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Chapter

Current Status of Antimicrobial Resistance and Prospect for New Vaccines against Major Bacterial Bovine Mastitis Pathogens

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Abstract

Economic losses due to bovine mastitis is estimated to be $2 billion in the United States alone. Antimicrobials are used extensively in dairy farms for prevention and treatment of mastitis and other diseases of dairy cattle. The use of antimicrobials for treatment and prevention of diseases of dairy cattle needs to be prudent to slow down the development, persistence, and spread of antimicrobial-resistant bacteria from dairy farms to humans, animals, and farm environments. Because of public health and food safety concerns regarding antimicrobial resistance and antimicrobial residues in meat and milk, alternative approaches for disease control are required. These include vaccines, improvements in housing, management practices that reduce the likelihood and effect of infectious diseases, management systems and feed formulation, studies to gain a better understanding of animal behavior, and the development of more probiotics and competitive exclusion products. Monitoring antimicrobial resistance patterns of bacterial isolates from cases of mastitis and dairy farm environments is important for treatment decisions and proper design of antimicrobial-resistance mitigation measures. It also helps to determine emergence, persistence, and potential risk of the spread of antimicrobial-resistant bacteria and resistome from these reservoirs in dairy farms to humans, animals, and farm environments.

Keywords: antimicrobial resistance, vaccines against mastitis, bovine mastitis, bacterial mastitis pathogens, bacterial pathogens, current status

1. Introduction

1.1 Antibiotic use in dairy farms and antimicrobial resistance

Economic losses due to bovine mastitis is estimated to be $2 billion in the United States alone [1]. Most studies showed that there is no widespread, emerging resistance among mastitis pathogens [2–4] in dairy farms. Some studies showed that the antimicrobial resistance of mastitis pathogens varies with dairy farms and bacterial species within and among dairy farms [4–9]. However, antimicrobial resistance patterns of human pathogenic bacteria and their resistome in dairy farms might be of significant concern.

On average, starting from calving (giving birth) dairy cow is milked (in lactation) for about 300 days and then dried off (stop milking) for about 60 days before they calve again. Under the ideal dairy farming condition, a dairy cow should become pregnant within 60 days of calving, and the lactation cycle continues (**Figure 1**). The goal of a dry period is to give them a break from milking so that milk-producing cells regenerate, multiply, and ready for the next cycle of lactation. The incidence of intramammary infection (IMI) by bacteria is high during the early dry period and transition periods [10]. In general, for a dairy cow, a transition period, also known as the periparturient period, is a time range from three weeks before parturition (non-milking time) until three weeks after calving (milking time). It is a transition time from non-milking to milking.

Dairy cows are susceptible to mastitis during early non-lactating (dry period) and transition periods [11, 12], especially new infection with environmental pathogens (*Streptococcus* spp. and coliform) are highest during the first two weeks after drying off and last two weeks before calving [13] compared to contagious mastitis pathogens such as *S. aureus* [14]. The incidence of intramammary infection is high during the early dry period because of an absence of hygienic milking practices such as pre-milking teat washing and drying [15], pre- and post-milking teat dipping in antiseptic solutions [16, 17], that are known to reduce teat end colonization by bacteria and infection. An udder infected during the early dry period usually manifests clinical mastitis during the transition period [18] because of increased production of parturition inducing immunosuppressive hormones [19], negative energy balance [12], and physical stress during calving [20].

Cows are naturally protected against intramammary infections during the dry period by physical barriers such as the closure of teat opening by smooth muscle (teat sphincter) and the formation of a keratin plug, fibrous structural proteins (scleroproteins) [21, 22], in the teat canal produced by teat canal epithelium [23]. Keratin contains a high concentration of fatty acids, such as lauric, myristic, and palmitoleic acids, which are associated with reduced susceptibility to infection and stearic, linoleic, and oleic acids that are associated with increased susceptibility to infection. Keratin also contains antibacterial proteins that can damage the cell wall of some bacteria by disrupting the osmoregulatory mechanism [23]. However, the
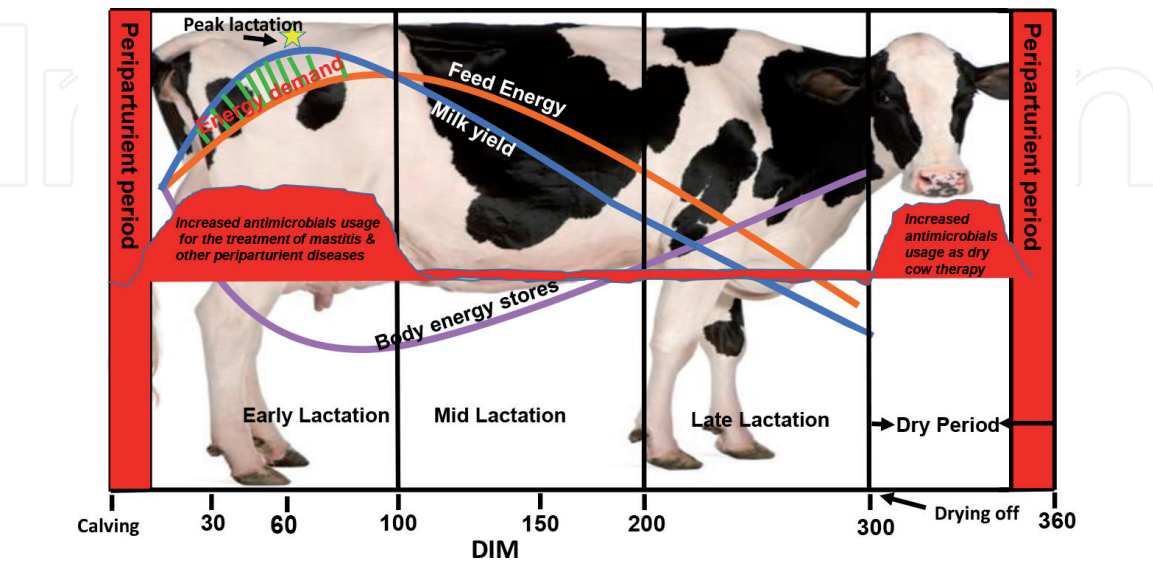

Figure 1.
Antimicrobials usage patterns during the lactation cycle. DIM: Days in milk, yellow star: Peak lactation at 60 DIM, green bars: Energy demand that requires the mobilization of body energy reserve at the expense of losing bodyweight, red bumps showed increased usage of antimicrobials.

time of teat canal closure varies among cows. Some studies showed that 50% of teat canals were classified as closed by seven days after drying off, 45% closed over the following 50–60 days after drying off, and 5% had not closed by 90 days after dry off [24]. Teats that do not form a plug-like keratin seal are believed to be most susceptible to infection. Infusion of long-acting antimicrobials into the udder at drying-off (dry cow therapy) has been the major management tool for the prevention of IMI during the dry period, as well as to clear IMI established during the previous lactation [24].

In the United States and many other countries at the end of lactation (at drying off), all cows regardless of their health status, are given an intramammary infusion of long-acting antimicrobials (blanket dry cow therapy) to prevent IMI by bacteria during the dry period [3, 25]. Because of increased concern on the use of blanket dry cow therapy for its role in driving antimicrobial resistance, selective dry cow therapy (intramammary infusion of antimicrobials into only quarters that have tendency or risk of infection) has been under investigation [26, 27]. Some recent studies showed that the use of bacteriological culture-based selective dry cow therapy at drying-off did not negatively affect cow health and performance during early lactation [26, 27]. In general, dairy farms are one of the largest users of antimicrobials including medically important antimicrobials [28]. Some of the antimicrobials used in dairy farms include beta-lactams (penicillins, Ampicillin, oxacillin, penicillin-novobiocin), extended-spectrum beta-lactams (third-generation cephalosporins, e.g., ceftiofur), aminoglycosides (streptomycin), macrolides (erythromycin), lincosamide (pirlimycin), tetracycline, sulfonamides, and fluoroquinolones [28–30]. Antimicrobials are also heavily used in dairy farms for the treatment of cases of mastitis [3, 25, 31] and other diseases of dairy cows such as metritis, retained placenta, lameness, diarrhea, pneumonia, [32–36] and neonatal calf diarrhea [37]. Over 90% of dairy farms in the US infuse all udder quarters of all cows with antimicrobial regardless of their health status [7, 25, 38]. According to dairy study in 2007 that was conducted in 17 major dairy states in the United States, 85.4% of farms use antibiotics for mastitis, 58.6% for lameness, 55.8% for diseases of the respiratory system, 52.9% for diseases of reproductive system, 25% for diarrhea or gastrointestinal infections and 6.9% for all other health problems [3, 25]. Cephalosporins were the most widely used antibiotics for the treatment of mastitis, followed by lincosamides and non-cephalosporin beta-lactam antibiotics [3, 25]. The two most commonly used antibiotics for dry cow therapy are Penicillin G/dihydrostreptomycin and cephalosporins [3, 25]. Antimicrobials were administered for the prevention and treatment of mastitis and other diseases of dairy cattle mainly through intramammary infusion and intramuscular route (USDA APHIS, 2009a). Antimicrobials infused into the mammary glands can be excreted to the environment through leakage of milk from the antimicrobial-treated udder or absorbed into the body and enter the blood circulation and biotransformed in the liver or kidney and excreted from the body through urine or feces into the environments [39–42]. Similarly, antimicrobials administered through parenteral routes for the treatment of acute or peracute mastitis or other diseases of dairy cows will enter the blood circulation and biotransformed in the liver or kidney and excreted from the body through urine or feces into the environments [39–42]. Therefore, both parenteral and intramammary administration of antibiotics has a significant impact on other commensals or opportunistic bacteria in the gastrointestinal tract of dairy cows and farm environments.

In addition to the use of antimicrobials for the prevention and treatment of mastitis and other diseases of dairy cattle, some farms also feed raw waste milk or pasteurized waste milk from antibiotic-treated cows to dairy calves. Feeding of

raw waste milk or pasteurized waste milk from antibiotic-treated cows to calves increases pressure on gut microbes such as *E. coli* to became antimicrobial-resistant [43–45]. Aust et al. [43] showed that the proportion of antimicrobial-resistant *E. coli,* especially cephalosporin-resistant *E. coli* isolates, was significantly higher in calves fed waste milk or pasteurized waste milk from antimicrobial treated cows than calves fed bulk tank milk from non-antibiotic treated cows. However, pasteurized waste milk from cows not treated with antimicrobials is acceptable to be feed to young calves [43] but it is not known if pasteurization prevents the transfer of antimicrobial-resistant genes to microbes in the calve's gut. Some studies also showed that feeding pasteurized waste milk from antimicrobial treated cows to calves increased the presence of phenotypic resistance to ampicillin, cephalothin, ceftiofur, and florfenicol in fecal *E. coli* compared with milk replacer-fed calves [45]. However, the presence of resistance to sulfonamides, tetracyclines, and aminoglycosides was common in dairy calves regardless of the source of milk, suggesting other driving factors for resistance development [45]. It has been suggested that antimicrobial residues present in waste milk have a non-specific effect at a lower taxonomical level [44]. Collectively, these non-prudent antimicrobials usage practices in dairy farms expose a large number of animals in dairy farms to antimicrobials and also increases the use of antimicrobials in dairy farms, which in turn creates intense pressure on microbes in animals' body especially commensal and opportunistic microbes in the gastrointestinal tract and farm environments. Some of these commensal bacteria in the animal body are serious human pathogens (e.g., *E. coli* 0157:H7). *Staphylococcus aureus* is one of the pathogens with a known ability to develop antimicrobial resistance and established *S. aureus* infections are persistent and difficult to clear. The failure to control these infections leads to the presence of reservoirs in the dairy herd, which ultimately leads to the spread of the infection and the culling of the chronically infected cows [46, 47].

Monitoring antimicrobial resistance patterns of bacterial isolates from cases of mastitis is important for treatment decisions and proper design of mitigation measures. It also helps to determine emergence, persistence, and potential risk of the spread of antimicrobial-resistant bacteria and resistome to human, animal, and environment [48, 49]. The prudent use of antimicrobials in dairy farms reduce emergence, persistence, and spread of antimicrobial-resistant bacteria and resistome from dairy farms to human, animal, and environment.

1.2 Transmission of antimicrobial-resistant bacteria from dairy farms to human

Most studies showed that there is no widespread, emerging resistance among mastitis pathogens [2–4] in dairy farms. However, dairy farms may serve as a source of antimicrobial-resistant human pathogenic bacteria. Extensive use of third-generation cephalosporins (3GCs) in dairy cattle for the prevention and treatment of mastitis [3, 25, 28] and other diseases of dairy cattle [31, 32] can result in the carriage of extended-spectrum beta-lactamase producing *Enterobacteriaceae* (ESBL Ent) [50, 51]. Third- and fourth-generation cephalosporins are commonly used for the treatment of invasive Gram-negative bacterial infections in humans [52–54]. In 2017, there were an estimated 197,400 cases of ESBL Ent among hospitalized patients and 9100 estimated deaths in the US alone [55]. Among *Enterobacteriaceae, Escherichia coli* (*E. coli*) is the most common bacteria that reside in the gut as normal microflora or opportunist pathogen of animals and humans. However, certain pathogenic strains can cause diseases such as mastitis in cattle, neonatal calf diarrhea in calves and hemorrhagic enteritis, and more life-threatening conditions such as hemolytic uremic syndrome and urinary tract infections in humans. New strains of multi-drug resistant foodborne pathogens that produce extended-spectrum

beta-lactamases that inactivate nearly all beta-lactam antibiotics have been reported [30]. Ceftiofur is the most common 3GC used in dairy cattle operations [56]. The 3GCs are also critically important antibiotics for the treatment of serious infections caused by *Enterobacteriaceae* such as *Escherichia coli (E. coli)* and *Salmonella* spp. in humans [57, 58]. The use of structurally and chemically similar antibiotics in dairy cattle production and human medicine may lead to co-resistance or cross-resistance [52–54]. Some of the species of Gram-negative environmental mastitis pathogens, such as *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Pseudomonas* spp., *Enterobacter* spp. are the greatest threat to human health due to the emergence of strains that are resistant to all or most available antimicrobials [59, 60].

The resistance of *Enterobacteriaceae* to 3GC is mainly mediated by the production of extended-spectrum beta-lactamase enzymes (ESBLs) that breakdown 3GC [61]. *E. coli* is one of the most frequently isolated *Enterobacteriaceae* carrying ESBL genes (bla_{CTX-M}, bla_{SHV}, $bla_{TEM,}$ and bla_{OXA}) families [62–64]. These ESBL genes are usually carried on mobile plasmids along with other resistance genes such as tetracycline, quinolones, and aminoglycosides. *E. coli* resides in the gastrointestinal tract of cattle as normal or opportunistic microflora, but some strains (for e.g., 0157:H7) cause serious infection in humans [58], indicating that cattle could serve as a reservoir of ESBLs producing *E. coli* (ESBLs *E. coli*) for human.

In the US, the occurrence of ESBLs *E. coli* in the dairy cattle was reported a decade ago from Ohio [52] and few previous studies reported the occurrence and an increase in the trend of ESBLs *E. coli* in the dairy cattle production system [52, 53, 65–67]. However, recent studies increasingly showed the rise of ESBLs *E. coli* in the cattle [51, 52, 65, 67]. Similarly, reports from the Center for Disease Control (CDC) showed a continuous increase in the number of community-associated human infections caused by ESBLs-producing *Enterobacteriaceae* [55]. This CDC report showed a 9% average annual increase in the number of hospitalized patients from ESBLs pathogens in six consecutive years (from 2012 to 2017). As a result, the human health sector tends to blame dairy farms that routinely use the 3GC for the rise of ESBLs pathogens such as *E. coli* [55, 68]. However, despite the general believe of possibility of transmission of antimicrobial-resistant bacteria from dairy farms to humans directly through contact or indirectly through food chain, there was no clear evidence-based data that showed the spread of antimicrobial-resistant bacteria from the dairy production system to humans. The opinion of the scientific community on the factors that drive the emergence and spread of antimicrobial-resistant bacteria also varies [69]. Transmission of an antimicrobial-resistant pathogen to humans could occur if contaminated unpasteurized milk and/or undercooked meat from culled dairy cows due to chronic mastitis is consumed [70]. So it is crucial to pasteurize milk or cook meat properly to reduce the risk of infection by antimicrobial-resistant bacteria [71]. It is not known, if pasteurization or proper cooking prevents the transfer of resistant genes from milk or meat to commensal or opportunistic bacteria in the human gastrointestinal tract (GIT), or the GIT of calves fed pasteurized waste milk. Mechanisms of antibiotic resistance gene transfer from resistant to susceptible bacteria are not well known, and killing resistant pathogens alone may not be good enough to prevent the transfer of the resistance gene. Non-prudent use of antimicrobials in dairy farms increases selection pressure, which could result in the emergence, persistence, and horizontal transfer of antimicrobial-resistant determinants from resistant to non-resistant bacteria. Bacteria exchange resistance genes through mobile genetic elements such as plasmids, bacteriophages, pathogenicity islands, and these genes may ultimately enter bacteria pathogenic to humans or commensal or opportunistic bacterial pathogens. The prudent use of antimicrobials in dairy farms requires identification of the pathogen causing

mastitis, determining the susceptibility/resistance of the pathogen, and proper dose, duration, and frequency of treatment to ensure effective concentrations of the antibiotic to eliminate the pathogen.

2. Prospects for effective vaccines against major bacterial mastitis pathogens

Despite decades of research to develop effective vaccines against major bacterial bovine mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, and *E. coli*, the effective intramammary immune mechanism is still poorly understood, perpetuating reliance on antibiotic therapies to control mastitis in dairy cows. Dependence on antimicrobials is not sustainable because of their limited efficacy [46, 47] and increased risk of emergence of antimicrobial-resistant bacteria that pose serious public health threats [4, 72–74]. Neither of the two currently available commercial Bacterin vaccines against *S. aureus* (**Table 1**), Lysigin® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) in the USA and Startvac® (Hipra, Girona, Spain) in Europe and other countries, confer protection from new intramammary infection under field trials as well as under controlled experimental challenge studies [75–81].

There are four commercial vaccines against *E. coli* mastitis which include 1) the Eviracor®J5 (Zoetis, Kalamazoo, MI), [82, 83], 2) Mastiguard®, 3) J-VAC® (Merial-Boehringer Ingelheim vet medical, Inc., Duluth, GA) and 4) ENDOVAC-Bovi® (IMMVAC) (Endovac Animal Health, Columbia, MO) (**Table 1**). The Endovac-bovi® is a cross-protective vaccine made of genetically engineered R/17 mutant strain of *Salmonella typhimurium* and the core somatic antigen mutant J-5 strain of *E. coli* combined with an immune-potentiating adjuvant (IMMUNEPlus®). Endovac-bovi significantly reduces diseases caused by Gram-negative bacteria producing various endotoxins and protects against *E. coli* mastitis and other endotoxin-mediated diseases caused by *E. coli*, *Salmonella*, *Pasteurella multocida*, and *Mannheimia hemolytica*. The UBAC® (Hipra, Amir, Spain) [84] is a recently developed vaccine against *S. uberis* mastitis with label claim of partial reduction in clinical severity of *S. uberis* mastitis.

Mastitis Pathogen	Vaccine	Vaccine component	Protective effect	Reference
	Commercial			
S. aureus	Lysigin®	Bacterin: Somatic antigen containing phage types I, II, III, IV with different strains of *S. aureus*	Reduced SCC, clinical mastitis, and chronic IMI	[85–87]
	"	"	Field-based studies concluded no such effect	[80, 81, 88–90]
	Startvac®	Bacterin: *E. coli* J5 and *S. aureus* CP type 8 with SAAC	Decreased duration of IMI, transmissibility of *S. aureus,* coliforms, and CNS	[77]
	"	"	Use of the vaccine was not associated with a decrease in mastitis	[75]

Mastitis Pathogen	Vaccine	Vaccine component	Protective effect	Reference
	Bestvac® Vs Startvac	herd-specific autologous vaccine compared with Startvac®	Both vaccines decreased herd prevalence of *S. aureus* mastitis but no other differences in terms of improvement of udder health	[78]
	Experimental			
	Whole-cell lysate	Bacterin encapsulated in biodegradable microspheres	Induced antibodies that were more opsonic for neutrophils and inhibited adhesion to mammary epithelium.	[91]
	Whole-cell lysate from two strains	Bacterin from two strains (α and α + β hemolytic) plus supernatants from non-hemolytic strain	Vaccinated cows had 70% protection from infection compared to less than 10% protection in control cows	[92]
	MASTIVAC I	Whole-cell lysate	Improved udder health in addition to specific protection against *S. aureus* infection	[93]
	Live pathogenic *S. aureus* through IM route	Live pathogenic *S. aureus*	Induce activation of immune cells in mammary gland and blood	[94]
	Fibronectin binding protein and clumping factor A	DNA primed and protein boosted	Induced cellular and humoral immune responses that provide partial protection against *S. aureus*	[95]
	Protein A of *S. aureus* with the green fluorescent protein	DNA	Induced humoral and cellular immune responses	[96]
	Plasmid encoding bacterial antigen β-gal	DNA	Induced humoral and cellular immune responses	[97]
	Polyvalent *S. aureus* Bacterin	Bacterin	Eliminated some cases of chronic intramammary *S. aureus* infections	[88]
	Lysigin® with three-isolates based experimental Bacterin	Bacterin	Lysigin reduced the clinical severity and duration of clinical disease. None of the experimental Bacterins has significant effects	[80]
	Polyvalent *S. aureus* Bacterin	Bacterin + antibiotic therapy	*S. aureus* intramammary infection cure rate increased	[89]
	Whole-cell lysate	Whole-cell trivalent vaccine containing CP types 5, 8 and 336 with FIA or Alum adjuvants	Elicited antibody responses specific to the 3 capsular polysaccharides	[98]

Mastitis Pathogen	**Vaccine**	**Vaccine component**	**Protective effect**	**Reference**
	CP conjugated to a protein and incorporated in polymicrospheres and emulsified in FIA	CP types 5, 8 and 336	Cows in both groups produced increased concentrations of IgG1, IgG2 antibodies, hyperimmune sera from immunized cows increased phagocytosis, decreased bacterial adherence to epithelial cells	[99]
	Polysaccharide-protein conjugates in FIA	Polysaccharide-protein conjugate		
	SASP or SCSP	Surface proteins	Induced partial protection	[100]
	Vaccination with Efb and LukM		Induced increased titers in serum and milk	[101]
	Inactivated Bacterin	Bacterin	Partial protection	[102]
S. uberis	**Commercial**			
	UBAC®	Extract from biofilm-forming strains of *S. uberis*	Reduce clinical signs, bacterial count, temperature, daily milk yield losses and increased the number of quarters with isolation and somatic cell count <200,000 cells/mL of milk	[84]
	Experimental			
	Killed *S. uberis cells*	Bacterin	Reduced numbers of homologous *S. uberis* in milk	[103]
	Killed bacterial cells	Bacterin of *S. uberis* and *S. agalactiae*	Parenteral vaccination has no effect on streptococcal mastitis	[104, 105]
	Live *S. uberis/* cutaneous route	Live *S. uberis*	Some protective effect only on the homologous strain	[106]
	GapC or chimeric CAMP factor	Protein	Reduction in inflammation	[107]
	PauA	protein	Partial protection	[108]
Coliform	**Commercial**			
	E. coli J5 Mastiguard® J Vac® Endovac-bovi® (IMMVAC)	Bacterin	Reduce bacterial counts in milk, duration of IMI and resulted in fewer clinical symptoms	[82, 83, 109–111]

SAAC: slime associated antigenic complex, SASP: Staphylococcus aureus surface proteins, SCSP: Staphylococcus chromogenes surface proteins, CP: Capsular polysaccharide, GapC: Glyceraldehyde-3-phosphate dehydrogenase C, pauA: plasminogen activator protein, FIA: Freund's incomplete adjuvant, Efb: fibrinogen-binding protein, LukM: leukocidin subunit M.

Table 1.
Commercialized and experimental vaccines against major bovine mastitis pathogens.

2.1 Intramammary immune mechanisms

Intramammary immunity can be induced locally in the mammary gland or systemically in the body and cross from the body into the mammary glands. Mammary gland pathogen that enters through teat opening interact with host innate defense system primarily with macrophages in the mammary gland. Macrophages recognize invading pathogens through its pattern recognition receptors (PRR) which binds to pathogen associated molecular patterns (PAMPs) and engulf and break down the foreign pathogen into small peptides and load on to MHC-II molecules move to the supramammary lymph nodes and display on its surface to the T cells. Naïve T cells bind with peptide on MHC-II molecule through its T- cell receptor and become activated and start secreting cytokines, which further stimulate B-cells to produce antibodies. Antibody produced by B-cells released into the blood circulation and depending on type of antibody may be released to the site of infection (e.g., IgG) and opsonize the infecting pathogen and subject them to destruction by opsono-phagocytic mechanisms. Antibodies may also remain on mucosal surfaces (e.g., IgA) and bind to invading pathogens and prevent them from binding to host cells or tissue and thereby prevent colonization and infection.

Intramammary infection (IMI) leads to increased somatic cell count in the milk or mammary secretion. Somatic cells are mainly white blood cells such as granulocytes (neutrophils, eosinophils, and basophils), monocytes or macrophages, and lymphocytes, which are recruited to the mammary glands in response to mammary gland infection to fight off infection. A small proportion of mammary epithelial cells that produce milk are also shed through milk and are included in the somatic cell count. So, somatic cells are white blood cells and mammary epithelial cells. Milk somatic cell count (SCC) increases when there is mammary gland infection (IMI) because of an inflammatory response to clear infection. In general, SCC is also an indicator of milk quality [112–116] because if there are few mammary pathogenic bacteria in the gland, the inflammatory response is less, and somatic cells recruitment into the gland is also low and vice versa. Bulk tank milk (BTM) is milk collected from all lactating dairy cows in a farm into a tank or multiple tanks. So BTSCC is somatic cell counts obtained from milk sample collected from a tank.

Intramammary infection may progress to clinical or subclinical mastitis [117]. Clinically infected udder usually treated with antimicrobial, whereas subclinically infected udder may not be diagnosed immediately and treated but remained infected and shedding bacteria through milk throughout lactation. The proportion of cure following treatment of mastitis varies and the variation in cure rate is multifactorial including cow factors (age or parity number, stage of lactation, and duration of infection, etc.), management factors (detection and diagnosis of infection and time from detection to treatment, availability of balanced nutrition, sanitation, etc.), factors related to antimicrobial use patterns (type, dose, route, frequency, and duration), and pathogen factors (type, species, number, pathogenicity or virulence, resistance to antimicrobial, etc.) [46, 118].

The dilution of effector humoral immune responses by large volume of milk coupled with the ability of mastitis causing bacteria to develop resistance to antimicrobials makes the control of mastitis very difficult. Therefore, the development of an alternative preventive tool such as a vaccine, which can overcome these limitations, has been a crucial focus of current research to decrease not only the incidence of mastitis but also the use of antimicrobials in dairy cattle farms. Most vaccination strategies against mastitis have focused on the enhancement of humoral immunity. Development of vaccines that induce an effective cellular

immune response in the mammary gland has not been well investigated. The ability to induce cellular immunity, especially neutrophil activation and recruitment into the mammary gland, is one of the key strategies in the control of mastitis, but the magnitude and duration of increased cellular recruitment into the mammary gland leads to a high number of somatic cells and poor-quality milk. So, effective balanced humoral and cellular immunity that clear intramammary infection in a short period of time is required. Several vaccine studies were conducted over the years under controlled experimental and field trials. The major bacterial bovine mastitis pathogens that have been targeted for vaccine development are *S. aureus*, *S. uberis,* and *E. coli* [119]. Most of these experimental and some commercial vaccines are Bacterins which are inactivated whole organism, and some vaccines contained subunits of the organism such as surface proteins [100], toxins, or polysaccharides.

2.2 Vaccine trials against *Staphylococcus aureus* mastitis

Staphylococcus aureus is one of the most common contagious mastitis pathogens, with an estimated incidence rate ranging from 43–74% [25, 38, 56, 120, 121]. *Staphylococcus chromogenes* is another increasingly reported coagulase-negative *Staphylococcus* species with an estimated quarter incidence rate of 42.7% characterized by high somatic cell counts [122–128]. In a study on conventional and organic Canadian dairy farms, coagulase-negative *Staphylococcus species* were found in 20% of the clinical samples [129]. Recently, mastitis caused by coagulase-negative *Staphylococcus species* increasingly became more problematic in dairy herds [125, 127, 130, 131].

Several staphylococcal vaccine efficacy trials showed that vaccination with Bacterin vaccines induced increased antibody titers in the serum and milk that are associated with partial protection [75–77, 80, 132–134] or no protection at all [78, 79, 81]. However, effective intramammary immune mechanisms against staphylococcal mastitis is still poorly understood. None of the commercially available Bacterin vaccines protects new intramammary infection [75, 77, 80, 81]. Dependence on antibiotics for the prevention and treatment of mastitis is not sustainable because of limited success [46, 47] and the emergence of antimicrobial-resistant bacteria that are major threat to human and animal health [72–74].

Despite several mastitis vaccine trials conducted against *S. aureus* mastitis [75, 77, 80, 88, 89, 91, 93–95, 97–99, 133] all field trials have either been unsuccessful or had limited success. There are two commercial vaccines for *Staphylococcus aureus* mastitis on the market, Lysigin® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) in the United States and Startvac® (Hipra S.A, Girona, Spain) in Europe and Canada [78]. None of these vaccines confer protection under field trials as well as under controlled experimental studies [75, 77, 80, 81]. Several field trials and controlled experimental studies have been conducted testing the efficacy of Lysigin® and Startvac®' and results from those studies have shown some interesting results, namely a reduced incidence, severity, and duration of mastitis in vaccinated cows compared to non-vaccinated control cows [75–77]. Contrary to these observations, other studies failed to find an effect on improving udder health or showed no difference between vaccinated and non-vaccinated control cows [78, 79]. None of these Bacterin-based vaccines prevents new *S. aureus* IMI [75, 77, 80, 81]. Differences found in these studies are mainly due to methodological differences (vaccination schedule, route of vaccination, challenge model, herd size, time of lactation, etc.) in testing the efficacy of these vaccines. It is critically important to have a good infection model that mimics natural infection and a model that has 100% efficacy in causing infection. Without a good challenge model, the results from vaccine efficacy will be inaccurate.

The Startvac® (Hipra, Girona, Spain) is the commercially available vaccine in Europe and is a polyvalent vaccine that contains *E. coli* J5 and *S. aureus* strain SP140 [119]. In a field trial, Freick et al. [78] compared the efficacy of Startvac® with Bestvac® (IDT, Dessau-Rosslau, Germany) another herd-specific autologous commercial vaccine in a dairy herd with a high prevalence of *S. aureus* and found that the herd prevalence of *S. aureus* mastitis was lower in the Startvac® and Bestvac® vaccinated cows compared to the control cows. However, there were no other differences in terms of improvement of udder health. These authors [78] concluded that vaccination with Startvac® and Bestvac® did not improve udder health. In another field efficacy study on Startvac® in the UK, Bradley et al. [75] found that Startvac® vaccinated cows had clinical mastitis with reduced severity and higher milk production compared to non-vaccinated control cows [75].

Similarly, Schukken et al. [77] evaluated effect of Startvac® on the development of new IMI and the duration of infections caused by *S. aureus* and CNS. These authors [77] found that vaccinated cows had decreased incidence rate and a shorter duration of *S. aureus* and CNS mastitis. Piepers et al. [76], also tested the efficacy of Startvac® through vaccination and subsequent challenge with a heterologous killed *S. aureus* strain and found that the inflammatory response in the vaccinated cows was less severe compared to the control cows. These authors [76] suggested that Startvac® elicited a strong Th2 immune response against *S. aureus* in vaccinated cows and was more effective at clearing bacteria compared to the control cows. Contrary to these observations, Landin et al. [135], evaluated the effects of Startvac® on milk production, udder health, and survival on two Swedish dairy herds with *S. aureus* mastitis problems and found no significant differences between the Startvac® vaccinated and non-vaccinated control cows on the health parameters they evaluated.

An experimental *S. aureus* vaccine made up of a combination of plasmids encoding fibronectin-binding motifs of fibronectin-binding protein (FnBP) and clumping factor A (ClfA), and plasmid encoding bovine granulocyte-macrophage-colony stimulatory factor, was used as a vaccine with a subsequent challenge with bacteria to test its protective effects [95]. These authors (Shkreta et al. 2004) found that their experimental vaccine-induced immune responses in the heifers that were partially protective upon experimental challenge [95]. Another controlled experimental vaccine efficacy study was conducted on the slime associated antigenic complex (SAAC) which is an extracellular component of *Staphylococcus aureus,* as vaccine antigen in which one group of cows were vaccinated with a vaccine containing a low amount of SAAC and another group with a high amount of SAAC and the unvaccinated group served as a control [136]. Upon intramammary infusion (challenge) with *S. aureus*, no difference in the occurrence of mastitis among all three groups despite the fact that the vaccine with high SAAC content induced higher production of antibodies compared to the vaccine with a low amount of SAAC [136]. Similarly, Pellegrino et al. [137], vaccinated dairy cows with an avirulent mutant strain of *S. aureus* and subsequently challenged with *S. aureus* 20 days after the second vaccination which resulted in no significant differences in the number of somatic cell count (SCC) or number of bacteria shedding through milk despite increased IgG antibody titer in the vaccinated cows compared to the control cows.

Some of the constraints affecting the successful development of effective mastitis vaccines are strain variation, the presence of exopolysaccharide (capsule, slime, biofilm) layer in most pathogenic strains of bacteria (*Staph. aureus*, *Strep. uberis*) which does not allow recognition of antibody-coated bacteria by phagocytic cells, dilution of immune effectors by milk [138, 139], the interaction between milk components and immune effectors [140] that reduce their effectiveness, and the ability

of most mastitis-causing bacteria to attach and internalize into mammary epithelial cells. Furthermore, evaluation of mastitis vaccines is complicated by the absence of uniform challenge study models, and lack of uniform route(s) of vaccination, time of vaccination, adjuvants, and challenge dose. There is an increasing need for development of better vaccines that overcome these problems. Most mastitis vaccines are killed whole bacterial cells (Bacterin) vaccines [75, 77, 80, 88, 89, 91–95, 97–99] that are difficult to improve because of difficulty to specifically identify an immunogenic component that induced partial or some protective effect. In this regard, some of the current efforts to use a mixture of purified surface proteins as vaccine antigens [100] to induce immunity than killed whole bacterial cells (Bacterin) is encouraging. A better understanding of natural and acquired immunological defenses of the mammary gland coupled with detailed knowledge of the pathogenesis of each mammary pathogen should lead to the development of improved methods of reducing the incidence of mastitis in dairy cows.

2.3 Vaccine trials against *Streptococcus uberis*

S. uberis is ubiquitous in the cow's environment accounting for a significant number of mastitis cases. It is found on-farm in water, soil, plant material, bedding, flies, hay, and feces [141]. As such, *S. uberis* is remarkably adaptable, affecting lactating and dry cows, heifers, and multiparous cows, causing clinical or subclinical mastitis, and even being responsible for persistent colonization without an elevation in the somatic cell count [142, 143]. It has been described as an environmental pathogen [108, 144–146] with potential as a contagious pathogen [142, 143, 147]. *S. uberis* has ability to persist within the mammary gland which lead to chronic mastitis that is difficult to treat [148]. Coliform bacteria are a major cause of clinical mastitis [149, 150]. A vaccine that prevents *S. uberis* mastitis is not available, control measures are limited to the implementation of good management practices. Recently vaccine efficacy trial with extract of biofilm-forming strains of *S. uberis* (UBAC®) (Hipra, Amir, Spain), was reported to reduce clinical severity [84]. It is not clear what kind of adative immunity is induced by UBAC® *S. uberis* vaccine [84] and it only conferred partial reduction in clinical severity of mastitis. Multiple intramammary vaccinations of dairy cows with killed *S. uberis* cells resulted in the complete protection from experimental infection with the homologous strain [103]. Similarly, subcutaneous vaccination of dairy cows with live *S. uberis* followed by intramammary booster vaccination with *S. uberis* cell surface extract protected against challenge with the homologous strain but was less effective against a heterologous strain [106]. Vaccination with *S. uberis* glyceraldehyde phosphate dehydrogenase C (GapC) protein induced immune responses that confer a significant reduction in inflammation post-challenge [107, 151]. The pauA is a plasminogen activator and also binds active protease plasmin [152]. It has been postulated that acquisition of plasmin may promote invasion [153]. Vaccination of dairy cows with PauA induced increased antibody titers that conferred reduction in clinical severity [154]. However, mutation of pauA did not alter ability to grow in milk or to infect lactating bovine mammary glands. It appears that the ability to activate plasminogen through PauA does not play a major role in pathogenesis of *S. uberis* to either grow in milk or infect bovine mammary gland [155].

S. uberis expresses several surface associated proteins such as *S. uberis* adhesion molecule (SUAM) and extracellular matrix binding proteins, which allow it to adhere to and internalize into mammary epithelial cells, successfully inducing IMI [156–158]. The *S. uberis* adhesion molecule (SUAM) plays a central role in the adherence of *S. uberis* to mammary epithelial cells [159–162]. Vaccination of dairy cows with SUAM induced strong immune reposes in vaccinated cows [163].

The immune serum from SUAM vaccinated cows prevented *S. uberis* adhesion and invasion into mammary epithelial cells *in vitro* [163]. In vivo infusion of mammary quarters of dairy cows with *S. uberis* pre-incubated with immune-serum from SUAM vaccinated cows reduced clinical severity [164]. The SUAM gene deletion mutant strain is less pathogenic to mammary epithelial cells [165] and to dairy cows [159]. Controlled experimental efficacy studies using SUAM as vaccine antigen to control *S. uberis* mastitis showed that SUAM is immunogenic but the induced immunity was not protective. Following experimental IMI challenge with *S. uberis*, clinical signs emerged at about 48 h, along with increased levels of inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 in milk at 60 h post-infection [166]. Adaptive immune response cytokines such as IFN-γ promotes a cell-mediated immune response by enhancing functions such as macrophage bacterial killing, antigen presentation, cytotoxic T cell activation, and increased IgG2 levels. The IL-4 expression is associated with the antibody-mediated response, which is generally linked to parasite resistance, allergic reactions, and increased levels of IgG1 [167, 168]. This partial protection by the SUSP vaccine can be improved with dose optimization, appropriate adjuvant, route of injection, and timing of vaccination.

In conclusion, it is clear that Bacterin vaccines have some protective effect against homologous strains, and single surface protein is not effective. Therefore; use of multiple surface proteins may induce better immunity that prevents clinical disease and production losses.

2.4 Vaccine trials against *E. coli* mastitis

Coliform bacteria are a major cause of clinical mastitis [149, 150]. Coliforms include the genera *Escherichia, Klebsiella*, and *Enterobacter* [169]. Eighty to ninety percent of coliform intramammary infection (IMI) develop clinical mastitis, and 10% will be severe and could lead to death [150]. *E. coli* usually infects the mammary glands during the dry period and progresses to inflammation and clinical mastitis during the early lactation with local and sometimes severe systemic clinical manifestations.

Iron is an essential nutrient for the growth of coliforms [170]. However, free iron is limited in the bovine milk because most iron is bound to citrate and to a lesser extent to lactoferrin, transferrin, xanthine oxidase, and some caseins [171] and maintained at concentrations below levels required to support coliform growth [172]. To overcome this limited iron source, coliforms express multiple iron transport systems [173], which include synthesis of siderophores (e.g., enterobactin, aerobactin, ferrichrome) that bind iron with high affinity [174], the expression of iron-regulated outer membrane proteins (IROMP) that binds to ferric siderophore complexes to transport into bacterial cell and enzymes to utlize the chelated iron [173]. The siderophores are too large (600 to 1200 Da) to pass through the porin channels of the bacterial outer membrane [175, 176]. Therefore, the siderophores require specific IROMP to enable their passage across the bacterial outer membrane into the periplasm [177, 178]. The enterobactin is a siderophore with the highest affinity for iron, and it is produced by most pathogenic *E. coli* and *Klebsiella* spp. [179–181]. The aerobactin is another siderophore that was detected in only 12% of *E. coli* isolated from mastitis cases [182]. Enterobactin is the primary siderophore of *Escherichia coli* and many other Gram-negative bacteria [183]. Coliform bacteria also developed the ability to take up iron directly from naturally occurring organic iron-binding acids, including citrate [173, 184]. The citrate iron uptake system requires ferric dicitrate for induction [184]. More than 0.1 mM citrate is required for the induction of this system under iron-restricted conditions [184]. The ferric

citrate transport system is the major iron acquisition system utilized by *E. coli* [173] to grow in the mammary gland. The mammary gland is an iron-restricted environment, and bovine milk contains approximately 7 mM citrate [185] which is ideal for induction of ferric citrate transport sytem.

Ferric enterobactin receptor, FepA, is an 81 kDa iron regulated outer membrane protein (IROMP), that binds to ferric enterobactin complex to transoport iron into the bacterial cell [186, 187]. Vaccination of dairy cows with FepA elicited an increased immunological response in serum and milk [188]. Bovine IgG directed against FepA inhibited the growth of coliform bacteria by interfering with the binding of the ferric enterobactin complex [189]. Ferric citrate receptor, FecA, is an 80.5-kDa IROMP that is responsible for the binding of ferric dicitrate [190] and transport into the bacterial cell. The FecA, is conserved among coliforms isolated from cases of naturally occurring mastitis [191]. The iron-regulated outer membrane proteins, FepA and FecA are ideal vaccine candidates because they are surface exposed, antigenic, and conserved among isolates from IMI.

Immunization of dairy cows with FepA induced significantly higher serum and whey anti-FepA IgG titers than in *E. coli* J5 vaccinates [188]. Results of *in vitro* growth inhibition studies demonstrated that antibody specific for blocking ferric enterobactin-binding site (anti-FepA) inhibited the growth of *E. coli* in vitro [192]. Cows immunized with FecA did have increased antibody titers in serum and mammary secretions compared with *E. coli* J5 immunization and unimmunized control cows [193, 194]. Antibody purified from colostrum inhibited the growth of *E. coli* when cultured in synthetic media modified to induce FecA expression [193]. Despite their antigenicity, the use of either FepA or FecA alone were not sufficient to prevent mastitis. The FecA and FepA are antigenically distinct [191].

Intramammary infection with *E. coli* induced expression and release of pro-inflammatory cytokines such as TNF-alpha, IL-8, IL-6, and IL-1 [195, 196]. Recently it has been shown with mouse mastitis models that IL-17A and Th17 cells are instrumental in the defense against *E. coli* IMI [197, 198]. However, the role of IL-17 in bovine *E. coli* mastitis is not well defined. Results of a recent vaccine efficacy study against *E. coli* mastitis suggested that cell-mediated immune response has more protective effect than humoral response [199]. The cytokine signaling pathways that lead to efficient bacterial clearance is not clearly defined.

The four coliform vaccines which include 1) J-5 Bacterin® (Zoetis, Kalamazoo, MI) [82, 83], 2) Mastiguard®, 3) J Vac® (Merial-Boehringer Ingelheim vet medical, Inc., Duluth, GA) and 4) Endovac-bovi® (IMMVAC) (Endovac Animal Health, Columbia, MO). Of the four coliform vaccines, J-5 Bacterin® and Mastiguard® are believed to have the same component, which is J5 Bacterin. The J Vac® is a different bacterin-toxoid. The Endovac-Bovi® contains mutant *Salmonella typhimurium* bacterin toxoid. All coliform mastitis vaccine formulations use gram-negative core antigens to produce non-specific immunity directed against endotoxin (LPS) [119]. The efficacy of these vaccines has been demonstrated in both experimental challenge trials and field trials in commercial dairy herds [109–111]. The principle of these bacterins is based upon their ability to stimulate the production of antibodies directed against common core antigens that gram-negative bacteria share. These vaccines are considered efficacious even though the rate of intramammary infection is not significantly reduced in vaccinated animals because they significantly reduce the clinical effects of the infection. Experimental challenge studies have demonstrated that J5 vaccines are able to reduce bacterial counts in milk and result in fewer clinical symptoms [109]. Vaccinated cows may become infected with gram-negative mastitis pathogens at the same rate as control animals but have a lower rate of development of clinical mastitis [111], reduced the duration of IMI [110], reduced production, culling, and death losses [200, 201].

There is an increasing need for the development of effective vaccines against major bacterial bovine mastitis pathogens. A better understanding of the natural and acquired immunological defenses of the mammary gland coupled with detailed knowledge of the pathogenesis of each mammary pathogen should lead to the development of improved methods of reducing the incidence of mastitis in dairy cows (**Table 1**).

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