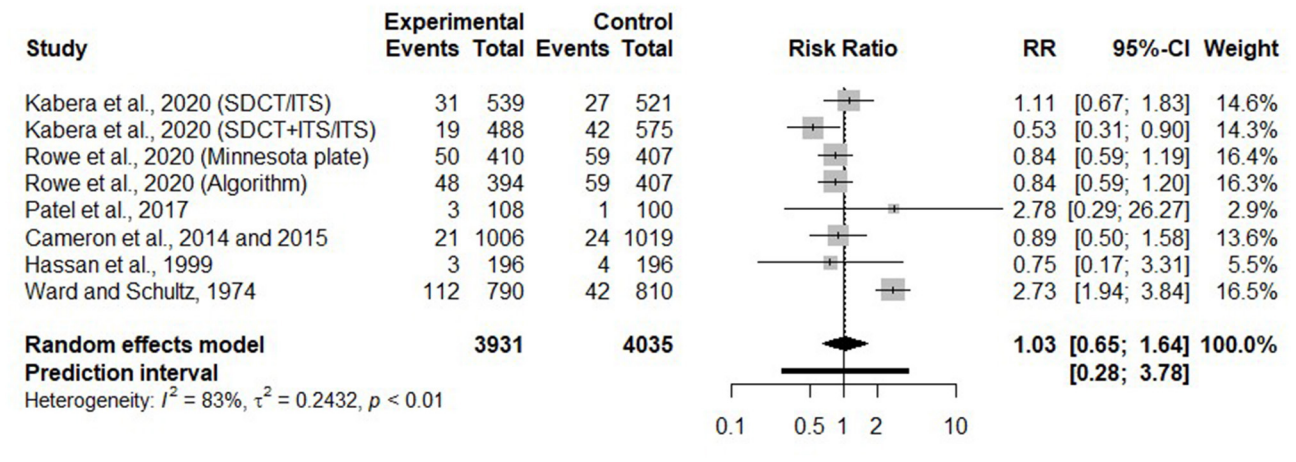


**FIGURE 7 |** Forest plots showing the effect of selective dry cow treatment compared with blanket dry cow therapy on risk of acquiring CM during the first 4 months of lactation.

(MD = −0.24 kg/day, 95% CI = −1.17, 0.70) between SDCT and BDCT.

### Effects of Selective Dry Cow Therapy on SCC in the Early Lactation

Five trials reported on SCC (transformed in ln SCC using the natural logarithm scale) during the first 4 months of the subsequent lactation. **Figure 9** presents the mean difference of ln SCC during the 1st months of lactation after a SDCT approach, in comparison with a BDCT, for each trial, as well as a summary measure. There was no difference in ln SCC during the 0–120 DIM period of the subsequent lactation (MD = 0.03, 95% CI = −0.09, 0.15) between SDCT and BDCT.

## Publication Bias

Contour-enhanced funnel plots for each outcome of comparison between SDCT and BDCT are presented in **Supplementary Figure 2**. However, because of the limited number of available trials, tests for funnel plot asymmetry could not be performed. Therefore, plots were evaluated visually, but it was not possible to identify putative missing studies.

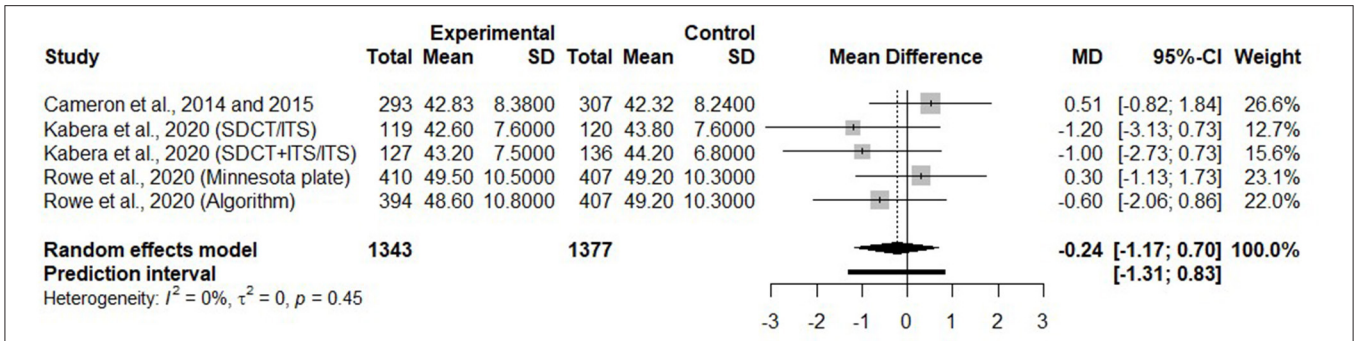


**FIGURE 8 |** Forest plot illustrating the mean difference in milk production (kg/day) during the first 4 months of lactation after a selective dry cow treatment approach, in comparison with a blanket dry cow therapy.

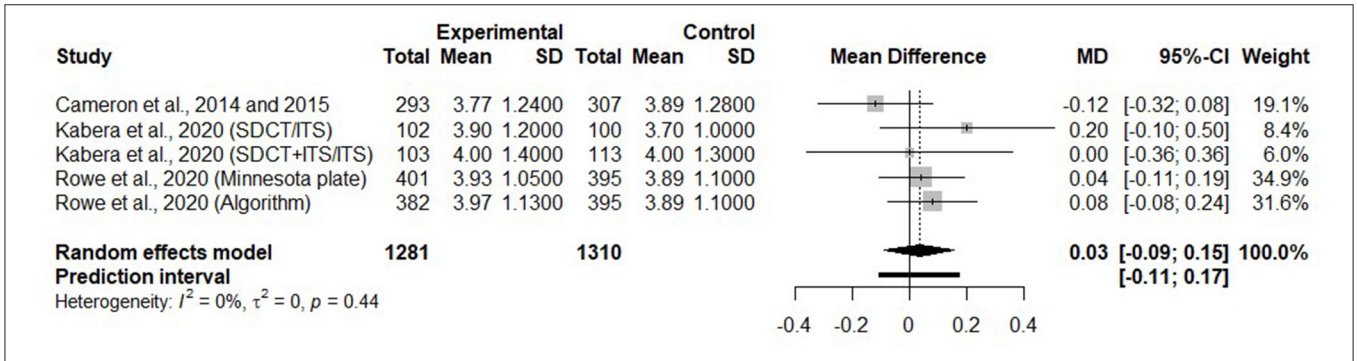


**FIGURE 9 |** Forest plot illustrating the mean difference in somatic cell counts (on a natural logarithm scale) during the first 4 months of lactation after a selective dry cow treatment approach, in comparison with a blanket dry cow therapy.

## Summary of Evidence

**Table 3** presents a GRADE evidence profile for the different outcomes comparing SDCT and BDCT. Our GRADE assessment indicated a high level of confidence for four of the six studied outcomes/comparisons: (1) risk of acquiring a new IMI in selective dry cow treated quarters/cows when an ITS was administered to healthy quarters; (2) prevalence of IMI, again when an ITS was administered to healthy quarters as part of the selective dry cow protocol; (3) milk yield in the subsequent lactation; and (4) ln SCC in the subsequent lactation.

# DISCUSSION

This systematic review was conducted to determine the efficacy of SDCT (anti-microbial treatment of infected quarters/cows solely) compared with BDCT (all quarters/all cows treated). It reports on SDCT as a potential alternative to BDCT. The main rationale for using a SDCT strategy is to reduce anti-microbial use. This, however, should be achieved, if possible, without any detrimental effect on udder health and milk production. Our results confirm that SDCT can help reduce the use of anti-microbials and that it can be without detrimental effects. However, this was only achieved when IMI incidence in untreated quarters was prevented using an ITS.

A comparable effect of SDCT and BDCT was reported by a review reporting on the prevalence of IMI at calving when all cows received an ITS (12). The same review, in agreement with us, reported a difference between SDCT and BDCT, when an ITS was not used to protect untreated quarters/cows. The importance of the use of ITS at dry off was reported by other previous reviews (8, 29, 30).

The current review also reported on acquisition and elimination of IMI during the dry period and on CM, milk yield, and ln SCC during the subsequent lactation. For all these outcomes, SDCT and BDCT were equivalent, as long as an ITS was used for untreated quarters. However, all trials which reported on milk yield and ln SCC used an ITS. Thus, for those two outcomes, it was not possible to measure the effect of SDCT when an ITS is not used for untreated quarters/cows.

There were small numbers of trials in both ITS categories for all outcomes, but low or no heterogeneity was observed in the ITS category for all tested outcomes (new IMI, prevalence of IMI at calving, and CM during the first 4 months of the subsequent lactation). For trials not using ITS, there was a high risk of new IMI and of IMI at calving in cows/quarters assigned to a SDCT protocol, compared with BDCT, but heterogeneity between trials was still important in this category. This maintenance of heterogeneity may be due not only to a small number of included

**TABLE 3 |** GRADE evidence profile: comparison between selective dry cow therapy (SDCT) and blanket dry cow therapy (BDCT) for curing intramammary infections (IMI) at dry off and preventing new IMI during the dry period.

| Outcome and comparison | Quality assessment | | | | | | Number of quarters (for IMI) or cows (for CM) | | Relative risk (95% CI) or Mean difference (95% CI) | Quality |
|---|---|---|---|---|---|---|---|---|---|---|
| | # trials (design) | Risk of bias | Inconsistency | Indirectness | Imprecision | Publication bias | BDCT | SDCT | | |
| **IMI incidence** | | | | | | | | | | |
| ITS to healthy Q/C[a] | 6 (RCT) | No serious | No serious | No serious | No serious | No serious | 855/4,713 | 884/4,665 | 1.04 (0.95, 1.13) | + + + +High |
| No ITS to healthy Q/C[b] | 2 (RCT) 3 (CT) | Very serious | Serious | No serious | No serious | No serious | 150/3,483 | 310/3,467 | 1.97 (1.52, 2.54) | + + – –Low |
| **Elimination of IMI** | 8 (RCT) 2 (CT) | No serious | Serious | No serious | No serious | No serious | 1,194/1,455 | 1,170/1,458 | 0.99 (0.96, 1.02) | + + + –Moderate |
| **IMI at calving** | | | | | | | | | | |
| ITS to healthy Q/C | 4 (RCT) | No serious | No serious | No serious | No serious | No serious | 847/4,032 | 866/4,013 | 1.02 (0.94, 1.11) | + + + +High |
| No ITS to healthy Q/C | 3 (RCT) 2 (CT) | Very serious | Serious | No serious | No serious | No serious | 394/3,638 | 631/3,617 | 1.48 (1.19, 1.84) | + + – –Low |
| **CM incidence** | 7 (RCT) 1 (CT) | No serious | Very serious | No serious | No serious | No serious | 258/4,035 | 287/3,931 | 1.03 (0.65, 1.64) | + + + –Moderate |
| **Milk yield** | 5 (RCT) | No serious | No serious | No serious | No serious | No serious | NA | NA | −0.24 (−1.17, 0.70)[c] | + + + +High |
| **ln SCC** | 5 (RCT) | No serious | No serious | No serious | No serious | No serious | NA | NA | 0.03 (−0.09, 0.15)[d] | + + + +High |

*CM, clinical mastitis; CI, confidence interval; RCT, randomized control trial; CT, control trial (not randomized or randomization not reported); ln SCC, natural logarithm of somatic cell counts.*

[a] *Where ITS was used for healthy quarters/cows at dry off.*

[b] *Where ITS was not used for healthy quarters/cows at dry off.*

[c] *Mean difference in milk yield (kg/day) or in SCC on the natural logarithm scale during the 1st months of the subsequent lactation.*

[d] *Mean difference in SCC on the natural logarithm scale during the 1st months of the subsequent lactation.*

trials but also to other unmeasured factors which may affect the effect estimated (12).

For all trials, cow- or quarter-level data were used in the meta-analysis, and therefore, clustering of quarters by cow or cows by herd was not accounted for. However, by considering a random effects approach, we accounted for clustering of individuals within different studies.

Regarding the reduction of antimicrobial use in dairy cows at dry off, we conclude that when SDCT is applied, antimicrobial use could be reduced by 66% (95% CI = 49, 80) compared with BDCT. For that outcome, a bimodal distribution was observed, with eight trials reporting proportions in the range of 43–68% and two trials with proportions of 81 and 96%. However, in these trials reporting proportions of 81 (24) and 96% (15), selection of treated quarters was based on a high NAGase (N-acetyl-beta-D-glucosaminidase) value or the occurrence of clinical mastitis during 1 month prior to drying off, respectively.

Moreover, 112 additional antimicrobial infusions during the dry period and early lactation were reported by Ward and Schultz (15). In total, 37 positive quarters including 10 clinical mastitis were reported by Hassan et al. (24) during the dry period in the selective group. These two latter SDCT approaches indeed resulted in very large reduction in antimicrobial usage at dry off, but also in substantial usage of antimicrobials during the dry period.

## Summary of Evidence

### Impact of Selective Dry Cow Therapy on Preventing the Acquisition of New Intramammary Infections During the Dry Period

Regarding the prevention of IMI over the dry period, we conclude with a high level of confidence that SDCT is as efficient as BDCT when an ITS (65% bismuth subnitrate) is used for untreated healthy quarters/cows at dry off. The efficacy of ITS in the prevention of IMI has been reported in previous reviews (8, 29–31). When an ITS was not used, we would conclude toward a higher risk of new IMI in SDCT compared with BDCT, but with a low level of confidence. These results suggest that, in the countries and through the different time periods where these studies were conducted, the infection pressure during the dry period was too important for leaving quarters completely unprotected (i.e., without antimicrobial and without ITS).

Regarding applying the selection at cow or quarter levels, we did not detect a difference between these SDCT approaches for IMI prevention. However, Halasa et al. (29) reported BDCT to be more protective of new IMI than SDCT when selection was based at the quarter level (RR = 2.01, 95% CI = 1.34, 3.02), but to no significant difference when selection was based at the cow level (RR = 0.52, 95% CI = 0.12, 2.31). In this latter review, however, SDCT protocols of the included studies did not include an ITS for untreated, healthy quarters or cows.

### Impact of Selective Dry Cow Therapy on the Elimination of Existing Intramammary Infections During the Dry Period

Regarding the elimination of existing IMI present at dry off, we conclude with a moderate level of confidence toward the

comparable efficiency of SDCT and BDCT. For that comparison, our level of confidence was mainly affected by the multimodal distribution observed for RR point estimates, with one trial reporting RR estimate of 1.28 (20), one trial with RR estimate of 0.52 (15), and eight trials with RR estimates in the 0.80–1.02 range. However, heterogeneity for this comparison was low ($I^2 = 32.8\%$) and the predicted RR interval was the same as the confidence interval of the effect size from the random effects model (0.96–1.03). A similar efficiency between SDCT and BDCT was also reported by Halasa et al. (32). When Ward and Schultz (15) was omitted, the RR was the same (RR = 0.99, 95% CI = 0.96, 1.03), but no heterogeneity was still seen in the analysis ($I^2 = 0$).

### Impact of Selective Dry Cow Therapy on Intramammary Infection Prevalence at Calving

Regarding IMI prevalence at calving, we concluded with a high level of confidence regarding the comparable efficiency of SDCT and BDCT, when an ITS (65% bismuth subnitrate) was used for untreated healthy quarters/cows. The same conclusion was reported by Winder et al. (12).

Conversely, when an ITS was not used, we had a low confidence in our general conclusions. The level of confidence was mainly affected by the bimodal distribution observed for RR point estimates and by a very serious ROB. Almost all trials included in this comparison were older (published between 1974 and 1999), and many of the important information on randomization (e.g., random sequence generation, allocation concealment) were not reported. As it was also reported by Winder et al. (12), when an ITS was not used, there was an increased risk of IMI at calving for SDCT compared with BDCT and a substantial heterogeneity was noted in this subgroup. The presence of a high residual heterogeneity indicates that there is more than one effect within the trials where an ITS was not used. The predicted RR interval within this subgroup was 0.74–2.93.

### Impact of Selective Dry Cow Therapy on Clinical Mastitis Incidence Early in the Subsequent Lactation

We have a moderate level of confidence regarding the equivalence of SDCT compared with BDCT for the reduction of CM in the following lactation. The level of confidence was affected by a bimodal distribution observed for the estimated RR. However, when we exclude two trials where an ITS was not used for untreated healthy quarters/cows at dry off, the heterogeneity was very low. The importance of ITS in the reduction of CM incidence in the subsequent lactation was reported by previous reviews (8, 30).

### Impact of Selective Dry Cow Therapy on Milk Yield and ln SCC During the Subsequent Lactation

Concerning milk yield and ln SCC during the subsequent lactation, we conclude with a high level of confidence regarding the comparable efficiency of SDCT and BDCT. However, only trials published between 2014 and 2020 and where ITS was used for untreated healthy quarters/cows at dry off were included in this comparison. None of the previous reviews reported on these two outcomes. In fact, those outcomes were not commonly reported in older studies. However, one of the included trials (7) reported SCC, but on an arithmetic scale which could not be compared with the logarithmic scale. We were not able to reach the authors to get these latter data on a logarithmic scale.

## Comparisons With Published Reviews

The fact that the review of Winder et al. (12) was conducted concurrently to our review provided an opportunity for comparing how our different methodologies affected the presented results. The most striking difference between the reviews are the outcomes analyzed. The main rationale for adopting selective dry cow treatment is the associated reduction in the use of antimicrobials. Quantifying this potential reduction was, in our opinion, essential. Likewise, the risk of CM, milk yield, and SCC in the early next lactation are also important parameters to quantify, to better inform producers considering moving to a selective treatment approach. Finally, although IMI incidence and elimination rates are somewhat captured by measuring IMI prevalence at calving, reporting on these indices provides a better understanding of the underlying biological processes. Our analyses indeed confirmed that the increased IMI prevalence at calving in SDCT protocols when an ITS is not used was mainly caused by an increased IMI incidence in untreated quarters.

Beyond the different outcomes presented, our different methodologies also affected article selection. Three articles included in (12) were not included in our review. The first article (33) was excluded from our review because the antimicrobials used were not specified. Furthermore, it was not clear whether infected cows in the selective group received the same antimicrobial as the cows in the blanket group. When the first author was contacted, he confirmed that each farm used the intramammary antibiotic which was normally used before the trial, but he could not confirm that cows of the same herd and allocated to the selective or blanket groups received the same antimicrobial, as the antimicrobials used could have been modified by a farmer during the study. The second article (34) was excluded, as we considered that cows in the SDCT and BDCT groups were managed differently. In fact, in this latter study, cows in the BDCT group were teat dipped after each milking, while in the SDCT group, they were not teat dipped. Thus, the study of Robinson et al. (34) actually compared blanket dry cow therapy with teat disinfection vs. selective dry cow therapy and no teat disinfection. Moreover, Winder et al. (12) included the Serieys and Roguinsky (35) paper, while our review considered the Roguinsky and Serieys (20) paper. These two papers reported on results of the same trial. The 1977 paper was judged more complete by our team and was, therefore, chosen for inclusion. The results presented in the 1975 and 1977 papers differed slightly and this resulted in (12) using 23/82 quarters with an IMI at calving for blanket treated cows for the Roguinsky's study while our review considered 23/72 infected quarters at calving for blanket treated cows for that same study. Finally, one paper (27) published after the publication of the review of Winder et al. (12) was included in our review and used for comparing prevalence of IMI at calving.

Another difference between our review and that of Winder et al. (12) was observed in the numbers extracted from the

study of Cameron et al. (5). In their review, Winder et al. (12) mentioned 164/1,130 and 160/1,157 quarters with a prevalent IMI at calving for the SDCT and BDCT groups, respectively. These numbers were incorrectly extracted in the review of Winder et al. (12). In the paper of Cameron et al. (5), these numbers are indeed presented, but they represented the new IMI risk over the dry period, not the post-calving IMI risk, which were presented in a different table. These numbers were 179/1,130 and 177/1,157 quarters with a prevalent IMI at calving for the SDCT and BDCT groups, respectively.

Overall, these differences in selected studies and in data extraction between reviews resulted in very small differences in the estimated summary measures. Using data from 3,750 quarters, Winder et al. (12) reported a summary risk ratio (95% CI) of 1.09 (0.92, 1.28) when comparing the risk of IMI at calving using selective dry cow therapy with a teat sealant for untreated quarters compared with blanket dry cow therapy. Using data from 8,045 quarters, we reported a risk ratio of 1.03 (0.97, 1.09). On the other hand, we were also able to report on the reduction of antimicrobial usage, IMI incidence risk, and IMI elimination risk, as well as CM incidence, milk yield, and ln SCC in the beginning of the subsequent lactation.

Beyond the review of Winder et al. (12), two other previously published meta-analyses (29, 32) have investigated the comparison of SDCT vs. no dry cow treatment or SDCT vs. BDCT for the prevention of new IMI and elimination of existing IMI during the dry period. In our review, only studies comparing SDCT and BDCT were retained. Thus, only a small number of articles used in the reviews of Halasa et al. (29, 32) are included in this review (7, 22–24). Moreover, none of the studies included in the comparison of SDCT and BDCT (7, 22–24, 36) had used ITS for untreated, healthy quarters or cows.

### Limitations

A small number of trials were included in our review. Those trials were published over a wide period of time (1974–2020). Herd-level inclusion criteria were not reported for six trials. For trials which did, herds were selected with a low BTSCC (<250,000 cells/ml of milk) (5, 6, 25, 27, 28) or a wide range in BTSCC (100,000–400,000 cells/ml of milk) (21, 22). Moreover, Cameron et al. (5, 25) reported on cows with a SCC <200,000 cells/ml on the last three DHI tests and no CM on the same period.

Most reviewed studies (mostly the more recent ones) and, in particular, studies where ITS was used for healthy and untreated quarters were conducted in herds with a relatively low bulk-tank SCC <250,000 cells/ml. For herds with higher bulk milk SCC, there would probably be a higher prevalence of IMI at dry off and especially a higher prevalence of contagious pathogens. Thus, there might be an increased risk of IMI during the dry period for quarters that were not treated at dry off, regardless of receiving an ITS. So, the results of this review should be extrapolated to low SCC herds (BTSCC <250,000 cells/ml) only.

There were also differences in the definition of IMI used across different trials, and the time when the post-calving samples were collected also varied between studies (**Supplementary Table 1**). These differences in IMI definition could be one of the important causes for the heterogeneity of effect observed between studies.

We initially planned to investigate the effect of randomization (randomized vs. non-randomized trials) in our meta-regressions. However, there was no information on randomization for four studies (20–24). They reported that subjects were allocated randomly, but the description of the randomization process was not detailed. In our descriptive work, these studies were, therefore, classified simply as controlled trials. These studies with no mention of randomization were, however, mostly older studies. Perhaps, at that time, it was not common to mention randomization in the text. Thus, it is unclear whether these studies were truly non-randomized or if the information on randomization was simply lacking in the text. To avoid inappropriate categorization, we did not conduct meta-regression based on reporting or not a randomization.

Meta-regression suggested that the use of teat sealants for quarters/cows not treated with an antimicrobial could explain part of the heterogeneity in the original analysis and reduces the negative impact of SDCT on udder health and milk production in the subsequent lactation. More research would be needed to investigate other factors explaining heterogeneity in the effect estimates.

Another potential limitation was the language restriction, as only articles in English and French were evaluated for inclusion in our review. Thus, we could hypothesize that additional articles would possibly have been included if this restriction was not applied. Also, because of a small number of included trials in each comparison, the potential publication bias could not be thoroughly investigated.

## CONCLUSION

From the available literature, we can conclude that, for low SCC herds (BTSCC < 250,000 cells/ml), SDCT is as efficient as BDCT for curing existing IMI at dry off, preventing new IMI during the dry period, and preventing CM in the beginning of the subsequent lactation if ITS (65% bismuth subnitrate) is used for healthy, untreated quarters/cows. Moreover, milk yield and ln SCC in the beginning of the subsequent lactation would not differ between quarters treated using a selective or a blanket treatment approach. Finally, we can conclude that the use of SDCT would have an important impact on the use of anti-microbials at dry off in dairy cows.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

FK co-ordinated the team and was responsible for searching articles from databases and drafting the manuscript. MA was responsible for searching articles from conference proceedings. FK, J-PR, and SD were responsible for extracting data and assessing ROB and their analyses. All authors reviewed

and provided feedback on the manuscript, contributed to the development of the review protocol, and selection of eligible articles.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.688450/full#supplementary-material

## REFERENCES

1. Neave FK, Dodd FH, Kingwill RG, Westgarth DR. Control of mastitis in the dairy herd by hygiene and management. *J Dairy Sci*. (1969) 52:696–707. doi: 10.3168/jds.S0022-0302(69)86632-4
2. Bradley AJ, Green MJ. A study of the incidence and significance of intramammary enterobacterial infections acquired during the dry period. *J Dairy Sci*. (2000) 83:1957–65. doi: 10.3168/jds.S0022-0302(00)75072-7
3. Bradley AJ, Green MJ. The importance of the non-lactating period in the epidemiology of intramammary infection and strategies for prevention. *Vet Clin North Am Food Anim Pract*. (2004) 20:547–68. doi: 10.1016/j.cvfa.2004.06.010
4. Green MJ, Green LE, Medley GF, Schukken YH, Bradley AJ. Influence of dry period bacterial intramammary infection on clinical mastitis in dairy cows. *J Dairy Sci*. (2002) 85:2589–99. doi: 10.3168/jds.S0022-0302(02)74343-9
5. Cameron M, McKenna SL, MacDonald KA, Dohoo IR, Roy JP, Keefe GP. Evaluation of selective dry cow treatment following on-farm culture: risk of post-calving intramammary infection and clinical mastitis in the subsequent lactation. *J Dairy Sci*. (2014) 97:270–84. doi: 10.3168/jds.2013-7060
6. Kabera F, Dufour S, Keefe G, Cameron M, Roy JP. Evaluation of quarter-based selective dry cow therapy using Petrifilm on-farm milk culture: A randomized controlled trial. *J Dairy Sci*. (2020) 103:7276–87. doi: 10.3168/jds.2019-17438
7. Rindsig RB, Rodewald RG, Smith AR, Spahr SL. Complete vs. selective dry cow therapy for mastitis control. *J Dairy Sci*. (1978) 61:1483–97. doi: 10.3168/jds.S0022-0302(78)83753-9
8. Dufour S, Wellemans V, Roy JP, Lacasse P, Ordonez-Iturriaga A, Francoz D. Non-antimicrobial approaches at drying-off for treating and preventing intramammary infections in dairy cows. Part 1. Meta-analyses of efficacy of using an internal teat sealant without a concomitant antimicrobial treatment. *Anim Health Res Rev*. (2019) 20:86–97. doi: 10.1017/S1466252319000070
9. Huxley JN, Green MJ, Green LE, Bradley AJ. Evaluation of the efficacy of an internal teat sealer during the dry period. *J Dairy Sci*. (2002) 85:551–61. doi: 10.3168/jds.s0022-0302(02)74108-8
10. Sanford CJ, Keefe GP, Dohoo IR, Leslie KE, Dingwell RT, DesCoteaux L, et al. Efficacy of using an internal teat sealer to prevent new intramammary infections in non-lactating dairy cattle. *J Am Vet Med Assoc*. (2006) 228:1565–73. doi: 10.2460/javma.228.10.1565
11. Vanhoudt A, van Hees-Huijps K, van Knegsel ATM, Sampimon OC, Vernooij JCM, Nielen M, et al. Effects of reduced intramammary antimicrobial use during the dry period on udder health in Dutch dairy herds. *J Dairy Sci*. (2018) 101:3248–60. doi: 10.3168/jds.2017-13555
12. Winder CB, Sargeant JM, Kelton DF, Leblanc SJ, Duffield TF, Glanville J, et al. Comparative efficacy of blanket vs. selective dry-cow therapy: a systematic review and pairwise meta-analysis. *Anim Health Res Rev*. (2019) 20:217–28. doi: 10.1017/S1466252319000306
13. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *J Clin Epidemiol*. (2009) 62:1006–12. doi: 10.1136/bmj.b2535
14. Affix M, Kabera F, Styron H, Roy JP, Header LC, Godden S, et al. Antimicrobial-based dry cow therapy approaches for cure and prevention of intramammary infections: a protocol for a systematic review and meta-analysis. *Anim Health Res Rev*. (2018) 19:74–8. doi: 10.1371/journal.pmed.1000097
15. Ward GE, Schultz LH. Incidence and control of mastitis during the dry period. *J Dairy Sci*. (1974) 57:1341–9. doi: 10.3168/jds.S0022-0302(74)85065-4
16. Higgins JPT, Sterne J, Slavonic J, Hróbjartsson A, Boutron I, Reeves B, et al. A revised tool for assessing risk of bias in randomized trials. In: Chandler J, McKenzie J, Boutron I, Welch V, editors. *Cochrane Methods Cochrane Database of Systematic Reviews*. (Suppl 1). Oxford: John Wiley & Sons, Ltd. (2016). p. 29–31.
17. Langan D, Higgins JPT, Jackson D, Bowden J, Veroniki AA, Kontopantelis E, et al. A comparison of heterogeneity variance estimators in simulated random-effects meta-analyses. *Res Synth Methods*. (2019) 10:83–98. doi: 10.1002/jrsm.1316
18. Knapp G, Hartung J. Improved tests for a random effects meta-regression with a single covariate. *Stat Med*. (2003) 22:2693–710. doi: 10.1002/sim.1482
19. Guyatt GH, Oxman AD, Kunz R, Brozek J, Alonso-Coello P, Rind D, et al. GRADE guidelines 6. Rating the quality of evidence–imprecision. *J Clin Epidemiol*. (2011) 64:1283–93. doi: 10.1016/j.jclinepi.2011.01.012
20. Roguinsky M, Serieys F. Treatment of mastitis in dry cows: treatment of all cows vs. treatment of infected cows only (author's transl). *Ann Rech Vet*. (1977) 8:327–31.
21. Browning JW, Mein GA, Barton M, Nicholls TJ, Brightling P. Effects of antibiotic-therapy at drying off on mastitis in the dry period and early lactation. *Aust Vet J*. (1990) 67:440–2. doi: 10.1111/j.1751-0813.1990.tb03055.x
22. Browning JW, Mein GA, Brightling P, Nicholls TJ, Barton M. Strategies for mastitis control–dry cow therapy and culling. *Aust Vet J*. (1994) 71:179–81. doi: 10.1111/j.1751-0813.1994.tb03383.x
23. Williamson JH, Woolford MW, Day AM. The Prophylactic Effect of a Dry-Cow Antibiotic against Streptococcus-Uberis. *N Z Vet J*. (1995) 43:228–34. doi: 10.1080/00480169.1995.35898
24. Hassan Z, Daniel RCW, O'Boyle D, Frost AJ. Effects of dry cow intramammary therapy on quarter infections in the dry period. *Vet Rec*. (1999) 145:635–9. doi: 10.1136/vr.145.22.635
25. Cameron M, Keefe GP, Roy JP, Stryhn H, Dohoo IR, McKenna SL. Evaluation of selective dry cow treatment following on-farm culture: milk yield and somatic cell count in the subsequent lactation. *J Dairy Sci*. (2015) 98:2427–36. doi: 10.3168/jds.2014-8876
26. Patel K, Godden SM, Royster EE, Timmerman JA, Crooker BA, McDonald N. Pilot study: impact of using a culture-guided selective dry cow therapy

program targeting quarter-level treatment on udder health and antibiotic use. *Bovine Practitioner*. (2017) 51:48–57. doi: 10.21423/bovine-vol51no1p48-57
27. Rowe SM, Godden SM, Nydam DV, Gorden PJ, Lago A, Vasquez AK, et al. Randomized controlled non-inferiority trial investigating the effect of 2 selective dry-cow therapy protocols on antibiotic use at dry-off and dry period intramammary infection dynamics. *J Dairy Sci*. (2020) 103:6473–92. doi: 10.3168/jds.2019-17728
28. Rowe SM, Godden SM, Nydam DV, Gorden PJ, Lago A, Vasquez AK, et al. Randomized controlled trial investigating the effect of 2 selective dry-cow therapy protocols on udder health and performance in the subsequent lactation. *J Dairy Sci*. (2020) 103:6493–503. doi: 10.3168/jds.2019-17961
29. Halasa T, Osteras O, Hogeveen H, van Werven T, Nielen M. Meta-analysis of dry cow management for dairy cattle. Part 1. Protection against new intramammary infections. *J Dairy Sci*. (2009) 92:3134–49. doi: 10.3168/jds.2008-1740
30. Rabiee AR, Lean IJ. The effect of internal teat sealant products (Teatseal and Orbeseal) on intramammary infection, clinical mastitis, and somatic cell counts in lactating dairy cows: a meta-analysis. *J Dairy Sci*. (2013) 96:6915–31. doi: 10.3168/jds.2013-6544
31. Robert A, Seegers H, Bareille N. Incidence of intramammary infections during the dry period without or with antibiotic treatment in dairy cows–a quantitative analysis of published data. *Vet Res*. (2006) 37:25–48. doi: 10.1051/vetres:2005047
32. Halasa T, Nielen M, Whist AC, Osteras O. Meta-analysis of dry cow management for dairy cattle. Part 2. Cure of existing intramammary infections. *J Dairy Sci*. (2009) 92:3150–7. doi: 10.3168/jds.2008-1741
33. Seeth MT, Wente N, Paduch JH, Klocke D, Vries EMD, Hoedemaker M, et al. Different selective dry cow therapy concepts compared to blanket antibiotic dry cow treatment. *Tieraerztliche Praxis Ausgabe Grosstiere Nutztiere*. (2017) 45:343–9. doi: 10.15653/TPG-170208
34. Robinson TC, Jackson ER, Marr A. Within herd comparison of teat dipping and dry cow therapy with only selective dry cow therapy in six herds. *Vet Rec*. (1983) 112:315–9. doi: 10.1136/vr.112.14.315
35. Serieys F, Roguinsky M. Comparative tests with dry cow therapy for some or all cows in a herd. *Ann Bull*. (1975) 85:349–51.
36. Robinson TC, Jackson ER, Marr A. Mastitis incidence in quarters with different infections status at drying off and calving in two treatment groups. *Br Vet J*. (1988) 144:166–73. doi: 10.1016/0007-1935(88)90049-8

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# Milk Macrophage Function in Bovine Leukemia Virus-Infected Dairy Cows

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The implications of bovine leukemia virus (BLV) on innate and adaptive immune responses have been widely investigated; however, the effects of BLV on mammary gland immunity require further investigation. The present study investigated the viability, phagocytic capacity, and intracellular production of reactive oxygen and nitrogen species (RONS) by macrophages in milk samples from dairy cows naturally infected with BLV with or without persistent lymphocytosis (PL). No effect of BLV infection in the overall number of macrophages per milliliter and in the percentage of viable macrophages among overall milk viable cells was found. Furthermore, BLV-infected dairy cows had a higher frequency of viable milk macrophages, while healthy animals had a tendency toward a higher percentage of apoptotic milk macrophages. The percentage of milk macrophages that phagocytosed *Staphylococcus aureus* in seronegative animals was higher than that in BLV-infected dairy cows. No effect of BLV infection on the intracellular RONS production and the intensity of phagocytosis by milk macrophages was observed. Thus, this study provides new insights into the implications of BLV infections in the bovine mammary gland.

**Keywords: enzootic bovine leukosis, deltaretrovirus, immune response, mastitis, dairy cattle**

## INTRODUCTION

Bovine leukemia virus (BLV) belongs to the family *Retroviridae* of the oncogenic genus *Deltaretrovirus*, which has tropism for B cells (1–4), although, it could infect and persist in other cell types, such as macrophages (1, 2). It is the etiological agent of enzootic bovine leukosis (EBL), which is widely disseminated in dairy herds in several countries (5). For the most part, the infectious implications of EBL remain asymptomatic, and these infections are termed aleukemic (AL). However, ∼20–30% of BLV-infected dairy cows have dramatic hematological changes known

as persistent lymphocytosis (PL), which is characterized by a non-malignant polyclonal expansion of B lymphocytes. However, its malignant presentation is rare and results in lymphoma of B cells (2).

It is understandable that different viruses have direct implications on the function of the innate and adaptive immune systems (4–6). Hereupon, several studies have investigated the effects of BLV infection on lymphocyte subpopulations (2, 4, 5, 7, 8), functions of neutrophils (4, 6, 9), mammary epithelial cells (10–12), and monocytes (13). However, to the best of our knowledge, the effects of BLV infection on the function of milk macrophages and their viability are only speculative.

Eventually, the impact of chronic viral diseases with low lethality, especially BLV, is important when correlated with comorbidities, such as mastitis. This disease remains one of the main health problems in dairy herds, and it has a direct impact on milk quality and public health due to the transmission of pathogens and antimicrobial residues in milk (4). In this sense, some studies pointed to an association between BLV in herds with a high prevalence of mastitis (14, 15), the severity of clinical mastitis (16), and reduced milk production due to immunosuppression caused by BLV leading to opportunistic infections, which are also seen in other viral infections (17). Infection by BLV also negatively impacts the culling rate of dairy cattle, and AL animals may show decreased productivity and profitability (18). Thus, these findings raised questions about the effects of BLV on mammary gland immunity, in which macrophages are the main population present in milk from health mammary glands (19–24), thus being the first defense cells to come into contact with the pathogen that causes mastitis.

Thus, considering the importance of macrophages to the mammary gland during an infectious process, the present study investigated the phagocytic capacity and intracellular production of reactive oxygen and nitrogen species (RONS) by milk macrophages and their viability in BLV naturally infected dairy cows with or without PL.

# MATERIALS AND METHODS

## Animals, Experimental Design, and Sample Collection

The present study used 57 mammary quarters of 19 Holstein dairy cows from a commercial herd at different stages of lactation. Immediately postpartum animals were not used. The following exclusion criteria were applied because of the effects of bacterial mastitis pathogens on mammary gland immunity (4): (1) udder quarters with clinical mastitis detected by the strip cup test and clinical examination; (2) bacteriologically positive quarters; and (3) udder quarters with high somatic cell count (SCC) (>200,000 cells $mL^{-1}$). All dairy cows were in their second or third lactation.

Blood sera from all animals were tested for BLV using an agar gel immunodiffusion assay (AGID) (Tecpar®, Curitiba, Brazil) and ELISA (cat. n. 284-5, VMRD Pullman Inc., Pullman, WA, USA) using the gp51 antigen. The animals were divided into three groups according to the results of the serological tests and the leukogram: (1) eight healthy dairy cows (24 mammary quarters) seronegative for BLV and without hematological alterations (25); (2) six BLV-infected dairy cows (16 mammary quarters) without hematological alterations (25), so-called AL; and (3) five BLV-infected dairy cows (17 mammary quarters) with PL. The BLV-infected cows were classified as having PL when their lymphocyte counts exceeded $1 \times 10^4$ $\mu L^{-1}$ and their leukocyte counts exceeded $1.5 \times 10^4$ $\mu L^{-1}$, as established by Thurmond et al. (26).

One hundred ten days after the first blood collection for the serodiagnosis of EBL, additional blood samples were collected for hematological examinations and serodiagnosis for the BLV to confirm the PL. At this time, 110 days after the first collection, milk samples were also collected for SCC, bacteriological culture, and functional analysis of macrophages determined by flow cytometry.

First, for milk sampling, the strip cup test was performed to detect the presence of any obviously abnormal macroscopic alterations in milk. Afterwards, pre-dipping was performed and immediately after drying using a sheet of paper towel for each teat. After discarding the first three milk streams, the teat sphincter was scrubbed with 70% alcohol-moistened cotton, and individual quarter milk samples were aseptically collected in sterile flasks for microbiological analysis. Finally, milk samples were collected for SCC and evaluation of macrophage function and viability. The samples were kept at 4°C until arrival at the laboratory. Milk samples for bacteriological analysis were stored at −20°C for a maximum of 30 days until analysis.

Subsequently, each sample was coded and randomized, and the analyses were performed so that the researcher was not aware of the BLV status of the animal from which the sample was collected.

## Leukogram

The total leukocyte count was determined using an automated cell counter (ABX VET ABC, Horiba ABX Diagnostic®, Montpellier, France). The differential leukocyte count was performed using a routine blood smear.

## Bacteriological Examination of Milk

Bacteriological analyses were performed by culturing 10 μL of milk on 5% defibrinated sheep blood agar plates. The plates were incubated at 37°C for 72 h. For bacterial identification, macroscopic observation of colony morphology, Gram staining, and biochemical tests were performed (27). A milk sample was considered positive when there was the growth of one or more colonies (>100 CFU $mL^{-1}$) (28, 29).

## Determination of the Somatic Cell Count

Milk samples for SCC determination were collected in 40-mL sterile flasks containing micropellets of the Bronopol preservative (2-bromo-2-nitropane-1,3-diol). Subsequently, SCC was performed with the automated somatic cell counter Somacount 300 (Bentley Instruments, Chaska, MN, USA).

## Separation of Milk Cells

The separation of milk cells was performed as described by Blagitz et al. (30, 31). In summary, 1 L of milk was diluted in 1 L of buffered saline solution (PBS). After centrifugation at

1000 × *g* for 15 min, the fat layer and the supernatant were discarded. The cell pellet was then washed again with 30 mL of PBS solution and centrifuged at 400 × *g* for 10 min. Subsequently, the cells were resuspended in 1 mL of RPMI-1640 cell culture medium (cat. n. R7638, Sigma Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (Cultilab, Brazil), and subsequently, the cells were counted in a Neubauer chamber. Cell viability was first assessed by trypan blue exclusion. The milk cells were then diluted with cell culture medium containing 10% fetal bovine serum at a concentration of $2 \times 10^6$ $mL^{-1}$ viable milk cells. Then, 100 μL of the milk cell suspension (containing $2 \times 10^5$ cells) was transferred to tubes suitable for flow cytometry analysis for further flow cytometric analysis.

## Detection of Apoptosis by Flow Cytometry

Apoptosis of milk macrophages was determined by double staining with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) by flow cytometry analysis using a commercial kit (cat. n. K2350, APOPTEST-FITC, Dako Cytometrion, Netherlands), as previously described by Della Libera et al. (4) and Souza et al. (32). Initially, $2 \times 10^5$ milk cells were resuspended in 100 μL of binding buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing anti-annexin-V FITC antibody and incubated at room temperature for 20 min in the dark. Subsequently, the macrophages were labeled with a CD14-specific mAb, as described below. Immediately before the flow cytometry analysis, 5 μL of a 250 μg $mL^{-1}$ PI solution was added. Cells negative for annexin-V FITC and PI were considered alive (**Supplementary Figure 1**). Cells that were reactive to FITC-labeled annexin-V but negative for PI were classified as apoptotic (**Supplementary Figure 1**). Finally, cells positive for both annexin-V FITC and PI were regarded as late apoptotic or necrotic (33) (**Supplementary Figure 1**).

The readings of the samples were performed using a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems™, San Diego, USA) with argon (excitation 488 nm) and diode (excitation 635 nm) lasers. Here, 20,000 cells, excluding most of the debris, were examined per sample. FlowJo software (Tree Star Inc., Ashland, USA) was used to analyze the data.

## Preparation of *Staphylococcus aureus* Stained With PI

The staining of heat-killed *S. aureus* (ATCC 25923) with PI was prepared as proposed by Hasui et al. (34) and slightly modified by Della Libera et al. (4).

## Intracellular Production of Rons

The intracellular production of RONS was performed by flow cytometry, as previously described (4, 31, 32), using 2′,7′ dichlorofluorescein diacetate ($DCFH_2$-DA) as a probe. The various types of RONSs (hydrogen peroxide, peroxynitrite, nitric oxide, hydroxyl radicals, and peroxyl) oxidize $DCFH_2$-DA into DCF, which is fluorescent and can be detected by flow cytometry (35). Briefly, $2 \times 10^5$ viable milk cells were incubated with 200 μL of $DCFH_2$-DA (0.3 mM, cat. n. D6883, Sigma Aldrich, St. Louis, USA) for 30 min at 37°C and 800 μL of PBS. Subsequently, 2 mL of 3 mM EDTA was added. Next, the macrophages were labeled with a CD14-specific mAb, as described below. Finally, the samples were centrifuged at 400 × *g* for 10 min, and the supernatant was discarded, and the leukocytes were resuspended in 300 μL of PBS.

Finally, 20,000 cells, except for most cellular debris, were examined per sample. The readings of the samples were performed using a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems™, San Diego, USA) with argon (excitation 488 nm) and diode (excitation 635 nm) lasers. FlowJo software (Tree Star Inc., Ashland, USA) was used to analyze the data. The data are presented as the percentage of macrophages ($CD14^+$ cells; **Supplementary Figure 1**) that produced RONSs (percentage of fluorescent cells), and the geometric mean fluorescence intensity (GMFI) indicates the intensity of RONS production of each cell given by the measurement of fluorescence intensity. The results were corrected for autofluorescence content using non-stained milk cells from milk samples from the same mammary quarter.

## Phagocytosis

The phagocytosis assay was performed by flow cytometry using PI-conjugated *S. aureus*, as previously described (4, 32, 36). Briefly, $2 \times 10^5$ viable milk cells were incubated with 100 μL of PI-conjugated *S. aureus* for 30 min at 37°C and 900 μL of PBS. Subsequently, 2 mL of 3 mM EDTA was added to drastically reduce the number of bacteria adhering to the cell membrane that could be mistakenly considered phagocytized (34, 37). Next, the macrophages were labeled with a CD14-specific mAb, as described below. Finally, the samples were centrifuged at 400 × *g* for 10 min, and the supernatant was discarded, and the leukocytes were resuspended in 300 μL of PBS.

Finally, 20,000 cells, excluding most cellular debris, were examined per sample. The readings of the samples were performed using a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems™, San Diego, USA) with argon (excitation 488 nm), and diode (excitation 635 nm) lasers. FlowJo software (Tree Star Inc., Ashland, USA) was used to analyze the data. The data are presented as the percentage of macrophages ($CD14^+$ cells) that phagocytized PI-stained bacteria (percentage of fluorescent cells; **Supplementary Figure 1**), and the GMFI indicates the number of bacteria phagocytized per macrophage by measuring the fluorescence intensity, which is correlated with the number of phagocytized bacteria per cell. The results were corrected for autofluorescence content using non-stained milk cells from milk samples from the same mammary quarter.

## Identification of Milk Macrophages

Initially, the cells were incubated with 1 μL of mouse anti-bovine CD14 mAb (clone MM61A; VMRD Pullman, Pullman, USA) for 30 min at room temperature. Immediately after, 1 mL of PBS was added to the specific cytometry tube, and the samples were centrifuged at 400 × *g* for 8 min, and the supernatant was discarded. Subsequently, 1 μL of secondary antibody allophycocyanin-conjugated goat anti-mouse IgG1

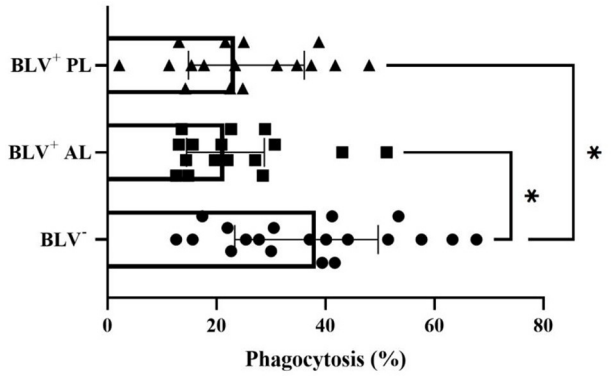


**FIGURE 1 |** Percentage (individual values and median ± interquartile range) of milk macrophages that phagocytized *S. aureus* from health mammary glands in bovine leukemia virus (BLV) seronegative ($BLV^-$) and in BLV-infected ($BLV^+$) dairy cows without (AL) and with persistent lymphocytosis (PL). *$P < 0.05$.

(APC; cat. n. A10541, Invitrogen, Carlsbad, USA) was added to the samples, which were incubated for 30 min at room temperature. Then, 1 mL of the PBS solution was added to the cell suspension, which was centrifuged at 400 × g for 8 min, and the supernatant was discarded. The macrophages were identified using flow cytometry based on cells' CD14 positivity (**Supplementary Figure 1**). Finally, 300 μL of PBS was added to the samples that were analyzed by flow cytometry (BD FACSCalibur, Becton Dickinson Immunocytometry System™, San Diego, USA). The number of macrophages were determined by multiplying the percentage of overall macrophages ($CD14^+$ cells) per the number of milk somatic cells per milliliter. To determine the percentage of macrophages ($CD14^+$) among viable cells in quarter milk samples, dead cells ($PI^+$) were excluded from the analysis. A negative control (unstained), fluorochrome-conjugated secondary antibody control, and single-stained samples were prepared for the compensation controls. FlowJo software (Tree Star Inc., Ashland, USA) was used to analyze the data.

## Statistical Analysis

The distributions of all variables were analyzed using normal probability plots obtained by the Shapiro and Wilk tests. As all data presented high coefficient of variation, we carried out a logarithmic transformation ($Log_{10}$). First, interclass correlation at the cow and quarter levels was calculated to determine the strength of clustering, as previously described by McGraw and Wong (38). All variables presented ICC value >0.60 at both cow and udder quarter levels, apart from the percentage of apoptotic, late apoptotic/necrotic, and viable milk macrophages. The data were analyzed using ANOVA followed by the *post-hoc* Student–Newnan–Keuls test (39). The model of mammary quarters and cows nested within cows was considered (36). Statistical analyses were performed using the statistical software InfoStat (Cordoba, Argentina). The results are presented as the mean ± standard error. The significance level was set at $P \leq 0.05$.

# RESULTS

The SCC, days in lactation, and parity (data not shown) values did not differ among groups. No effect of BLV infection in the overall number of macrophages per milliliter ($P = 0.87$) and in the percentage of viable macrophages ($P = 0.09$) among overall viable milk cells was found. In the present study, the percentage of milk macrophages that phagocytosed *S. aureus* in BLV-seronegative animals was higher than AL BLV-infected dairy cows ($P = 0.008$) (**Figure 1**) and those with PL ($P = 0.015$) (**Figure 1**). However, the percentage of milk macrophages that produced RONS ($P = 0.22$), the GMFI of intracellular RONS ($P = 0.14$), and the GMFI *S. aureus* phagocytosis ($P = 0.79$) did not differ among groups.

The AL BLV-infected dairy cows (**Figure 2A**) and those with PL (**Figure 2A**) had a higher frequency of viable milk macrophages ($P = 0.03$), while healthy animals had a tendency toward higher percentage of apoptotic milk macrophages ($P = 0.06$) (**Figure 2B**), although, no significant difference on the percentage of necrotic/late apoptotic milk macrophages was found ($P = 0.48$).

# DISCUSSION

Although, most studies on BLV demonstrate its impact on the adaptive immune system, some studies show that BLV infection negatively affects the performance of monocytes (5, 13, 40); however, to the best of our knowledge, no study has investigated the impact of BLV infection on the function of milk macrophages. In this concern, although BLV persists mainly in B cells, monocytes and macrophages could be infected by BLV and act as a reservoir for BLV (1, 41).

Macrophages have a pivotal role in the initiation, maintenance, and resolution of inflammation (42). These cells represent the main leukocyte population in milk from mammary glands of healthy cows (21, 22, 24), in which the percentage of the milk macrophages found here was similar to the previous values reported by Sarikaya et al. (21, 22) and Takano et al. (24), but a little bit higher than others (43, 44). Therefore, macrophages are the first to find possible invasive pathogens. Macrophages play an important role in the defense of the mammary gland and, among various functions, they act on phagocytosis mechanisms, antigen presentation, lactoferrin synthesis, complement system factors, N-acetyl-β-D-glucosaminidase, and cytokines, in addition to removing cellular debris and apoptotic neutrophils (45–47).

Phagocyte's viability and their capacity to produce ROS and phagocytosis bacteria are inextricably connected. However, even though we expected a higher macrophage functionality in milk quarter samples with a higher percentage of viable cells, BLV infection impaired *S. aureus* macrophage phagocytosis while having a higher frequency of viable macrophages. Similarly, we have shown a reduction in the percentage of *S. aureus* phagocytosis by blood monocytes in cows infected with BLV with PL (5). Furthermore, we also have demonstrated that monocytes from cows with PL showed a lower phagocytosis of zymosan particles of *Saccharomyces cerevisiae* than both BLV-seronegative and AL BLV-infected dairy cows (13). Therefore, although BLV

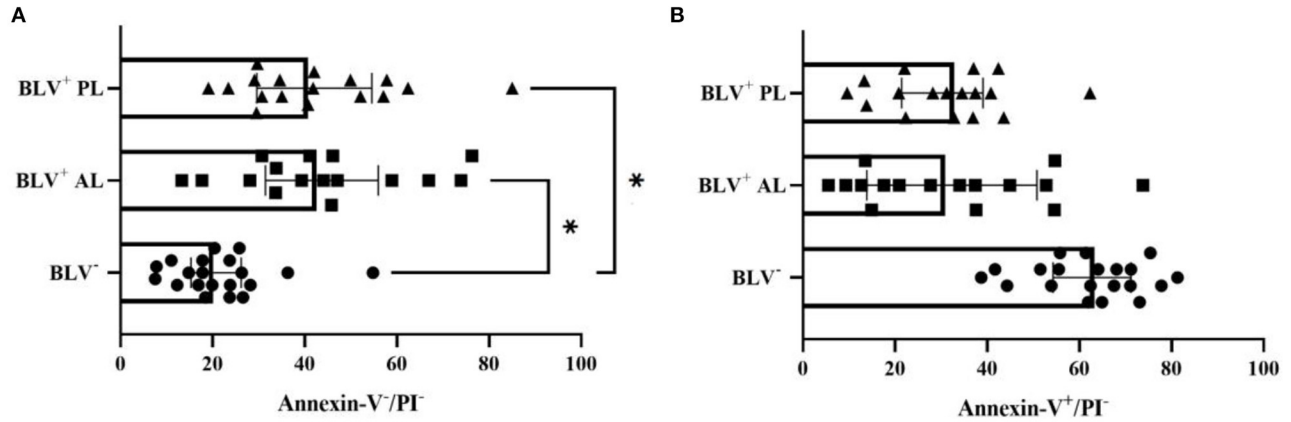


**FIGURE 2 |** Percentage (individual values and median ± interquartile range) of viable (Annexin-V$^-$/PI$^-$) **(A)** and apoptotic (Annexin-V$^+$/PI$^-$) **(B)** milk macrophages from health mammary glands in bovine leukemia virus (BLV) seronegative (BLV$^-$) and in BLV-infected (BLV$^+$) dairy cows without (AL) and with persistent lymphocytosis (PL). *$P < 0.03$.

infection affects the phagocytic capacity of blood monocytes only in animals with PL, our findings showed that the function of milk macrophages is impaired in BLV-infected animals regardless of the manifestation of PL. Furthermore, Werling et al. (48) showed that CD14$^+$ blood monocytes from BLV-infected dairy cows had less Fcγ-receptor mediated phagocytosis, which could, at least in part, explain our results. Although, regarding our outcomes, it appears that this dysfunction is preserved in milk macrophages, it needs to be further investigated.

Although, the mechanism by which BLV interferes with milk macrophage apoptosis is unknown, we assume that inhibition of milk macrophage apoptosis could be related to the intracellular levels of glutathione that is correlated with BLV-associated protection against apoptosis, which could be linked to the decrease of oxidation and subsequent impairment of apoptosis (49), although, this mechanism in milk macrophages needs to be further elucidated. Furthermore, we also hypothesized that the higher percentage of milk macrophages in BLV-infected cows with PL may be associated, at least in part, with the expression of the *Bcl-2* and *Bcl2 L1* genes involved in apoptotic inhibition (48), in line with the higher viability of milk macrophages found here in BLV-infected dairy cows. Although, it is not yet known exactly how BLV alters cell death and growth mechanisms, there is evidence to suggest that BLV can affect signaling pathways for cell growth and death (2, 40).

Overall, our results indicate that BLV infection may favor alternatively activated macrophages (M2 macrophages), as lower *S. aureus* phagocytosis was found, but this hypothesis needs to be further investigated. In this context, M2 macrophages was associated with lower phagocytosis of bacterial pathogens (50, 51). Furthermore, the lower frequency of apoptotic milk macrophages could be also explained by higher efferocytosis capability of M2 macrophages (52) in BLV-infected dairy cows, which lead to the phagocytic removal of apoptotic cells by the recognition of the "eat me" signals (e.g., exposure of phosphatidylserine) from cells undergoing apoptosis (52, 53). In fact, BLV was associated with IL-10 production by peripheral blood mononuclear cells (54), which, in turn, favors M2 polarization (53). Additionally, M2 macrophages were associated with a worse clinical prognosis of adult T-cell leukemia/lymphoma caused by human T-cell leukemia virus type 1 (55), a virus closely related to BLV (2). Indeed, viruses usually evolve mechanisms to enhance M2 macrophages, which exert potent immunosuppressive effects (56).

Furthermore, we should regard that our macrophage activities were carried out in the context where macrophage activities could be modified by other cell types, as all milk cells are present in functional assays. Similar to our study, many studies have used all milk cells in their assays to investigate activities of a particular cell type (4, 29–33, 36), as have long been performed with blood cells (so-called whole blood assay), which has largely been used and regarded as simple, reproducible, and clinically relevant, allowing us to *ex vivo* assess immune functions (57, 58).

An important aspect that could affect our outcomes is the interdependence of udder quarters and the fact that some quarters had IMIs, which could be a bias of our data analysis due to the effect of IMIs on immune response of the healthy neighborhood mammary gland quarters (4). In this concern, to deal with it, as the immune response between quarters within cow is not an independent process (36, 59), we considered it in the statistical analysis by determining the intraclass correlation (60), and using a model of udder quarters and cows nested with cows (36), as previously proposed.

Therefore, despite the limited number of animals used in the present study, our findings demonstrate that BLV infection can negatively affect the immunity of the mammary gland, which could predispose animals to coinfections or superinfections and can augment the severity of infections, as previously described for bovine udder health (4, 15, 16, 61, 62). Thus, it is clear that BLV infection can impact the phagocytic capacity of macrophages against mastitis-causing pathogens (e.g., *S. aureus*) and may favor susceptibility to infections or decrease their ability to

eliminate intramammary infection (spontaneous cure). Thus, our study strengthens the idea that the impact of this chronic disease with low lethality is underestimated due to its association with comorbidities.

## CONCLUSION

The present study provides crucial information on the implications of BLV infections in the mammary gland and negatively impacts the functionality of milk macrophages in BLV-infected animals. In addition, this study highlights the importance of controlling BLV infections due to their indirect effects on the emergence of secondary infections, such as mastitis.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Bioethic Commission of the Faculty of Veterinary Medicine and Animal Science of University of São Paulo. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

EL drafted and edited the manuscript. MB performed the experiments and designed the studies. CB participated in flow cytometry analysis. AA, AF, HF, and ER provided technical help and edited the manuscript. AD designed and supervised the studies. FS performed the analysis, designed the studies, and edited the manuscript. SD and MS performed all statistical analysis and edited the manuscript. All authors have read and agreed to the published version of this manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.650021/full#supplementary-material

**Supplementary Figure 1 |** Successive gating scheme for assessment the functional analysis of milk macrophages ($CD14^+$ cells). Recording of scatter and fluorescent properties were performed in a standard population excluding most cell debris **(A)**. Then, the $CD14^+$ cells (macrophages) were analyzed based on their cytoplasmic granularity and mean fluorescence intensity following a two-step fluorescent immunolabeling protocol using primary anti-bovine monoclonal antibody (Ab) specific for $CD14^+$ cells identification and secondary Abs coupled to long-wavelength fluorescent probe [FL4-H; **(B)**]. Afterwards, the percentage of CD14-positive cells that intracellularly produced reactive oxygen species **(C)** and phagocytosed *Staphylococcus aureus* **(D)** were determined. Furthermore, annexin-V-fluorecein isothiocynate (FL1-H) vs. propidium iodide (FL3-H) dot plot was performed to assess the percentage of apoptotic (Q1) and viable (Q4) CD14-positive events in milk **(E)**.

## REFERENCES

1. Schwartz I, Bensaid A, Polack B, Perrin B, Berthelemy M, Levy D. *In vivo* leukocyte tropism of bovine leukemia virus in sheep and cattle. *J Virol.* (1994) 68:4589–96. doi: 10.1128/JVI.68.7.4589-4596.1994
2. Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, et al. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology.* (2007) 4:18. doi: 10.1186/1742-4690-4-18
3. Della Libera AMMP, Blagitz MG, Batista CF, Latorre AO, Stricagnolo CR, Souza FN. Quantification of B cells and T lymphocytes subsets in bovine leukemia virus infected dairy cows. *Semina Cien Agrar.* (2012) 33:1487–94. doi: 10.5433/1679-0359.2012v33n4p1487
4. Della Libera AMMP, Souza FN, Batista CF, Santos BP, Azevedo LF, Sanchez EMR, et al. Effects of bovine leukemia virus infection on milk neutrophil function and the milk lymphocyte profile. *Vet Res.* (2015) 46:2. doi: 10.1186/s13567-014-0125-4
5. Blagitz MG, Souza FN, Batista CF, Azevedo LFF, Sanchez EMR, Diniz SA, et al. Immunological implications of bovine leukemia virus infection. *Res Vet Sci.* (2017) 114:109–16. doi: 10.1016/j.rvsc.2017.03.012
6. Souza FN, Blagitz MG, Latorre AO, Sanchez EMR, Batista CF, Weigel RA, et al. Intracellular reactive oxygen species production by polymorphonuclear leukocytes in bovine leukemia virus-infected dairy cow. *J Vet Sci.* (2012) 74:221–5. doi: 10.1292/jvms.11-0246
7. Suzuki S, Konnai S, Okagawa T, Ikebuchi R, Shirai T, Sunden Y, et al. Expression analysis of Foxp3 in T cells from bovine leukemia virus infected cattle. *Microbiol Immunol.* (2013) 57:600–4. doi: 10.1111/1348-0421.12073
8. Nieto Farias MV, Souza FN, Lendez PA, Martínez-Cuesta L, Santos KR, Della Libera AMMP, et al. Lymphocyte proliferation and apoptosis of lymphocyte subpopulations in bovine leukemia virus-infected dairy cows with high and low proviral load. *Vet Immunol Immunopathol.* (2018) 206:41–8. doi: 10.1016/j.vetimm.2018.10.012
9. Rademacher R, Sodomkova D, Vanasek J. Alkaline phosphatase in neutrophil granulocytes of the peripheral blood of healthy and leukotic cattle. *Vet Med.* (1977) 22:673–7
10. Cuesta LM, Nieto Farias MV, Lendez PA, Barone L, Pérez SE, Dolcini G, et al. Stable infection of a bovine mammary epithelial cell line (MAC-T) with bovine leukemia virus (BLV). *Virus Res.* (2018) 256:11–6. doi: 10.1016/j.virusres.2018.07.013
11. Cuesta LM, Nieto Farias MV, Lendez PA, Rowland RRR, Maureen AS, Cheuquepán Valenzuela FA, et al. Effect of bovine leukemia virus on bovine mammary epithelial cell. *Virus Res.* (2019) 271:197678. doi: 10.1016/j.virusres.2019.197678

12. Cuesta LM, Liron JP, Nieto Farias MV, Dolcini GL, Ceriani MC. Effect of bovine leukemia virus (BLV) infection on bovine mammary epithelial cells RNA-seq transcriptome profile. *PLoS ONE.* (2020) 15:1–12. doi: 10.1371/journal.pone.0234939
13. Azedo MR, Blagitz MG, Souza FN, Benesi FJ, Della Libera AMMP. Avaliação funcional de monócitos de bovinos naturalmente infectados pelo vírus da leucose bovina. *Arq Bras Vet Zoot.* (2011) 63:1131–40. doi: 10.1590/S0102-09352011000500013
14. Emanuelson U, Scherling K, Petterson H. Relationship between herd bovine leukemia virus infection status and reproduction, disease incidence, and productivity in Swedish dairy herds. *Prev Vet Med.* (1992) 12:121–31. doi: 10.1016/0167-5877(92)90075-Q
15. Rinaldi M, Li RW, Capuco AV. Mastitis associated transcriptomic disruptions in cattle. *Vet Immunol Immunopathol.* (2010) 138:267–79. doi: 10.1016/j.vetimm.2010.10.005
16. Watanabe A, Murakami H, Kakinuma S, Murao K, Ohmae K, Isobe N, et al. Association between bovine leukemia virus proviral load and severity of clinical mastitis. *J Vet Med Sci.* (2019) 81:1431–7. doi: 10.1292/jvms.19-0285
17. Norby B, Bartlett PC, Byrem TM, Erskine RJ. Effect of infection with bovine leukemia virus on milk production in Michigan dairy cows. *J Dairy Sci.* (2015) 99:2043–52. doi: 10.3168/jds.2015-10089
18. Erskine RJ, Bartlett PC, Byrem TM, Render CL, Febvay C, Houseman JT. Association between bovine leukemia virus, production, and population age in Michigan dairy herds. *J Dairy Sci.* (2012) 95:727–34. doi: 10.3168/jds.2011-4760
19. Lee CS, Wooding FB, Kemp P. Identification, properties, and differential counts of cell populations using electron microscopy of dry cow secretion, colostrum and milk from normal cows. *J Dairy Res.* (1980) 47:39–50. doi: 10.1017/S0022029900020860
20. Miller RH, Paape MJ, Fulton LA. Variation in milk somatic cells of heifers at first calving. *J Dairy Sci.* (1991) 74:3789–90. doi: 10.3168/jds.S0022-0302(91)78570-6
21. Sarikaya H, Prgomet C, Pfaffl MW, Bruckmaier RM. Differentiation of leukocytes in bovine milk. *Milchwissenschaft.* (2004) 59:586–9.
22. Sarikaya H, Werner-Misof C, Atzkern M, Bruckmainer RM. Distribution of leucocyte populations, milk composition, in milk fractions of healthy quarters in dairy cows. *J Dairy Res.* (2005) 72:486–92. doi: 10.1017/S0022029905001317
23. Merle R, Schröder A, Hamann J. Cell function in bovine mammary gland: a preliminary study on the interdependence of healthy and infected udder quarters. *J Dairy Res.* (2007) 74:174–9. doi: 10.1017/S002202990600238X
24. Takano PV, Blagitz MG, Mira CS, Batista CF, Della Libera AMMP, Souza FN. Estudo comparativo das diferentes técnicas empregadas na contagem diferencial de leucócitos no leite. *Pesq Vet Bras.* (2018) 38:773–8. doi: 10.1590/1678-5150-pvb-5252
25. Divers TJ, Peek SF. *Rebhunn's Diseases of Catlle*. Amsterdam: Elsevier (2018). p. 704.
26. Thurmond MC, Carter RL, Picanso JP, Stralka K. Upper-normal prediction limits of lymphocyte counts for cattle not infected with bovine leukemia virus. *Am Vet Res.* (1990) 51:466–70.
27. Oliver SP, González RN, Hogan JS, Jayarao BM, Owens WE. *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality*. Verona: National Mastitis Council (2004).
28. Piepers S, De Meulemeester L, de Kruif A, Opsomer G, Barkema, HW, De Vliegher S. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J Dairy Res.* (2007) 74:478–83. doi: 10.1017/S0022029907002841
29. Piepers S, De Vliegher S. Oral supplementation of medium-chain fatty acids during the cry period supports the neutrophil viability of peripartum dairy cows. *J Dairy Res.* (2013) 80:309–18. doi: 10.1017/S0022029913000228
30. Blagitz MG, Souza FN, Santos BP, Batista CF, Parra AC, Azevedo LFF, et al. Function of milk polymorphonuclear neutrophils leukocytes in bovine mammary glands infected with Corynebacterium bovis. *J Dairy Sci.* (2013) 96:3750–7. doi: 10.3168/jds.2012-6370
31. Blagitz MG, Souza FN, Batista CF, Azevedo LF, Benites NR, Melville PA, et al. The neutrophil function lymphocyte profile of milk from bovine mammary glands infected with Streptococcus dysgalactiae. *J Dairy Res.* (2015) 82:460–9. doi: 10.1017/S0022029915000308
32. Souza FN, Blagitz MG, Batista CF, Takano PV, Gargano RG, Diniz SA, et al. Immune response in nonspecific mastitis: what can it tell us? *J Dairy Sci.* (2020) 103:5376–86. doi: 10.3168/jds.2019-17022
33. Santos BP, Souza FN, Blagitz MG, Batista CF, Bertagnon HG, Diniz SA, et al. Blood and milk polymorphonuclear leukocyte and monocyte/macrophage functions in naturally caprine arthritis encephalitis virus infection in dairy goats. *Vet Immunol Immunopahtol.* (2017) 188:21–6. doi: 10.1016/j.vetimm.2017.04.009
34. Hasui M, Hirabayashi Y, Kobayashi Y. Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. *J Immunol Methods.* (1989) 117:53–8. doi: 10.1016/0022-1759(89)90118-X
35. Shehat MG, Tigno-Aranjuez J. Flow cytometric measurement of ROS production in macrophages in response to FcγR cross-linking. *J Vis Exp.* (2019) 145:e59167. doi: 10.3791/59167
36. Blagitz MG, Souza FN, Batista CF, Diniz SA, Azevedo LFF, Silva MX, et al. Flow cytometric analysis: interdependence of healthy infected udder quarters. *J Dairy Sci.* (2015) 98:2401–8. doi: 10.3168/jds.2014-8727
37. Batista CF, Souza FN, Santos KR, Ramos Sanchez EM, Reis LC, Bertagnon HG, et al. R-Phycoerythrin-labeled Mannheimia haemolytica for the simultaneous measurement of phagocytosis and intracellular reactive oxygen species production in bovine blood and bronchoalveolar lavage cells. *Vet Immunol Immunopathol.* (2018) 196:53–9. doi: 10.1016/j.vetimm.2017.12.004
38. McGraw KO, Wong SP. Forming inferences about some intraclass correlation coefficients. *Psych Methods.* (1996) 1:30–46. doi: 10.1037/1082-989X.1.1.30
39. Newman D. The distribution of range in samples from a normal population, expressed in terms of an independent estimate of standard deviation. *Biometrika.* (1939) 31:20–30. doi: 10.1093/biomet/31.1-2.20
40. Frie MC, Coussens PM. Bovine leukemia virus: a major threat to proper immune responses in cattle. *Vet Immunol Immunopathol.* (2015) 163:103–14. doi: 10.1016/j.vetimm.2014.11.014
41. Doménech A, Goyache J, Llames L, Jesús Payá M, Suárez G, Gómez-Lucía E. *In vitro* infection of cells of the monocytic/macrophage lineage with bovine leukaemia virus. *J Gen Virol.* (2000) 81:109–18. doi: 10.1099/0022-1317-81-1-109
42. Sladek Z, Rysanek D. Apoptosis of resident and inflammatory macrophages before and during the inflammatory response of the virgin bovine mammary gland. *Acta Vet Scand.* (2010) 52:1–13. doi: 10.1186/1751-0147-52-12
43. Pilla R, Malvisi M, Snel GGM, Schwarz D, Konig S, Czerny C-P, et al. Differential cell count as an alternative method to diagnose dairy cow mastitis. *J Dairy Sci.* (2013) 96:1653–60. doi: 10.3168/jds.2012-6298
44. Gonçalves JL, Lyman RL, Hockett M, Rodriguez R, Santos MV, Anderson KL. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. *J Dairy Res.* (2017) 84:309–17. doi: 10.1017/S0022029917000267
45. Johnston RB Jr. Monocytes and macrophages. *N Engl J Med.* (1988) 318:747–52. doi: 10.1056/NEJM198803243181205
46. Carneiro DMVF, Domingues PF, Vaz AK. Imunidade inata da glândula mamária bovina: resposta à infecção. *Ciência Rural.* (2009) 39:1934–43. doi: 10.1590/S0103-84782009005000106
47. Bastos CR, Blagitz MG, Souza FN, Batista CF, Stricagnolo CR, Azedo MR, et al. Viabilidade celular, fagocitose e espraiamento de fagócitos mononucleares, e liberação de peróxido de hidrogênio por leucócitos de glândulas mamárias bovina sadias e infectadas. *Pesq Vet Bras.* (2012) 32:850–4. doi: 10.1590/S0100-736X2012000900006
48. Werling D, Howard CJ, Niederer E, Straub OC, Saalmuller A, Langhans W. Analysis of the phenotype and phagocytic activity of monocytes/macrophages from cattle infected with the bovine leukemia virus. *Vet Immunol Immunopathol.* (1998) 62:185–95. doi: 10.1016/S0165-2427(98)00074-9
49. Alcaraz TS, Kerkhofs P, Reichert M, Kettmann R, Willems L. Involvement of glutathione as a mechanism of indirect protection against spontaneous *ex vivo* apoptosis associated with bovine leukemia virus. *J Virol.* (2004) 78:6180–9. doi: 10.1128/JVI.78.12.6180-6189.2004
50. Varin A, Mukhopadhyay S, Herbein G, Gordon S. Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signaling and cytokine secretion. *Blood.* (2010) 115:353–62. doi: 10.1182/blood-2009-08-236711

51. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. *Allergy.* (2011) 66:396–403. doi: 10.1111/j.1398-9995.2010.02498.x
52. Zhong X, Lee HN, Kim SH, Park SA, Kim W, Cha Y-N, et al. Myc-nick promotes efferocytosis through M2 macrophage polarization during resolution of inflammation. *FASEB J.* (2018) 32:5312–25. doi: 10.1096/fj.201800223R
53. Linton MF, Babaev VR, Huang J, Linton EF, Tao H, Yancey PG. Macrophage apoptosis and efferocytosis in the pathogenesis of atherosclerosis. *Circ J.* (2016) 80:2259–68. doi: 10.1253/circj.CJ-16-0924
54. Trueblood ES, Brown WC, Palmer GH, Davis WC, Stone DM, McElwain TF. B-Lymphocyte proliferation during bovine leukemia virus-induced persistent lymphocytosis is enhanced by T-Lymphocyte-Derived Interleukin-2. *J Virol.* (1998) 72:3169–77. doi: 10.1128/JVI.72.4.3169-3177.1998
55. Komohara Y, Niino D, Saito Y, Ohnishi K, Horlad H, Ohshima K, et al. Clinical significance of CD163+ tumor-associated macrophages in patients with adult T-cell leukemia/lymphoma. *Cancer Sci.* (2013) 104:945–51. doi: 10.1111/cas.12167
56. Sang Y, Miller LC, Blecha F. Macrophage polarization in virus-host interactions. *J Clin Cell Immunol.* (2015) 6:311. doi: 10.4172/2155-9899.1000311
57. Naghizadeh M, Larsen FT, Wattrang E, Norup LR, Dalgaard TS. Rapid whole blood assay using flow cytometry for measuring phagocytic activity of chicken leukocytes. *Vet Immunol Immunopat hol.* (2019) 207:53–61. doi: 10.1016/j.vetimm.2018.11.014
58. Reiné J, Rylance J, Ferreira DM, Pennington SH, Welters ID, Parker R, et al. The whole blood phagocytosis assay: a clinically relevant test of neutrophil function and dysfunction in community-acquired pneumonia. *BMC Res Notes.* (2020) 13:203. doi: 10.1186/s13104-020-05034-0
59. Barkema HW, Schukken YH, Lam TJGM, Galligan DT, Beiboer ML, Brand A. Estimation of interdependence among quarters of the bovine udder with subclinical mastitis and implications for analysis. *J Dairy Sci.* (1997) 80:1592–9. doi: 10.3168/jds.S0022-0302(97)76089-2
60. Paixão MG, Abreu LR, Richert R, Ruegg PL. Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis. *J Dairy Sci.* (2017) 100:1–12. doi: 10.3168/jds.2017-12547
61. Almeida IC, Almeida SLH, Barioni G, Donatele DM, Clipes RC, Glória LS, et al. Seroprevalence and influence of bovine leukemia virus on the incidence of mastitis in dairy herds. *Acta Sci Vet.* (2021) 49:1783. doi: 10.22456/1679-9216.109458
62. Erskine RJ, Corl CM, Gandy JC, Sordillo LM. Effect of infection with bovine leukosis virus on lymphocyte proliferation and apoptosis in dairy cattle. *Am J Vet Res.* (2011) 72:1059–64. doi: 10.2460/ajvr.72.8.1059


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# A Comparative Study on Changes in Total and Differential Milk Cell Counts, Activity, and Expression of Milk Phagocytes of Healthy and Mastitic Indigenous Sahiwal Cows

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Milk somatic cell counts (SCCs) have been used as a gold standard to monitor mammary health as well as an indicator of raw milk quality. The present work was undertaken to compare the changes in the milk SCC, milk differential leukocyte counts (DLCs), phagocytic activity (PA) of milk neutrophils and macrophages (by nitroblue tetrazolium assay), extracellular trap formation (PicoGreen assay) and mRNA expression of various genes in milk neutrophils and macrophages (reverse transcription–polymerase chain reaction), and milk plasma cortisol concentration (enzyme-linked immunosorbent assay) in healthy, subclinical mastitis (SCM), and clinical mastitis (CM) cows. Milk was collected from healthy, SCM, and CM cows grouped based on their SCCs and California mastitis test with eight cows in each group. Milk SCC was estimated by SCC counter, and DLC was done after staining the milk slide under a microscope at 100×. Total SCCs in healthy, SCM, and CM cows were on an average of 128.30, 300.3, and 694.40 × $10^3$ cells/mL, respectively. Milk DLCs indicated a lower percentage of macrophage and lymphocytes and a higher ($p < 0.05$) percentage of neutrophils in SCM and CM compared to healthy milk. The percentage of mature segmented neutrophils was lower, whereas immature band neutrophils were higher ($p < 0.05$) in the SCM and CM groups as compared to healthy cows. The viability, *in vitro* PA, and extracellular trap formation of neutrophils were lower ($p < 0.05$) in SCM and CM milk samples as compared to healthy samples. However, the PA of macrophage remained unchanged in all the studied groups. The relative mRNA expression of Toll-like receptors (TLR2, TLR4), myeloperoxidase, and interleukin 2α (IL-2α) receptor (CD25) were minimum in healthy samples and increased ($p < 0.05$) with the progress of mammary inflammation. However, CD44 decreased ($p < 0.05$), and CD62L remained unchanged in mastitis as compared to healthy cows. Plasma cortisol concentrations were higher ($p < 0.05$) in mastitis as compared to healthy cows and were negatively correlated with the number of milk macrophages and the functions of milk phagocytes. Estimation of total SCC, milk DLC, and activity of milk phagocytes is essential for effective control and prevention of incidence of mastitis in dairy cows.

**Keywords: mammary gland, SCC, DLC, macrophage, neutrophil, NETs**

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## INTRODUCTION

Mastitis, the inflammation of mammary gland, is one of the most costly and widespread diseases occurring in dairy cows worldwide (1, 2). The dairy sector, particularly in developing countries such as India, is facing massive economic losses because of mastitis, which can be attributed to reduced milk production; milk contamination with antibiotic residues; treatment, culling, or death of chronically infected cows; and many more (3, 4). Also, this disease possesses a serious zoonotic potential as it is associated with the shedding of various mastitis-causing microbes and their toxins in the infected milk (5). Although there is a number of pathogens that can cause mammary infection, *Staphylococcus aureus* is the most common bacteria that are responsible for chronic mastitis in dairy cows worldwide (6–8). This pathogen is exceedingly difficult to control by treatment alone. However, effective control can be gained through the prevention of new infections, which can be achieved by monitoring milk somatic and differential cell counts (9, 10).

Milk somatic cells (SCs) consist of milk-producing epithelial cells and leukocytes transmigrated to the mammary tissue. The count of somatic cell counts (SCCs) in milk is widely accepted as a novel method for the diagnosis of mammary infection in dairy cows (4, 11). Moreover, the proportions of various white cells in milk can give a deep insight into the severity and stage of mammary infection (12–14). Besides, the fluctuations in the proportions of various immune cells of milk during mammary infection may indicate an infection with specific groups of microbes and reflect the potential of mammary immune response (15–18). Milk phagocytes (neutrophils and macrophages) are the primary immune cells present in milk and represent the innate arm of mammary immune response during mammary infection (19, 20). Both neutrophils and macrophages perform phagocytosis, which is one of the fundamental mechanisms to ingest and intra-cellularly kill various invading pathogens during mammary infection (1, 21). Research in recent years revealed that neutrophils can also entrap and inactivate the invading pathogens by the release of extracellular traps (ETs), which composed mainly of DNA, histones, and many other antimicrobial proteins (22–24).

Sahiwal (SW) cows are considered one of the highest milk producers amongst the Indian native breed of cattle and are admirably adapted to the harsh climatic conditions of the tropics. Since these are high milk producers, they are regularly encountered with mammary infection (17, 21). Studying the changes in the milk somatic and differential leukocyte counts (DLCs) in healthy and mastitis cows and their impact on the cellular functions of the main arm of innate immunity (milk phagocytes) can provide critical information essential for effective control and treatment of mastitis. Therefore, the present study was designed to study and compare the milk SCC, DLC, and the cellular activity of milk phagocytes in term of phagocytosis and neutrophil extracellular trap (NET) formation in healthy, SCM, and CM SW cows naturally infected with *S. aureus*. An effort has also been made to investigate the changes in the expression of the key genes and receptors that mediate critical functions of neutrophils and macrophages.

## MATERIALS AND METHODS

### Animal Selection, Sampling, and Management of the Cows

The approval of all the experiments carried out in this research work was obtained from the Animal Ethics Committee of the NDRI according to the Committee for the Purpose of Control and Supervision of Experiments on Animals rules, laid down by the Government of India. A total of 24 indigenous SW cows were selected from the Livestock Research Centre (LRC) of the National Dairy Research Institute, Karnal, India. All the experimental cows were high yielder (>10 kg/d) and in their early stage of lactation (days in milk <90). The cows were multiparous with an average body condition score of 3.5. Evaluation of the health status of mammary gland and classifying cows into three groups of healthy ($n = 8$), subclinical mastitis (SCM) ($n = 8$), and clinical mastitis (CM; $n = 8$) was done based on several diagnostic tests such as California Mastitis Test (CMT), milk SCC, and electrical conductivity (EC). The groups were classified as follows: healthy group (CMT score = 1, EC = 5.90 mS/cm, and SCC $<200 \times 10^3$ cells/mL), SCM group (CMT score = 2, EC = 6.25 mS/cm, and SCC = 200–500 $\times 10^3$ cells/mL), and CM group (CMT score = 3, EC = 7.20 mS/cm, and SCC >500 $\times 10^3$ cells/mL). Also, several other clinical symptoms of clinical mammary infection were considered during the selection of CM group such as swelling and pain in the infected quarters, fever, and abnormal alteration in milk characteristics including changes in the color, consistency, and blood in milk. Three milk samples were collected from each animal at 1-day intervals. Composite milk samples representing all four quarters were collected from healthy cows. However, the milk samples were collected from the affected quarters only in the case of SCM and CM groups. The aforementioned tests were repeated daily to make sure the health status of mammary gland is still the same. These cows were fed with *ad libitum* green fodder and concentrate diet (20% crude protein and 70% total digestible nutrient) as per practices followed in the LRC-NDRI for early lactating cows. Fresh and clean tap water was provided at all times of the day.

### Estimation of Somatic and DLCs of Milk

Several diagnostic tests such as CMT, milk SCC, and EC were performed to classify the experimental cows into various groups based on their udder health as described by Alhussien and Dang (25). For the CMT procedure, 2 mL of milk sample was loaded in the CMT paddle kit and mixed with 2 mL of CMT reagent (DeLaval Pvt. Ltd., India) in each cup. The mixture of both milk sample and the reagent was rotated for <1 min, and the result was recorded as follows: score 1 (negative, the mixture remains unchanged), score 2 (trace or slight to distinct gel formation), and score 3 (strong and clear gel formation). The measurement of SCC and EC in milk samples was performed using a Lactoscan milk analyzer (Milkotronic Ltd., Stara Zagora, Bulgaria), which utilize a digital system to measure the milk concentration of the fluorescent cells as described by Alhussien and Dang (4). Counting of milk SCC was also crosschecked microscopically as described by Panda et al. (16). Briefly, 96% ethyl alcohol was used for fixation (3 min), xylene was used to remove fat (10 min), and

methylene blue dye for staining (15 min). Differential leukocyte counting (DLC) of milk was done to estimate the percentage of various immune cells secreted in milk, including macrophages, neutrophils, and lymphocytes as per the method described by Dang et al. (26). Using the same SCC smear, milk DLC of various immune cells (macrophages, neutrophils, and lymphocytes), and the type of neutrophil (mature with segmented nuclei and immature with band nuclei), staining was carried out in 30 fields under oil immersion at 100× (Olympus IX51 microscope): DLC of a particular leukocyte (%) = (no of that specific leukocyte / total no of leukocytes) × 100.

## Mastitis-Causing Bacteria

Milk samples obtained from healthy, SCM, and CM groups were studied for the presence of possible mastitis-causing bacteria including the major organisms mainly responsible for mammary infection in Indian breed of cattle under tropical conditions. These include *S. aureus*, *Streptococcus agalactiae*, and *Escherichia coli*, which were studied by pour plate method using Baird–Parker agar, blood agar, and eosin–methylene blue agar as described by Alhussien and Dang (17). Briefly, the milk samples were incubated at 37°C for 24–48 h until the appearance of colonies. Characterization of the isolates was carried out using negative staining and Gram staining. Gram-positive bacteria (*S. aureus* and *S. agalactiae*) were distinguished and confirmed by several approaches including cellular arrangement, catalase production, Hotis test, and coagulase test. More than 50% of the milk samples isolated from SCM and CM cows were positive for *S. aureus*. Milk samples that were positive for other pathogens such as *S. agalactiae* and *E. coli* were excluded. Milk samples that were positive only for *S. aureus* were selected for further processing. Milk samples collected from healthy cows did not display any bacterial content and were completely free of infection.

## Isolation of Milk Phagocytes

The isolation of milk phagocytes (neutrophils, macrophages) was carried out using the gradient density method as described in our earlier studies (17, 27). Briefly, milk sample (200 mL) was centrifuged, and the obtained cell pellet was washed and suspended in Dulbecco phosphate-buffered saline (PBS, Himedia, India Pvt. Ltd.) containing 0.5 mg/mL gelatin. For milk neutrophil isolation, 3 mL each of Histopaque 1077 and Histopaque 1119 (Sigma–Aldrich, St. Louis, MO, USA) was carefully layered above each other, followed by layering the leukocyte suspension (3 mL) over the Histopaque 1077. Centrifugation (2,000 × *g*, 20 min, 4°C) was carried out, and the milk neutrophils were harvested at the interface of the Histopaque 1119 and Histopaque 1077 layers. For milk macrophage isolation, the leukocyte pellet was dissolved in 5 mL of Dulbecco PBS, and the cell suspension was layered on 4 mL Histopaque 1083 (Sigma–Aldrich). Centrifugation was then carried out (300 × *g*, 15 min, 4°C), and milk macrophages were harvested at the interface of PBS and the Histopaque 1083. The obtained milk phagocytes (neutrophil/macrophage) were washed with PBS and suspended in RPMI media (Sigma–Aldrich) for further processing. The number and viability of milk phagocytes were evaluated by trypan blue method using hemocytometer (Reinfeld, Germany). The purity of milk phagocytes was >96% as evaluated by May–Grünwald–Giemsa staining under oil immersion at 100× (Olympus IX51 microscope).

## Phagocytic Activity of Milk Neutrophils and Macrophages

The phagocytic activity (PA) of milk phagocytes was estimated under *in vitro* conditions using nitroblue tetrazolium (NBT) assay as described in our earlier studies (17, 27). Briefly, RPMI-1640 media was used to adjust the number of milk neutrophils/macrophages to $5 \times 10^6$/mL in a flat-bottomed tissue culture plate (Coster; Sigma–Aldrich, USA). The milk phagocytes cells were stimulated using zymosan and NBT (Sigma–Aldrich, USA), followed by an incubation step for 3 h in a humidified $CO_2$ incubator (37°C, 95% air and 5% $CO_2$). Finally, the optical density (OD) was measured at 540 nm by an enzyme-linked immunosorbent assay (ELISA) reader (MutiSkan GO; Thermo Scientific, Finland).

## Quantification of Milk NETs

The release of extracellular traps from milk neutrophils was estimated by PicoGreen, a DNA-binding dye (Invitrogen; Thermo Scientific, USA) as described earlier (24, 28). For NET study, RPMI-1640 medium lacking phenol red and having 2% fetal bovine serum (Thermo Scientific, USA) was used to suspend the isolated milk neutrophils. Approximately $2 \times 10^5$ live cells of milk neutrophils/well were seeded (in triplicate) in a 96-well flat-bottomed tissue culture plate (Coster; Sigma–Aldrich, USA) using the aforementioned RPMI-1640 medium. The milk neutrophils of healthy, SCM, and CM animals were incubated with zymosan (1 mg/mL) in 200-μL volume (3 h, 37°C, 5% $CO_2$). Micrococcal nuclease (5 U/well; Sigma–Aldrich, USA) was then added and incubation (10 min) was done followed by centrifugation (700 × *g*, 5 min). Thereafter, 100 μL/well of supernatant was transferred into 96-well flat-bottomed black polystyrol microplates (Greiner Bio-one, Germany). PicoGreen reagent (Invitrogen; Thermo Scientific, USA) was diluted (1:200) in 10 mM Tris base buffered with 1 mM EDTA and then added 100 μL/well followed by an incubation step for 5 min in the dark. Automated multiplate reader was used to estimate the formation of NETs in arbitrary fluorescence units (AFU) at excitation and emission wavelengths of 480 and 525 nm, respectively. Unstimulated milk neutrophils suspended in RPMI medium lacking phenol red were used as negative controls. To prove the DNA nature of extracellular traps of neutrophils, 90 U of DNase I (Sigma–Aldrich, USA) was added to the culture of milk neutrophils and zymosan approximately 15 min prior to the completion of the incubation period.

## Real-Time Gene Expression Study

Total RNA was isolated from milk neutrophils and milk macrophages using RNeasy Mini Kit (Qiagen, India Pvt. Ltd.). DNase 1, RNase-free (Qiagen, India Pvt. Ltd.) was used to

**TABLE 1 |** Details of various primers used in the study.

| Genes | Sequence (5′→ 3′) | Accession no. | Size (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| TLR2 | F: GCCTTGACCTGTCCAACAAT<br>R: GACCTGAACCAGGAGGATGA | NM174197.2 | 199 | 59 |
| TLR4 | F: GGCATCATCTTCATCGTCCT<br>R: CTGGACTCTGGGGTTTACCA | AY634630.1 | 178 | 59 |
| MPO | F: TCGATACCAACCTATGCAGCCCAA<br>R: ATTTGGTTCTGGCGGTTCAGCTTC | NM_001113298.1 | 147 | 59 |
| CD25 | F: ACATCGGCAGTGGTCTCAG<br>R: GAACCTCCACATCAGCAAGC | NM_174358.2 | 60 | 58 |
| CD44 | F: CTGTCAACAGTAGGAGAAGGTGTG<br>R: TCCTCCATGGTTCCATTCCCATTG | NM_174013.3 | 73 | 58 |
| CD62L | F: TCCAGAACCAACCTGTCGAGTG<br>R: TCCATGGTTCCCAAATCGGGTTC | NM_174182.1 | 66 | 58 |
| GAPDH | F: GGGTCATCATCTCTGCACCT<br>R: GGTCATAAGTCCCTCCACGA | NM_001034034 | 176 | 59 |
| β-Actin | F: CATCGCGGACAGGATGCAGAAAGC<br>R: GCGCGATGATCTTGATCTTCATTG | NM_173979.3 | 71 | 58 |

*F, forward; R, reverse; TLR2 and TLR4, Toll-like receptors; MPO, myeloperoxidase; CD25, CD44, CD62L, cluster of designation molecules; GAPDH and β-actin, housekeeping genes.*

eliminate the genomic as per the manufacturer's protocol. Agarose gel (1.6%) electrophoresis was used to assess the integrity of RNA samples, and the purity of RNA samples was assessed using Biospec-nano Spectrophotometer (Shimadzu Corp., Kyoto, Japan) based on the OD absorption ratio at $\frac{\lambda 260}{\lambda 280}$ in which the ratio of 2 was considered as pure RNA. The isolated RNA samples were then stored at −80°C until further processing. An amount of 1 μg for each RNA sample was reverse transcribed into cDNA in a 20 μL reaction mixture using cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol. Primers of housekeeping genes (β-actin, GAPDH), Toll-like receptors (TLR2, TLR4), myeloperoxidase (MPO), and the cluster of designation receptors (CD25, CD44, CD62L) were adapted from the available literature and are shown in **Table 1**. The relative mRNA expression of TLR2, TLR4, and MPO was studied in both neutrophils and macrophages. However, the relative expression of CD25, CD44, and CD62L was studied only in neutrophils. SYBR Green Master Mix kit (Thermo Scientific, USA) was used to amplify the cDNA samples in a Roche Light Cycler 480 instrument following the protocol described earlier (17, 27). A no-template control was included, and the data were normalized using two housekeeping genes, GAPDH and β-actin. The healthy control group of cows was taken as a calibrator for which the relative mRNA expression of all the studied genes was estimated. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification of the studied genes in milk phagocytes as described elsewhere (29).

## Quantification of Plasma Milk Cortisol

Plasma milk cortisol was quantified in the skimmed milk as per the method described elsewhere (27, 30). The concentrations of plasma cortisol were estimated using a competitive format of bovine cortisol hormone ELISA kit (Cusabio Biotech Co., Ltd.) as per the manufacturer's protocols. The minimum detectable dose of cortisol was <0.049 ng/mL, and the detection range was 0.049–200 ng/mL. The intra-assay and interassay coefficients of variance were <8 and <10%, respectively.

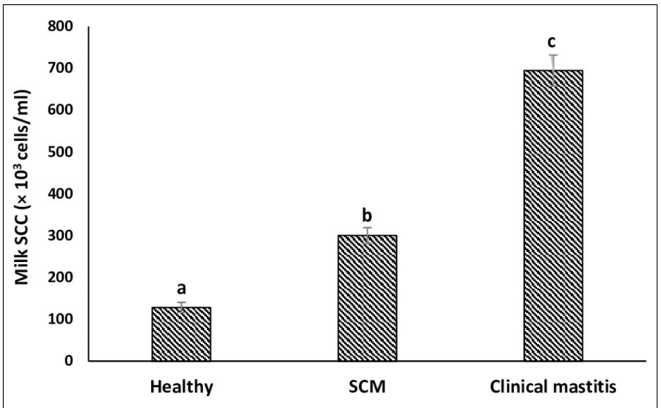


**FIGURE 1 |** Milk somatic cell count (SCC) in healthy, subclinical mastitis (SCM) and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with superscript letters (a–c).

## Statistical Analysis

The analysis of the data was performed by repeated-measures one-way analysis of variance using SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). The pairwise comparison was performed using the Tukey multiple-comparisons test. To analyze the correlations between milk SCC and various studied parameters, SAS 9.1 was used to calculate Pearson correlation co-efficients and the corresponding $P$ values; $p < 0.05$ was considered statistically significant.

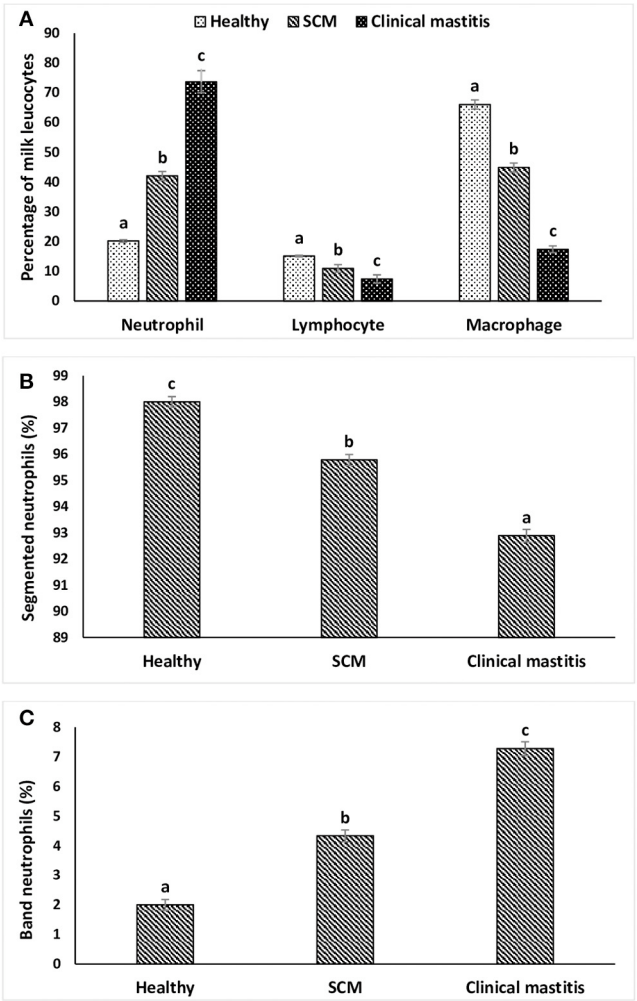


**FIGURE 2 |** Differential milk leukocyte counts. **(A)** The percentage of milk neutrophils, macrophages, and lymphocytes; **(B)** segmented neutrophils; **(C)** band neutrophils in healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with superscript letters (a–c).

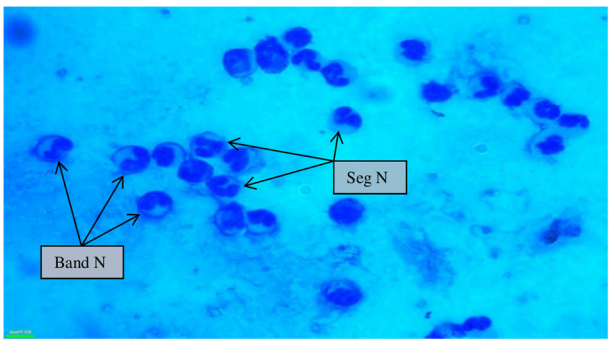


**FIGURE 3 |** Milk somatic cell counts in mastitis cows (100×) showing a maximum influx of banded neutrophils along with segmented neutrophils during mastitis. Seg N, segmented neutrophils; band N, banded neutrophils.

# RESULTS

## Milk SCC and DLCs

SCs were counted in healthy animals and animals diagnosed with SCM and CM. The number of SCs ($\times 10^3$ cells/mL) in healthy samples of milk was 128.30 ± 12.10, which were found to be significantly increased ($p < 0.05$) in samples of milk from animals with SCM, and the highest values were recorded in milk collected from the CM group (694.40 ± 37.20) (**Figure 1**). The percentage of milk neutrophils was 20.16 ± 0.40% in healthy milk increased ($p < 0.05$) in SCM (42.01 ± 1.50) and attained the maximum values in the CM group (73.66 ± 3.73) (**Figure 2A**). Milk macrophages and lymphocytes displayed different patterns compared to milk neutrophils in which they were highest in the healthy group and decreased ($p < 0.05$) with the progress of mammary infection. Macrophage was the dominant leukocyte in healthy milk (macrophage = 66.03% vs. neutrophil = 20.16%), both macrophage and neutrophils were dominant in SCM milk (macrophage = 44.84% vs. neutrophils = 42.01%). However, neutrophils represent the major leukocytes in milk collected from the CM group (neutrophils = 73.66% vs. macrophage = 17.39%) (**Figure 2A**). The percentage of the mature form of milk neutrophils (segmented type) was maximum in healthy milk (98 ± 0.20) and decreased significantly ($p < 0.05$) with the progress of mammary inflammation, with the lowest values recorded in the CM group (92.89 ± 0.24) (**Figure 2B**). However, the immature type (band neutrophils) followed an inverse pattern of that of segmented neutrophils in which it was lowest in healthy milk (2.0 ± 0.18) and increased significantly ($p < 0.05$) in mastitis milk (7.28 ± 0.23) (**Figures 2C**, **3**). Milk SCC was positively correlated ($p < 0.05$) with the percentage of milk neutrophils and the immature band type of neutrophil, as well as the concentration of milk plasma cortisol. However, it was negatively correlated ($p < 0.05$) with the percentage of milk macrophage, segmented neutrophils, and the PA of neutrophils (**Table 2**).

## Viability and PA of Milk Phagocytes

To investigate the impact of increased SCC and progress of mammary infections on the life span and activity of milk phagocytes, the viability and PA of neutrophils and macrophages were studied in healthy, SCM, and CM cows. The viability of milk phagocytes was highest in healthy milk (macrophage = 96.25% vs. neutrophils = 95.06%), decreased ($p < 0.05$) in the SCM group, and reached the lowest values in mastitis group (macrophage = 87.35% vs. neutrophils = 75.92%). Although the percentage of milk macrophages and neutrophils decreased dramatically in the mastitis group as compared to the healthy group, the decrease in the percentage of live cells was greater in neutrophils as compared to macrophages (**Figure 4A**). The PA of milk neutrophils was highest in the healthy group (0.85 ± 0.01), decreased in the SCM group, and continued to decline ($p < 0.05$), with the progress of mammary infection reaching the lowest values in the CM group (0.31 ± 0.03). Unlike neutrophils, the PA of milk macrophages remained unchanged in the SCM and CM groups as compared to the healthy group. Moreover, the

**TABLE 2 |** Correlation of milk SCC with milk DLC, phagocytic activity (PA) of phagocytes, NET formation, and plasma cortisol in healthy and mastitis cows.

| Parameters | SCC | PMN | Seg PMN | Band PMN | L | M | PA of PMN | PA of M | NETs | Cortisol |
|---|---|---|---|---|---|---|---|---|---|---|
| SCC | 1 | | | | | | | | | |
| PMN | 0.89* | 1 | | | | | | | | |
| Seg PMN | −0.86* | −0.91* | 1 | | | | | | | |
| Band PMN | 0.84* | 0.92* | −0.89* | 1 | | | | | | |
| L | −0.82* | −0.91* | 0.87* | −0.84* | 1 | | | | | |
| M | −0.89* | −0.98* | 0.91* | −0.92* | 0.91* | 1 | | | | |
| PA of PMN | −0.88* | −0.95* | 0.90* | −0.90* | 0.84* | 0.95* | 1 | | | |
| PA of M | −0.01 | −0.11 | 0.06 | −0.02 | 0.13 | 0.11 | 0.06 | 1 | | |
| NETs | −0.87* | −0.96* | 0.90* | −0.92* | 0.90* | 0.96* | 0.93* | 0.10 | 1 | |
| Cortisol | 0.87* | 0.89* | −0.84* | 0.84* | −0.83* | −0.89* | −0.88* | −0.04 | −0.84* | 1 |

*$p < 0.01$.
*PMN, neutrophil; L, lymphocyte; M, macrophage; Seg PMN, segmented neutrophil.*

PA of milk macrophage was higher ($p < 0.05$) than that of milk neutrophils in all the groups of cows (**Figure 4B**). The increase in the SCC and neutrophil percentage was negatively associated ($p < 0.05$) with the viability, PA, and NET formation of milk neutrophils (**Table 2**).

## NET Release by Milk Neutrophils

To explore the impact of mammary infection on the ability of milk neutrophils to release extracellular traps, NETosis was quantified using PicoGreen-derived fluorescence intensities. Measurement of the concentration of extracellular DNA ($\times 10^3$ AFU) in the supernatant of milk neutrophils revealed NET formation in response to stimulation with zymosan. The concentration of extracellular DNA in milk neutrophils isolated from the healthy group of cows was the highest ($8.57 \pm 0.14$). However, it decreased ($p < 0.05$) in the SCM group and continued to decrease with the progress of mammary infection reaching the lowest values ($3.58 \pm 0.04$) in CM group (**Figure 5**).

## Expression of Different Receptors and Genes in Milk Neutrophils and Macrophages

This experiment was to investigate the impact of different degrees of mammary infections on the expression of major genes and receptors essential for defense functions of milk phagocytes. The relative mRNA expression of TLR2, TLR4, and MPO has been presented in **Figure 6A** for milk neutrophils and in **Figure 6B** for milk macrophages. Relative mRNA expression of TLR2, TLR4, and MPO in milk neutrophils and macrophages was the lowest in the healthy group of cows. However, it increased ($p < 0.05$) significantly with the initiation of mammary infection in the SCM group and reached the highest values in the CM group. Although the relative mRNA expression of TLR2 was higher in milk macrophages compared to neutrophils, the mRNA expression of TLR4 and MPO was significantly higher ($p < 0.05$) in neutrophils compared to macrophages (**Figure 6**). The relative mRNA expression of CD molecules (CD25, CD44, CD62L) on milk neutrophils is presented in **Figure 7**. The relative mRNA expression of CD25 was lowest in healthy milk and increased ($p < 0.05$) with the development of mammary infection reaching the highest values in the CM group. The expression of CD44 increased ($p < 0.05$) during the initial stage of mammary

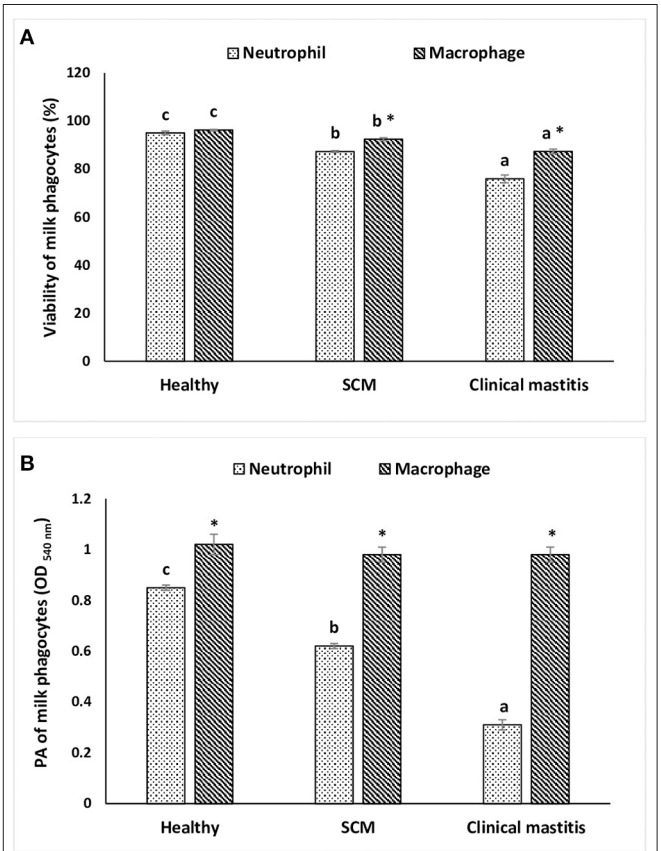


**FIGURE 4 |** The viability **(A)** and *in vitro* phagocytic activity **(B)** of milk phagocytes in healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with superscript letters (a–c). *Statistical difference ($p < 0.05$) within the same group.

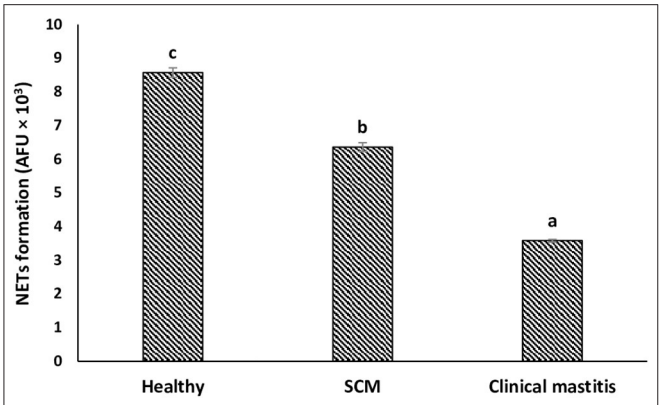


**FIGURE 5 |** NET release by milk neutrophils of healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Extracellular DNA quantifications of supernatants from milk neutrophils following zymosan treatment (1 mg/mL, 3 h) using fluorometric DNA quantification assay (PicoGreen). Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with small superscript letters (a–c).

infection in SCM. However, it decreased ($p < 0.05$) with the progress of mammary infection in the CM group as compared to the healthy group. Unlike all the previous genes and receptors, the relative mRNA expression of CD62L on milk neutrophils remained unaltered in all the studied groups of cows (**Figure 7**).

## Milk Plasma Cortisol

The concentration (in ng/mL) of milk plasma cortisol was minimum (1.60 ± 0.11) in the milk samples collected from the healthy group of cows. However, it started increasing ($p < 0.05$) during the subclinical form of mammary infection (2.50 ± 0.12) and continued to increase with the progress of mammary inflammation reaching the maximum values in the CM group (3.93 ± 0.11) (**Figure 8**).

# DISCUSSION

Bovine mastitis, an inflammation of the mammary gland, is known to be the most significant disease in dairy cattle globally. It does not only affect animal health but also put a huge economic burden on the dairy industry due to milk production losses, low milk quality, and increased cost of treatments (3, 4). However, the best strategy to combat mastitis is its prevention from occurrence. Following some proper management practices in dairy farms, diagnosing mastitis at the earliest and understanding the underlying mechanism of mammary immune response to invading pathogens are some key aspects for prevention (21, 25, 31, 32). However, mastitis is usually caused by multiple infectious agents, and to fight against the infections, the mammary gland has a line of defense system called SCs, which comprise white blood cells and milk epithelial cells. Owing to a pathogenic insult, the number of SCs in milk increases, and monitoring of SCC (number of cells per mL of milk) helps in determining the mammary inflammation (4, 11).

In our study, we observed that a significant increment in SCC during udder inflammation with the highest number was recorded in CM cows. The reason for rising SCC might be a protective mean to kill the pathogens, and this is associated with the severity and duration of mammary inflammation (33). Nonetheless, to differentiate between healthy and mastitis milk,

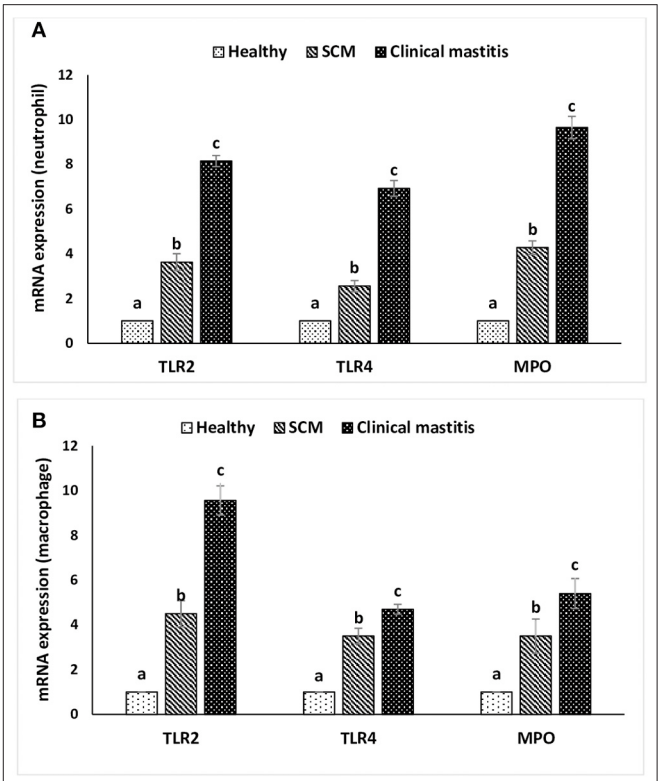


**FIGURE 6 |** Relative mRNA expression of toll-like receptors (TLR2, TLR4) and myeloperoxidase (MPO) in milk neutrophils **(A)** and macrophages **(B)** of healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with small superscript letters (a–c).

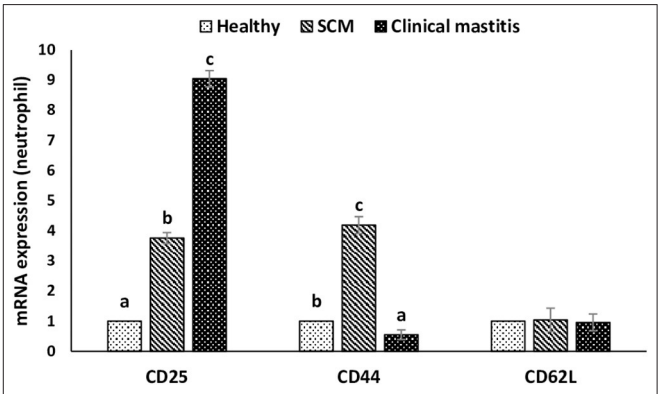


**FIGURE 7 |** Relative mRNA expression of various cluster of designation receptors (CD25, CD44, CD62L) on milk neutrophils of healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with small superscript letters (a–c).

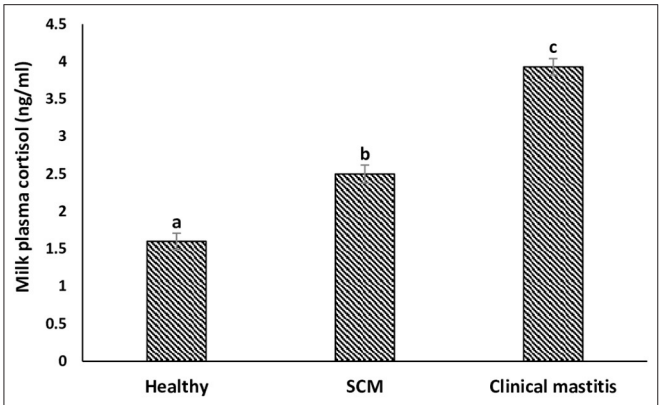


**FIGURE 8 |** Milk plasma cortisol concentration (ng/mL) in healthy, subclinical mastitis (SCM), and clinical mastitis Sahiwal cows. Data were expressed as mean ± SEM. Means statistically ($p < 0.05$) different between the groups are shown with small superscript letters (a–c).

every country has set a limit for SCC, and in Indian condition, healthy mammary gland should have 150 × $10^3$ SCs/mL of milk, whereas a value higher than this denotes mammary health disturbance (4, 26). However, various factors could influence the count of SCs including the stage of lactation, parity, season, frequency of milking, and physiological and environmental stress (4, 27, 34). Moreover, diurnal variation in the milk SCC has been reported in cows and buffaloes (35, 36). Therefore, differential cell counts can be used as an alternative approach for a more detailed analysis of the health status of mammary gland (37, 38). DLC indicates the percentage of individual cell populations such as neutrophils, macrophages, and lymphocytes, which play critical roles during intramammary infection (39). Usually, healthy udder comprises predominantly macrophages, whereas elevated neutrophil percentage is characteristic of the infected udder (15, 38).

Macrophage and neutrophils have a common precursor of origin and known to be the professional phagocytes that work in a coordinated manner during an inflammatory immune response (40). Macrophages are sentinel cells that recognize the intruding pathogens and release various chemoattractants upon activation. The chemical messengers subsequently activate neutrophils and facilitate their transmigration from blood to the inflamed tissue (1, 4). This signaling cascade justifies the drastic rise in the number of milk neutrophils with the progress of mammary infection observed in our study. Paape et al. (1) also reported that neutrophils rapidly transmigrate to the inflamed mammary gland, and their number may go up to 90% during mastitis. In accordance with our findings, Gulbe et al. (41) demonstrated decreased percentage of milk macrophage in sub-clinically infected mammary glands. We speculate that the absolute number of milk macrophages did not change significantly as the decrease in their percentage may be attributed to the parallel increase in the number of neutrophils.

The severity of infection not only increases the rate of neutrophil production from bone marrow but also reduces its maturation time, and it leads to the left shift, that is, release of immature neutrophils/band cells (1). We found a higher percentage of band cells in mastitis cows in comparison to healthy and SCM cows. Nevertheless, the immature neutrophils cannot function normally as compared to mature neutrophils, which comprise the disease resistivity of infected animals. Also, the pathological condition of mammary gland influences the viability of neutrophil and delineates the tissue retention of neutrophil after recruitment (42). Lower viability and activity of milk phagocytes isolated from mastitis milk are correlated with the pathogens and their components, which are known to influence the life span and functions of milk phagocytes (43). The present study was limited to mastitis naturally induced by *S. aureus* as this pathogen is responsible for the majority of mastitis cases in the study area (44). In our previous study, we reported lower inflammatory responses and impaired neutrophil activities during Gram-positive bacterial infections (*S. agalactiae* and *S. aureus*) as compared to Gram-negative bacterial infections (*E. coli*), which may explain the association between *S. aureus* infection and chronic mastitis (17). Also, we reported pathogen-dependent modulation of neutrophil viability in which the viability of milk neutrophils decreased with the progress of mammary infection and reached the lowest values in *S. aureus*–infected cows compared to mastitis induced by *S. agalactiae* or *E. coli* (17).

The main function of neutrophils is to kill the invading pathogens, and neutrophils can do it through several effector mechanisms including phagocytosis, excretion of granules contents of antimicrobial peptides/proteins, and NETs (1, 23, 45). Phagocytosis involves the respiratory burst, and *in vitro* PA is an effective parameter to investigate when evaluating mastitis resistance (21). There was a significant decrease in the PA of milk neutrophils in mastitis group as compared to SCM and healthy groups. The reason might be due to the negative effect of cortisol on PA as the concentration of milk cortisol was the highest in mastitis cows. Cortisol exerts its pleiotropic effects via the cytoplasmic receptors, which are present in various immune cells, and one of the effects is impairment of reactive oxygen species production (18). In the present study, we observed no decrease in the PA of milk macrophages in association with the increased cortisol concentration and the decreased macrophage viability in SCM and CM milk samples. Previous studies showed that dexamethasone promotes phagocytosis and bacterial killing ability of monocytes/macrophages challenged with various pathogens, and the blocking of glucocorticoid receptor could impair their PA (46). This can partially explain the maintained level of PA of milk macrophage during mammary infection, but more studies are warranted to explore this mechanism in bovine mastitis.

MPO is one of the effector lysosomal enzymes present in the azurophilic granules of neutrophils, and it gets released by activated neutrophils during mammary infections (47). During neutrophils' respiratory burst, MPO mediates the transformation of hydrogen peroxide into hypochlorous acid. This process is essential during phagocytosis for efficient killing and elimination of microorganisms invading mammary tissue (45, 47). Moreover, MPO is one of the main proteins released during the extrusion of NETs by neutrophils. Although the expression of MPO

increased in milk neutrophils isolated from SCM and CM cows, the PA and NET formation decreased in these cows. This is because phagocytosis and NET formation are multistep processes that involve many essential molecules and pathways, and the impairment of any step could impair the whole process. Although MPO was always studied in neutrophils, a recent study revealed that bovine monocytes are also expressing MPO comparable to neutrophils (48). In the present study, the expression of MPO in milk macrophages was comparable to milk neutrophils in healthy mammary gland and increased significantly in SCM and CM samples. However, the expression of MPO in neutrophils isolated from CM cows was double the expression of MPO in macrophages isolated from the same group. This indicates that this enzyme is more important for the functions of neutrophil especially NET release during mammary infections.

NETs are extracellular structures of DNA framework decorated with histones and many granular proteins including MPO and neutrophil elastase. This defense mechanism exhibited by neutrophils is critical for the control and elimination of pathogens (22–24). Recent studies have proved that milk neutrophils can perform NETs in response to mammary infections in sheep and cows (49, 50), but the variation in NET formation by bovine neutrophil in relation to changes in milk SCC and DLC has not been reported. The ability of milk neutrophils to perform NETs under *in vitro* condition was maximum in healthy cows in association with lower SCC and minimum cortisol concentration. However, the increases in SCC, neutrophil percentage, and cortisol concentration with the progress of mammary infection were associated with diminished NET formation. The decreased NET release by milk neutrophils of infected cows could be because these cells have already been subjected to *in vivo* activation, utilized part of their energy resources, and the increased percentage of immature band neutrophils. This also can explain the mitigated innate immune response and the longer time required to clear infection in SCM and CM cows.

TLRs are the pathogen recognition receptors that help in the recognition of a wide range of pathogen-associated molecular patterns and elicit the innate immune response. TLR2 and TLR4 are known to recognize different bacterial structures such as TLR2 ligands, that is, peptidoglycan of Gram-positive bacteria, and TLR4 ligands, that is, lipopolysaccharide of Gram-negative bacteria. Higher expression of TLR2 and TLR4 in neutrophil during mastitis might indicate higher recognition and elevated immune response against the microbes (49). We observed a significant increase in the expression of TLR2 and TLR4 in both neutrophils and macrophages with the progress of mammary infection. Griesbeck-Zilch et al. (51) reported that the expression of TLR2 significantly increases during mastitis induced by *S. aureus*, which facilitates effective recognition and elimination of the Gram-positive bacteria by the host immune cells. Underhill et al. (52) reported that TLR2 is recruited to macrophage phagosomes and discriminates between pathogens. Moreover, a point mutation in TLR2 abrogates the inflammatory responses to yeast and Gram-positive bacteria, but not to Gram-negative bacteria. However, a recent study reported that soluble TLR2 and full-length TLR2 are released by human macrophages in response to lipopolysaccharide challenge, which may contribute to glucocorticoids-induced immunosuppression and chronic infections (53). We observed that the expression of TLR2 was double the expression of TLR4 in both neutrophils and macrophages during mastitis, which reflects the role of TLR2 in mediating an effective immune response against *S. aureus*. However, the increase in the expression of TLR4 in milk phagocytes isolated from SCM and CM mastitis was also significant, which indicates that this receptor is also critical for the elimination of the invading pathogens. Similarly, Gonen et al. (54) reported that adoptive transfer of TLR4-expressing macrophage restricted the invasion of epithelial cells by *E. coli* in a murine model of acute mastitis.

CD25 is the main receptor for IL-2, and the higher expression of CD25 in bovine neutrophils was reported as a potential biomarker of inflammation during various diseases such as mastitis (55). IL-2 is a pro-inflammatory cytokine secreted by type 1 helper cells and amplifies the immune response against both Gram-positive and Gram-negative bacteria. Recently, we have reported increased concentrations of plasma IL-2 during mastitis induced by both *S. aureus* and *E. coli* (17). In the present study, the increased expression of CD25 is essential to mediate the signaling of IL-2 in milk neutrophil during mammary infection. Adhesion molecules such as selectin and integrins are necessary for effective neutrophil recruitment to the sites of inflammation. The very first step of recruitment is the capturing/tethering, which is mediated by the CD62L (L-selectin), which helps in slowing down of neutrophil and allowing the neutrophil to roll along with the vascular endothelial cell (56). However, for tight binding with the endothelium, integrin is required, and for that, neutrophil should shed CD62L after proteolytic cleavage (57). This might be the probable reason for the lower expression of CD62L observed in the milk neutrophils of all the groups.

CD44 is a non-specific accessory adhesion molecule that has been reported to play a critical role in mediating the trafficking of leukocyte to extra lymphoid sites of inflammation (58, 59). Higher expression of CD44 in milk neutrophils isolated from SCM cows may be important for the recruitment of neutrophils to the mammary tissue during the initial stage of infection. After neutrophils perform their functions at the site of infection, they get removed by macrophages, and CD44 serves as an apoptotic signal for the macrophages (60). Pathogen-dependent modulation of CD44 expression has been reported by Harp et al. (58), in which they found increased expression of CD44 on milk lymphocytes during *Streptococcus uberis* mastitis but not with *Serratia marcescens*. Lower expression of CD44 in neutrophils isolated from mastitis cows in our study has two explanations. First, neutrophils were isolated at the beginning of mastitis occurrence, and it could be too early for macrophages to start clearing neutrophils as the removal of apoptotic neutrophils occurs after the inflammation subsides. Second, it could be induced by *S. aureus* to impair CD44 signaling and the recruitment of immune cells, which leads to mammary

tissue injury and chronic inflammation usually observed in mastitis caused by this pathogen. Similarly, Cairns et al. (61) demonstrated that decreased expression of CD44 on neutrophils and monocytes during systemic lupus erythematosus impairs neutrophil clearance by macrophage and increases disease severity.

## CONCLUSIONS

The present study highlights the importance of SCC and DLC as novel methods for monitoring the health status of mammary gland in dairy cows. DLC reveals the alteration in milk leukocyte population without an increase in total cell number and thus could identify infected quarters more efficiently than SCC. The increase in the SCC and percentage of milk neutrophils during subclinical and clinical forms of mammary infection was associated with reduced phagocytosis and extracellular trap formation. Milk cortisol was positively correlated with the number of milk neutrophils and expression of TLRs, MPO, and CD25, whereas it was negatively correlated with the PA of neutrophils coming in milk. This may increase the chance of CM in cows; however, this interplay needs to be explored further in a large number of animals particularly with respect to the functions and gene expression of milk phagocytes. This is essential to improve animal welfare and increase farmer income as well as milk quality and production.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by the approval of all the experiments carried out in this research work was obtained from the Animal Ethics Committee of the NDRI according to the CPCSEA rules, laid down by the Government of India.

## AUTHOR CONTRIBUTIONS

MA and AD designed the study. MA performed all the experiments, analyzed the data, prepared the figures, wrote, and revised the manuscript. AD supervised the project and provided the funds. BSKP helped in sample collection and wrote some of the paper. All authors read and approved the final version of the manuscript.

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## REFERENCES

1. Paape MJ, Bannerman DD, Zhao X, Lee JW. The bovine neutrophil: structure and function in blood and milk. *Vet Res.* (2003) 34:597–627. doi: 10.1051/vetres:2003024
2. Curone G, Filipe J, Cremonesi P, Trevisi E, Amadori M, Pollera C, et al. What we have lost: mastitis resistance in Holstein Friesians and in a local cattle breed. *Res Vet Sci.* (2018) 116:88–98. doi: 10.1016/j.rvsc.2017.11.020
3. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet Res.* (2003) 34:475–91. doi: 10.1051/vetres:2003027
4. Alhussien MN, Dang AK. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: an overview. *Vet World*. (2018) 11:562–77. doi: 10.14202/vetworld.2018.562-577
5. Richards VP, Zadoks RN, Bitar PD, Lefébure T, Lang P, Werner B, et al. Genome characterization and population genetic structure of the zoonotic pathogen, *Streptococcus canis*. *BMC Microbiol.* (2012) 12:293. doi: 10.1186/1471-2180-12-293
6. Schukken YH, Bronzo V, Locatelli C, Pollera C, Rota N, Casula A, et al. Efficacy of vaccination on *Staphylococcus aureus* and coagulase-negative *staphylococci* intramammary infection dynamics in 2 dairy herds. *J Dairy Sci*. (2014) 97:5250–64. doi: 10.3168/jds.2014-8008
7. Monistero V, Graber HU, Pollera C, Cremonesi P, Castiglioni B, Bottini E, et al. Staphylococcus aureus isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes. *Toxins*. (2018) 10:247. doi: 10.3390/toxins10060247
8. Latorre AA, Pachá PA, González-Rocha G, San Martín I, Quezada-Aguiluz M, Aguayo-Reyes A. On-farm surfaces in contact with milk: the role of *Staphylococcus aureus*-containing biofilms for udder health and milk quality. *Foodborne Pathog. Dis.* (2020) 17:44–51. doi: 10.1089/fpd.2019.2704
9. Alhussien M, Manjari P, Mohammed S, Sheikh AA, Reddi S, Dixit S, et al. Incidence of mastitis and activity of milk neutrophils in Tharparkar cows reared under semi-arid conditions. *Trop Anim Health Prod*. (2016) 48:1291–5. doi: 10.1007/s11250-016-1068-8
10. Rainard P, Foucras G, Fitzgerald JR, Watts JL, Koop G, Middleton JR. Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. *Transbound Emerg Dis*. (2018) 65:149–165. doi: 10.1111/tbed.12698
11. Halasa T, Kirkeby C. Differential somatic cell count: value for udder health management. *Front Vet Sci.* (2020) 7:1153. doi: 10.3389/fvets.2020.609055
12. Dosogne H, Vangroenweghe F, Mehrzad J, Massart-Leen AM, Burvenich C. Differential leukocyte count method for bovine low somatic cell count milk. *J Dairy Sci.* (2003) 86:828–34. doi: 10.3168/jds.S0022-0302(03)73665-0
13. Gonçalves JL, Lyman RL, Hockett M, Rodriguez R, dos Santos MV, Anderson KL. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. *J Dairy Res.* (2017) 84, 309–17. doi: 10.1017/S0022029917000267
14. Zecconi A, Vairani D, Cipolla M, Rizzi N, Zanini L. Assessment of subclinical mastitis diagnostic accuracy by differential cell count in individual cow milk. *Ital J Anim Sci.* (2019) 18:460–5. doi: 10.1080/1828051X.2018.1533391
15. Paudyal S, Pena G, Melendez P, Roman-Muniz IN, Pinedo PJ. Relationships among quarter milk leukocyte proportions and cow and quarter-level variables under different intramammary infection statuses. *Transl Anim Sci.* (2018) 2:231–40. doi: 10.1093/tas/txy065
16. Panda BS, Mohapatra SK, Alhussien MN, Dang AK. Amount of milk neutrophil percentage and associated CD molecular changes on the compositional and technological properties of milk. *Open Biotechnol J*. (2019) 13:129–36. doi: 10.2174/1874070701901301 29
17. Alhussien MN, Dang AK. Pathogen-dependent modulation of milk neutrophils competence, plasma inflammatory cytokines and milk quality during intramammary infection of Sahiwal (*Bos indicus*) cows. *Microb Pathog.* (2018) 121:131–8. doi: 10.1016/j.micpath.2018.05.029

18. Alhussien MN, Dang AK. Interaction between stress hormones and phagocytic cells and its effect on the health status of dairy cows: a review. *Vet World*. (2020) 13:1837–48. doi: 10.14202/vetworld.2020.1837-1848
19. Piccinini R, Bronzo V, Moroni P, Luzzago C, Zecconi A. Study on the relationship between milk immune factors and *Staphylococcus aureus* intramammary infections in dairy cows. *J Dairy Res.* (1999) 66:501–10. doi: 10.1017/S0022029999003751
20. Rinaldi M, Moroni P, Paape MJ, Bannerman DD. Differential alterations in the ability of bovine neutrophils to generate extracellular and intracellular reactive oxygen species during the periparturient period. *Vet J.* (2008) 178:208–13. doi: 10.1016/j.tvjl.2007.07.030
21. Dang AK, Prasad S, De K, Pal S, Mukherjee J, Sandeep IV. Effect of supplementation of vitamin E, copper and zinc on the *in vitro* phagocytic activity and lymphocyte proliferation index of peripartum Sahiwal (*Bos indicus*) cows. *J Anim Physiol Anim Nutr*. (2013) 97:315–21. doi: 10.1111/j.1439-0396.2011.01272.x
22. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
23. Delgado-Rizo V, Martínez-Guzmán MA, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M, et al. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol.* (2017) 8:81. doi: 10.3389/fimmu.2017.00081
24. Alhussien MN, Dang AK. JAK3 and PI3K mediates the suppressive effects of interferon tau on neutrophil extracellular traps formation during peri-implantation period. *J Reprod Immunol.* (2021) 45:103321. doi: 10.1016/j.jri.2021.103321
25. Alhussien MN, Dang AK. Sensitive and rapid lateral-flow assay for early detection of subclinical mammary infection in dairy cows. *Sci Rep*. (2020) 10:11161. doi: 10.1038/s41598-020-68174-0
26. Dang AK, Kapila S, Singh C, Sehgal JP. Milk differential cell counts and compositional changes in cows during different physiological stages. *Milchwissenschaft*. (2008) 63:239–42.
27. Alhussien MN, Dang AK. Impact of different seasons on the milk somatic and differential cell counts, milk cortisol and neutrophils functionality of three Indian native breeds of cattle. *J Therm Biol*. (2018) 78:27–35. doi: 10.1016/j.jtherbio.2018.08.020
28. Wilson-Welder JH, Frank AT, Hornsby RL, Olsen SC, Alt DP. Interaction of bovine peripheral blood polymorphonuclear cells and Leptospira species; Innate responses in the natural bovine reservoir host. *Front Microbiol*. (2016) 7:1110. doi: 10.3389/fmicb.2016.01110
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262
30. Sgorlon S, Fanzago M, Guiatti D, Gabai G, Stradaioli G, Stefanon B. Factors affecting milk cortisol in mid lactating dairy cows. *BMC Vet Res*. (2015) 11:1. doi: 10.1186/s12917-015-0572-9
31. Gurjar A, Gioia G, Schukken Y, Welcome F, Zadoks R, Moroni P. Molecular diagnostics applied to mastitis problems on dairy farms. *Vet Clin Food Anim Pract*. (2012) 28:565–76. doi: 10.1016/j.cvfa.2012.07.011
32. Brenaut P, Lefèvre L, Rau A, Laloë D, Pisoni G, Moroni P, et al. Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to Staphylococcus aureus. *Vet Res.* (2014) 45:16. doi: 10.1186/1297-9716-45-16
33. Gussmann M, Kirkeby C, Schwarz D, Farre M, Halasa T. A simulation study to investigate the added value in using differential somatic cell count as an additional indicator for udder health management in dairy herds. *Prev Vet Med*. (2020) 182:105090. doi: 10.1016/j.prevetmed.2020.105090
34. Harmon RJ. Physiology of mastitis and factors affecting somatic cell counts. *J Dairy Sci.* (1994) 77:2103–12. doi: 10.3168/jds.S0022-0302(94)77153-8
35. Bombade K, Kamboj A, Alhussien MN, Mohanty AK, Dang AK. Diurnal variation of milk somatic and differential leukocyte counts of Murrah buffaloes as influenced by different milk fractions, seasons and parities. *Biol Rhythm Res.* (2018) 49:151–63. doi: 10.1080/09291016.2017.1345472
36. Alhussien MN, Dang AK. Diurnal rhythm in the counts and types of milk somatic cells, neutrophil phagocytosis and plasma cortisol levels in Karan Fries cows during different seasons and parity. *Biol Rhythm Res.* (2018) 49:187–99. doi: 10.1080/09291016.2017.1350442
37. Schwarz D, Diesterbeck US, König S, Brügemann K, Schlez K, Zschöck M, et al. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *J Dairy Res*. (2011) 78:448–55. doi: 10.1017/S0022029911000574
38. Pilla R, Malvisi M, Snel GG, Schwarz D, König S, Czerny CP, et al. Differential cell count as an alternative method to diagnose dairy cow mastitis. *J Dairy Sci.* (2013) 96:1653–60. doi: 10.3168/jds.2012-6298
39. Damm M, Holm C, Blaabjerg M, Bro MN, Schwarz D. Differential somatic cell count-A novel method for routine mastitis screening in the frame of dairy herd improvement testing programs. *J Dairy Sci.* (2017) 100:4926–40. doi: 10.3168/jds.2016-12409
40. Kumar KP, Nicholls AJ, Wong CH. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res.* (2018) 371:551–65. doi: 10.1007/s00441-017-2753-2
41. Gulbe G, Pilmane M, Saulite V, Donina S, Jermolajevs J, Peškova L, et al. Cells and cytokines in milk of subclinically infected bovine mammary glands after the use of immunomodulatory composition GLP 810. *Mediators Inflamm*. (2020) 2020:8238029. doi: 10.1155/2020/8238029
42. Mehrzad J, Duchateau L, Burvenich C. Viability of milk neutrophils and severity of bovine coliform mastitis. *J Dairy Sci.* (2004) 87:4150–62. doi: 10.3168/jds.S0022-0302(04)73558-4
43. Boutet P, Boulanger D, Gillet L, Vanderplasschen A, Closset R, Bureau F, et al. Delayed neutrophil apoptosis in bovine subclinical mastitis. *J Dairy Sci.* (2004) 87:4104–14. doi: 10.3168/jds.S0022-0302(04)73553-5
44. Hegde R, Isloor S, Prabhu KN, Shome BR, Rathnamma D, Suryanarayana VV, et al. Incidence of subclinical mastitis and prevalence of major mastitis pathogens in organized farms and unorganized sectors. *Indian J Microbiol*. (2013) 53:315–20. doi: 10.1007/s12088-012-0336-1
45. Alhussien MN, Dang AK. Potential roles of neutrophils in maintaining the health and productivity of dairy cows during various physiological and physiopathological conditions: a review. *Immunol Res*. (2019) 67:21–38. doi: 10.1007/s12026-019-9064-5
46. Desgeorges T, Caratti G, Mounier R, Tuckermann J, Chazaud B. Glucocorticoids shape macrophage phenotype for tissue repair. *Front Immunol.* (2019) 10:1591. doi: 10.3389/fimmu.2019.01591
47. Cooray R, Petersson C G, Holmberg O. Isolation and purification of bovine myeloperoxidase from neutrophil granules. *Vet Immunol Immunopathol.* (1993) 38:261–72. doi: 10.1016/0165-2427(93)90086-J
48. Depreester E, Meyer E, Demeyere K, Van Eetvelde M, Hostens M, Opsomer G. Flow cytometric assessment of myeloperoxidase in bovine blood neutrophils and monocytes. *J Dairy Sci.* (2017) 100:7638–47. doi: 10.3168/jds.2016-12186
49. Swain DK, Kushwah MS, Kaur M, Patbandha TK, Mohanty AK, Dang AK. Formation of NET, phagocytic activity, surface architecture, apoptosis and expression of toll like receptors 2 and 4 (TLR2 and TLR4) in neutrophils of mastitic cows. *Vet Res Commun*. (2014) 209:209–19. doi: 10.1007/s11259-014-9606-1
50. Pisanu S, Cubeddu T, Pagnozzi D, Rocca S, Cacciotto C, Alberti A, et al. Neutrophil extracellular traps in sheep mastitis. *Vet Res.* (2015) 46:59. doi: 10.1186/s13567-015-0196-x
51. Griesbeck-Zilch B, Meyer HH, Kühn CH, Schwerin M, Wellnitz O. *Staphylococcus aureus* and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J Dairy Sci.* (2008) 91:2215–24. doi: 10.3168/jds.2007-0752
52. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature*. (1999) 401:811–5. doi: 10.1038/44605
53. Hoppstädter J, Dembek A, Linnenberger R, Dahlem C, Barghash A, Fecher-Trost C, et al. Toll-like receptor 2 release by macrophages: an anti-inflammatory program induced by glucocorticoids and lipopolysaccharide. *Front Immunol.* (2019) 10:1634. doi: 10.3389/fimmu.2019.01634
54. Gonen E, Vallon-Eberhard A, Elazar S, Harmelin A, Brenner O, Rosenshine I, et al. Toll-like receptor 4 is needed to restrict the invasion of *Escherichia coli* P4 into mammary gland epithelial cells in a murine model of acute mastitis. *Cell Microbiol.* (2007) 9:2826–38. doi: 10.1111/j.1462-5822.2007.00999.x

55. Zoldan K, Moellmer T, Schneider J, Fueldner C, Knauer J, Lehmann J. Increase of CD25 expression on bovine neutrophils correlates with disease severity in post-partum and early lactating dairy cows. *Dev Comp Immunol.* (2014) 47:254–63. doi: 10.1016/j.dci.2014.08.002
56. Ivetic A, Hoskins Green HL, Hart SJ. L-selectin: a major regulator of leukocyte adhesion, migration and signaling. *Front Immunol.* (2019) 10:1068. doi: 10.3389/fimmu.2019.01068
57. Zarbock A, Ley K. Neutrophil adhesion and activation under flow. *Microcirculation*. (2009) 16:31–42. doi: 10.1080/10739680802350104
58. Harp JA, Waters TE, Goff JP, Bannerman DD, Paape MJ. Expression of lymphocyte homing and adhesion molecules during intramammary infection of cows with *Serratia marcescens* or *Streptococcus uberis*: correlation with bacterial colonization and clinical signs. *Vet Immunol Immunopathol.* (2006) 109:13–21. doi: 10.1016/j.vetimm.2005.06.018
59. Gonen E, Nedvetzki S, Naor D, Shpigel NY. CD44 is highly expressed on milk neutrophils in bovine mastitis and plays a role in their adhesion to matrix and mammary epithelium. *Vet Res.* (2008) 39:29. doi: 10.1051/vetres:2008005
60. Takazoe K, Tesch GH, Hill PA, Hurst LA, Jun Z, Lan HY, et al. CD44-mediated neutrophil apoptosis in the rat. *Kidney Int.* (2000) 58:1920–30. doi: 10.1111/j.1523-1755.2000.00364.x
61. Cairns AP, Crockard AD, McConnell JR, Courtney PA, Bell AL. Reduced expression of CD44 on monocytes and neutrophils in systemic lupus erythematosus: relations with apoptotic neutrophils and disease activity. *Ann Rheum Dis.* (2001) 60:950–5. doi: 10.1136/ard.60.10.950

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# Metformin Inhibits Lipoteichoic Acid–Induced Oxidative Stress and Inflammation Through AMPK/NRF2/NF-κB Signaling Pathway in Bovine Mammary Epithelial Cells

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The objective of this research was to explore the effect of metformin on the lipoteichoic acid (LTA)–induced mastitis model using isolated primary bovine mammary epithelial cells (PBMECs). The PBMECs were exposed to either 3 mM metformin for 12 h as a metformin group (MET) or 100 μg/mL LTA for 6 h as LTA group (LTA). Cells pretreated with 3 mM metformin for 12 h followed by washing and 100 μg/mL LTA exposure for 6 h served as the MET + LTA group. Phosphate-buffered saline was added to cells as the control group. PBMECs pretreated with different metformin doses were analyzed by a flow cytometry (annexin V–fluorescein isothiocyanate assay) to detect the cell apoptotic rate. We performed quantitative reverse transcriptase–polymerase chain reaction and Western blot analysis to evaluate the inflammatory and oxidative responses to metformin and LTA by measuring cellular cytotoxicity, mRNA expression, and protein expression. Immunofluorescence was used to evaluate nuclear localization. The results showed that the gene expression of *COX2*, *IL-1β*, and *IL-6* significantly increased in the cells challenged with LTA doses compared to control cells. In inflammatory PBMECs, metformin attenuated LTA-induced expression of inflammatory genes nuclear factor κB (NF-κB) p65, tumor necrosis factor α, cyclooxygenase 2, and interleukin 1β, as well as the nuclear localization and phosphorylation of NF-κBp65 protein, but increased the transcription of nuclear factor erythroid 2–related factor 2 (Nrf2) and Nrf2-targeted antioxidative genes heme oxygenase-1 (HO-1) and Gpx1, as well as the nuclear localization of HO-1 protein. Importantly, metformin-induced activation of Nrf2 is AMP-activated protein kinase (AMPK)–dependent; as metformin-pretreated PBMECs activated AMPK signaling via the upregulation of phosphorylated AMPK levels, cell pretreatment with metformin also reversed the translocation of Nrf2 that was LTA inhibited. This convergence between AMPK and Nrf2 pathways is essential for the

anti-inflammatory effect of metformin in LTA-stimulated PBMECs. Altogether, our results indicate that metformin exerts anti-inflammation and oxidative stress through regulation of AMPK/Nrf2/NF-κB signaling pathway, which highlights the role of AMPK as a potential therapeutic strategy for treatment of bovine mastitis.

**Keywords: metformin, AMPK signaling, antioxidant, anti-inflammation, bovine mammary epithelium cells**

## INTRODUCTION

Mastitis is a frequent and costly bovine mammary disease in the milk production industry (1), which seriously affects animal health and production ability and causes a huge economic loss to the dairy industry (2). *Staphylococcus aureus* is a frequently isolated Gram-positive bacteria that induce mastitis (3, 4). Approximately one-third of clinical and subclinical mastitis cases are caused by *S. aureus* infection in dairy cattle, characterized by less severe inflammation and is sometimes asymptomatic (5). Lipoteichoic acid (LTA), a bacterial endotoxin embedded in the cytoderm of *S. aureus,* is a critical factor known to activate inflammatory responses (6) and affect lactation in the mammary gland of cows (7, 8). Toll-like receptors (TLRs), particularly TLR2, are involved in LTA detection derived from Gram-positive bacteria (9, 10), and TLR2 activation leads to nuclear factor κB (NF-κB) activation, which regulates the expression of proinflammatory cytokines, such as *IL1B*, *TNFA*, and *IL6* (11).

AMP-activated protein kinase (AMPK), a sensor of intracellular energy status, is an attractive target for suppressing inflammation. Indeed, evidence shows that AMPK activation can decrease oxidative stress and inhibit inflammation (12). However, mechanistic connections between AMPK and inflammation have limited links with the NF-κB pathway (13). NF-κB and mitogen-activated protein kinase signaling pathways regulate cytokines and chemokine expression, which are essential immune mediators during inflammation (14). On sensing redox system imbalance, AMPK exerts a beneficial effect in the prevention of reactive oxygen species (ROS) accumulation to alleviate oxidative stress (15, 16). Notably, the AMPK pathway shares distinct crosstalk with the antioxidant response, specifically, nuclear factor erythroid 2–related factor 2 (Nrf2). Recently, the direct phosphorylating effect of AMPK on Nrf2 has been identified (17). Many studies have supported the notion that the Nrf2 antioxidant pathway is downstream to AMPK (18, 19). Whether AMPK plays a positive action in inhibiting oxidative stress and inflammation in bovine mammary epithelial cells (BMECs) remains unknown.

To date, treatment and prevention of bovine mastitis relied on antibiotics. However, the growing concerns about antibiotic therapies being linked with the emergence of drug-resistant bacteria, have found the industry to look to alternative safe and available antibacterial treatment. Metformin (Met), a derivative of biguanide, was initially developed from natural compounds found in the plant *Galega*. It is officially known as French lilac or goat's rue and is one of the most classic and standard first-line therapies commonly used to treat type 2 diabetes for nearly 60 years (20). Met exerts its effect through targeting multiple pathways such as activating AMPK and inhibiting the mTOR, HER2, and NF-κB pathways (21). Studies indicate the dual role of AMPK activation using its inducers such as Met on the tumor necrosis factor α (TNF-α) levels in different tissues of rats (22). In mice, experiments indicated that induction of AMPK by its inducers leads to the inhibition of *COX2*, *TNFα*, *IL-6*, and *iNOS* through suppressing NF-κB nuclear translocation in neurons (23). Studies in the rat reported that Met activates Nrf2 in an AMPK-dependent manner and exerts antioxidant and anti-inflammatory effects under global cerebral ischemia; moreover, pretreatment with Met enhanced the level of glutathione and catalase activities compared with those in the ischemic group (24). Activation of AMPK by Met stabilized Nrf2 levels, and this result leads to the protective role of Met in oxidative stress. Therefore, pretreatment with Met increased Nrf2 expression sufficiently to induce antioxidant response element (ARE) genes, which subsequently activate antioxidant related factors such as heme oxygenase-1 (HO-1), glutathione, and catalase (25). In this study, we aimed to investigate the potential effect of Met on the LTA-induced mastitis model using isolated BMECs, with respect to the activation of the AMPK signaling pathway and inhibition of inflammatory responses and oxidative stress through the suppression of NF-κB and Nrf2 signaling.

## MATERIALS AND METHODS

### Materials

Met was purchased from Sigma (D150959; Sigma–Aldrich, St. Louis, MO, USA) with a purity of >97%. LTA (derived from *S. aureus*) used in these experiments was purchased from Sigma (L2515; Sigma–Aldrich).

### Ethics Statement

All experimental procedures were approved by the Animal Experiment Committee of Yangzhou University (YZUDWLL-202003-209), following the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of China, 1988) published by the Ministry of Science and Technology, China, in 2004. All of the experimental protocols were performed in accordance with the approved guidelines and regulations.

### Primary BMEC Isolation and Culture

Mammary gland biopsy was selected from three peak lactation dairy cows (26). After phosphate-buffered saline (PBS) washing, fat tissue and connective tissue were removed. BMECs were separated by the tissue block method followed by purification via differential digestion and cryopreservation after

subculturing. Cells were incubated in Dulbecco modified eagle medium (DMEM)/F12 supplemented with 10% (vol/vol) fetal bovine serum including 5 μg/mL bovine insulin and 10 KU/L cyan/streptomycin. The resuscitated mammary epithelial cells (MECs) were cultured in an incubator at 37°C, 5% $CO_2$, and suitable humidity. The medium was changed every 48 h. The BMECs were digested with 0.25% trypsin for passaging, and the growth of cells was observed using an inverted microscope (26, 27).

BMECs are primary cells isolated from the mammary glands of dairy cows. The primary cells can genuinely reflect the situation in the cow. To our knowledge, research of lactation function in MECs is mainly on milk protein (the main component is casein) gene expression. β-Casein is a marker protein representing the lactating function of MEC (28). Our research group who determined both mRNA level and protein level of β-casein in BMECs indicated that BMEC possesses secretory capacity. Therefore, confirmation of BMECs maintained lactating functions and can study gene expression of milk protein and milk secretion mechanism in the mammary gland.

The culture of primary bovine mammary epithelial cells (PBMECs) was performed as described previously (26, 29). Briefly, mammary tissue obtained from three lactating dairy cows without incidence of clinical disease was used for cell isolation and purification. The BMECs cultured in basal medium contained DMEM/F12 (catalog number: 11320082, American Thermo Fisher, Waltham, MA, USA) and 10% fetal bovine serum, and various cytokines (e.g., 5 μg/mL bovine insulin, 10 kU/L cyan/streptomycin) (catalog no. 7120-30; Invitrogen, Carlsbad, CA, USA). The resuscitated BMECs were cultured at 37°C, 5% $CO_2$, and proper humidity. When the cell confluence reached 80%, after infection of small RNA chemical synthesis reagents, cells were collected 48 h later for the following analyses. Three replicates for each treatment were used. All experiments were performed with cells at the four to six passages. Cells ($2 \times 10^5$) were seeded in six-well plates with overnight incubation in complete medium (90% RPMI 1640, 8119417; Gibco, CA, USA), 10% fetal bovine serum, and antibiotics (penicillin 100 IU/mL; streptomycin 100 μg/mL). All medium supplements were from Gibco (Thermo Fisher Scientific, CA, USA). The cells were maintained at 37°C and 5% $CO_2$ in a humidified incubator until reaching confluence.

## Experimental Design

The inflammation model was tested and optimized concerning LTA and Met concentrations. Dose dependence for Met to choose a suitable treatment for the following experiments was as follows: PBMECs pretreated with graded concentrations of Met (1, 2, 3, 5, and 10 mM) for 12 h were used to investigate the apoptosis rate by flow cytometry analysis (**Figure 1A**). Also, Western blot analysis was used to determine the optimum concentration of Met among Met doses of 0, 1, 3, 5, 10, and 15 mM (**Figure 1B**). Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and Western blot analysis were used to measure LTA dose-dependence (**Figure 2**): Western blot analysis for PBMECs induced by LTA with various concentrations (0, 10, 20, 30, 40, and 100 μg/mL) for 6 h. For qRT-PCR, PBMECs were induced by LTA doses (0, 50, and 100 μg/mL) for 6 h, as shown in **Figures 2A–C**.

PBMECs were challenged with LTA to stimulate the *in vitro* setting that existed in mastitis. A total of 5 μg of LTA was dissolved in 5 mL of RPMI 1640 (CCM) to serve as a stock solution (1 μg/mL). Four treatments were set as follows: a control group (Control) supplied with PBS only; LTA treatment refers to the cells administered with LTA at a concentration of 100 μg/mL for 6 h; cells pretreated with Met were subsequently primed with or without LTA as the MET + LTA group or MET group. For Met treatments, the PBMECs were treated with Met at a dose of 3 mM according to the dose-dependent assay for 12 h.

## Flow Cytometry Analysis

The apoptotic status of PBMECs was evaluated by measuring the exposure of phosphatidylserine on the cell membranes using annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining [50]. The BD Pharmingen annexin V–FITC Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used for the apoptosis assay. PBMECs ($2 \times 10^4$ cells/well) were plated in a 12-well plate, and after 24-h incubation, the cells were treated with graded concentrations of Met (0, 1, 2, 3, 5, and 10 mM) for 12 h and harvested. The cell pellets were washed twice after centrifugation with cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, pH 7.4) and suspended in 100 μL of 1× binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). The cells were incubated with 5 μL annexin V–FITC and 10 μL PI at room temperature for 15 min in the dark. After the incubation, 400 μL of 1×binding buffer was added to each tube. The cells were analyzed immediately by Epics XL-MCL Flow Cytometry (Beckman–Coulter, Cassina de Pecchi, Italy).

## RNA Isolation, cDNA, and Gene Expression Using Quantitative Real-Time PCR Analysis

After removing the cell culture, the cells were washed with PBS twice, and the TRIzol reagent (catalog no. 9108; Takara, Dalian, China) was added to isolate the total RNA in cells, and a NanoDropND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to value the concentration and quality of the isolated RNA. One microgram of total RNA was added in the reaction system to reverse transcribe into cDNA by a Prime Script RT Master Mix Kit (catalog no. RR036A; Takara), as described in the manufacturer's instructions. Related primers were designed with Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA), and the primer sequences were provided in **Table 1**. The efficiency of primers was evaluated before use. Each cDNA sample was amplified through qRT-PCR using the SYBR Premix Ex Taq Kit (catalog no. DRR420A; Takara) on an ABI 7300 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde phosphate dehydrogenase (*GAPDH*), *RPS9*, and *UXT* were used as an internal control of the mRNA expression in our experiments. Fold changes of the related mRNAs were quantified by the $2^{-\Delta\Delta Ct}$ method (30).

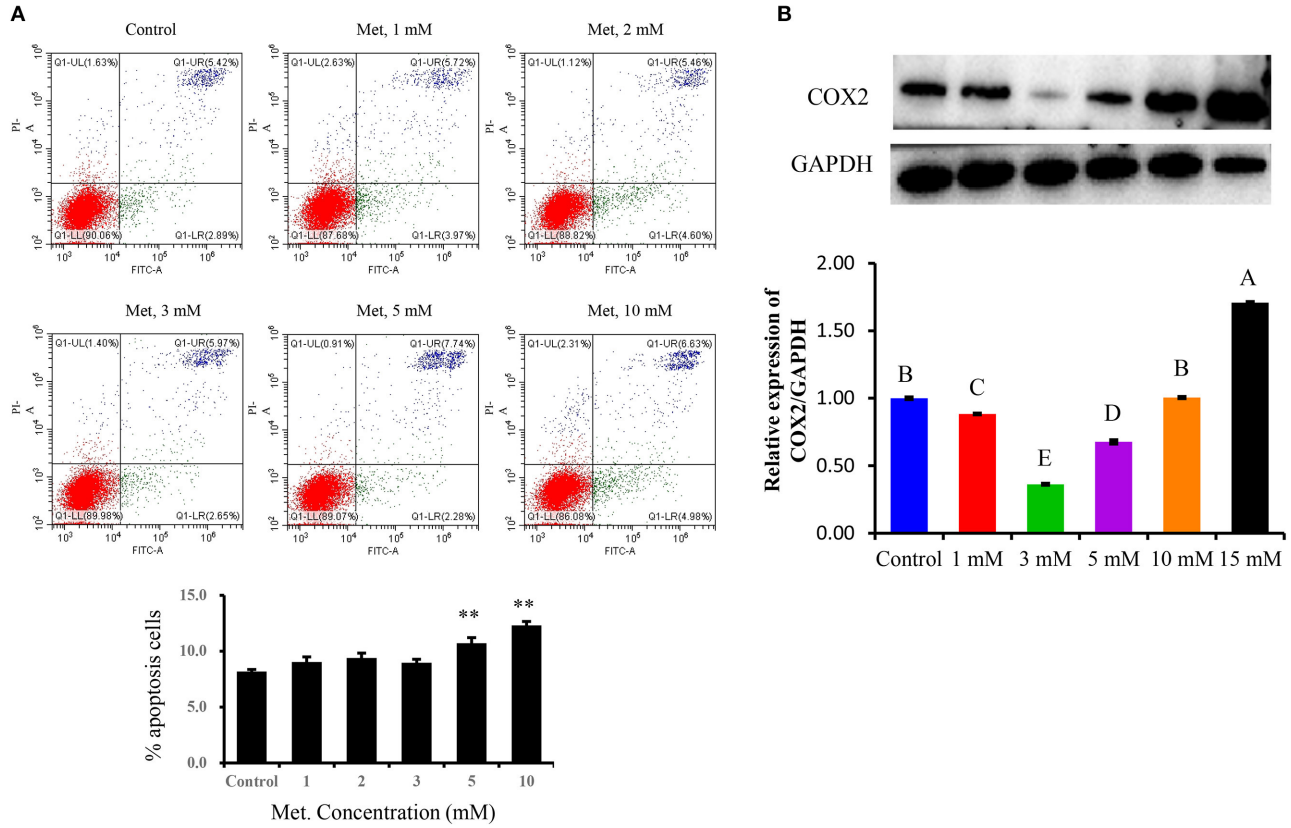


**FIGURE 1 |** Determination of the optimal metformin concentration in the study. Metformin was used to investigate its effect on growth and apoptosis in PBMECs. PBMECs were pretreated with the indicated concentrations of metformin (1, 2, 3, 5, and 10 mM) and incubated for 12 h; apoptotic cells were measured using flow cytometry, a statistical graph of the apoptotic death rates is shown in **(A)**. Western blot analysis was used to determine the optimum concentration of metformin among (1, 3, 5, 10, and 15 mM) metformin doses **(B)**. Values represent means ± SEM, different uppercase letters (A–E) on the top of bars indicate significant differences ($n = 3$, $p < 0.05$). **indicates $p < 0.01$ vs the Control group.

## Western Blot Analysis

Western blot was performed using the protocols described previously (26). Briefly, PBMECs were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and then were transferred onto nitrocellulose (NC) membranes (Bio Trace, Pall Corp, Port Washington, NY, USA). After blocking with 7% skim milk or 5% bovine serum albumin (BSA) [used for phosphorylated protein) in Tris-buffered saline with Tween (TBST) for 2 h at room temperature, the NC membranes were incubated overnight at 4°C with the primary antibodies for P-AMPK, AMPK, cyclooxygenase 2 (COX-2), P65, P-NRF2, HO-1, Gpx1, and GAPDH]. Rabbit antibodies were purchased from Beyotime (#AA393-1, #AF1627, #AF1924, #AF1234, #AF1609, #AF1333, #AF0162, and AF1186). Interleukin 1β (IL-1β) was purchased from Bioss ANTIBODIES (AL08133511/bs-6319R). P-P65 (#3033) and NRF2 (#1271T) were purchased from Cell Signaling Technology (Danvers, MA, USA). After washing with TBST, six times 5 min each the next day, the blots were incubated at room temperature for 2 h with corresponding goat anti-rabbit secondary antibody, purchased from Abcam (ab6712), and were diluted 1:5,000. After washing with TBST six times 5 min each and chemiluminescence, bands were detected and analyzed with Bio-Rad Gel Doc 2000 system analysis software (Bio-Rad, Hercules, CA, USA). GAPDH was used as a reference in our present study. The protein expression was quantitatively analyzed using ImageJ software.

## Immunofluorescence Analysis

Ten thousand PBMECs per well ($2 \times 10^4$ cells/well) were seeded in 12-well plates. The cells were incubated overnight and then treated according to the experimental design. For the immunofluorescence procedure, PBS (200 μL/well) was used to wash the coverslips covered by cells three times, and then 4% paraformaldehyde (500 μL/well) was added to fix the structure of cells for 15 min. Cells were washed three times with PBS and then perforated with 0.3% Triton X-100 (T9284; Sigma–Aldrich) for 15 min at room temperature to increase the permeability. After washing with PBS three times, the surface of cells was blocked for 1 h with 1× PBS/5% BSA/0.3% Triton-100 blocking buffer at room temperature. The resultant cells were incubated with the anti-p65, anti-COX2, and anti–HO-1 primary antibodies (same as those used in the Western blot analysis) in antibody

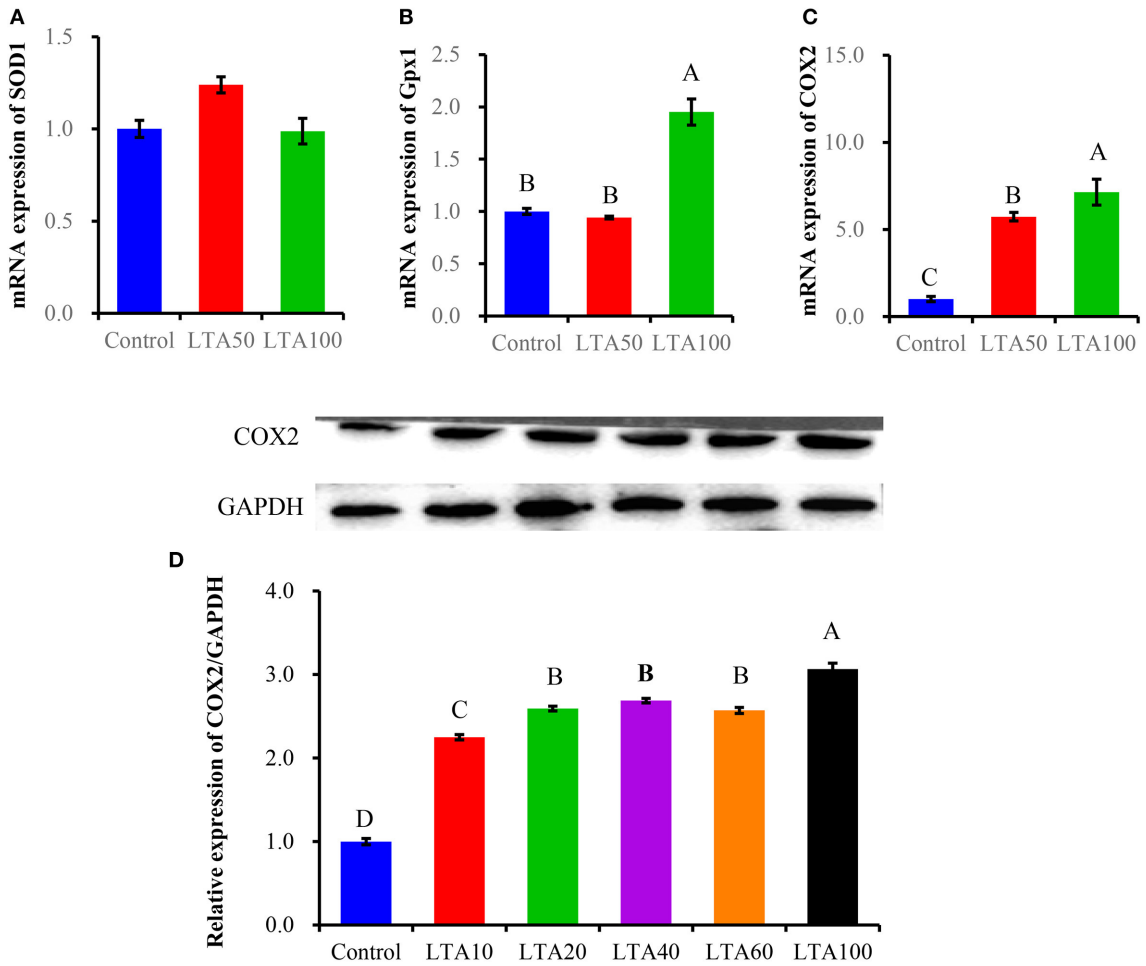


**FIGURE 2 |** Dose-dependent effect of LTA in PBMECs. Effect of lipoteichoic acid on SOD1 and Gpx1 antioxidant enzyme and COX2 expressions measured in PBMECs to determine the suitable LTA dose by qRT-PCR **(A–C)**. For optimum LTA dose confirmation, the expression of COX2 protein was measured in PBMECs stimulated with LTA (100 μg/mL) **(D)**. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) is used as a control. Graphs show the densitometric analysis (protein/GAPDH) from three independent experiments. Values represent means ± SEM. LTA, lipoteichoic acid. Different uppercase letters (A–D) on the top of bars indicate significant differences ($n = 3$, $p < 0.05$) among the groups.

**TABLE 1 |** Sequence of primers used in this study.

| Genes | Sequence (5′-3′) | References |
|---|---|---|
| Gpx1 | F: CCCCTGCAACCAGTTTGG, R: GAGCATAAAGTTGGGCTCGAA | |
| SOD1 | F: GGCTGTACCAGTGCAGGTCC, R: GCTGTCACATTGCCCAGGT | |
| COX2 | F: CGTTTTCTCGTGAAGCCCTATG, R: CTCCATGGCATCTATGTCTCCAT | |
| GAPDH | F: GGGTCATCATCTCTG CACCT, R: GGTCATAAGTCCCTCCACGA | (29) |

*The reverse transcription–generated cDNA encoding Gpx1 (glutathione peroxidase 1), SOD1 (superoxide dismutase-1), COX2 (cyclooxygenase 2), GAPDH (glyceraldehyde-3 phosphate dehydrogenase) was amplified by RT-PCR using the above selective primers. F, forward primer (5′-3′); R, reverse primer (3′-5′).*

buffer (1×PBS/1% BSA/0.3% Triton X-100) at 4°C overnight, followed by washing three times with PBS, and then were stained with the FITC-conjugated goat anti-rabbit antibody (A0562; Beyotime) for 1 h in the dark at 37°C. The cells were rinsed three times with PBS gently. The nucleus of the cell was stained by DAPI (1 μg/mL) (D8417; Sigma–Aldrich) for 5 min and then washed three times with PBS. The expression of p65, COX2, and HO-1 was visualized using a DMi8 Microsystems GmbH (Leica, Wetzlar, Germany).

## Statistical Analysis

Data were reported as the means ± standard error of the means. Statistical differences between groups and treatments were determined by one-way analysis of variance with Duncan

multiple-range tests by IBM SPSS 20.0 Statistics for Windows (IBM Inc., New York, NY, USA). $P < 0.05$ was considered as statistically significant. Study experiments were performed in triplicate, with three replicates in each experiment.

# RESULTS

## Effect of MET on the PBMEC Apoptosis and Met Dose Dependence by Flow Cytometry Analysis

To optimize the Met doses, the flow cytometry analyses of PBMECs pretreated with graded concentrations of Met (1, 2, 3, 5, and 10 mM) for 12 h were used to investigate the apoptosis rate by using an annexin V–FITC assay. As shown in **Figure 1A**, the apoptotic death rate of PBMECs pretreated with Met doses (1, 2, and 3 mM) did not differ from control ($P > 0.05$), whereas compared with the respective control, Met at 5 and 10 mM have induced PBMECs' apoptotic death rate ($P < 0.01$). Concerning early apoptotic death rates of PBMECs, the proportion of living cells pretreated with 3 mM Met showed statistically similar protective effects to control cells (**Figure 1A**, $P > 0.05$). Therefore, in the subsequent experiments, the dose of Met 3 mM was chosen as a suitable treatment dose.

## Optimization of Met Concentration by Western Blot Analysis

To confirm the proper dose of Met in this study, we used Western blot analysis to determine the optimum concentration of Met among (0, 1, 3, 5, 10, and 15 mM) Met doses (**Figure 1B**). Based on COX2 protein expression analysis, 3 mM of Met showed a dramatic decrease compared to the control group ($P < 0.05$).

## Optimizing LTA Concentration by Using qRT-PCR and Western Blot Analysis in PBMECs

qRT-PCR and Western blot analysis were used to measure LTA dose dependence (**Figure 2**). We measured mRNA expressions of superoxide dismutase-1 (*SOD1*), glutathione peroxidase 1 (*Gpx1*), and *COX2* in PBMECs induced by LTA at different doses (0, 50, and 100 μg/mL) for 6 h, as shown in **Figures 2A–C**. Moreover, COX2 protein level was measured by using Western blot analysis for PBMECs induced by LTA with various concentrations (0, 10, 20, 30, 40, 100, and 100 μg/mL) for 6 h. Our result showed that PBMECs stimulated with LTA at doses of 0, 50, and 100 μg/mL for 6 h; the *SOD1* expression level revealed no significant difference between groups ($P > 0.05$) (**Figure 2A**). The Gpx1 protein level had no significant difference at 50 μg/mL of LTA compared to the control group ($P < 0.05$) (**Figure 2B**). Our results indicated that mRNA levels of *COX2* increased significantly in LTA treatments at both 50 and 100 μg/mL compared to the control group ($P < 0.05$) (**Figure 2C**). Furthermore, to confirm this, we tested *COX2* protein expression by using Western blot; we observed that the *COX2* level was increased significantly with LTA treatment at 100 μg/mL (**Figure 2D**). Therefore, from these results, we show that LTA at a dose of 100 μg/mL was a suitable concentration to be used for the following experiments.

## Met Induced Activation of AMPK Signaling Pathway Examined in LTA-Induced PBMECs

The protein expressions related to AMPK signaling is shown in **Figure 3**. The protein levels of phosphorylated AMPKα (P-AMPKα) and ACCα were examined using Western blot analysis. Compared with the control group, the ratio of P-AMPK to total AMPK and the ratio of phosphorylated ACCα to total ACCα ($P < 0.05$) were increased following Met pretreatment ($P < 0.05$). LTA stimulation downregulated phosphorylation of both AMPKα and ACCα compared with the control group ($P < 0.05$). However, the phosphorylated AMPKα and ACCα proteins were increased following pretreatment with Met, which indicated that Met reversed the AMPK signaling inactivation due to LTA treatment ($P < 0.05$). The expression of phosphorylated AMPK was also significantly increased in cells pretreated with Met followed by LTA treatment as compared with the LTA group ($P < 0.05$).

## Pretreatment With Met Suppressed the Inflammatory Response of PBMECs Caused by LTA

We pretreated the PBMECs with 3 mM Met for 12 h and then stimulated with LTA (100 μg/mL) for 6 h. The protein expressions related to the inflammatory response were measured (**Figure 4**). Interestingly, we found that the cellular levels of p-p65, IL-1β, TNF-α, and COX2 increased in PBMECs challenged with LTA compared with the control group ($P < 0.05$). However, Met suppressed the expression of phosphorylated p65 (**Figure 4A**) and downregulated the expression of COX2 (**Figure 4B**), IL-1β (**Figure 4C**), and TNF-α (**Figure 4D**) induced by LTA PBMECs. As seen in **Figure 4**, compared with the control group, the expression of phospho-p65 in LTA group was prominently increased ($P < 0.05$). However, PBMECs pretreated with 3 mM Met alone or Met combined with the LTA had remarkably lower phospho-p65 than that in control group (**Figure 4A**). Consistently, the expression levels of COX2, IL-1β, and TNF-α were upregulated in response to LTA stimulation ($P < 0.05$). However, pretreatment with 3 mM Met reversed the LTA induction of cytokines (IL-1β, TNF-α) and COX2 expression (**Figures 4B–D**).

This study also showed the translocation of NF-κB p65 and subsequently the induction of COX2 in nuclei after LTA induced inflammatory response in PBMECs or pretreatment with Met to reverse the process. Immunofluorescence microscopy was used to visualize the fluorescence signal. FITC fluorescence was used to visualize NF-κB and COX2 (green), and DAPI was used to visualize the nuclei (blue). As our data showed (**Figure 4E**), compared with control cells, the strongest signal was NF-κB p65, and the green fluorescence was predominantly located in the nuclei in PBMECs stimulated with LTA treatment (**Figure 4**). However, for cells pretreated with 3 mM Met, there was less NF-κB p65 signal, and it was mainly in the cytoplasm, which

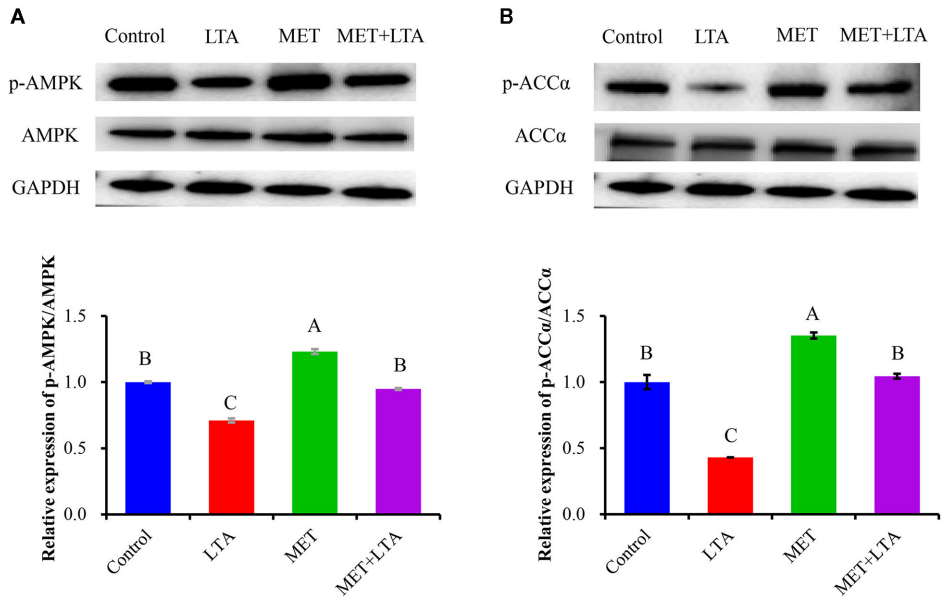


**FIGURE 3 |** Expression of proteins related to the AMPK signaling pathway. The expression of AMPK signaling–related proteins was measured by Western blot analysis in PBMECs treated with metformin 3 mM for 12 h, and LTA (100 μg/mL) for 6 h. Graphs show densitometric analysis (expressed as P-AMPK ratio to AMPK and P-ACC to ACC) from each band from three independent experiments. Values represent means ± SEM. LTA, lipoteichoic acid; MET, metformin. Different uppercase letters (A–C) on the top of bars indicate significant differences ($n = 3$, $p < 0.05$) vs. among the groups. This study measured the phosphorylation of both **(A)** AMPK and **(B)** ACC expression level.

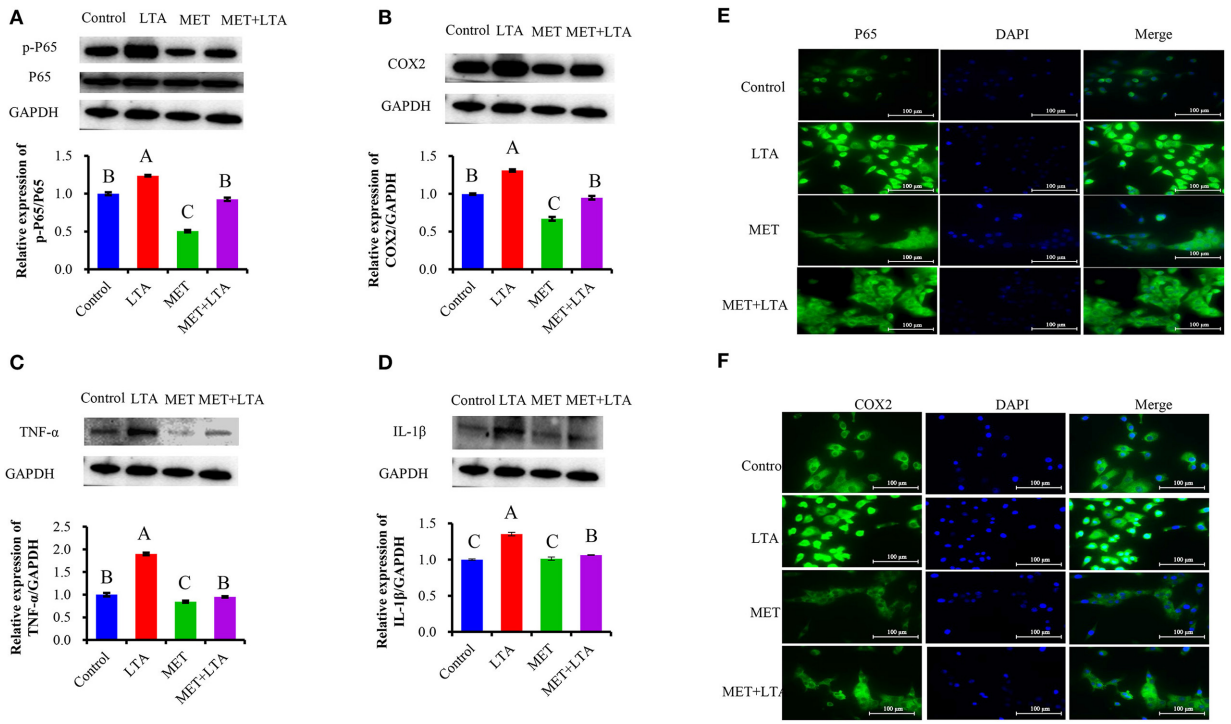


**FIGURE 4 |** Anti-inflammatory effect of metformin pretreated cells followed by LTA challenge. The expression of NF-κB–signaling related proteins was measured by Western blot analysis in bovine PBMECs pretreated with 3 mM metformin for 12 h and challenged with 100 μL/mL LTA for 6 h. The study measured NF-κB p65 **(A)**, COX2 **(B)**, IL-1β **(C)**, and TNF-α **(D)**, the expression level in the whole-cell lysates. GAPDH was used to normalize the protein expression. Immunofluorescence analysis for NF-κB p65 and COX2 (FITC) was performed, and the nuclear was stained with dye DAPI (blue). **(E, F)** Immunoblots and acquisition of intensity from the respective blots. Western blots shown are the representative image of three independent experiments. LTA, lipoteichoic acid; MET, metformin. Different uppercase letters (A–C) on the top of bars indicate significant differences ($n = 3$, $p < 0.05$) among the groups.

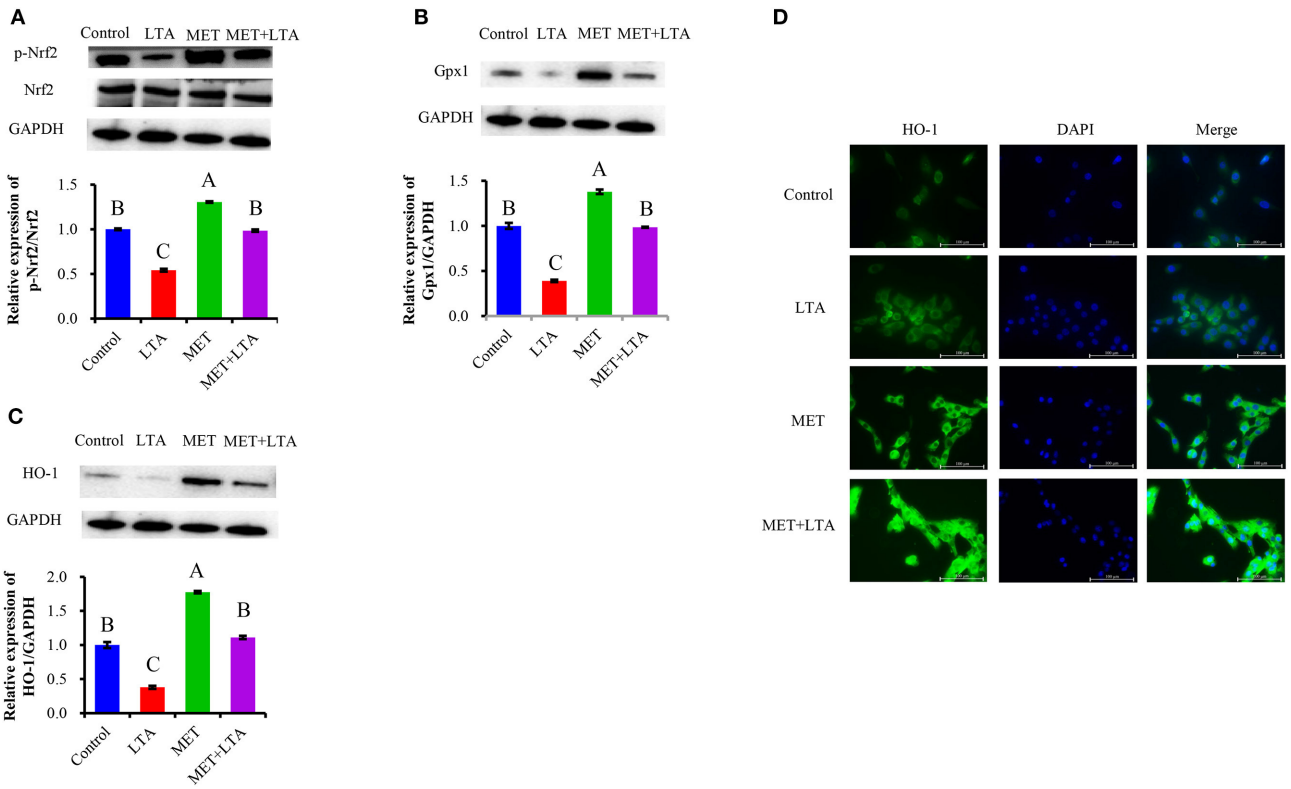


**FIGURE 5 |** Antioxidative effect of metformin in cells induced by LTA challenge. The expression of Nrf2 signaling–related proteins were measured by Western blot analysis in PBMECs pretreated with 3 mM metformin for 12 h and challenged with 100 μL/mL LTA for 6 h. The study measured the NRF2 **(A)** and its downstream Gpx1 **(B)** and HO-1 **(C)** protein expression. GAPDH was used to normalize the protein expression. Immunofluorescence analysis for HO-1 (FITC) was performed to visualize HO-1 (green), and the nuclear was stained with dye DAPI (blue). **(D)** Immunoblots and acquisition of intensity from the respective blots. Western blots shown are the representative image of three independent experiments. All results are expressed as the means ± SEM. LTA, lipoteichoic acid; MET, metformin. Different uppercase (A–C) on the top of bars indicate significant differences ($n = 3$, $p < 0.05$) vs. among the groups.

was similar to the control treatment. These results indicated that Met played an anti-inflammatory role by preventing the transformation of p65 into the active form, phospho-p65, and translocation into the nucleus. As a result, COX2, the downstream target gene of NF-κB p65 after translocation into the nucleus resulted in a quantitative increase of COX2 expression (**Figure 4F**).

## Met Regulates NRF2 Pathway and Its Downstream-Regulated HO-1 and Gpx1 Proteins Examined in LTA-Challenge PBMECs

We examined Nrf2 and phosphorylated Nrf2 (p-Nrf2) and its downstream HO-1 and Gpx1 protein expression in terms of genes and their coding proteins related to oxidative stress to confirm the antioxidant effects of Met. Western blot analysis demonstrated that Nrf2 protein level was decreased in the PBMECs stimulated with LTA; however, pretreatment with Met upregulated the level of Nrf2 that was downregulated by LTA treatment in PBMECs (**Figure 5A**). Moreover, the expression of Gpx1 (**Figure 5B**) and HO-1 (**Figure 5C**) activation, which decreased in response to LTA stimulation, was significantly upregulated by pretreatment with Met ($P < 0.05$).

To confirm the results from Western blots analysis, the current study determined the downstream protein expression (HO-1) of Nrf2 in the mammary cell using immunofluorescence. FITC fluorescence was used to visualize HO-1 (green), and DAPI was used to visualize the nuclei (blue). As our results showed (**Figure 5D**), because of location change of upstream kinase of HO-1 after LTA induced oxidative response or pretreatment Met in PBMECs. The weakest signal was HO-1, and the green fluorescence was predominantly located in the cytoplasm in the LTA treatment. For cells pretreated with 3 mM Met, there was the strongest HO-1 signal, and it was mainly in the nuclei, which was similar to the control group.

# DISCUSSION

The key motivation of this study was to determine if activation of AMPK via Met has a potential effect in suppressing oxidative stress and inflammatory responses in an LTA-induced mastitis model using isolated BMECs (**Figure 6**). It is not surprising that Met has been demonstrated to induce an antioxidant

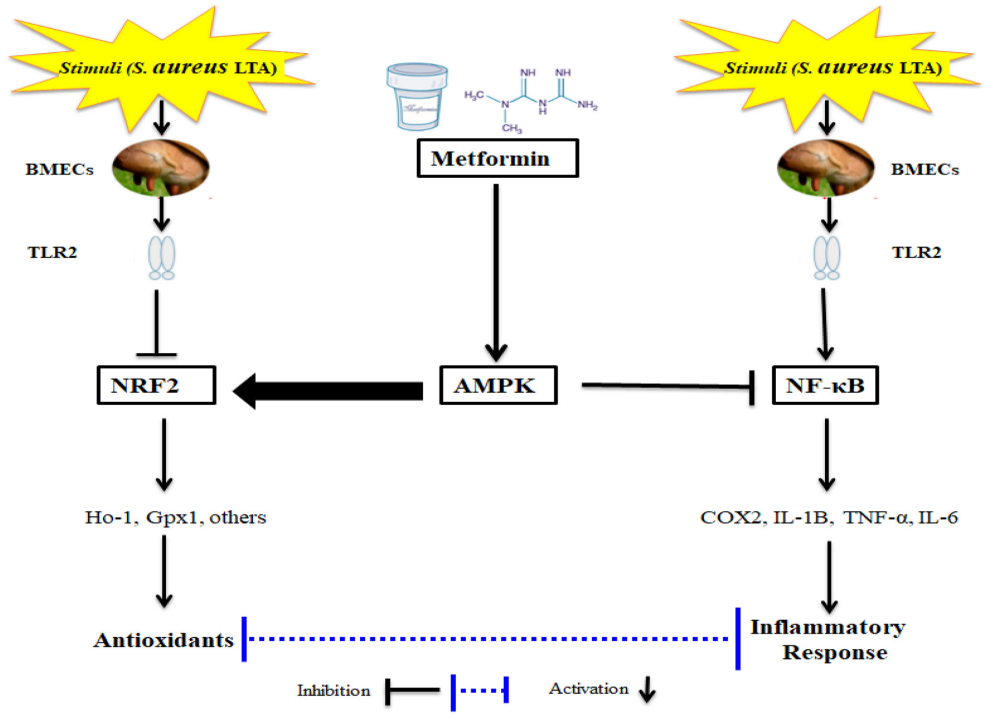


**FIGURE 6 |** Schematic Representations of Metformin and LTA.

response; however, the effect of Met on inflammation and cellular antioxidant protein expression induced by LTA in the PBMECs has not been characterized. Therefore, it is necessary to determine the functional and novel role of AMPK together with the application of Met, which would provide novel strategies besides antibiotics for veterinarians to intervene during cases of acute mastitis. The current study demonstrated a protective effect of Met on LTA-induced inflammation in mammary cells through specific activation of AMPK signaling and inhibition of NF-κB signaling. In addition to the anti-inflammatory role, Met might be involved in the activation of Nrf2 pathway; this activation is markedly AMPK-dependent in LTA-stimulated oxidative stress in PBMECs *in vitro*. Uncovering the functional relationship between AMPK and Nrf2 pathways is of significance because it reveals a novel link between energy homeostasis and inflammation suppression. This finding may substantially contribute to the development of new therapeutic approaches for inflammatory diseases such as mastitis.

LTA, an endotoxin of bacteria embedded in the cytoderm of *S. aureus*, is released during the cell proliferation process and/or after death (12) and was identified to activate inflammatory responses (31). Interestingly, *S. aureus* and its endotoxins' internalization into PBMECs were critical factors associated with the active state of NF-κB and induction of inflammatory responses and affected lactation in the mammary gland of cows (7, 8, 32). Research has demonstrated that LTA inhibited cell proliferation in PBMECs.

As a sensor of intracellular energy status, AMPK is an attractive target for inflammation control. Indeed, there is emerging evidence showing that AMPK activation can decrease oxidative stress and inhibit inflammation (12); another researcher reported that activation of AMPK decreases the production of ROS (33). However, mechanistic connections between AMPK and inflammation have been limited to a link with the NF-κB pathway (13). Several lines of evidence have shown that chemical activators of AMPK decreased NF-κB–mediated transcription in endothelial cells (12). Met exerts its effect by targeting multiple pathways such as activating AMPK and inhibiting mTOR, HER2, and NF-κB pathways (21). Met can induce AMPK phosphorylation, protein kinase c, and mitogen-activated protein kinase directly (34). In this study, LTA stimulation downregulated phosphorylation of both AMPKα and its downstream gene ACCα in PBMECs. However, the levels of phosphorylated AMPKα and ACCα proteins were increased following pretreatment with Met, which indicated that Met reversed the inactivation of AMPK signaling as a result of LTA treatment. The phosphorylation of AMPK expression was also significantly increased under the combined administration of Met with LTA compared to the LTA treatment. Collectively, Met addition activated AMPK signaling via the regulation of phosphorylated AMPK levels. Results of our study agreed with previous research that indicated Met could induce AMPK phosphorylation (34). Other studies showed the dual role of AMPK activation using its inducers such as Met on TNF-α levels in different tissues (22). In mouse neurons, it was indicated that

induction of AMPK by its inducers leads to inhibition of COX2, inducible nitric oxide synthase expression and decreased TNF-α, IL-6, and IL-1β through suppression of NF-κB p65 nuclear translocation (23).

NF-κB transcription factor is a key regulator of inflammation and immune responses (35). Met decreased NF-κB activity and reduced proinflammatory cytokine secretion (21), for instance; pretreatment of rats with Met attenuated cellular levels of NF-κB, COX2, and TNF-α, which are considered essential proteins involved in the inflammation pathway (24). Those findings were in part replicated in the current study because Met suppressed the expression of P-p65 in the PBMECs challenged with LTA. The NF-κB downstream protein expression of COX2, IL-1β, and TNF-α upregulated in response to LTA stimulation; however, pretreatment with 3 mM Met reduced IL-1β, TNF-α, and COX2 expression. The results of this study may indicate the protective role of Met in regulating the NF-κB pathway as a potential therapeutic way to treat bovine inflammation.

Another point to be discussed is that it is necessary to expand the investigation on signaling networks other than the NF-κB pathway for inflammation suppression, together with the unequivocal action of Nrf2-mediated antioxidant pathway on ROS clearance. Thus, searching the central probability of a new pathway for inflammation suppression, the downstream pattern of the AMPK pathway might be considered a molecular mechanism. Given this situation and the promising importance of the relationship between energy and redox equilibrium, we planned this line of study by asking specific questions: Is there is potential and functional crosstalk between NRF2 and AMPK pathways? If such crosstalk exists, how do they interact together in inflammation suppression? Studies have previously reported that activation of Nrf2 or AMPK signaling elicit patently overlapping phenotypic cellular or organismal responses and are alongside activated by several natural molecules, indicating cooperation (36, 37). Accordingly, an AMPK-driven boost of the Nrf2 signaling axis has already been confirmed in several studies (38, 39). Only one study has demonstrated direct phosphorylation of Nrf2 by AMPK in an *in vitro* enzyme assay at position Ser 558 (human Nrf2) (40). AMPK phosphorylates Nrf2 at the Ser 550 residue, which, in conjunction with AMPK mediated GSK3 inhibition, promotes nuclear accumulation of Nrf2 for ARE-driven gene transactivation (40). Serving as a stress sensor, AMPK exerts a beneficial effect in the prevention of ROS accumulation to alleviate oxidative stress (15, 16). On sensing redox system imbalance, notably, the AMPK pathway shares distinct association with an antioxidant response, Nrf2, a master regulator that has been activated to upregulate antioxidant gene expression (38). The underlying mechanism is demonstrated to be the induction of AMPK on Nrf2 and its downstream target HO-1 (41, 42). A study using mouse embryonic fibroblasts has shown the AMPK-triggered direct phosphorylation effect on Nrf20 (17). Therefore, to investigate the potential for a functional interaction, we examined the effect of Met on oxidative and inflammatory stress by utilizing LTA-stimulated PBMECs in the present study. Lines of evidence have shown that activation of AMPK by Met stabilized Nrf2 levels, and this phenomenon leads to protection. Therefore, pretreatment with Met increased Nrf2 expression sufficiently to induce ARE genes, which subsequently activated antioxidant related factors such as HO-1, glutathione, and catalase (25). In our work, Nrf2 at the protein level was decreased in the PBMECs challenged with LTA. However, pretreatment with Met upregulated the level of Nrf2 and HO-1 that was downregulated by LTA treatment in PBMECs. Importantly, pretreatment with Met also reversed the translocation of Nrf2 that induced by LTA. To our knowledge, the results of this study indicated the effect of Met on Nrf2 protein in PBMECs induced by LTA, and it may provide an opportunity for a new mechanism on functional molecules capable of interfering with the binding and activation of the Nrf2 pathway.

## CONCLUSION

In the LTA dose-dependent experiment, the gene expression level of COX2, IL-1β, and IL-6 increased significantly in LTA-challenged PBMECs relative to those in control cells. The protein level of NrF2 was decreased in the cells treated with LTA. Met addition activated AMPK signaling via the regulation of phosphorylated AMPK level. Additionally, in the PBMECs challenged with LTA, the pretreatment with Met attenuated cellular levels of NF-κB, TNF-α, COX2, and IL-1β. Moreover, pretreatment of PBMECs with Met upregulated Nrf2 and HO-1 protein expressions, which were downregulated by LTA treatment. Importantly, pretreatment with Met also reversed the translocation of Nrf2 induced by LTA. Altogether, our results indicate that Met exerts the anti-inflammation and antioxidant effects through regulation of AMPK/NrF2/NF-κB signaling pathway (**Figure 6**), which highlights the role of AMPK as a potentially therapeutic target for the treatment of *S. aureus*–induced bovine mastitis.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

AA and TX conceived and designed the experiments. AA and XL performed the experiments. AA and IA analyzed the data. AA, ZC, and AI contributed in figure format. AA, ML, and TX were major contributor in writing the manuscript. ZY support to the financial cost throughout the experiment and generate the ideas. All authors read and approved the final manuscript.

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## REFERENCES


1. Günther J, Liu S, Esch K, Schuberth HJ, Seyfert HM. Stimulated expression of TNF-α and IL-8, but not of lingual antimicrobial peptide reflects the concentration of pathogens contacting bovine mammary epithelial cells. *Vet Immunol Immunopathol.* (2010) 135:152–7. doi: 10.1016/j.vetimm.2009.11.004
2. Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet Res.* (2003) 34:475–91. doi: 10.1051/vetres:2003027
3. Jensen K, Günther J, Talbot R, Petzl W, Zerbe H, Schuberth HJ, et al. *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters. *BMC Genomics.* (2013) 14:36. doi: 10.1186/1471-2164-14-36
4. Hertl JA, Schukken YH, Bar D, Bennett GJ, González RN, Rauch BJ, et al. The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in Holstein dairy cows. *J Dairy Sci.* (2011) 94:4863–77. doi: 10.3168/jds.2010-4000
5. Pumipuntu N, Kulpeanprasit S, Santajit S, Tunyong W, Kong-Ngoen T, Hinthong W, et al. Screening method for *Staphylococcus aureus* identification in subclinical bovine mastitis from dairy farms. *Vet World.* (2017) 10:721–6. doi: 10.14202/vetworld.2017.721-726
6. Wall SK, Hernández-Castellano LE, Ahmadpour A, Bruckmaier RM, Wellnitz O. Differential glucocorticoid-induced closure of the blood-milk barrier during lipopolysaccharide- and lipoteichoic acid-induced mastitis in dairy cows. *J Dairy Sci.* (2016) 99:7544–53. doi: 10.3168/jds.2016-11093
7. Wu Y, Chen J, Sun Y, Dong X, Wang Z, Chen J, et al. PGN and LTA from *Staphylococcus aureus* induced inflammation and decreased lactation through regulating DNA methylation and histone H3 acetylation in bovine mammary epithelial cells. *Toxins.* (2020) 12:238. doi: 10.3390/toxins12040238
8. Mount JA, Karrow NA, Caswell JL, Boermans HJ, Leslie KE. Assessment of bovine mammary chemokine gene expression in response to lipopolysaccharide, lipotechoic acid + peptidoglycan, and CpG oligodeoxynucleotide 2135. *Can J Vet Res.* (2009) 73:49–57.
9. Hacine-Gherbi H, Denys A, Carpentier M, Heysen A, Duflot P, Lanos P, et al. Use of toll-like receptor assays for the detection of bacterial contaminations in icodextrin batches released for peritoneal dialysis. *Toxicol Rep.* (2017) 4:566–73. doi: 10.1016/j.toxrep.2017.10.004
10. Kell DB, Pretorius E. No effects without causes: the iron dysregulation and dormant microbes hypothesis for chronic, inflammatory diseases. *Biol Rev.* (2018) 93:1518–57. doi: 10.1111/brv.12407
11. Liu L, Pang XL, Shang WJ, Xie HC, Wang JX, Feng GW. Over-expressed microRNA-181a reduces glomerular sclerosis and renal tubular epithelial injury in rats with chronic kidney disease via down-regulation of the TLR/NF-κB pathway by binding to CRY1. *Mol Med.* (2018) 24:49. doi: 10.1186/s10020-018-0045-2
12. Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S, et al. Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. *Clin Exp Immunol.* (2009) 156:542–51. doi: 10.1111/j.1365-2249.2009.03924.x
13. Salminen A, Hyttinen JMT, Kaarniranta K. AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan. *J Mol Med.* (2011) 89:667–76. doi: 10.1007/s00109-011-0748-0
14. Calzado M, Bacher S, Schmitz ML. NF-κB inhibitors for the treatment of inflammatory diseases and cancer. *Curr Med Chem.* (2007) 14:367–76. doi: 10.2174/092986707779941113
15. Wang S, Song P, Zou MH. AMP-activated protein kinase, stress responses and cardiovascular diseases. *Clin Sci.* (2012) 122:555–73. doi: 10.1042/CS20110625
16. Ceolotto G, Gallo A, Papparella I, Franco L, Murphy E, Iori E, et al. Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. *Arterioscler Thromb Vasc Biol.* (2007) 27:2627–33. doi: 10.1161/ATVBAHA.107.155762
17. Matzinger M, Fischhuber K, Pölöske D, Mechtler K, Heiss EH. AMPK leads to phosphorylation of the transcription factor Nrf2, tuning transactivation of selected target genes. *Redox Biol.* (2020) 29:101393. doi: 10.1016/j.redox.2019.101393
18. Yaku K, Matsui-Yuasa I, Konishi Y, Kojima-Yuasa A. AMPK synergizes with the combined treatment of 1′-acetoxychavicol acetate and sodium butyrate to upregulate phase II detoxifying enzyme activities. *Mol Nutr Food Res.* (2013) 57:1198–208. doi: 10.1002/mnfr.201200809
19. Iwasaki K, Ray PD, Huang BW, Sakamoto K, Kobayashi T, Tsuji Y. Role of AMP-activated protein kinase in ferritin H gene expression by resveratrol in human T cells. *Biochemistry.* (2013) 52:5075–83. doi: 10.1021/bi400399f
20. Marshall SM. 60 years of metformin use: a glance at the past and a look to the future. *Diabetologia*. (2017) 60:1561–5. doi: 10.1007/s00125-017-4343-y
21. Lei Y, Yi Y, Liu Y, Liu X, Keller ET, Qian CN, et al. Metformin targets multiple signaling pathways in cancer. *Chin J Cancer.* (2017) 36:17. doi: 10.1186/s40880-017-0184-9
22. Kewalramani G, Puthanveetil P, Wang F, Kim MS, Deppe S, Abrahani A, et al. AMP-activated protein kinase confers protection against TNF-α-induced cardiac cell death. *Cardiovasc Res.* (2009) 84:42–53. doi: 10.1093/cvr/cvp166
23. Lu J, Wu DM, Zheng YL, Hu B, Zhang ZF, Shan Q, et al. Quercetin activates AMP-activated protein kinase by reducing PP2C expression protecting old mouse brain against high cholesterol-induced neurotoxicity. *J Pathol.* (2010) 222:199–212. doi: 10.1002/path.2754
24. Ashabi G, Khalaj L, Khodagholi F, Goudarzvand M, Sarkaki A. Pre-treatment with metformin activates Nrf2 antioxidant pathways and inhibits inflammatory responses through induction of AMPK after transient global cerebral ischemia. *Metab Brain Dis.* (2015) 30:747–54. doi: 10.1007/s11011-014-9632-2
25. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene.* (1998) 17:3145–56. doi: 10.1038/sj.onc.1202237
26. Chen Z, Chu S, Wang X, Sun Y, Xu T, Mao Y, et al. MiR-16a regulates milk fat metabolism by targeting large tumor suppressor kinase 1 (LATS1) in bovine mammary epithelial cells. *J Agric Food Chem.* (2019) 67:11167–78. doi: 10.1021/acs.jafc.9b04883
27. Chen Z, Zhang Y, Zhou J, Lu L, Wang X, Liang Y, et al. Tea tree oil prevents mastitis-associated inflammation in lipopolysaccharide-stimulated bovine mammary epithelial cells. *Front Vet Sci.* (2020) 7:496. doi: 10.21203/rs.3.rs-18655/v1
28. Huynh HT, Robitaille G, Turner JD. Establishment of bovine mammary epithelial cells (MAC-T): An *in vitro* model for bovine lactation. *Exp Cell Res.* (1991) 197:191–9. doi: 10.1016/0014-4827(91)90422-Q
29. Ma N, Chang G, Huang J, Wang Y, Gao Q, Cheng X, et al. Cis-9, trans-11-conjugated linoleic acid exerts an anti-inflammatory effect in bovine mammary epithelial cells after *Escherichia coli* stimulation through NF-κB signaling pathway. *J Agric Food Chem.* (2019) 67:193–200. doi: 10.1021/acs.jafc.8b05500
30. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* (2001) 29:e45. doi: 10.1093/nar/29.9.e45
31. Bougarn S, Cunha P, Harmache A, Fromageau A, Gilbert FB, Rainard P. Muramyl dipeptide synergizes with *Staphylococcus aureus* lipoteichoic acid to recruit neutrophils in the mammary gland and to stimulate mammary epithelial cells. *Clin Vaccine Immunol.* (2010) 17:1797–809. doi: 10.1128/CVI.00268-10
32. Oviedo-Boyso J, Barriga-Rivera JG, Valdez-Alarcón JJ, Bravo-Patiño A, Cárabez-Trejo A, Cajero-Juárez M, et al. Internalization of *Staphylococcus aureus* by bovine endothelial cells is associated with the activity state of NF-κB and modulated by the pro-inflammatory cytokines TNF-α and IL-1β. *Scand J Immunol.* (2008) 67:169–76. doi: 10.1111/j.1365-3083.2007.02056.x
33. Li C, Wang Y, Li L, Han Z, Mao S, Wang G. Betaine protects against heat exposure–induced oxidative stress and apoptosis in bovine mammary epithelial cells via regulation of ROS production. *Cell Stress Chaperones.* (2019) 24:453–60. doi: 10.1007/s12192-019-00982-4
34. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. The antidiabetic drug metformin suppresses HER2 (erbB-2) oncoprotein overexpression via inhibition of the mTOR effector p70S6K1 in human breast carcinoma cells. *Cell Cycle.* (2009) 8:88–96. doi: 10.4161/cc.8.1.7499
35. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspect Biol.* (2009) 1:a001651. doi: 10.1101/cshperspect.a001651

36. Habib SL, Yadav A, Kidane D, Weiss RH, Liang S. Novel protective mechanism of reducing renal cell damage in diabetes: Activation AMPK by AICAR increased NRF2/OGG1 proteins and reduced oxidative DNA damage. *Cell Cycle.* (2016) 15:3048–59. doi: 10.1080/15384101.2016.1231259
37. Kim T, Davis J, Zhang AJ, He X, Mathews ST. Curcumin activates AMPK and suppresses gluconeogenic gene expression in hepatoma cells. *Biochem Biophys Res Commun.* (2009) 388:377–82. doi: 10.1016/j.bbrc.2009.08.018
38. Mo C, Wang L, Zhang J, Numazawa S, Tang H, Tang X, et al. The crosstalk between Nrf2 and AMPK signal pathways is important for the anti-inflammatory effect of Berberine in LPS-stimulated macrophages and endotoxin-shocked mice. *Antioxidants Redox Signal.* (2014) 20:574–88. doi: 10.1089/ars.2012.5116
39. Zimmermann K, Baldinger J, Mayerhofer B, Atanasov AG, Dirsch VM, Heiss EH. Activated AMPK boosts the Nrf2/HO-1 signaling axis - A role for the unfolded protein response. *Free Radic Biol Med.* (2015) 88:417–26. doi: 10.1016/j.freeradbiomed.2015.03.030
40. Joo MS, Kim WD, Lee KY, Kim JH, Koo JH, Kim SG. AMPK facilitates nuclear accumulation of Nrf2 by phosphorylating at serine 550. *Mol Cell Biol.* (2016) 36:1931–42. doi: 10.1128/MCB.00118-16
41. Wu W, Wang S, Liu Q, Wang X, Shan T, Wang Y. Cathelicidin-WA attenuates LPS-induced inflammation and redox imbalance through activation of AMPK signaling. *Free Radic Biol Med.* (2018) 129:338–53. doi: 10.1016/j.freeradbiomed.2018.09.045
42. Liu XM, Peyton KJ, Shebib AR, Wang H, Korthuis RJ, Durante W. Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. *Am J Physiol Hear Circ Physiol.* (2011) 300:H84–93. doi: 10.1152/ajpheart.00749.2010

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# Effect of Weeping Teats on Intramammary Infection and Somatic Cell Score in Dairy Goats

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Mastitis is the most common disease affecting dairy goats and causing economic losses. Although it is accepted that increased somatic cell count (SCC) is mainly a response to infection, its reliability for subclinical mastitis detection in goats is controversial. Indeed, many physiological and extrinsic variables can increase SCC, including breed, parity, age, stage of lactation, seasonal variations, and milking methods. In some animals, milk-secreting tissue is present in the wall of the teat and, in some instances, milk can filter through pores in the skin to the udder surface. This condition is known as "weeping teat" (WT). In these animals, mammary tissue might be prone to develop bacterial infections, although limited information is provided. Weeping teat seems to have a genetic background and is reported to be especially found in goat breeds selected for high milk production. Moreover, it is observed a genetic correlation between WT and decreased milk yield as well as increased somatic cell scores (SCS). Since information on this topic is very limited, this study aimed at investigating any possible relationship between WT, high SCC, and the presence of bacteria in goat milk. Alpine goat farms in Northern Italy were selected based on the presence of WT. Each herd was divided into two age-matched groups, identified as case (WT+) and control (WT–). Half-udder milk samples were collected aseptically at three timepoints; bacteriological analysis was performed, and SCC were determined and transformed in SCS. There was a positive association between SCS and the presence of bacteria in milk ($P = 0.037$) overall, whereas WT udder defect was associated with positive bacterial culture in just one herd ($P = 0.053$). Thus, this herd was further investigated, repeating the sampling and the analysis on the following year. The positive association between high SCS and the presence of bacteria in milk was then confirmed ($P = 0.007$), whereas no association with WT condition was found. These results indicate that WT defect is usually unrelated to both the outcome of milk bacterial culture and SCS. As a side outcome, we could confirm the role of bacterial infection in increasing SCS.

**Keywords: goat, weeping teat, udder defect, milk, microbiology, somatic cell count**

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## INTRODUCTION

Mastitis is the most common disease affecting dairy goats and represents the main cause of economic losses due to various factors, including the decrease in milk yield and quality as well as the increase of the associated treatment costs.

Somatic cell count (SCC) is the most used indicator of udder health status in cows, but its reliability for subclinical mastitis detection in goats is controversial. Therefore, milk SCC threshold value established for cows is not suitable for goats (1).

Koop et al. (2) showed that high SCC in goat milk is not always associated with a positive bacterial culture. However, various studies reported that increased SCC in goats is mainly a response to infection, thus prevention of IMI can contribute to control SCC in milk (3–5). Rupp et al. (6) provided further evidence that SCC is related to subclinical mastitis in goats, as they observed a positive association between somatic cell score (SCS) and bacterial counts in milk. Moreover, goats with repeated bacteriologically negative udders had the lowest SCS. The degree of the inflammatory reaction may also depend on the microorganisms involved. Rupp et al. (7) detected significant differences between udder halves infected by major pathogens (such as *Staphylococcus aureus*, *Enterobacteriaceae*, *Streptococcus* spp., and *Mycoplasma* spp.), and those infected by minor pathogens [such as non-aureus staphylococci (NAS), *Corynebacterium* spp., and *Micrococcus* spp.], which presented lower SCC. In addition, caprine arthritis-encephalitis virus (CAEV) infection was suggested as a possible cause of increased SCC as well (8, 9).

According to Plummer and Plummer (10), in some animals, milk-secreting tissue may be present in the wall of the teat and, in some instances, milk can filter through tiny pores in the skin to the surface of the udder or the teat, in absence of any invisible orifice. This condition is known as "weeping teat" (WT) and is characterized by the presence of milk cysts in goats, in which the accumulated milk may come out to the outside (11). Weeping teat animals can be easily identified by the presence of milk on the outer surface of the udder, especially right before milking. Seykora and McDaniel (12), hypothesized that this condition may contribute to the developing of bacterial infections, as milk, passing through the pores of the skin onto the external surface of the udder, would facilitate the entry of bacteria into the udder itself; therefore, it can be predicted that this porous tissue might be prone to developing bacterial infections and mastitis. Nevertheless, no data on health effects associated to this condition are available. Differently, other two outcomes can occur: milk may communicate with the teat cistern without visual evidence of the presence of this tissue or it may accumulate forming subcutaneous cysts if the secretory tissue does not have an opening.

Currently, very limited data are available on either the frequency of WT or its economic impact in goat farms. In Italy, genetic evaluations for type traits of dairy goats started in 2000, providing information on possible defects with potential functional impact, including the presence/absence of WT. The WT condition is reported to be especially associated with goat breeds selected for high milk production (10, 13). In Italy, mammary gland abnormality has been reported in around 4 and 13% of primiparous Saanen and Alpine females kidding from 2009 to 2014, respectively (14), with an observed incidence, respectively, of 3.6 and 7.5% for primiparous Saanen and Alpine goats. However, this proportion could be underestimated because of voluntary culling or inaccurate evaluation of WT. Biffani et al. (14) observed genetic correlation between WT and milk production or SCS, but the standard error of the estimates was very large. In particular, primiparous Alpine goats showed a loss of 0.046 kg/day milk in comparison with normal does, while SCS increased 0.26–0.21 in pluriparous or primiparous WT animals, respectively (13).

Since information on the role played by WT on the occurrence of intramammary infections is almost unknown, the present case-control study aimed at investigating the possible association between WT, the increase of SCC, and the presence of bacteria in milk of Alpine goats reared in Italy.

## MATERIALS AND METHODS

### Herds and Sampling

Four Alpine goat farms located in Lombardy region (Northern Italy) and registered in genealogical herd books of Associazione Nazionale della Pastorizia (ASSONAPA, Rome, Italy) were selected based on the phenotypic presence of WT. Alpine goats were chosen as they have a higher frequency of WT compared to the Saanen breed. Herd size ranged from 39 to 116 lactating goats (mean $\pm$ *S.E.*: 65.3 $\pm$ 17.3). The prevalence of WT in the four herds was 13.6, 14.1, 7.2, and 9.9%. Goats were housed indoor on permanent straw litter, with occasional access to outdoor pasture, and were milked twice/day. All the goats in their second, third or fourth lactation presenting WTs were included as case groups (WT+); the same number of goats, matched with WT+ for age and parity, was recruited as control group (WT–). We decided to exclude parities higher than the fourth one, as older goats show usually more intramammary infections than younger ones. Three WT-goats were culled during the trial period, and therefore were excluded from final analysis. Our follow-up study was performed in 2018, and then repeated in a single herd (herd A) during the following year, to further investigate it.

Half udder milk samples were collected from goats in their second, third, or fourth lactation at three timepoints, at the beginning, in the middle, and at the end of lactation. Samples were taken before milking with an aseptic procedure, by disinfecting the teat with wipes containing chlorhexidine, discarding a few streams of milk from the teat (foremilk), and collecting 10 ml of milk into sterile tubes. After collection, samples were immediately placed on ice and then transported chilled at +4°C to the laboratory.

### Bacteriological Analysis and Somatic Cell Counting

Bacteriological analysis was performed with standard techniques on the day of sampling. In detail, for each sample, 10 μl of milk was plated onto blood agar supplemented with 5% defibrinated bovine blood using a sterile inoculating loop. Plates were then incubated at 37°C and analyzed after 24 and 48 h. Colonies grown

**TABLE 1 |** Results of bacteriological analysis and SCC determination on half-udder milk samples from 49 Alpine goats (23 cases and 26 controls) collected in 2018 at three timepoints (at the beginning, in the middle and at the end of lactation).

| | | | | First sampling | | | | Second sampling | | | | Third sampling | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Left | | Right | | Left | | Right | | Left | | Right | |
| Herd | Animal ID | No. of lactations | Group | SCC* | Bacteria | SCC* | Bacteria | SCC* | Bacteria | SCC* | Bacteria | SCC* | Bacteria | SCC* | Bacteria |
| A | 1 | 2 | Case | 14 | / | 39 | / | 306 | / | 157 | *S.* sp. | 22 | *S. chromogenes* | 144 | Polymicrobic |
| A | 2 | 2 | Case | 82 | *S. caprae* | 469 | / | 252 | *S. caprae* | 250 | *S. caprae* | 73 | / | 124 | / |
| A | 3 | 2 | Case | 288 | *S. caprae* | 240 | *S. caprae* | 267 | *S. caprae* | 254 | *S. caprae* | 31 | / | 49 | *S. haemolyticus* |
| A | 4 | 2 | Case | 13 | / | 14 | / | 85 | / | 578 | / | 178 | *S. chromogenes* + *S. warneri* | 361 | / |
| A | 5 | 2 | Case | 384 | *S.* sp. | 622 | *S. caprae* | 751 | *S.* sp. | 388 | Polymicrobic | 256 | *S. capitis* | 261 | / |
| A | 6 | 3 | Case | 519 | *S. caprae* | 171 | / | 149 | / | 418 | / | 508 | / | 578 | *S. chromogenes* |
| A | 7 | 2 | Case | 776 | / | 53 | Polymicrobic | 8,211 | / | 1,793 | / | 255 | / | 670 | / |
| A | 8 | 4 | Case | 1,223 | / | 91 | / | 781 | / | 73 | / | 1,301 | / | 936 | / |
| A | 9 | 2 | Control | 43 | / | 52 | / | 791 | / | 554 | / | 597 | / | 182 | / |
| A | 10 | 2 | Control | 1,166 | *S.* sp. | 364 | *E. faecalis* | 289 | / | *128* | / | 297 | / | 343 | / |
| A | 11 | 2 | Control | 33 | / | 27 | / | 2,577 | / | 153 | / | 57 | Polymicrobic | 61 | / |
| A | 12 | 2 | Control | 43 | / | 11 | / | 86 | / | 13 | / | 21 | / | 16 | *S. chromogenes* |
| A | 13 | 2 | Control | 1 | / | 8 | / | 11 | / | 7 | / | 4 | / | 1 | / |
| A | 14 | 2 | Control | 103 | / | 142 | / | 270 | / | 370 | / | 75 | / | 19 | / |
| A | 15 | 4 | Control | 79 | / | 384 | *S. caprae* | 1,684 | / | 3,065 | / | 408 | *S. chromogenes* | 295 | *S. capitis* |
| A | 16 | 4 | Control | n.d. | n.d. | n.d. | n.d. | 181 | *S. caprae* | 93 | Polymicrobic | 343 | / | 368 | / |
| A | 17 | 3 | Control | 168 | / | 173 | / | 1,044 | / | 1,276 | / | 472 | / | 351 | / |
| B | 18 | 4 | Case | 25 | / | 191 | / | 4 | / | 237 | / | 1,428 | / | 2,845 | / |
| B | 19 | 4 | Case | 164 | *S.* sp. | 370 | / | 2,988 | / | Blind teat | | 1,602 | / | Blind teat | |
| B | 20 | 4 | Case | 71 | / | 50 | / | 3,185 | / | 635 | / | 4,811 | / | 1,724 | / |
| B | 21 | 4 | Control | 31 | / | 45 | / | 3,787 | / | 406 | / | 4,859 | / | 2,898 | / |
| B | 22 | 4 | Control | 175 | / | 181 | *S.* sp. | 748 | / | 367 | *S.* sp. | 882 | / | 582 | / |
| B | 23 | 4 | Control | 2,475 | *S. chromogenes* | 28 | / | 795 | / | 171 | / | 224 | / | 354 | / |
| C | 24 | 2 | Case | 28 | / | 24 | / | 47 | / | 106 | / | 159 | / | 2,271 | / |
| C | 25 | 2 | Case | 648 | *S.* sp. | 115 | / | 170 | *S. capitis* | 1,479 | / | 733 | / | 953 | / |
| C | 26 | 2 | Case | 797 | / | 3,564 | *S. aureus* | 714 | / | 534 | *S. aureus* | 511 | *S. capitis* | 1,954 | *S. aureus* |
| C | 27 | 3 | Control | 8 | / | 8 | / | 323 | *S. capitis* | 1 | / | 473 | *S. capitis* | 72 | / |
| C | 28 | 2 | Control | 116 | / | 80 | / | 643 | / | 43 | / | 2,405 | | 352 | / |
| C | 29 | 2 | Control | 380 | *S.* sp. | 277 | *S.* sp. | 315 | / | 272 | / | 665 | *S. capitis* | 292 | / |
| C | 30 | 2 | Control | 59 | / | 54 | / | 1 | / | 1 | / | 35 | *S. warneri* | 1,363 | / |

*(Continued)*

**TABLE 1 |** Continued

| | | | | First sampling | | | | Second sampling | | | | Third sampling | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Left | | Right | | Left | | Right | | Left | | Right | |
| **Herd** | **Animal ID** | **No. of lactations** | **Group** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** |
| C | 31 | 3 | Control | 486 | *S.* sp. | 365 | / | 1,718 | *S. aureus* | 127 | / | | Culled | | Culled |
| D | 32 | 4 | Case | 74 | / | 159 | *S. epidermidis* | 813 | / | 5,244 | / | 231 | / | 451 | *S. epidermidis* |
| D | 33 | 4 | Case | 1 | / | 1 | / | 145 | / | 163 | / | 567 | / | 125 | / |
| D | 34 | 2 | Case | 1 | / | 283 | *S. chromogenes* | 170 | / | 375 | / | 7 | / | 4 | *S. chromogenes* |
| D | 35 | 2 | Case | 1 | / | 4 | / | 152 | / | 122 | / | 10 | / | 12 | / |
| D | 36 | 2 | Case | 40 | / | 7 | / | 113 | / | 111 | / | 22 | / | 10 | *S. chromogenes* |
| D | 37 | 3 | Case | 81 | *S. epidermidis* | 20 | / | 280 | *S. epidermidis* | 167 | / | 557 | *S. epidermidis* | 32 | *S. epidermidis* |
| D | 38 | 3 | Case | 51 | / | 267 | / | 551 | / | 704 | / | 16 | / | 27 | / |
| D | 39 | 3 | Case | 1 | / | 1 | / | 11 | / | 11 | / | 3 | / | 6 | / |
| D | 40 | 4 | Case | 192 | / | 237 | / | 1,781 | / | 967 | / | 614 | / | 573 | *S. chromogenes* |
| D | 41 | 3 | Control | 5 | / | 3 | / | 82 | / | 73 | / | 136 | / | 186 | / |
| D | 42 | 3 | Control | 166 | / | 152 | / | 19 | / | 15 | / | 11 | / | 8 | / |
| D | 43 | 4 | Control | 1 | / | 1 | / | 38 | / | 27 | / | 583 | / | 134 | / |
| D | 44 | 2 | Control | 2 | / | 1 | / | 1,723 | / | 414 | *S. epidermidis* | 433 | *S. capitis* | 65 | *S. chromogenes* |
| D | 45 | 4 | Control | 1 | / | 2 | / | 118 | / | 151 | / | 606 | / | 752 | / |
| D | 46 | 4 | Control | 116 | *S. caprae* | 259 | / | 51 | / | 66 | / | 221 | / | 308 | / |
| D | 47 | 2 | Control | 185 | / | 66 | / | 41 | / | 46 | / | 272 | *S. epidermidis* | 98 | / |
| D | 48 | 2 | Control | 95 | / | 101 | / | 104 | / | 197 | / | | Dried | | Dried |
| D | 49 | 3 | Control | 1,010 | *S. chromogenes* | 239 | / | 995 | / | 212 | / | 4,009 | *S. chromogenes* | 249 | / |

**Expressed as cells/μl; n.d., no data available; S. sp., Staphylococcus specie; /, bacteriologically negative.*

on agar plates were isolated and identified following National Mastitis Council guidelines (15), then confirmed by API system. A sample was defined as polymicrobic when more than two distinct colony types were present. The presence of *Mycoplasma* spp. was not investigated, because contagious agalactia of goats is a notifiable disease and no case was officially reported since years. Somatic cell count was determined as well, using a Somacount$^{TM}$ 150 (Bentley Instruments, Minnesota, USA). Cell counts were expressed as cells/μl.

## Statistical Analyses

A general linear mixed model was used to investigate the effect of WT phenotypes and SCC on the observed microbiology outcome (MO).

The general model (Model 1) was:

$$Model\ 1: MO = wt + sampling + parity + SCS + animal + herd + error$$

where MO was the dependent variable considered as a binomial trait (0 = no infection , 1 = at least one infected teat); wt (two classes) is the presence/absence of a WT phenotype; sampling (three classes) is the milk sampling at the beginning, the middle, and the end of lactation; parity (three classes) is the parity class; SCS is the mean of the SCC of the two teats transformed to SCS as

$$SCS = Log_2\left(\frac{SCC}{100}\right) + 3$$

according to Shook (16); animal is the random permanent environmental effect; herd (4 classes) is the herd where data were collected.

Model 1 was fitted to complete data. Successively, a data subset (A) was created including only records from herd A. The same model, hereinafter called model 2, was fitted after excluding the herd effect. Lastly, dataset A was additionally subset in two datasets, namely B and C. Dataset B included records collected in year 2018, while dataset C included records collected in 2019. Model 2 was fitted to both dataset B and C.

The general linear mixed model was fitted using the function *glmer* of the package "lme4" implemented in the R environment[1] for statistical programming. Odds ratio have been calculated as exponential of the results of the respective linear general mix model in R statistical environment. All graphical representations were produced using R[1].

To further corroborate our results, we performed bootstrap resampling to calculate the 95% Confidence Intervals (C.I.) of the estimates of the effects included in the models (17). All bootstrap analyses were performed with the R libraries boot (18, 19), based on 5,000 bootstrap replicates. Further libraries were used, tidyverse, knitr, tidyr, and broom.

# RESULTS

In 2018, a total of 286 half-udder milk samples were collected from 49 Alpine goats (23 cases and 26 controls). The results of bacteriological analysis and SCC determination are shown in **Table 1**. Overall, most of isolates were NAS (91.7%), the most prevalent being *Staphylococcus chromogenes* (23.6%), *Staphylococcus caprae* (21.8%), unidentified NAS (*Staphylococcus* sp., 20%), and *Staphylococcus epidermidis* (14.5%). Non-aureus staphylococci species varied from herd to herd. In particular, *S. caprae* was isolated mainly in herd A, whereas *S. epidermidis* just in herd D. *S. chromogenes* was equally distributed in three herds, being absent in herd C. *S. aureus* was isolated only in herd C, in two animals. One of them was infected at all the sampling points (goat n. 26), while the other one (n. 31) was positive for *S. aureus* at two supplemental samplings carried out by the farmer and was then culled.

Overall SCS mean value in 2018 was 3.8 ± 2.12. Regarding the presence/absence of WT, the SCS-value was 4.1 or 3.5, respectively. When the udder half and the microbiological outcome was considered, the mean value of SCS was 4.6 or 4.3 in the bacteriologically positive left or right half udders, respectively, decreasing to 3.6 or 3.2 in the negative halves. The presence of bacteria in milk was significantly associated with SCS ($P = 0.037$), but neither with WT udder defect, nor to parity or sampling time (**Table 2**). Considering herd A only, an almost significant effect of WT udder defect on the response to bacterial culture was observed, with a mean SCS-value of 4.4 ± 1.7 in WT udders (median value 4.5) and 3.5 ± 2.3 in normal glands (median value 3.9; $P = 0.053$; **Figure 1**, **Table 3**). The box plot of SCS in cases and controls in herd A is shown in **Figure 1**.

Therefore, herd A was further investigated on the following year (2019). A total of 109 half-udder milk samples were collected from 19 goats (9 cases and 10 controls), including the surviving goats sampled in 2018, plus three new animals. The results are shown in **Table 4**.

Most of the isolates were NAS (90.5%), identified almost exclusively as *S. caprae* (78.9%). No contagious microorganism was detected.

In 2019, the presence of bacteria in milk was no more associated with the WT udder defect, whereas the effect on SCS became statistically significant ($P = 0.008$; **Table 5**). The box plot of SCS and bacterial infections in herd A in 2019 is shown in **Figure 2**.

Pooling together data collected in 2018 and 2019 in Herd A, no significant effect of SCS nor WT udder on intramammary bacterial infections was observed (**Table 6**).

**TABLE 2 |** Results of the linear general mix model on four herds sampled in 2018 on fixed effects in Model 1.

| Effect | Odds ratio | C.I. LL | C.I. UL | *P*-value |
|---|---|---|---|---|
| Weeping teat | 2.809 | 0.934 | 8.439 | 0.066 |
| No. of sampling | 0.789 | 0.459 | 1.357 | 0.392 |
| No. of lactation | 1.068 | 0.526 | 2.170 | 0.853 |
| Somatic cell score | 1.284 | 1.014 | 1.624 | 0.037* |

*Odds ratio, 95% lower confidence intervals (C.I. LL), 95% upper confidence intervals (C.I. UL), and p-values are reported.*
*$P \leq 0.05$.

[1]www.r-project.org

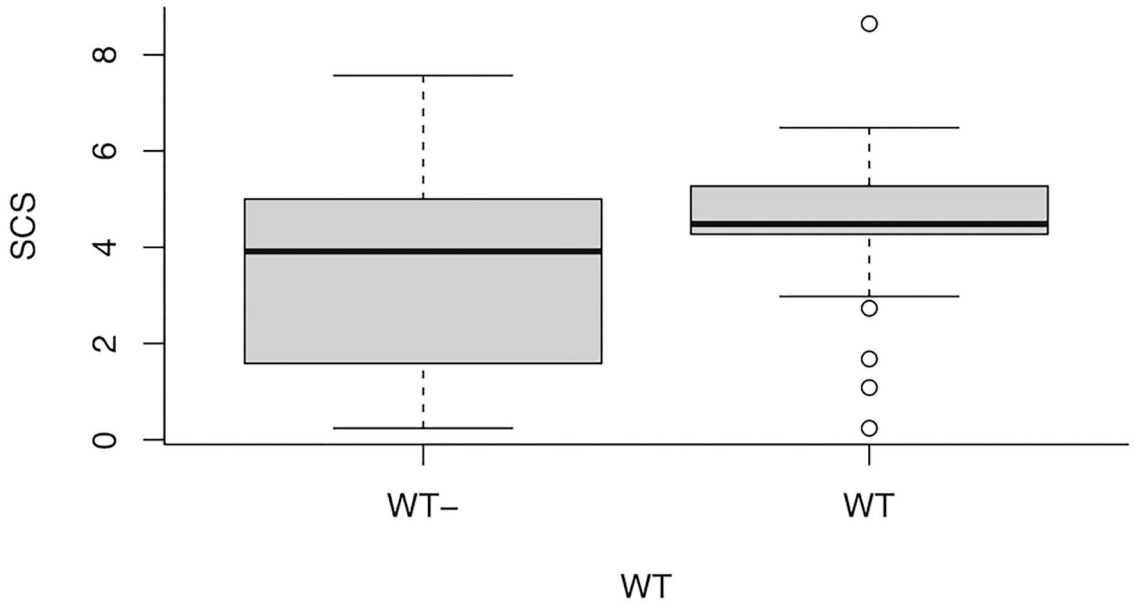


**FIGURE 1 |** Box plot of somatic cell score (SCS) in goats with (WT+) and without (WT–) weeping teats in herd A in 2018.

**TABLE 3 |** Results of the linear general mix model in herd A sampled in 2018 on fixed effects in Model 2.

| Effect | Odds ratio | C.I. LL | C.I. UL | *P*-value |
|---|---|---|---|---|
| Weeping teat | 9.765 | 0.970 | 98.313 | 0.053 |
| No. of sampling | 1.223 | 0.488 | 3.963 | 0.660 |
| No. of lactation | 0.889 | 0.199 | 3.962 | 0.870 |
| Somatic cell score | 1.099 | 0.669 | 1.805 | 0.711 |

*Odds ratio, 95% lower confidence intervals (C.I. LL), 95% upper confidence intervals (C.I. UL), and p-values are reported.*

The results of the bootstrap C.I. estimates confirmed all previous significant findings. In detail, significant association for SCS levels and MO was confirmed in the analysis of the four Herds sampled in 2018. Furthermore, association between MO and WT was confirmed in herd A in 2018 and between MO and SCS in 2019. All non-significant effects in all models were further confirmed (**Supplementary Tables 1–4**).

## DISCUSSION

The importance of dairy goats has significantly increased during last decades, providing an alternative to dairy cow products for human consumption (20). A healthy mammary gland is essential for dairy farms and directly correlated with milk yield and quality.

Various pathological conditions of the udder in goats have been described, including supernumerary and abnormal teats, gynecomastia, precocious udders, and fibrocystic disease (11, 21). Among udder defects, information about the WT is extremely limited, but raises concern about its possible role in the development of mastitis. Yet, both its etiology and consequences are not fully understood. In this respect, our study provides new evidence suggesting that WT may be usually unrelated to both the outcome of milk bacterial culture and SCS. However, in one herd out of four we found a positive association of WT defect with positive bacterial culture, although this was not further confirmed in the following year.

In order to define the udder health status, SCC is the most used indicator in cows, but its ability to predict subclinical mastitis in goats has been questioned. Indeed, average SCC values in goats are higher than those in cattle and sheep, since they are influenced by several physiological and environmental factors, such as parity, stage of lactation, season, and milk yield (22–24). Our results highlighted significantly higher SCS in goat udders presenting bacterial infections, independently of parity, season, or managerial factors. This result was in accordance with different studies reporting increased SCC in goats in response to infection (2–5).

Various bacteria can be implicated in goat subclinical mastitis. The most frequently isolated bacteria in the four herds were NAS, mostly *S. chromogenes* and *S. caprae*, followed by unidentified Staphylococci and *S. epidermidis*. Accordingly, in the literature NAS are the most prevalent bacteria isolated from udder halves in goats and appear to behave as minor and opportunistic pathogens (2, 4, 25). Also Koop et al. (2) reported that NAS species have a high prevalence in goat mastitis, and cause persistent infections.

**TABLE 4 |** Results of bacteriological analysis and SCC determination on half-udder milk samples from 19 Alpine goats (9 cases and 10 controls) collected in herd A in 2019 at three timepoints (at the beginning, in the middle, and at the end of lactation).

| | | | | First sampling | | | | Second sampling | | | | Third sampling | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Left | | Right | | Left | | Right | | Left | | Right | |
| **Herd** | **Animal ID** | **N. of lactations** | **Group** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** | **SCC*** | **Bacteria** |
| A | 1 | 3 | Case | 30 | / | 49 | / | 32 | / | 393 | / | 197 | / | 123 | / |
| A | 2 | 3 | Case | 5,089 | / | 1,402 | / | 575 | Polymicrobic | 720 | Polymicrobic | 674 | *S. caprae* | 477 | *S. caprae* |
| A | 3 | 3 | Case | 163 | *S. caprae* | 197 | *S. caprae* | 1,171 | *S. caprae* | 1,272 | *S. caprae* | 563 | / | 507 | / |
| A | 4 | 3 | Case | 5 | / | 1,314 | *Str*. sp. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| A | 5 | 3 | Case | 19 | / | 5 | / | 428 | / | 949 | / | 608 | / | 631 | / |
| A | 6 | 4 | Case | 225 | / | 309 | Polymicrobic | 468 | / | 624 | *S. epidermidis* | 186 | / | 287 | / |
| A | 7 | 3 | Case | 283 | / | 63 | / | 593 | / | 820 | / | 202 | Polymicrobic | 309 | / |
| A | 8 | 5 | Case | 4,123 | / | 84 | / | 353 | / | 156 | / | 215 | / | 542 | / |
| A | New1 | 6 | Case | 39 | *S. caprae* | 293 | *S.* sp. | 194 | / | 546 | / | 640 | / | 1,042 | / |
| A | 9 | 3 | Control | 42 | / | 28 | / | 273 | / | 522 | / | 136 | / | 155 | / |
| A | 10 | 3 | Control | 209 | *S. caprae* | blind teat | | 402 | *S. caprae* | 373 | / | 878 | *S. caprae* | 135 | / |
| A | 12 | 3 | Control | 142 | / | 72 | *S. chromogenes* | 72 | / | 98 | / | 197 | / | 72 | / |
| A | 13 | 3 | Control | 2 | / | 4 | / | 12 | / | 24 | / | 1 | / | 1 | / |
| A | 14 | 3 | Control | 36 | / | 79 | / | 238 | / | 343 | / | 185 | / | 219 | / |
| A | 15 | 5 | Control | 326 | / | 439 | *S. caprae* | 1,790 | / | 847 | *S. caprae* | 841 | *S. caprae* | 797 | / |
| A | 16 | 5 | Control | 21 | / | 35 | Polymicrobic | 53 | / | 57 | / | 1 | / | 5 | / |
| A | 17 | 4 | Control | 28 | / | 26 | / | 635 | / | 580 | / | 45 | *S.* sp. | 3,050 | *S. lentus* |
| A | New2 | 4 | Control | 2,887 | / | 65 | / | 1,017 | *S. caprae* | 1 | / | 712 | *S. caprae* | 48 | / |
| A | New3 | 6 | Control | 114 | / | 61 | / | 303 | / | 283 | / | 109 | / | 206 | / |

*The sampled goats include the surviving goats sampled in 2018, plus three new animals.*

**Expressed as cells/μl; n.d., no data available; S. sp., Staphylococcus specie; Str. sp., Streptococcus specie; /, bacteriologically negative sample.*

Among NAS, *S. caprae* was one of the most frequently isolated Staphylococci, followed by *S. epidermidis*, *Staphylococcus xylosus*, *S. chromogenes,* and *Staphylococcus simulans*. Analogously, Rupp et al. (6), reported NAS as the prevalent agents of goat mastitis with a decreasing frequency of isolation from *S. xylosus*, to *S. caprae* and *S. epidermidis*.

## CONCLUSIONS

In conclusion, our results cannot confirm the hypothesis that WT udder condition facilitates the entry of bacteria into the udder and that WT goats are more likely to develop localized bacterial infections. However, we cannot exclude that the WT defect could represent a risk for the health of the udder of dairy goats, when associated with particular conditions. Indeed, in our follow-up study only a single herd showed a significant effect of WT on intramammary infection just in the first year, that could not be confirmed in the following year. Additionally, our results showed that the presence of bacteria in milk is positively related with the increase in SCS, despite the physiological increase during lactation. It is necessary to extend the research to a larger number of farms in order to investigate the reasons for this variability and understand if and when the presence of WT could represent a risk for the health of goat's udder.

## INTERPRETATIVE SUMMARY

Mastitis is the most common disease affecting dairy goats and causing economic losses. Although it is accepted that

**TABLE 5 |** Results of the linear general mix model on herd A sampled in 2019 on fixed effects in Model 2.

| Effect | Odds ratio | C.I. LL | C.I. UL | *P*-value |
|---|---|---|---|---|
| Weeping teat | 0.336 | 0.081 | 1.393 | 0.148 |
| No. of sampling | 0.798 | 0.338 | 1.886 | 0.608 |
| No. of lactation | 1.161 | 0.527 | 2.558 | 0.709 |
| Somatic cell score | 1.847 | 1.176 | 2.900 | 0.008** |

*Odds ratio, 95% lower confidence intervals (C.I. LL), 95% upper confidence intervals (C.I. UL), and p-values are reported.*
***P ≤ 0.01.*

**TABLE 6 |** Results of the linear general mix model on herd A sampled in 2018 and 2019 on fixed effects in Model 2.

| Effect | Odds ratio | C.I. LL | C.I. UL | *P*-value |
|---|---|---|---|---|
| Weeping teat | 2.198 | 0.412 | 11.722 | 0.366 |
| No. of sampling | 1.032 | 0.554 | 1.924 | 0.919 |
| No. of lactation | 0.678 | 0.231 | 1.991 | 0.480 |
| Somatic cell score | 1.307 | 0.938 | 1.821 | 0.113 |
| Year | 1.133 | 0.127 | 10.054 | 0.911 |

*Odds ratio, 95% lower confidence intervals (C.I. LL), 95% upper confidence intervals (C.I. UL), and p-values are reported.*

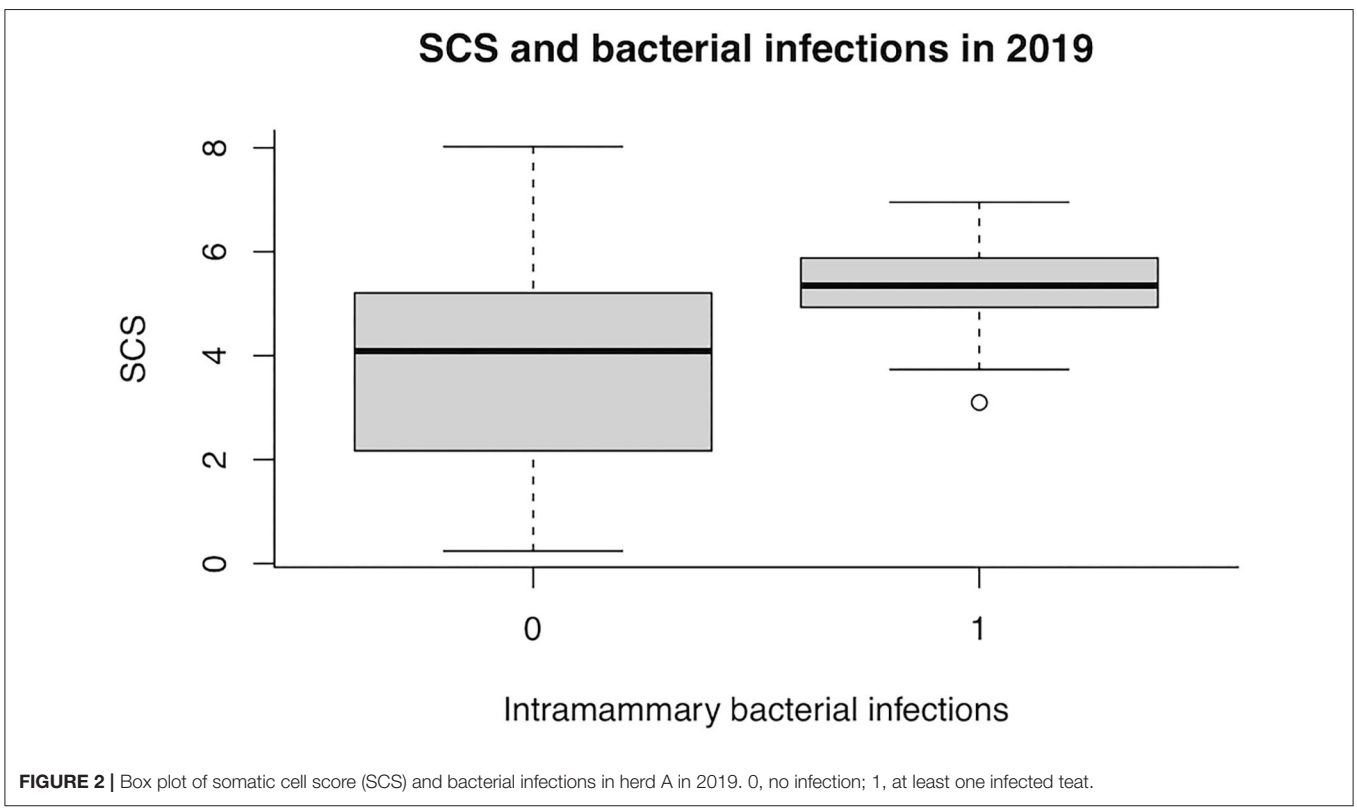


**FIGURE 2 |** Box plot of somatic cell score (SCS) and bacterial infections in herd A in 2019. 0, no infection; 1, at least one infected teat.

increased SCSs is mainly a response to infection, its reliability for subclinical mastitis detection in goats is controversial, since it is influenced by many physiological and extrinsic variables, including breed, parity, age, stage of lactation, seasonal variations, and milking methods.

In some animals, milk-secreting tissue is present in the wall of the teat and, in some instances, milk can filter through pores in the skin to the surface of the udder. This condition is known as "weeping teat," and it is hypothesized that the mammary gland might be prone to develop bacterial infections, although very few information is provided. Our results cannot exclude that the WT defect could represent a risk for udder health of dairy goats, when associated with particular conditions. Indeed, in our follow-up study only a single herd showed a significant effect of WT on intramammary infection, and this was not confirmed by further investigations. As a side outcome, our results showed that the presence of bacteria in milk is positively related with the increase in SCS, despite the physiological increase during lactation.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

Ethical review and approval was not required for the animal study because Our study could not be classified as a clinical one and we did not apply interventions outside of routine care, since we just collected milk during routine milking.

## AUTHOR CONTRIBUTIONS

GM, SB, and RP: design of study and experiments. AG, GB, and SM: laboratory and field activities. AG, GM, SM, and RP: analysis of results, data interpretation, and manuscript drafting. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.622063/full#supplementary-material

## REFERENCES


1. Persson Y, Olofsson I. Direct and indirect measurement of somatic cell count as indicator of intramammary infection in dairy goats. *Acta Vet Scand.* (2011). 53:15. doi: 10.1186/1751-0147-53-15
2. Koop G, De Vliegher S, De Visscher A, Supré K, Haesebrouck F, Nielen M, et al. Differences between coagulase-negative Staphylococcus species in persistence and in effect on somatic cell count and milk yield in dairy goats. *J Dairy Sci*. (2012) 95:5075–84. doi: 10.3168/jds.2012-5615
3. Poutrel B, de Crémoux R, Ducelliez M, Verneau D. Control of intramammary infections in goats: impact on somatic cell counts. *J Anim Sci.* (1997) 75:566–70. doi: 10.2527/1997.752566x
4. Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X. Mastitis of dairy small ruminants. *Vet Res.* (2003) 34:689–716. doi: 10.1051/vetres:2003030
5. Moroni P, Pisoni G, Ruffo G, Boettcher PJ. Risk factors for intramammary infections and relationship with somatic-cell counts in Italian dairy goats. *Prev Vet Med.* (2005) 69:163–73. doi: 10.1016/j.prevetmed.2004.10.013
6. Rupp R, Huau C, Caillat H, Fassier T, Bouvier F, Pampouille E, et al. Divergent selection on milk somatic cell count in goats improves udder health and milk quality with no effect on nematode resistance. *J Dairy Sci.* (2019) 102:5242–53. doi: 10.3168/jds.2018-15664
7. Rupp R, Clément V, Piacere A., Robert-Granié C, Manfredi, E. Genetic parameters for milk somatic cell score and relationship with production and udder type traits in dairy Alpine and Saanen primiparous goats. *J Dairy Sci*. (2011) 94:3629–34. doi: 10.3168/jds.2010-3694
8. Nord K, Adnøy T. Effects of infection by caprine arthritis-encephalitis virus on milk production of goats. *J Dairy Sci.* (1997) 80:2391–7. doi: 10.3168/jds.S0022-0302(97)76190-3
9. Sánchez A, Contreras A, Corrales JJ, Marco JC. Relationships between infection with caprine arthritis encephalitis virus, intramammary bacterial infection and somatic cell counts in dairy goats. *Vet Rec.* (2001) 148:711–4. doi: 10.1136/vr.148.23.711
10. Plummer PJ, Plummer C. Diseases of the mammary gland. In: Pugh DG, Baird N, editors. *Sheep and Goat Medicine*. Saunders: Elsevier (2012). pp. 442–465.
11. Matthews J, editor. Diseases of the mammary gland. In: *Diseases of the goat. 4th Ed.* Chichester: John Wiley and Sons, Ltd (2016). pp. 198–201.
12. Seykora AJ, McDaniel BT. Udder and teat morphology related to mastitis resistance: a review. *J Dairy Sci.* (1985) 68:2087–93. doi: 10.3168/jds.S0022-0302(85)81072-9
13. Biffani S, Minozzi G, Piccinini R, Castiglioni B, Grande S, Fresi P, et al. Effect of weeping udder on the level of somatic cell counts and production traits in Italian Alpine and Saanen dairy goats. In: *International Bovine Mastitis Conference In Proc. National Mastitis Council.* Milan (2018). doi: 10.13140/RG.2.2.19023.38569
14. Biffani S, Tiezzi F, Fresi P, Stella A, Minozzi G. Genetic parameters of weeping teats in Italian Saanen and Alpine dairy goats and their relationship with milk production and somatic cell score. *J Dairy Sci.* (2020) 103:9167–76. doi: 10.3168/jds.2020-18175
15. Hogan JS, Gonzales RN, Harmon RJ, Nickerson SC, Oliver SP, Pankey JW, et al. *Laboratory Handbook on Bovine Mastitis, revised ed*. Madison WI: National Mastitis Council Inc. (1999). P. 222.
16. Shook GE. Genetic improvement of mastitis through selection on somatic cell count. *Vet Clin North Am Food Anim Pract.* (1993) 9:563–81. doi: 10.1016/s0749-0720(15)30622-8
17. Efron B, Tibshirani R. *An Introduction to the Bootstrap*. New York, NY: Chapman and Hall, Inc. (1993).
18. Canty A, Ripley BD. *Boot: Bootstrap R (S-Plus) Functions. R package version 1.3-25.* (2020). Available online at: http://CRAN.R-project.org/package=boot (accessed December 27, 2020).
19. Davison AC, Hinkley DV. *Bootstrap Methods and Their Applications*. Cambridge: Cambridge University Press (1997). Available online at: http://statwww.epfl.ch/davison/BMA/ (accessed January 11, 2021).
20. Lérias JR, Hernández-Castellano LE, Suárez-Trujillo A, Castro N, Pourlis A, Almeida AM. The mammary gland in small ruminants: major morphological and functional events underlying milk production–a review. *J Dairy Res.* (2014) 81:304–18. doi: 10.1017/S0022029914000235
21. Yeruham I, Sharir B, Friedman S, Perl S. Cystic dilation of the teat sinuses in doe goats. *Vet Rec.* (2005) 156:844. doi: 10.1136/vr.156.26.844
22. Paape MJ, Poutrel B, Contreras A, Marco JC, Capuco AV. Milk somatic cells and lactation in small ruminants. *J Dairy Sci.* (2001). 84(Suppl.):E237–44. doi: 10.3168/jds.S0022-0302(01)70223-8

23. Jimenez-Granado R, Sanchez-Rodriguez M, Arce C, Rodriguez-Estevez V. Factors affecting somatic cell count in dairy goats: a review. *Span J Agric Res.* (2014) 12:133–50. doi: 10.5424/sjar/2014121-3803
24. Tedde V, Bronzo V, Puggioni GMG, Pollera C, Casula A, Curone G, et al. Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation. *J Dairy Res.* (2019) 86:217–21. doi: 10.1017/S0022029919000335
25. Dore S, Liciardi M, Amatiste S, Bergagna S, Bolzoni G, Caligiuri V, et al. Survey on small ruminant bacterial mastitis in Italy, 2013–2014. *Small Rumin Res.* (2016) 141:91–3. doi: 10.1016/j.smallrumres.2016.07.010

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Clinical Mastitis Incidence in Dairy Cows Housed on Recycled Manure Solids Bedding: A Canadian Cohort Study

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Bedding can affect mammary health of dairy cows. The objectives of this study were to evaluate clinical mastitis incidence in cows housed on recycled manure solids bedding and, more specifically, to determine which pathogens were involved. We followed 26 recycled manure solids farms and 60 straw-bedded farms as a comparative group during 1 year (2018–2019). For each episode of clinical mastitis, defined as a visual alteration of the milk, with or without local or systemic signs of infection, producers sampled aseptically the affected quarter, provided some details about the animal, and sent the sample to the research team at the Université de Montréal. We received and analyzed 1,144 milk samples. The samples were cultured according to the National Mastitis Council guidelines and the different colony phenotypes were subsequently identified with mass spectrometry. In 54.6% of CM cases, a single phenotype of bacteria was cultured (pure culture), while two different phenotypes were found in 16.8% of the samples (mixed culture), and no growth was observed in 14.4% of the samples. Samples with three or more phenotypes were considered contaminated and were not included in the pathogen-specific analyses (14.3% of the submitted samples). The most frequently identified bacterial species in pure and mixed culture in farms using recycled manure solids were *Streptococcus uberis* (16.0%), *Escherichia coli* (13.8%), *Klebsiella pneumoniae* (13.2%), *Streptococcus dysgalactiae* (6.2%), and *Staphylococcus aureus* (3.4%). In straw farms, the most frequent species were *S. aureus* (16.6%), *S. uberis* (11.0%), *E. coli* (9.1%), *S. dysgalactiae* (8.0%), and *K. pneumoniae* (1.1%). The incidence of clinical mastitis (all cases together) was not higher in recycled manure solids farms (14.0 cases/100 cow-year; 95% CI: 8.3–23.7) compared with straw-bedded farms (16.3 cases/100 cow-year; 95% CI: 9.0–29.6). However, *K. pneumoniae* clinical mastitis episodes were 7.0 (95% CI: 2.0–24.6) times more frequent in recycled manure solids farms than in straw farms. Adjusted least square means estimates were 1.6 *K. pneumoniae* clinical

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mastitis cases/100 cow-year (95% CI: 0.8–3.4) in recycled manure solids farms vs. 0.2 cases/100 cow-year (95% CI: 0.1–0.6) in straw-bedded farms. *Klebsiella pneumoniae* clinical mastitis is in general severe. Producers interested in this bedding alternative need to be aware of this risk.

**Keywords: recycled manure solids, dairy cows, bedding, housing, clinical mastitis**

## INTRODUCTION

Dairy producers are interested in alternative bedding products that may be less expensive to buy or produce, easily available in large quantities, and secure for animals and humans. There is a growing interest in Eastern Canada to use recycled manure solids (RMS) as bedding. This product is already used in many countries such as the United States, United Kingdom, and the Netherlands. However, there is no consensus on the best technique to produce RMS to ensure its safety. The method used to produce RMS and the different climates where it is used will influence its physicochemical and, possibly, its microbiological characteristics (1, 2). Recycled manure solids is an organic bedding with a high moisture content and, therefore, represents a favorable environment for bacterial growth. Indeed, previous studies demonstrated the high bacterial content of this product (3, 4) and its potential to sustain bacterial growth (5).

There are few studies on the association between RMS usage and animal health. It was demonstrated that *Cryptosporidium* parasites were found more frequently from the feces of cows in RMS farms compared with cows housed on straw bedding (6). In the same project, the presence of *Listeria monocytogenes* and *Salmonella* spp. was more frequently detected in RMS bedding samples, showing that the processing methods used to produce RMS were not efficient to eliminate these zoonotic pathogens[1]. There are also some concerns about the survival of *Mycobacterium avium* ssp. *paratuberculosis* throughout the RMS maturation process (7).

Associations between the use of RMS bedding and the risk of clinical mastitis (CM) were evaluated in two studies that yielded conflicting results. In an experimental study in one dairy facility of 309 primiparous Holstein cows, associations between type of bedding (RMS or sand) and CM incidence could not be highlighted (8). In a second study, 1,600 cows housed together in one farm were followed using an observational study design, and in this study, the use of RMS was associated with a greater risk of CM with an odds ratio of 2.1, compared with cows housed on sand (9).

There are anecdotal reports from veterinarians and producers about an increased incidence of CM and particularly CM caused by *Klebsiella* spp. on farms using RMS bedding. There are two main hypotheses explaining this potential increase in CM incidence. First, this bedding may contain an increased amount of *Klebsiella* spp. before usage compared with more conventional bedding. However, this hypothesis was refuted in two studies which observed that unused RMS, on average, contained less *Klebsiella* spp. than straw (4). Another possible explanation is that this CM may be due to the ability of *Klebsiella* spp. to multiply in this type of bedding during its use in the stalls. Indeed, the ability of RMS to support the growth of *Klebsiella pneumoniae* and *Enterococcus faecium* was investigated and shown to be superior to those of sand and wood products (5).

An increased incidence of *Klebsiella* spp. CM on farms using RMS was not yet confirmed in the scientific literature. Moreover, only one study have described CM incidence in a large number of farms using RMS bedding (10). Finally, pathogen-specific CM incidence was never reported on RMS farms. The objectives of this study were, therefore, to describe the total incidence of CM in dairy cows housed on recycled manure solids bedding and CM incidence by the main bacterial species and to compare these to herds using a more conventional type of bedding, straw. The results presented in this paper are part of a larger study on RMS farms, and results about milk quality, parasite survival, and bedding bacteriological analyses can be found elsewhere (6, 11).

## MATERIALS AND METHODS

### Ethical Statement

This project was approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine (University de Montréal; protocol 17-Rech-1886). This paper was elaborated using the "STROBE-Vet statement" guidelines (12).

### Herd Recruitment

A list of farms using RMS was generated by contacting RMS equipment dealers and veterinarians and through social media. Straw farms were recruited with the help of Québec Dairy Herd Improvement Association (DHIA, Lactanet, Ste-Anne de Bellevue, QC, Canada). For both type of herds, to be eligible, farmers needed to be located within 250 km of the research facilities (St-Hyacinthe, QC), to have used the same bedding for >6 months prior to the farm visit, and for the straw farms, to be enrolled in a DHIA milk recording program. This latter condition was added for another part of the study on subclinical mastitis. We aimed at recruiting ∼90 farms. This number was determined by an *a priori* power estimation. We estimated that, using 90 herds (20%: RMS bedding vs. 80%: straw bedding), milking an average of 50 cows, and a baseline probability of clinical mastitis of 20%, we would have >95% power to detect a difference of probability of mastitis corresponding to an odds ratio ≥1.4. For this calculation, we did not account for clustering of cows by herd. Thus, the real power is likely to be smaller than 95%.

All potential farms were contacted by telephone between July and December 2017 to verify their eligibility and willingness to participate. Basic demographic information such as the number

[1]Beauchemin J, Fréchette A, Thériault W, Dufour S, Fravalo P, Thibodeau A. Comparison of microbiota of recycled manure solids and straw bedding used in dairy farms in Eastern Canada. *J Dairy Sci*. (2021).

of milking cows in the herd, type of bedding used, and for RMS farms, which equipment they used were also gathered from herds that were excluded, to study their similarities with participants and assess the presence of a selection bias.

## Sample Collection and Bacteriological Analyses

Farm visits were described elsewhere (6, 11). Briefly, farms were visited once and producers had to answer a standardized questionnaire about their bedding management. Methods used to produce bedding were recorded and bedding samples as well as bulk tank milk samples were collected. Herd size was recorded as the number of lactating cows. We also recorded housing type (free or tie stall), time since the last renovations of the stalls (in years), and bedding thickness defined as shallow bedding ($<$10 cm of depth) or deep bedding ($\geq$10 cm of depth). These latter covariables were pre-identified using directed acyclic graph as putative confounders of the association between bedding type and CM incidence.

During 1 year following the initial visit, producers were asked to sample aseptically each quarter of cows experiencing a CM. Farmers had to provide information regarding the identification of the cow, its parity, the position of the quarter affected, and the severity of the CM. For the latter, farmers had to categorize CM events as score 1 (abnormal milk only), score 2 (abnormal milk and udder, without systemic signs), or score 3 (systemic signs of illness such as fever, depression, and anorexia) as described by Sears and McCarthy (13). Two consecutives cases of CM in the same quarter of a cow were considered distinct if they were $\geq$8 days apart (14). Samples were sent on ice to the Faculty of Veterinary Medicine Laboratory (St-Hyacinthe, QC, Canada). The bacteriological analyses were realized following the National Mastitis Council guidelines (15). Briefly, 0.01 ml of milk was plated on blood agar and incubated for 24–48 h at 35°C. The sample was then classified as negative (no growth), pure intramammary infection (IMI) (one single phenotype of CFU), mixed IMI (two types of CFU), or contaminated ($\geq$3 types of CFU). An IMI was defined as the isolation of $\geq$100 CFU/ml of a given phenotype. Pure and mixed IMI bacterial isolates where then identified by mass spectrometry (MALDI-TOF) using the database of the manufacturer (BDAL-8468) and a custom database validated specifically for staphylococci identification (16). Isolates needed to be identified to the species level (vs. genus level solely) to be retained for pathogen-specific statistical analyses.

## Statistical Analyses

The number of CM episodes was compiled for each farm, as well as the number of severe (score 3) CM episodes. Finally, we compiled the CM episodes by specific pathogens. To account for the varying herd size and the exact time period of follow-up, the number of milking cows in each herd and the length of the follow-up period were also compiled.

Most CM studies have to deal with different levels of the compliance of producers for reporting CM cases and/or submitting samples. To investigate this potential bias, we used two different approaches to estimate the herd animal-time denominator used to adjust CM incidence. First, we used a common approach which is the exact number of milking cows and the exact period of follow-up (17) to compute the number of animal-year at risk of the herd.

Then, as a sensitivity analysis, we also estimated the follow-up period using the interval between the first and last sampling dates as the definition. Thus, with this alternative method, farms who did not send any samples or that sent only one sample during the 1 year study period were excluded (i.e., they would contribute 0 animal-year at risk). Moreover, farms that may have sent $>$one sample but then stopped sending samples at some point in time would be included, but with a shorter time at risk period (i.e., only the time between the first and last sent samples would be compiled). Then, we computed the number of cow-year at risk of the herd for each farm by multiplying the number of milking cows of the herd by the follow-up time. Using this alternative method allowed to exclude producers who sent $<$2 samples during the study period and weighted down producers who possibly stopped sending samples during the study.

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics were used to explore relations between predictors. To compare the incidence of CM in RMS and straw farms, we used a binomial negative model with the number of CM cases on a given farm as the outcome (firstly total number of CM cases, then severe CM cases only, then CM cases by bacterial species), type of bedding used (RMS or straw) as the main predictor, and the natural logarithm of the number of cow-year at risk as an offset term. In this model, we also included a number of putative confounding variables as predictors: housing type (free or tie stall), time since the last renovations of the stalls (in years), bedding thickness (deep or shallow bedding), and herd size (number of milking cows). With such a model, we could thus compute the CM incidence ratio (IR) between RMS- and straw-bedded farms, after adjusting for these confounders, simply by exponentiating the bedding coefficient. Moreover, the mean estimated CM incidence (in cases/100 cow-year) for a given type of herd could be computed simply by adding the intercept and the coefficients corresponding to that farm description, then exponentiating the results and, finally, multiplying the results by 100 cows (to obtain an incidence per 100 cow-year). Finally, all models were ran twice, initially using the complete follow-up period to compute the animal-time at risk of the herd and, then, using the animal-time at risk computed using the alternative method.

The assumption of linearity of the relation between quantitative predictors (time since the last renovation of the stalls and herd size) and the outcome (logarithmic transformation of the incidence ratio) was verified with the addition of polynomial terms (square and cubic terms) after centering the predictor. If the polynomial terms were significant ($p < 0.05$), the polynomial presentation of the variable was retained in the final model. If overdispersion was observed in the data (Pearson chi-square $>$ 1.2), robust variance was used. Significance level was fixed at $p \leq 0.05$. Data and the SAS code used to construct the models are publicly available at https://doi.org/10.5683/SP2/KIEMHY.

# RESULTS

## Herds Description

We obtained a list of 49 RMS and 139 straw farms and recruited 27 and 61 RMS and straw farms, respectively. Reasons for exclusion of RMS farms were as follows: four had recently changed their bedding type to non-RMS bedding, 11 were outside the defined geographic location, one did not use RMS under the milking cows, and six could not be reached despite several attempts. From the 139 straw farms interested in the project, 61 were selected on their ability to provide computerized health records.

We visited the farms between January 15th and July 10th, 2018. The farms recruited have been described in Lasprilla-Mantilla et al. (6) and Gagnon et al. (11). Briefly, recruited RMS farms had 55–900 lactating cows (median 111) and straw farms had 43–229 lactating cows (median 65). An automatic milking system was in used in 37% of RMS farms and in 3% of straw farms. Furthermore, 59% of RMS farms and 98% of straw farms participated regularly in a DHI program. Even though participating in a DHI program was an inclusion criterion in straw herds, one farm did not record any data (i.e., dropped out of DHI) during the follow-up period. In the 27 RMS farms, 26 used a separation process as first step for producing RMS bedding. One RMS farm used an anaerobic digester as the first step and a separation process as the second and last step. From the 26 farms who did a separation first, one used the solid fraction immediately after separation, two used a rotative drum to turn into compost the solid fraction, 10 allowed the solid fraction to mature in a heap, and 13 allowed the solid fraction to mature in an enclosed container. During the monitoring year, two farms (one RMS and one straw) burned and five others (one RMS and four straw) dropped out of the project. Two of these farms (one RMS and one straw) dropped off very early and without sending any milk samples. They were, therefore, excluded from the analyses. For the three other farms, data collected until they left the project were used in the analyses. When we used the alternative follow-up period (from the first to the last sampling dates) to compute the animal-time at risk, 28% of RMS farms and 35% of straw-bedded farms had a follow-up time of 6 months or less. The characteristics of the general herds are reported in **Table 1**.

**TABLE 1 |** Description of the 26 recycled manure solids farms and 60 straw farms.

| | RMS bedding Median (range) | Straw bedding Median (range) |
|---|---|---|
| Follow-up period in years | 1.0 (0.4–1.0) | 1.0 (0.2–1.0) |
| Number of milking cows | 111 (55–900) | 65 (43–229) |
| Number of years since the last renovations of the stalls | 3.0 (0.1–23.0) | 10.0 (0.0–70.0) |
| Proportion of freestall | 70.4[a] | 3.3[a] |
| Proportion of deep bedding (≥10 cm) | 38.5[a] | 0.0[a] |

[a] *Proportion (in %).*

## Milk Samples

We received 1,247 samples during the study period (**Figure 1**). We excluded 11 samples because they were collected on the same mammary gland quarter <8 days since the last CM episode. From the 1,236 remaining samples, there were no information about CM severity in 69 (5.6%) of the samples, 92 (7.4%) were submitted as score 0 (no clinical mastitis), 492 (39.8%) as score 1, 426 (34.5%) as score 2, and 157 (12.7%) as score 3. We excluded the 92 samples for which the producers explicitly reported a severity score of 0. Samples with no reported severity were, however, retained. We observed a pure IMI in 624 (54.5%) of the 1,144 remaining samples, a mixed IMI in 192 (16.8%), and no growth in 165 (14.4%). Of the collected samples, 163 (14.2%) were considered contaminated. The proportion of contaminated samples was not associated with bedding type (chi-square test; $p = 0.89$).

The most frequent recovered pathogens (in pure or mixed IMI) by bedding type and severity are reported in **Table 2**. Briefly, in RMS farms, the most frequent pathogens were *Streptococcus uberis* (16.0%), *Escherichia coli* (13.8%), *K. pneumoniae* (13.2%), *Streptococcus dysgalactiae* (6.2%), and *Staphylococcus aureus* (3.4%). In straw-bedded farms, *S. aureus* (16.6%), *S. uberis* (11.0%), *E. coli* (9.1%), *S. dysgalactiae* (8.0%), and *K. pneumoniae* (1.1%) were the most frequent pathogens. Clinical mastitis episodes due to coliforms (*K. pneumoniae* and *E. coli*) were more often severe. Clinical mastitis episodes due to *S. uberis*, *S. dysgalactiae*, or *S. aureus* were, in general, mainly mild or moderate (**Table 2**).

## Effect of Bedding on CM Incidence

Clinical mastitis (all cases) incidence distribution estimated using a period at risk extending from start to end of the study is illustrated in **Figure 2**. Unconditional least square means (LSM) estimates (i.e., not adjusted for putative confounding variables) are presented as **Supplementary Table 1**. After adjusting for potential confounders, there was no statistical difference in the general CM incidence between the two farm types. A LSM estimate of 14.0 cases/100 cow-year (95% CI: 8.3, 23.7) was obtained for RMS farms, whereas a LSM of 16.3 cases/100 cow-year (95% CI: 9.0, 29.6) was obtained for straw farms (**Table 3**). Moreover, there was no difference in the incidence of severe CM episodes (severity score 3) between the two types of farm. A LSM of 2.1 severe cases/100 cow-year (95% CI: 1.1, 4.1) was obtained for RMS farms, and straw farms had a LSM of 1.6 severe cases/100 cow-year (95% CI: 0.8, 3.4).

The estimated incidence of *K. pneumoniae* CM, however, was higher in RMS farms with a LSM of 1.6 cases/100 cow-year (95% CI: 0.8, 3.4) compared with 0.2 cases/100 cow-year (95% CI: 0.1, 0.6) for straw farms. For this comparison, a 7.0 times (95% CI: 2.0, 24.6) higher incidence was observed in RMS farms. There was no significant difference between the two types of farms regarding the incidence of CM due to *S. uberis*, *E. coli*, *S. dysgalactiae*, or *S. aureus*.

## Sensitivity Analysis

When using the alternative approach for computing period at risk, we ended up excluding 8 RMS farms and 11

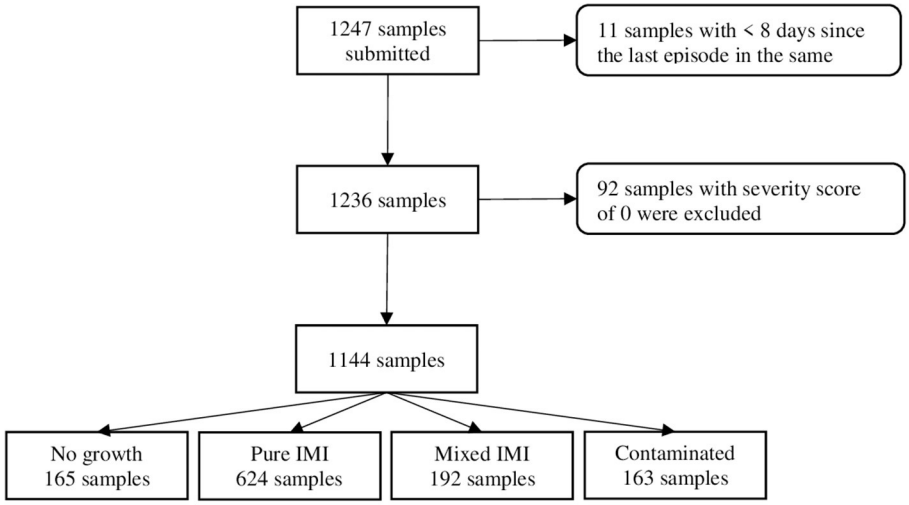


**FIGURE 1 |** Flow chart representing clinical mastitis samples submitted and retained in a study comparing clinical mastitis incidence in 26 farms using recycled manure solids bedding and 60 farms using straw bedding.

**TABLE 2 |** Percentage (number) of clinical mastitis cases by bedding type, bacterial species, and severity among 1,144 clinical mastitis cases obtained on 26 farms using recycled manure solid (RMS) bedding and 60 farms using straw bedding.

| Category | RMS bedding | | | | | Straw bedding | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total[a] | Severity[b] | | | | Total[a] | Severity[b] | | | |
| | | Unknown | Mild | Moderate | Severe | | Unknown | Mild | Moderate | Severe |
| All cases | 100 (356) | 13.2 (47) | 28.7 (102) | 34.3 (122) | 23.9 (85) | 100 (788) | 2.8 (22) | 49.5 (390) | 38.6 (304) | 9.1 (72) |
| *Streptococcus uberis* | 16.0 (57) | 7.0 (4) | 28.0 (16) | 59.6 (34) | 5.3 (3) | 11.0 (87) | 1.1 (1) | 37.9 (33) | 57.5 (50) | 3.4 (3) |
| *Escherichia coli* | 13.8 (49) | 8.2 (4) | 10.2 (5) | 32.7 (16) | 49 (24) | 9.1 (72) | 1.4 (1) | 12.5 (9) | 37.5 (27) | 48.6 (35) |
| *Klebsiella pneumoniae* | 13.2 (47) | 6.4 (3) | 8.5 (4) | 31.9 (15) | 53.2 (25) | 1.1 (9) | 0 (0) | 33.0 (3) | 33.0 (3) | 33.0 (3) |
| *Streptococcus dysgalactiae* | 6.2 (22) | 18.2 (4) | 40.9 (9) | 22.7 (5) | 18.2 (4) | 8.0 (63) | 0.0 (0) | 34.9 (22) | 55.6 (35) | 9.5 (6) |
| *Staphylococcus aureus* | 3.4 (12) | 8.3 (1) | 33.3 (4) | 25 (3) | 33.3 (4) | 16.6 (131) | 3.8 (5) | 55.7 (73) | 37.4 (49) | 3.1 (4) |
| Other pathogen | 19.4 (69) | 23.2 (16) | 34.8 (24) | 26.1 (18) | 15.9 (11) | 28.6 (225) | 2.7 (6) | 57.8 (130) | 35.1 (79) | 4.4 (10) |
| No growth | 16.9 (60) | 6.7 (4) | 48.3 (29) | 30 (18) | 15.0 (9) | 13.3 (105) | 1.9 (2) | 61.0 (64) | 28.6 (30) | 8.6 (9) |

[a] *Proportion of clinical mastitis cases in % (absolute number of cases) within a type of farm where a given bacterial species was found. Note that the sum within a column may add to more than 100% since some samples may yield more than one bacterial species.*

[b] *Distribution of severity of clinical mastitis cases in % (absolute number of cases) within a type of farm and for a given bacterial species. Severity was scored as described before (13) as follows: unknown, mild (1; abnormal milk only), moderate (2; abnormal quarter), or severe (3; abnormal cow).*

straw-bedded farms that sent <2 samples during the study period (**Table 4**). Using this alternative approach, the general estimated CM incidence was different between the two groups (**Supplementary Table 2**) with a LSM estimate of 26.5 cases/100 cow-year (95% CI: 19.2, 36.6) for RMS farms and a LSM of 46.2 cases/100 cow-year (95% CI: 30.2, 70.8) for straw-bedded farms. Furthermore, the total incidence of severe CM was not different between the two farms groups with a LSM estimate of 10.2 cases/100 cow-year (95% CI: 6.9, 15.0) in RMS farms and a LSM of 13.6 cases/100 cow-year (95% CI: 7.1, 26.1) in straw-bedded farms.

As in our first approach, the estimated incidence of CM due to *K. pneumoniae* was significantly higher in RMS farms with a LSM of 3.4 cases/100 cow-year (95% CI: 1.6, 7.1) compared with 0.6 cases/100 cow-year (95% CI: 0.2, 1.6) in straw farms. This was equivalent to a 5.9 times (95% CI: 1.6, 21.2) higher incidence in RMS farms. There was still no difference between the two groups concerning the incidence of CM due to *S. uberis*, *E. coli*, *S. dysgalactiae*, or *S. aureus*.

## DISCUSSION

To our knowledge, this is the first study to report pathogen-specific CM incidence on RMS farms. In our study, the proportion of contaminated samples was similar to a previously published work (18) and was not associated with the bedding type. Using the approach most often used in mastitis research to compute total CM incidence (i.e., considering the complete time period of follow-up), we did not observe a statistically

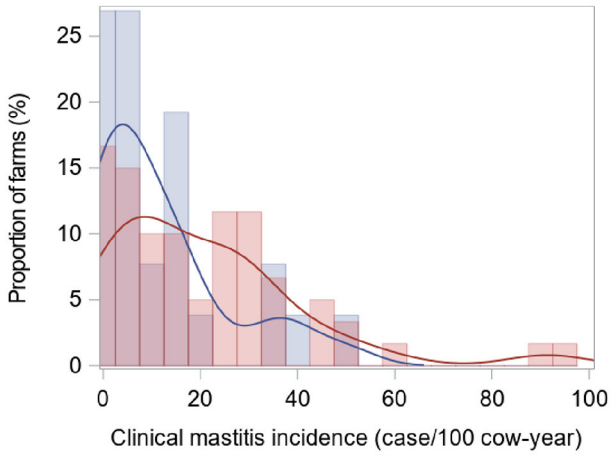


**FIGURE 2 |** Clinical mastitis incidence distribution estimated using a period at risk extending from start to end of the study in a study comparing 26 farms using recycled manure solids bedding (blue) and 60 farms using straw bedding (red).

**TABLE 3 |** Least square means clinical mastitis incidence estimates (in cases/100 cow-year) and incidence ratio (IR) between 26 RMS farms and 60 straw-bedded farms and computed using a binomial negative model.

| Clinical mastitis category | Estimated incidence in cases/100 cow-year (95% CI) | | IR (95% CI) |
|---|---|---|---|
| | RMS farms | Straw farms | |
| All clinical mastitis | 14.0 (8.3–23.7) | 16.3 (9.0, 29.6) | 0.9 (0.4–1.9) |
| Severe clinical mastitis | 4.0 (2.5–6.3) | 2.7 (1.6–4.6) | 1.5 (0.7–3.1) |
| By bacterial species involved | | | |
| *Klebsiella pneumoniae* | 1.6 (0.8–3.4)[a] | 0.2 (0.1–0.6)[b] | 7.0 (2.0–24.6) |
| *Streptococcus dysgalactiae* | 0.6 (0.2–1.2) | 0.8 (0.3–1.8) | 0.7 (0.3–1.9) |
| *Escherichia coli* | 0.5 (0.2–1.2) | 0.7 (0.3–1.5) | 0.8 (0.3–2.0) |
| *Streptococcus uberis* | 0.4 (0.1–1.5) | 0.8 (0.3–2.5) | 0.5 (0.1–2.3) |
| *Staphylococcus aureus* | 0.4 (0.1–1.2) | 0.9 (0.3–3.0) | 0.4 (0.1–1.4) |

*A period at risk extending from start to end of the study was used to compute the animal-time denominator. Models were adjusted for confounding by housing type, time since the last renovation of the stalls, bedding thickness, and herd size. Means within a row with different superscripts are statistically different.*

**TABLE 4 |** Variation of the number of cows-year at risk in the two groups (26 RMS farms and 60 straw farms) when using different follow-up periods.

| | | RMS bedding Median (range) | Straw bedding Median (range) |
|---|---|---|---|
| Cow-year at risk | Method 1 | 109 (55–900) | 64 (10–229) |
| | Method 2 | 63 (0–230) | 45 (0–109) |

*Method 1 corresponds to a follow-up period of 1 year, and method 2 is a follow-up period calculated as the interval between the first and last sampling dates.*

significant association between the use of RMS as bedding and the incidence of CM. This finding is in agreement with those of an experimental study realized at the University of Wisconsin-Madison (8). In both studies, quarter milk was sampled and analyzed for each CM event, rather than just relying on the farm records for analysis of incidence. In the Rowbotham and Ruegg (8) study, environmental *Streptococcus*, *E. coli*, and *Klebsiella* spp. were identified in 50% of CM culture-positive samples. In that latter study, however, pathogen-specific CM incidence was not reported.

In our study, we were able to investigate the most common CM pathogens. Prior to conducting this study, veterinarians and producers in our province anecdotally reported *K. pneumoniae* CM outbreaks in RMS farms. This hypothetical higher incidence of *K. pneumoniae* CM in RMS farms was confirmed in the current study. One hypothesis to explain these mastitis episodes is that, right from the start, RMS bedding may contain a higher concentration of *K. pneumoniae* than straw. In a parallel study conducted on the same herds during the same period, we observed that unused RMS contained lower concentrations of *Klebsiella* spp. than unused straw. However, at the end of the usage cycle (prior to removal from the stall), the concentrations of *Klebsiella* spp. were similar between the two bedding types. Another hypothesis is that the growth rate of *Klebsiella* spp. would be higher in RMS bedding than in other bedding types. Thus, despite lower bacterial concentrations to start with, the rapid growth of *Klebsiella* spp. in this bedding type after contamination with feces would quickly lead to increased concentrations of *Klebsiella* spp. A previous study demonstrated the high potential of RMS for supporting the growth of *Klebsiella* spp. (5). Still, Beauchemin et al. also reported similar concentrations of *Klebsiella* spp. in samples using RMS and straw bedding. Thus, RMS did not seem to lead to a riskier environment (based solely on bacterial concentration) for *Klebsiella* spp. CM, even at the end of the usage cycle. Some other properties of this bedding, for instance its ability to stick to the teats, may better explain the higher *K. pneumoniae* CM incidence. This latter hypothesis, however, was not investigated in the current study.

Clinical mastitis episodes due to *K. pneumoniae* are usually severe (19). In our study, CM episodes due to *K. pneumoniae* were moderate or severe in 66% of cases in straw-bedded farms and in 85% of cases in RMS farms. In one study, they estimated a loss of 700 kg of milk in a multiparous cow experiencing clinical mastitis due to *Klebsiella* spp. at 30 days in milk (20). In comparison, a CM due to *E. coli* was causing a loss of 354 kilos. Moreover, cows experiencing *Klebsiella* spp. CM had a 22.3 times greater risk of culling than healthy cows (21).

Nevertheless, in our study, there was no difference in the total incidence of severe CM episodes between the two types of bedding. Since *K. pneumoniae* is just one of the multiple bacterial species that can cause severe CM, this result is not surprising. For instance, *E. coli* is another pathogen that was responsible for severe mastitis and *E. coli* CM incidence was similar between the two types of beddings.

When we used the alternative follow-up period (from the first to the last sampling dates) to compute the animal-time at risk, there were significantly more cases of CM in straw farms. Since herd size varied as function of bedding type, the impact

of this more restrictive follow-up period affected differentially the incidence denominator for straw- and RMS-bedded herds. Consequently, since our results on the total CM incidence are affected by the method used to compute them, we can hardly conclude on whether the general CM incidence varied between the two groups of farms. The most commonly used approach in CM research (i.e., considering herds with few CM cases reported as herds with a low CM incidence) would conclude on similar CM incidence between bedding types. The more conservative approach (where herds reporting few CM cases or reporting for a limited period of time would be considered non-compliant and excluded) would conclude to a larger general CM incidence in straw-bedded farms. Nevertheless, regardless of the method used, the general CM incidence was never higher in RMS herds. Moreover, our results on species-specific CM incidence appeared to be robust and consistent between both computation methods.

A strength of our study was the number of participating farms and the number of cows recruited. To our knowledge, this is the largest number of herds and cows assembled to study the effect of RMS bedding on CM incidence. This is also the first time, to our knowledge, that pathogens responsible of CM were identified. We can now confirm that there are some differences in the pathogen patterns causing CM according to the bedding type. However, since this is an observational study, our study presents some limitations.

First, the sampling strategy was not random and some regions were overrepresented due to our proximity criteria. However, to our knowledge, most of the farms using RMS bedding in these regions and during this period were recruited. The bias may be more important regarding recruitment of farms using straw bedding, since we selected only herds enrolled in DHIA for that group. This criterion was not used for RMS farms. Producers enrolled in DHIA may be more concerned about udder health of their cows than the general population of dairy farmers, possibly generating a bias when measuring the association between bedding used and CM incidence. Nevertheless, a good proportion of RMS farms were also enrolled in DHIA, thus limiting the magnitude of this potential bias.

Second, the exposition to each type of bedding was not randomly assigned and many confounding factors were possibly operating within these farms. We were able to include in our models some potentially important confounding factors. Thus, our incidence estimates were adjusted for some of the other differences that we observed between RMS- and straw-bedded farms. Nevertheless, some residual confounding is likely to be present. Our results would have to be confirmed using an experimental study design where cows from one or many farms would be randomly assigned to different bedding types while monitoring pathogen-specific CM incidence.

Finally, a well-known challenge in studies on CM is the relatively low compliance of farmers for recording CM episodes, which may represent an information bias. We hypothesize that this lack of reporting was similar in the two groups of farms. In our study, to improve the reporting of CM episodes, we covered the costs for all the milk analyses conducted during the year of follow-up, provided timely results (i.e., <2 days) to the herd veterinarian, and called all participants every 4 months to keep them engaged and motivated. Moreover, using the alternative method for computing time of follow-up allowed for the exclusion of some herds that were possibly low-compliance herds.

In the future, experimental studies could help in confirming the results observed in this observational study. For instance, a randomized controlled trial or a crossover study design conducted in one or a few large herds and over a sufficiently long period of time (since *Klebsiella* spp. CM is an uncommon health event) would be of great value to confirm these initial findings.

## CONCLUSION

The general incidence of CM and of severe CM was not higher in RMS- compared with straw-bedded herds. However, the distribution of bacterial species causing the CM cases was different. The incidence ratio of CM due to *K. pneumoniae* was seven times greater in RMS farms than in straw farms. These mastitis cases are usually very severe. Producers interested to adopt this type of bedding must be aware of this risk.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://doi.org/10.5683/SP2/KIEMHY.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the Faculty of Veterinary Medicine, University de Montréal. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## SUPPLEMENTARY MATERIAL

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## REFERENCES

1. Jorgensen K, Jensen LS. Chemical and biochemical variation in animal manure solids separated using different commercial separation technologies. *Bioresour Technol.* (2009) 100:3088–96. doi: 10.1016/j.biortech.2009.01.065
2. Husfeldt AW, Endres MI, Salfer JA, Janni KA. Management and characteristics of recycled manure solids used for bedding in Midwest freestall dairy herds. *J Dairy Sci.* (2012) 95:2195–203. doi: 10.3168/jds.2011-5105
3. Patel K, Godden SM, Royster E, Crooker BA, Timmerman J, Fox L. Relationships among bedding materials, bedding bacteria counts, udder hygiene, milk quality, and udder health in US dairy herds. *J Dairy Sci.* (2019) 102:10213–34. doi: 10.3168/jds.2019-16692
4. Robles I, Kelton DF, Barkema HW, Keefe GP, Roy JP, von Keyserlingk MAG, et al. Bacterial concentrations in bedding and their association with dairy cow hygiene and milk quality. *Animal.* (2020) 14:1052–66. doi: 10.1017/S1751731119002787
5. Godden S, Bey R, Lorch K, Farnsworth R, Rapnicki P. Ability of organic and inorganic bedding materials to promote growth of environmental bacteria. *J Dairy Sci.* (2008) 91:151–9. doi: 10.3168/jds.2007-0415
6. Lasprilla-Mantilla MI, Wagner V, Pena J, Frechette A, Thivierge K, Dufour S, et al. Effects of recycled manure solids bedding on the spread of gastrointestinal parasites in the environment of dairies and milk. *J Dairy Sci.* (2019) 102:11308–16. doi: 10.3168/jds.2019-16866
7. Leach KA, Archer SC, Breen JE, Green MJ, Ohnstad IC, Tuer S, et al. Recycling manure as cow bedding: potential benefits and risks for UK dairy farms. *Vet J.* (2015) 206:123–30. doi: 10.1016/j.tvjl.2015.08.013
8. Rowbotham RF, Ruegg PL. Associations of selected bedding types with incidence rates of subclinical and clinical mastitis in primiparous Holstein dairy cows. *J Dairy Sci.* (2016) 99:4707–17. doi: 10.3168/jds.2015-10675
9. Harrison E, Bonhotal J, Schwarz M. *Using Manure Solids as Bedding*. Ithaca, NY: Cornell Waste Management Institute (2008).
10. Husfeldt AW, Endres MI. Association between stall surface and some animal welfare measurements in freestall dairy herds using recycled manure solids for bedding. *J Dairy Sci.* (2012) 95:5626–34. doi: 10.3168/jds.2011-5075
11. Gagnon M, Hamelin L, Frechette A, Dufour S, Roy D. Effect of recycled manure solids as bedding on bulk tank milk and implications for cheese microbiological quality. *J Dairy Sci.* (2020) 103:128–40. doi: 10.3168/jds.2019-16812
12. Sargeant JM, O'Connor AM, Dohoo IR, Erb HN, Cevallos M, Egger M, et al. Methods and processes of developing the strengthening the reporting of observational studies in epidemiology-veterinary (STROBE-Vet) statement. *J Food Prot.* (2016) 79:2211–9. doi: 10.4315/0362-028X.JFP-16-016
13. Sears PM, McCarthy KK. Diagnosis of mastitis for therapy decisions. *Vet Clin North America Food Anim Pract.* (2003) 19:93–108. doi: 10.1016/S0749-0720(02)00074-9
14. Jamali H, Barkema HW, Jacques M, Lavallee-Bourget EM, Malouin F, Saini V, et al. Invited review: incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. *J Dairy Sci.* (2018) 101:4729–46. doi: 10.3168/jds.2017-13730
15. NMC. *Laboratory Handbook on Bovine Mastitis*. 3rd ed. New Prague: NMC (2017).
16. Cameron M, Perry J, Middleton JR, Chaffer M, Lewis J, Keefe GP. Short communication: evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. *J Dairy Sci.* (2018) 101:590–5. doi: 10.3168/jds.2017-13226
17. Olde Riekerink RG, Barkema HW, Kelton DF, Scholl DT. Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci.* (2008) 91:1366–77. doi: 10.3168/jds.2007-0757
18. Makovec JA, Ruegg PL. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J Dairy Sci.* (2003) 86:3466–72. doi: 10.3168/jds.S0022-0302(03)73951-4
19. Schukken Y, Chuff M, Moroni P, Gurjar A, Santisteban C, Welcome F, et al. The "other" gram-negative bacteria in mastitis: Klebsiella, serratia, and more. *Vet Clin North Am Food Anim Pract.* (2012) 28:239–56. doi: 10.1016/j.cvfa.2012.04.001
20. Grohn YT, Wilson DJ, Gonzalez RN, Hertl JA, Schulte H, Bennett G, et al. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *J Dairy Sci.* (2004) 87:3358–74. doi: 10.3168/jds.S0022-0302(04)73472-4
21. Grohn YT, Gonzalez RN, Wilson DJ, Hertl JA, Bennett G, Schulte H, et al. Effect of pathogen-specific clinical mastitis on herd life in two New York State dairy herds. *Prev Vet Med.* (2005) 71:105–25. doi: 10.1016/j.prevetmed.2005.06.002

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# Prevalence of Mastitis Pathogens and Antimicrobial Susceptibility of Isolates From Cattle and Buffaloes in Northwest of Pakistan

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Mastitis is the most prevalent disease of dairy animals, imparting huge economic losses to the dairy industry. There is always a dire need to monitor the prevalence of mastitis, its bacteriology, and evaluation of antimicrobial susceptibilities for mastitis control and prevention. Therefore, the objectives of this study were to investigate: (i) the prevalence of mastitis in cattle and buffaloes; (ii) identification of bacteria associated with mastitis; (iii) antimicrobial susceptibility of bacterial isolates. Milk samples ($n$ = 1,566) from cattle ($n$ = 1,096) and buffaloes ($n$ = 470) were processed for detection of mastitis using the California mastitis test in the year 2018–19. A total of 633 mastitic milk samples were further processed for bacteriology and antimicrobial susceptibility testing by the disc diffusion method. Overall, the prevalence of clinical and subclinical mastitis was 17 and 57% in both species. Clinical mastitis was higher in cattle (20%) compared to buffaloes (11%), whereas subclinical was higher in buffaloes (66%) than cattle (53%). Besides, month-wise prevalence was higher in hot and humid months in both species. *Staphylococci* spp. (34%) were the most predominant bacterial isolates from mastitic milk, followed by *Escherichia coli* (19.4%), *Streptococci* spp. (9%), and *Klebsiella* spp. (8%). Most of the bacteria were susceptible to gentamicin (92%) and enrofloxacin (88%), when a panel of 16 different antimicrobials was tested. Nevertheless, most of the isolates were resistant to sulphamethoxazole (99%), lincomycin (98%), oxytetracycline (89%), ampicillin (86%), and doxycycline (85%). This study concludes a high prevalence of mastitis caused by *Staphylococcal* spp. in cattle and buffaloes belonging to the northwest of Pakistan, and gentamicin and enrofloxacin might be appropriate antimicrobial agents in the treatment of bovine mastitis.

**Keywords: bovine mastitis, bacteriology, antimicrobial susceptibility, cattle, buffaloes**

# INTRODUCTION

Mastitis (inflammation of the mammary gland) is one of the most prevalent diseases of dairy animals (1). It is the most costly and devastating disease to the dairy industry because it imparts vast economic losses, compromising the health and welfare of animals, and due to its adverse effects on the quality and quantity of milk (1). Cattle and buffaloes (*Bubalus bubalis*) are the major dairy animals in Pakistan. The disease is associated with considerable alterations in milk chemical composition with a significant decrease in milk synthesis and changes in cell permeability (2). Bacterial pathogens are majorly (70%) involved in the etiology of mastitis. Besides, minor causes (30%) include non-infectious agents such as physical trauma, mechanical injuries to the gland, etc. (3). More than 135 types of bacterial species have been reported to be associated with bovine mastitis, but 20 different pathogenic bacteria are most commonly involved in mastitis of dairy animals (3, 4). The most common mastitis-causing bacterial pathogens are *Staphylococcus aureus*, *Escherichia coli*, *coagulase-negative staphylococci* (CNS), *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Streptococcus agalactiae* (4–6).

Generally, antimicrobial agents are used to treating bacterial infections including intramammary infections (IMI) in livestock. In dairy animals, mastitis and reproductive disorders are some of the most important reasons for frequent and prolonged use of antimicrobials (7). Quite usual, the treatment of mastitis takes a longer duration due to relapse and failure to flush out those bacteria which are resistant to a wide range of antimicrobials. The therapy of mastitis should be as efficient and quick as possible; however, there is a rapid increase in the prevalence of multidrug-resistant bacteria globally (5). According to the World Health Organization (WHO), an efficient monitoring system for the antimicrobial resistance of various bacterial pathogens is urgently required at national levels to fulfill the requirements of international standards (8). Data regarding the prevalence of bovine mastitis and the knowledge of pathogens causing mastitis is important to chalk out prevention and control strategies and to adopt appropriate therapeutic protocols (4).

In Pakistan, national surveillance programs for monitoring the prevalence of bovine mastitis and antimicrobial resistance in bacterial pathogens are lacking. Only some discrete studies were conducted in different areas of Pakistan (9, 10). Thus, there is always a dire need to monitor the prevalence of mastitis at local levels, as well as its etiology, and to evaluate antimicrobial susceptibility of bacterial isolates. Therefore, this study was carried out with the objectives to investigate: (i) the prevalence of bovine mastitis in cattle and buffaloes located in the northwest of Pakistan; (ii) detection of bacterial etiology of mastitis; and (iii) antimicrobial susceptibility of the bacterial isolates.

# MATERIALS AND METHODS

## Statement of Ethics

This cross-sectional prevalence study was performed in accordance with ethical guidelines of Veterinary Research Institute Peshawar, Khyber Pakhtunkhwa.

## Sample Collection and Processing

Milk samples were aseptically collected by local dairy farmers within our connection according to standard procedures. Dairy farmers were properly guided for collection of milk samples from their cattle and buffaloes (Bubalus bubalis). Milk samples ($n$ = 1,566) were collected from cattle ($n$ = 1,096) and buffaloes ($n$ = 470) by their owners or by local veterinarians and processed for the detection of mastitis at the Mastitis section, Center of Microbiology and Biotechnology, Veterinary Research Institute Peshawar, Khyber Pakhtunkhwa, Pakistan, from July 2018 to June 2019. Milk samples were mostly brought by smallholding dairy farmers having one to three animals. According to data provided by the farm owners, most of the farms were semi-Paka, made of sand and bricks, or Kacha, made of sand and mud. Farmers were guided to collect aseptical milk samples according to the standard protocols of the National Mastitis Council. They were advised to collect composite milk samples in duplicate per animal. Dairy milk samples were brought by farmers from their dairy cattle ($n$ = 1,096) and buffaloes ($n$ = 470), belonging to different areas of Khyber Pakhtunkhwa province including districts Peshawar ($n$ = 744), Charsada ($n$ = 125), Nowshera ($n$ = 57), Khyber ($n$ = 67), Swabi ($n$ = 38), and other areas ($n$ = 65). The sample size from different areas was proportional to the local total number of cattle and buffaloes.

## Clinical and Subclinical Mastitis Detection

Dairy farmers were guided to recognize clinical mastitis from the clinical signs and abnormal changes in milk (10). Subclinical mastitis was tested using the California Mastitis Test (CMT), according to the recommendations of commercially available CMT Kit (Techni. Vet., Inc. USA). In addition, the scoring system of CMT was considered (scoring at +, ++, and + + +, corresponding to mildly positive, moderately positive, and strongly positive, respectively), indicating the intensity of subclinical mastitis in cattle and buffaloes. Month-wise and area-wise prevalence of mastitis in cattle and buffaloes were also enumerated.

## Identification of Bacterial Isolates Associated With Mastitis

A total of 633 mastitic milk samples were further processed for the identification of bacteria at the Bacteriology section of the Center of Microbiology and Biotechnology. Bacteria were identified up to the genus levels as described previously (11). Standard protocols were adopted to carry out bacteriology of milk samples according to the guidelines of the National Mastitis Council (NMC, http://www.nmconline.org/wp-content/uploads/2016/09/Procedures-for-CollectingMilk-Samples.pdf and University of MN Laboratory for Udder Health, https://www.vdl.umn.edu/sites/vdl.umn.edu/files/sample-collection-guide-withpictures.pdf). Additionally, presumptively identified *E. coli* isolates were further confirmed using molecular assay by thermal cycler (Bio-Rad T100$^{TM}$) using forward primers (5′-TGG TAA TTA CCG ACG AAA ACG GC-3′) and reverse primers (5′-ACG CGT GGT TAC AGT CTT GCG-3′), targeting *uidA* at 62°C annealing temperature as described by Tantawiwat et al. (12).

**TABLE 1 |** The prevalence of bovine mastitis in cattle and buffaloes.

| Species | Samples processed | Clinical mastitis | | Subclinical mastitis | | Blood in milk | |
|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % |
| Cattle | 1,096 | 220 | 20.1 | 582 | 53.1 | 51 | 4.7 |
| Buffaloes | 470 | 51 | 10.9 | 312 | 66.4 | 19 | 4.0 |
| Total | 1,566 | 271 | 17.3 | 894 | 57.1 | 70 | 4.5 |

**TABLE 2 |** The severity of subclinical mastitis in cow and buffaloes.

| Species | Samples processed | Traces | | Mild | | Moderate | | Severe | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % | *n* | % |
| Cattle | 582 | 08 | 1.4 | 84 | 14.4 | 91 | 15.6 | 399 | 68.6 |
| Buffaloes | 312 | 14 | 4.5 | 65 | 20.8 | 63 | 20.2 | 170 | 54.5 |
| Total | 894 | 22 | 2.5 | 149 | 16.7 | 154 | 17.2 | 569 | 63.7 |

## Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the bacterial isolates from cases of clinical and subclinical mastitis was performed on Mueller-Hinton agar (Difco™) using the Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standard Institute (13). A panel of 16 different commercially available discs of antimicrobial agents (Oxoid™, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) was tested. The antimicrobial agents included ampicillin, amoxicillin, amoxicillin + clavulanate, cefotaxime + clavulanate, oxytetracycline, doxycycline, gentamicin, kanamycin, lincomycin, streptomycin, erythromycin, norfloxacin, enrofloxacin ciprofloxacin, chloramphenicol, and sulphamethoxazole.

## Statistical Analysis

The data were analyzed by STATA software using descriptive analysis. Chisq test was applied to compare the prevalence and severity of clinical mastitis between samples from cattle and buffaloes.

# RESULTS

## Prevalence of Mastitis in Cattle and Buffaloes

The overall prevalence of clinical and subclinical mastitis was 17 and 57% in milk samples from cattle and buffaloes belonging to various areas of Khyber Pakhtunkhwa province (**Table 1**). The highest prevalence of clinical mastitis (20%) was noted in cattle, whereas subclinical mastitis was 53% in cattle belonging to various breeds, mostly crossbred Holstein Frisian and Jersey breeds. However, the prevalence of subclinical (66%) was highest in buffaloes, while clinical mastitis was 11%. Blood mixed milk samples were detected in 4.7% ($n = 51$) and 4% ($n = 19$) cows and buffaloes, respectively (**Table 1**). The prevalence of clinical mastitis in cattle was significantly higher than that in buffaloes ($p < 0.5$), while the prevalence of subclinical mastitis in cattle was significantly lower than that in buffaloes ($p < 0.5$).

The results also revealed that strongly positive intensity (+ + +) was noted in 64% cases of subclinical mastitis, followed by moderate intensity (++; 17%) and mild intensity (+; 17%) in cattle and buffaloes using California Mastitis Test. The higher prevalence of strongly positive subclinical mastitis (+ + +) was noted in cattle (69%), compared to buffaloes (55%), as shown in **Table 2**. The prevalence of strongly positive subclinical mastitis in cattle was significantly higher than that of buffaloes ($p < 0.5$).

## Month-Wise and Area-Wise Prevalence of Mastitis

Month-wise prevalence of bovine mastitis in cattle and buffaloes of Khyber Pakhtunkhwa is shown in **Table 3**. Highest prevalence of mastitis was noted in the hot and humid months of July, August, and September in cattle and buffaloes. However, the lowest prevalence of mastitis in cattle was observed in May, but in buffalos, the prevalence of mastitis was lowest in the month of November (**Table 3**). The area-wise highest prevalence of mastitis was observed in cattle (76.9%) and buffaloes (80.2%) of district Peshawar, followed by the Sawabi and Nowshera districts (**Table 4**).

## Bacteriology of Mastitic Milk Samples

**Table 5** shows the prevalence of different bacterial isolates from mastitis-positive cases of cattle and buffaloes of Khyber Pakhtunkhwa. The results showed the most common isolated bacteria were *Staphylococci* spp. (34%), followed by *Escherichia coli* (19%), *Streptococci* spp. (9%), and *Klebsiella* spp. (8%). Minor bacteria recovered from mastitic milk samples were *Salmonella* spp. (2%), *Proteus* spp. (1%), and *Candida* spp. (0.6%). However, no growth was observed in 16% of mastitic milk samples. A total of 186 (19%) *E. coli* isolates were confirmed by PCR amplification of *uidA* gene with amplicon size of 147 bp.

## Antimicrobial Susceptibility Testing

The results of antimicrobial susceptibility testing of the bacterial isolates are shown in **Table 6**. Gentamicin, enrofloxacin, and ciprofloxacin showed the highest susceptibility to various

**TABLE 3 |** Month-wise prevalence of bovine mastitis in cattle and buffaloes.

| Months | Cow | | | Buffaloes | | |
|---|---|---|---|---|---|---|
| | Samples | *n* | % | Samples | *n* | % |
| January | 75 | 47 | 62.7 | 87 | 67 | 77.0 |
| February | 57 | 47 | 82.5 | 41 | 33 | 80.5 |
| March | 60 | 45 | 75.0 | 39 | 27 | 69.2 |
| April | 106 | 85 | 80.2 | 45 | 38 | 84.4 |
| May | 122 | 56 | 45.9 | 26 | 23 | 88.5 |
| June | 73 | 51 | 69.9 | 8 | 7 | 87.5 |
| July | 65 | 55 | 84.6 | 09 | 8 | 88.9 |
| August | 168 | 120 | 71.4 | 36 | 33 | 91.7 |
| September | 134 | 114 | 85.1 | 27 | 23 | 85.2 |
| October | 72 | 55 | 76.4 | 54 | 40 | 74.1 |
| November | 95 | 74 | 77.9 | 53 | 26 | 49.1 |
| December | 69 | 53 | 76.8 | 45 | 38 | 84.4 |

**TABLE 4 |** Area-wise prevalence of bovine mastitis in cattle and buffaloes.

| Areas | Cow | | | Buffaloes | | |
|---|---|---|---|---|---|---|
| | *n* | Positive | % | *n* | Positive | % |
| Peshawar | 744 | 572 | 76.9 | 358 | 287 | 80.2 |
| Charsada | 125 | 82 | 65.6 | 21 | 12 | 57.1 |
| Nowshera | 57 | 37 | 64.9 | 69 | 52 | 75.4 |
| Khyber | 67 | 42 | 62.7 | 03 | 02 | 67.0 |
| Sawabi | 38 | 29 | 76.0 | 15 | 08 | 53.3 |
| Other areas* | 65 | 40 | 61.5 | 04 | 02 | 50.0 |

**Other areas included scattered samples from Kohat, Karak, Bannu, Hazara, Dara Adam Kheil, etc.*

**TABLE 5 |** The bacteriology of milk ($n = 633$) of cattle and buffaloes suffering from mastitis.

| Bacteria isolated | Numbers (*n*) | Percentage (%) |
|---|---|---|
| *Staphylococci* spp. | 215 | 34.0 |
| *Escherichia coli* | 186 | 19.4 |
| *Streptococcus* spp. | 58 | 09.2 |
| *Klebsiella* spp. | 50 | 07.9 |
| *Salmonella* spp. | 14 | 02.2 |
| *Proteus* spp. | 07 | 01.1 |
| *Candida* spp. | 04 | 00.6 |
| Culture negative | 99 | 15.6 |

bacterial isolates from milk of cows and buffaloes suffering mastitis. Overall, 92% of the bacterial isolates were susceptible to gentamicin, followed by 88% susceptibility to enrofloxacin and 79% to ciprofloxacin. On the contrary, the highest resistance was noted against sulphamethoxazole (99%), followed by lincomycin (98%), oxytetracycline (89%), ampicillin (86%), and doxycycline (85%).

## DISCUSSION

Mastitis is one of the most prevalent diseases of dairy animals all over the world (1), including Pakistan (11, 14, 15). In the current study, 20 and 53% prevalence of clinical and subclinical mastitis was noted in cattle, whereas it was 11 and 66% in buffaloes belonging to different areas of Khyber Pakhtunkhwa. This prevalence of subclinical mastitis was quite higher than the previous reports of 35% subclinical mastitis in cattle of district Muzaffar Gharr (14), 30% in cattle of district Lahore (9), and 44% in Punjab (11), and our previously reported 42% subclinical mastitis in buffaloes of district D. I. Khan (10). This considerable increase in the prevalence of bovine mastitis is alarming, which might be because dairy farming is rapidly growing in Khyber Pakhtunkhwa, and it is reported that mastitis is significantly increasing with an increase in the number of dairy animals (10). The other reasons might be that the number of milk samples processed and areas under study in the present study were considerably more than the other studies. The prevalence of clinical mastitis was in line with previous studies which reported 11.5% clinical mastitis in cattle of the Jammu and Kashmir region (15) and 11% in Nili Ravi buffaloes (10). However, Mustafa

**TABLE 6 |** Antimicrobial susceptibility of bacterial isolates from mastitic cattle and buffaloes.

| Antimicrobial agents | Concentration (μg) | Susceptible isolates | | Resistant isolates | |
|---|---|---|---|---|---|
| | | *n* | % | *n* | % |
| Ampicillin | 10 | 35 | 13.9 | 217 | 86.1 |
| Amoxicillin | 10 | 68 | 34.0 | 132 | 66.0 |
| Amoxicillin/clavulanate | 20/10 | 66 | 38.4 | 106 | 61.6 |
| Cefotaxime/clavulanate | 30/10 | 35 | 14.6 | 205 | 85.4 |
| Oxytetracycline | 30 | 26 | 10.7 | 216 | 89.3 |
| Doxycycline | 30 | 34 | 16.8 | 168 | 83.2 |
| Gentamicin | 10 | 205 | 91.5 | 19 | 08.5 |
| Kanamycin | 30 | 92 | 52.0 | 85 | 48.0 |
| Lincomycin | 10 | 4 | 02.0 | 204 | 98.0 |
| Streptomycin | 10 | 38 | 32.8 | 78 | 67.2 |
| Erythromycin | 15 | 50 | 22.3 | 174 | 77.7 |
| Norfloxacin | 10 | 149 | 65.4 | 79 | 34.6 |
| Enrofloxacin | 10 | 233 | 88.3 | 31 | 11.7 |
| Ciprofloxacin | 05 | 192 | 79.3 | 50 | 20.7 |
| Chloramphenicol | 30 | 212 | 84.1 | 40 | 15.9 |
| Sulphamethoxazole | 23.75 | 2 | 00.7 | 291 | 99.3 |

et al. (9) and Sharif et al. (16) reported a higher prevalence of clinical mastitis in cattle and buffaloes. They reported a higher prevalence of 40% and 61% of clinical mastitis in buffaloes and cattle. The highest prevalence of clinical mastitis was observed in cattle followed by buffaloes, which is also in line with a previous study (9). This might be attributed to the decreased immunity of exotic and crossbred cattle in Pakistan. However, as buffaloes are local to the environment, they are more resistant to various diseases including mastitis. In addition, 4.7 and 4.0% blood-mixed milk samples were observed in cows and buffaloes, which is in agreement with other studies conducted in this region (9, 16). The prevalence of bovine mastitis in cattle was also in partial agreement with several global studies conducted in India (17), China (4), Ethiopia (18), and Poland (19). Additionally, the highest prevalence of mastitis was noted in the hot and humid months of summer in both species. Similar findings were also observed by Sinha et al. (17); they reported the highest incidence of mastitis during the monsoon season. The prevalence of mastitis varied with different regions, the highest prevalence being noted in the cattle (81%) and buffaloes (82%) of district Peshawar. Previous studies also reported that the prevalence of mastitis varied with geography (10, 11).

Bacteriology of mastitic milk samples from cattle and buffaloes showed that *Staphylococci* spp. (34%) and *E. coli* (19%) were the most frequently isolated bacterial pathogen from cattle and buffaloes suffering mastitis. This was followed by *streptococci* spp. (9%), *Klebsiella* spp. (8%), *Salmonella* spp. (2%), and *Proteus* spp. (1%). This was in agreement with the other studies conducted in various regions of Pakistan (9, 11, 15, 20, 21), which also predominantly isolated *Staphylococci* spp. and *E. coli* from cases of bovine mastitis. We identified *E. coli* isolates ($n = 186$) by molecular assay using PCR amplification of *uidA* gene. This was in agreement with our previous work (5) and the study of Tantawiwat et al. (12). In a recent large-scale Chinese study, Song et al. (22) also reported that staphylococci spp. were the main pathogens associated with mastitis in cattle. Similarly, Bhat et al. (15) reported that *Staphylococcus aureus* (61%) was the most prevalent bacteria isolated from the mastitic cattle of Jammu and Kashmir, followed by *E. coli* (13%), coagulase-negative staphylococci (13.04%), *Streptococcus uberis* (4.35%), and *Streptococcus dysgalactiae* (8.69%). Gao et al. (4) and Ali et al. (5) also reported these pathogens from Chinese cattle. However, in another large-scale Chinese study by Gao et al. (4), they concluded that *E. coli* (14%) were the most prevalent bacteria isolated cases of bovine clinical mastitis followed by *Klebsiella* spp. (13%), coagulase-negative staphylococci (10%), *Streptococcus dysgalactiae* (11%), *Staphylococcus aureus* (10%), *Streptococcus agalactiae* (3%), and *Streptococcus uberis* (2%). This might be because the bacteria causing mastitis are changing with topographical and management conditions.

In the current study, gentamicin (92%), enrofloxacin (88%), and ciprofloxacin (79%) showed the highest susceptibility to various bacterial isolates from milk of cows and buffaloes suffering mastitis. However, the highest resistance of bacterial isolates was observed against sulphamethoxazole (99%), lincomycin (98%), oxytetracycline (89%), ampicillin (86%), and doxycycline (85%). This was in partial agreement with the study of Iqbal et al. (20); they also reported that gentamicin, enrofloxacin, norfloxacin, and kanamycin were the most effective antimicrobial drugs against the isolated bacteria from bovine mastitis (20). Similarly, several other studies also reported the highest susceptibility of bacterial isolates toward aminoglycosides including gentamicin, fluoroquinolone like enrofloxacin, and ciprofloxacin (11, 16). Notably, gentamicin and enrofloxacin antibiotics are listed in the approved list of antimicrobial agents approved for veterinary medicine by the World Health

Organization and World Organization for Animal Health (23). This study, describes for the first time the prevalence of clinical and subclinical mastitis in a large number of dairy cattle and buffaloes belonging to different areas of Khyber Pakhtunkhwa, and identifies different bacterial pathogens associated with mastitis and their antimicrobial susceptibility profiles. Thus, this study would be beneficial for scientists, researchers, and clinicians working with the dairy sector to sketch prevention and control strategies and to adopt appropriate therapeutic protocols for bovine mastitis.

## CONCLUSIONS

This study concludes the high prevalence of clinical and subclinical mastitis in cattle and buffaloes belonging to the northwest of Pakistan. *Staphylococci* spp. was the major bacterial pathogen associated with mastitis. This indicates unhygienic and poor management practices at the local farms. In addition, based on susceptible antimicrobial profiling of the isolated bacterial pathogens from cases of bovine mastitis, gentamicin and enrofloxacin might be appropriate antimicrobial agents in the treatment of bovine mastitis.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of Veterinary Research Institute Peshawar, Khyber Pakhtunkhwa. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

TA, AR, MA, and GL designed and conceived the study. TA, Kamran, IW, and RU performed the experiments. TA, PS, and BH analyzed the data and wrote the article. BH and GL critically reviewed and revised the manuscript. All authors read and approved the final version.

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## REFERENCES

1. De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW. Invited review: mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. *J Dairy Sci.* (2012) 95:1025–40. doi: 10.3168/jds.2010-4074
2. Schukken YH, Hertl J, Bar D, Bennett GJ, González RN, Rauch BJ, et al. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. *J Dairy Sci.* (2009) 92:3091–105. doi: 10.3168/jds.2008-1557
3. Bradley AJ, Green MJ. Bovine mastitis: an evolving disease. *Vet J.* (2002) 164:116–28. doi: 10.1053/tvjl.2002.0724
4. Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L, et al. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *J Dairy Sci.* (2017) 100:4797–806. doi: 10.3168/jds.2016-12334
5. Ali T, Ur Rahman S, Zhang L, Shahid M, Zhang S, Liu G, et al. ESBL-producing *Escherichia coli* from cows suffering mastitis in China contain clinical class 1 integrons with CTX-M linked to ISCR1. *Front Microbiol.* (2016) 7:1931. doi: 10.3389/fmicb.2016.01931
6. Yang X, Wang D, Li J, Meng X, Wei Y, Tuerxun G, et al. Molecular epidemiology and characteristics of *Streptococcus agalactiae* isolated from bovine mastitis in large dairy herds of China. *Pak Vet J.* (2020) 40:301–6. doi: 10.29261/pakvetj/2020.025
7. Nobrega DB, De Buck J, Naqvi SA, Liu G, Naushad S, Saini V, et al. Comparison of treatment records and inventory of empty drug containers to quantify antimicrobial usage in dairy herds. *J Dairy Sci.* (2017) 100:9736–45. doi: 10.3168/jds.2017-13116
8. WHO. *World Health Organization: Antimicrobial Resistance: Global Report on Surveillance*. WHO, Geneva, Switzerland (2014). Available online at: http://www.who.int/drugresistance/documents/surveillancereport/en/.
9. Mustafa YS, Awan FN, Zaman T, Chaudhry SR, Zoyfro V. Prevalence and antibacterial susceptibility in mastitis in buffalo and cow in and around the district Lahore-Pakistan. *Pak J Pharm.* (2011) 24:29–33. doi: 10.14456/ku-bufbu.2013.42
10. Ali T, Rahman A, Qureshi MS, Hussain MT, Khan MS, Uddin S. Effect of management practices and animal age on incidence of mastitis in Nili Ravi buffaloes. *Trop Anim Health Pro.* (2014) 46:1279–85. doi: 10.1007/s11250-014-0641-2
11. Ali MA, Ahmad MD, Muhammad K, Anjum AA. Prevalence of sub clinical mastitis in dairy buffaloes of Punjab, Pakistan. *J Anim Plant Sci.* (2011) 21:477–80. doi: 10.4081/ijas.2011.e41
12. Tantawiwat S, Tansuphasiri U, Wongwit W, Wongchotigul V, Kitayaporn D. Development of multiplex PCR for the detection of total coliform bacteria for Escherichia coli and Clostridium perfringens in drinking water. *Southeast Asian J Trop Med Public Health.* (2005) 36:162–9.
13. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*. Wayne, PA: Clinical and Laboratory Standard Institute (2014). CLSI document M*100*–S124.
14. Bachaya HA, Raza MA, Murtaza S, Akbar IUR. Subclinical bovine mastitis in Muzaffar Garh district of Punjab (Pakistan). *J Anim Plant Sci.* (2011) 21:16–9. doi: 10.4081/ijas.2011.e11
15. Bhat AM, Soodan JS, Singh R, Dhobi IA, Hussain T, Dar MY, et al. Incidence of bovine clinical mastitis in Jammu region and antibiogram of isolated pathogens. *Vet World.* (2017) 10:984–9. doi: 10.14202/vetworld.2017.984-989
16. Sharif A, Muhammad G, Sharif MA. Mastitis in buffaloes. *Pak J of Zool.* (2009) 9:479–90.
17. Sinha MK, Thombare NN, Mondal B. Subclinical mastitis in dairy animals: Incidence, economics, and predisposing factors. *Sci World J.* (2014) 2014:1–4. doi: 10.1155/2014/523984
18. Birhanu M, Leta S, Mamo G, Tesfaye S. Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC Res Notes.* (2017) 10:767. doi: 10.1186/s13104-017-3100-0

19. Sztachańska M, Barański W, Janowski T, Pogorzelska J, Zduńczyk S. Prevalence and etiological agents of subclinical mastitis at the end of lactation in nine dairy herds in north-East Poland. *Pol J Vet Sci*. (2016) 19:119–24. doi: 10.1515/pjvs-2016-0015
20. Iqbal M, Khan MA, Daraz B, Saddique U. Bacteriology of mastitic milk and *in vitro* antibiogram of the isolates. *Pak Vet J*. (2004) 24:161–4.
21. Pumipuntu N, Tunyong W, Chantratita N, Diraphat P, Pumirat P, Sookrung N, et al. *Staphylococcus spp*. associated with subclinical bovine mastitis in central and northeast provinces of Thailand. *Peer J*. (2019) 7:e6587. doi: 10.7717/peerj.6587
22. Song X, Huang X, Xu H, Zhang C, Chen S, Liu F, wt al. The prevalence of pathogens causing bovine mastitis and their associated risk factors in 15 large dairy farms in China: an observational study. *Vet Microbiol*. (2020) 247:108757. doi: 10.1016/j.vetmic.2020.108757
23. WHO. *World Health Organization: Critically Important Antimicrobials for Human Medicine 5th Revision, 2016.* (2016). Available online at: https://apps.who.int/iris/handle/10665/112642a.

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# Spatio-Temporal Variation in the Prevalence of Major Mastitis Pathogens Isolated From Bovine Milk Samples Between 2008 and 2017 in Ontario, Canada

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An understanding of the spatio-temporal distribution of several groups of mastitis pathogens can help to inform programs for the successful control and management of mastitis. However, in the absence of an active surveillance program such information is not readily available. In this retrospective study we analyzed passive surveillance data from a diagnostic laboratory with an aim to describe the spatio-temporal trend of major mastitis pathogens between 2008 and 2017 in Ontario dairy cattle. Data for all milk culture samples submitted to the Animal Health Laboratory (AHL) at the University of Guelph between 2008 and 2017 was accessed. Descriptive analyses were conducted to identify the major pathogens and Chi-square goodness-of-fit tests were used to compare between multiple proportions. Likewise, univariable logistic regression analysis was performed to determine if there was a change in the probability of isolating the major mastitis pathogens depending on geography or time. Seasonality was assessed by calculating the seasonal relative risk (RR). Of a total of 85,979 milk samples examined, more than half of the samples (61.07%) showed no growth and the proportion of samples that showed no growth almost halved during the study period. Of the samples (36.21%, $n = 31{,}133$) that showed any growth, the major bacterial pathogens were *Staphylococcus aureus* (15.60%), Non-aureus Staphylococci (NAS) (5.04%), *Corynebacterium* spp. (2.96%), and *Escherichia coli* (2.00%). Of the NAS, the major species reported were *Staphylococcus chromogenes* (69.02%), *Staphylococcus simulans* (14.45%), *Staphylococcus epidermidis* (12.99%), and *Staphylococcus hyicus* (2.13%). A temporal change in the prevalence of contagious pathogens like *S. aureus* and *Corynebacterium* spp. was observed with an increasing odds of 1.06 and 1.62, respectively. Likewise, except for *Trueperella pyogenes*, the prevalence of all the major environmental mastitis pathogens increased during the study period. The isolation of most of the pathogens peaked in summer, except for *S. aureus*, *T. pyogenes,* and *Streptococcus dysgalactiae* which peaked in spring months. Interestingly, a regional

pattern of isolation of some bacterial pathogens within Ontario was also observed. This study showed a marked spatio-temporal change in the prevalence of major mastitis pathogens and suggests that a regional and seasonal approach to mastitis control could be of value.

**Keywords: spatio-temporal variation, prevalence, mastitis pathogens, Canada, bovine**

## INTRODUCTION

Mastitis, the inflammation of the mammary gland, is one of the most important production limiting diseases in dairy cattle (1). Farmers have prioritized mastitis as the second most important dairy cattle disease based on the National Dairy Survey in Canada (2). Although the case fatality rate for mastitis is low, it adversely affects farm profitability and can result in significant economic losses (1, 3). Costs due to both clinical and sub-clinical forms of the disease include production costs, treatment costs, and prevention costs (3–5). In addition, there are indirect costs to farmers due to the penalty imposed on milk with a high somatic cell count (6). While the relative importance of the cost components may vary according to the clinical forms of the disease, losses associated with both clinical and sub-clinical forms of mastitis can amount to an average of Canadian $662 per milking animal per annum for Canadian dairy farmers (5). Mastitis is also important from an animal welfare point of view as clinical mastitis causes pain in the animals (7).

The relationships between pathogen, animal, and farm environment and management factors play an important role in the causation, and hence management and treatment of mastitis (8, 9). The management of mastitis is particularly challenging as mastitis in dairy cattle is caused by several groups of pathogens like the most frequently isolated *Staphylococcus aureus*, and opportunistic pathogens like *Streptococcus* species, *Escherichia coli*, *Klebsiella* species, and coagulase-negative Staphylococci (3, 9). The relative frequency of isolation of these pathogens can vary temporally or spatially and can also depend on the production stage of the animal and the clinical form of the disease (2, 10–15). The treatment of mastitis in cows involves the use of antimicrobials via intramammary and/or parenteral routes, which has been associated with antimicrobial resistance of the mastitis pathogens and future treatment failure (16). This is especially important when antibiotics are used in dairy cows as blanket dry cow therapy without the identification of the causative pathogen and its antimicrobial sensitivity profile (17–20). Antimicrobial resistance (if developed against antibiotics that are critically important for human use) will be detrimental to public health as some of the mastitis pathogens are also human pathogens, opportunistic human pathogens, or can transmit antimicrobial resistance genes to human pathogens. Therefore, information on the pathogens isolated from animals with mastitis will (1) improve situational awareness related to mastitis pathogens in the Ontario dairy population, (2) support the design of mastitis control plans, and (3) help clinicians to identify the hazards that are present in the Ontario dairy environment.

While many cross sectional studies have documented the most common mastitis causing pathogens in Ontario (2, 10–15), understanding how the prevalence of these pathogens has changed over time is an important area of focus. In this study, we estimate the prevalence of mastitis pathogens and describe the overall spatio-temporal trend for the major mastitis pathogens between 2008 and 2017 in Ontario dairy cattle.

## METHODS

This retrospective study examined laboratory data for all milk culture samples submitted to the Animal Health Laboratory (AHL) at the University of Guelph between 2008 and 2017. These milk samples comprise samples that were routinely submitted to the laboratory for only culture or culture and susceptibility testing. Samples were submitted to the AHL by veterinarians as a part of mastitis testing and are expected to represent a sample from a quarter of an animal udder, while some of the samples were composite samples. Additional information on the clinical stage of the disease, and/or treatment history of the animal was not available.

### Bacterial Isolation

At the AHL, a standard microbiological isolation technique was followed to isolate bacteria from a milk sample. Briefly, 10 μl of milk were inoculated on Columbia blood agar (BA) and, if <20 milk samples were submitted, also on MacConkey (MAC) agar. Blood agar plates were incubated at 35°C in the presence of 5% $CO_2$ whereas MAC agar plates were incubated at ambient air at 35°C. All plates were checked for the presence of bacterial growth after 24 and 48 h of incubation. In addition, all milk samples were incubated at 35°C aerobically up to 5 days if no bacterial growth was detected on the initial culture. After 5 days of incubation the milk samples were re-plated on BA only and incubated and checked for the presence of bacterial growth as specified above. Before 2011 bacterial identification was done biochemically following standard operating procedures at AHL whereas from 2011 until 2017 bacterial identification was achieved using matrix assisted time-of-flight mass spectroscopy (MALDI-TOF MS). Briefly, individual bacterial colonies were smeared on stainless steel plate and covered by matrix [α-cyano-4-hydroxycinnamic acid (HCCA)]. The results were read using the Bruker MALDI Biotyper. All major mastitis pathogens were reported. If no bacterial growth was detected the results were reported as no bacterial growth. If bacterial growth was detected but there were no major mastitis pathogens present the results were reported as no bacterial pathogens. Overgrowth with mold

and/or *Proteus* spp. and numerous bacterial species was reported as overgrowth with contaminants.

## Data Management and Statistical Analysis

The milk culture data obtained from AHL was tabulated using a spreadsheet (Microsoft® Excel® for Office 365). A single entry represents a test performed on a milk sample from a single mammary quarter, or composite milk from all or some quarters of an animal. Duplicate entries were removed. It was expected that the milk samples tested at the AHL would be representative of milk samples tested for mastitis in the province of Ontario, Canada as the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA) subsidizes the laboratory costs for samples submitted to the AHL. Hence, only milk samples submitted from farms with an Ontario address were retained in the dataset. In this study we retained the test results corresponding to general bacteriological examination of the milk samples.

For qualitative analysis, pathogens that constituted at least 1.5% of the total isolates were selected for further analysis except for *Streptococcus dysgalactiae* which was included because of its known importance as a mastitis pathogen. This included bacteria representing contagious mastitis pathogens (*S. aureus* and *Corynebacterium* spp.), environmental mastitis pathogens [*E. coli*, non-*aureus* Staphylococci (NAS), *Trueperella pyogenes,* and *S. dysgalactiae*], environmental mastitis pathogen that can also act as contagious mastitis pathogen (*Streptococcus uberis*) (21), fungi (all yeast, mold, and other fungus spp. aggregated), and algae (*Prototheca* spp.) that are frequently isolated from mastitis milk samples and are important causes of bovine mastitis (2, 10–15). The pathogens of genus *Corynebacterium* (*Corynebacterium bovis*, *Corynebacterium ulcerans,* and all other *Corynebacterium* spp.) were aggregated as *Corynebacterium* spp. and treated as contagious mastitis pathogen as the majority of the isolates reported at the species level included the contagious mastitis pathogen *Corynebacterium bovis*. Likewise, NAS includes all Staphylococci other than *S. aureus*, like *Staphylococcus agnetis*, *Staphylococcus capitis*, *Staphylococcus caprae*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, *Staphylococcus sciuri*, *Staphylococcus simulans*, *Staphylococcus xylosus,* and those not reported at species level. While most of these pathogens that are grouped as NAS are coagulase negative (formerly grouped as coagulase negative staphylococci), some others are coagulase positive and coagulase variable Staphylococci.

Date of sample submission was categorized by both month and season—Winter (Dec 21–March 19), Spring (March 20–June 19), Summer (June 20–Sep 21), and Autumn/Fall (Sep 22–Dec 20). Year was modeled as a continuous variable. Likewise, the location of the submitting clinic was categorized into Eastern Ontario, Central Ontario, Southwestern Ontario, and Northern Ontario regions (22). The clinics in Toronto was included in Central Ontario. We used R (23) to perform all the statistical analysis. Chi squared goodness-of-fit tests were used to compare between multiple proportions. Univariable logistic regression analysis was performed to determine if the probability of isolating the major mastitis pathogens (outcome) changed with year, month, and/or geography (24). Seasonal relative risk (RR) and the confidence interval (CI) was calculated as described by Brookhart et al. and is interpreted as a RR measure that compares the month with highest incidence to that of the month with the lowest incidence also called Peak-Low Ratio analysis (25). Statistical significance was determined at $P < 0.05$.

# RESULTS

Between 2008 and 2017, a total of 85,979 milk samples were submitted to the AHL for general bacteriological examination resulting in 91,802 test results. The 85,979 samples included 92 composite milk samples.

## Frequency of the Major Mastitis-Associated Pathogens

Between January 1, 2008 and December 31, 2017, the AHL performed milk culture on 85,979 milk samples from dairy cattle in the province of Ontario. The number of samples subjected to milk culture are shown in **Table 1**. Over the years, the average number of milk samples submitted by a clinic was approximately reduced by half. Consequently, the number of samples tested more than halved from 13,339 to 5,891, resulting in a corresponding decrease in the test results.

More than half of the milk samples (61.07%, $n = 85,979$) showed no growth of any group of pathogens. The peak season for observing no growth in milk culture was summer with a RR of 1.21 (95% CI: 1.12–1.31) compared to that in winter. During the study period, the proportion of samples that showed no growth almost halved in the year 2017 when compared to the year 2008 (**Figure 1**). This overall decreasing trend with each year was found to be significant with an odds ratio (OR) of 0.82 (95% CI: 0.81–0.82).

Less than half of the samples (36.21%, $n = 85,979$) showed any growth, while 2.73% of the samples showed contamination. The overall RR of observing contamination was significantly higher during summer compared to that in winter (RR: 21.69, 95% CI: 2.65–177.68). While the proportion of samples showing contamination fluctuated during the study period, an overall decrease in proportion per year was observed (OR = 0.93, 95% CI: 0.91–0.94) compared to the baseline year of 2008. During the study period, the proportion of isolation of bacteria and algae from a milk sample doubled (**Figure 1**). Of the uncontaminated milk culture results, 89.60% of the samples ($n = 31,133$) yielded bacterial isolates only, while 1.31% showed mixed cultures of bacteria, fungi and/or algae. Among the major pathogens isolated from the milk samples, most of them were bacterial pathogens (**Figure 2**) and the top five bacterial species constituted more than two-thirds (78.20%) of the total isolates. Among the fungi, most of the isolates were yeasts (99.56%, $n = 1,607$). Among the *Corynebacterium* spp., 14.02% (359) of the isolates were reported as *Corynebacterium bovis*, three as *Corynebacterium ulcerans* while rest of the isolates were not reported at the species level. Likewise, of the 5.04% of NAS, less than a half of the NAS (44.36%, $n = 4,337$) were reported at species level and of those identified at species level, most of them comprised *S. chromogenes*

**TABLE 1 |** The final data included a total of 85,979 milk samples that were cultured between 2008 and 2017 in the Animal Health Laboratory, Ontario, Canada.

| Year | Number of sample submissions | Average number of samples per submission | Total number of samples tested | Total milk culture test results | Average number of test results per sample |
|---|---|---|---|---|---|
| 2008 | 1,929 | 6.91 | 13,339 | 13,473 | 1.01 |
| 2009 | 1,786 | 5.18 | 9,249 | 9,375 | 1.01 |
| 2010 | 1,711 | 5.36 | 9,169 | 9,281 | 1.01 |
| 2011 | 1,905 | 5.40 | 10,289 | 10,373 | 1.01 |
| 2012 | 2,376 | 4.21 | 9,992 | 10,161 | 1.02 |
| 2013 | 1,964 | 4.45 | 8,747 | 8,943 | 1.02 |
| 2014 | 1,637 | 4.05 | 6,625 | 7,988 | 1.21 |
| 2015 | 1,883 | 3.35 | 6,312 | 7,365 | 1.17 |
| 2016 | 1,866 | 3.41 | 6,366 | 7,459 | 1.17 |
| 2017 | 1,769 | 3.33 | 5,891 | 7,384 | 1.25 |
| Total | 18,826 | 4.57 | 85,979 | 91,802 | 1.07 |

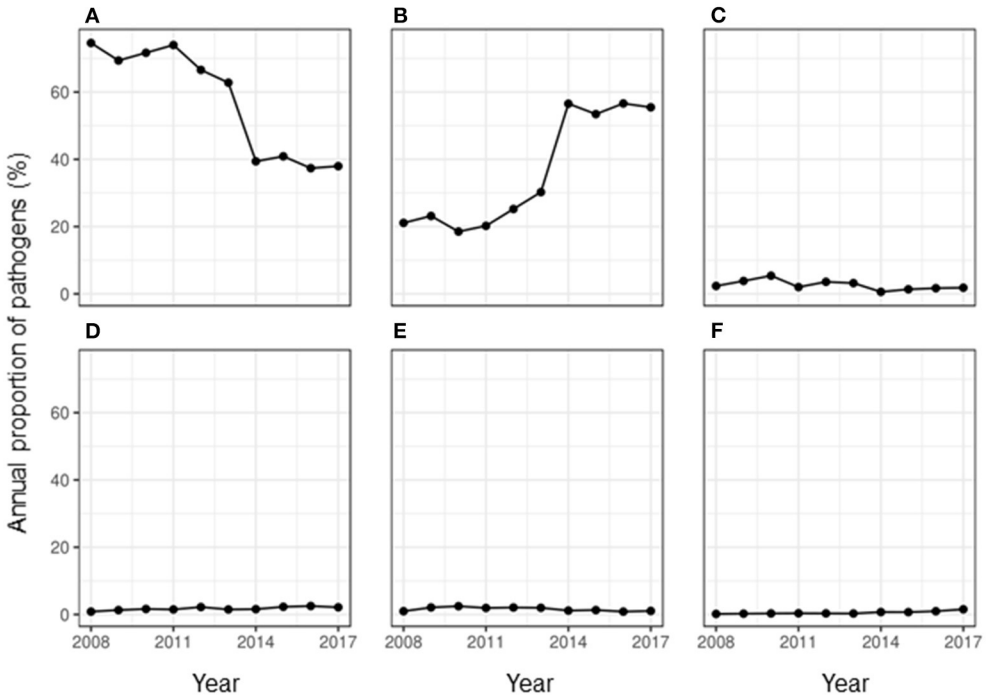


**FIGURE 1 |** Annual proportion of the major classes of pathogens isolated from milk samples submitted to the University of Guelph, Animal Health Laboratory, Ontario, Canada between 2008 and 2017. **(A)** No growth, **(B)** bacteria, **(C)** contamination, **(D)** algae, **(E)** fungus, and **(F)** mixed growth.

(69.02%), *S. simulans* (14.45%), *S. epidermidis* (12.99%), and *S. hyicus* (2.13%).

## Temporal Change in Frequency of Isolation of Major Mastitis Pathogens in Milk Samples in Ontario

### Contagious Mastitis Pathogens

During the study period, there was a fluctuating trend in the proportion of contagious mastitis pathogens isolated from milk. When the number of samples positive for a pathogen was assessed, there was a significant overall increase in the proportion of samples positive for the major contagious mastitis pathogens during the study period (**Figure 2**). In the year 2008, 15.27% of all the milk samples that were cultured isolated *S. aureus*. While there was a decrease in the proportion of samples isolating *S. aureus* in the year 2010 and 2011, an overall increase in prevalence of *S. aureus* was observed during the study period, such that in 2017, *S. aureus* was isolated from 22.27% of the milk samples. While the prevalence of *Corynebacterium* spp. remarkably increased in the milk samples. during the study period, the positivity rate of *Streptococcus agalactiae* almost halved (**Figure 2**). Likewise, the prevalence of *S. uberis*, some strains of which are known to act as a contagious pathogen,

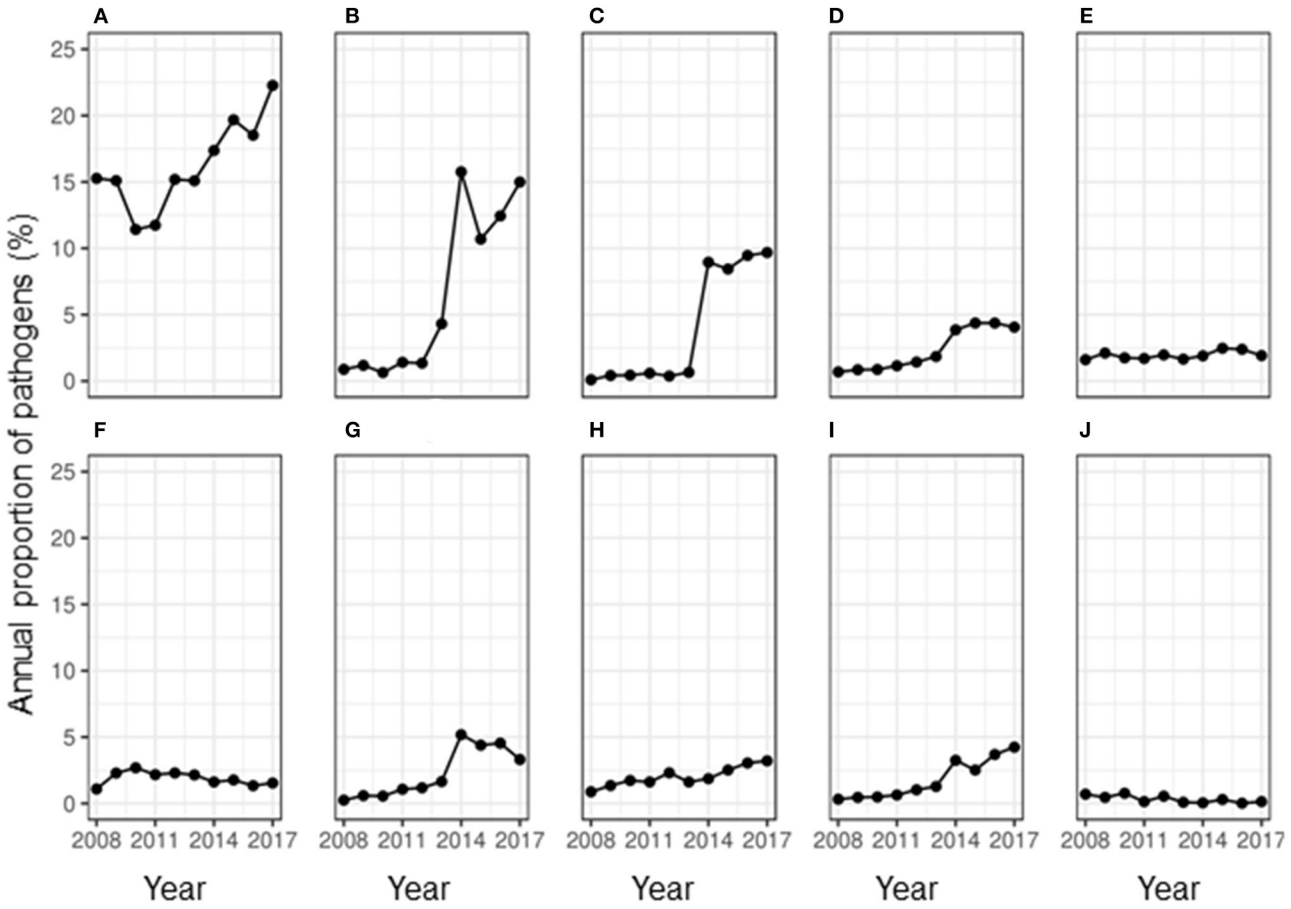


**FIGURE 2 |** Annual proportion of major mastitis pathogens isolated from 85,988 milk samples between 2008 and 2017 in Ontario, Canada. **(A)** *Staph. aureus*, **(B)** non-aureus Staphylococci, **(C)** Corynebacterium spp., **(D)** *E. coli*, **(E)** *Trueperella pyogenes*, **(F)** Algae, **(G)** *Streptococcus uberis*, **(H)** Fungus, **(I)** *Strep. dysgalactiae*, and **(J)** *Streptococcus agalactiae*.

also remarkably increased in the milk samples. during the study period (**Figure 2**). The annual odds of increase were 1.06 for *S. aureus* and 1.62 for *Corynebacterium* spp. (**Table 2**).

### Environmental Mastitis Pathogens

**Figure 2** show that there was an increase in the proportion of each major environmental pathogen in the milk samples from the year 2008 to 2017. There was a corresponding 17-fold increase in the prevalence of NAS, an environmental pathogen (**Figure 2**) during the study period. Likewise, the proportion of samples positive for *E. coli* increased more than 5-fold from 0.69 to 4.06% and the samples positive for *S. dysgalactiae* increased more than 13-fold during this time. On contrary, the proportion of samples positive for *T. pyogenes* remained similar throughout the study period. The annual odds of increase of prevalence per year were significant for all the environmental mastitis pathogens (**Table 2**).

### Minor Mastitis Pathogens

**Figure 2** shows that the proportion of samples positive for Algae (*Prototheca* spp.) changed from 1.09% in the year 2008 to 1.54% in the year 2017 without any significant increase or decrease per year from the base year of 2008 (OR = 0.99; 95% CI: 0.97–1.01, *P*-value = 0.22). Likewise, the proportion of samples positive for fungi increased from <1 to 3.21% during the study period with an annual odds of increase of 1.13 (95% CI: 1.11–1.15, *P*-value < 0.01) from the year 2008.

## Seasonality

**Tables 3–5** show variation in the monthly frequency of isolation of the mastitis pathogens. The odds of increase or decrease of the prevalence of the pathogens during the months of year compared to that in January showed that there was an increasing odds of the prevalence of pathogens like *S. aureus* during March and April and decreasing odds of isolation during the summer months of August to September (**Tables 6–8**). Likewise, a higher odds of isolating NAS and *E. coli* from milk was observed in the spring and summer months compared to that in January. In contrast, the odds of isolating *S. dysgalactiae* from a milk sample was lower during summer, although this observation was not statistically significant. While compared to January the odd of isolating fungus was higher for both the summer months (July–September) and autumn months (October and November), the

**TABLE 2 |** Result of univariable regression analysis of the proportion of samples positive for major mastitis pathogens by year.

| Pathogens | Odds ratio (95% Confidence interval) | *P*-value (Chi square) |
|---|---|---|
| *Staphylococcus aureus* | 1.06 (1.05, 1.07) | <0.01 |
| Non-*aureus* Staphylococci | 1.47 (1.45, 1.49) | <0.01 |
| *Corynebacterium* spp. | 1.62 (1.59, 1.65) | <0.01 |
| *Escherichia coli* | 1.27 (1.25, 1.29) | <0.01 |
| *Trueperella pyogenes* | 1.03 (1.01, 1.05) | <0.01 |
| *Streptococcus dysgalactiae* | 1.36 (1.33, 1.39) | <0.01 |
| Algae (*Prototheca* spp.) | 0.99 (0.97, 1.01) | 0.22 |
| Fungi | 1.13 (1.11, 1.15) | <0.01 |
| *Streptococcus uberis* | 1.33 (1.31, 1.36) | <0.01 |
| Contamination | 0.93 (0.91, 0.94) | <0.01 |
| No growth | 0.82 (0.81, 0.82) | <0.01 |

*P, Probability.*

highest odds of isolating algae from a milk sample was observed in winter (February) when compared to that in January.

Likewise, Peak-Low Ratio analysis (**Table 9**) showed that during a year the isolation of most of the pathogens peaked in summer, except for *S. aureus*, *T. pyogenes,* and *S. dysgalactiae* which peaked in spring.

## Spatial Change in Frequency of Isolation of Major Mastitis Pathogens in Milk Samples at Ontario

**Tables 3–5** show that frequency of isolation of the major pathogens varied by geographical regions, however, such variation was only significant for some of the pathogens (**Table 10**). Compared to Central Ontario, in Eastern Ontario, there was a significantly increased odds of samples being positive for *S. aureus*, and *Corynebacterium* spp. while there were decreased odds of a sample being positive for *S. uberis*. In all regions of Ontario, there was an increased odds of isolation of *E. coli*, and *S. dysgalactiae* compared to Central Ontario. In contrast, compared to that of Central Ontario, a significant decreasing odds of isolation of algae and fungi was noted in Eastern Ontario (**Table 10**).

# DISCUSSION AND CONCLUSIONS

In this retrospective study we have described the frequently isolated pathogens from milk samples in Ontario, Canada and evaluated the major trends for nine major mastitis pathogens. This information will facilitate spatial and temporal comparisons with other studies. The study result is not intended to be a diagnostic guide for veterinarians managing the treatment of bovine mastitis as such decisions should be based on testing of individual samples as far as practically possible. However, information on the most frequently isolated pathogens from milk samples and the general trend in their occurrence will be useful for veterinarians as a reference for decision making in the therapy of mastitis.

The major pathogens responsible for mastitis in Ontario have changed significantly over the years examined in this study. The previously important pathogens have been replaced by pathogens which were considered minor pathogens in the past. While this trend is like that in other countries and other parts in Canada, the findings from this study highlight some differences from other studies. This decreasing trend could be attributed to control efforts toward the causative agent of communicable mastitis. However, control efforts should be continued to reduce not only the major contagious pathogens in Ontario but also environmental, minor, and emerging mastitis pathogens.

While *S. aureus*, *S. uberis*, and *E. coli* were more frequently isolated from mastitis milk samples in the Atlantic Provinces of Canada between 1994 and 2013 (26), this study shows that in Ontario NAS were more frequently isolated than *E. coli*. In contrast to our study finding, in a Canada wide study *Enterobacteriaceae* (*E. coli* and *Klebsiella* spp.) accounted for more than a quarter of isolates from mastitis milk (10). This study also showed that over the years NAS have increasingly been isolated from the milk samples. At the species level, *S. chromogenes* was the dominant NAS species similar to what was noted in a Canada wide study, the US study and some European studies, however regional variation in the type of NAS species prevalence was noted (27–29). Understanding of the prevalence of NAS at species level is necessary from a disease control and management point of view as infection by some of these species of NAS has been known to be persistent and hence a source of infection to other animals in the herd. Whereas, other species like *S. simulans* are more of an environmental pathogen (29, 30).

This study shows the low prevalence of some important emerging mastitis pathogens. There was a consistent presence of a potential pathogen *Aerococcus* spp. in Ontario milk samples since 2014. While no confirmatory evidence is available, *Aerococcus viridans* has been implicated in subclinical bovine mastitis (31). Likewise, in this study at least 1% of the isolates were identified as *Prototheca* spp., an algae responsible for incurable mastitis. *Prototheca* spp. is an emerging pathogen in Canada with it not been reported between 2003 and 2005 (6). A case-control study in 2011 however determined mean within-herd prevalence of *Prototheca* spp of 5.1% (32). The present study also reported a low prevalence of an important contagious mastitis pathogen *Mycoplasma* in milk. In a Canada wide study conducted between 2003 and 2005, no *Mycoplasma* was detected in milk samples from clinically infected animals (6). A low prevalence of *Mycoplasma* in bulk milk samples was reported in a study from Prince Edward Island, Canada (33). In the current study, no *Mycoplasma* was detected in milk samples from 2008 to 2012, however, between 2012 and 2018 a total of 98 isolates were identified. While Canada has historically maintained a low prevalence of *Mycoplasma* compared to other countries (34), it may represent an emerging concern to be monitored.

This study reports interesting seasonal trends of isolation of major mastitis pathogen and clustering of these pathogens by the location of the clinic submitting the samples indicating

**TABLE 3 |** Temporal (monthly) and spatial change in the proportion of bovine milk samples positive for various mastitis pathogens between 2008 and 2017 in Ontario, Canada.

| Parameters | | Percentage of samples showing growth of different mastitis pathogens | | | Total samples per category |
|---|---|---|---|---|---|
| Category | | *Staphylococcus aureus* | *Corynebacterium* spp. | *Streptococcus uberis* | |
| Month | January | 7.90 | 4.32 | 5.94 | 6,728 |
| | February | 7.85 | 6.99 | 7.49 | 7,001 |
| | March | 10.48 | 7.81 | 7.93 | 7,172 |
| | April | 9.85 | 10.76 | 7.68 | 6,648 |
| | May | 7.78 | 8.09 | 0.85 | 6,461 |
| | June | 8.90 | 7.15 | 8.42 | 7,539 |
| | July | 10.00 | 10.80 | 11.64 | 9,151 |
| | August | 9.11 | 11.54 | 12.94 | 9,595 |
| | September | 7.96 | 7.54 | 8.79 | 7,439 |
| | October | 7.64 | 7.62 | 7.86 | 7,009 |
| | November | 6.73 | 11.58 | 7.06 | 6,073 |
| | December | 5.80 | 5.81 | 5.39 | 5,163 |
| Location | Central Ontario | 8.35 | 8.13 | 8.85 | 7,165 |
| | Eastern Ontario | 28.00 | 31.92 | 20.25 | 22,220 |
| | Northern Ontario | 2.93 | 2.87 | 2.04 | 1,952 |
| | Southwestern Ontario | 60.72 | 57.09 | 68.85 | 54,642 |
| Total (*N*) | | 13,411 | 2,547 | 1,615 | 85,979 |

**TABLE 4 |** Temporal (monthly) and spatial change in the proportion of bovine milk samples positive for various environmental mastitis pathogens between 2008 and 2017 in Ontario, Canada.

| Parameters | | Number of samples showing growth of different environmental mastitis pathogens (% of total samples) | | | | Total samples per category |
|---|---|---|---|---|---|---|
| Category | | Non-*aureus* Staphylococci | *E. coli* | *Trueperella pyogenes* | *Streptococcus dysgalactiae* | |
| Month | January | 5.42 | 5.86 | 8.82 | 7.47 | 6,728 |
| | February | 5.26 | 4.06 | 18.76 | 5.11 | 7,001 |
| | March | 9.25 | 5.63 | 9.12 | 13.22 | 7,172 |
| | April | 10.72 | 4.76 | 9.74 | 10.23 | 6,648 |
| | May | 7.06 | 8.53 | 8.33 | 9.05 | 6,461 |
| | June | 8.97 | 10.74 | 8.21 | 8.89 | 7,539 |
| | July | 16.16 | 13.23 | 8.70 | 8.42 | 9,151 |
| | August | 13.19 | 14.39 | 8.76 | 9.91 | 9,595 |
| | September | 9.68 | 11.38 | 6.80 | 7.32 | 7,439 |
| | October | 6.11 | 8.59 | 7.96 | 7.00 | 7,009 |
| | November | 4.82 | 7.78 | 8.33 | 7.08 | 6,073 |
| | December | 3.37 | 5.05 | 6.49 | 6.29 | 5,163 |
| Location | Central Ontario | 5.53 | 2.26 | 7.90 | 4.64 | 7,165 |
| | Eastern Ontario | 18.75 | 18.69 | 25.05 | 21.95 | 22,220 |
| | Northern Ontario | 1.38 | 1.74 | 2.57 | 3.54 | 1,952 |
| | Southwestern Ontario | 74.34 | 77.31 | 64.48 | 69.87 | 54,642 |
| Total (*n*) | | 4,337 | 1,723 | 1,633 | 1,271 | 85,979 |

the possible necessity of seasonal and regional mastitis control approaches. However, caution should be taken while interpreting this as the clustering could be due to stochastic effect of sample submission by the clinics in the geography as some of the clinics conduct milk culture themselves and some sample submission could be to identify the pathogens that they are not capable of doing at their laboratories. In contrast to our study, a Danish study reported that the incidence rate of *S. aureus*, *E. coli*

**TABLE 5 |** Temporal (monthly) and spatial change in the proportion of samples positive for algae and fungi in bovine milk samples between 2008 and 2017 in Ontario, Canada.

| Parameters | Category | Number of samples showing growth of different minor mastitis pathogens (% of total samples per year) | | Total samples per category |
|---|---|---|---|---|
| | | Algae (*Prototheca* spp.) | Fungi (several spp.) | |
| Month | January | 8.47 | 5.97 | 6,728 |
| | February | 12.00 | 6.34 | 7,001 |
| | March | 10.05 | 7.15 | 7,172 |
| | April | 5.48 | 6.22 | 6,648 |
| | May | 7.92 | 5.22 | 6,461 |
| | June | 9.20 | 7.34 | 7,539 |
| | July | 8.47 | 12.06 | 9,151 |
| | August | 9.26 | 13.18 | 9,595 |
| | September | 8.17 | 12.44 | 7,439 |
| | October | 6.89 | 10.20 | 7,009 |
| | November | 7.19 | 7.84 | 6,073 |
| | December | 6.89 | 6.03 | 5,163 |
| Location | Central Ontario | 9.45 | 10.07 | 7,165 |
| | Eastern Ontario | 20.72 | 28.54 | 22,220 |
| | Northern Ontario | 1.89 | 0.56 | 1,952 |
| | Southwestern Ontario | 67.95 | 60.82 | 54,642 |
| Total (*n*) | | 1,641 | 1,608 | 85,979 |

**TABLE 6 |** Regression analysis of the proportion of samples positive for various mastitis pathogens by month.

| Categories of explanatory variable month | Outcome as proportion of samples positive for each of the mastitis pathogens | | |
|---|---|---|---|
| | *Staphylococcus aureus* | Corynebacterium spp. | *Streptococcus uberis* |
| | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 |
| January | 1.00 | 1.00 | 1.00 |
| February | 0.95 (0.86, 1.04) | 1.57 (1.24, 2.00)* | 1.21 (0.93, 1.59) |
| March | 1.30 (1.19, 1.42)* | 1.72 (1.36, 2.18)* | 1.25 (0.96, 1.64) |
| April | 1.33 (1.21, 1.45)* | 2.59 (2.07, 3.25)* | 1.31 (1.00, 1.72) |
| May | 1.03 (0.94, 1.13) | 1.98 (1.57, 2.51)* | 1.56 (1.21, 2.03)* |
| June | 1.01 (0.92, 1.10) | 1.49 (1.17, 1.90)* | 1.27 (0.98, 1.66) |
| July | 0.92 (0.84, 1.00) | 1.86 (1.5, 2.34)* | 1.46 (1.14, 1.87)* |
| August | 0.78 (0.71, 0.85)* | 1.9 (1.53, 2.38)* | 1.54 (1.21, 1.97)* |
| September | 0.9 (0.82, 0.98)* | 1.59 (1.26, 2.03)* | 1.34 (1.04, 1.75)* |
| October | 0.92 (0.83, 1.01) | 1.71 (1.36, 2.18)* | 1.27 (0.98, 1.67) |
| November | 0.93 (0.85, 1.03) | 3.07 (2.47, 3.85)* | 1.32 (1.01, 1.74)* |
| December | 0.95 (0.86, 1.05) | 1.78 (1.39, 2.28)* | 1.18 (0.88, 1.59) |

**Represent significant odds ratios.*

(pastured herd), and *S. dysgalactiae* clinical mastitis was found to be the highest in the winter months (35). Dairy herds in Ontario are predominantly of tie stall types (36) which could be one reason for this unique observation as it was found in a previous study that in case of confined herds, incidence rate of *E. coli* clinical mastitis was higher in summer than in winter (35). The frequency of isolation of *S. uberis* was higher not only in summer, similar to what was found in the Danish study where the highest incidence rate of *S. uberis* clinical mastitis was observed in summer (August), which was associated with pasture (35), but also in autumn.

The spatio-temporal change in the test results could also be a result of a decrease in sample submissions over the years possibly as a result of greater use of in-clinic identification techniques, a decrease in farms and/or animals over the years (37), improvement in the culture techniques, adoption of improved sample collection and transportation, and implementation of mastitis control measures on farm.

**TABLE 7 |** Regression analysis of the proportion of samples positive for environmental mastitis pathogens by month.

| Categories of explanatory variable month | Outcome as proportion of samples positive for each of the mastitis pathogens | | | |
|---|---|---|---|---|
| | Non-*aureus* Staphylococci | *Escherichia coli* | *Trueperella pyogenes* | *Streptococcus dysgalactiae* |
| | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) Overall *P*-value ≤ 0.01 |
| January | 1.00 | 1.00 | 1.00 | 1.00 |
| February | 0.93 (0.77, 1.12) | 0.66 (0.49, 0.90)* | 0.95 (0.75, 1.2) | 0.65 (0.47, 0.90)* |
| March | 1.64 (1.39, 1.93)* | 0.9 (0.68, 1.19) | 0.97 (0.77, 1.22) | 1.67 (1.30, 2.17)* |
| April | 2.08 (1.77, 2.44)* | 0.82 (0.61, 1.10) | 1.12 (0.89, 1.41) | 1.39 (1.07, 1.82)* |
| May | 1.37 (1.16, 1.64)* | 1.53 (1.18, 1.98)* | 0.98 (0.78, 1.25) | 1.27 (0.96, 1.67) |
| June | 1.5 (1.28, 1.78)* | 1.65 (1.30, 2.11)* | 0.83 (0.65, 1.05) | 1.06 (0.81, 1.40) |
| July | 2.29 (1.97, 2.67)* | 1.68 (1.33, 2.13)* | 0.72 (0.57, 0.91)* | 0.83 (0.63, 1.09) |
| August | 1.75 (1.5, 2.05)* | 1.74 (1.38, 2.21)* | 0.69 (0.55, 0.87)* | 0.93 (0.71, 1.22) |
| September | 1.65 (1.41, 1.95)* | 1.78 (1.40, 2.27)* | 0.69 (0.54, 0.89)* | 0.88 (0.66, 1.18) |
| October | 1.09 (0.91, 1.3) | 1.42 (1.10, 1.83)* | 0.86 (0.68, 1.1) | 0.9 (0.67, 1.20) |
| November | 0.98 (0.81, 1.19) | 1.48 (1.14, 1.92)* | 1.05 (0.83, 1.33) | 1.05 (0.78, 1.40) |
| December | 0.8 (0.65, 0.99)* | 1.12 (0.84, 1.50) | 0.96 (0.74, 1.23) | 1.1 (0.81, 1.48) |

**Represent significant odds ratios.*

**TABLE 8 |** Regression analysis of the proportion of samples positive for mastitis pathogens, no growth, and contamination by month.

| Categories of explanatory variable month | Outcome as proportion of samples positive for mastitis pathogens, contamination, or no growth | | | |
|---|---|---|---|---|
| | Algae (Prototheca spp.) | Fungi (several genus) | Contamination | No growth |
| | Odds ratio (95% confidence interval) overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) overall *P*-value ≤ 0.01 | Odds ratio (95% confidence interval) overall *P*-value ≤ 0.01 |
| January | 1.00 | 1.00 | 1.00 | 1.00 |
| February | 1.37 (1.10, 1.71)* | 1.02 (0.77, 1.35) | 0.67 (0.50, 0.91)* | 1.02 (0.95, 1.09) |
| March | 1.12 (0.89, 1.40) | 1.13 (0.86, 1.48) | 0.79 (0.60, 1.05) | 0.75 (0.70, 0.81)* |
| April | 0.65 (0.50, 0.85)* | 1.06 (0.80, 1.40) | 0.80 (0.60, 1.06) | 0.76 (0.71, 0.82)* |
| May | 0.97 (0.76, 1.24) | 0.91 (0.68, 1.22) | 2.01 (1.59, 2.57)* | 0.81 (0.75, 0.86)* |
| June | 0.97 (0.77, 1.22) | 1.10 (0.84, 1.44) | 2.96 (2.38, 3.71)* | 0.72 (0.67, 0.77)* |
| July | 0.73 (0.58, 0.93)* | 1.50 (1.17, 1.92)* | 3.55 (2.88, 4.41)* | 0.68 (0.64, 0.73)* |
| August | 0.76 (0.60, 0.96)* | 1.56 (1.23, 2.00)* | 3.31 (2.69, 4.12)* | 0.79 (0.74, 0.85)* |
| September | 0.87 (0.68, 1.11) | 1.91 (1.50, 2.45)* | 2.83 (2.27, 3.55)* | 0.80 (0.75, 0.86)* |
| October | 0.78 (0.60, 1.00) | 1.66 (1.29, 2.14)* | 1.49 (1.17, 1.92)* | 0.90 (0.84, 0.97)* |
| November | 0.94 (0.73, 1.20) | 1.46 (1.12, 1.92)* | 0.67 (0.49, 0.91)* | 0.87 (0.81, 0.94)* |
| December | 1.06 (0.82, 1.36) | 1.32 (0.99, 1.76) | 1.14 (0.86, 1.52) | 0.93 (0.86, 1.00) |

**Represent significant odds ratios.*

This study is based on passively collected data which have limitations. It is a non-systematic study and since laboratory submission requires a veterinarian, the decision to submit a sample will be influenced by the farmer. Likewise, information on farm type, age of animal, their lactational status, clinical form of the disease, and/or treatment history is not known. The availability of this information would have improved our understanding of seasonal and regional variations that were observed in this study. Additionally, some of the submissions may have been a result of a research study on mastitis or on-farm surveillance. However, information was not available to identify such submissions. In this study, we have considered that all the samples were from cattle in either clinical or sub-clinical stages of mastitis. A subset of samples may have originated from animals with a history of treatment failure. Likewise, the samples could be subject to selection bias and likely underrepresent milk samples from mastitis cattle in Ontario as many clinics and farms have their own regular mastitis testing programs in place. Likewise, some findings can also be attributed to the improvement in diagnostic techniques at the AHL as since 2011

**TABLE 9 |** Seasonal relative risk of isolation of major mastitis pathogens between 2008 and 2017 in Ontario, Canada.

| Isolates | Peak/Low ratio (95% confidence interval) | Peak season |
|---|---|---|
| *Staphylococcus aureus* | 1.36 (1.16, 1.59) | Spring |
| *Corynebacterium* spp. | 1.35 (1, 1.93) | Summer |
| Non-*aureus* Staphylococci | 2.76 (1.96, 3.89) | Summer |
| *Escherichia coli* | 3.04 (1.72, 5.38) | Summer |
| *Trueperella pyogenes* | 1.12 (1, 1.73) | Spring |
| *Streptococcus dysgalactiae* | 1.38 (1, 2.29) | Spring |
| Fungi | 1.08 (1, 1.68) | Spring |
| Algae (*Prototheca* spp.) | 2.11 (1.27, 3.49) | Summer |
| Contamination | 21.69 (2.65, 177.68) | Summer |
| No growth | 1.21 (1.12, 1.31) | Summer |

**TABLE 10 |** Univariable regression analysis of the proportion of samples showing contamination or no growth and those positive for major contagious and environmental mastitis pathogens by location.

| Pathogens | Locations | Odds ratio (95% confidence interval) | *P*-value |
|---|---|---|---|
| *Staphylococcus aureus* | Eastern Ontario | 1.10 (1.02, 1.18)* | <0.01 |
| | Northern Ontario | 1.36 (1.20, 1.54)* | |
| | Southwestern Ontario | 0.94 (0.88, 1.01) | |
| | Central Ontario | 1.00 | |
| *Streptococcus uberis* | Eastern Ontario | 0.73 (0.60, 0.90)* | <0.01 |
| | Northern Ontario | 0.84 (0.57, 1.22) | |
| | Southwestern Ontario | 1.02 (0.86, 1.22) | |
| | Central Ontario | 1.00 | |
| *Corynebacterium* spp. | Eastern Ontario | 1.28 (1.10, 1.49)* | <0.01 |
| | Northern Ontario | 1.31 (0.99, 1.71) | |
| | Southwestern Ontario | 0.92 (0.79, 1.07) | |
| | Central Ontario | 1.00 | |
| Non-*aureus* Staphylococcus | Eastern Ontario | 1.1 (0.95, 1.27) | <0.01 |
| | Northern Ontario | 0.92 (0.68, 1.21) | |
| | Southwestern Ontario | 1.81 (1.59, 2.07)* | |
| | Central Ontario | 1.00 | |
| *Escherichia coli* | Eastern Ontario | 2.69 (1.95, 3.81)* | <0.01 |
| | Northern Ontario | 2.85 (1.75, 4.59)* | |
| | Southwestern Ontario | 4.57 (3.37, 6.39)* | |
| | Central Ontario | 1.00 | |
| *Trueperella pyogenes* | Eastern Ontario | 1.02 (0.84, 1.25) | 0.64 |
| | Northern Ontario | 1.2 (0.83, 1.69) | |
| | Southwestern Ontario | 1.07 (0.89, 1.29) | |
| | Central Ontario | 1.00 | |
| *Streptococcus dysgalactiae* | Eastern Ontario | 1.53 (1.16, 2.05)* | <0.01 |
| | Northern Ontario | 2.84 (1.91, 4.19)* | |
| | Southwestern Ontario | 1.99 (1.54, 2.62)* | |
| | Central Ontario | 1.00 | |
| *Prototheca* spp. (Algae) | Eastern Ontario | 0.70 (0.58, 0.85)* | <0.01 |
| | Northern Ontario | 0.73 (0.49, 1.06) | |
| | Southwestern Ontario | 0.94 (0.80, 1.12) | |
| | Central Ontario | 1.00 | |
| Fungus | Eastern Ontario | 0.91 (0.76, 1.10) | <0.01 |
| | Northern Ontario | 0.20 (0.09, 0.37)* | |
| | Southwestern Ontario | 0.79 (0.67, 0.94)* | |
| | Central Ontario | 1.00 | |
| Contamination | Eastern Ontario | 1.09 (0.95, 1.26) | <0.01 |
| | Northern Ontario | 0.76 (0.56, 1.02) | |
| | Southwestern Ontario | 0.70 (0.61, 0.81)* | |
| | Central Ontario | 1.00 | |
| No growth | Eastern Ontario | 0.87 (0.82, 0.92)* | <0.01 |
| | Northern Ontario | 0.87 (0.78, 0.96)* | |
| | Southwestern Ontario | 0.85 (0.81, 0.90)* | |
| | Central Ontario | 1.00 | |

**Represent significant odds ratios.*

spectrometric techniques are being used to identify bacterial species. While the use of an in-house clinic lab can be attractive for improved timeliness, AHL is one of the major laboratories in Ontario, and Ontario laboratory samples are subsidized making submission more economically attractive. Therefore, the milk samples are expected to be representative of mastitis milk samples in Ontario. In addition, the representativeness of these samples could also have been affected by farm economics and disease outbreaks as they have been shown to influence laboratory sample submissions (38).

It is a valid expectation that the culture of milk originating from a mastitis cow would focus on all the pathogens that are known to cause mastitis. Likewise, the pathogens that were isolated and hence reported might come from the milk, or the environment of the animal (skin, farm, milking machine, human handlers, etc.). Nevertheless, knowledge of this is important as it is known that some of the environmental pathogens can persist in the farm environment and be a constant source of infection for the animals. Despite all these limitations, there is a value of the information obtained from these passive surveillance data.

Bulk milk samples are used to estimate the prevalence of mastitis pathogens in a region (33, 39). However, the information they provide can be of limited value as the probability of detecting a pathogen will be reduced many fold by the dilution effect of the bulk milk and the inherent limit of detection of culture techniques especially if only a small fraction of animals are infected in a herd. Testing milk samples from individual quarters is valuable but is more costly making prevalence studies challenging. In this context, information on the prevalence of mastitis pathogens can be obtained using passive surveillance data generated by a diagnostic laboratory, as has been attempted in this study.

A significant number of samples had a culture negative test result, and this reduced significantly during the study period. Reduction in no growth is possibly due to the improvement in sampling of milk and culture techniques. However, it is also possible that the clinics submitted the higher proportion of culture positive samples to the AHL for confirmation. Likewise, the diversification of culturing techniques would have enabled the capacity to identify the presence of pathogens like *Mycoplasma* which require special media. Given the low prevalence of *Mycoplasma* in Canada and particularly Ontario, this however looks less likely. Often culture negative test results are attributed to infection by pathogens which are short lived

such as *E. coli* and pathogens which are shed in a cyclical manner such as *S. aureus* if a single sample has been tested (40, 41). The simultaneous reduction of the proportion of samples positive for *E. coli* and *S. aureus* could explain the reduction in culture negative test results. Likewise, as previously mentioned, some of the data in this study may come from research studies like a case-control study conducted in 2011, which might have overestimated the culture negative test result (32).

The present study confirms that the prevalence of mastitis pathogens that do not respond to antibiotic treatment is substantial in the milk samples obtained from Ontario cattle between 2008 and 2017 similar to what was observed Canada wide, in the United States, and in the European countries (2, 3, 42, 43). Likewise, we observed that a large proportion of the mastitis milk was negative on culture as reported previously (24). Thus, the use of antibiotics in most instances of mastitis without the identification of the pathogen and their antimicrobial sensitivity result can be unnecessary. The knowledge of the major pathogens prevalent in Ontario farms will aid veterinarians in evaluating their mastitis treatment protocols and management decision making thereby reducing the unnecessary use of antibiotics, as mastitis is considered one of the main reasons for antibiotic use on a dairy farm (44, 45), and hence improving the future prognosis of mastitis treatment. Similarly, this approach can be adopted in other geographies for estimating the prevalence of major mastitis pathogens by utilizing the passive surveillance data originating from diagnostic laboratories.

## DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: On request, the de-identified dataset used in this study can be made available for solely scholarly purposes. Requests to access these datasets should be directed to agreer@uoguelph.ca.

## AUTHOR CONTRIBUTIONS

KA, GB, DS, and AG contributed to the concept of the study and data analysis. KA conducted the data analysis and wrote the initial draft of the manuscript. All the authors contributed to and approved the final manuscript.

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## REFERENCES

1. Jamali H, Barkema HW, Jacques M, Lavallée-Bourget EM, Malouin F, Saini V, et al. Invited review: incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. *J Dairy Sci.* (2018) 101:4729–46. doi: 10.3168/jds.2017-13730
2. Reyher KK, Dufour S, Barkema HW, Des Côteaux L, DeVries TJ, Dohoo IR, et al. The National Cohort of Dairy Farms—a data collection platform for mastitis research in Canada. *J Dairy Sci.* (2011) 94:1616–26. doi: 10.3168/jds.2010-3180
3. Cobirka M, Tancin V, Slama P. Epidemiology and classification of mastitis. *Animals.* (2020) 10:2212. doi: 10.3390/ANI10122212
4. Hogeveen H, Van Der Voort M. Assessing the economic impact of an endemic disease: the case of mastitis. *Rev Sci Tech Off Int Epiz.* (2017) 36:217–26. doi: 10.20506/rst.36.1.2623
5. Aghamohammadi M, Haine D, Kelton DF, Barkema HW, Hogeveen H, Keefe GP, et al. Herd-level mastitis-associated costs on Canadian dairy farms. *Front Vet Sci.* (2018) 5:100. doi: 10.3389/fvets.2018.00100
6. Blowey R, Edmondson P. *Mastitis Control in Dairy Herds*. Oxfordshire: CABI Publishing (2010). doi: 10.1079/9781845935504.0000
7. Leslie KE, Petersson-Wolfe CS. Assessment and management of pain in dairy cows with clinical mastitis. *Vet Clin North Am Food Anim Pract.* (2012) 28:289–305. doi: 10.1016/j.cvfa.2012.04.002
8. Barkema HHW, Schukken YYH, Zadoks RN. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci.* (2006) 89:1877–95. doi: 10.3168/jds.S0022-0302(06)72256-1
9. Zigo F. Vasil' M, Ondrašovičová S, Výrostková J, Bujok J, Pecka-Kielb E. Maintaining optimal mammary gland health and prevention of mastitis. *Front Vet Sci.* (2021) 8:607311. doi: 10.3389/FVETS.2021.607311
10. Riekerink RGMO, Barkema HW, Kelton DF, Scholl DT. Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci.* (2008) 91:1366–77. doi: 10.3168/jds.2007-0757
11. Sargeant J. Qualitative research part II: participants, analysis, and quality assurance. *J Grad Med Educ.* (2012) 4: 1–3. doi: 10.4300/JGME-D-11-00307.1
12. Sargeant JM, Scott HM, Leslie KE, Ireland MJ, Bashiri A. Clinical mastitis in dairy cattle in Ontario: frequency of occurrence and bacteriological isolates. *Can Vet J.* (1998) 39:33–8.
13. Saini V, McClure JT, Léger D, Keefe GP, Scholl DT, Morck DW, et al. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. *J Dairy Sci.* (2012) 95:4319–32. doi: 10.3168/jds.2012-5373
14. Saini V, McClure JT, Scholl DT, DeVries TJ, Barkema HW. Herd-level relationship between antimicrobial use and presence or absence of antimicrobial resistance in gram-negative bovine mastitis pathogens on Canadian dairy farms. *J Dairy Sci.* (2013) 96:4965–76. doi: 10.3168/jds.2012-5713
15. Naqvi SA, De Buck J, Dufour S, Barkema HW. Udder health in Canadian dairy heifers during early lactation. *J Dairy Sci.* (2018) 101:3233–47. doi: 10.3168/JDS.2017-13579
16. Saini V, McClure JT, Scholl DT, DeVries TJ, Barkema HW. Herd-level relationship between antimicrobial use and presence or absence of antimicrobial resistance in gram-negative bovine mastitis pathogens on Canadian dairy farms. *J Dairy Sci.* (2013) 96:4965–76. doi: 10.3168/JDS.2012-5713
17. Oliveira L, Ruegg PL. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. *J Dairy Sci.* (2014) 97:5426–36. doi: 10.3168/jds.2013-7756
18. Anonymous. *Prescription Drug List*. Available online at: https://www.canada.ca/en/health-canada/services/drugs-health-products/drug-products/prescription-drug-list/list.html#a2 (accessed March 29, 2019).
19. Saini V, McClure JT, Léger D, Dufour S, Sheldon AG, Scholl DT, et al. Antimicrobial use on Canadian dairy farms. *J Dairy Sci.* (2012) 95:1209–21. doi: 10.3168/JDS.2011-4527
20. WHO. *WHO List of Critically Important Antimicrobials for Human Medicine (WHO CIA List)*. (2019). Available online at: https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/ (accessed May 21, 2019).

21. Zadoks RN, Gillespie BE, Barkema HW, Sampimon OC, Oliver SP, Schukken YH. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. *Epidemiol Infect.* (2003) 130:335–49. doi: 10.1017/S0950268802008221
22. *First Character of the Postal Code and Corresponding Province, Territory or Region*. Available online at: https://www150.statcan.gc.ca/n1/pub/92-195-x/2011001/other-autre/pc-cp/tbl/tbl9-eng.htm (accessed January 13, 2021).
23. *R Core Team. R: A Language and Environment for Statistical Computing*. (2019). Available online at: https://www.r-project.org/
24. Makovec JA, Ruegg PL. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J Dairy Sci.* (2003) 86:3466–72. doi: 10.3168/jds.S0022-0302(03)73951-4
25. Brookhart MA, Rothman KJ. Simple estimators of the intensity of seasonal occurrence. *BMC Med Res Methodol.* (2008) 8:67. doi: 10.1186/1471-2288-8-67
26. Awosile BB, Heider LC, Saab ME, McClure JT. Antimicrobial resistance in mastitis, respiratory and enteric bacteria isolated from ruminant animals from the Atlantic Provinces of Canada from 1994–2013. *Can Vet J.* (2018) 59:1099.
27. Condas LAZ, De Buck J, Nobrega DB, Carson DA, Naushad S, De Vliegher S, et al. Prevalence of non-*aureus* Staphylococci species causing intramammary infections in Canadian dairy herds. *J Dairy Sci.* (2017) 100:5592–612. doi: 10.3168/JDS.2016-12478
28. Jenkins SN, Okello E, Rossitto P V, Lehenbauer TW, Champagne J, Penedo MCT, et al. Molecular epidemiology of coagulase-negative Staphylococcus species isolated at different lactation stages from dairy cattle in the United States. *PeerJ.* (2019) 2019:e6749. doi: 10.7717/peerj.6749
29. 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. *Front Vet Sci.* (2021) 8:658031. doi: 10.3389/fvets.2021.658031
30. Piessens V, Van Coillie E, Verbist B, Supré K, Braem G, Van Nuffel A, et al. Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. *J Dairy Sci.* (2011) 94:2933–44. doi: 10.3168/JDS.2010-3956
31. Sun M, Gao J, Ali T, Yu D, Zhang S, Khan SU, et al. Characteristics of *Aerococcus viridans* isolated from bovine subclinical mastitis and its effect on milk SCC, yield, and composition. *Trop Anim Health Prod.* (2017) 49:843–9. doi: 10.1007/s11250-017-1271-2
32. Pieper L, Godkin A, Roesler U, Polleichtner A, Slavic D, Leslie KE, et al. Herd characteristics and cow-level factors associated with *Prototheca mastitis* on dairy farms in Ontario, Canada. *J Dairy Sci.* (2012) 95:5635–44. doi: 10.3168/jds.2011-5106
33. Olde Riekerink RGM, Barkema HW, Veenstra S, Poole DE, Dingwell RT, Keefe GP. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. *Can Vet J.* (2006) 47:567–72.
34. Wilson DJ, Goodell G, Justice-Allen A, Smith ST. Herd-level prevalence of Mycoplasma spp mastitis and characteristics of infected dairy herds in Utah as determined by a statewide survey. *J Am Vet Med Assoc.* (2009) 235:749–54. doi: 10.2460/javma.235.6.749
35. Riekerink RGMO, Barkema HW, Stryhn H. The effect of season on somatic cell count and the incidence of clinical mastitis. *J Dairy Sci.* (2007) 90:1704–15. doi: 10.3168/jds.2006-567
36. *Dairy Barns by Type - Canadian Dairy Information Centre (CDIC)*. Available online at: https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/farm-statistics/dairy-barns-by-type/?id=1502467060775 (accessed January 13, 2021).
37. *Number of Dairy Cows and Heifers by Province - Agricultural Industry Market Information System (AIMIS) - Agriculture and Agri-Food Canada (AAFC)*. Available online at: https://aimis-simia-cdic-ccil.agr.gc.ca/rp/index-eng.cfm?action=pR&r=219&pdctc= (accessed January 13, 2021).
38. O'Sullivan T, Friendship R, Pearl DL, McEwen B, Ker A, Dewey C. The association between submission counts to a veterinary diagnostic laboratory and the economic and disease challenges of the Ontario swine industry from 1998 to 2009. *Prev Vet Med.* (2012) 106:275–83. doi: 10.1016/j.prevetmed.2012.03.005
39. Bauman CA, Barkema HW, Dubuc J, Keefe GP, Kelton DF. Canadian National Dairy Study: herd-level milk quality. *J Dairy Sci.* (2018) 101:2679–91. doi: 10.3168/jds.2017-13336
40. Sears PM, Smith BS, English PB, Herer PS, Gonzalez RN. Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. *J Dairy Sci.* (1990) 73:2785–9. doi: 10.3168/jds.S0022-0302(90)78964-3
41. Smith KL, Hogan JS. Environmental mastitis. *Vet Clin North Am Food Anim Pract.* (1993) 9:489–98. doi: 10.1016/S0749-0720(15)30616-2
42. Boireau C, Cazeau G, Jarrige N, Calavas D, Madec JY, Leblond A, et al. Antimicrobial resistance in bacteria isolated from mastitis in dairy cattle in France, 2006–2016. *J Dairy Sci.* (2018) 101:9451–62. doi: 10.3168/JDS.2018-14835
43. Fernández G, Barreal ML, Pombo MB, Ginzo-Villamayor MJ, González-Manteiga W, Prieto A, et al. Comparison of the epidemiological behavior of mastitis pathogens by applying time-series analysis in results of milk samples submitted for microbiological examination. *Vet Res Commun.* (2013) 37:259–267. doi: 10.1007/S11259-013-9570-1
44. APHIS. *Antibiotic Use on U.S. Dairy Operations, 2002 and 2007*. (2008).
45. Saini V, McClure JT, Léger D, Dufour S, Sheldon AG, Scholl DT, et al. Antimicrobial use on Canadian dairy farms. *J Dairy Sci.* (2012) 95:1209–21. doi: 10.3168/jds.2011-4527


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# Intracellular *Staphylococcus aureus* Infection Decreases Milk Protein Synthesis by Preventing Amino Acid Uptake in Bovine Mammary Epithelial Cells

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*Staphylococcus aureus* (*S. aureus*) is one of the main pathogens in cow mastitis, colonizing mammary tissues and being internalized into mammary epithelial cells, causing intracellular infection in the udder. Milk that is produced by cows that suffer from mastitis due to *S. aureus* is associated with decreased production and changes in protein composition. However, there is limited information on how mastitis-inducing bacteria affect raw milk, particularly with regard to protein content and protein composition. The main purpose of this work was to examine how *S. aureus* infection affects milk protein synthesis in bovine mammary epithelial cells (BMECs). BMECs were infected with *S. aureus*, and milk protein and amino acid levels were determined by ELISA after *S. aureus* invasion. The activity of mTORC1 signaling and the transcription factors NF-κB and STAT5 and the expression of the amino acid transporters SLC1A3 and SLC7A5 were measured by western blot or immunofluorescence and RT-qPCR. *S. aureus* was internalized by BMECs *in vitro*, and the internalized bacteria underwent intracellular proliferation. Eight hours after *S. aureus* invasion, milk proteins were downregulated, and the level of BMECs that absorbed Glu, Asp, and Leu from the culture medium and the exogenous amino acids induced β-casein synthesis declined. Further, the activity of mTORC1 signaling, NF-κB, and STAT5 was impaired, and *SLC1A3* and *SLC7A5* were downregulated. Eight hours of treatment with 100 nM rapamycin inhibited NF-κB and STAT5 activity, *SLC1A3* and *SLC7A5* expression, and milk protein synthesis in BMECs. Thus mTORC1 regulates the expression of *SLC1A3* and *SLC7A5* through NF-κB and STAT5. These findings constitute a model by which *S. aureus* infection suppresses milk protein synthesis by decreasing amino acids uptake in BMECs.

**Keywords: *Staphylococcus aureus*, amino acid, milk protein synthesis, bovine mammary epithelial cells, amino acid transporters, mTORC1**

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## INTRODUCTION

Bovine milk is an important source of nutrients, with diverse functions in humans, serving as a source of essential amino acids, providing immunological defense, and stimulating the absorption of nutrients (1, 2). Milk contains a wide array of proteins, which can be broadly classified into caseins and whey proteins. In bovine milk, caseins include αS1-, αS2-, β-, and κ-CN, and whey proteins include α-lactalbumin (α-LA), β-lactoglobulin (β-LG), serum albumin and immunoglobulins (3). Caseins are assembled in micelles, whereas whey proteins are soluble (4, 5). Casein is the principal protein in bovine milk, accounting for 75 to 80% of all proteins, and whey protein constitutes 15 to 20% (4, 6, 7). Increasing efforts have been undertaken to understand the regulatory mechanism of milk protein synthesis and improve protein concentrations in bovine milk.

The milk protein content in raw milk is governed by several factors, including the stage of lactation, nutrition supply, and disease (1). Mastitis, an intramammary type of inflammation, is a highly prevalent disease in dairy cows that causes significant economic losses in the bovine dairy industry. *Staphylococcus aureus* (*S. aureus*) is one of the main pathogens in bovine mastitis, with cell-bound properties on the surface that render the bacteria capable of adherence and invasion and secreted virulence factors that facilitate spread of the infection (8) and are often associated with cases of clinical mastitis (CM) and subclinical mastitis (SM) (8–12).

Milk that is produced by cows with mastitis due to *S. aureus* undergoes losses in production and changes in protein, that are abundance of cultured pathogens in milk-dependent (13, 14) or days in milk (DIM)-dependent (15). During mastitis, the protein composition is altered in the milk proteome (16, 17), wherein casein levels decrease in bovine milk, resulting in a lower yield, casein degradation, an imbalance between micellar and soluble casein, and changes in the stability and texture in fermented products (18–20). Moreover, the microbiological quality of raw milk is critical with regard to the quality of the final dairy product (19, 21). In recent years, researchers worldwide have conducted much work on improving the nutrient composition of milk to ensure milk quality and safety (1, 22, 23). However, there is limited information on how mastitis bacteria affects raw milk, particularly its protein content and protein composition.

Mechanistic (mammalian) target of rapamycin (mTOR) complex 1 (mTORC1) is the master regulator of cell growth and metabolism, responding to various environmental cues, including amino acids (24, 25). In addition to serving as the basic elements for protein synthesis, amino acids are irreplaceable for mTORC1 activation (26, 27), which recruits mTORC1 to the lysosomal surface, where it is activated (28–31). Data from the past several years have shown that several types of amino acids in lysosomes and the cytosol can be sensed by mTORC1 (32–35). mTORC1 is believed the most important regulator of protein synthesis, particularly translation, in all mammalian cells, through its downstream effectors, S6K1 and 4EBP1 (25, 36).

Bovine mammary epithelial cells (BMECs) synthesize and secrete milk and thus have been used widely as an *in vitro* cellular model to study the synthesis of milk protein in the udder of dairy cows (37–39). Recent work in mammary epithelial cells of dairy livestock has demonstrated the regulation of milk protein synthesis by mTORC1 (40–43). To synthesize milk protein, BMECs require the uptake of amino acids from extracellular fluid to improve the availability of intracellular amino acids, resulting in mTORC1 signaling activation (44–46); amino acid transporters are then used to concentrate amino acids in cells (46–48). Although mTORC1 and amino acid transporters are involved in milk protein synthesis (49–51), the effect of bacterial infection, particularly *S. aureus*, on mTORC1 signaling, amino acid uptake, and milk protein synthesis is unknown in BMECs.

To determine the mechanism by which intracellular infection by *S. aureus* affects milk protein synthesis in BMECs, we examined the uptake of amino acids; mTORC1 function in amino acid transporter expression; the expression of *CSN2* and its product, β-casein; *LALBA* and its protein, α-lactalbumin (α-LA); and *BLG* and its product, β-lactoglobulin (β-LG) in BMECs *in vitro* and measured the levels of β-caseins, α-LA, and β-LG in the cell culture medium. The purpose of this study was to develop a model by which intracellular infection by *S. aureus* suppresses milk protein synthesis, in which internalized bacteria inhibit mTORC1 activation and then prevent amino acid uptake in BMECs.

## MATERIALS AND METHODS

### Ethics Statement

All experimental procedures with animals were conducted according to the guidelines for the care and use of experimental animals that have been established by the Inner Mongolia University Animal Care and Use Committee.

### Primary BMEC Culture

Primary BMECs were isolated and identified as described (52). Briefly, mammary tissue was obtained from Chinese Holstein cows after being slaughtered on a commercial cattle slaughter farm. After surgical removal of mammary tissue from the slaughtered cow, it was placed in sterile, ice-cold phosphate-buffered saline (PBS) that was supplemented with 300 U/mL penicillin G and 100 mg/mL streptomycin (Sigma-Aldrich, Inc., USA) and transported immediately to the laboratory. Purified primary BMECs were isolated and maintained in DMEM/F12 medium (Hyclone Laboratories, Inc., Logan, UT, USA) that contained 10% fetal bovine serum. Cells were cultured in 25 $cm^2$ tissue culture flasks at 37°C in humidified air with 5% $CO_2$. P2 to P4 BMECs that were in the logarithmic growth phase were used for all experimental assays.

**Abbreviations:** mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; BMECs, bovine mammary epithelial cells; CM, clinical mastitis; SM, subclinical mastitis; NF-κB, nuclear factor kappa-B; STAT, signal transducer and activator of transcription.

## Reagents and Antibodies

Glu (Cat# G8415), Asp (Cat# A7219), and Leu (Cat# L8912) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). β-casein (Cat# EIA06975Bo) was purchased from Wuhan Xinqidi (Wuhan Xinqidi Biological Technology Co. Ltd. Wuhan, China). Rapamycin (Cat# 53123-88-9) was purchased from Gene Operation (Gene Operation, Ann Arbor, MI, USA). Rapamycin was dissolved in ethanol (Sigma-Aldrich, Inc., USA) to a stock concentration of 50 mg/mL, stored at −20°C, and diluted to the appropriate final concentration with culture medium before use. The concentration of ethanol in the final solution did not exceed 0.5% (v/v) in any experiment. β-mercaptoethanol (Cat# M8211) was obtained from Solarbio (Solarbio Science & Technology, Co., Ltd. Beijing, China). Dil (Cat# KGMP002) was purchased from KeyGEN (KeyGEN BioTECH, Co., Ltd. Jiangsu, China). DAPI (Cat# C1005) and Hoechst 33342 Staining Kit (Cat# C1022) were acquired from Beyotime (Beyotime Biotechnology, Co., Ltd. Shanghai, China). Annexin V-FITC/PI Cell Apoptosis Detection Kit (Cat# FA101) was purchased from TransGen (TransGen Biotech Co. Ltd. Beijing, China). Alexa Fluor® 594 Phalloidin (Cat# A12381) was purchased from Invitrogen (Invitrogen, Carlsbad, New Mexico, USA). 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were purchased from Sigma (Sigma-Aldrich, Inc. St. Louis, MO, USA).

Anti-4EBP1 (Cat# ab2606), anti-p-mTOR (Ser2448) (Cat# ab109268), anti-mTOR (Cat# ab32028), goat anti-rabbit (Cat# ab136817), and goat anti-mouse (Cat# ab205719) were purchased from Abcam (Abcam plc 330 Cambridge Science Park, Cambridge, UK). Anti-p-S6 (Ser240/244) (Cat# 5364s), anti-p-4EBP1 (Thr37/46) (Cat# 2855s), anti-p-NF-κB p65 (Ser536) (Cat# 3033), anti-NF-κB p65 (Cat# 8242), and anti-p-STAT5 (Tyr694) (Cat# 4322) were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc., Beverley, MA, USA). Anti-S6 (Cat# sc-74459) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., 10410 Finnell Street Dallas, Texas 75220 USA). Anti-STAT5A (Cat# 13179-1-AP), anti-SLC1A3 (Cat# 20785-1-AP), anti-SLC7A5 (Cat# 13752-1-AP) and anti-Caspase 3 (Cat# 66470-2-Ig) were purchased from Proteintech (Proteintech Group, Inc., 5500 Pearl Street, Suite 400 Rosemont, IL 60018, USA). Goat FITC-conjugated anti-rabbit IgG (Cat# 115-095-003) and FITC-conjugated anti-mouse IgG (Cat# 115-095-146) were purchased from Jackson (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Anti-β-actin (Cat# A5441) was purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, MO, USA).

## Spread Plate Method

BMECs were infected with *S. aureus* (ATCC 27543) for 2 h at an MOI of 30, and the extracellular bacteria were killed and lysed with antibiotics and lysozyme. Intracellularly infected cell cultures were continued and maintained in medium for 2, 4, and 8 h. The cells were lysed, and the number of intracellular bacteria was determined by spread plate method.

## Bacterial Staining

BMECs were seeded on a slide and incubated overnight. Bacteria (*S. aureus*) were washed with PBS and then incubated with CFSE [5 (6)-carboxyfluorescein diacetate N-succinimidyl ester] at 4°C for 15 min. The stained bacteria were centrifuged for 10 min at 3,000 × g at 4°C 3 times. Cells were infected by the stained bacteria at an MOI of 30 for 2 h, washed three times with PBS, and fixed with 4% paraformaldehyde for 20 min. After being treated with 1% Triton X-100 for 5 min, the cells were stained with Alexa Fluor® 594 Phalloidin for 1 h in the dark, washed three times with PBS, and counterstained with 100 μl DAPI for 3 min to assess the nuclear morphology. Finally, the slide was mounted with glycerin for examination under a laser scanning confocal microscope (NIKON A1R, Nikon Corp., Tokyo, Japan).

## TEM

BMECs were infected with bacteria (*S. aureus*) for 2 h at an MOI of 30, and the extracellular bacteria were killed and lysed with antibiotics and lysozyme for 2 h. The infected cells were washed three times with PBS, centrifuged for 10 min at 3,000 × g at 4°C, and fixed with 2.5% glutaraldehyde overnight, the precipitation was wrapped in the 1% agarose. Agarose blocks with samples avoid light post fixed with 1% $OsO_4$ in 0.1 M PB (pH 7.4) for 2 h at room temperature. The tissues were sequentially fed with 30%-50%-70%-80%-95%-100%-100% alcohol and dehydrated for 20 min each time, 100% acetone twice, 15 min each time. Resin penetration and embedding as followed: Acetone, EMBed 812 = 1:1 for 2–4 h at 37°C; Acetone, EMBed 812 = 1:2 overnight at 37°C; pure EMBed 812 for 5–8 h at 37°C; Pouring the pure EMBed 812 into the embedding models and insert the tissues into the pure EMBed 812, and then keep in 37°C overnight. The embedding models with resin and samples were moved into 65°C to polymerize for more than 48 h. The resin blocks were cut to 60–80 nm thin on the ultra microtome, and the tissues were fished out onto the 150 meshes cuprum grids with formvar film, and the 2% uranium acetate saturated alcohol solution avoid light staining for 8 min and then rinsed in 70% ethanol for three times. 2.6% Lead citrate avoid $CO_2$ staining for 8 min. After dried by the filer paper, the cuprum grids were put into the grids board and dried overnight at room temperature. Finally, the samples were examined by TEM (Hitachi HT7700, Hitachi, Ltd., Tokyo, Japan) to detect intracellular bacteria.

## ELISA

BMECs were seeded into 6-well plates, incubated until 80% confluence, and treated with the indicated conditions, including infection with *S. aureus*; serum and amino acid starvation, followed by amino acid stimulation and rapamycin.

To examine how *S. aureus* invasion suppresses milk protein synthesis, BMECs were infected with *S. aureus* for 2 h, and the extracellular bacteria were killed and lysed with antibiotics and lysozyme. The intracellularly infected cells were continued in culture and maintained in medium for 8 h. Cell culture supernatants were collected to measure extracellular β-casein, α-lactalbumin, and β-lactoglobulin using ELISA kits (Wuhan Xinqidi Biological Technology Co. Ltd. Wuhan, China) per the manufacturer's instructions. Intracellularly infected cells were harvested with trypsin and centrifuged to remove the supernatant, and cell lysates were prepared through five freeze-thaw cycles. The total protein concentration of the control and

treatment groups was standardized by adjusting the volume of the protein lysate. An equal volume of each total protein lysate was analyzed for β-casein, α-lactalbumin, and β-lactoglobulin by ELISA.

To determine how rapamycin treatment decreases milk protein synthesis, BMECs were treated with 100 nM rapamycin for 8 h, the cell culture supernatants were collected, the cells were harvested, and cell lysates were prepared and standardized. The levels of extracellular and intracellular β-casein, α-lactalbumin, and β-lactoglobulin were analyzed by ELISA.

To examine how exogenous amino acids induce casein synthesis, BMECs were serum-starved for 16 h, amino acid-starved for 1 h, and then stimulated with amino acids for 1 h. Control and treated cells were harvested, and cell lysates were prepared and standardized. The level of intracellular β-casein was analyzed by ELISA.

To study the suppression of amino acid uptake by *S. aureus*, BMECs were serum-starved for 16 h, amino acid-starved for 1 h, stimulated with amino acids for 1 h, and infected intracellularly with *S. aureus* for 8 h. Cell culture supernatants were collected to measure Glu, Asp, and Leu using ELISA kits (Wuhan Xinqidi Biological Technology Co. Ltd. Wuhan, China).

To examine how *S. aureus* invasion suppresses amino acid induced-casein synthesis, four groups of BMECs were compared: control, amino acid induction (Glu, Asp, Leu), *S. aureus* invasion (8 h), and amino acid stimulation with bacterial infection. BMECs were serum starved for 16 h, amino acid-starved for 1 h, stimulated with amino acids for 1 h, and infected intracellularly with *S. aureus* for 8 h. Control and treated cells were harvested, and cell lysates were prepared and standardized. Intracellular β-casein was analyzed by ELISA.

To determine the contents of alpha-hemolysin (Hlα) and Plasmin, BMECs were infected with *S. aureus* for 2 h, and the extracellular bacteria were killed and lysed with antibiotics and lysozyme. The contents of α-hemolysin (Hla), and Plasmin in cell medium and in cells were measured after infection 8 h using ELISA kits (Wuhan Xinqidi Biological Technology Co. Ltd. Wuhan, China). To determine whether β-casein was directly degraded by *S. aureus* in culture medium, $1.5 \times 10^3$ CFU/mL *S. aureus* were inoculated into DMEM/F12 medium which β-casein was dissolved to the final concentration of 1 μg/mL, and maintained in 37°C. The content of β-casein was determined after 8 h.

Absorbance at 450 and 630 nm was read on a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA). All measurements were performed in triplicate, and the mean value of the 3 independent measurements was used for statistical analysis.

## Western Blot

Western blot was used to measure the indicated proteins and phosphorylated proteins as described (53). Briefly, BMECs were managed as four groups, i.e., control cells (uninfected cells), cells were infected by *S. aureus* 2, 4, and 8 h, respectively. Four groups of cells were culture in medium simultaneously, and then three infected groups were inoculated with *S. aureus* at different time points and continued in co-culture. Finally, the cells were harvested with trypsin at the same time. The harvested BMECs were washed with cold PBS, and lysed in cell lysis buffer. The lysis buffer comprised 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, PMSF, and phosphatase inhibitors. Equal amounts (40 μg) of protein were electrophoresed on 10% (w/v) sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated with the primary antibody. Peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) reagent were used to detect the signals with the Western Blotting System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The bands were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA).

## RT-qPCR

RT-qPCR was performed to measure *EAAT1/GLAST/SLC1A3 and LAT1/SLC7A5* in BMECs in the treatment and control groups. Cells were infected with *S. aureus* for 2, 4, and 8 h or treated with 100 nM rapamycin for 8 h, and total RNA was extracted from untreated and treated cells. Total RNA was prepared with RNAiso Plus per the manufacturer's instructions (9109, TaKaRa Co. Ltd., Dalian, China). Briefly, the cells were washed with PBS and lysed in RNAiso Plus, and chloroform was added to the cell lysates for homogenization; the top aqueous layer was transferred to a new tube after centrifugation, and isopropanol was added to the supernatant and mixed well. Total RNA was precipitated by centrifugation, and the pellet was dissolved in RNase-free water.

mRNA was reverse-transcribed with oligo $(dT)_{12-18}$ primer using the AMV first Strand cDNA Synthesis Kit (Takara Co. Ltd., China). cDNA sequences were amplified with the primers in **Supplementary Table S1**. The KAPA SYBP FAST qPCR Kit Optimized for LightCycler 480 (KAPA, Inc., Boston, MA, USA) was used for the PCR with the primers (**Supplementary Table S1**), according to the manufacturer's instructions. The program comprised an initial denaturation step at 95°C for 5 min; 40 cycles of 95°C for 5 s, 54°C for 30 s, and 72°C for 20 s; and a final extension of 72°C for 10 min. Three technical replicates were run in each experiment. 2-ΔΔCT values were calculated to determine expression levels, and the qPCR results were compared by student's *t*-test between untreated and treated groups. Three independent experiments were performed.

## Immunofluorescence

Cells were seeded onto a slide, incubated overnight, and infected with *S. aureus* for 2, 4, and 8 h. After being washed with PBS and fixed with 4% paraformaldehyde for 15 min, the cells were blocked with 1% BSA for 1 h. Then, the cells were incubated with primary antibodies against SLC1A3 and SLC7A5, p-STAT5 and p-NF-κB p65 at 4°C overnight and FITC-labeled goat anti-rabbit IgG for 1 h at room temperature. DAPI was used to stain the nucleus. Finally, the slide was mounted with glycerin and examined under a laser scanning confocal microscope (NIKON A1R, Nikon Corp., Tokyo, Japan).

## Adhesion Assays

Adhesion assay of *S. aureus* was achieved in two phases. First, BMECs were infected with *S. aureus* at MOI 30 for 30, 60, and 90 min, respectively. End of infection, BMECs were continued

and maintained for 8 h in medium with antibiotics and lysozyme. After incubation, BMECs were harvested with trypsin and washed softly three times with PBS to remove extracellular dead bacteria, and then lysed using lysis buffer. The number of intracellular bacteria was determined by bacterial colony count. Second, BMECs were infected with *S. aureus* for 30 min at MOI 30, and then the cells were cultured for 8 h in a medium with antibiotics and lysozyme. After incubation, BMECs were harvested with trypsin and washed softly three times with PBS to remove non-adherent bacteria, remaining bacteria considered to be adherent but not internalized in cells, and then lysed using lysis buffer. The bacteria were evaluated by bacterial colony count, which were considered as adherent bacteria.

## Apoptosis Analysis

BMECs were infected with *S. aureus* at MOI 30 for 2 h, then the antibiotics and lysozyme were used to kill and lyse the extracellular bacteria. The intracellularly infected cells were continued in culture and maintained in medium for 8 h, and then the apoptosis were assessed with the Hoechst 33342 Staining Kit and FITC Annexin V Apoptosis Detection Kit according to the manufacturer's instructions, respectively. Following treatment, cells were stained with Hoechst for 5 min and washed with PBS, followed by observation under a fluorescence microscope (Observer A1, Zeiss, Oberkochen, Germany). For the flow cytometry assay, cells were collected after treatment, and washed with PBS, and then stained with FITC-Annexin V and PI. Cells were subsequently analyzed using flow cytometry (Cytoflex, Beckman, CA, USA).

## Statistical Analysis

Statistical analyses were conducted using SPSS PASW Statistics for Windows, v18.0 (SPSS Inc.: Chicago, IL, USA). Data were analyzed using standard parametric statistics and one-way ANOVA, followed by Tukey's method. Data are expressed as mean ± SD. The results are presented as the average of at least 3 independent experiments. Western blot results were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA). Statistical significance was accepted when $p \leq 0.05$.

# RESULTS

## *Staphylococcus aureus* Invasion Suppresses Milk Protein Synthesis and Prevents Uptake of Exogenous Amino Acids in BMECs

In order to confirm *S. aureus* can be internalized by BMECs, we infected BMECs with *S. aureus* for 2 h at MOI 30 and then killed and lysed the extracellular bacteria with antibiotics and lysozyme. The infected cells were maintained in medium for 2-8 h, and the bacteria were evaluated intracellularly and extracellularly by bacterial colony count. The results showed that $1.5 \times 10^3$ CFU/mL − $5.1 \times 10^3$ CFU/mL were counted in whole BMEC lysates, whereas extracellular bacteria were not found in the culture medium (**Supplementary Table S2**), indicating that intracellular *S. aureus* proliferated. Further, to confirm *S. aureus* invasion of BMECs, we stained *S. aureus* with fluorescent dye and observed the bacteria under a laser scanning confocal microscope (LSCM) (**Supplementary Figure S1A**). *S. aureus* was also found in cytosolic vacuoles in BMECs by TEM (**Supplementary Figure S1B**). These results indicate that *S. aureus* was internalized by BMECs *in vitro*.

To examine whether *S. aureus* invasion suppresses the synthesis of milk protein in BMECs, cells were infected with *S. aureus* at 30 MOI for 2 h, and extracellular bacteria were then killed and lysed with antibiotics and lysozyme. The infected cells were maintained in medium for 8 h, and β-casein, α-lactalbumin, and β-lactoglobulin were determined by ELISA. The levels of β-casein, α-lactalbumin and β-lactoglobulin decreased intracellularly (**Figures 1A–C**) and in medium (**Figures 1D–F**), indicating that *S. aureus* invasion inhibits the synthesis of milk protein in BMECs.

Considering milk protein can be degraded by endogenous protease or bacterial enzymes, to eliminate the possibility of milk proteins were degraded by these enzymes, we first determined the level of endogenous protease Plasmin by ELISA in *S. aureus* infected cells, and found that the level of Plasmin was not increased in the culture medium and in cells of the *S. aureus*-infected cells, compared to control (**Supplementary Figure S2A**). Next, to determine whether β-casein was directly degraded by *S. aureus* during infection of 8 h, we simulated the conditions under which *S. aureus* infected BMECs, i.e., $1.5 \times 10^3$ CFU/mL *S. aureus* (**Supplementary Table S2**) were inoculated into DMEM/F12 medium which β-casein, and the content of β-casein was determined by ELISA after 8 h. Comparing to the control, the level of β-casein did not show significant decline in infected group (**Supplementary Figure S2B**). These data indicate that the decrease in milk protein content was caused by intracellular infection of *S. aureus*, rather than by both endogenous and bacterial enzymes. Then, to eliminate the possibility of apoptosis induced by *S. aureus* leading to the decrease of milk protein, we examined apoptosis 8 h after *S. aureus* infection, and the results showed that no apoptosis was found in BMECs (**Supplementary Figures S3A–C**), suggesting that the decrease of milk protein was not caused by apoptosis.

Epithelial cells are the central component of bovine mammary alveoli, which produce milk during lactation. Mammary epithelial cells are considered to derive amino acids from blood to synthesize milk proteins. Thus, we tested whether *S. aureus* invasion prevents cells from absorbing amino acids from the culture medium. Control and *S. aureus*-infected BMECs were subjected to serum and amino acid starvation, after which Glu, Asp, and Leu were added to the medium and measured by ELISA. The levels of Glu, Asp, and Leu in the medium of *S. aureus*-infected cells was significantly higher than that in the control (**Figures 2A–C**), indicating that *S. aureus* prevents BMECs from taking up amino acids from the culture medium.

## Exogenous Amino Acids Induce Casein Synthesis and mTORC1 Activation

Amino acids initiate mTORC1 signaling to promote protein synthesis. Thus, we speculated that mTORC1 activation and

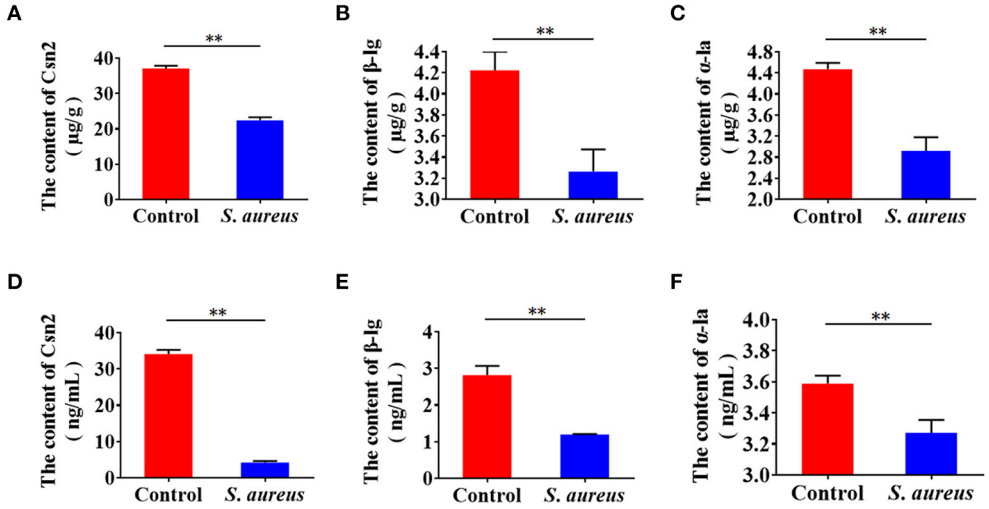


**FIGURE 1 |** *Staphylococcus aureus* invasion suppresses milk protein synthesis and secretion in BMECs 8 h after infection. **(A–C)** Levels of intracellular Csn2 (β-casein), β-lg, and α-la. **(D–F)** Levels of Csn2 (β-casein), β-lg and α-la in cell culture medium. $^{**}p < 0.01$. $n = 3$ independent experiments.

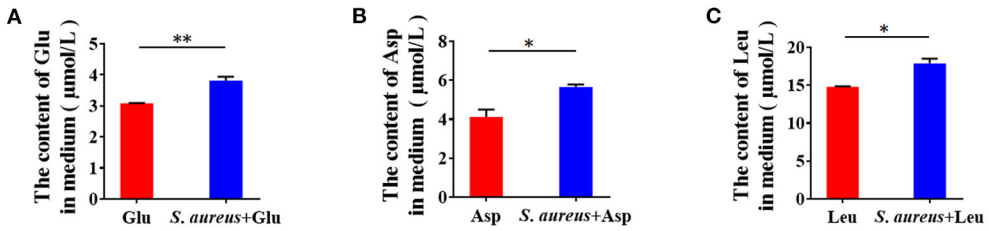


**FIGURE 2 |** *Staphylococcus aureus* invasion prevents amino acid uptake in BMECs 8 h after infection. **(A–C)** Glu **(A)**, Asp **(B)**, and Leu **(C)** content in medium. $^{*}p < 0.05$; $^{**}p < 0.01$. $n = 3$ independent experiments.

milk protein synthesis are stimulated by exogenous amino acids in BMECs. We treated serum- and amino acid-starved cells with Glu, Asp, and Leu and measured mTORC1 activation and β-casein (Csn 2) in BMECs. The results showed that mTORC1 activation (**Figures 3A–C**) and β-casein (**Figure 3D**) were increased in BMECs, demonstrating that exogenous amino acids initiate mTORC1 activation and induce β-casein (Csn 2) synthesis.

## *Staphylococcus aureus* Invasion Suppresses Amino Acid Induced-Casein Synthesis

To characterize the suppression of amino acid-induced casein synthesis by *S. aureus*, β-casein was measured in four groups of BMECs: control, amino acid-treated (Glu, Asp, Leu), *S. aureus* invasion, and *S. aureus* invasion with amino acids (Glu, Asp, Leu). Exogenous amino acids significantly increased β-casein concentrations, an effect that *S. aureus* infection mitigated (**Figures 4A–C**). These data indicate that *S. aureus* invasion suppresses amino acid-induced casein synthesis.

## *Staphylococcus aureus* Invasion Downregulates Amino Acid Transporter Genes and the Phosphorylation of NF-κB, STAT5, mTOR, and S6

To validate the underlying mechanism by which *S. aureus* invasion prevents amino acid uptake and amino acid-induced casein synthesis in BMECs, we examined the expression of *SLC1A3*(*EAAT1/GLAST*) and *SLC7A5*(*LAT1*) by RT-qPCR. The mRNA levels of *SLC1A3* and *SLC7A5* increased at 2 and 4 h and declined at 8 h in *S. aureus*-invaded BMECs (**Figures 5A,B**). SLC1A3 and SLC7A5 were detected by western blot and immunofluorescence, following the same trend as the mRNA levels in *S. aureus*-infected BMECs (**Figures 5C–E**). These results suggest that *S. aureus* invasion impairs the expression of amino acid transporter genes at the mRNA and protein levels in BMECs.

Based on the findings, we speculated that certain transcription factors that are related to these genes are also impaired in *S. aureus*-invaded BMECs. To identify transcription factors for *SLC1A3* and *SLC7A5*, we performed a bioinformatic analysis, which predicted NF-κB and STAT5 (**Supplementary Figure S4**, **S5**). Further, we examined the phosphorylation of NF-κB

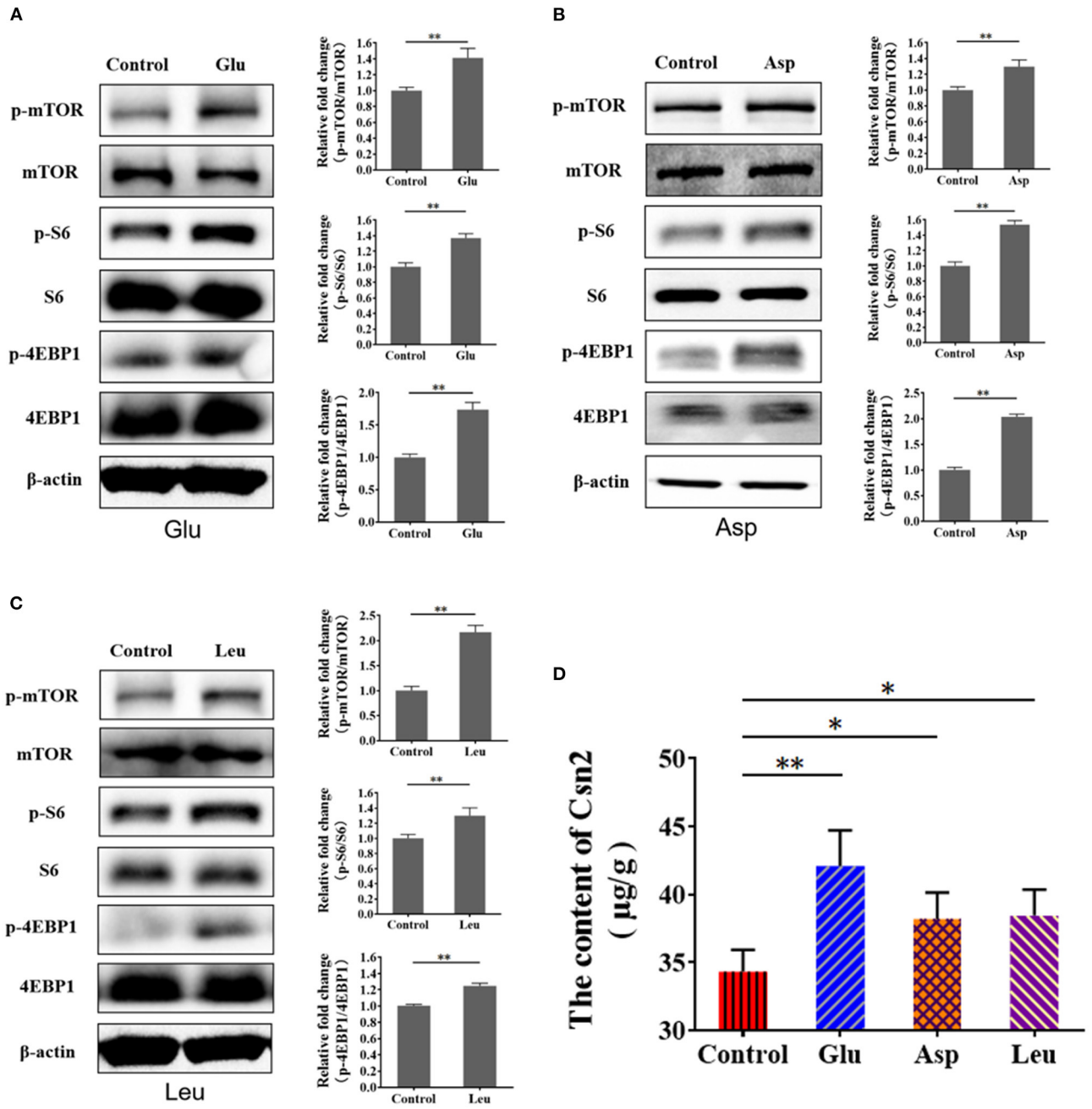


**FIGURE 3 |** Exogenous amino acid promotes mTORC1 activation and CSN2 (β-casein) synthesis. **(A–C)** Western blot of mTORC1 activation after BMEC stimulation with Glu **(A)**, Asp **(B)**, and Leu **(C)**. Phosphorylation of mTOR, S6, and 4EBP1. **(D)** Intracellular Csn2 (β-casein). The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). $^{*}p < 0.05$; $^{**}p < 0.01$. $n = 3$ independent experiments.

and STAT5 and nuclear localization by western blot and immunofluorescence. We found that the phosphorylation of NF-κB and STAT5 was reduced, and the nuclear translocation of phosphorylated NF-κB p65 and STAT5 was also attenuated at 8 h after bacterial infection (**Figures 6A–C**). These data suggest that *SLC1A3* and *SLC7A5* expression is directed by NF-κB and STAT5 in BMECs. The experiments above demonstrate that exogenous amino acids induce mTORC1 signaling and that *S. aureus* invasion prevents the uptake of exogenous amino acids. Thus, we speculated that *S. aureus* invasion decreases the activity of mTORC1 in BMECs. In *S. aureus*-infected BMECs, the phosphorylation of mTOR and S6 fell 8 h after

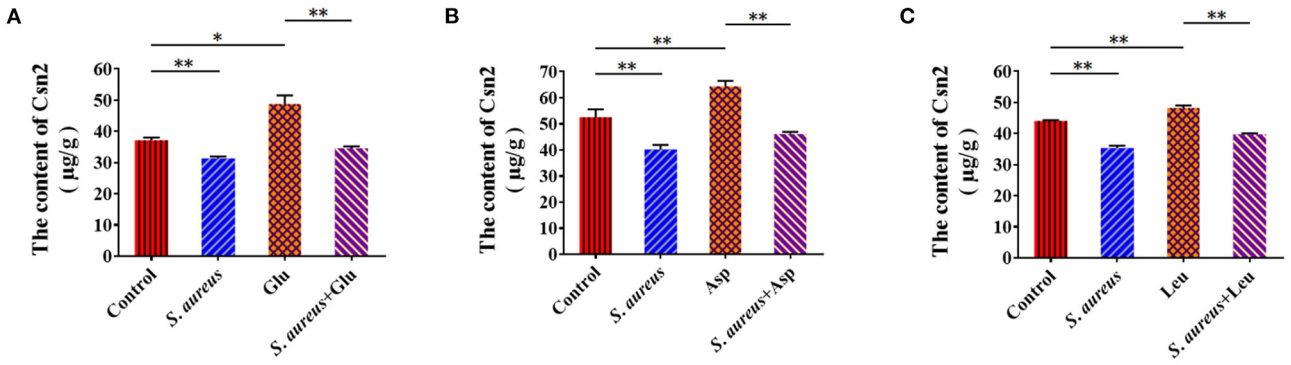


**FIGURE 4 |** *Staphylococcus aureus* invasion suppresses amino acid induced-casein synthesis. Intracellular Csn2 (β-casein) content **(A)** Glu, **(B)** Asp, and **(C)** Leu. *$p < 0.05$; **$p < 0.01$. $n = 3$ independent experiments.

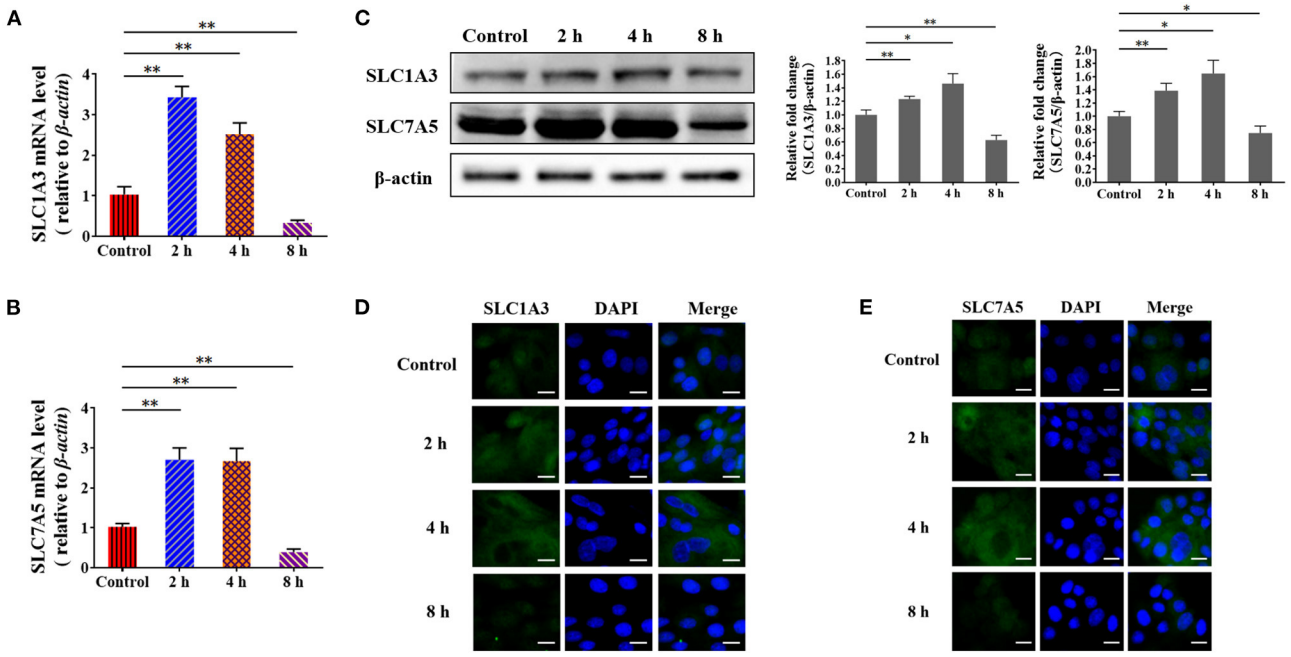


**FIGURE 5 |** *Staphylococcus aureus* infection impairs the expression of amino acid transporter genes at 8 h. **(A,B)** mRNA levels of *SLC1A3* **(A)** and *SLC7A5* **(B)** in *S. aureus*-infected BMECs. **(C)** Protein levels of SLC1A3 and SLC7A5 in *S. aureus*-infected BMECs. **(D,E)** Immunofluorescence assay of SLC1A3 **(D)** and SLC7A5 **(E)** in infected BMECs and control. Representative confocal microscopy images of SLC1A3 and SLC7A5 (Green) in cells that were co-stained with DAPI (blue). Scale bars represent 20 μm. The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). *$p < 0.05$; **$p < 0.01$. $n = 3$ independent experiments.

invasion (**Figure 6D**), indicating that mTORC1 is involved in the expression of *SLC1A3* and *SLC7A5*.

Considering non-invasive *S. aureus* is also very important in mastitis, to eliminate the possibility of *S. aureus* adhesion to affect the activity of mTORC1, and NF-κB and STAT5, and the expression of SLC1A3 and SLC7A5, we first examined adhesion of *S. aureus* to BMECs, and found that the bacteria only adhered to BMECs but failed to internalize within 30 min. Next, BMECs were infected with *S. aureus* for 30 min, and then the cells were cultured for 8 h in a medium with antibiotics and lysozyme. The content of β-casein, α-lactalbumin, and β-lactoglobulin in cell culture medium was determined by ELISA, and the expression of the targeting proteins by Western blot. The results showed that there was no significant difference between the bacterial adhesion group and the control group (**Supplementary Figures S6A–C**), indicating that *S. aureus* adhesion has no effect on the milk protein synthesis, mTORC1 signaling, the activity of NF-κB p65 and STAT5, and the expression of SLC1A3 and SLC7A5. Further, to eliminate the possibility of *S. aureus* toxin effect on milk protein synthesis, alpha-hemolysin (Hlα), which is the most abundant toxin in *S. aureus*, was determined by ELISA after infection

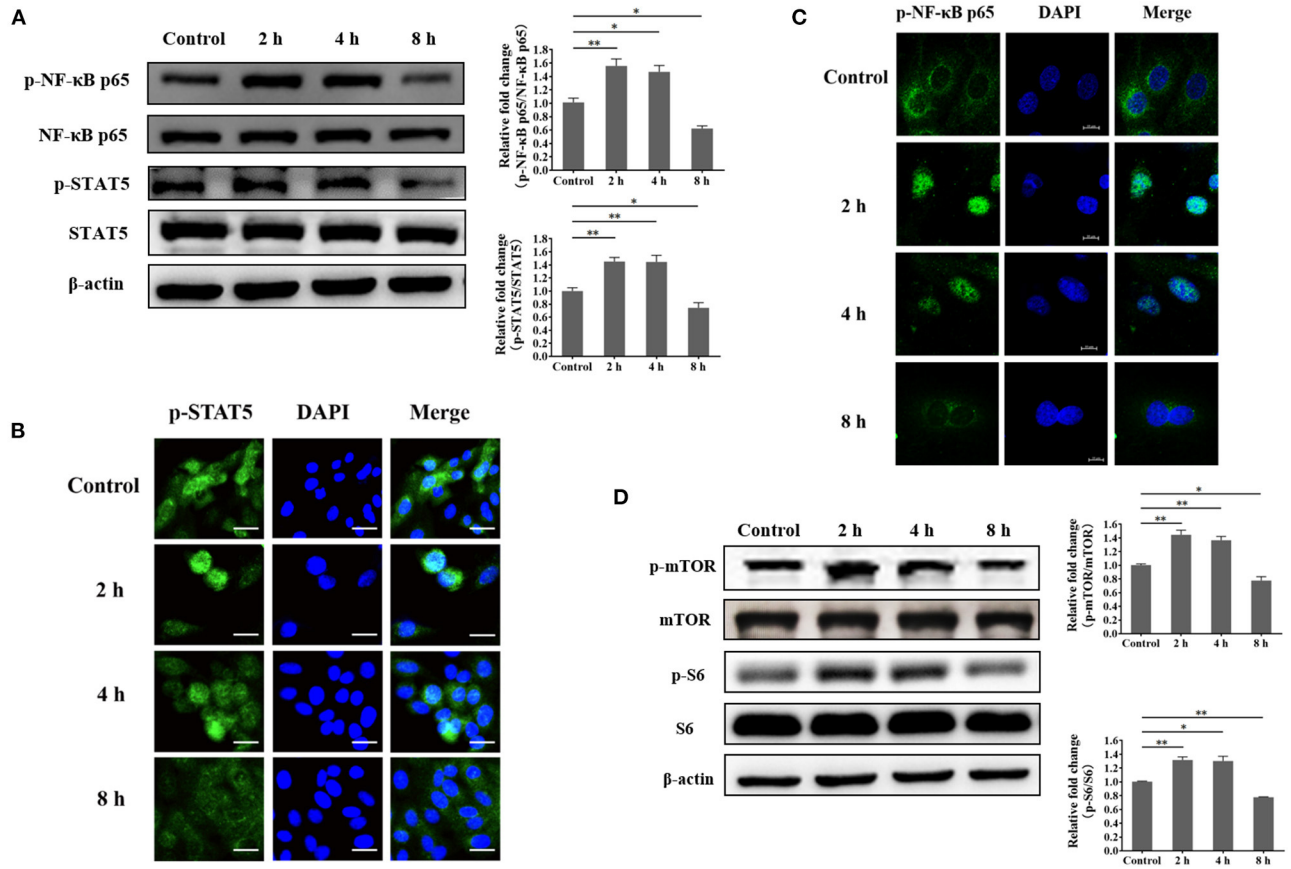


**FIGURE 6 |** *Staphylococcus aureus* infection suppresses the phosphorylation of NF-κB p65, STAT5, mTOR, and S6 at 8 h. **(A)** Phosphorylation of NF-κB p65 and STAT5 in *S. aureus*-invaded BMECs. **(B)** Nuclear localization of phosphorylated STAT5 in *S. aureus*-invaded BMECs. Representative confocal microscopy images of the level of p-STAT5 (Green) in cells that were co-stained with DAPI (blue). Scale bars represent 20 μm. **(C)** Nuclear localization of phosphorylated NF-κB p65 in *S. aureus*-invaded BMECs. Representative confocal microscopy images of the level of p-NF-κB p65 (Green) in cells that were co-stained with DAPI (blue). Scale bars represent 10 μm. **(D)** mTORC1 signaling in *S. aureus*-invaded BMECs. The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). $^{*}p < 0.05$; $^{**}p < 0.01$. $n = 3$ independent experiments.

8 h. The results showed that α-hemolysin was not detectable both in cell medium and cells (**Supplementary Figure S7**), suggesting that toxins were not produced by *S. aureus* within 8 h.

## mTORC1 Regulates the Expression of *SLC1A3* and *SLC7A5* Through NF-κB and STAT5 in BMECs

To verify the mTORC1 pathway regulates the expression of *SLC1A3* and *SLC7A5* through NF-κB and STAT5 is being affected during *S. aureus* infection, and demonstrate the function of mTORC1 signaling in the expression of *SLC1A3* and *SLC7A5* in BMECs, cells were managed as four groups, i.e., control cells, cells were infected by *S. aureus* 8 h, cells were treated by 100 nM rapamycin 8 h and cells were both infected by *S. aureus* and treated by 100 nM rapamycin 8 h, respectively. The phosphorylation of NF-κB and STAT5 and *SLC1A3* and *SLC7A5* expression were examined by western bolt and RT-qPCR. The results showed that the phosphorylation of mTOR, S6, NF-κB, and STAT5 was inhibited by *S. aureus* and rapamycin in BMECs (**Figure 7A**), and *SLC1A3* and *SLC7A5* mRNA and protein were downregulated by *S. aureus* and rapamycin (**Figures 7A,B**), indicating that mTORC1 pathway was inhibited during the *S. aureus* infection, and mTORC1 regulates the expression of *SLC1A3* and *SLC7A5* via NF-κB and STAT5.

Next, to confirm the function of mTORC1 in milk protein synthesis, we measured β-casein, α-lactalbumin, and β-lactoglobulin intracellularly and in the culture medium. Milk proteins synthesis (**Figures 8A–C**) and secretion (**Figures 8D–F**) were lower, indicating that mTORC1 signaling controls milk protein synthesis in BMECs. These data demonstrate that mTORC1 governs milk protein synthesis by regulating the expression of *SLC1A3* and *SLC7A5* in BMECs.

## DISCUSSION

*S. aureus* is the most prevalent microorganism in intramammary infections (IMIs) in dairy herds. This bacteria enters the udder and colonizes mammary tissues or invades cells, including

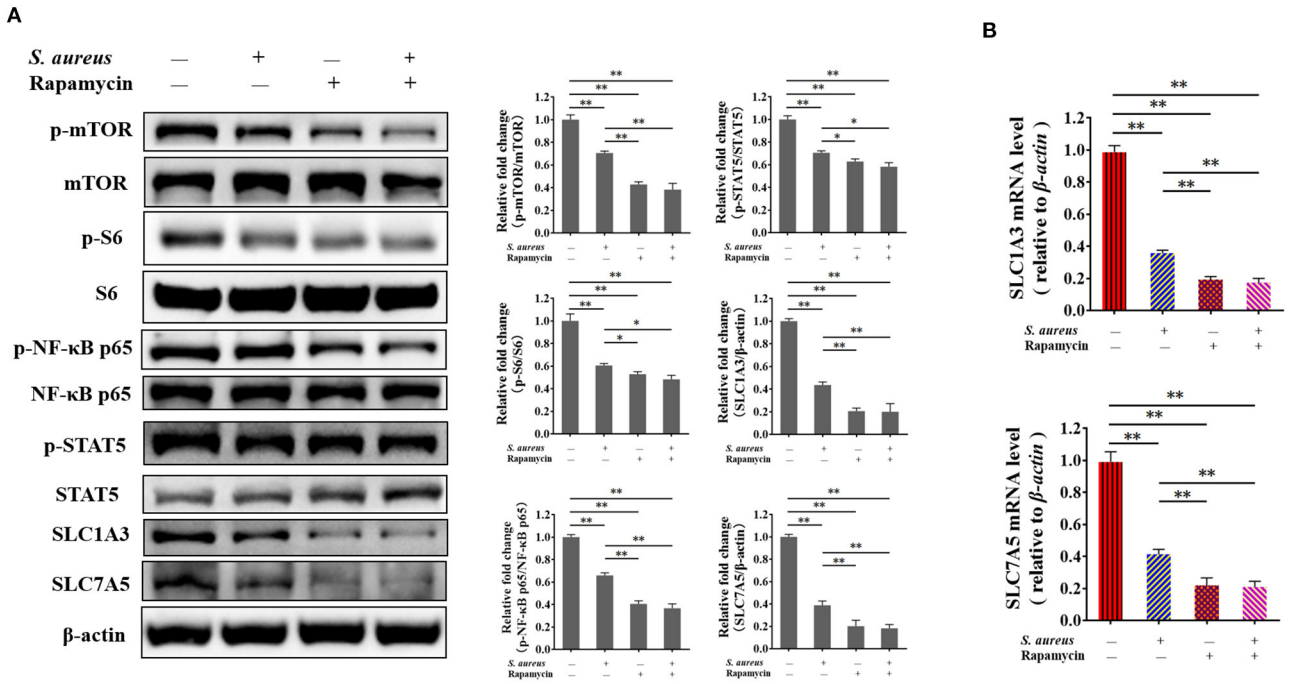


**FIGURE 7 |** Inhibition of mTORC1 by *S. aureus* infection and rapamycin attenuates the phosphorylation of NF-κB p65 and STAT5 and the expression of *SLC1A3* and *SLC7A5*. The phosphorylation of mTOR, S6, NF-κB p65, and STAT5 was detected by western blot, and *SLC1A3* and *SLC7A5* were examined by RT-qPCR and western blot. **(A)** Phosphorylation of mTOR, S6, NF-κB p65, and STAT5 and levels of SLC1A3 and SLC7A5 in different BMECs treatment groups. **(B)** mRNA levels of *SLC1A3* and *SLC7A5* in different BMECs treatment groups. mTORC1 pathway was inhibited during *S. aureus* infection. The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). $^{*}p < 0.05$; $^{**}p < 0.01$. $n = 3$ independent experiments.

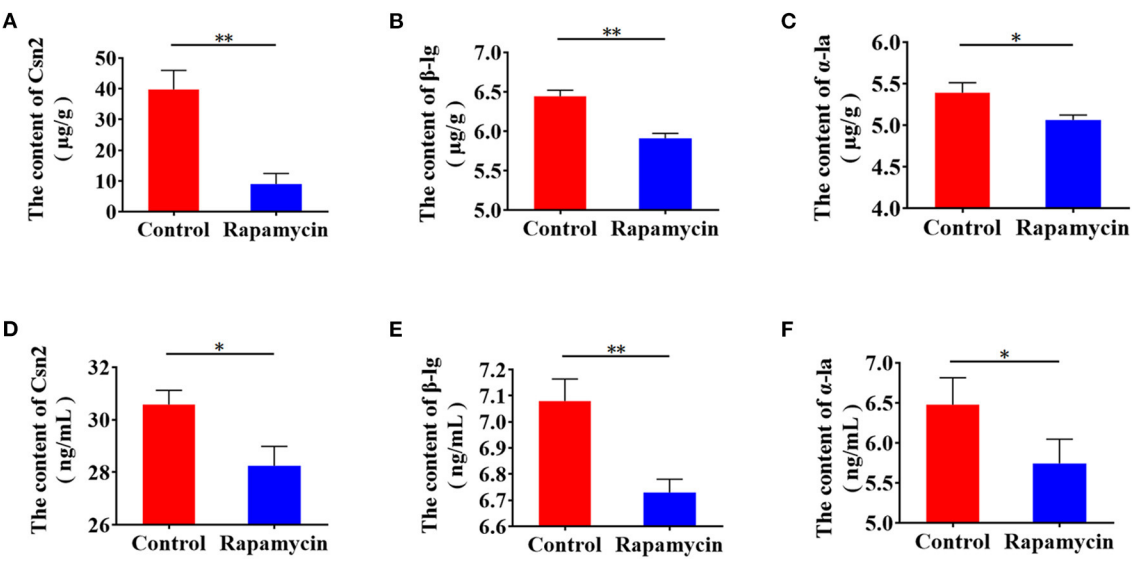


**FIGURE 8 |** Rapamycin suppresses milk protein synthesis. **(A–C)** Intracellular Csn2 (β-casein), β-lg, and α-la after rapamycin treatment for 8 h by ELISA. **(D–F)** Csn2 (β-casein), β-lg, and α-la in cell culture medium. $^{*}p < 0.05$; $^{**}p < 0.01$. $n = 3$ independent experiments.

mammary epithelial cells (54, 55). Bacteria that live in cells often cause subclinical and chronic mastitis due to their resistance to antibiotics and ability to evade phagocytosis by neutrophils (56, 57). This mastitis decreases milk production and milk quality in dairy cows (13, 14). In this study, we found that *S. aureus* causes intracellular infections in BMECs *in vitro*. *S. aureus*

was internalized by BMECs over 8 h, decreasing milk protein synthesis. Further, *S. aureus* invasion affected mTORC1 signaling, and mTORC1 was activated 2 and 4 h after bacterial invasion but inhibited at 8 h. This pattern of mTORC1 activation is consistent with that of milk protein synthesis. These data indicate that the decrease in milk protein synthesis due to *S. aureus* invasion is related to mTORC1 signaling.

It is believed that a few mechanisms are involved in the decreased of milk production and milk quality in dairy cows suffer from mastitis (58), e.g., toxins, and endogenous and bacterial proteases (18, 59–61). In the present study, we found that α-hemolysin, which is the most abundant toxin in *S. aureus*, was not detectable both in cell medium and cells (**Supplementary Figure S7**), and the level of endogenous protease Plasmin was not increased in *S. aureus* infected cells (**Supplementary Figure S2A**). Meanwhile, we found that $1.5 \times 10^3$ CFU/mL *S. aureus* were inoculated into medium with β-casein for 8 h, the level of β-casein did not show significant decline in infected group (**Supplementary Figure S2B**). These data mean that the depression of milk protein synthesis were not caused by toxins or endogenous and bacterial enzymes. Moreover, it is known that non-invasive *S. aureus* strains are also very important in *S. aureus*-mastitis. In our study, we examined the time point which *S. aureus* only adhered to BMECs but failed to internalize by referring to the Ménard's method (62). We found that *S. aureus* adhesion has no effect on the milk protein synthesis, mTORC1 signaling, the activity of NF-κB p65 and STAT5, and the expression of SLC1A3 and SLC7A5 (**Supplementary Figure S6**). These data demonstrated that intracellular infection of *S. aureus* caused the depression of milk protein synthesis. However, the limitation is that only one intracellular *S. aureus* strain was being consider in our work. Although the adhesion experiment was carried out in the present study, we need to reconsider non-invasive *S. aureus* strain along our work.

BMECs synthesize and secrete milk in mammary tissue and must derive exogenous amino acids from extracellular fluid to synthesize milk protein (44–46), for which various types of membrane amino acid transporters take up amino acids (47, 48). SLC (solute carrier) transporters function in many essential processes, including nutrient uptake, ion influx/efflux, and waste disposal (63). SLC1A3, also known as EAAT1 ($Na^+$-dependent excitatory amino acid transporter 1) and GLAST (glutamate–aspartate transporter), has glutamate and aspartate as substrates (47). SLC7A5, also called LAT1 (L-type amino acid transporter 1), is the transport-competent unit of the LAT1/CD98 heterodimeric amino acid transporter (64) and is indispensable as a transporter of essential amino acids to maintain cell growth and protein synthesis (48). In recent years, it has been reported that amino acid transporters are related to milk protein synthesis (50, 51), but whether bacterial infection of BMECs affects the uptake of amino acid and amino acid transporter expression is unknown. In our study, we examined the expression of SLC1A3 and SLC7A5, where SLC1A3 is the transporter of Glu and Asp, and SLC7A5 is the transporter of Leu (47). Meanwhile, it is known that Glu, Asp and Leu are associated with lactation in dairy cows (65). Thus, the cells were treated with amino acids Glu, Asp and Leu, respectively, to evaluate their effects on milk protein synthesis in BMECs. Furthermore, *S. aureus* infection attenuated the expression of the amino acid transporter genes *SLC1A3* and *SLC7A5* and prevented BMECs from deriving Glu, Asp, and Leu from the culture medium, impeding amino acid induced-casein synthesis. These data indicate that *S. aureus* infection downregulates amino acid transporter genes, which are important in milk protein synthesis in BMECs.

NF-κB is a key transcription factor of inflammation-related genes and regulates the expression of EAAT1 in primary rat astrocytes and human astrocytes (66). STAT5 is critical in prolactin-induced beta-casein transcription in rodents and bovine mammary explant cultures (67), and LPS inactivates STAT5 in mouse mammary glands (68). In our study, NF-κB and STAT5 were inactivated 8 h after *S. aureus* invasion. Based these data, we conclude that *S. aureus* infection prevents amino acid uptake to suppress milk protein synthesis through impaired expression of *SLC1A3* and *SLC7A5,* which is mediated by NF-κB and STAT5 in BMECs.

## CONCLUSION

*S. aureus* can be internalized by BMECs *in vitro*, and the internalized bacteria can undergo intracellular proliferation. Milk proteins were suppressed 8 h after *S. aureus* invasion. *S. aureus* invasion downregulated the amino acid transporter genes *SLC1A3* and *SLC7A5*, impaired absorption of amino acids by BMECs from the culture medium, decreased exogenous amino acid-induced β-casein synthesis, and attenuated mTORC1 signaling. Rapamycin inhibited the activation of NF-κB and STAT5, the expression of *SLC1A3* and *SLC7A5,* and milk protein synthesis. The mechanism by which *S. aureus* infection depresses milk protein synthesis in BMECs is likely *S. aureus* invasion-mediated attenuation of mTORC1 signaling and SLC1A3 and SLC7A5 expression, resulting in suppression of amino acid uptake and milk protein synthesis.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Inner Mongolia University Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

ZW and YW proposed the initial experiments and analyzed the experimental feasibility. YC, YM, and XF performed the experiments. TL participated in the design of experimental technical route. XY cultured the primary BMECs. QJ, RY, and

XC analyzed the experimental data together. YC wrote the final manuscript. YW revised the final manuscript. All authors approved the final article.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.756375/full#supplementary-material

**Supplementary Figure S1 |** *Staphylococcus aureus* invades BMECs. BMECs were infected with *S. aureus* for 2 h, and the cells were cultured in medium supplemented with antibiotics and lysozyme to kill and lyse the extracellular bacteria. **(A)** Intracellular *S. aureus* (green) stained with CFSE by laser confocal microscopy; BMEC nuclei were co-stained with DAPI (blue), and actin was stained with phalloidin (red). Scale bars represent 20 μm. **(B)** *S. aureus* was internalized by BMECs, based on micrographs obtained by TEM; several important observations are magnified. Red arrows indicate *S. aureus*. a Control, uninfected BMECs, b *S. aureus* in cytosolic vacuoles by TEM in BMECs. Scale bars represent 2 μm. $N = 3$ independent experiments.

**Supplementary Figure S2 |** *Staphylococcus aureus* adhesion does not affect the contents of protease Plasmin in BMECs 8 h after infection, and β-casein was not degraded by *S. aureus*. **(A)** Levels of Plasmin (Pla) in intracellular and cell culture medium. **(B)** Levels of Csn2 (β-casein) in co-incubated culture medium. ns $p > 0.05$. $n = 3$ independent experiments.

**Supplementary Figure S3 |** Cell apoptosis was not found in BMECs after *Staphylococcus aureus* infection 8 h. **(A)** Hoechst assay was used to examine apoptosis in infected BMECs and control. **(B)** FITC annexin V apoptosis detection was used to examine apoptosis in infected BMECs and control. **(C)** The expression levels of Caspase 3 and cleaved Caspase 3 in BMECs were examined by Western blotting. The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). ns $p > 0.05$. $n = 3$ independent experiments.

**Supplementary Figure S4 |** Putative transcription factor binding sites (TFBSs) for NF-κB and STAT5 in the promoters of *SLC1A3* and *SLC7A5*. NF-κB TFBS in the promoter sequences of **(A)** *SLC1A3* and **(B)** *SLC7A5*. STAT5a TFBS in the promoter sequences of **(C)** *SLC1A3* and **(D)** *SLC7A5*.

**Supplementary Figure S5 |** Putative transcription factor binding motifs (TFBMs) for NF-κB and STAT5 in the promoters of *SLC1A3* and *SLC7A5* (red boxes represent motif). NF-κB TFBM in the promoter sequence of **(A)** *SLC1A3* (−1878, −1848, −1797, −1780,−1749, −1276, −524, −102, −80, 79, 80, $p < 0.001$) and **(B)** *SLC7A5* (−159,−211,−396, −507,−605, −692, −737, −782, −1073, −1284, −1322, −1371,−1573, $p < 0.001$). STAT5a TFBM in the promoter sequence of **(C)** *SLC1A3* (-1432, −1254,−1005,−844,−442, −354, −114, 75, $p < 0.001$) and **(D)** *SLC7A5* (-1018,−1067,−1206,−1768, $p < 0.001$). TFBM of **(E)** NF-κB and **(F)** STAT5a in the Jaspar Database.

**Supplementary Figure S6 |** *Staphylococcus aureus* adhesion has no effect on the milk protein synthesis, mTORC1 signaling, the activity of NF-κB p65 and STAT5, and the expression of SLC1A3 and SLC7A5. **(A)** Levels of Csn2 (β-casein), β-lg and α-la in cell culture medium. **(B,C)** Phosphorylation of mTOR, S6, NF-κB p65, and STAT5 and the expression of SLC1A3 and SLC7A5. ns $p > 0.05$. $n = 3$ independent experiments.

**Supplementary Figure S7 |** *Staphylococcus aureus* toxin alpha-hemolysin (Hlα) was determined by ELISA after infection 8 h. The α-hemolysin was not detectable both in cell medium and cells. ns $p > 0.05$. $n = 3$ independent experiments.

**Supplementary Table S1 |** The target genes and primers for qPCR.

**Supplementary Table S2 |** The colon number of *S. aureus* in BMECs and cells medium ($\bar{x} \pm$ SD).

## REFERENCES

1. Ramírez-Rivera EJ, Rodríguez-Miranda J, Huerta-Mora IR, Cárdenas-Cágal A, Juárez-Barrientos JM. Tropical milk production systems and milk quality: a review. *Trop Anim Health Prod.* (2019) 51:1295–305. doi: 10.1007/s11250-019-01922-1
2. Singhal S, Baker RD, Baker SS. A comparison of the nutritional value of cow's milk and nondairy beverages. *J Pediatr Gastroenterol Nutr.* (2017) 64:799–805. doi: 10.1097/MPG.0000000000001380
3. Farrell HM Jr, Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK, et al. Nomenclature of the proteins of cows' milk–sixth revision. *J Dairy Sci.* (2004) 87:1641–74. doi: 10.3168/jds.S0022-0302(04)73319-6
4. Maurmayr A, Pegolo S, Malchiodi F, Bittante G, Cecchinato A. Milk protein composition in purebred Holsteins and in first/second-generation crossbred cows from Swedish Red, Montbeliarde and Brown Swiss bulls. *Animal.* (2018) 12:2214–20. doi: 10.1017/S1751731117003640
5. Bonfatti V, Grigoletto L, Cecchinato A, Gallo L, Carnier P. Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *J Chromatogr A.* (2008) 1195:101–6. doi: 10.1016/j.chroma.2008.04.075
6. Brinkmann J, Jagannathan V, Drögemüller C, Rieder S, Leeb T, Thaller G, et al. Genetic variability of the equine casein genes. *J Dairy Sci.* (2016) 99:5486–97. doi: 10.3168/jds.2015-10652
7. Holt C, Carver JA, Ecroyd H, Thorn DC. Invited review: Caseins and the casein micelle: their biological functions, structures, and behavior in foods. *J Dairy Sci.* (2013) 96:6127–46. doi: 10.3168/jds.2013-6831
8. Whist AC, Osterås O, Sølverød L. Association between isolation of *Staphylococcus aureus* one week after calving and milk yield, somatic cell count, clinical mastitis, and culling through the remaining lactation. *J Dairy Res.* (2009) 76:24–35. doi: 10.1017/S0022029908003592
9. Heikkilä AM, Liski E, Pyörälä S, Taponen S. Pathogen-specific production losses in bovine mastitis. *J Dairy Sci.* (2018) 101:9493–504. doi: 10.3168/jds.2018-14824
10. Botaro BG, Cortinhas CS, Dibbern AG. e Silva LF, Benites NR, Dos Santos MV. Staphylococcus aureus intramammary infection affects milk yield and SCC of dairy cows. *Trop Anim Health Prod.* (2015) 47:61–6. doi: 10.1007/s11250-014-0683-5

11. Tesfaye GY, Regassa FG, Kelay B. Milk yield and associated economic losses in quarters with subclinical mastitis due to *Staphylococcus aureus* in Ethiopian crossbred dairy cows. *Trop Anim Health Prod.* (2010) 42:925–31. doi: 10.1007/s11250-009-9509-2
12. Gröhn YT, Wilson DJ, González RN, Hertl JA, Schulte H, Bennett G, et al. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *J Dairy Sci.* (2004) 87:3358–74. doi: 10.3168/jds.S0022-0302(04)73472-4
13. Nagasawa Y, Kiku Y, Sugawara K, Tanabe F, Hayashi T. Exfoliation rate of mammary epithelial cells in milk on bovine mastitis caused by *Staphylococcus aureus* is associated with bacterial load. *Anim Sci J.* (2018) 89:259–66. doi: 10.1111/asj.12886
14. Reksen O, Sølverød L, Østerås O. Relationships between milk culture results and milk yield in Norwegian dairy cattle. *J Dairy Sci.* (2007) 90:4670–8. doi: 10.3168/jds.2006-900
15. Kayano M, Itoh M, Kusaba N, Hayashiguchi O, Kida K, Tanaka Y, et al. Associations of the first occurrence of pathogen-specific clinical mastitis with milk yield and milk composition in dairy cows. *J Dairy Res.* (2018) 85:309–16. doi: 10.1017/S0022029918000456
16. Mansor R, Mullen W, Albalat A, Zerefos P, Mischak H, Barrett DC, et al. A peptidomic approach to biomarker discovery for bovine mastitis. *J Proteomics.* (2013) 85:89–98. doi: 10.1016/j.jprot.2013.04.027
17. Hogarth CJ, Fitzpatrick JL, Nolan AM, Young FJ, Pitt A, Eckersall PD. Differential protein composition of bovine whey: a comparison of whey from healthy animals and from those with clinical mastitis. *Proteomics.* (2004) 4:2094–100. doi: 10.1002/pmic.200300723
18. Johansson M, Åkerstedt M, Li S, Zamaratskaia G, Sternesjö Lundh Å. Casein breakdown in bovine milk by a field strain of *Staphylococcus aureus*. *J Food Prot.* (2013) 76:1638–42. doi: 10.4315/0362-028X.JFP-13-112
19. Åkerstedt M, Wredle E, Lam V, Johansson M. Protein degradation in bovine milk caused by *Streptococcus agalactiae*. *J Dairy Res.* (2012) 79:297–303. doi: 10.1017/S0022029912000301
20. Kitchen BJ. Review of the progress of dairy science: bovine mastitis: milk compositional changes and related diagnostic tests. *J Dairy Res*. (1981) 48:167–88. doi: 10.1017/S0022029900021580
21. Paludetti LF, Kelly AL, O'Brien B, Jordan K, Gleeson D. Microbiological quality of milk from farms to milk powder manufacture: an industrial case study. *J Dairy Res.* (2019) 86:242–7. doi: 10.1017/S0022029919000347
22. Aryana KJ, Olson DW, A. 100-Year Review: Yogurt and other cultured dairy products. *J Dairy Sci.* (2017) 100:9987–10013. doi: 10.3168/jds.2017-12981
23. Miglior F, Fleming A, Malchiodi F, Brito LF, Martin P, Baes CF, et al. 100-Year Review: Identification and genetic selection of economically important traits in dairy cattle. *J Dairy Sci.* (2017) 100:10251–71. doi: 10.3168/jds.2017-12968
24. Chen J, Ou Y, Yang Y, Li W, Xu Y, Xie Y, et al. KLHL22 activates amino-acid-dependent mTORC1 signaling to promote tumorigenesis and ageing. *Nature.* (2018) 557:585–9. doi: 10.1038/s41586-018-0128-9
25. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell.* (2017) 169:361–71. doi: 10.1016/j.cell.2017.03.035
26. Mutvei AP, Nagiec MJ, Hamann JC, Kim SG, Vincent CT, Blenis J. Rap1-GTPases control mTORC1 activity by coordinating lysosome organization with amino acid availability. *Nat Commun.* (2020) 11:1416. doi: 10.1038/s41467-020-15156-5
27. Rabanal-Ruiz Y. Otten EG1, Korolchuk VI. mTORC1 as the main gateway to autophagy. *Essays Biochem.* (2017) 61:565–84. doi: 10.1042/EBC20170027
28. Demetriades C, Doumpas N, Teleman AA. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell.* (2014) 156:786–99. doi: 10.1016/j.cell.2014.01.024
29. Menon S, Dibble CC, Talbott G, Hoxhaj G, Valvezan AJ, Takahashi H, et al. Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell.* (2014) 156:771–85. doi: 10.1016/j.cell.2013.11.049
30. Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol.* (2008) 10:935–45. doi: 10.1038/ncb1753
31. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science.* (2008) 320:1496–501. doi: 10.1126/science.1157535
32. Gu X, Orozco JM, Saxton RA, Condon KJ, Liu GY, Krawczyk PA, et al. SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science.* (2017) 358:813–8. doi: 10.1126/science.aao3265
33. Saxton RA, Chantranupong L, Knockenhauer KE, Schwartz TU, Sabatini DM. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. *Nature.* (2016) 536:229–33. doi: 10.1038/nature19079
34. Jewell JL, Kim YC, Russell RC, Yu FX, Park HW, Plouffe SW, et al. Differential regulation of mTORC1 by leucine and glutamine. *Science.* (2015) 347:194–8. doi: 10.1126/science.1259472
35. Rebsamen M, Pochini L, Stasyk T, de Araújo ME, Galluccio M, Kandasamy RK, et al. SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature.* (2015) 519:477–81. doi: 10.1038/nature14107
36. Zhang Y, Nicholatos J, Dreier JR, Ricoult SJ, Widenmaier SB, Hotamisligil GS, et al. Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature.* (2014) 513:440–3. doi: 10.1038/nature13492
37. Hu L, Chen Y, Cortes IM, Coleman DN Dai H, Liang Y, et al. Supply of methionine and arginine alters phosphorylation of mechanistic target of rapamycin (mTOR), circadian clock proteins, and α-s1-casein abundance in bovine mammary epithelial cells. *Food Funct.* (2020) 11:883–94. doi: 10.1039/C9FO02379H
38. Zhang MC, Zhao SG, Wang SS, Luo CC, Gao HN, Zheng N, et al. D-Glucose and amino acid deficiency inhibits casein synthesis through JAK2/STAT5 and AMPK/mTOR signaling pathways in mammary epithelial cells of dairy cows. *J Dairy Sci.* (2018) 101:1737–46. doi: 10.3168/jds.2017-12926
39. Qi H, Meng C, Jin X, Li X, Li P, Gao X. Methionine promotes milk protein and fat synthesis and cell proliferation via the SNAT2-PI3K signaling pathway in bovine mammary epithelial cells. *J Agric Food Chem.* (2018) 66:11027–33. doi: 10.1021/acs.jafc.8b04241
40. Dai W, Zhao F, Liu J, Liu H. ASCT2 is involved in SARS-mediated β-casein synthesis of bovine mammary epithelial cells with methionine supply. *J Agric Food Chem.* (2020) 68:13038–45. doi: 10.1021/acs.jafc.9b03833
41. Liu Y, Hou J, Zhang M, Seleh-Zo E, Wang J, Cao B, et al. circ-016910 sponges miR-574-5p to regulate cell physiology and milk synthesis via MAPK and PI3K/AKT-mTOR pathways in GMECs. *J Cell Physiol.* (2020) 235:4198–216. doi: 10.1002/jcp.29370
42. Zhao Y, Yan S, Chen L, Shi B, Guo X. Effect of interaction between leucine and acetate on the milk protein synthesis in bovine mammary epithelial cells. *Anim Sci J.* (2019) 90:81–9. doi: 10.1111/asj.13125
43. Sigl T, Meyer HH, Wiedemann S. Gene expression analysis of protein synthesis pathways in bovine mammary epithelial cells purified from milk during lactation and short-term restricted feeding. *J Anim Physiol Anim Nutr (Berl).* (2014) 98:84–95. doi: 10.1111/jpn.12039
44. Luo C, Zheng N, Zhao S, Wang J. Sestrin2 negatively regulates casein synthesis through the SH3BP4-mTORC1 pathway in response to aa depletion or supplementation in cow mammary epithelial cells. *J Agric Food Chem.* (2019) 67:4849–59. doi: 10.1021/acs.jafc.9b00716
45. Cant JP, Kim JJM, Cieslar SRL, Doelman J. Symposium review: Amino acid uptake by the mammary glands: Where does the control lie? *J Dairy Sci.* (2018) 101:5655–66. doi: 10.3168/jds.2017-13844
46. Bionaz M, Loor JJ. Gene networks driving bovine mammary protein synthesis during the lactation cycle. *Bioinform Biol Insights.* (2011) 5:83–98. doi: 10.4137/BBI.S7003
47. Yahyaoui R, Pérez-Frías J. Amino acid transport defects in human inherited metabolic disorders. *Int J Mol Sci.* (2019) 21:E119. doi: 10.3390/ijms21010119
48. Lin Y, Duan X, Lv H, Yang Y, Liu Y, Gao X, et al. The effects of L-type amino acid transporter 1 on milk protein synthesis in mammary glands of dairy cows. *J Dairy Sci.* (2018) 101:1687–96. doi: 10.3168/jds.2017-13201
49. Dai W, Zhao F, Liu J, Liu H. Seryl-tRNA synthetase is involved in methionine stimulation of β-casein synthesis in bovine mammary epithelial cells. *Br J Nutr.* (2020) 123:489–98. doi: 10.1017/S0007114519002885

50. Duan X, Lin Y, Lv H, Yang Y, Jiao H, Hou X. Methionine induces LAT1 expression in dairy cow mammary gland by activating the mTORC1 signaling pathway. *DNA Cell Biol.* (2017) 36:1126–33. doi: 10.1089/dna.2017.3792
51. Saad A, Bijian K, Qiu D, da Silva SD, Marques M, Chang CH, et al. Insights into a novel nuclear function for Fascin in the regulation of the amino-acid transporter SLC3A2. *Sci Rep.* (2016) 6:36699. doi: 10.1038/srep36699
52. Guo Z, Zhao K, Feng X, Yan D, Yao R, Chen Y, et al. mTORC2 regulates lipogenic gene expression through PPAR?? to control lipid synthesis in bovine mammary epithelial cells. *BioMed Research International.* (2019) 5196028. doi: 10.1155/2019/5196028
53. Guo Z, Cheng X, Feng X, Zhao K, Zhang M, Yao R, et al. The mTORC1/4EBP1/PPARγ axis mediates insulin-induced lipogenesis by regulating lipogenic gene expression in bovine mammary epithelial cells. *J Agric Food Chem.* (2019) 67:6007–18. doi: 10.1021/acs.jafc.9b01411
54. Piccinini R, Tassi R, Daprà V, Pilla R, Fenner J, Carter B, et al. Study of *Staphylococcus aureus* collected at slaughter from dairy cows with chronic mastitis. *J Dairy Res.* (2012) 79:249–55. doi: 10.1017/S002202991200009X
55. Bohl LP, Isaac P, Breser ML, Orellano MS, Correa SG, Tolosa de Talamoni NG, et al. Interaction between bovine mammary epithelial cells and planktonic or biofilm Staphylococcus aureus: The bacterial lifestyle determines its internalization ability and the pathogen recognition. *Microb Pathog.* (2021) 152:104604. doi: 10.1016/j.micpath.2020.104604
56. Rajeeve K, Das S, Prusty BK, Rudel T. Chlamydia trachomatis paralyses neutrophils to evade the host innate immune response. *Nat Microbiol.* (2018) 3:824–35. doi: 10.1038/s41564-018-0182-y
57. Watkins KE, Unnikrishnan M. Evasion of host defenses by intracellular *Staphylococcus aureus*. *Adv Appl Microbiol.* (2020) 112:105–41. doi: 10.1016/bs.aambs.2020.05.001
58. Martins L, Barcelos MM, Cue RI, Anderson KL, Santos MVD, Goncalves JL. Chronic subclinical mastitis reduces milk and components yield at the cow level. *J Dairy Res.* (2020) 87:98–305. doi: 10.1017/S0022029920000321
59. Lahteenmaki K, Kuusela P, Korhonen TK. Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev.* (2001) 25:531–52. doi: 10.1016/S0168-6445(01)00067-5
60. Kelly AL, O'Flaherty F, Fox PF. Indigenous proteolytic enzymes in milk: A brief overview of the present state of knowledge. *Int Dairy J.* (2006) 16:563–72. doi: 10.1016/j.idairyj.2005.10.019
61. Livney YD, Schwan AL, Dalgleish DG. A study of beta-casein tertiary structure by intramolecular crosslinking and mass spectrometry. *J Dairy Sci.* (2004) 87:3638–47. doi: 10.3168/jds.S0022-0302(04)73502-X
62. Ménard G, Bonnaure-Mallet M, Donnio PY. Adhesion of Staphylococcus aureus to epithelial cells: an *in vitro* approach to study interactions within the nasal microbiota. *J Med Microbiol.* (2020) 69:1253–61. doi: 10.1099/jmm.0.001248
63. Song W, Li D, Tao L, Luo Q, Chen L. Solute carrier transporters: the metabolic gatekeepers of immune cells. *Acta Pharm Sin B.* (2020) 10:61–78. doi: 10.1016/j.apsb.2019.12.006
64. Napolitano L, Scalise M, Galluccio M, Pochini L, Albanese LM, Indiveri C. LAT1 is the transport competent unit of the LAT1/CD98 heterodimeric amino acid transporter. *Int J Biochem Cell Biol.* (2015) 67:25–33. doi: 10.1016/j.biocel.2015.08.004
65. Mackle TR, Dwyer DA, Ingvartsen KL, Chouinard PY, Ross DA, Bauman DE. Evaluation of whole blood and plasma in the interorgan supply of free amino acids for the mammary gland of lactating dairy cows. *J Dairy Sci.* (2000) 83:1300–09. doi: 10.3168/jds.S0022-0302(00)74996-4
66. Karki P, Kim C, Smith K, Son DS, Aschner M, Lee E. Transcriptional regulation of the astrocytic excitatory amino acid transporter 1 (EAAT1) via NF-kappa B and Yin Yang 1 (YY1). *J Biol Chem.* (2015) 290:23725–37. doi: 10.1074/jbc.M115.649327
67. Yang J, Kennelly JJ, Baracos VE. The activity of transcription factor Stat5 responds to prolactin, growth hormone, and IGF-I in rat and bovine mammary explant culture. *J Anim Sci.* (2000) 78:3114–25. doi: 10.2527/2000.78123114x
68. Kobayashi K, Oyama S, Uejyo T, Kuki C, Rahman MM, Kumura H. Underlying mechanisms involved in the decrease of milk secretion during *Escherichia coli* endotoxin induced mastitis in lactating mice. *Vet Res.* (2013) 44:119. doi: 10.1186/1297-9716-44-119


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Genetic Characterization of *Staphylococcus aureus* From Subclinical Mastitis Cases in Dairy Cows in Rwanda

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Whole-genome sequencing was carried out on 30 *Staphylococcus (S.) aureus* isolates from dairy cows with subclinical mastitis from all five provinces of Rwanda. Twenty-five of the isolates produced enough sequence to be analyzed using core genome multilocus sequence typing (cg-MLST). The isolates group into three main clusters. The largest cluster contain isolates of sequence type (ST) 152 ($n = 6$) and the closely related ST1633 ($n = 2$). These sequence types have previously mainly been encountered in humans. The isolates of the second-largest cluster belong to ST5477 ($n = 5$),so far exclusively isolated from cows in Rwanda. The third cluster consists of isolates of ST97 ($n = 4$), which is a well-known bovine-adapted sequence type. These three clusters were all widespread over the country. Isolates of the usually human-adapted sequence types 1 ($n = 2$) and 5 ($n= 1$) were found and a single isolate of ST2430, previously found among humans in Africa. Finally, four isolates of novel sequence types were found: ST7108 ($n = 2$), ST7109 ($n = 1$), and ST7110 ($n = 1$). The blaZ penicillin resistance gene was found in 84% of the isolates and was in all cases corroborated by phenotypic resistance determination. Five (20%) of the isolates carried a tetracycline resistance gene, tet(K) or tetM, and three of these five also displayed phenotypic resistance while two isolates carried a tetM-gene but were yet tetracycline susceptible. Seven (28%) isolates carried the dfrG gene conferring resistance to trimethoprim. Four of these isolates indeed were resistant to trimethoprim while three isolates were sensitive. The str gene conferring resistance to aminoglycosides was found in three isolates; however, none of these displayed resistance to gentamycin. Our data revealed a high diversity of the sequence types of *S. aureus* isolates from cows with subclinical mastitis in Rwanda. Two major clusters of ST97 and ST5477 are likely to be bovine adapted and cause mastitis while the third cluster of ST152 usually have been found in humans and may signify a recent transmission of these types from human to cows, for example from hand milking. The high prevalence of this sequence type among dairy cows may pose zoonotic threat. The sequence types were widely

distributed without any geographic correlation. Penicillin resistance, the most common type of resistance with a prevalence over 80%, but also tetracycline and trimethoprim resistance were displayed by several isolates.

**Keywords: subclinical, core genome multilocus sequence typing, antibiotic resistance, AMR, whole genome sequencing**

## INTRODUCTION

Bovine mastitis is a common and costly disease on dairy farms that not only affects milk yields but also milk quality. Typically, mastitis is an inflammation of the mammary gland due to microorganisms invading the udder, but also physical or chemical trauma of the mammary gland could be the origin of udder inflammation.

*Staphylococcus* (*S.*) *aureus* is among the microorganisms that cause both clinical and subclinical mastitis (SCM) and is characterized by its reoccurring and chronic type of mastitis (1). Its contagious nature means that the infected udder becomes a reservoir for the bacterium which is transmitted to uninfected animals in the herd mainly during milking (2). In addition, the pathogen is hard to cure and eradicate in herds because of its ability to persist in cow environment and to colonize skins or mucosal epithelia (3). Reservoirs of *S. aureus* include teat skin, external orifices, housing, feedstuffs, humans, non-bovine animals, air, equipment, bedding, insects, and water (4). The bacteria are spread to uninfected quarters by teat cup liners, milkers' hands, wash cloths, contaminated floor/bedding, and flies.

*S. aureus* is common in mastitis cases in east African countries, where implementation of the ten-point mastitis control plan is still lacking (5–8). However, *S. aureus* is a major cause of mastitis also in developed countries which have successfully implemented mastitis control plans for decades (9, 10). This highlights the need for new ways to study *S. aureus* infection dynamics with the aim to control and limit the infection. Genotyping is one of the ways to understand the characteristics of strains of *S. aureus* for several reasons. For example, the cure rate of mastitis caused by *S. aureus* is very variable and may depend on prevalent genotypes (11). Haveri et al. (12) implied that persistence of mastitis infection depended on genotypes. Furthermore, the virulence and spread of *S. aureus* is also strain dependent (3). Antimicrobial resistance in *S. aureus* is increasingly becoming a problem worldwide, and therefore, it is important to monitor mechanisms of resistance in this pathogen in order to guide therapy and collect knowledge of resistant strains in different ecological niches (13).

DNA-based methods that are used in strain typing such as multilocus sequence typing (MLST) yield standardized results that can be compared across laboratories using databases (14). This molecular typing method has been used to show that some strains more commonly cause mastitis and/or intramammary infections (IMI) than others (15). Whilst MLST has a greater discriminatory power than non-sequencing-based methods, it still only uses seven genes to assess the relatedness between strains of *S. aureus*. Core genome (cg-) MLST is a recently developed method that typically utilizes whole genome sequencing (WGS) and around 2,000-gene target from the core genome, allowing much greater discrimination and reliability when typing and comparing strains. In cg-MLST, table-top sequencing platforms such as Illumina HiSeq can be used to generate WGS data which can then either be assembled *de novo* or mapped to a reference genome for the organism, and the strains can be typed in this way (16). Using cg-MLST, the strains can be grouped into clonal complexes (CCs) based on how many alleles of each of the genes being assessed they share with other strains. This, too, is an assessment of relatedness as the strains within a CC will share a common lineage. In this study, therefore, cg-MLST was used for the bioinformatic analysis of *S. aureus* strains utilizing Ridom SeqSphere+ (Ridom GmbH, Münster, Germany) for determination of the minimum spanning tree describing the relatedness of the strains. To the best of our knowledge, this is the first study carried out to show genetic diversity or relatedness of *S. aureus* from SCM cases in Rwanda.

## MATERIALS AND METHODS

### *Staphylococcus aureus* Isolates

Thirty *S. aureus* isolates from SCM cases in dairy cows in Rwanda collected from 2016 to 2017 were included in this study. A SCM case was defined as a quarter with score of $\geq$3 on a 1–5 scale in the California Mastitis Test for milk samples. Six isolates were selected from each of the five provinces (Kigali, Eastern, Northern, Western, and Southern) of Rwanda. From each province, isolates were randomly selected from individual herds as to simulate natural distribution with the exclusion criterium to select only one sample from each farm. No information about antibiotic use was gathered or known. More information about the collection of isolates have been given elsewhere for samples collected in the Kigali province (6) and the other four provinces (7). Once selected, $\sim$1 μL of each of the samples was cultured on 5% bovine blood agar plates and incubated at 37°C overnight. The colonies were evaluated based on the expected *S. aureus* morphology and partial or complete hemolysis. Cultures with non-uniform colonies were sub-cultured and incubated overnight at 37°C to obtain pure cultures. All cultures were stored at 4°C.

### DNA Extraction

Prior to DNA extraction, isolates were cultured on horse blood agar plates to verify their purity. The EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extractions. Approximately 1 μL of pure colony from each of the 30 strains was suspended in 180 μL Digestion Buffer G2, plus 20 μL lysozyme (50 mg/mL; Sigma-Aldrich) and 10 μL lysostaphin

(5 mg/mL; Sigma-Aldrich) and incubated at 37°C for 1 h and 30 min. Automated DNA extraction was then carried out using the EZ1 Advanced or Advanced XL robot (Qiagen) following the manufacturer's instructions, with a final elution volume of 50 μL. The extracted DNA was immediately stored at −20°C. DNA concentrations were adjusted to the range 5–15 ng/μL suitable for sequencing using a Qubit® 2.0 fluorometric analysis double-stranded DNA high sensitivity kit (Thermo Fisher Science, Massachusetts, United States).

## Sequencing of *S. aureus* Isolates

All library preparation and sequencing was carried out at Clinical Genomics Stockholm facility at Science for Life Laboratory (Stockholm, Sweden) using an Illumina Novaseq 6000 instrument with a S4 flow cell. Twenty-five of 30 samples produced sufficient sequence data for bioinformatic analysis. No further investigations were carried out on the five failed samples. The successfully sequenced samples had a mapping rate in the range 84.5–96.6% to the NCTC 8,325 strain (GenBank accession NC_007795). The percentage of base pairs with a coverage better than 100 were in the range 89.6–93.8.

## Bioinformatics Analysis of Sequences

### Sequence Assembly and Genotyping

Sequence assembly was carried using the UniCycler pipeline (17). UniCycler employs read error correction and optimizes *de novo* assembly by SPAdes (18). In addition, UniCycler removes errors in the assembly by using pilon (19). The UniCycler, SPAdes, and pilon versions were v0.4.8-beta, 3.13.0, and 1.23, respectively. Minimum spanning trees were calculated by SeqSphere + version 5.1.0 (20) using the assembled contigs obtained from UniCycler for the 25 isolates with the seed genome with GenBank accession NC_002951.2 and the *S. aureus* cg-MLST version 1.3 containing 1,861 loci (https://www.cgmlst.org/ncs/schema/141106/). The criteria for identification were 100% aligned length and 90% identity. For all 25 strains, 1,692 loci were found and used for creating a minimum spanning tree. Strains with <200 different alleles were considered as members of a cluster. Sequence types (STs) are defined by alleles from the following standard set of *S. aureus* MLST genes: *arcC*, *aroE*, *gpF*, *gmk*, *pta*, *tpi*, and *yqiL* (21). MLST profiles were determined at the PubMLST.org website (https://pubmlst.org/organisms/staphylococcus-aureus) which is an open-access, curated database that integrate population sequence data with provenance and phenotype information (22).

### Detection of Antibiotic Resistance Genes

The UniCycler sequences assemblies were used to detect antibiotic resistance genes by utilizing of the Resfinder 3.2 (23) web server (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity and coverage threshold of 90 and 60%, respectively. In addition, the UniCycler assemblies were analyzed with the resistance gene identifier service of the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/analyze/rgi) to detect antibiotic resistance genes with the search in "Perfect" and "strict" mode only (24). The results using the two databases were consistent except that aminoglycoside resistance only were found with Resfinder. In parallel, the 25 *S. aureus* isolates were tested for antimicrobial susceptibility by determination of minimum inhibitory concentration (MIC) using a micro-dilution method according to recommendations from the Clinical and Laboratory Standards Institute using VetMIC™ panels (SVA, Uppsala, Sweden). Isolates were tested for susceptibility to penicillin, tetracycline, trimethoprim, and gentamicin.

# RESULTS

## Core Genome Multilocus Sequence Typing Results

Three main clusters can be discerned among the 25 isolates (**Figure 1**). The largest cluster contains isolates of sequence type (ST) 152 ($n = 6$) and the closely related ST1633 ($n = 2$), which differ from ST152 in a single allele. The ST152/1663 isolates were found in all provinces except the Southern.

The second largest cluster ($n = 5$) consists of ST5477. The present data indicate that also this sequence type is geographically dispersed in Rwanda since it is found in all provinces except the Eastern.

A third cluster consist of isolates that belong to the well-known bovine adapted CC97 clonal complex (15, 25–28). This sequence type was found in the Kigali, Southern, and Western provinces and thus also display large geographical spread.

There are two isolates of ST1 and one isolate of ST5, which typically are human adapted. In addition, there are four isolates of novel sequence types. Two of these are of ST7108, which differ fromST20 with a single mutation and one isolate each of sequence type ST7109 and ST7110. ST7109 differ from ST101 with a single mutation while ST7110 is most closely related to ST5476, which is a sequence type represented by a single isolate in pubMLST obtained from a mastitis case in Rwanda 2018 (29). All sequence types found in the present study are listed in **Table 1**.

Overall, there is no geographic association that can be discerned for the sequence types of the isolates in the present study.

## Antibiotic Resistance

Results of genotypic and phenotypic resistance determinations are presented in **Table 1**. The BlaZ gene, which confer penicillin resistance, was prevalent at a level of 84% and was in all cases supported by MIC phenotypic resistance results. Four of the isolates (16%) were resistant to tetracycline. There is not full accordance between the presence of tet(M) or tetK genes and the observed resistance pattern (**Table 1**). For two tetracycline sensitive isolates, either the tetK or the tet(M) gene is found from the NGS-data, while one resistant isolate lack both genes. The dfrG gene which encodes trimethoprim resistance was present for seven isolates but only four of these actually displayed resistance, thus the prevalence was 16%. The Str genes which encodes resistance among aminoglycosides including gentamicin was found in three isolates, but all isolates were still susceptible to gentamicin.

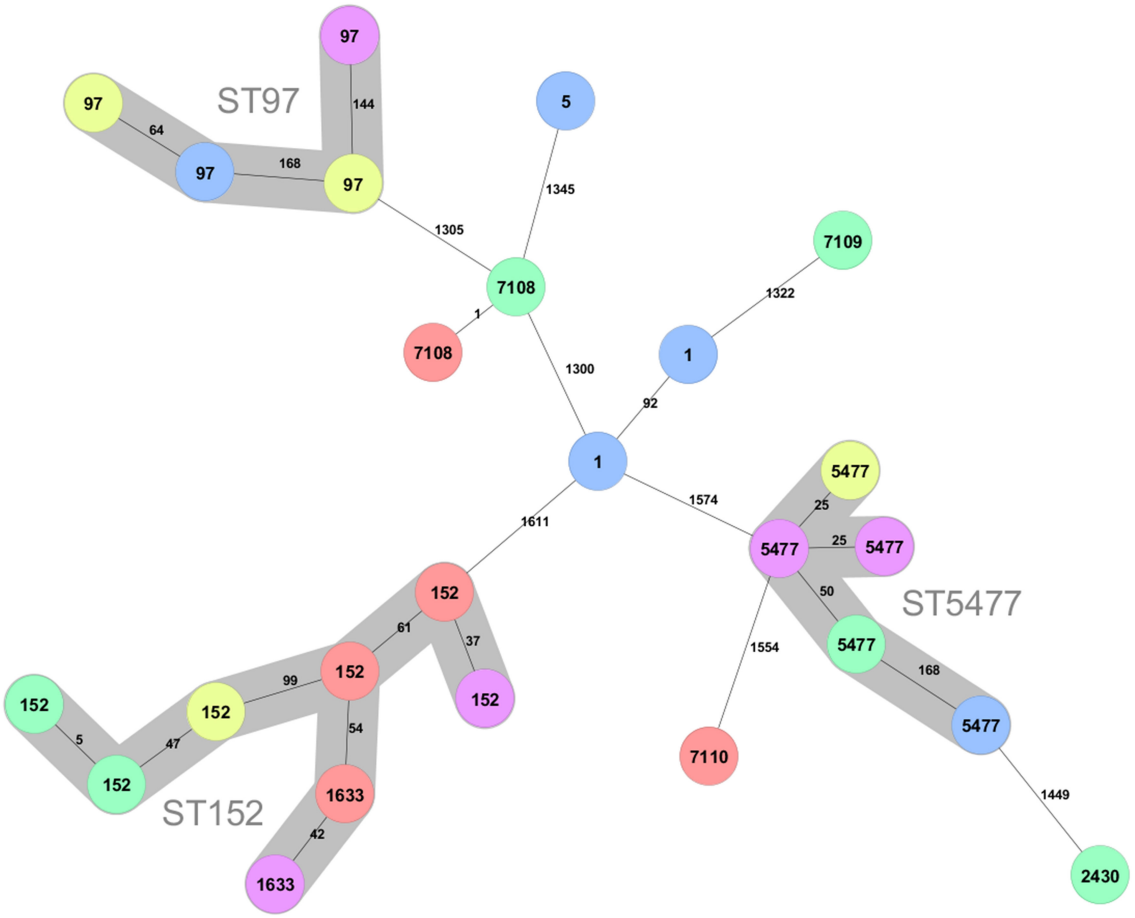


**FIGURE 1 |** Minimum spanning tree created for the 25 *S. aureus* strains from the Kigali (yellow), Eastern (red), Western (pink), Northern (green), and Southern (blue) province of Rwanda. The tree was created using 1,692 loci. Clusters connecting isolates with less the 200 different loci are indicated with background colors and named with the ST number in the cluster. The ST of the isolates are shown on the nodes and the number of different alleles between pairs of isolates are shown at the connecting lines.

## DISCUSSION

The present study is of limited scope encompassing only 25 *S. aureus* isolates from SCM cases in Rwanda, but this still has increased the number of *S. aureus* isolates from Rwanda in pubMLST almost tenfold. However, the samples have been collected in roughly equal numbers from all provinces of the country, which allows some important observations to be made even from this limited sample set. It is clear from the cg-MLST data that the isolates mainly fall into three clusters. Not unexpectedly, one cluster belongs to the clonal complex CC97, which is common among cattle all over the world. Four samples, collected in three different provinces, belong to this cluster.

A second, presumably also bovine adapted, cluster is of ST5477. This sequence type has, except for the five isolates of the present study, only been isolated from a mastitis case in the Musanze district in the Northern Province of Rwanda (29). In the same study, *S. aureus* of sequence type ST5476 was isolated from another mastitis case in the same district. This ST has five identical alleles and is most closely related to the novel sequence type ST7110, found in the present work. A second singleton in the present data, also with a ST5477 isolate as the closest neighbor in the minimum spanning tree, is a ST2430, although only distantly related (**Figure 1**). ST2430 was first discovered when isolated from inpatients in Thika, Kenya in 2014 (30), but was later also found in an isolate from 1995 from a pyomyositis case in Uganda in (31). Single locus variants of this sequence type are found all over the world but invariably from human hosts (31). Thus, the current evidence indicates that there exists a novel bovine clonal complex in the Rwanda region related to ST5477, while ST2430 are more likely a transfer from humans. The ST7110 and ST5476 might represent a second bovine adapted cluster since all entries in pubMLST with three or more alleles identical to ST5476 have been isolated from cows with ST7110 (present study), ST5475 andST5476 isolated in Rwanda, and ST3591 isolated from a milk sample 2009 in Kenya (31).

The third and largest cluster, surprisingly, is constituted of ST152 and ST1633, which is a single locus variant of ST152. ST152 was first isolated from humans in Europe (32) and have subsequently been shown to be an important and prevalent

**TABLE 1 |** Antibiotic resistance profiling of the *S. aureus* strains obtained from subclinical mastitis cases in dairy cows from five regions in Rwanda.

| Strain | Province of isolation | ST | Resistance [Genotype (+/–), Phenotype (R/S)] | | | |
|---|---|---|---|---|---|---|
| | | | Penicillin (blaZ) | Tetracycline (tet(K)/tetM) | Trimethoprim (dfrG) | Gentamicin (str) |
| JB1 | Kigali | 97 | +, R | +/–, R | –, S | +, S |
| JB3 | Kigali | 152 | +, R | –/–, R | –, S | –, S |
| JB4 | Kigali | 5,477 | +, R | +/–, R | –, S | –, S |
| JB5 | Kigali | 97 | +, R | –/–, S | –, S | –, S |
| JB7 | Eastern | 7,108 | –, S | –/–, S | –, S | –, S |
| JB9 | Northern | 2,430 | +, R | –/+, S | +, S | –, S |
| JB10 | Northern | 152 | +, R | –/–, S | –, S | –, S |
| JB11 | Eastern | 152 | +, R | –/–, S | +, R | –, S |
| JB12 | Southern | 5,477 | +, R | –/–, S | –, S | –, S |
| JB13 | Southern | 1 | –, S | –/–, S | –, S | –, S |
| JB15 | Southern | 97 | +, R | –/–, S | –, S | –, S |
| JB16 | Southern | 1 | +, R | –/–, S | –, S | –, S |
| JB17 | Northern | 5,477 | +, R | –/–, S | –, S | –, S |
| JB18 | Northern | 7,109 | +, R | –/–, S | –, S | –, S |
| JB19 | Western | 1,633 | +, R | –/–, S | +, S | –, S |
| JB21 | Southern | 5 | +, R | –/–, S | +, R | –, S |
| JB23 | Eastern | 1,633 | +, R | –/–, S | +, R | –, S |
| JB24 | Western | 5,477 | +, R | –/–, S | –, S | –, S |
| JB25 | Northern | 152 | +, R | –/–, S | –, S | –, S |
| JB26 | Western | 152 | +, R | –/–, S | +, R | –, S |
| JB30 | Eastern | 7,108 | –, S | –/–, S | –, S | –, S |
| JB31 | Western | 5,477 | +, R | –/–, S | –, S | +, S |
| JB33 | Western | 97 | +, R | +/–, S | –, S | +, S |
| JB34 | Eastern | 152 | +, R | –/–, S | +, S | –, S |
| JB35 | Eastern | 7,110 | –, S | +/–, R | –, S | –, S |
| | Prevalence (phenotype): | | 84% (0.7–0.98) | 16% (0.02–0.30) | 16% (0.02–0.30) | 0% |

*For the phenotypic antibiotic resistance prevalence, a 95% confidence interval is given calculated by:* $p \pm 1.96 * \sqrt{p * (p-1)/n}$, *where p is the sampled prevalence and n is the sample size.*

sequence type infecting humans in many African countries (30, 33–41). These ST152 strains with a local predominance in African countries are typically of spa-type t355, Panton-Valentine leucocidin (PVL) positive and methicillin susceptible (MSSA). To our knowledge, infections of bovids with ST152 in Africa have not been reported. However, Mekkonen et al. (42) reported three PVL+ isolates from dairy cows from north-western Ethiopia with spa type t355, which are typical features of ST152 and indeed all eight ST152/ST1633 bovine isolates of the present work carry the lukF-PV and lukS-PV genes signifying PVL positivity, and they are all of spa-type t355 (data not shown). The large prevalence of ST152 (PVL+) among dairy cows in Rwanda is serious since it may pose a public health risk *via* zoonotic transfer of pathogen strains to humans. Since hand milking is still prevalent in Rwanda, it is possible that human ST152 strains initially have infected the cows. These strains were distributed over almost all districts of Rwanda (**Table 1**), and apparently, bovine ST152 is prevalent all over the country. It can be hypothesized that since management of herds are similar and include hand milking and lack of post milking teat dipping, there will be opportunities for human contact with animals in absence of consistent disinfection. This will facilitate transmission of human adapted pathogens to the dairy cows during milking. Similar management means that pattern of transmission is the same across regions and that is why there were positive identification of ST152/1633 in all regions included in the study. Interestingly, when screening milk and dairy products in southern Italy for MRSA, Basanisi et al. (43) found that PVL encoding ST152 (t355) accounted for 67.5% of all MRSA isolates ($n = 40$). In fact, ST152 isolates have also sporadically been isolated from humans in Europe and are usually methicillin resistant (44–47). It has been suggested that ST152 is an originally African lineage which first acquired PVL and subsequently after introduction to Europe also have acquired methicillin resistance (41). Although the number of isolates is low in the present study, the geographic spread of sampling still makes it very likely that the prevalence of MSSA ST152 (PVL+) in the bovine population of Rwanda is high. Taken together with the locally high prevalence of MRSA

ST152 (PVL+) in dairy and milk products in the Apulia region of southern Italy (43), it indicates that ST152 have capacity to establish in the bovine population. Since many investigations only genotype MRSA strains, the possibility exists that MSSA ST152(PVL+) is underdiagnosed both in Europe and Africa. Due to the often-high pathogenicity of PVL+ *S. aureus* strains, it is important to further investigate the epidemiology and prevalence of ST152.

Three novel sequence types were discovered in the present work (**Figure 1**, **Table 1**). Besides ST7110, which might represent a bovine adapted complex (see above), the two others are denoted ST7108 and ST7109, which differ with a single mutation from ST20 and ST101, respectively. Two other sequence types represented among the isolates are ST1 and ST5. All these STs have primarily been associated with globally dispersed human infections (26, 27) although they occasionally also are found in bovine isolates (25, 26, 48).

Penicillin resistance was the most common type of resistance with a prevalence over 80%. The high prevalence of penicillin resistance is of concern since it may imply treatment failures of *S. aureus* IMI in dairy cows (49). The prevalence of penicillin resistance found here (84%) for isolates from Rwanda is similar to the 86% that recently was observed for isolates derived from dairy cows in north-western Ethiopia (42). However, tetracycline and trimethoprim resistance were both found among 16% of the isolates (**Table 1**), which is a significantly lower fraction than the 54 and 79%, respectively, observed in the study from Ethiopia (42). These differences should be interpreted with caution due to the small number of isolates in the present study. The Str genes which encodes aminoglycoside 6-adenylyltransferase and confer resistance among aminoglycosides were found in three isolates, which all, despite this, were susceptible for gentamicin. Since mechanisms of antimicrobial resistance is complex, it is possible to detect resistance genes in susceptible isolates, for example due to lacking, but crucial, accessor genes, or to find phenotypic resistance when there are no resistance genes (13).

In summary, among *S. aureus* isolates collected from milk samples from SCM diagnosed cows, three genotype clusters dominate presumably of bovine-adapted sequence types ST152/1633, ST97, and ST5477. Of these, ST152/1633 may pose a potential zoonotic threat since the sequence type frequently are encountered among humans in Africa while ST5477 so far appear to be a sequence type local to east Africa and Rwanda. Since tetracycline and penicillin are used to treat mastitis in the region (50), one can speculate that their frequent use and lack of biosecurity and mastitis control program in dairy cows in Rwanda (6) have contributed to the high resistance levels reported in this study.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI SRA BioProject, accession no: PRJNA767102.

## ETHICS STATEMENT

This animal study was reviewed and approved by the Research Screening and Ethics Clearance Committee (RSEC-C) of the College of Agriculture Animal Sciences and Veterinary Medicine, University of Rwanda (UR-CAVM).

## AUTHOR CONTRIBUTIONS

JN, YP, RB, and KA planned the study. JN carried out the sampling and sample preparations. ML performed bioinformatic analysis of sequence data. JN and ML wrote the manuscript that was revised by KA, RB, and YP. All authors read and approved the final manuscript.

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## REFERENCES

1. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023
2. Capurro A, Aspán A, Ericsson Unnerstad H, Persson Waller K, Artursson K. Identification of potential sources of *Staphylococcus aureus* in herds with mastitis problems. *J Dairy Sci.* (2010) 93:180–91. doi: 10.3168/jds.2009-2471
3. Rainard P, Foucras G, Fitzgerald JR, Watts JL, Koop G, Middleton JR. Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. *Transbound Emerg Dis.* (2018) 65(Suppl 1):149–65. doi: 10.1111/tbed.12698
4. Roberson JR, Fox LK, Hancock DD, Gay JM, Besser TE. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. *J Dairy Sci.* (1994) 77:3354–64. doi: 10.3168/jds.S0022-0302(94)77277-5
5. Mekonnen SA, Koop G, Melkie ST, Getahun CD, Hogeveen H, Lam T. Prevalence of subclinical mastitis and associated risk factors at cow and herd level in dairy farms in North-West Ethiopia. *Prev Vet Med.* (2017) 145:23–31. doi: 10.1016/j.prevetmed.2017.06.009

6. Ndahetuye JB, Persson Y, Nyman AK, Tukei M, Ongol MP, Båge R. Aetiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. *Trop Anim Health Prod.* (2019) 51:2037–44. doi: 10.1007/s11250-019-01905-2
7. Ndahetuye JB, Twambazimana J, Nyman AK, Karege C, Tukei M, Ongol MP, et al. A cross sectional study of prevalence and risk factors associated with subclinical mastitis and intramammary infections, in dairy herds linked to milk collection centers in Rwanda. *Prev Vet Med.* (2020) 179:105007. doi: 10.1016/j.prevetmed.2020.105007
8. Mpatswenumugabo JP, Bebora LC, Gitao GC, Mobegi VA, Iraguha B, Kamana O, et al. Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu Districts, Rwanda. *J Vet Med.* (2017) 2017:8456713. doi: 10.1155/2017/8456713
9. Ericsson Unnerstad H, Lindberg A, Persson Waller K, Ekman T, Artursson K, Nilsson-Öst M, et al. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. *Vet Microbiol.* (2009) 137:90–7. doi: 10.1016/j.vetmic.2008.12.005
10. Tenhagen BA, Koster G, Wallmann J, Heuwieser W. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J Dairy Sci.* (2006) 89:2542–51. doi: 10.3168/jds.S0022-0302(06)72330-X
11. Lundberg A, Aspán A, Nyman A, Ericsson Unnerstad H, Persson Waller K. Associations between bacterial genotype and outcome of bovine clinical *Staphylococcus aureus* mastitis. *Acta Vet Scand.* (2014) 56:2. doi: 10.1186/1751-0147-56-2
12. Haveri M, Taponen S, Vuopio-Varkila J, Salmenlinna S, Pyorala S. Bacterial genotype affects the manifestation and persistence of bovine *Staphylococcus aureus* intramammary infection. *J Clin Microbiol.* (2005) 43:959–61. doi: 10.1128/JCM.43.2.959-961.2005
13. Yang F, Wang Q, Wang X-r, Wang L, Li X-p, Luo J-y, et al. Genetic characterization of antimicrobial resistance in Staphylococcus aureus isolated from bovine mastitis cases in Northwest China. *J Integr Agric.* (2016) 15:2842–7. doi: 10.1016/S2095-3119(16)61368-0
14. Enright MC, Spratt BG. Multilocus sequence typing. *Trends Microbiol.* (1999) 7:482–7. doi: 10.1016/S0966-842X(99)01609-1
15. Smith EM, Green LE, Medley GF, Bird HE, Fox LK, Schukken YH, et al. Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. *J Clin Microbiol.* (2005) 43:4737–43. doi: 10.1128/JCM.43.9.4737-4743.2005
16. Cunningham SA, Chia N, Jeraldo PR, Quest DJ, Johnson JA, Boxrud DJ, et al. Comparison of whole-genome sequencing methods for analysis of three methicillin-resistant *Staphylococcus aureus* outbreaks. *J Clin Microbiol.* (2017) 55:1946–53. doi: 10.1128/JCM.00029-17
17. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol.* (2017) 13:e1005595. doi: 10.1371/journal.pcbi.1005595
18. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol.* (2012) 19:455–77. doi: 10.1089/cmb.2012.0021
19. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS ONE.* (2014) 9:e112963. doi: 10.1371/journal.pone.0112963
20. Kohl TA, Diel R, Harmsen D, Rothganger J, Walter KM, Merker M, et al. Whole-genome-based mycobacterium tuberculosis surveillance: a standardized, portable, and expandable approach. *J Clin Microbiol.* (2014) 52:2479–86. doi: 10.1128/JCM.00567-14
21. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol.* (2000) 38:1008–15. doi: 10.1128/JCM.38.3.1008-1015.2000
22. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLSTorg website and their applications Wellcome. *Open Res.* (2018) 3:124. doi: 10.12688/wellcomeopenres.14826.1
23. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother.* (2012) 67:2640–4. doi: 10.1093/jac/dks261
24. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* (2017) 45:D566–D73. doi: 10.1093/nar/gkw1004
25. Boss R, Cosandey A, Luini M, Artursson K, Bardiau M, Breitenwieser F, et al. Bovine *Staphylococcus aureus*: subtyping, evolution, and zoonotic transfer. *J Dairy Sci.* (2016) 99:515–28. doi: 10.3168/jds.2015-9589
26. Shepheard MA, Fleming VM, Connor TR, Corander J, Feil EJ, Fraser C, et al. Historical zoonoses and other changes in host tropism of *Staphylococcus aureus*, identified by phylogenetic analysis of a population dataset. *PLoS ONE.* (2013) 8:e62369. doi: 10.1371/journal.pone.0062369
27. Weinert LA, Welch JJ, Suchard MA, Lemey P, Rambaut A, Fitzgerald JR. Molecular dating of human-to-bovid host jumps by *Staphylococcus aureus* reveals an association with the spread of domestication. *Biol Lett.* (2012) 8:829–32. doi: 10.1098/rsbl.2012.0290
28. Leijon M, Atkins E, Persson Waller K, Artursson K. Longitudinal study of *Staphylococcus aureus* genotypes isolated from bovine clinical mastitis. *J Dairy Sci.* (2021) 104:11945. doi: 10.3168/jds.2021-20562
29. Antok FI, Mayrhofer R, Marbach H, Masengesho JC, Keinprecht H, Nyirimbuga V, et al. Characterization of antibiotic and biocide resistance genes and virulence factors of Staphylococcus species associated with Bovine Mastitis in Rwanda. *Antibiotics.* (2019) 9:1. doi: 10.3390/antibiotics9010001
30. Aiken AM, Mutuku IM, Sabat AJ, Akkerboom V, Mwangi J, Scott JA, et al. Carriage of *Staphylococcus aureus* in Thika level 5 hospital, Kenya: a cross-sectional study. *Antimicrob Resist Infect Control.* (2014) 3:22. doi: 10.1186/2047-2994-3-22
31. Anonymous. *PubMLST*. (2019). Available online at: https://pubmlst.org/bigsdb?db=pubmlst_saureus_isolates&page=profiles
32. Mueller-Premru M, Strommenger B, Alikadic N, Witte W, Friedrich AW, Seme K, et al. New strains of community-acquired methicillin-resistant *Staphylococcus aureus* with panton-valentine leukocidin causing an outbreak of severe soft tissue infection in a football team. *Eur J Clin Microbiol Infect Dis.* (2005) 24:848–50. doi: 10.1007/s10096-005-0048-0
33. Shittu A, Oyedara O, Abegunrin F, Okon K, Raji A, Taiwo S, et al. Characterization of methicillin-susceptible and -resistant staphylococci in the clinical setting: a multicentre study in Nigeria. *BMC Infect Dis.* (2012) 12:286. doi: 10.1186/1471-2334-12-286
34. Amissah NA, Buultjens AH, Ablordey A, van Dam L, Opoku-Ware A, Baines SL, et al. Methicillin resistant *Staphylococcus aureus* transmission in a Ghanaian burn unit: the importance of active surveillance in resource-limited settings. *Front Microbiol.* (2017) 8:1906. doi: 10.3389/fmicb.2017.01906
35. Conceição T, Coelho C, Silva IS, de Lencastre H, Aires-de-Sousa M. *Staphylococcus aureus* in former Portuguese colonies from Africa and the Far East: missing data to help fill the world map. *Clin Microbiol Infect.* (2015) 21:842 e1–e10. doi: 10.1016/j.cmi.2015.05.010
36. Egyir B, Guardabassi L, Sorum M, Nielsen SS, Kolekang A, Frimpong E, et al. Molecular epidemiology and antimicrobial susceptibility of clinical *Staphylococcus aureus* from healthcare institutions in Ghana. *PLoS ONE.* (2014) 9:e89716. doi: 10.1371/journal.pone.0089716
37. Eibach D, Nagel M, Hogan B, Azuure C, Krumkamp R, Dekker D, et al. Nasal carriage of *Staphylococcus aureus* among children in the Ashanti Region of Ghana. *PLoS ONE.* (2017) 12:e0170320. doi: 10.1371/journal.pone.0170320
38. Jaton L, Pillonel T, Jaton K, Dory E, Prod'hom G, Blanc DS, et al. Common skin infection due to panton-valentine leucocidin-producing *Staphylococcus aureus* strains in asylum seekers from Eritrea: a genome-based investigation of a suspected outbreak. *Clin Microbiol Infect.* (2016) 22:739 e5–8. doi: 10.1016/j.cmi.2016.05.026
39. Kraef C, Alabi AS, Peters G, Becker K, Kremsner PG, Rossatanga EG, et al. Co-detection of panton-valentine leukocidin encoding genes and cotrimoxazole resistance in *Staphylococcus aureus* in Gabon: implications for HIV-patients' care. *Front Microbiol.* (2015) 6:60. doi: 10.3389/fmicb.2015.00060
40. Ruffing U, Alabi A, Kazimoto T, Vubil DC, Akulenko R, Abdulla S, et al. Community-associated *Staphylococcus aureus* from Sub-Saharan Africa and Germany: a cross-sectional geographic correlation study. *Sci Rep.* (2017) 7:154. doi: 10.1038/s41598-017-00214-8
41. Ruimy R, Maiga A, Armand-Lefevre L, Maiga I, Diallo A, Koumare AK, et al. The carriage population of *Staphylococcus aureus* from Mali is

composed of a combination of pandemic clones and the divergent panton-valentine leukocidin-positive genotype ST152. *J Bacteriol.* (2008) 190:3962–8. doi: 10.1128/JB.01947-07
42. Mekonnen SA, Lam T, Hoekstra J, Rutten V, Tessema TS, Broens EM, et al. Characterization of *Staphylococcus aureus* isolated from milk samples of dairy cows in small holder farms of North-Western Ethiopia. *BMC Vet Res.* (2018) 14:246. doi: 10.1186/s12917-018-1558-1
43. Basanisi MG, La Bella G, Nobili G, Franconieri I, La Salandra G. Genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk and dairy products in South Italy. *Food Microbiol.* (2017) 62:141–6. doi: 10.1016/j.fm.2016.10.020
44. Dermota U, Müller-Premru M, Švent-Kučina N, Petrovič Z, Ribič H, Rupnik M, et al. Survey of community-associated-methicillin-Resistant *Staphylococcus aureus* in Slovenia: identification of community-associated and livestock-associated clones. *Int J Med Microbiol.* (2015) 305:505–10. doi: 10.1016/j.ijmm.2015.05.002
45. Krziwanek K, Metz-Gercek S, Mittermayer H. Trends in the occurrence of MRSA strains in Upper Austria from 2006 to (2009). *Clin Microbiol Infect.* (2011) 17:920–3. doi: 10.1111/j.1469-0691.2010.03376.x
46. Baig S, Johannesen TB, Overballe-Petersen S, Larsen J, Larsen AR, Stegger M. Novel SCCmec type XIII (9A) identified in an ST152 methicillin-resistant *Staphylococcus aureus*. *Infect Genet Evol.* (2018) 61:74–6. doi: 10.1016/j.meegid.2018.03.013
47. Brauner J, Hallin M, Deplano A, De Mendonca R, Nonhoff C, De Ryck R, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* clones circulating in Belgium from 2005 to 2009: changing epidemiology. *Eur J Clin Microbiol Infect Dis.* (2013) 32:613–20. doi: 10.1007/s10096-012-1784-6
48. Hata E, Katsuda K, Kobayashi H, Uchida I, Tanaka K, Eguchi M. Genetic variation among *Staphylococcus aureus* strains from bovine milk and their relevance to methicillin-resistant isolates from humans. *J Clin Microbiol.* (2010) 48:2130–9. doi: 10.1128/JCM.01940-09
49. Barkema HW, Schukken YH, Zadoks RN. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci.* (2006) 89:1877–95. doi: 10.3168/jds.S0022-0302(06)72256-1
50. Ssajjakambwe P, Bahizi G, Setumba C, Kisaka SM, Vudriko P, Atuheire C, et al. Milk hygiene in rural Southwestern Uganda: prevalence of mastitis and antimicrobial resistance profiles of bacterial contaminants of milk and milk products. *Vet Med Int.* (2017) 2017:8710758. doi: 10.1155/2017/8710758

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# Biological Characteristics and Pathogenicity of *Helcococcus ovis* Isolated From Clinical Bovine Mastitis in a Chinese Dairy Herd

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*Helcococcus ovis* (*H. ovis*) was first reported in ovine subclinical mastitis milk and post-mortem examination organs in Spain and the United Kingdom in 1999; subsequently, it appeared in cattle, horse, goat, and human. However, isolation and characterization of the pathogen from clinical bovine mastitis is unknown. The objective of this study was to identify the pathogen in clinical bovine mastitis. A total of four strains were isolated from bovine mastitis milk samples from a Chinese dairy farm, and they were identified as *H. ovis* by microscopic examination and 16S rRNA gene sequencing. Phylogenetic tree was constructed using 16S rRNA gene, and the isolates were closely related to other China strains and strains from Japan. The growth speed of the *H. ovis* isolated was relatively slower than *Streptococcus agalactiae*, and the phenotypic characteristics were similar to *H. ovis* CCUG37441 and CCUG39041 except to lactose. The isolates were sensitive to most of the common used antimicrobials. The *H. ovis* isolates could lead to mild murine mastitis alone and induce severe mastitis when co-infected with *Trueperella pyogenes* in the murine mammary infection model constructed.

**Keywords: pathogenicity, phylogeny, antimicrobial resistance profile, *Helcococcus ovis*, clinical bovine mastitis**

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## INTRODUCTION

Bovine mastitis is one of the most costly diseases in dairy industry due to milk discarding and related treatment costs, as well as culling of cows (1–3).

In China, the most frequently isolated mastitis pathogens were *Escherichia coli* (14.4%), *Klebsiella* spp. (13.0%), coagulase-negative staphylococci (11.3%), *Streptococcus dysgalactiae* (10.5%), and *Staphylococcus aureus* (10.2%) (4). Once these pathogens enter the mammary gland and detected by epithelial cells together with resident macrophages, the cells will release inflammatory cytokines such as TNF-α (tumor necrosis factor–alpha), IL-1β (interleukin-1 beta), humoral components (alexin, antibodies, antimicrobial peptides) are also participate in the process, and then, more leukocytes (neutrophils and lymphocytes) will be attracted under the chemotaxis, and more cytokines be synthesized and secreted; in the end, bovine mastitis is induced till the mastitis pathogens are eliminated (5).

Traditional diagnosis for bovine mastitis includes visual examination, California Mastitis Test, and Somatic Cells Counting. Pathogens isolation and identification are help for mastitis diagnosis

and treatment; whereas, due to characteristics of microorganism, some of them are hard to isolate, which limits the research of such mastitis pathogens. Because of the limits of farm diagnostic tools in China, certain uncommon mastitis pathogens cannot be accurately identified on site. This might result in false negative results for mastitis diagnosis.

*Helcococcus ovis* (*H. ovis*) was first isolated from colonies mixed with *Trueperella pyogenes* (*T. pyogenes*) and *Staphylococcus* spp. from the lung, liver, spleen, and mastitis milk of two sheep in the United Kingdom and Spain (6), respectively. Subsequently, studies indicated that it can induce many other diseases in species of animals, even in human being, which suggest that it may be a zoonotic pathogen. *H. ovis* was also isolated from cows with abortions (7), puerperal metritis (8, 9), valvular endocarditis (10), horses with pulmonary abscess (11), and sheep with pleuritis and bronchopneumonia (12, 13).

Bacteriology methods and biochemistry tests are usually used to identify the biology characteristics of *H. ovis*; results indicated that this bacterium is a catalase-negative, facultatively anaerobic, and gram-positive cocci; and it often co-infected with other bacteria such as *Staphylococci* and *T. pyogenes*.

To the best of our knowledge, only four isolates of *H. ovis* were genome sequenced by Cunha et al. (8), whose results indicated that the guanine cytosine (GC) content of *H. ovis* was about 27.5%, the number of 33 tRNAs was identified in *H. ovis*, and all four isolates of *H. ovis* contained a ribosomal protection gene (*tetB*) and major facilitator superfamily (MFS) efflux gene (*tetA*), which confer resistance to tetracyclines. In addition, one of the isolates contained AcrEF-TolC, which can confer resistance to fluoroquinolones, cephalosporins, cephamycins, and penams (8).

Recently, *H. ovis* was found in human with pyogenic disease. The patient was reported to have a contact history with wool and cowhide leather before the disease and recovered after treatments of cefotaxime and ornidazole. The *H. ovis* was isolated from the samples collected from the skin around the eyes of the patient. Further antimicrobial resistance test indicated that the *H. ovis* strain is susceptible to penicillin, ampicillin, teicoplanin, ceftriaxone, vancomycin, and linezolid (14). Schwaiger et al. (15) reported coexistence of *H. ovis* with *T. pyogenes* detected by PCR; however, they failed to isolate the bacteria from the samples.

The objective of this study is to describe the *H. ovis* isolates first found in clinical mastitis cases in China, to determine the phylogeny relation to *H. ovis* strains isolated from other species, and investigate the antimicrobial resistance profiles and pathogenicity.

# MATERIALS AND METHODS

## Statement of Ethics

All experiments followed the Regulations of Experimental Animals (2008) promulgated by China Ministry of Science and Technology. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yunnan Agricultural University (Approval No: IACUC-20132030301).

## Isolation and Identification

During the daily etiology examination of clinical mastitis milk samples in a dairy farm in Hebei province, four blood agar plates with tiny and transparent colonies were classified as gram-positive *cocci* but could not be further classified into *Staphylococcus* spp. nor *Streptococcus* spp. by a Streptococcal grouping kit (Lancefield's classification kit, Hopebio, Qingdao, China) test.

To identify these isolates, the four blood agar plates were delivered to the Mastitis Diagnostic Laboratory in China Agriculture University (Beijing). The pin-point colonies were transplanted onto blood agar plates and were incubated in 37°C until 36 h. Heavy growth of two types of colonies was observed, one was tiny and transparent, whereas the other type was white. All the two types of colonies were exposed to gram-staining and microscope examination.

Partial 16S rRNA gene (1,300 bp) amplification (forward primer: 5′-TACCTTGTTACGACTT-3′; reverse primer: 5′-AGAGTTTGATCCTGGCTCAG-3′) (16) and sequencing were conducted, and these sequences were BLAST with the available sequences in GenBank. The phylogenetic tree of these *H. ovis* isolates was constructed using the clustal V method (DNASTAR Lasergene-Megalign, version 7.1).

## Growth Curve of *H. ovis*

The growth curves of *H. ovis* and *S. agalactiae* were assessed simultaneously as *S. agalactiae* is one of the typical $G^+$ cocci pathogens of bovine mastitis. The volume of 1 ml of culture media of each isolate was incubated into 100-ml brain heart infusion broth with 5% fetal bovine serum and placed on a constant temperature shaker (37°C, 220 rpm). The volume of 3 ml of bacterial suspension was collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h, respectively, for optical density (OD) examination at 600 nm in a UV spectrophotometer (Jingke Scientific Instrument Co., Ltd., Shanghai, China).

## Biochemistry Test

Biochemical tests were performed on the four isolates using an HBI biochemical kit according to the manufacturer's instructions (Hopebio, Qingdao, China). Isolates were plated onto tryptic soy blood agar, and then, single colony was suspended into saline equivalent to a turbidity of 0.5 McFarland standard. The volume of 200 μl of saline suspension was added into each micro biochemical pool and then incubated at 37°C for 24 h. The culture medium will turn yellow when the test is positive to ribose, mannitol, sorbitol, lactose, raffinose, maltose, melibiose, and sucrose; red to urease; modena to hippurate hydrolysis; and blue to esterase.

## Antimicrobial Resistance Test

Antimicrobial resistance tests were performed using the broth microdilution method with *Streptococcus pneumoniae* ATCC 49619 as quality control strain according to the Clinical and Laboratory Standards Institute (17). Common used antimicrobials for bovine mastitis treatment and in human medicine was selected for antimicrobial resistance testing, which includes penicillin, cefalexin, ceftiofur, oxacillin, clindamycin,

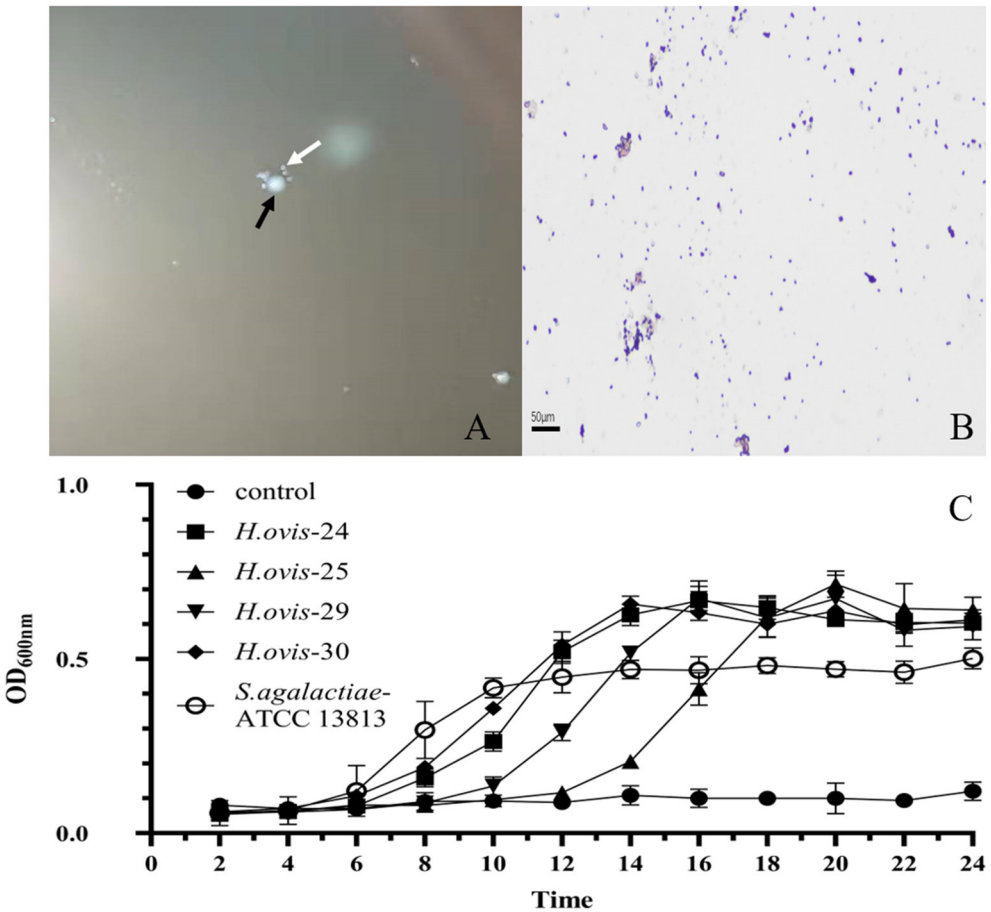


**FIGURE 1 |** Morphological characteristics and growth curves of *Helcococcus ovis*. **(A)** White arrow shows the tiny, transparent colony; the black arrow indicates white colony. **(B)** Gram-positive cocci (400×). **(C)** Gram-positive irregularity rod-shaped bacteria (400×).

**TABLE 1 |** Information of four isolates of *Helcococcus ovis*.

| Place | Isolate identity | Quarter | Mastitis grade (20) | Date | Pathogen |
|---|---|---|---|---|---|
| Hebei | 24 | Left rear | II | July 31, 2019 | *H. ovis, T. pyogenes* |
| Hebei | 25 | Left front | III | July 31, 2019 | *H. ovis, T. pyogenes* |
| Hebei | 29 | Left rear | II | July 31, 2019 | *H. ovis, T. pyogenes* |
| Hebei | 30 | Left front | I | July 31, 2019 | *H. ovis, T. pyogenes* |

tetracycline, enrofloxacin, daptomycin, erythromycin, and vancomycin (18). The dosage was based on previous research (14). Plates were incubated at 37°C in a humidified atmosphere for 24 h. The minimum inhibitory concentration (MIC) of each strain was defined as the lowest concentration of an antimicrobial that completely inhibited growth in broth (no growth) during the 24-h incubation. The MIC was determined as per the CLSI's guidelines. The concentrations of the antimicrobials agents range from 0.015 to 16 μg/ml. Isolates were classified following the clinical breakpoints described in CLSI (17).

## Murine Mammary Infection Model of *H. ovis*

Pregnant (20 days of gestation) 6- to 8-week-old SPF BALB/c mice (SiPeiFu Laboratory Animal Technology, Beijing, China) were used to determine the pathogenic role of *H. ovis* during intramammary infection, as described before (19). On the third day after parturition, mice were anesthetized with intramuscular injection of Zoletil 50 (50 mg/kg; Virbac, Carros, France). Four groups ($n = 5$ per group) of mice were allocated with three challenge groups (12, 24, and 36 h) and one negative control

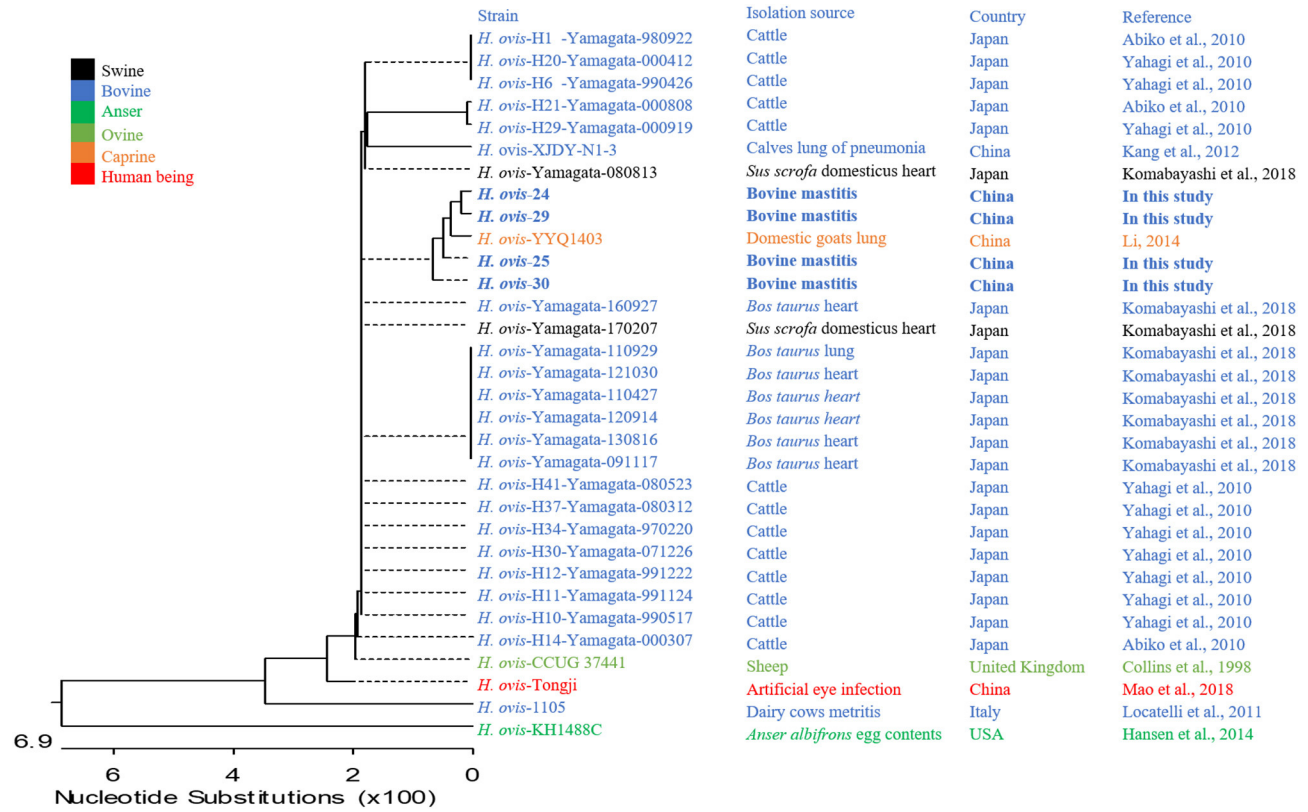


**FIGURE 2 |** Phylogenetic analysis of four strains of *Helcococcus ovis*. Bold fonts indicate *Helcococcus ovis* isolates in this study.

group [sterile phosphate buffered saline (PBS)]. Teat ducts of both the L4 (left) and R4 (right) abdominal mammary glands were exposed under a binocular stereoscopic microscope, and 100 μl of bacterial suspension ($10^5$ CFU/ml) was injected using a syringe with a 34G blunt needle (19). The clinical signs and pathological changes of each mouse were observed and recorded. Then, at 12, 24, and 36 h after challenge (five mice for each time point), the murine mammary gland was separated, part of mammary gland tissue (0.1 g) was separated into sterile tube under a germ-free environment to measure the bacterial burden, and the other part of mammary gland tissue was separated and fixed in 5% of paraformaldehyde to conduct histological evaluation.

## Murine Mammary Co-infection Model *H. ovis* and *T. pyogenes*

To illustrate the pathogenicity of the co-infection of *H. ovis* and *T. pyogenes*, four groups ($n = 5$ per group) of the abovementioned mice were allocated with three challenge groups (*H. ovis*, *T. pyogenes*, and *H. ovis* and *T. pyogenes*) and one negative control group (sterile PBS). The challenge method and dosage is same ($10^5$ CFU/ml) with the single infection model above. The clinical signs and pathological changes of each mouse were observed and recorded. Murine blood was collected for serum separation to conduct ELISA at 24 h after challenge. Then, at 24 h after challenge (five mice for each time point), histological evaluation and immunohistochemical assay were conducted, as in the single challenge model above.

## ELISA

The IL-16 in blood was measured by ELISA kits (MULTI SCIENCES, Hangzhou, Jiangsu, China) according to the manufacturer's instructions. The absorbance was read at 450 nm by Multiskan MK3 (Thermo-Fisher Scientific, Waltham, MA, USA). All absorbance results were normalized by standard curves.

## Histological Analysis and Immunohistochemical Assay

After embedded in paraffin wax, and sectioned and stained with hematoxylin-eosin, histological evaluation was performed to assess tissue necrosis, polymorphonuclear neutrophilic granulocyte inflammation (i.e., neutrophilic inflammation), and lymphocytic inflammation. For immunohistochemical assays, the sections were incubated with rabbit immunoglobulin G (IgG) against mouse IL-1β or TNF-α and then with a HRP-conjugated secondary antibody. A microscope (Olympus, Tokyo, Japan) was used for the examination.

**TABLE 2 |** Biochemical characteristics of the four *Helcococcus ovis* isolates.

| Phenotypic characteristic | Reported *H. ovis* (10) | | *H. ovis* in this study | | | |
|---|---|---|---|---|---|---|
| | CCUG37441 | CCUG39041 | 24 | 25 | 29 | 30 |
| Ribose | − | − | − | − | − | − |
| Mannitol | − | − | − | − | − | − |
| Sorbitol | − | − | − | − | − | − |
| Lactose | − | − | + | + | + | + |
| Raffinose | − | − | − | − | − | − |
| Maltose | + | + | + | + | + | + |
| Melibiose | − | − | − | − | − | − |
| Sucrose | − | − | − | − | − | − |
| β-Mannosidase | − | − | − | − | − | − |
| Urease | − | − | − | − | − | − |
| Hippurate hydrolysis | − | − | − | − | − | − |
| Esterase | + | + | − | − | − | − |

**TABLE 3 |** Antimicrobial resistance profiles of the four *Helcococcus ovis* isolates.

| Antimicrobials | Isolate MIC (μg ml$^{-1}$) | | | | Breakpoints (μg ml$^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | Hebei-24 | Hebei-25 | Hebei-29 | Hebei-30 | S | I | R |
| Penicillin | 0.015 | 0.015 | 0.015 | 0.015 | ≤0.12 | — | ≥0.25 |
| Cefalexin | 1.000 | 1.000 | 1.000 | 0.500 | — | — | ≥8 |
| Ceftiofur | <0.015 | <0.015 | <0.015 | <0.015 | — | — | ≥8 |
| Oxacillin | 0.015 | <0.015 | <0.015 | <0.015 | ≤2 | — | ≥4 |
| Clindamycin | 0.250 | 0.250 | 0.250 | 0.250 | ≤0.5 | 1-2 | ≥4 |
| Tetracycline | 8.000 | 0.500 | 0.500 | 0.500 | ≤4 | 8 | ≥16 |
| Enrofloxacin | 4.000 | 0.500 | 1.000 | 0.500 | ≤2 | 4 | ≥8 |
| Daptomycin | 0.25 | 0.015 | 0.25 | 0.25 | ≤1 | — | — |
| Erythromycin | <0.015 | <0.015 | <0.015 | <0.015 | ≤0.5 | 1-4 | ≥8 |
| Vancomycin | 0.125 | 0.125 | 0. 250 | 0.125 | ≤2 | 4-8 | ≥16 |

*S, sensitive; I, intermediate; R, resistant. Breakpoints refer to Staphylococcus aureus (17) that were used as (14) demonstrated: penicillin ≥ 0.25μg ml$^{-1}$; cefalexin ≥ 8μg ml$^{-1}$; oxacillin ≥ 4μg ml$^{-1}$; clindamycin ≥ 4μg ml$^{-1}$; tetracycline ≥ 16μg ml$^{-1}$; enrofloxacin ≥ 8μg ml$^{-1}$; daptomycin ≥ 1μg ml$^{-1}$; erythromycin ≥ 8μg ml$^{-1}$; and vancomycin ≥ 16μg ml$^{-1}$. Unshaded cells indicate that the MIC value was sensitive, light gray shading indicates intermediate.*

## Statistical Analysis

The prevalence of *H. ovis* and its 95% confidence interval (95% CI) was calculated by using online tool VassarStats (http://www.vassarstats.net/).

All experiments (growth curve determination, bacterial burden, and ELISA) consisted of three independent repeats, and results were analyzed using SPSS 20 and GraphPad Prism 8. 0. 2. Data were assessed using one-way analysis of variance (one- way ANOVA). The data were expressed as the mean ± SD. *P*-values $< 0.05$ were significant.

# RESULTS

## Bacteria Isolation and Identification

The small and transparent colonies were single or in pair gram-positive *cocci*, whereas the white colonies were gram-positive single irregular rod-shaped bacteria (**Figure 1**). The gram-positive *cocci* were identified as *H. ovis*, whereas the rod-shaped bacteria were identified as *T. pyogenes* [information of the four *H. ovis* isolates (prevalence: 11.76%, 95% CI: 4.67–26.62%) was shown in **Table 1**]. The sequences of the *H. ovis* isolates were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers MT758192.1, MT758194.1, MT758195.1, and MT758196.1. All the four isolates (bold fonts) were closely related to the strains isolated from goat (*H. ovis*–YYQ1403) and bovine case (*H. ovis*–XJDY-N1-3) in China; meanwhile, they were also closely related to strains isolated from swine and bovine cases in Japan (e.g., *H. ovis*–Ymagata-080813 and *H. ovis*–Ymagata-160927) (**Figure 2**).

## Growth Curve of *H. ovis*

The growth curve of the 4 *H. ovis* isolates consisted of a lag phase (∼6 h), a log phase (∼10 h), and a stationary phase. The *H. ovis* isolates have a long lag phase and relatively higher $OD_{600nm}$ value comparing with *Streptococcus agalactiae* (**Figure 1D**).

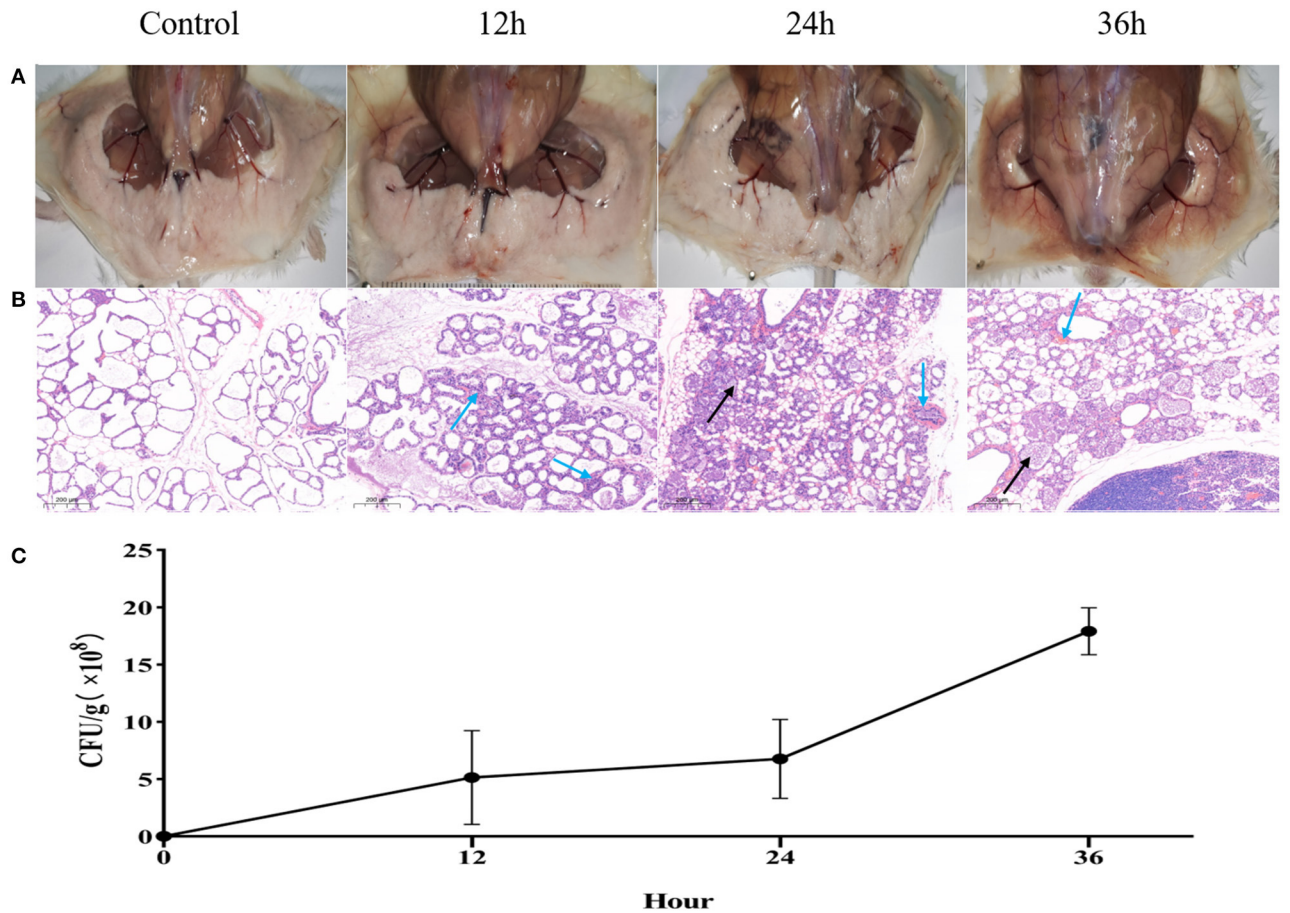


**FIGURE 3 |** Inflammation and histological evaluation and bacterial burden of murine mammary gland after intramammary inoculation with *Helcococcus ovis*. **(A)** Pathological changes in murine mammary glands challenged with *Helcococcus ovis*. **(B)** *Helcococcus ovis* infection induced slight murine mastitis: infiltration of inflammatory cells (mainly neutrophils) into gland alveoli (black arrow) and stromal hyperplasia and progressive mammary alveolar damage (blue arrow). **(C)** Bacterial burden in mammary glands of mice (up to 36 h after inoculation). Each time point represents five mice in each of the four groups. Data are mean ± SD of three independent experiments.

## Biochemistry Test

The ribose, mannitol, sorbitol, raffinose, melibiose, sucrose, β-mannosidase, urease, hippurate, hydrolysis, and esterase reactions were negative for the four isolates while positive to lactose and maltose (**Table 2**). The biochemistry test characterization of the *H. ovis* isolates was same to the standard strain of *H. ovis* CCUG 37441 and CCUG 39041 except for lactose and esterase.

## Antimicrobial Resistance Test

As shown in **Table 3**, results showed that all isolates were susceptible to penicillin, cefalexin, ceftiofur, oxacillin, clindamycin, erythromycin, and vancomycin; except, one isolate (Hebei-24) was intermediate to tetracycline and enrofloxacin.

## Inflammation of Murine Mammary Gland Infected by *H. ovis*

Swollen and hyperemia mammary glands were observed 12 h after challenge with *H. ovis*, and more profound pathological changes observed after 24 and 36 h (**Figure 3A**) after challenge. For histological characteristics of murine mammary glands (see **Figure 3B**). Slight infiltrations of inflammatory cells and progressive mammary alveolar damage were observed, stromal hyperplasia appeared in the infected mammary gland 12 and 24 h after challenge, large quantity of lymphocytes formed a tumor-like structure after 36 h, and degeneration and necrosis of mammary epithelial cells were observed. The bacterial load of the *H. ovis* isolates in the murine mammary gland tissue was $4.6 \times 10^8$ CFU/g after 12-h challenge and gradually increased gradually to $6.8 \times 10^8$ CFU/g and $1.8 \times 10^9$ CFU/g at 24 and 36 h after challenge (**Figure 3C**).

## Pathogenicity of Co-infection of *H. ovis* and *T. pyogenes*

At 24-h after challenge, the bacterial load was even among the three infected groups (**Figure 4C**). Swollen, hyperemia, and edema mammary gland were observed after individual challenge with *H. ovis* and *T. pyogenes* separately; severer pathological

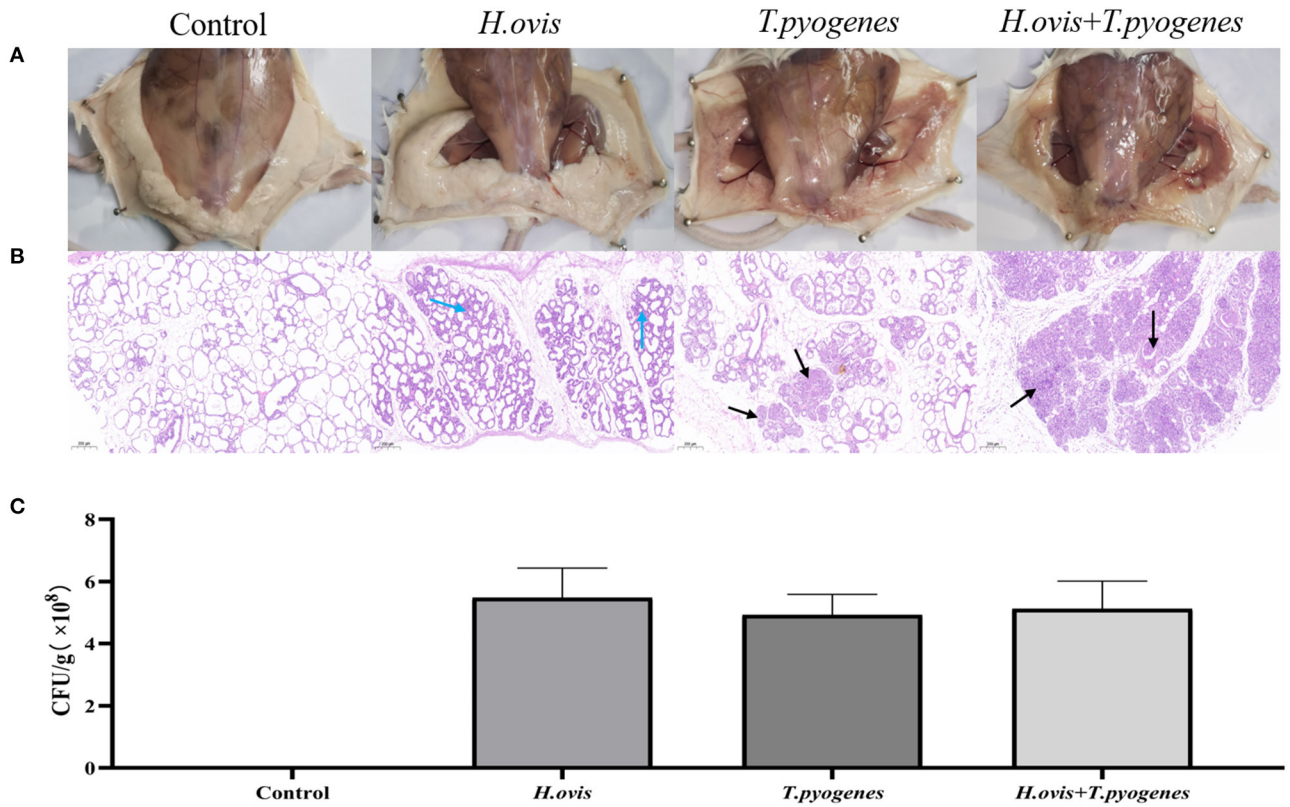


**FIGURE 4 |** Inflammation and histological evaluation and bacterial burden of murine mammary gland after intramammary inoculation with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. **(A)** Pathological changes in murine mammary glands challenged with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. **(B)** *Helcococcus ovis* induced murine mastitis, with stromal hyperplasia and mammary alveolar damage (blue arrow) after infection. *Trueperella pyogenes* induced murine mastitis, with an increased infiltration of inflammatory cells (mainly neutrophils) into the gland alveoli (black arrow) and progressive mammary alveolar damage after infection. *Helcococcus ovis* and *Trueperella pyogenes* co-infection induced murine mastitis, with an increasing number of inflammatory cells (mainly neutrophils) infiltrated into the gland alveoli (black arrow), stromal hyperplasia, and progressive mammary alveolar damage after infection. **(C)** Bacterial burden in mammary glands of mice (24 h after inoculation). Each group represents five mice in each of the four groups. Data are mean ± SD of three independent experiments.

changes exhibited in the group co-infected by *H. ovis* and *T. pyogenes* (**Figure 4A**). Histological examination revealed severer inflammation in the co-infection group than the individual challenge group: stromal hyperplasia, slight infiltrations of inflammatory cells, and mammary alveolar damage exhibited in *H. ovis* infection group; numerous inflammatory cells infiltrations and mammary alveolar damage were observed in *T. pyogenes* infection group; whereas massive infiltrations of inflammatory cells and stromal hyperplasia together with mammary alveolar damage exhibited in the *H. ovis* and *T. pyogenes* co-infection group (**Figure 4B**).

The intensity of brown color revealed that TNF-α and IL-1β in murine mammary gland was upregulated in the co-infection group than the *H. ovis* and *T. pyogenes* individual infectiongroups in immunohistochemical analysis. The expression of IL-16 in murine serum was increased significantly in the co-infection group than in the individual infection group by the ELISA test results (**Figure 5**).

## DISCUSSION

To the best of our knowledge, this study is the first report of *H. ovis* isolated from bovine mastitis in China, although the prevalence was relatively low. In China, there were only four publications of *H. ovis*, which includes three cases in goat and bovine pneumonia (18, 21, 22) and one in human artificial eye, which raised concern over the zoonotic property of *H. ovis* (14). More mastitis milk samples should be invesgated and etiological research need to be conducted to confirm the pathogenicity of *H. ovis* in bovine mastitis and other infection diseases according to Koch's postulates. Rothschild (11) assumed that this bacteria is one of the skin microbiota of cows, which also required further study. The colony of *H. ovis* is so tiny that it may be ignored by laboratory technicians when mixes with other major bacteria or milk droplets during routine milk sample examination. Similar with mycoplasma, it is a critical mastitis pathogen but difficult to isolate due to slow growth and tiny colonies, which makes it

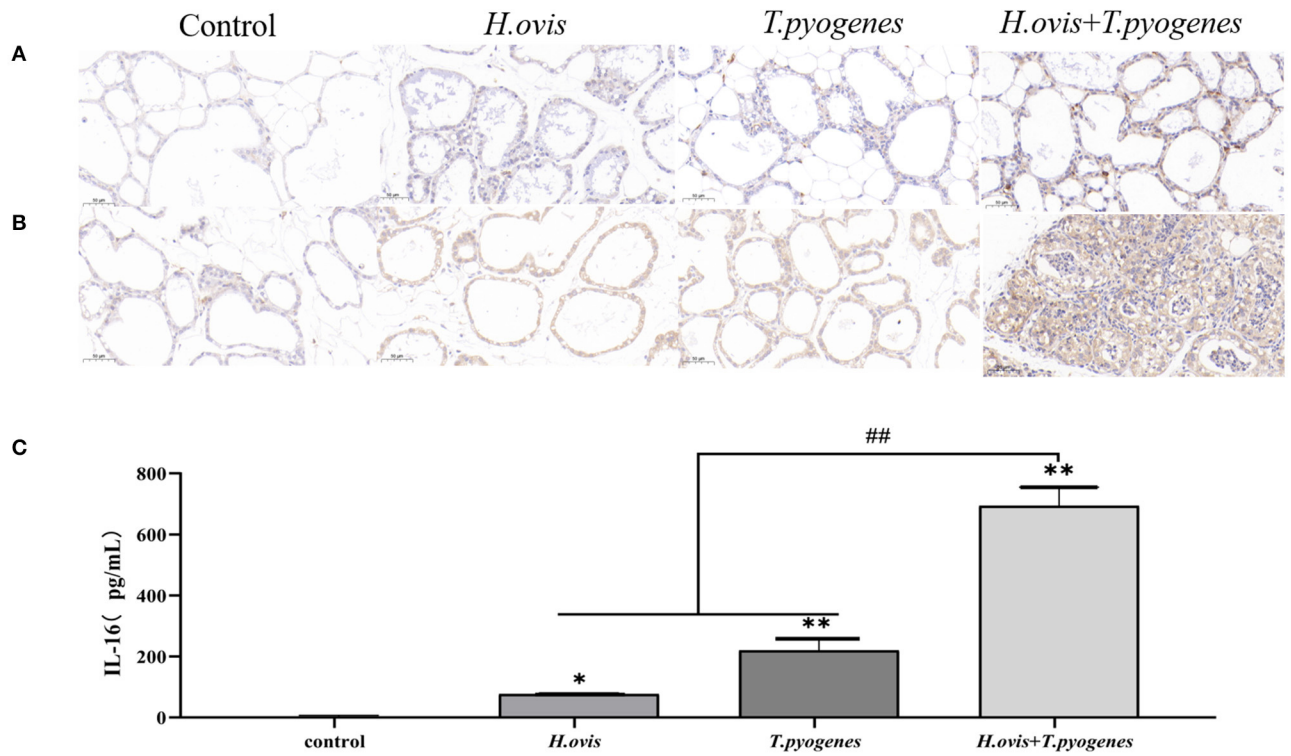


**FIGURE 5 |** Immunohistochemical (IHC) detection of IL-1β and TNF-α in murine mammary glands and enzyme linked immunosorbent assay (ELISA) detection of IL-16 in murine serum after intramammary inoculation with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. **(A)** IHC detection of IL-1β in murine mammary glands challenged with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. **(B)** IHC detection of TNF-α in murine mammary glands challenged with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. **(C)** ELISA detection of IL-16 in murine serum challenged with *Helcococcus ovis*, *Trueperella pyogenes*, *Helcococcus ovis*, and *Trueperella pyogenes*. Data are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}$ and $\#\#P < 0.01$.

hard to diagnose and research (23). Molecular diagnostics can overcome this and, at the same time, etiology investigation of an emerging pathogen is also pivotal.

Bovine mastitis is predominantly caused by bacteria; therefore, antimicrobials are extensively applied to mastitis prevention and treatment (19), which raised the concern of AMR that threatens human health (18). In this study, the resistance of the four isolates to daptomycin was observed, and one of the isolates was intermediary resistant to tetracycline and enrofloxacin, which were common used in treatments against bovine mastitis. Genome sequencing of *H. ovis* isolated from bovine puerperal metritis in previous research has indicated that the isolates in that study contained tetracycline-resistant gene: a ribosomal protection gene (*tetB*) and MFS efflux gene (*tetA*), and one of the isolates contained AcrEF-TolC, an inner membrane proton that confers resistance to fluoroquinolones, cephalosporins, cephamycins, and penams (8). However, in our study, only slight resistance to antimicrobials was demonstrated, which may indicated that the isolates were rarely exposed to antimicrobial treatments and their emergence in mastitis milk was recent.

The phenotypic characteristics difference of the four isolates with CCUG 37441 and CCUG 39041 may be due to the fact that isolates were isolated from mastitis milk and that lactose was the main carbohydrate source. Because of the unreliability of biochemical methods, they can only be used as auxiliary inspection method, and molecular identification is required to confirm it (14).

The murine model of intramammary challenge with bovine mastitis pathogens has been successfully used to assess bacterial infection and tissue damage (19). The murine model of *H. ovis* may improve our understanding on the correlation between this bacteria and bovine mastitis, the treatment efficacy applied in clinical trials, as well as the relationship among the bacteria, bovine immune response, and lactation (24). The mammary epithelial cells and the resident macrophages are the first line to interact with pathogens at the onset of mastitis, and then, cytophagocytosis is activated, and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8 are released. Consequently, more immunocytes such as neutrophils and lymphocytes are gathered to the inflammation site, and then, more inflammatory cytokines secreted and inflammatory worsened (5).

In this study, the pathogenicity of *H. ovis* to mice was examnied at different periods after challenge. The slight infiltrations of inflammatory cells, stromal hyperplasia, mild mammary alveolar damage, and bacteria burden in tissue

indicated that *H. ovis* cannot cause severe mastitis. Disease caused by the co-infection of *H. ovis* and other pathogens have been reported before (6, 9, 10), whereas the pathogenicity of the co-infection *H. ovis* infection has not been researched. To discover the pathogenicity of the co-infection of *H. ovis* and *T. pyogenes*, an advanced study was conducted. Individual infection by *T. pyogenes* leads to typical bovine mastitis (such as the symptoms of redness, swelling, heat, pain, and dysfunction of mammary glands, as well as massive infiltration of inflammatory cells, and mammary alveolar damage) in this study, which was consistent with previous study that *T. pyogenes* was distributed in variaty of animals and can lead to bovine mastitis (25). Although the co-infection of *H. ovis* and *T. pyogenes* induced severer mastitis than individual infections by each of the two bacteria, the murine mammary gland alveolar was filled with rupt epithelial cells and neutrophils. Leukocytes are always circulating in the blood around mammary gland; when the barriers of mammary gland are broke through by bacteria, the epithelial cells are damaged, cytokines such as IL-1β and TNF-α are released, and chemotaxis moves neutrophils into alveolar to eliminate the invading pathogen. When the infection occurs and neutrophils apoptosis being delayed, it helps limiting the extent of infection (5). The infection of *H. ovis* and *T. pyogenes* also increased the IL-16 concentration in murine serum. As IL-16 is secreted by T cells and is chemoattractant to $CD4^+$T cells, the two bacteria may be able to activate cellular immunity. The interaction among microbiota plays a crucial role in biofilm forming, metabolism, and pathogenicity (26–29). Stipkovits also demonstrated the synergy between *Mycoplasma arginini* (*M. arginini*) and *Streptococcus dysgalactiae* (*Strep. dysgalactiae*); due to the ability of *M. arginini* to inhibit the T cells growth and the cytotoxic T cells activity, *M. arginini* infection does not produce clinical signs of mastitis but it can induce severe mastitis together with *Strep. dysgalactiae* (30). In this study, *H. ovis* was isolated from samples that *T. pyogenes* exist, and the co-infection of *H. ovis and T. pyogenes* caused much serious murine mastitis than individual infection by *T. pyogenes*; meanwhile, the challenge with *H. ovis* alone only caused mild mastitis, which means that *H. ovis* may be able to induce immunosuppression in cows or improve the inflammation.

## CONCLUSIONS

The *H. ovis* isolates in our research were closely related to other strains isolated from China and the strains from Japan, the growth speed of the isolates was relatively slower than *S. agalactiae*, and the phenotypic characteristics were similar to CCUG37441 and CCUG39041 except to lactose; isolates were sensitive to most of antimicrobials; *H. ovis* could lead to mild murine mastitis and could induce severe mastitis when co-infected with *T. pyogenes*. As an emerging pathogen, it draws importance when designing mastitis control plans against *H. ovis*.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Yunnan Agricultural University (Approval No: IACUC-20132030301).

## AUTHOR CONTRIBUTIONS

The study was designed by KL, LZ, and XG. The experiments were performed by YL and PC. The data were analyzed by JG, WQ, and BH, who also drafted the manuscript, which was reviewed and revised by ZD and GL. The final version was read and approved by all authors.

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## REFERENCES

1. Shaheen M, Tantary HA, Nabi SU. A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. *J Adv Dairy Res.* (2016) 1:1000150. doi: 10.4172/2329-888X.1000150
2. Krömker V, Leimbach S. Mastitis treatment-reduction in antibiotic usage in dairy cows. *Reprod Domest Anim.* (2017) 3:21–9. doi: 10.1111/rda.13032
3. Gussmann M, Denwood M, Kirkeby C, Farre M, Halasa T. Associations between udder health and culling in dairy cows. *Prev Vet Med.* (2019) 171:104751. doi: 10.1016/j.prevetmed.2019.104751
4. Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L, et al. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *J Dairy Sci.* (2017) 100:4797–806. doi: 10.3168/jds.2016-12334
5. Alhussien MN, Dang AK. Potential roles of neutrophils in maintaining the health and productivity of dairy cows during various physiological and physiopathological conditions: a review. *Immunol Res.* (2019) 67:21–38. doi: 10.1007/s12026-019-9064-5
6. Collins MD, Falsen E, Foster G, Monasterio LR, Dominguez L. Fernandez-Garazabal JF. *Helcococcus ovis* sp. *nov,* a gram-positive organism from sheep. *Int J Syst Bacteriol.* (1999) 4:1429–32. doi: 10.1099/00207713-49-4-1429
7. AHVLA's disease surveillance report. Bovine abortions caused by *Helcococcus ovis. Vet Rec.* (2014) 175:38–41. doi: 10.1136/vr.g4376
8. Cunha F, Jeon SJ, Kutzer P, Jeong KC, Galvão KN. Draft genome sequences of *Helcococcus ovis* strains isolated at time of metritis diagnosis from the uterus of holstein dairy cows. *Microbiol Resour Announc.* (2019) 8:e00402–19. doi: 10.1128/MRA.00402-19
9. Locatelli C, Scaccabarozzi L, Pisoni G, Bronzo V, Casula A, Testa F, et al. *Helcococcus kunzii* and *Helcococcus ovis* isolated in dairy cows with puerperal metritis. *J Gen Appl Microbiol.* (2013) 59:371–4. doi: 10.2323/jgam.59.371
10. Kutzer P, Schulze C, Engelhardt A, Wieler LH, Nordhoff M. *Helcococcus ovis*, an emerging pathogen in bovine valvular endocarditis. *J Clin Microbiol.* (2008) 46:3291–5. doi: 10.1128/JCM.00867-08

11. Rothschild CM, Oaks JL, Schaupp JK, Rurangirwa FR, Sellon DC, Hines MT. *Helcococcus ovis* isolated from a pulmonary abscess in a horse. *J Clin Microbiol.* (2004) 42:2224–6. doi: 10.1128/JCM.42.5.2224-2226.2004
12. Zhang Y, Cui J, Parkinson A, Hayes J, Ott K, Byrum B. Isolation of *Helcococcus ovis* from sheep with pleuritis and bronchopneumonia. *J Vet Diagn Invest.* (2009) 21:164–6. doi: 10.1177/104063870902100130
13. Li R, Yang Y, Tang Q. Diagnosis and treatment of mixed infection of *Pasteurella* and *Helcococcus ovis* in Nubian goats (in Chinese). *Chin J Vet Med.* (2015) 52:48–9.
14. Mao L, Chen Z, Lu Y, Yu J, Zhou Y, Lin Q, et al. *Helcococcus ovis* in a patient with an artificial eye: a case report and literature review. *BMC Infect Dis.* (2018) 18:401. doi: 10.1186/s12879-018-3310-7
15. Schwaiger K, Wimmer M, Huber-Schlenstedt R, Fehlings K, Hölzel CS, Bauer J. Hot topic: Bovine milk samples yielding negative or nonspecific results in bacterial culturing–the possible role of PCR-single strand conformation polymorphism in mastitis diagnosis. *J Dairy Sci.* (2012) 95:98–101. doi: 10.3168/jds.2011-4700
16. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol.* (2008) 74:2461–70. doi: 10.1128/AEM.02272-07
17. Clinical and Laboratory Standard Institute. *Performance Standards for Antimicrobial Susceptibility Testing.* Wayne, PA: CLSI (2019). p. M100–S124.
18. Cheng J, Qu W, Barkema HW, Nobrega DB, Gao J, Liu G, et al. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *J. Dairy Sci.* (2019) 102:2416–26. doi: 10.3168/jds.2018-15135
19. Chen P, Qiu Y, Liu G, Li X, Cheng J, Liu K, et al. Characterization of *Streptococcus lutetiensis* isolated from clinical mastitis of dairy cows. *J Dairy Sci.* (2021) 104:702–14. doi: 10.3168/jds.2020-18347
20. Adkins PR, Middleton JR. *Laboratory Handbook on Bovine Mastitis.* Madison, WI: National Mastitis Council Incorporated (2017).
21. Zhou W, Li N, Wei X, Hu J, Xia T, Wang Z, et al. Isolation and identification of *Helcococcus ovis* from bovine (in Chinese). *Chin J Prev Vet Med.* (2012) 34:584–6.
22. Guo H. Diagnosis and treatment of a case of mixed infected of *Mycoplasma bovine* and *Helcococcus ovis* pneumonia (in Chinese). *China Cattle Sci.* (2015) 41:91–2.
23. Liu Y, Zhou M, Xu S, Khan MA, Shi Y, Qu W, et al. Mycoplasma bovis-generated reactive oxygen species and induced apoptosis in bovine mammary epithelial cell cultures. *J Dairy Sci.* (2020) 103:10429–45. doi: 10.3168/jds.2020-18599
24. Ingman WV, Glynn DJ, Hutchinson MR. Mouse models of mastitis - how physiological are they? *Int Breastfeed J.* (2015) 10:12. doi: 10.1186/s13006-015-0038-5
25. Rzewuska M, Kwiecień E, Chrobak-Chmiel D, Kizerwetter-Swida M, Stefańska I, Gieryńska M. Pathogenicity and virulence of *Trueperella pyogenes*: a review. *Int J Mol Sci.* (2019) 20:2737. doi: 10.3390/ijms20112737
26. Bäumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature.* (2016) 535:85–93. doi: 10.1038/nature18849
27. Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. *Nat Rev Microbiol.* (2010) 8:340–9. doi: 10.1038/nrmicro2313
28. Røder HL, Sørensen SJ, Burmølle M. Studying bacterial multispecies biofilms: where to start? *Trends Microbiol.* (2016) 24:503–13. doi: 10.1016/j.tim.2016.02.019
29. Rakoff-Nahoum S, Coyne MJ, Comstock LE. An ecological network of polysaccharide utilization among human intestinal symbionts. *Curr Biol.* (2014) 24:40–9. doi: 10.1016/j.cub.2013.10.077
30. Stipkovits L, Somogyi M, Asvanyi B, Toth A, Szathmary S. Short communication: role of Mycoplasma arginini in mastitis caused by *Streptococcus dysgalactiae*. *J Dairy Sci.* (2013) 96:1661–7. doi: 10.3168/jds.2012-5669


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# The Prevalence of *Klebsiella* spp. Associated With Bovine Mastitis in China and Its Antimicrobial Resistance Rate: A Meta-Analysis

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Understanding distribution of bovine mastitis pathogen *Klebsiella* spp. can contribute to the treatment decision and the control within programs of bovine mastitis, we conducted a meta-analysis to investigate the epidemiology and antimicrobial resistance rates of *Klebsiella* spp. associated with bovine mastitis in China. Three databases, namely, PubMed, Google scholar, and China National Knowledge Infrastructure database, were utilized to obtain relevant publications. According to PRISMA reporting standards, a total of 38 publications were included in the research, among them, 7 papers included an AMR test. The pooled prevalence of *Klebsiella* spp. was 5.41% (95% CI: 3.87–7.50%). Subgroup analysis revealed that the prevalence was higher in South China (8.55%, 95% CI: 3.57–19.09%) than in North China (4.22%, 95% CI: 2.46–7.14%), in 2010–2020 (7.45%, 95% CI: 5.29–110.40%) than in 2000–2010 (3.14%, 95% CI: 1.90–15.14%), and in the clinical bovine mastitis cases (7.49%, 95% CI: 3.71–14.54%) than in the subclinical cases (4.03%, 95% CI: 1.55–10.08%). The pooled AMR rate revealed that *Klebsiella* spp. were most resistant to sulfonamides (45.07%, 95% CI: 27.72–63.71%), followed by tetracyclines (36.18%, 95% CI: 23.36–51.34%), aminoglycosides (27.47%, 95% CI: 17.16–40.92%), β-lactams (27.35%, 95% CI: 16.90–41.05%), amphenicol (26.82%, 95% CI: 14.17–44.87%), lincosamides (21.24%, 95% CI: 7.65–46.75%), macrolides (20.98%, 95% CI: 7.20–47.58%), polypeptides (15.51%, 95% CI: 6.46–32.78%), and quinolones (7.8%, 95% CI: 3.25–17.56%). The climate difference between South and North China and the natural pathogenicity of *Klebsiella* spp. may be the primary reasons for its distribution, and the prevalence of *Klebsiella* spp. indicated that the genus is an increasing hazard to the dairy industry. The prevalence of AMR in China is commonly higher than in the European countries and Canada, this is a very important concern for strategy programs to control bovine mastitis caused by *Klebsiella* spp. in China.

**Keywords: bovine mastitis, *Klebsiella* spp., epidemiology, antimicrobial resistance, meta-analysis**

# INTRODUCTION

Mastitis is one of the costliest diseases in the dairy industry due to the discarding of milk and expenses of treatments, including the culling of cows (1–3). *Klebsiella* spp. are the major gram-negative pathogens that cause mastitis (4–7), and the concern for their pernicHiousness to the dairy industry in China has increased in recent years (8). In recent research, *Klebsiella* spp. was isolated from 13% of clinical bovine mastitis samples collected from dairy farms in China (9). *Klebsiella* spp. mastitis is prolonged with a severe and long-lasting duration of intramammary infection and is often accompanied by a considerable decrease in milk production (10); this condition shows no desirable response to antimicrobial treatments (5, 11). Consequently, cows with *Klebsiella* spp. mastitis are more likely to be culled compared with cows with other types of mastitis (12–15).

Antimicrobials are still the major option for the treatment of mastitis (16). However, the abuse of antimicrobials increases the risks of antimicrobial resistance (AMR) in bacteria, which is a worldwide public health concern (17, 18). Alvarez-Uria et al. predicted that a considerable proportion of *Klebsiella pneumoniae* will likely be resistant to carbapenems and third-generation cephalosporin in most parts of the world by 2030 (19). The "National action plan to combat animal resources antimicrobial resistance (2017–2020). Beijing: China Ministry of Agriculture and Rural Affairs; 2017," is one of the national protocols for standardizing veterinary medication, along with strict biosecurity, sterile standard, and the prudent use of antimicrobials to release the pressure of transmission of antimicrobial-resistant pathogens. Wang et al. (20) reported that the policy and decreased use of colistin in agriculture had a significant effect on the reduction of colistin resistance in animals and humans in China.

Investigation of the epidemiology and AMR profiles of *Klebsiella* spp. can contribute to treatment decisions and optimization of *Klebsiella* spp. control programs (21). Numerous publications focused on the AMR of other major bovine mastitis pathogens in China, including *S. aureus* and *E. coli* (22, 23), whereas the meta-analysis can overcome the insufficient spatial and temporal distribution of *Klebsiella* spp.

# MATERIALS AND METHODS

## Literature Search

**Figure 1** illustrates the relevant steps and results of the literature retrieval. For a previously published review, a comprehensive and systematic literature search was conducted by two independent reviewers on 23 May 2021, utilizing the PubMed (http://www.pubmed.gov), Google scholar (https://scholar.google.com), and China National Knowledge Infrastructure (CNKI) databases (https://www.cnki.net/) to identify the literature focusing on Klebsiella mastitis in cows. The subject heading "bovine mastitis AND bacteria" was used to find all trials on this topic written in the English or Chinese language. The time was set from 2000 to 2021 to assure the timeliness of the subsequent meta-analytic investigation.

## Inclusion and Exclusion Criteria

As reported previously (24), our study was in accordance with PRISMA reporting standards (25), specific exclusion criteria were defined to exclude articles that did not describe clinical trials (e.g., descriptive, or *in vitro* studies). Two authors reviewed all abstracts and then performed a full-text review of articles for eligibility independently, the agreement between the two reviewers for inclusion of articles was good ($\kappa = 0{\cdot}86$). The excluded publications included review articles, articles did not meet the inclusion criteria due to wrong indexation ("off topic"), out of the considered time period, small sample size (less than three samples), exclusion of Klebsiella, undeclared bacterial identification method, samples containing non-mastitis diseases, undeclared sample size or number of bacterial isolates, and unobtainable through the internet. Then, the two reviewers extracted the data from included articles independently. Retrieval and management of references were performed with Excel (Office 16 for Windows, Microsoft Office, New York, USA) (**Table 1**).

## Statistical Analysis

Data were extracted from individual studies using a predesigned form obtaining data on the author, year, province, the number of samples, the number of Klebsiella isolates, mastitis grade (clinical and subclinical mastitis criterion: Laboratory handbook on bovine mastitis and National Mastitis Council), bacterial identification methods, the number of antibiotic-resistant isolates, and laboratory procedures. The same two reviewers independently, and in duplicate, assessed the methodological quality of each individual study based on the prespecified study quality indicators adapted from the Downs and Black checklist.

The numbers of *Klebsiella* spp., antimicrobial-resistant isolates, and mastitis milk samples within individual studies were calculated for their proportion. Resistance was considered a dichotomous outcome, as classified by individual primary studies. Isolates with intermediate susceptibility were classified as susceptible.

Meta-analyses were performed separately for *Klebsiella* spp. prevalence and their AMR rates. This procedure was performed by using the "meta" and "metafor" package in R (Version 4.0.5) and only conducted if four or more studies were considered because between-study variance cannot be estimated accurately when it is less than this number and may result in biased pooled estimates after the meta-analysis.

We pooled the prevalence of *Klebsiella* spp. using random effects models. Subgroup meta-analyses were conducted for isolation time, isolate regions, and mastitis grade to illustrate the heterogeneity between the included studies.

For the AMR studies, we pooled analyses within nine groups: β-lactams, quinolones, aminoglycosides, tetracyclines, polypeptides, sulfonamides, amphenicol, macrolides, and lincosamides.

A publication bias test was performed by using "Egger" test, and the funnel plot was created. Sensitivity analysis was conducted by using "leave-one-out" analyses. Both of them were conducted by using the "meta" and "metafor" packages in R (version 4.0.5).

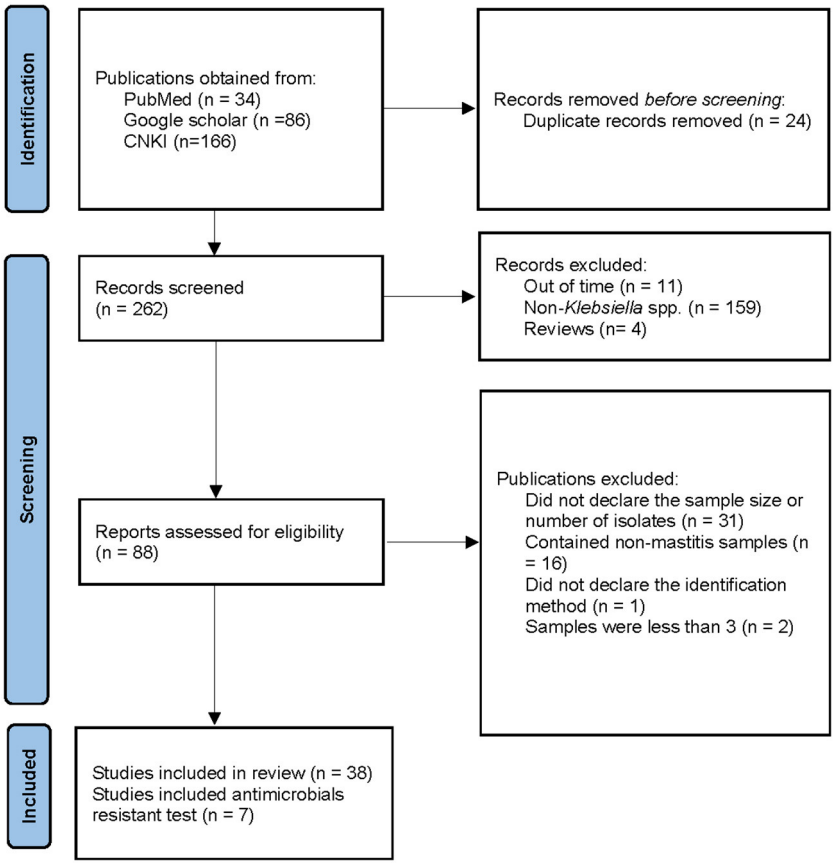


**FIGURE 1 |** Search and selection criteria for literatures. CNKI means Chinese National Knowledge Infrastructure.

# RESULTS

## Inclusion of Publications

A total of 34, 86, and 166 articles were obtained from PubMed, Google scholar, and CNKI, respectively. Among them, 24 publications were duplicated, a total of 31 publications were excluded because they did not declare the sample size nor the number of bacterial isolates, and two publications used a small sample size, which were excluded. In addition, 16 articles contained non-mastitis cases, 159 publications did not contain *Klebsiella* spp. cases, 11 articles were beyond the considered period (before 2000), four articles were reviews, and one article did not declare the identification method. Therefore, these publications were denied. Finally, a total of 38 full-textt publications were included in our research, of which 7 covered the AMR test (**Figure 1**).

As for the 38 publications, two publications did not describe the sample collected location exactly, they were included and given our focus on the prevalence of Klebsiella throughout the country. A total of seven publications obtained clinical and subclinical samples, and 14 did not describe the grade of mastitis. However, we still included them in our research because we focused on the whole condition of bovine mastitis (**Table 1**).

## Prevalence of *Klebsiella* spp.

The pooled prevalence of *Klebsiella* spp. was 5.41% (95% CI: 3.87–7.50%). An evident heterogeneity was observed ($I^{2=}$ 95%, $\tau^2 = 0.965$, $P<0.01$). Therefore, a subgroup analysis was conducted to explore the sources of heterogeneity (**Figure 2**).

## Subgroup Analysis

We divided the research articles into subgroups based on the research period (2000–2010 vs. 2010–2020), sample sites (North China vs. South China), and mastitis grade (clinical mastitis vs. subclinical mastitis). The pooled prevalence values of *Klebsiella* spp. were 3.14 and 7.45% (2000–2010 vs. 2010–2020, **Figure 3**); 7.49 and 4.03% (clinical mastitis vs. subclinical mastitis, **Figure 4**); 4.22 and 8.55% (North China vs. South China, **Figure 5**), respectively.

## Publication Bias of the Prevalence of *Klebsiella* spp.

The funnel plot (**Figure 6**) exhibited an even distribution of the studies around the mean effect size, which suggested that the publication bias was not evident.

**TABLE 1 |** Information of literatures included in our study.

| Author | Publication year | Samples | Identification assay[1] | Case | Grade[2] | Region[3] | AMR method[4] |
|---|---|---|---|---|---|---|---|
| Linzheng Jiang | 2020 | 31 | 16S | 2 | – | S | K–B |
| Lan Liu | 2009 | 32 | other | 9 | – | NS | – |
| Ling Wang | 2020 | 37 | 16S | 12 | C | S | – |
| Xiujuan Ye | 2004 | 44 | other | 3 | – | S | – |
| Ridong Guo | 2015 | 45 | other | 3 | C | NS | – |
| Qiuyun Zhao | 2016 | 48 | other | 1 | C | NS | – |
| Mingxu Zhou | 2019 | 50 | 16S | 13 | S | S | K–B |
| Chengyi Zhou | 2007 | 50 | other | 3 | S | S | – |
| Le Wang | 2019 | 53 | 16S | 6 | C | NS | K–B |
| Jing Wang | 2018 | 57 | other | 13 | C | NS | – |
| Jin Li | 2014 | 58 | other | 3 | C | NS | – |
| Wei Liu | 2006 | 60 | other | 2 | – | NS | – |
| Dongyang He | 2006 | 64 | other | 2 | CS | S | – |
| Ning Zhu | 2020 | 71 | other | 8 | – | S | MIC |
| Xurong Wang | 2012 | 76 | other | 3 | – | NS | – |
| Zhiyuan Wang | 2002 | 85 | other | 1 | CS | NS | – |
| Haiping Deng | 2007 | 100 | other | 2 | – | NS | – |
| Yonghua Qi | 2006 | 102 | other | 3 | – | NS | – |
| Huiyun Zhao | 2020 | 110 | 16S | 2 | C | NS | – |
| Guiying Wang | 2008 | 115 | other | 3 | C | NS | – |
| Jidong Zhang | 2006 | 150 | other | 9 | CS | – | – |
| Lijun Wu | 2019 | 165 | 16S | 2 | S | S | – |
| Jie Tan | 2014 | 166 | other | 5 | CS | NS | – |
| Xiaohui Feng | 2019 | 200 | 16S | 32 | – | NS | MIC |
| Yingying Ge | 2019 | 210 | other | 5 | – | NS | – |
| Airi Ha | 2018 | 212 | other | 55 | – | NS | – |
| Javed Memon | 2012 | 217 | 16S | 11 | S | NS | – |
| Yuxiang Shi | 2020 | 245 | 16S | 45 | C | NS | K–B |
| Huarong Song | 2009 | 260 | other | 6 | S | NS | – |
| Hongsheng Li | 2002 | 280 | 16S | 4 | – | NS | – |
| Xinpu Li | 2015 | 302 | 16S | 1 | C | NS | – |
| Bo Yang | 2009 | 370 | 16S | 4 | S | NS | – |
| Jia Cheng | 2020 | 916 | 16S | 206 | CS | NS | MIC |
| Zhe Zhang | 2019 | 1122 | 16S | 18 | – | – | – |
| Xiangbin Song | 2020 | 1153 | 16S | 23 | CS | NS | – |
| Limei Wang | 2007 | 1456 | other | 13 | CS | NS | – |
| Sanping Bo | 2014 | 1716 | other | 78 | – | NS | – |
| Jian Gao | 2017 | 3190 | 16S | 426 | C | NS | – |

*1.16S means 16S rDNA sequencing. 2. C, clinical bovine mastitis; S, subclinical bovine mastitis; CS, clinical and subclinical bovine mastitis. 3. S, South China; N, North China; NS, North and South China. 4. K–B, disk diffusion, MIC, broth microdilution. "–" means the information was not declared in the original articles.*

## Antimicrobial Resistant Rate of *Klebsiella* spp.

The pooled resistant rates were as follows: β-lactams, 27.35% (95% CI: 11.73–24.79%); quinolones, 7.8% (95% CI: 3.25–17.56%); aminoglycosides, 27.47% (95% CI: 17.16–40.92%); tetracyclines, 36.18% (95% CI: 23.36–51.34%); polypeptides, 15.51% (95% CI: 6.46–32.78%); sulfonamides, 45.07% (95% CI: 27.72–63.71%); amphenicol, 26.82% (95% CI: 14.17–44.87%); macrolides, 20.98% (95% CI: 7.20–47.58%); lincosamides, 22.24% (95% CI: 7.65–46.75%) (**Figure 7**).

## Publication Bias of the AMR Rate of *Klebsiella* spp.

The funnel plot (**Figure 8**) exhibited an even distribution of the studies around the mean effect size, which suggested a negligible publication bias.

# DISCUSSIONS

Bovine mastitis is the costliest disease in the dairy industry (26). *Klebsiella* spp. is important pathogens causing bovine

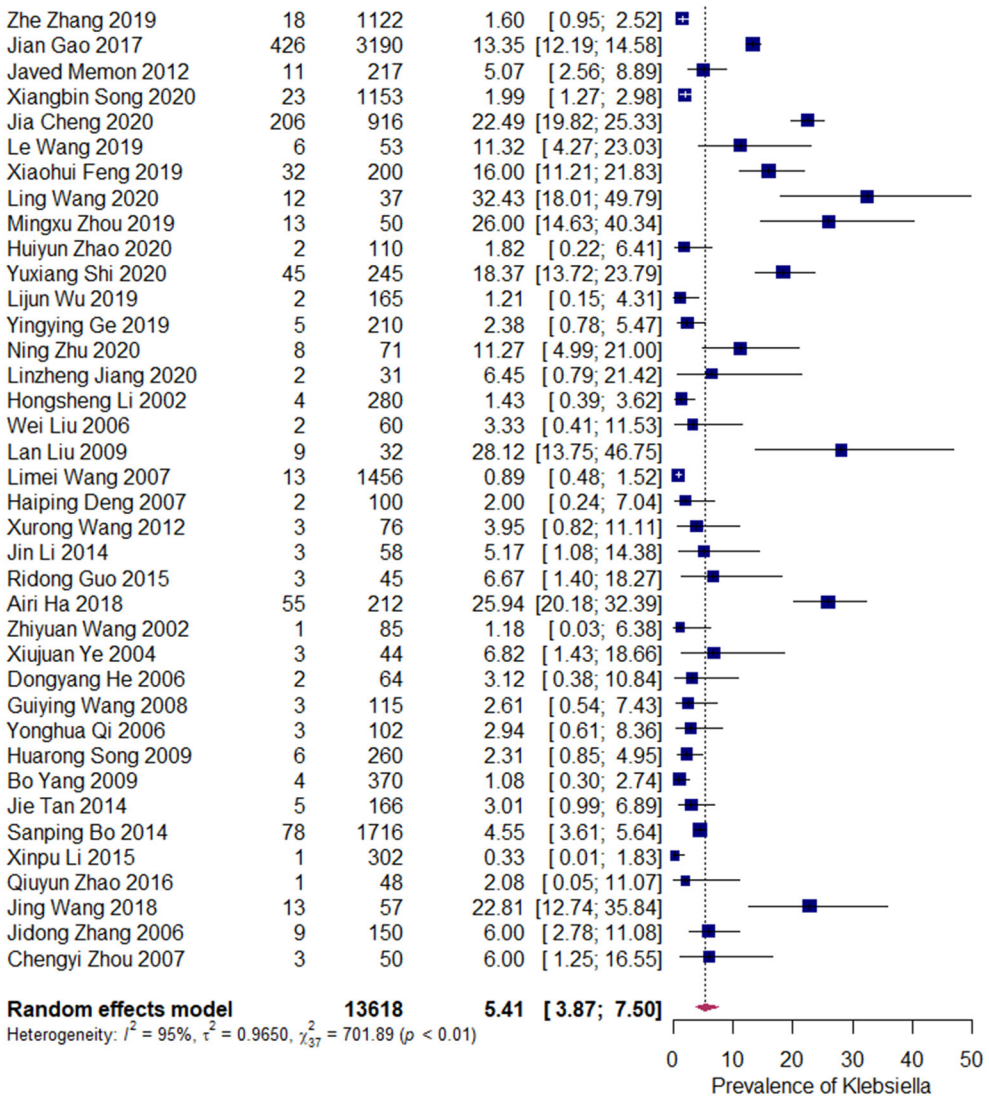


**FIGURE 2 |** Prevalence of *Klebsiella* spp. Each navy blue square represents the mean prevalence for a study. The upper and lower limit of the line connected to the square represents the upper and lower 95% CI for the prevalence. The size of the square reflects the relative weighting of the study to the overall prevalence estimate, with larger squares representing greater weight. The broken vertical line represents overall prevalence and the diamond at the bottom represents the overall prevalence (location of the diamond) and its 95% CI (width of the diamond). Cases refers to the *Klebsiella* spp. containing samples. "Author year" refers to the first author and publication year of the publication (the same below).

mastitis and human infection (27, 28). Understanding the prevalence and AMR profiling of bovine mastitis, *Klebsiella* spp. may contribute to therapeutic interventions and preventive strategies.

A total of 38 publications, 13,618 samples, and 1,037 isolates were pooled in our study. The pooled prevalence of *Klebsiella* spp. was 5.41% (95% CI: 3.87–7.50%). Subgroup meta-analysis indicated that the prevalence of *Klebsiella* spp. in South China was higher than that in North China, that in subclinical mastitis was lower than that in clinical mastitis, and that in 2000–2010 was lower than that in 2010–2020.

Our results revealed that the pooled prevalence of *Klebsiella* spp. was relatively lower than those of previous studies conducted in China (13 and 9.78%) (9, 29). Meanwhile, the prevalence of *Klebsiella* spp. in South China (8.5%) was twice that in North China (4.2%). Environmental sources, such as alleyways, holding pens, and sawdust and shavings in bedding, are important sources of *Klebsiella* spp. (National Mastitis Council, 1999; (28, 30). Our results were consistent with those of Gao et al., whose results have revealed that the prevalence of *Klebsiella* spp. in Northwest China is lower than that in South China, and that in winter was lower than that in summer; this finding is attributed

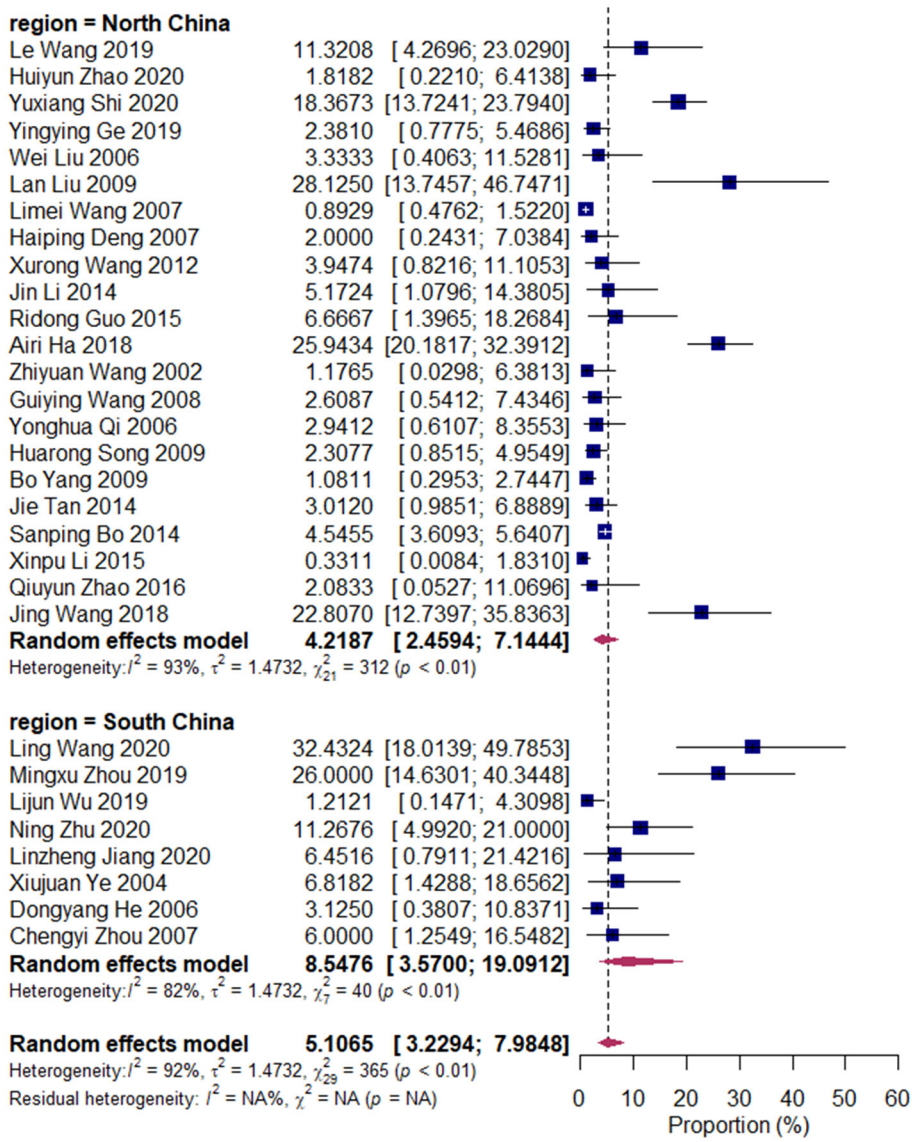


**FIGURE 3 |** Prevalence of *Klebsiella* spp. in North and South China. Subgroups were created according to the sampling region in China (North China/South China). The two upper diamonds represent the overall prevalence within the respective subgroups, whereas the diamond at the bottom represents the overall prevalence of all studies (the same below).

to the dry and cold weather in North China, which is unsuitable for environmental microorganisms (9).

In recent years, *Klebsiella* spp. mastitis, which is attributed to the fecal shedding of *Klebsiella* spp., increased the concern for herds that use inorganic bedding (31). *Klebsiella pneumoniae* is an endophyte of several plants, such as wheat, corn, and alfalfa, and it can act as milk cow feed; bacteria can be found inside the plants without external fecal contamination (32–34). Consequently, the oral intake of *Klebsiella* spp. can be due to the plants used for feed or to fecal contamination of feed and water, whereas fecal shedding of *Klebsiella* spp. results in the contamination of the environment of cows. Such sources provide dairy herd managers and veterinarians with additional control points for the prevention of *Klebsiella* spp. mastitis. The increased prevalence of *Klebsiella* from 2000 to 2020 raised the concern for this important mastitis pathogen.

Cheng et al. (35) revealed that *Klebsiella* spp. can induce severe and long-term infection in the bovine milk gland, and can give rise to clinical bovine mastitis more than the subclinical version. Our results also revealed that the prevalence of *Klebsiella* spp. in clinical mastitis is higher than that in subclinical bovine mastitis.

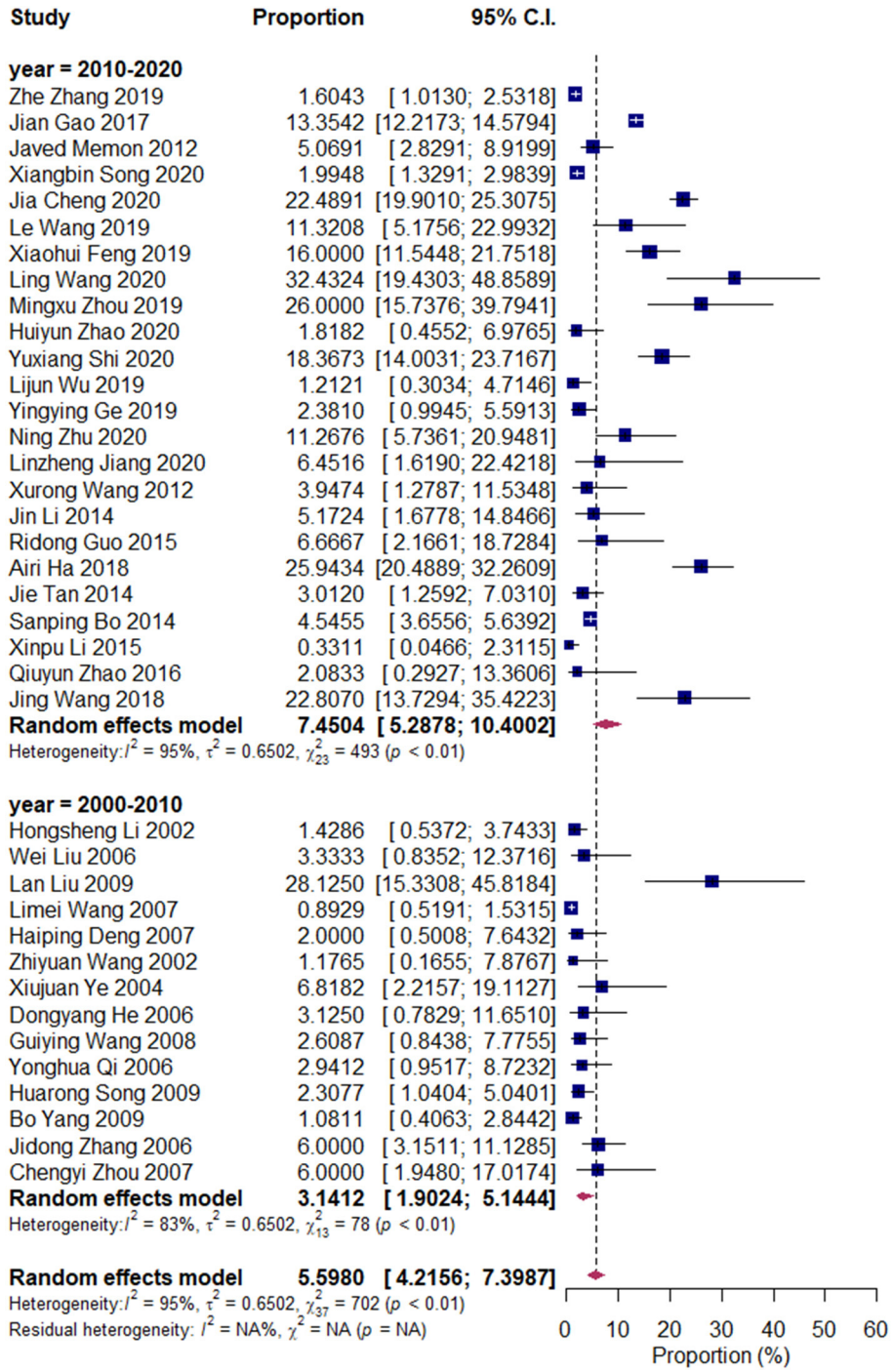


**FIGURE 4 |** Prevalence of *Klebsiella* spp. in the period of 2000–2010 and 2010–2020. Subgroups were created according to the publication year (2000–2010/2010–2020).

*Klebsiella* spp. can induce bovine mastitis; antimicrobial treatment is normally used for mastitis prevention and control (7).

The misuse of antimicrobials can increase the risk of AMR and threaten public health. (36). In our research, the AMR of *Klebsiella* spp. against nine kinds of frequently used antimicrobials (β-lactams, quinolones, aminoglycosides, tetracyclines, polypeptides, sulfonamides, amphenicol, macrolides, and lincosamides) was determined. We first pooled nationwide studies that were conducted to determine

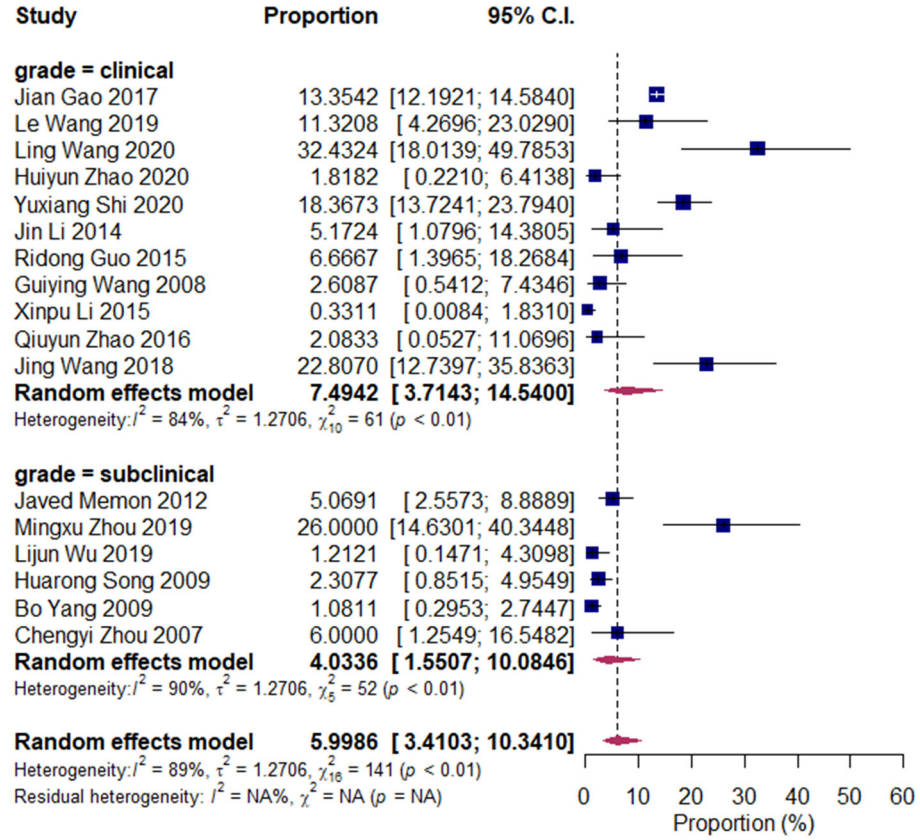


**FIGURE 5 |** Prevalence of *Klebsiella* spp. isolated in clinical mastitis and subclinical mastitis cases. Subgroups were created according to the bovine mastitis grade (clinical/subclinical).

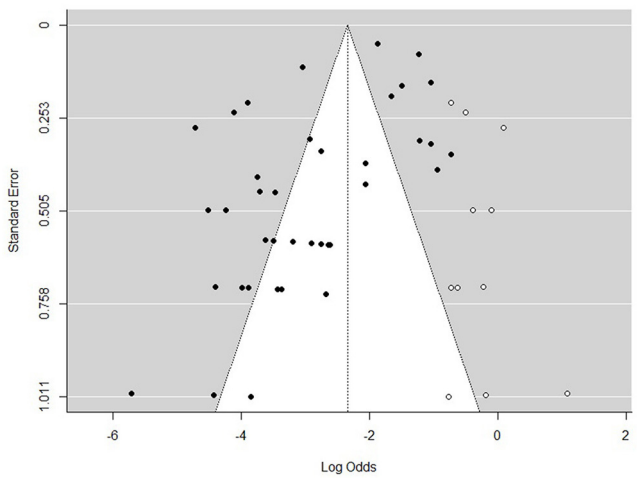


**FIGURE 6 |** Funnel plot of publication bias of prevalence of *Klebsiella* spp.

the AMR of *Klebsiella* spp. isolated from bovine mastitis in China. For these nine antimicrobials, the resistance rate of sulfonamides was the highest (45.07%), followed by tetracyclines (36.18%), aminoglycosides (27.47%), β-lactams (27.34%), amphenicol (26.82%), lincosamides (21.24%), macrolides (20.98%), polypeptides (15.51%), and quinolones (7.80%).

In a previous study conducted by Saini et al. (36), the resistance to sulfonamides (11.7%) and β-lactams (17.3%) was the highest, but the values were still lower than that in our research. In another study conducted in Canada, *Klebsiella* spp. was the main pathogen resistant to tetracyclines (19%) and streptomycin (38%) (37). The high percentages of AMR of *Klebsiella* spp. against sulfonamides, β-lactams, amphenicol, lincosamides, macrolides, and polypeptides in that study were not observed, similar to the observations in European countries (38), in which the resistance to streptomycin was higher than that in our research; however, the resistance to tetracyclines was lower than that in our study. The use of sulfonamides and tetracyclines in husbandries had been forbidden by the Chinese government. However, the observed AMR rate was still high, which indicated that the AMR mechanism was still harbored by *Klebsiella* spp. aminoglycosides and β-lactams should raise the most concern when used in treating bovine mastitis. Tetracyclines are one of the extensively used antimicrobials among dairy farms. Its AMR has been a serious problem before, but with the increased awareness of public health in society, the Chinese government imposed a ban on the use of antimicrobials as growth

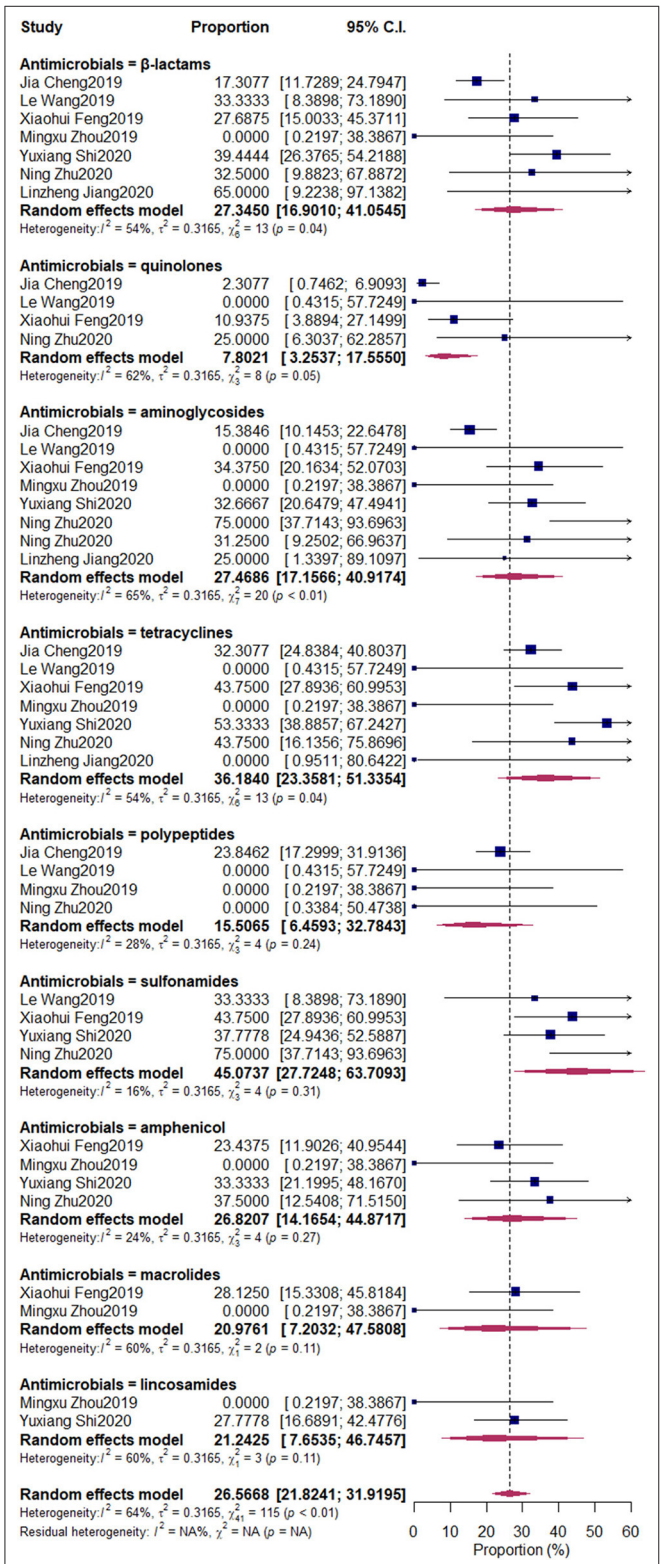


**FIGURE 7 |** Antimicrobial-resistant rate of *Klebsiella* spp. Subgroups were created according to the different kinds of antimicrobials. The nine upper diamonds represent the overall antimicrobial resistance (AMR) rate within the respective subgroups, whereas the diamond at the bottom represents the overall AMR rate of all studies.

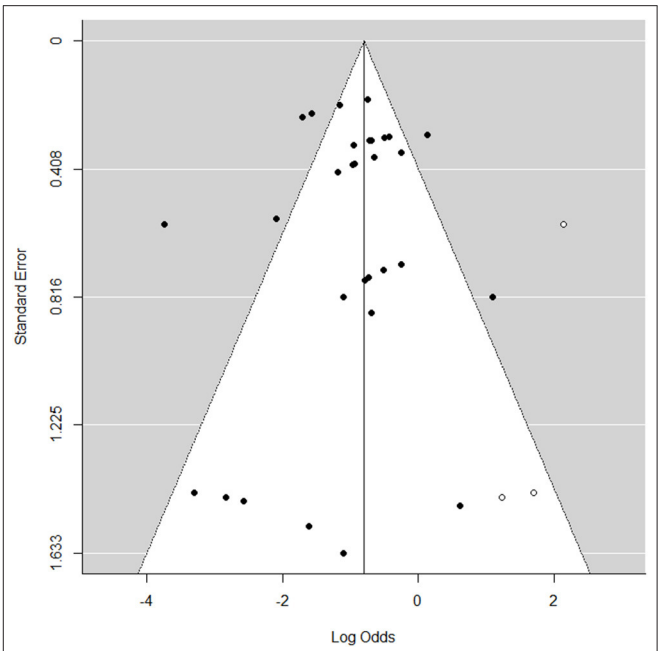


**FIGURE 8 |** Funnel plot publication bias of antimicrobial resistance rate of *Klebsiella* spp.

promoters in the husbandry industry, which restricted the AMR of pathogens.

Fuenzalida and Ruegg (39) indicated that the cure rate for *Klebsiella pneumoniae* mastitis was 21% greater in 8-day than in 2-day intramammary ceftiofur group. A previous study conducted in China by Cheng et al. (35) reported that *Klebsiella* spp. were also highly resistant to amoxiclav (38%), with a value higher than that in the study of Schukken et al. (11) in the USA. Our results also indicated that the AMR of bovine mastitis-associated *Klebsiella* spp. against β-lactams in China was as high as 27.34%. Yang et al. (40) reported that the β-lactam resistance gene *bla*CTM-M-1 located on pC5-like plasmids can be responsible for the resistance against ceftiofur for bovine mastitis treatment. Schukken et al. (11) suggested that the antimicrobial treatment of *Klebsiella* spp. bovine mastitis has a minimal value, and heteropathy for clinical symptoms should be the primary goal. A recent study indicated that third-generation cephalosporin and carbapenems will be ineffective against a large proportion of *Klebsiella* spp. in most parts of the world by 2030 (19), which should raise the concern for the AMR of *Klebsiella* spp. associated with bovine mastitis.

The results in our study were consistent with those of Cheng et al. (35), whose results indicated that the AMR occurrence rates of five common bovine mastitis pathogens, including *Klebsiella* spp., in China were higher than those in the European countries (41). The occurrence rate of AMR among bovine mastitis pathogens differs among various countries (42), and this condition can be attributed to complex reasons, such as the national guidelines for proper antibiotics

usage, veterinarian prescription patterns, and pharmaceutical marketing strategies (43, 44). Hence, our results should raise the concern about the AMR of bovine mastitis Klebsiella spp. in Chinese dairy herds. There are still limitations including few databases retrieval and publication time substituting sample time in our manuscript, which should make improvements in the future.

## CONCLUSIONS

The pooled prevalence of *Klebsiella* spp. was 5.41% (95% CI: 3.87–7.50%). Subgroup analysis revealed that the incidence was higher in South China, from 2010 to 2020, and in clinical bovine mastitis cases, and the reason is attributed to the climate between South and North China and the natural pathogenicity of *Klebsiella* spp. The pooled AMR rates showed that *Klebsiella* spp. were most resistant to sulfonamides, followed by tetracyclines, aminoglycosides, β-lactams, amphenicol, lincosamides, macrolides, polypeptides, and quinolones, which should raise the most concern when used in treating bovine mastitis.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

KL designed the study, analyzed the data, and wrote the article. LZ and XG performed the literature research and review. WQ critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Shaheen M, Tantary HA, Nabi SU. A treatise on bovine mastitis: Disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. *J. Adv. Dairy Res.* (2016) 1:1000150. doi: 10.4172/2329-888X.1000150
2. Gussmann M, Denwood M, Kirkeby C, Farre M, Halasa T. Associations between udder health and culling in dairy cows. *Prev. Vet. Med*. (2019) 171:104751. doi: 10.1016/j.prevetmed.2019.104751
3. Krömker V, Leimbach S. Mastitis treatment-reduction in antibiotic usage in dairy cows. *Reprod Domest Anim.* (2017) 3:21–9. doi: 10.1111/rda.13032
4. Wenz JR, Barrington GM, Garry FB, McSweeney KD, Dinsmore RP, Goodell G, et al. Bacteremia associated with naturally occurring acute coliform mastitis in dairy cows. *J. Am. Vet. Med. Assoc*. (2001) 219:976–981. doi: 10.2460/javma.2001.219.976
5. Roberson JR, Warnick LD, Moore G. Mild to moderate clinical mastitis: Efficacy of intramammary amoxicillin, frequent milk-out, a combined intramammary amoxicillin, and frequent milk-out treatment versus no treatment. *J Dairy Sci.* (2004) 87:583–92. doi: 10.3168/jds.S0022-0302(04)73200-2
6. Olde Riekerink RGM, Barkema HW, Kelton DF, Scholl DT. Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci.* (2008) 91:1366–77. doi: 10.3168/jds.2007-0757
7. Oliveira L, Hulland C, Ruegg PL. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. *J Dairy Sci.* (2013) 96:7538–49. doi: 10.3168/jds.2012-6078
8. Cheng J, Zhang J, Han B, Barkema HW, Cobo ER, Kastelic JP. *Klebsiella pneumoniae* isolated from bovine mastitis is cytopathogenic for bovine mammary epithelial cells. *J Dairy Sci.* (2020) 103:3493–504. doi: 10.3168/jds.2019-17458
9. Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *J Dairy Sci.* (2017) 100:4797–806. doi: 10.3168/jds.2016-12334
10. Gröhn YT, Wilson DJ, Gonzalez RN, Hertl JA, Schulte H, Bennett G. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *J Dairy Sci.* (2004) 87:3358–74. doi: 10.3168/jds.S0022-0302(04)73472-4
11. Schukken Y, Chuff M, Moroni P, Gurjar A, Santisteban C, Welcome F. *The "other" gram-negative bacteria in mastitis: Klebsiella, Serratia, and more Vet Clin North Am Food Anim Pract.* (2012) 28:239–56. doi: 10.1016/j.cvfa.2012.04.001
12. Erskine RJ, Bartlett PC, VanLente JL, Phipps CR. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. *J. Dairy Sci.* (2002) 85:2571–5. doi: 10.3168/jds.S0022-0302(02)74340-3
13. Gröhn YT, Gonzalez RN, Wilson DJ, Hertl JA, Bennett G, Schulte H. (2005). *Effect of pathogen-specific clinical mastitis on herd life in two New York State dairy herds Prev Vet Med.* (2005) 71:105–25. doi: 10.1016/j.prevetmed.2005.06.002
14. Wilson DJ, Gröhn YT, Bennett GJ, Gonzalez RN, Schukken YH, Spatz J. Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. *J Dairy Sci.* (2007) 90:4282–8. doi: 10.3168/jds.2007-0160
15. Podder MP, Rogers L, Daley PK, Keefe GP, Whitney HG. *Klebsiella* species associated with bovine mastitis in Newfoundland. *PLoS ONE.* (2014) 9:e106518. doi: 10.1371/journal.pone.0106518
16. Keefe G. Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitis. *Vet Clin North Am Food Anim Pract.* (2012) 28:203–16. doi: 10.1016/j.cvfa.2012.03.010
17. Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges, and responses. *Nat Med.* (2004) 10:S122–9. doi: 10.1038/nm1145
18. Sarmah AK, Meyer MT. Boxall AB. A global perspective on the use, sales, exposure pathways, occurrence, fate, and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere.* (2006) 65:725–59. doi: 10.1016/j.chemosphere.2006.03.026
19. Alvarez-Uria G, Gandra S, Mandal S. Laxminarayan R. Global forecast of antimicrobial resistance in invasive isolates of *Escherichia coli* and *Klebsiella pneumoniae. Int J Infect Dis.* (2018) 68:50–3. doi: 10.1016/j.ijid.2018.01.011
20. Wang Y, Xu C, Zhang R, Chen Y, Shen Y, Hu F. Changes in colistin resistance and mcr-1 abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study. *Lancet Infect Dis.* (2020) 20:1161–71. doi: 10.1016/S1473-3099(20)30149-3
21. Kaczorek E, Małaczewska J, Wójcik R, Rekawek W, Siwicki AK. Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland. *J Dairy Sci.* (2017) 100:6442–53. doi: 10.3168/jds.2017-12660

22. Gao J, Ferreri M, Yu F, Liu X, Chen L, Su J. Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. *Vet J*. (2011) 192:550–2. doi: 10.1016/j.tvjl.2011.08.030
23. Ali T, Rahman SU, Zhang L, Shahid M, Han D, Gao J. Characteristics and genetic diversity of multi-drug resistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolated from bovine mastitis. *Oncotarget*. (2017) 8:90144–63. doi: 10.18632/oncotarget.21496
24. Moher D, Liberati A, Tetzlaff J, Altman DG, Group PRISMA. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann Intern Med*. (2009) 151:264–9. doi: 10.7326/0003-4819-151-4-200908180-00135
25. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*. (2021) 372:n71. doi: 10.1136/bmj.n71
26. Aghamohammadi M, Haine D, Kelton DF, Barkema HW, Hogeveen H, Keefe GP. Herd-level mastitisassociated costs on Canadian dairy farms. *Front Vet Sci*. (2018) 5:100. doi: 10.3389/fvets.2018.00100
27. Munoz MA, Bennett GJ, Ahlstrom C, Griffiths HM, Schukken YH, Zadoks RN. Cleanliness scores as indicator of *Klebsiella* exposure in dairy cows. *J Dairy Sci*. (2008) 91:3908–16. doi: 10.3168/jds.2008-1090
28. Zadoks RN, Griffiths HM, Munoz MA, Ahlstrom C, Bennett GJ, Thomas E. Sources of *Klebsiella* and *Raoultella* species on dairy farms: be careful where you walk. *J Dairy Sci*. (2011) 94:1045–51. doi: 10.3168/jds.2010-3603
29. Yang Y, Peng Y, Jiang J, Gong Z, Zhu H, Wang K. Isolation and characterization of multidrug-resistant *Klebsiella pneumoniae* from raw cow milk in Jiangsu and Shandong provinces, China. *Transbound Emerg Dis*. (2021) 68:1033–9. doi: 10.1111/tbed.13787
30. Kristula MA, Dou Z, Toth JD, Smith BI, Harvey N, Sabo M. Evaluation of free-stall mattress bedding treatments to reduce mastitis bacterial growth. *J Dairy Sci*. (2008) 91:1885–92. doi: 10.3168/jds.2007-0603
31. Munoz MA, Ahlstrom C, Rauch BJ, Zadoks RN. Fecal shedding of *Klebsiella pneumoniae* by dairy cows. *J Dairy Sci*. (2006) 89:3425–30. doi: 10.3168/jds.S0022-0302(06)72379-7
32. Chelius MK, Triplett EW. Immunolocalizatoin of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. *Appl Environ Microbiol*. (2000) 66:783–7. doi: 10.1128/AEM.66.2.783-787.2000
33. Dong Y, Iniguez AL, Ahmer BM, Triplett EW. Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativum* and *Medicago truncatula*. *Appl Environ Microbiol*. (2003) 69:1783–90. doi: 10.1128/AEM.69.3.1783-1790.2003
34. Iniguez AL, Dong Y, Triplett EW. Nitrogen fixation in wheat provided by Klebsiella pneumoniae 342. *Mol Plant Microbe Interact*. (2004) 17:1078–85. doi: 10.1094/MPMI.2004.17.10.1078
35. Cheng J, Qu W, Barkema HW, Nobrega DB, Gao J, Liu G. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *J Dairy Sci*. (2019) 102:2416–26. doi: 10.3168/jds.2018-15135
36. Saini V, McClure JT, Leger D, Dufour S, Sheldon AG, Scholl DT, et al. Antimicrobial use on Canadian dairy farms. *J Dairy Sci*. (2012) 95:1209–21. doi: 10.3168/jds.2011-4527
37. Massé J, Dufour S, Archambault M. Characterization of *Klebsiella* isolates obtained from clinical mastitis cases in dairy cattle. *J Dairy Sci*. (2020) 103:3392–400. doi: 10.3168/jds.2019-17324
38. de Jong A, El Garch F, Simjee S, Moyaert H, Rose M, Youala M, et al. Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. *Vet. Microbiol*. (2018) 213:73–81. doi: 10.1016/j.vetmic.2017.11.021
39. Fuenzalida MJ, Ruegg PL. Negatively controlled, randomized clinical trial to evaluate use of intramammary ceftiofur for treatment of nonsevere culture-negative clinical mastitis. *J Dairy Sci*. (2019) 102:3321–38. doi: 10.3168/jds.2018-15497
40. Yang Y, Higgins CH, Rehman I, Galvao KN, Brito IL, Bicalho ML. Genomic diversity, virulence, and antimicrobial resistance of *Klebsiella pneumoniae* strains from cows and humans. *Appl Environ Microbiol*. (2019) 85:e02654–e02618. doi: 10.1128/AEM.02654-18
41. Thomas V, de Jong A, Moyaert H, Simjee S, El Garch F, Morrissey I, et al. Antimicrobial susceptibility monitoring of mastitis pathogens isolated from acute cases of clinical mastitis in dairy cows across Europe: VetPath results. *Int J Antimicrob Agents*. (2015) 46:13–20. doi: 10.1016/j.ijantimicag.2015.03.013
42. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J Antimicrob Chemother*. (2014) 69:827–34. doi: 10.1093/jac/dkt443
43. ECDC, EFSA. ENA. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food producing animals. *EFSA J*. (2015) 13:4006. doi: 10.2903/j.efsa.2015.4006
44. Adkins PR, Middleton JR. *Laboratory Handbook on Bovine Mastitis*. National Mastitis Council, Inc., New Prague, Minnesota (2017).


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