Chapter

Mastitis in Small Ruminants

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Abstract

Bacterial mastitis in small ruminants is a complex disease, with massive economic loss in dairy sheep/goat industry due to poor productivity. The current mastitis prevention strategy relies on culling of infected ewes or does and or the use of antimicrobial agents to eliminate the bacterial infection. This has a potential risk for developing antibiotic resistant bacteria, posing human health risk from consumption of raw sheep or goat dairy products. Existing experimental and licensed vaccines on the market are ineffective against reducing the risk of mastitis in herds or flocks. Raising the needs for development of improved vaccines against mastitis for use in sheep and goats. This review examines, current understanding of the pathological processes and immunological responses against bacterial mastitis, using *S. aureus* as an example. By highlighting the protective defense mechanism induced in the udder against *S. aureus* mastitis. Based on evidence from published studies on pathological process and protective immune response mechanism, the need for improved vaccines for prevention of mastitis in small ruminant is highlighted and the development of a vaccine capable of enhancing immune response mechanism, that reduce the establishment of intramammary infection through induction of local IgA, IgG2 and Th17 immune responses is proposed.

Keywords: Mastitis, *S. aureus*, *Pathogenesis*, pathophysiology, vaccination

1. Introduction

Mastitis is a complex disease that results in inflammation of the mammary glands due to infection or mechanical injury. Host, pathogen, and environmental predisposing factors play major role in the development of mastitis. Most cases of mastitis of small ruminant occur as a result of bacterial intramammary infection (IMI), which are generally a contagious infection resulting from mammary and cutaneous carriages of bacterial agents and or spreading of bacteria during the milking process. The inflammatory response induced by the host is aimed at removing the irritant and repairing damaged mammary gland tissues to ensure normal functioning of the udder. Inability of the host to control IMI leads to persistent inflammatory response (chronic mastitis) that leads to premature culling of the affected ewes [1], loss of udder function, reduced milk yield [2], and quality [3, 4] and occasionally death. In addition, reduced milk production also affects livestock productivity, as it results in lower growth rates of suckling lambs [1]. As such, mastitis of small ruminant has a significant economic impact in the livestock industry. Mastitis in small ruminants caused by bacterial intramammary infections presents in two forms i.e., subclinical mastitis or clinical mastitis as depicted in **Table 1**, with varying severity from acute infections that last for short period of time to chronic

Ruminant	Inflammatory Changes		
	Milk	**Clinical Manifestation**	**Udder**
Sheep	No visible abnormality; high bacterial count; reduced milk production; SCC > 500 x10^3 cells/mL; changes in milk composition	Subclinical	No visible signs of inflammation;
	Visible changes in milk e.g., may be blood tinged or yellow,may be thick, "lumpy", or very watery	Clinical	Visible abnomalities in the udder; udder may be firm, swollen etc. the gravity of the abnomality varies based on disease severity.
	May contains clots, flakes, or discolored secretion	Subacute (mildly clinical)	Swollen, red udders, hot and painful to the touch; hard sensitive udder;
	Reduced milk secretion, contains clots, flakes, or discolored secretion; appears watery	Acute (sudden onset of inflammation, can be fatal)	Swollen; hot; red and painful to touch
	Abnormal milk appearance; bloody fluid	Peracute (Severe inflammation, fatal or loss of affected udder)	Visibly abnormalities; swollen; cold; blue/black; may slough off; gangrenous
	May have no milk production; reduced yield and composition; contains purulent material (pus);	Chronic	Hard or lumpy; abscesses; may have scars; may be fibrotic; swollen teat; may contain a hard core of pus; asymmetrical appearance; enlarge or shrunken;
Goat	No visible abnormality; But laboratory test present with: high bacterial count; reduced milk production; reduced antioxidant content; changes in milk composition	Subclinical	No visible signs of inflammation;
	Visible abnormalities in the milk (varies based on severity); increase in whey proteins; increase in albumin concentration; a reduced lactose concentration and milk fat; increase electrical conductivity	Clinical mastitis	Visible abnormalities in the udder (varies based on severity)
	Contains clots, flakes, or discolored secretion;	Subacute (mildly clinical)	slightly swollen and tender
	Reduced milk secretion, contains clots, flakes, or discolored secretion; appears watery	Acute (sudden onset of inflammation, can be fatal)	Swollen, red udders, hot and painful to the touch
	Serum-like milk secretion. Milk may appear reddish and may contain gas	Peracute (Severe inflammation, fatal or loss of affected udder)	Swollen, discolored (reddish to purple/black), cold to touch; may be gangrenous
	Milk may contain flakes, purulent material (pus) and be discolored; Reduced yield and composition	Chronic (persistent infection, may be incurable and recurring)	Hard, fibrotic, abscesses shrunken or lumpy

Table 1.
The different forms of mastitis in small ruminant and plausible signs in relation to the severity of infection.

infection which are persistent and long term. Herein, we discuss mastitis in small ruminant, focusing on sheep and goat.

1.1 Sheep

The most common form of mastitis in sheep is subclinical mastitis, with a reported prevalence of 5–30% [5] and sometimes up to 50% [6]. Subclinical mastitis is difficult to identify, mainly due to lack of clinical signs. Subclinical mastitis can only be detected by milk bacteriological test or somatic cells count (SCC) (i.e., inflammatory cells and some epithelial cells) [7–10]. In sheep a SCC of >500 x 10^3 cells/mL of milk [7, 8] and or a positive California Mastitis Test (CMT) [9, 10] is considered subclinical mastitis. In most cases, ewes with subclinical mastitis appear healthy, but have decrease milk production [11, 12] and changes in the composition of milk due to the inflammation. Subclinical mastitis may affect lambs of infected ewes by causing them to have a poor growth rate, lower weaning weight and occasional death [11, 13]. As such, subclinical mastitis has significant financial implications for both dairy sheep flocks due to its impact on milk production and quality [11, 12] in meat-producing sheep flocks as it affects lambs growth rate and weaning weight [11, 13].

Subclinical mastitis due to bacterial IMI may progress to acute or chronic mastitis. Progression of subclinical mastitis to clinical mastitis can occur as a result of the following events. 1) Subclinical mastitis-causing bacteria can be transmitted from an infected under to an uninfected udder during milking as a result of poor hygiene practices by milkers whereby they can transmit the infecting bacteria from their hands or from using contaminated shared milking equipment and udder washcloths. These practices provide the bacteria access to the teat canal, where successful bacterial growth subsequently results in mastitis. 2) Nutritional deficiency adversely affects the animal host defense mechanism, and may promote disease progression to clinical mastitis. Katsafadou et al. [14], associated nutritional deficiencies with impaired leucocyte function or mammary defense. Here nutritional elements such as Selenium, Zinc, vitamin E and Vitamin A deficiencies have been linked to an increased risk of mastitis in ewes [14–16]. For example, Selenium and Vitamin E are important in maintaining neutrophil function [17], they are known to protect leucocytes against reactive oxygen species (ROS)-induced damage [14–16]. Zinc forms part of teat keratin and skin, it has been suggested that deficiency in Zinc and vitamin A negatively affects the integrity of the teat and epithelia [14–16]. This could allow the colonization of the teat by infecting bacteria, coupled with other deficiencies that result in the establishment of clinical mastitis.

Clinical mastitis usually occurs in less than 5% of mastitis cases in sheep [5, 10, 18]. However, clinical mastitis in sheep is often observed as sporadic cases or during occasional herd outbreaks [5, 10]. Clinical mastitis can transition from subacute to chronic with increasing disease severity. Clinical mastitis is easy to identify, it presents with clearly observable clinical signs and physical changes in the udder, such as blue discoloration of the udder. Udders with clinical mastitis are usually swollen and sometimes painful to the touch. sheep affected with clinical mastitis go off feed, are lethargic, and often refuse to allow their lambs to nurse, resulting in lower growth rates of suckling lambs. The appearance and composition of milk obtained from ewes affected by clinical mastitis is abnormal, it may be discolored, watery, may contain blood or serum, may be foul-smelling if it contains pus and has visible clots or flakes.

1.2 Goats

The prevalence of subclinical mastitis in goat is 5–45% [5, 19]; some authors suggest it's 15–40 times more prevalent than clinical mastitis [20]. As in other ruminants, subclinical mastitis in goat is difficult to identify by clinical signs. Subclinical mastitis in goat presents with high bacterial count in milk; reduced milk production and quality, as well as a high SCC. However, SCC are not reliable indicators of subclinical mastitis diagnosis in goat [21, 22]. Generally, healthy goats have a higher milk SCC compared to sheep, and other ruminants such as cows. In addition, the number of SCC in goat milk various based on stage of lactation, SCC has been reported to reach 3.6 x10^6 cells/ mL at end of lactation [23]. Some have reported a SCC ≥ 10^6 cells/mL as an indication of subclinical mastitis in goat; however, this set minimum is usually combined with a bacteriological test to confirm diagnosis. SCC alone is not used to diagnose subclinical mastitis in goats, as shown by Hussein et al. [21], SCC ≥ 10^6 cells/mL threshold was unable to differentiate subclinical mastitic goat from healthy goats, thus confirming the use of bacteriological test as the most reliable indicator of subclinical mastitis in goats [21].

In goat subclinical mastitis usually precedes clinical mastitis, as its act as a source of infection for healthy animals [19]. Clinical mastitis presents with visible abnormalities in the udder and or milk that varies based on the severity of the infection, as mentioned in **Table 1**. Clinical mastitis in goats is also classified into four groups based on disease severity, i.e. subacute, acute, peracute and chronic. As mentioned in **Table 1**, above, this ranges from a mild infection to severe, presenting with pain, heat, swelling, redness, and reduced and abnormal secretion such as clots, flakes, or watery milk. May develop fever, depression, weakness, anorexia and may be fatal.

2. Important bacterial pathogens for vaccine development against mastitis in small ruminants

Several bacterial agents are associated with clinical or subclinical mastitis in small ruminants [5]. A very exhaustive list of gram-positive and gram-negative bacteria has been implicated in mastitis of sheep and goats. However, for the purpose of this review, the most implicated organism with potential for commercial vaccine development against mastitis of both sheep and goats will be discussed.

2.1 Sheep

Over 30 bacterial species have been isolated from sheep with mastitis [1, 24–26]. The most implicated organisms in sheep mastitis are *Staphylococcus aureus* [24, 27, 28]; *Mannheimia* spp. [29] *Streptococcus* spp. [30, 31]; and non-*aureus* staphylococci.

S. aureus is a zoonotic, Gram-positive bacteria that occurs as a mammalian commensal and opportunistic pathogen. *S. aureus* is the most common cause of mastitis in sheep and the major mastitis-causing agent isolated in 70% of clinical mastitis cases in dairy flocks [10, 24]. It is responsible for about 40% of mastitis cases in ewes suckling lambs and 80% mastitis cases in milking ewes [24, 27, 32]. Cases of mastitis due to *S. aureus* ranges from subclinical mastitis to severe gangrenous mastitis. It is important to note that *S. aureus* subclinical mastitis infections are extremely difficult to treat, cure and are highly contagious. As such, animals with *S. aureus* mastitis are culled or milked last to prevent spread of infection to other members of the herd or flock.

Other non-aureus staphylococci, are associated with subclinical intramammary infections [33], of these, *Staphylococcus epidermidis* is the most common species associated with ovine mastitis [34].

M. haemolytica is an aerobic, non-motile, bipolar, gram-negative rods, non-spore-forming opportunistic bacterium carried in the nasal and nasopharyngeal cavities of healthy animals. *M. haemolytica* is the most common cause of mastitis in meat and wool sheep producing systems [1, 29, 32]. *M. haemolytica* causes severe clinical mastitis, where the infected mammary glands are greatly enlarged, tense, blue-black, with watery secretion containing flakes and widespread gangrenous necrosis of the udder [29]. In some flocks *M. haemolytica* mastitis is more significant than *S. aureus* induced mastitis, due to its transmission by suckling lambs [29]. As a result, pneumonia may be observed in suckling lambs of ewes with *M. haemolytica* mastitis. Based on a 2008 research study by Arsenault *et al*., [1], the prevalence of *M. haemolytica* clinical mastitis is similar to *S. aureus* mastitis in meat-producing flocks, thus making it a significant organism in the etiology of mastitis in sheep.

Streptococcus spp. are zoonotic, anaerobic, non-spore-forming, Gram-positive, catalase-negative, homofermentative, spherical or ovoid cocci that occur as single or in pairs or chains [23]. They are usually responsible for sporadic outbreaks of mastitis in sheep and goats [30, 31, 35, 36]. Mastitis due to *Streptococcus* spp. occurs at a rate of 23–31% in flocks [25, 37]. Mastitis caused by *Streptococcus* spp. are more frequent in machine-milked flocks [25, 38, 39] or as a result of poor hygiene during milking [40], suggesting that proper milking practices may reduce the incidence of mastitis due to streptococci. *S. agalactiae*, *S. uberis*, *S. dysagalactiae* and *S. equi subsp.zooepidemicus* are the most isolated *Streptococcus* spp. causing mastitis in dairy ruminants [41].

2.2 Goats

Several bacterial species have been isolated from goats with mastitis, for example. *Staphylococcus* spp.; *Streptococcus* spp.; *Bacillus* spp.; *Listeria* spp.*; Corynebacterium* spp.; *Pseudomonas* spp.; *Mycoplasma* spp.; *Mannheimia haemolytica; Clostridium perfringens* and *Escherichia coli* [5, 10, 22, 25, 41–45]. However, non-*aureus* staphylococci are the most prevalent causative agent of subclinical mastitis in goats [10, 22, 42, 46–48] and *Staphylococcus aureus* is considered to cause the highest pathogenicity in goat mastitis, along with minor prevalence of *Escherichia coli*, *Streptococcus agalactiae, Mannheimia haemolytica* [10, 22, 42, 45–49]. *S. aureus*-induced mastitis in goat ranges from subclinical to gangrenous mastitis, which is the most severe form of the disease [45, 47]. As with sheep mastitis, *S. aureus* mastitis are difficult to treat, infected goats act as reservoir and source of spread.

Non-aureus staphylococci are the most prevalent bacteria from goats with subclinical mastitis, with up to 100% incidence [10, 48, 50–52]. The most isolated bacteria from these species are *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus xylosus*, and *Staphylococcus caprae* [10, 52–54]. *S. chromogenes* and *S. epidemidis* are associated with higher milk SCC [53] compared to other non-*aureus* staphylococci, however, the increase in SCC is lower than in *S. aureus* mastitis [10]. *S. caprae* and *S. simulans* have been linked to persistent mastitis in goat [55]. These bacteria do not produce coagulase, an enzyme responsible for prothrombin activation leading to the coagulation of plasma [56, 57]. They are opportunistic bacteria with the ability to produce biofilms [58], which enables these bacteria to persist on milking equipment, serving as a source of spread to other animals in cases of poor hygiene and milking practices. Non-*aureus* staphylococci have been reported to carry a wide range of antimicrobial resistance genes, which allows for persistent infections [59].

For vaccine development against mastitis in sheep and goat, it is imperative that intension and efforts are made towards the development of a vaccine containing

bacteria that have the highest pathogenicity or prevalence and has potential to impact human safety due to their zoonotic nature such as; *S. aureus*.

3. Pathogenesis of bacterial intramammary infection in small ruminant, using *S. aureus* as an example

The pathogenesis of bacterial intramammary infections in small ruminants is very complex. It is dependent on the infecting bacteria, bacterial virulence factors, and the interaction of these virulence factors with the host immunological response in the udder. Of the various bacteria known to cause mastitis in sheep and goats, *S. aureus* is used in this paper as an example to describe the pathogenesis of intramammary infection in sheep and goats, with emphasis on pathological processes that are essential for vaccine development.

The pathogenesis of *S. aureus* mastitis is very complex. It is associated with various surface proteins and virulence factors that are differentially expressed at various phases of the infection. This process entails three key steps, that is adhesion, invasion and evasion. In brief, the first step in the pathogenesis process is adhesion to epithelial cells and extracellular matrix, which permits the bacteria to avoid being flushed out of the udder from milk flux pressure [60]. During this step *S. aureus* expresses several virulent factors involved in adhesion, such as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM), e.g. protein A, elastin-binding proteins, collagen-binding protein etc.; surface-associated capsule (which inhibits phagocytosis and promotes adhesion); peptidoglycan (which activates complement); teichoic acids (involved in adhesion and colonization, cell division and biofilm formation) [60–64]. Here, the formation of biofilm protects *S. aureus* from host immune response or antibiotics [65, 66]. In the second step of this process, *S. aureus* again expresses different virulence factors to establish infection by invasion into host cells and tissues. This step or phase entails penetration and destruction of mammary glands tissues by the bacteria and involves the expression of the following virulence factors: haemolysins (i.e., alpha, beta, gamma & delta, these lyse cells); leukocidin (damages polymorphonuclear leucocytes); panton-valentine leukocidin (a β-pore-forming toxins); enterotoxins (heat stable toxin); epidermolytic toxin (this is a serine protease that causes splitting of desmosomes or intercellular bridges in the stratum granulosum); toxic shock syndrome toxin (TSST-1, causes leakage of endothelial cells and penetration of mucosal barrier) [60–64]. Together, these virulence factors result in damage to the epithelium of the cistern, duct and alveoli and perpetuate the infection, eventually leading to the clinical signs observed during mastitis. Subsequent to these events or perhaps in unison, the final step in the pathogenetic process is an evasion of the host immune response. Here, *S. aureus* escapes the host immune response by producing the various virulence factors that helps it not only to evade but also modulate the host immune response in its favor. For example: enzymes such as coagulase (that activates a coagulase reacting factor (CRF), which is believed to coat bacteria with fibrin to prevent opsonisation and phagocytosis); staphylokinase (fibrinolysin); Hyaluronidase (hyaluronic acid, which facilitates the spread of *S. aureus* through tissues); deoxyribonuclease; lipase; phospholipases; proteases; and again other already expressed virulence factors that were released during the adhesion and invasion process, these are continuously differentially expressed as required to anchor the infection and avoid elimination of *S. aureus* by mammary gland immune responses. *S. aureus* virulence factors target the main cells involved in mammary immunity, such as neutrophils and macrophages by counteracting their actions [60].

3.1 Immune response and pathological process in the mammary gland

3.1.1 Teat and teat cistern

The teat canal is the first physical barrier that bacterial agents meet before they can spread into the mammary glands [67–70]; protection against bacterial agents is provided by bacteriostatic fatty acids that are present in the keratin plug in the teat canal [68–70]. As such, invading microbial organisms are trapped in the lining of the teat canal by these hydrophobic lipids. The trapped microbial organisms are flushed out together with teat canal epithelial cells during the first outflow of milk. In addition, ewes teat are known to close 20 to 30 minutes; however, total closure occurs two hours post milking [69], as part of the first line of defense against invading microbial organisms. For this reason, it is recommended to move ruminants to clean areas after milking and provide feed to avoid laying down and exposure to contaminating environmental microbial organisms until teats end close [69]. If bacteria gain access to the teat canal, the bacterial adherence property is used to establish infection. As mention previously, using *S. aureus* as an example, MSCRAMM and capsular proteins are differentially expressed to permits attachments of the bacteria to the epithelial tissues. This mechanism is not only employed by *S. aureus* but other mastitis causing bacteria such as, M. *haemolytica* [71], *Streptococcus* spp. [72]. Therefore, adherence of microbial agent to teat epithelial tissue permits them to invade or penetrate this protective barrier and migrate to the teat duct.

3.1.2 The teat duct

Once the bacteria reach the teat duct, a cascade of complex sequence of events determines the outcome of the immune response induced. Here, somatic cells/leukocytes present in milk and component of the innate immune response in the teat duct act as a defense against any invading bacteria that has managed to by-passed the physical barrier in the teat cistern. The milk leukocytes act as phagocyte and secrete an array of immune-related components in milk, such as cytokines (e.g. TNF-α, IFN-γ, GM-CSF, IL-8, and IL-12), chemokines, reactive oxygen species (ROS), and antimicrobial peptides (Lactoferrin, defensins, and cathelicidins) [73]. In addition, inducible lymphoid nodules (containing B and T lymphocytes, as well as immune cells that express major histocompatibility complex (MHC) class II) that are present in the teat duct act in synergy with viable milk leucocytes to get rid of the invading bacteria. Based on this, an array of multiple cells are involved in the immune response of the teat duct against invading bacteria, these includes neutrophils, macrophages, αβ T cells, γδ T cells, B cells and inducible lymphoid cells etc. [69, 73].

A plausible scenario for the sequence of events that occurs when a bacteria is invading through the teat duct could be summarized as follows. As the bacteria invade the teat duct to colonize and establish infection, it releases a mirage of virulent factors, amongst these pathogen-associated molecular patterns (PAMPS) such as peptidoglycan and lipoteichoic acids in the case of *S. aureus*. These are recognized by Toll-like receptor (TLR)-2 on the surface of epithelial cells lining the teat ducts [70, 74, 75]. TRL2 stimulation leads to the release of IL-8; CCL2 and CCL4 [69, 74, 76]. IL-8 is a potent chemo-attractant and activating factor for neutrophils. CCL2 and CCL4 are chemoattractant for monocytes and macrophages [69, 76]. *S. aureus* PAMPs can also be recognized through formylated peptide receptors, mannose-binding lectins (MBL), ficolins, and complement molecules [70], resulting in the activation of the complement system leading to ingestion and killing of *S. aureus*. In addition, B cells in milk and milk macrophages process antigens from invading

bacteria and present these antigens in association with MHC class I or II on their membranes to different T cells.

As part of the innate response, activated neutrophils and tissue macrophages migrate into mammary glands to eliminate the invading bacteria and initiate the inflammatory response. The outcome of these early events results in increased neutrophils in the milk and an elevation in SCC, seen in subclinical mastitis, under normal state the udder tissue and milk mainly contain macrophages and during infection neutrophils are dominant in the udder tissue and milk [70].

Neutrophils/PMNs (Polymorphonuclear neutrophils) are the first recruited cells at the site of infection and are known to form part of the earliest protective response against bacterial mastitis in ruminants [77]. Their primary function is to engulf, phagocytose, and destroy invading bacteria. This is done through two pathways, the oxygen-dependent (respiratory burst, which includes the production of hydroxyl and oxygen radicals) pathway and or the oxygen-independent (which uses peroxidases, hydrolytic enzymes) pathway. Neutrophils also modulate vascular permeability and release a variety of inflammatory mediators that coordinate both the innate and adaptive immune response [69, 74]. In addition, neutrophils contain bactericidal peptides such as defensins; myeloperoxidase; S100-A9 protein, elastase; cathepsin types B, D, and G; procathepsins within their intracellular granules, these peptides can kill a variety of mastitis pathogens [69, 78–80]. However, neutrophil release oxidants and proteases are non-specific, as such they may also contribute to host mammary epithelium damage, and e.g., hydroxyl radicals may damage mammary epithelium [81]. Neutrophils undergo apoptosis or programmed cell death after completing their task and are removed by macrophages [81].

Milk macrophages and recruited macrophages (blood monocytes that differentiate in mammary tissue) act as antigen-presenting cells (APCs), by processing and presenting antigens to CD4+ T cells in association with MHC class II [82]. These macrophages ingest and phagocytose the invading bacteria, destroying them with proteases and ROS. However, as with neutrophils, macrophages also contribute to mammary gland epithelial damage due to their non-specific killing with proteases [83]. Macrophages have been shown to be inefficient in killing some mastitis pathogen by promoting their multiplication intracellularly [83].

In healthy udders, the predominating lymphocytes are αβ T cells, with a CD3+ CD8+ phenotype, that act as cytotoxic or suppressor T cells [84]. γδ T cells mediate cytotoxicity in a none restricted manner, with variable involvement of MHC molecules and also play a role in antibacterial immunity through production of granulysin [73, 85] expression of CD95L and TNF-related apoptosis-inducing ligand, that engage with several death receptors on target cells [85]. In addition, activated γδ T cells stimulate other immune cells e.g., dendritic cell maturation, through the production of TNF-α [85], IFN-γ, and IL-17 [86]. In mastitis, these cells form part of the early response as is reported in a mouse experimental mastitis study, where infection with *S. aureus*, induced an early influx of γδ T cells producing IL-17 into the mammary glands [87]. IL-17 activates mammary epithelial cells and enhances neutrophil infiltration through an expression of CXCL1, CXCL2, and CXCL5 chemokines. This enhances host clearance of the invading bacteria [87, 88]. It has also been suggested that γδ T cells plays a role in repairing damaged mammary gland tissues during and after mastitis. [89].

Whereas, αβ T cells recognize an antigen through membrane receptors, as such their specificity, diversity, and memory features are defined by the type of receptor they used to recognize antigens [73].

The efficiency of phagocytic killing culminating from events mentioned above determines the severity of the disease being established, i.e., the disease progression from subclinical mastitis to gangrenous mastitis.

Mastitis causing bacteria may by-pass the natural defense and innate immunity in the teat canal and establish infection in the intramammary area. Indeed, mastitis-causing organism, such as *S. aureus* secretes an array of virulence factors to facilitate invasion and deeper penetration into the mammary glands. *S. aureus* secretes cytolytic toxins such as α, β, γ, δ-haemolysins [90], phenol soluble modulins (PSMs) and bi-component leukocidins. These exert their role through pore-forming on host immune cell membrane, causing osmotic leakages of cell content, leading to lysis of neutrophils, monocytes, platelet, and erythrocytes. *S. aureus* also engages a wide range of virulence factors to restrain neutrophil activation, chemotaxis and phagocytosis and also target key host effector proteins that are released by host immune cells. For example, extracellular fibrinogen-binding (Efb) protein, coagulase (Coa), extracellular matrix-binding protein (Emp), extracellular adhesive protein (Eap), chemotaxis inhibitory protein (CHIPS) and staphylococcal complement inhibitor (SCIN) proteins. For example; Efb plays an immunosuppressive role by interfering with the complement system, it has been reported to significantly exacerbate *S. aureus* infections, impairs wound healing, and inhibit platelet aggregation and thrombus formation [91]. Through this mechanism, Efb facilitate *S. aureus* survival and persistent infection. CHIPS, is a potent inhibitor of neutrophil and monocyte chemotaxis towards C5a and formylated peptides [92]. Furthermore, macrophages also synthesize complements, such as component 3 (C3) in the mammary gland. These complements are involved in evoking and controlling the inflammatory process, bacterial opsonization and presentation, leukocytes recruitment and killing of microbial agents in the mammary glands [93, 94].

In a nutshell, *S. aureus* virulence factors promote the establishment of mastitis in the udder, through secretion of an array of virulence factors that facilitates adherence to mammary epithelium, invasion of mammary glands, and evasion of mounted host immune response mechanism against it by modulating counter-responses, e.g. *S. aureus* expresses T-cell superantigens such as, TSST-1, staphylococcal enterotoxin A, staphylococcal enterotoxin B, etc.) that bind to a specific subset of the variable Vβ chains of the T-cell receptor (TCR), leading to polyclonal proliferative responses and clonal deletion of T lymphocytes [95].

3.1.3 Alveoli

Mechanisms of the innate immune response previously described remain the same, but failure to resolve the induced inflammatory response lead to engagement of the adaptive immune response to eliminate the bacteria. *S. aureus* protective immune response mechanism entails both arms of the adaptive immune response, i.e. cell-mediated and humoral mediated arms of the immune response play a role in the clearance of bacterial mastitis. Numerous studies have shown the role of both T helper cells, cytotoxic T cells and B cell responses in clearance and resolving of bacterial infections. However, these cells have also been implicated in disease pathogenesis.

Using our previous scenario, once the bacteria reach the alveoli, the mammary epithelial cells lining acts as defense mechanism against the invading bacteria triggering a cascade of immunological responses directed against the invading bacteria's virulence factors. In addition, the magnitude of the induced immunological response determines the outcome of the inflammatory response (mastitis), i.e., the early expression of various inflammatory reaction modulators play a role in the severity of the subsequent inflammatory response e.g., IL-1, IL-8, IFN-γ, TNF-α, and G-CSF enhanced the activation of neutrophils, whereas IL-12, M-CSF, and GM-CSF stimulate enhanced activation of macrophages; IL-2 and IL-6 Stimulates

B cells differentiation, and so forth [96] and the type of effector T cell response induced is also related to the cytokines in its surrounding.

As the bacteria continue to proliferate in the alveoli, the mammary epithelial cells lining the alveoli have various role in protection against invading microbial organisms. For instance: 1) the mammary epithelial cells sense and recognizes microbial agents through pattern recognition receptors (PRRs), e.g., these cells are known to express TLR2 and TLR 4 [97, 98]. These cells recognize invading bacteria via the MyD88- dependent TLR (Toll-like receptor) signaling pathway [74, 79], 2) the mammary epithelial cells synthesize inflammatory mediators and antimicrobial peptides upon recognition of microbial organisms. According to various mastitis studies and reports, mammary epithelial cells produce cytokines such as, interleukin-8; chemokines such as, CCL5; β-defensins; haptoglobin; cathelicidin; lactoferrin; lysozyme, and serum amyloid A [42, 99–101]. The release of these inflammatory mediators activate local leukocytes that are normally present in the mammary gland tissue, such as macrophages, dendritic cells, and intra-epithelial lymphocytes, and also immune cells in milk and circulating immune cells, such as neutrophils (polymorphonuclear neutrophils (PMN)), and cytotoxic natural killer cells.

The adaptive immune response is initiated by activated macrophages; these produce cytokines and chemokines, antimicrobial peptides, such as, β-defensins and cathelicidins [102, 103]. In addition, macrophages process and present antigens through MHC class I or II mechanisms.

Macrophage present antigen through MHC class II to naïve circulating T helper cells. Upon activation, these cells subsequently differentiate into antigen-specific effector T helper cells based on the type of cytokine in the immediate surrounding, e.g., presence of IL-12 surrounding will induce polarization of CD4+ T helper 1 (Th1) cells, IL-4 will induce polarization of Th2 and a combination of IL-4, TGF-β, IL-22 induces Th9 polarization, whereas IL-1β, IL-6, TGF-β & or IL-23 combination induces polarization towards Th17 [104, 105] and so forth, thereby inducing specific local immune responses. The antigen-specific activated T-cell clonally expands into effector cells that produce specific cytokines which activate and induce polarization of other cells that participate in the immune response. These cells eventually differentiate into memory cells. Mastitis results in changes to cytokines concentration in the milk and udder; these changes are reported to differ based on the infecting bacteria. In *S. aureus* mastitis, protective immune responses that leads to eventual clearance of the bacteria, is facilitated by an initial increase in the numbers of activated Th17, Th1, Th2 cells associated with an increase in pro-inflammatory cytokines in the mammary gland, followed by an increase in Treg and anti-inflammatory cytokines IL-10 [88]. The study conducted in an *S. aureus* mastitis mice model showed that the frequency of these cells changed throughout the course of infection [88]. Whereby Th 17 cells producing IL-17 are increased in the early phase of a *S. aureus* mastitis infection, along with an increase in Th1 cells [88]. As previously mentioned, IL-17 enhances neutrophil infiltration facilitating bacterial clearance. IL-2, TNF-β and IFN-γ produced by Th1 cells promote the activation and proliferation of cytotoxic lymphocytes, natural killer cells, and macrophages. As the infection progress, the effector T helper cell response shifts to a Th 2 response, this is thought to limit the tissue damage due to the inflammatory response, Th2 secreted cytokines such as, IL-4 which regulate macrophage functions, and inflammatory cytokines were increased. IL-4 increase expression of IL-10 [106], which inhibits IL-17 expression [88, 107]. As part of the protective immune response against mastitis, Zhao et al. [88], reported that Treg cells and IL-10 tightly regulate the inflammatory response to mastitis, as observed after the peak of infection in mice *S. aureus* mastitis model.

Sometimes during infection, extracellular bacteria such as *S. aureus* avoid being targeted by the host response and persist by invading cells and multiplying within them, becoming an intracellular infection. In addition, phagocytosed *S. aureus* is able to replicate and multiply in phagocytic cells such as macrophages [108, 109]. Here, bacterial antigens are processed and presented on MHC class 1, where they are recognized for killing by antigen-specific cytotoxic T cells (CD8+), releasing *S. aureus* for the second round of opsonophagocytosis [110]. Activated antigen-specific CD8+ cytotoxic T-cells mainly produce IFN-γ and CD8+ suppressor T-cells produce regulatory IL-4. The severity of the disease outcome is dependent on the efficiency of phagocytic killing that occurs and this as already mentioned, is dependent on the early expression of the various inflammatory mediators [96]. Therefore, as the inflammatory response amplifies, with increasing migration of phagocytic cells and antigen-specific cytotoxic T cells to the site of infection, *S. aureus* has the ability to form abscess and release a wide variety of virulence factors such as haemolysins (alpha, beta, and delta) [60–63], T cells superantigens (enterotoxins, TSST-1) [60–63] and several others as mentioned in Section 3 above. These virulent factors enable the bacterium to evade detection by the immune system and inhibit the host immune response by destroying immune cells.

The humoral response plays an important role in the prevention and control of bacterial infections. Three different classes of immunoglobulins, i.e., IgG (subclass: IgG1, IgG2, IgG3), IgM, and IgA, play significant roles in mammary gland defense against bacterial pathogens. In sheep the predominant immunoglobulin G is IgG1, followed by IgG2 then IgG3, however this is dependent of the infecting organisms. IgG1 producing plasma cells are associated with a Th2 response whereas IgG2 producing cells are associated with a Th1 response. It has been suggested that immunoglobulins in colostrum and milk, are transported from blood into the mammary secretions as part of normal physiological process during colostrum and milk production or through leakage into the mammary gland during inflammation. For example, during normal physiological process, such as colostrum or milk production, blood derived IgG1 specific to intestinal antigens is trafficked into the mammary glands, blood derived IgG1 is produced by plasma cells derived from stimulated B lymphocytes of the Peyer's patches [111–114], and has no major role in intramammary infection. However, blood derived IgG2 leaks into the mammary glands during inflammation and is though to play a significant role in intramammary infection, as it is produced by plasma cells in the skin-associated lymphoid tissue and regional lymphoid tissues [111–114]. Blood derived IgG2 has specificity to bacterial antigens associated to skin infections [111–114]. In addition, during intramammary infection, antigen-activated plasma cells from regional lymphoid nodes present within the mammary glands produce IgA, IgM and IgG2 that are specific for antigens present in the mammary gland [111–114]. IgG1, IgG2 and IgM function by opsonising, invading bacterial pathogens and make them detectable by neutrophils and macrophages for opsonophagocytic destruction [115]. Phagocytosis by PMN is regarded as one of the most important defense mechanisms of the mammary gland. However, this defense mechanism can be hindered by toxins produced by *S. aureus* such as leukotoxin. IgA acts as a neutralizing antibody to protect the mammary gland against bacterial toxins [114]. In addition, IgA prevents the establishment of mastitis in the mammary gland through complement fixation, prevention of adhesion of pathogenic microbes to the endothelial lining by binding various adhesion receptors, and inhibition of bacterial metabolism by blocking enzymes [113], such as Staphyloccocus Enterotoxins. IgA also acts in bacterial agglutination, limiting bacterial dissemination and colonization [114, 116]. Immunoglobulin, specifically IgA, may play a very import role in protection and prevention of mastitis in small ruminants [113, 114].

4. Approach to new vaccine developments for the prevention of mastitis

Vaccination is a control strategy used to increase the adaptive immunity of the animal in order to prevent new infections. The purpose of using vaccines is to enhance immunity and reduce the reliance on the use of antimicrobial drugs (antibiotics), more so in the case of mastitis in sheep and goats, as the use of antibiotics in treatment may result in antimicrobial resistance, e.g., antibiotics resistance in *Staphylococcus* spp., such as methicillin resistant *Staphylococcus aureus*. This poses a human health risk, especially because most mastitis-causing bacteria are zoonotic, and some have been reported as cases in humans due to consumption of raw sheep and goat dairy product [117–121]. In addition, there are very few veterinary pharmaceutical products licensed for specific use in sheep and goats globally. Furthermore, the use of non-steroidal anti-inflammatory agents to alleviate clinical signs of mastitis and improve animal welfare [122], has no impact on milk quality. As such, alternatives strategies are needed to prevent mastitis in small ruminants. Several experimental vaccines against mastitis, based on formalin-inactivated whole cells, whole-cell lysate, polyvalent whole-cell Bacterin cultures of the vaccine strains or bacteria of interest, produced using old technologies, have been shown to play a role in mastitis prevention, by reducing the severity of clinical and subclinical mastitis, but does not reduce the incidence of the disease [123–126]. Although experimental vaccines against mastitis, based on formalin-inactivated whole cells, whole-cell lysate, polyvalent whole-cell Bacterin cultures of the vaccine strains [123–126], stimulates humoral immune responses, the levels of opsonizing antibodies in milk is poor or absent [127]. The lack of efficacy observed in conversional experimental vaccines may explain why mastitis vaccines for use in sheep and goats have not been developed further. Hence there are currently very few commercial vaccines licensed for use in sheep and goats. In addition, current vaccines on the market licensed against mastitis are mostly targeted at staphylococcal mastitis in bovine, there aren't many vaccines against mastitis targeting sheep and goats. Of the few vaccines against mastitis on the market, none of them are effective against mastitis but label claim indicate some effect. For example, Lysigin® (Boehringer Ingelheim) is the only vaccine against staphylococci in the US. While, Startvac (Hipra, spain) is the only vaccine licensed in Europe and few other coutries including Canada with label claim of some effect against *S. aureus, E. coli,* CNS. However, in controlled experimental studies their effects were none to very limited [127–130]. Another vaccine on the market is J5 vaccine from different manufacturer (zoetis, Boeringer, etc.) against *E. coli* mastitis. As with the other vaccines mentioned previously, this vaccine is also not very effective but claimed for some effect. Lastly, UBAC® (Hipra, Spain) with label claim against *S. uberis* mastitis is yet to be validated under field condition [130]. In comparison, only two licensed vaccine, Blue udder (Onderstepport biological products (OBP), South Africa), and Vimco ® (Hipra, Girona, Spain), targeting mastitis in sheep and goat are available on the market. As with the other mastitis vaccine, label claim of some effects against *S. aureus*, *M. heamolytica* and *S. aureus* respectively. Highlighting the need for the development of an efficacious mastitis vaccines for sheep and goat.
In the past 10 years, a wealth of knowledge on the pathogenesis of disease and protective immune response mechanisms against bacterial mastitis has been gained in ruminants. This knowledge needs to be applied in the development of an effective mastitis vaccine. Based on our current understanding of the immunological responses in the mammary gland of ewes against bacterial mastitis, as discussed above. The significant role played by antibody-mediated immune response, such as the importance of induction of locally produced antigen-specific IgA antibodies [131], and cell-mediated immune response geared towards a local Th17 response at

the onset of infection in preventing mastitis [87, 88, 132], supports the use of novel vaccines technologies in the improvement of already existing experimental vaccine. For example, already licensed vaccines for bacterial mastitis used in sheep and goats, could be improved in the following manner:

1. Inclusion of other prevalent mastitis-causing bacteria virulence factors such as toxins, surface proteins etc. In order to target more bacteria rather than focusing on one organism. For example, studies have shown that anti-leukotoxin antibodies have an important role in protection against mammary infection of ruminants. This was demonstrated through vaccination of ewes with partially purified leukotoxin and α-haemolysin, which conferred partial protection against an intramammary challenge with a mastitis-causing strain of *S. aureus* [133].

2. Use of delivery systems (formulation strategies and novel adjuvants) in order to stimulate the development of immunity towards a Th17 type response [132, 134] and stimulate local production of IgA and IgG2 responses [135]. In addition, to early recruitment of neutrophils. To induce Th17 responses in vaccines various adjuvants have been studied. For example; *S. pneumonia* whole cell antigen vaccine formulated in aluminum hydroxide enhanced the quality of antibodies and Th17 CD4+ T cell response [136]. In TB infections Cyclic dinucleotides (CDNs) adjuvanted vaccine has be shown to elicit a Th17 immune response correlating with enhanced protection against infections [137]. The bacterial components, muramyl dipeptide (MDP) a NOD2 ligand has been shown to induce Th17 response [138], lipopolysaccharide (LPS) a TLR4 ligand induces Th17 [139]. Therefore, prospective mastitis vaccine aiming on eliciting a Th17 response, maybe formulated in currently used adjuvants such as aluminum hydroxide gel in combinations with TRL ligands, such as TLR4 or TLR8/7 ligands; NOD2 ligands and CDNs. Alternatively, these ligands could also be formulated in combination with novel nanoemulsion oil and water adjuvants for the development of efficacious vaccine.

3. Exploring alternative vaccination routes, such as mucosal vaccine administration, in order to achieve the desired immune response, for example, in cow vaccination route have an impact on the subsequent immune response [132, 140, 141]. For example, studies, have shown that intramammary administration of antigens (e.g., inactivated *S. aureus*) in non-lactating ewe enhance the kinetics of neutrophil influx with no involvement of complement in the immunological response.

4. Use of newer technologies, such as biofilm matrix polysaccharides, have also been used to induce protective immune response against *S. aureus* mastitis in ewes [142]. Vaccines developed using this approach offers some degree of improved efficacy against *S. aureus* mammary infection and mastitis [143]. Mastitis Vaccines licensed for sheep such as, the Vimco ® vaccine based on biofilm-producing *Staphylococcus* has been shown to reduce the incidence of mastitis in sheep [144]. In addition, omics technologies could be harnessed to fully characterize immunological responses in mastitis and identify relevant vaccine candidates for more efficacious vaccine development against mastitis causing bacteria.

5. Conclusion

Lack of effective vaccines against mastitis in sheep and goat has long been attributed to lack of knowledge on the disease pathogenesis and protective immune

response mechanism required. In the past decades, a wealth of knowledge has been gained on the pathological processes leading to mastitis in sheep and goat caused by the most prevalent pathogenic bacteria, i.e. *Staphylococcus* spp. Using *Staphylococcus* spp. as an example, we now know that the pathological processes leading to sub-clinical and clinical mastitis depends on bacterial virulence factors and the induced host immune response. The pathogenesis of *S. aureus* mastitis entail three processes, i.e. adhesion, invasion and evasion. During these three processes *S. aureus* differentially expresses virulence factors that aids colonization of the host mammary glands. In addition, we now have a better understanding of which virulence factors target the main cells involved in mammary immunity and how their actions are counteracted by the bacteria. We have also gained more understanding of the immune response required to limit *S. aureus* infection. Although we do not fully know the mechanisms of the protective immune response in the mammary glands of ruminants and still do not know how to induce such a protective response. Our current knowledge, points to a local protective response that most likely entail early recruitment of neutrophils to control bacterial inversion and IgG2 antibodies isotypes, and to a potential role for IgA. In addition, a local cellular response geared towards a Th17 immunity plays a role in bacterial cleareance and neutrophil recruitment. This knowledge could be used to improve current conventional experimental vaccines against mastitis in small ruminants by employing immunostimulatory adjuvants or delivery systems capable of stimulating a local Th17 responses, by using TLR4, 7/8; NOD2 and CDNs ligands in adjuvant formulations.

Due to the lack of efficacy observed with conventional vaccines, research on the development of efficacious mastitis vaccine for small ruminant can be fast track by exploiting rapidly advancing omics technologies and developing immunological tools (reagents) for characterization of ruminant adaptive immune response in great detail. Reverse vaccinology approaches could be used to discover candidate vaccine antigens from mastitis causing bacteria. Omics technologies can also be applied to gain understanding on the protective adaptive immune response to mastitis infections by mapping relevant antigen through transcriptomics and proteomics, and characterizing antibody and T-cell repertoires through immuno-proteomics. Data generated from these approach may reveal correlates of protection to which vaccination strategies can be based.

Conflict of interest

The author declares no conflict of interest.

Notes/Thanks/Other declarations

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