

Modulation of the bovine mammary gland

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SUMMARY

This chapter provides an overview of how bovine mammary gland immunity can be modulated to resist or eliminate intramammary infection (IMI). Although the mammary gland of the cow evolved to nourish the newborn calf and support its diet during the first year of life, dairy cattle genetics and nutrition have been dramatically manipulated to allow production of copious amounts of milk for human consumption. The stresses associated with the high-producing Holstein cow, however, have rendered her more susceptible to diseases such as mastitis. Nature has provided her with innate anatomical defenses to repel mastitis-causing bacteria, and once bacteria enter the mammary gland, various cellular and molecular defenses play a role in removing the invading pathogens. In addition, the cow adapts to specific bacteria by eliciting antibodies and immune cells that function to destroy these pathogens. Research has demonstrated that these innate and adaptive immune mechanisms can be modulated to various degrees. Vaccination, dietary supplementation, and immune stimulation have been used experimentally as well as commercially to enhance the composition, magnitude, and efficiency of the bovine immune system with varying degrees of success to prevent the establish of IMI.

INTRODUCTION

Mastitis is an inflammation of the mammary gland usually caused by bacteria, resulting in reduced milk yield and quality. This disease continues to be the most devastating disease affecting dairy cows, and the associated losses continue to present a serious economic burden to producers. Current control practices are based on proper milking time hygiene, reduced exposure to environmental pathogens, antibiotic treatment of clinical cases, and nonlactating cow therapy. Although these practices have reduced the occurrence of IMI, recent estimates suggest that mastitis affects one-third of all cows in an average of 1.5 quarters. Thus, newer and more advanced approaches for control are needed if further progress is to be made. Fortunately, the cow has a natural ability to resist IMI, and mechanisms to enhance or “modulate” her immune system are needed to assist the animal in defending itself against bacterial invasion. Such defenses of the mammary gland against mastitis-causing pathogens are mediated by several anatomical, cellular, and soluble protective factors. Once bacteria successfully penetrate the teat orifice, it is the efficiency of these defense mechanisms that determines the resistance or susceptibility of the gland to new IMI.

There are certain times of the lactation cycle when mammary gland defenses fail to operate properly and cows become susceptible to mastitis. Strategies aimed at enhancing immune systems of the mammary gland during these periods of immune dysfunction would greatly affect the ability of the cow to resist infection. This chapter reviews the immune system of the bovine mammary gland, and how it may be controlled or modulated to better manage mastitis in dairy cattle.

ANATOMY AND PHYSIOLOGY OF THE MAMMARY GLAND

A basic knowledge of mammary gland anatomy, physiology, and milk secretion facilitates an understanding of processes associated with microbial invasion, and how the gland can be modulated to prevent establishment of new cases of mastitis and eliminate existing IMI.

Basic Mammary Structure

The udder is a modified exocrine skin gland composed of 4 separate functional mammary glands or quarters: 2 fore and 2 rear quarters, each drained by a separate teat. The interior of each quarter from ventral to dorsal regions is composed of a teat cistern, gland cistern, milk ducts, and glandular tissue (Figure 1). The glandular

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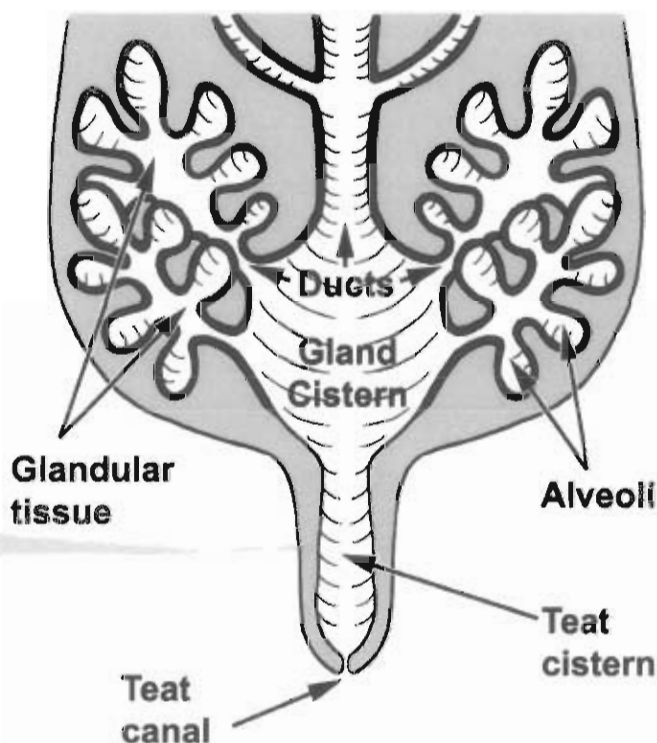


Figure 1. Structure of a bovine mammary quarter illustrating the teat canal, teat and gland cisterns, milk ducts, and glandular tissue composed of millions of microscopic alveoli.

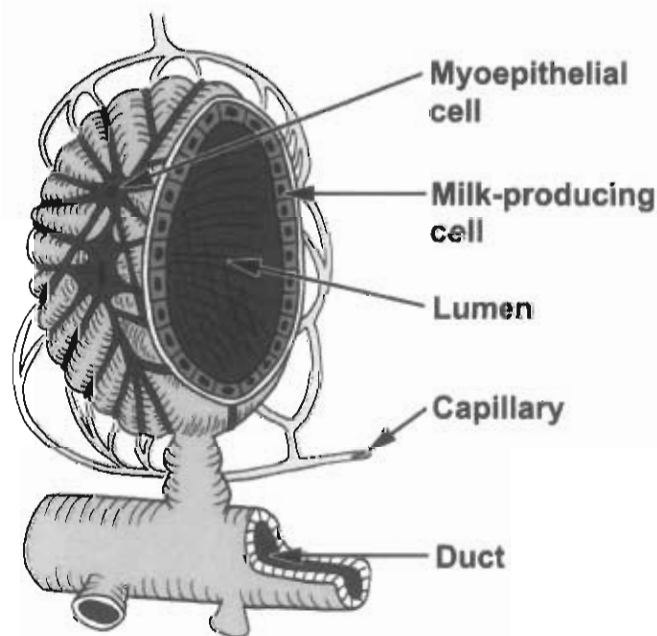


Figure 2. Anatomy of an alveolus lined by milk-producing cells that encloses a lumen. Capillaries supply nutrients to milk-producing cells, and surrounding myoepithelial cells contract in response to the let-down response and oxytocin release, expelling milk through a duct.

or secretory tissues contain millions of microscopic globe-like structures called alveoli (Figure 2), each of which is lined with milk-producing epithelial cells and surrounded by myoepithelial cells that contract and squeeze milk from the alveolus during milking.

Milk Synthesis, Secretion, and Removal

Blood vessels supply nutrients to each alveolus, where epithelial cells convert them into protein, fat, and lactose. These synthesized milk components are stored in the cells along with minerals and vitamins. During the secretory process, all components are released from epithelial cells and are stored in the alveolar lumina, ducts, and cisterns as milk between milkings. The accumulated product is removed from alveolar lumina through milk ducts in conjunction with the milk ejection reflex, or milk let-down, during the milking process. This reflex is caused by the contraction of myoepithelial cells surrounding the alveoli due to oxytocin, which is released from the posterior pituitary at the base of the brain in response to the stimuli that the cow receives associated with milking. Oxytocin binds to surface receptors on the myoepithelial cells surrounding the alveoli, causing them to contract and squeeze or eject milk from the alveolar lumina into their small draining ducts. It is important to note that alveoli, myoepithelial cells, and associated blood vessels are very delicate and can be damaged as a result of infection or inflammation processes. The small ducts that drain the milk-producing alveoli converge into larger ducts that drain into the gland and teat cisterns near the ventral portion of each quarter. The teat cistern terminates distally at the teat canal, the structure through which milk is removed from the mammary quarter.

Protective Tissues of the Teat End

The teat canal is also the structure through which microorganisms enter the mammary gland to establish an IMI (Figure 3) and measures 5 to 13 mm in length. Its diameter ranges from 0.4 mm at the distal end to 1.63 mm at the proximal end, where it terminates at the teat cistern in the region known as Fürstenberg's rosette. The teat duct lumen is occluded with keratin, which is formed by the continual sloughing of the epithelial cells lining the duct. Keratin seals the teat duct between milkings and serves as a physical barrier to bacterial invasion, particularly during the nonlactating period when the canal becomes completely blocked with this substance. The fatty acids and proteins contained in keratin are also antibacterial against some species of microorganisms; for example, gram-positive

bacteria are more susceptible than gram-negatives to the bactericidal effects of fatty acids.

The teat canal is surrounded by circular and longitudinal bundles of smooth muscle fibers, which create a sphincter that functions to maintain tight closure of the duct between milkings. In the contracted state, the muscle fibers prevent leakage of milk from the milk-engorged gland by compressing the teat duct keratin sufficiently to prevent bacterial penetration. This teat sphincter relaxes during milking to accommodate the flow of milk.

DEVELOPMENT OF MASTITIS

Invasion by Bacteria

Intramammary infection occurs after microorganisms, such as bacteria, pass through the teat duct, multiply in the teat and gland cisterns, and progress upward to the milk-producing tissues, eventually causing inflammation. These microorganisms breach the teat duct in several ways during the milking process as well as during the intermilking period. For example, during machine milking, the lower teat skin surfaces are exposed to contaminating bacteria remaining in teat cup liners from cows that were previously milked. Immediately after the teat cups are removed, bacteria remaining on the distal teat surfaces pool and concentrate at the teat opening, remaining in an opportunistic position to cause mastitis. The teat duct remains dilated (open) for an

hour before the teat sphincter muscle fully contracts. While dilated, the teat duct acts like a capillary tube, drawing residual milk with contaminating bacteria into the ductal lumen and up into the interior of the mammary gland. This is why postmilking teat antisepsis is so important—the germicide kills mastitis-causing bacteria remaining on the teat end before they enter the teat duct. Moreover, because the teat duct remains dilated after milking, cows should be offered feed and should remain standing during this time, rather than lying down in mud, manure, or bedding contaminated with environmental bacteria. Teats are continuously exposed to environmental mastitis bacteria between milkings, so the pathogen load increases on teat skin throughout the intermilking period, which increases the chances of developing a new IMI. To reduce bacterial numbers on teat skin and subsequent IMI, predipping of teats before milking is practiced to kill and remove most of these bacteria before milking units are attached to the mammary gland.

Inflammatory Response

Bacteria multiplying within the cisternal spaces, ducts, and milk-producing alveoli, as well as their metabolic byproducts and toxins, are recognized as foreign by the cow's immune system. The cellular immune system includes white blood cells or leukocytes composed of neutrophils, macrophages, and lymphocytes. The presence of bacteria signals the leukocytes already present in milk (mainly macrophages and neutrophils), also known as somatic cells, to engulf and kill the pathogens, and also serves as a chemoattractant to recruit more leukocytes from the blood stream (mainly neutrophils) into the infected quarter to attack the bacteria, subsequently increasing the somatic cell count (SCC). This influx of leukocytes from the blood constitutes the inflammatory response. If the pre-existing leukocyte population and those recruited from the blood are successful in killing all invading bacteria, then the infection is resolved, leukocyte recruitment from the blood slows, and the SCC in the affected quarter returns to preinfection levels. However, if bacteria survive the initial influx of leukocytes, then chronic inflammation continues as more leukocytes are recruited, and a constant battle ensues between bacteria and neutrophils as the infection becomes established (Ryman et al., 2015).

Establishment of Infection

Bacteria successfully cause IMI when leukocytes and other soluble factors can no longer prevent their multiplication. The adherence of some bacteria to tis-

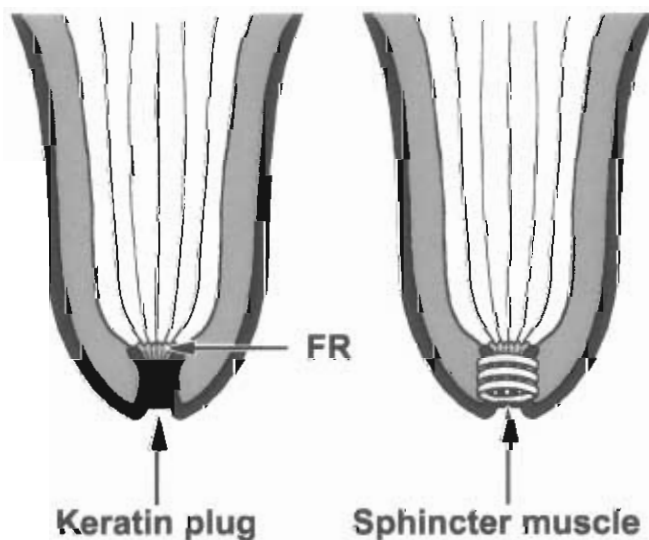


Figure 3. Structure of the teat illustrating the junction of the teat canal and teat cistern at Fürstenberg's rosette (FR). The teat canal is occluded with a keratin plug and surrounded by a sphincter muscle.

sues lining the interior of the mammary gland enhances establishment of infection, especially during lactation when the contents of the mammary gland are periodically flushed during each milking. *Streptococcus agalactiae* and *Staphylococcus aureus* adhere well to tissues lining the milk collecting spaces. *Escherichia coli* do not adhere but multiply rapidly in quarters with low SCC.

During the initial stages of bacterial infection, the tissues that line the large milk-collecting ducts and cisterns suffer small areas of damage. Then, the microorganisms enter small ducts and alveolar areas of the lower portions of the gland and continue to produce toxins and other tissue irritants. This results in the recruitment of additional leukocytes, mainly neutrophils, into the affected tissue area to destroy invading pathogens, but their intense migration into the infected quarter is detrimental to milk-producing tissues.

Mammary Tissue Response

After neutrophils cross blood vessels and move through mammary tissues toward infected tissue sites, they accumulate around the alveoli, ducts, and cisterns before entering the milk. Neutrophils move across the tissues lining these areas by squeezing between cells and passing through damaged regions. During migration, neutrophils release antibacterial chemicals that cause local destruction of milk-producing cells. Once neutrophils enter milk, they are dependent, in part, on random collisions to make contact with bacteria for engulfment, requiring high concentrations of neutrophils to be effective. The ratio of SCC to bacteria may be 10,000:1 in case of *Staph. aureus* mastitis.

The presence of bacteria, toxins, leukocytes, and other byproducts of inflammation in the affected area may cause the remaining healthy milk-producing cells to revert to a resting state and become nonproductive. In addition, tissue debris, leukocytes, and bacteria form clots that occlude the ducts that drain areas of secretory tissue. If bacteria are eliminated, inflammation subsides, occluded ducts are opened, and milk composition returns to normal in several days.

The mechanisms used by an injured quarter to redevelop secretory ability are largely unknown, but damaged milk-producing cells may repair themselves, resting cells may become active again, and healthy uninfected tissue areas may increase synthetic activity and compensate for nonproductive tissues, resulting in return of milk production. However, if infection persists and ducts remain occluded, milk accumulates in alveoli, exerting pressure on milk-producing cells. These cells revert to a resting state or may be destroyed, depending on the severity of infection. After destruction, alveolar

structures are permanently replaced by scar tissue, leading to reduced milk yield in the current as well as in subsequent lactations. See Akers and Nickerson (2011) for further information on mastitis and its impact on mammary structure and function.

MAMMARY IMMUNITY

The ability of cows to resist the establishment of new IMI depends on the efficiency of the mammary immune system. Immune components consist of a complex system of tissues, cells, and molecules that work together to defend against mastitis-causing pathogens and can be broadly classified as innate and adaptive immunity. Innate immunity includes resistance mechanisms that can be triggered within seconds to minutes of microbial challenge and can be localized within affected tissues or activated and quickly recruited from the bloodstream to the site of infection by numerous stimuli. The physical barrier of the teat end and a variety of soluble and cellular mechanisms facilitate the innate responses of the mammary gland. Conversely, the adaptive immune system can take several days to become fully activated, but it is capable of a more specific response to select bacterial factors that cause disease. Immunoglobulins, macrophages, dendritic cells, and several lymphoid populations mediate recognition of specific pathogenic factors. Because of the "memory" of certain lymphocytes, adaptive immune responses can be amplified by repeated exposure to a particular pathogen (*Staph. aureus*, for example). The adaptive immune response is the foundation of the vaccine strategies discussed below. In the mammary gland, both innate and adaptive protective factors are highly interactive and coordinated to provide optimal protection from mastitis-causing pathogens. Indeed, both systems are needed to not only prevent bacterial invasion of the mammary gland but also eliminate existing IMI and restore mammary tissues to normal function (Aitken et al., 2011).

Innate Immunity

Innate immunity is the initial line of defense when the mammary gland is first exposed to mastitis-causing pathogens, its hallmark being inflammation. In response to infection, the mammary gland can develop either a subclinical or acute inflammatory response depending on the invading pathogen. When innate immunity functions properly, however, pathogens are readily eliminated shortly after the initial invasion, and the inflammatory response is quickly resolved. Innate defenses consist of anatomical barriers provided by the teat, tissue and milk leukocytes, cellular receptors

located on different mammary cell populations, and a variety of noncellular molecular factors (Table 1).

Anatomical Defenses

Pathogens must gain entrance to the mammary gland to cause mastitis, so the teat end is considered the first line of localized defense against invading bacteria. The teat end impedes bacterial penetration by the aforementioned keratin plug and sphincter muscle.

Innate Cellular Defenses

The antibacterial activities of the mammary gland microenvironment can serve to inhibit the establishment of disease if bacteria are successful at overcoming the local defenses of the teat end. Both resident and newly recruited mammary gland leukocytes play an essential role during the early stages of pathogenesis. In healthy uninfected quarters, lymphocytes and macrophages are the predominant leukocyte types with relatively low numbers of neutrophils. The SCC in healthy glands are often <10,000/mL but can increase to >1,000,000/mL within just a few hours of bacterial invasion, and the major leukocyte types during inflammation are neutrophils. The promptness and magnitude of neutrophil migration into milk is considered a major determining factor for the establishment of new IMI (Paape et al., 2000).

An important innate defense mechanism facilitated by leukocytes is the ingestion and killing of bacteria, a process referred to as phagocytosis (Figure 4), carried out primarily by neutrophils and macrophages, although dendritic cells are also capable of phagocytosis. Phagocytic leukocytes engulf bacteria and encapsulate them within a cytoplasmic vacuole called a phagolysosome. Bacteria are destroyed within these

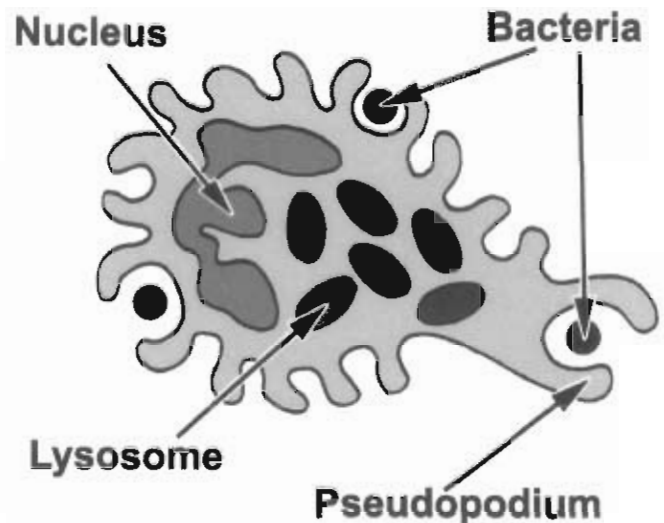


Figure 4. Illustration of a neutrophil using its pseudopodium to phagocytose bacteria, which will be internalized and killed by lysosomes that contain antibacterial substances.

phagolysosomes using high concentrations of toxic oxygen radicals and digestive enzymes and are then exocytosed from the phagocyte. Neutrophils and macrophages ingest fat, casein, and other milk components that render them less effective at phagocytosing bacteria. The phagocytic and bactericidal activities of these cells are especially diminished during the periparturient period and are thought to be an underlying cause of increased susceptibility to mastitis during this time. However, phagocytic and bactericidal capabilities can be increased substantially in the presence of opsonic antibody for specific pathogens (Rainard and Riollet, 2006), which can be enhanced by immunization.

Neutrophil extracellular trap (NET) formation is an additional innate antimicrobial defense mechanism.

Table 1. Summary of innate defense mechanisms

Type	Factor	Mechanism ¹
Anatomical	Teat end	Sphincter muscles provide a mechanical barrier to hinder bacterial entry Keratin impedes bacterial growth and entry into the gland Fürstenburg's rosette is densely populated with leukocytes; defense not yet defined
Cellular	Neutrophils Macrophages Dendritic cells Natural killer cells Epithelial cells	Phagocytosis and killing of bacteria; secretes antibacterial factors; forms NETs Phagocytosis and killing of bacteria; secretes cytokines and oxylipids Phagocytosis and cytokine production Non-immune lymphocytes that secrete antibacterial proteins upon activation Expression of TLR that sense the presence of bacteria in the mammary gland
Soluble	Cytokines Complement Lactoferrin	Proinflammatory factors that enhance the magnitude and activity of leukocytes Bacteriolytic and facilitates phagocytosis Bacteriostatic properties due to iron sequestration

¹NET = neutrophil extracellular traps; TLR = toll-like receptors.

Pathogen stimulation of neutrophils triggers the release of nuclear material (DNA, histones) as well as granular proteins and extracellular fibers that trap and kill microbes. Formation of NETs may be of particular importance to the mammary gland due to the ability to function in the presence of milk in contrast to other neutrophil functions that are suppressed in that environment (Lippolis et al., 2006).

Natural killer (NK) cells are a subpopulation of lymphocytes that also may play an important role in innate immunity. They are large, granular lymphocytes that have potent cytotoxic and bactericidal activity against both gram-positive and gram-negative bacteria. Indeed, NK cells isolated from bovine mammary tissue exhibit potent bactericidal activity against *Staph. aureus* and *E. coli*, and therefore could be an important aspect of innate defense in preventing mastitis, especially during the periparturient period (Aitken et al., 2011); however, their role has not been well established in vivo.

The ability to sense the presence of bacteria within the mammary gland is an essential component of early host defense, and any cell capable of facilitating this recognition can effectively stimulate the innate immune response. Both immune and nonimmune cells in the mammary gland possess receptors that can interact with bacterial components to send out alarm signals that microbes have invaded the cow's tissues. An example of these receptors are the toll-like receptors (TLR) expressed on leukocytes, endothelial cells, epithelial cells, fibroblasts, and adipocytes. Following pathogen recognition and binding, TLRs transmit signals to initiate innate immune responses through expression of cytokines and other inflammatory mediators.

Innate Molecular Factors

Neutrophils are the predominant leukocyte type during the initial inflammatory response, and they kill bacteria by either oxygen-dependent or protein-mediated mechanisms. The oxygen-dependent system occurs during the ingestion process, in which there is a major burst of oxidative metabolism, resulting in the production of reactive oxygen intermediates—a metabolic process known as respiratory burst. These microbicidal oxidizing enzymes are located within phagolysosomes and oxidize bacterial membrane lipids, causing their destruction. The primary enzymes involved in catalyzing the oxidation process are myeloperoxidase and superoxide dismutase (Paape et al., 2000).

In addition, bacteria may become exposed to and destroyed by protein-mediated mechanisms, including lysozyme, a variety of cationic proteins, and lactoferrin, which are proteins stored within neutrophil lysosomes.

Lactoferrin (Lf) is among the best-characterized antimicrobial proteins, and concentrations in milk fluctuate during the lactation cycle, with the highest concentrations observed in the fully involuted gland. The ability of Lf to bind soluble iron in milk is the basis of its most important biological activities. First, it can act as a transport protein by moving the bound iron to a different area within the host. In addition, the iron-binding capability of Lf greatly reduces soluble ferric iron available to multiplying bacteria, preventing the production of dismutase, an enzyme produced by bacteria to counteract superoxide radicals generated by the host. Collectively, the iron-binding capacity of Lf results in a bacteriostatic effect, resulting in greatly reduced bacterial multiplication rates. Lactoferrin can also have direct bactericidal effects on certain mastitis-causing pathogens, and it plays a role in lymphocyte and macrophage function. However, the bacteriostatic and antibacterial properties of Lf are depressed in the presence of citrate, which chelates iron into a form that bacteria can utilize. Citrate levels in milk tend to be very low during involution, when the mammary gland is resistant to IMI, but increase substantially at calving, when susceptibility to IMI increases. Thus, a direct correlation exists between changes in citrate and Lf ratios in milk and susceptibility to new IMI (Aitken et al., 2011).

Several other soluble factors are associated with innate mammary gland defenses. Complement is a collection of proteins present in serum and milk, which can affect both innate and adaptive immunity. Many of the biological activities of complement are mediated through complement receptors located on a variety of cells, resulting in lysis of bacterial target cells. Gram-negative bacteria such as *E. coli* are especially sensitive to complement-mediated lysis. Complement also functions in concert with a specific antibody as an opsonin, which promotes bacterial phagocytosis and intracellular killing by neutrophils and macrophages. Concentrations of complement are highest in colostrum, inflamed glands, and during involution, but lowest during lactation; therefore, it may play a minor bactericidal role (Rainard and Riollet, 2006).

Cytokines are a heterogeneous group of low-molecular-weight glycoproteins secreted by immune and nonimmune cells, and these molecules are known to regulate all aspects of inflammation and immunity. Cytokines regulate the intensity and duration of the cow's response to IMI by regulating (enhancing or inhibiting) the activation, proliferation, and differentiation of cells involved in the immune response. Therefore, cytokines can influence both innate and adaptive immunity in the mammary gland, and include interleukins (IL), inter-

ferons (IFN), chemokines, colony-stimulating factors (CSF), and tumor necrosis factors (TNF).

Cytokines can indirectly influence the severity and duration of mastitis by regulating the promptness of the leukocyte migratory response and the efficiency of phagocytes at the site of infection (Bannerman, 2009). Many different cell populations in the mammary gland secrete cytokines. As an example, mammary epithelial cells and macrophages are among the first to become activated by bacterial toxins and other factors associated with bacterial colonization, and to start producing a variety of cytokines. Certain macrophage-derived cytokines, including TNF- α , IL1, IL6, and IL8, regulate the magnitude and duration of leukocyte infiltration into infected tissues. If neutrophils are able to migrate rapidly from the blood into the mammary gland and effectively eliminate bacteria, then the recruitment of leukocytes will decrease and SCC will return to preinfection levels. However, if bacteria persist, then the inflammatory response will continue to an acute or chronic state. Prolonged or excessive migration of leukocytes from the blood causes considerable damage to mammary parenchymal tissues, resulting in reduced milk production. The overexpression of TNF- α and IL1 during acute inflammation is also directly correlated with morbidity and mortality associated with coliform mastitis. One of the most potent activators of neutrophil and macrophage functions after their migration into the mammary gland is IFN- γ , which is produced by activated T lymphocytes (Ryman et al., 2015).

Another important group of immune signaling molecules are a family of potent lipid-derived mediators referred to as oxylipids, which are capable of regulating all aspects of the initiation and resolution of the inflammatory response. Whereas many immune and nonimmune cell populations are capable of producing oxylipids, macrophages and endothelial cells are a major source within most tissues. Oxylipid biosynthesis is initiated when macrophages or endothelial cells encounter bacterial factors or other inflammatory stimuli such as cytokines. Within minutes of exposure to these proinflammatory agonists, polyunsaturated fatty acids are liberated from cell membrane phospholipids. These fatty acid substrates are then oxidized nonenzymatically by reactive oxygen species (ROS) or through different enzymatic routes including cyclooxygenase, lipoxygenase, and cytochrome P450 pathways to produce a variety of oxylipids such as prostaglandins, thromboxanes, leukotrienes, and lipoxins. Depending on the timing and magnitude of expression, certain oxylipid profiles can either enhance or resolve the inflammatory response during mastitis (Sordillo, 2016).

ADAPTIVE IMMUNITY

Although adaptive immunity takes longer to develop following microbial exposure, it becomes increasingly important if pathogens are able to evade the innate defense system. Specific immune responses are elicited to particular antigenic challenges associated with bacterial pathogens. An amazing feature of the immune system is the ability of a host to recognize and respond to billions of unique antigens that they may encounter. If a host encounters the same antigen more than once, a heightened state of immune reactivity occurs because of immunological memory. Compared with the first exposure to a particular antigen, a memory response will be much faster and considerably stronger, will last longer, and be more effective in clearing the pathogen. It also is important that an inappropriate specific immune response does not occur against the host's own antigens. For this reason, the immune system is able to distinguish self from nonself and selectively react to only foreign antigens. Genetically diverse, membrane-bound proteins called major histocompatibility complex (MHC) molecules assist in this recognition. A specific immune response will only occur if antigens are combined with an MHC molecule on the surface of certain cells, a process referred to as antigen presentation.

Adaptive Cellular Defenses

Generation of effective specific immunity involves 2 types of cells, lymphocytes and antigen-presenting cells (Table 2). Lymphocytes recognize bacterial antigens through membrane receptors specific to the invading pathogen, and mediate the defining attributes of adaptive immunity including specificity, diversity, memory, and self/nonself recognition. The T and B cells are 2 distinct subsets of lymphocytes, which differ in function and protein products. The T cells can be further subdivided into $\alpha\beta$ T cells, which include CD4⁺ (T helper, Th) and CD8⁺ (T cytotoxic, Tc) cells, and $\gamma\delta$ T cells. The Th cells can be refined further based on functional groupings that include Th1, Th2, Th17, and regulatory T cells (Tregs). The Th cells produce cytokines in response to recognition of antigen-MHC complexes on antigen-presenting cells (B cells and macrophages). When activated, Th cells produce a variety of cytokines, which play an important role in activating both T and B cells, macrophages, neutrophils, and various other immune cells involved in innate and adaptive immune responses. For example, the cytokines IFN- γ and IL2 are thought to enhance some nonspecific cellular activities such as phagocytosis and intracellular killing.

Table 2. Summary of adaptive defense mechanisms

Type	Subpopulation	Function
Macrophages		Antigen presentation
Dendritic cells		Antigen presentation
T Lymphocytes	Th cells	Produce immunoregulatory cytokines following antigen recognition with major histocompatibility complex (MHC) molecules; memory cells following antigen recognition
	Tc cells	Lysis of damaged host cells when complexed with MHC molecules; produce cytokines that downregulate leukocyte functions
	$\gamma\delta$ T cells	Biological role in the mammary gland is speculative
B Lymphocytes	Mature B cell	Displays membrane bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions.
	Plasma cell	Terminally differentiated B cells that synthesize and secrete antibody against a specific antigen
Natural killer cells		Large granular lymphoid cells that synthesize and secrete antibacterial proteins following activation
Immunoglobulins	IgG ₁	Selectively transported into mammary secretions; opsonizes bacteria to enhance phagocytosis
	IgG ₂	Transported into secretions during neutrophil diapedesis; opsonizes bacteria to enhance phagocytosis
	IgA	May cause bacterial agglutination; prevents bacterial adhesion; neutralizes toxins
	IgM	Complement (C) fixation, opsonization agglutination, toxin neutralization, opsonic for neutrophils in presence of C

Cytotoxic T cells recognize and eliminate microbes via antigen presentation in conjunction with MHC molecules, and may act as scavengers that remove damaged mammary secretory cells. Similar to the Th cells, suppressor functions of Tc cells are thought to modulate the immune response by the cytokines that they produce. The Tc cells also have a potential role in defending mammary cells against intracellular infections and surface-exposed antigens. The suppressor functions of Tc cells can become activated in infected mammary glands or during the periparturient period, and may contribute to impaired local defenses under these circumstances.

The biological functions of $\gamma\delta$ T cells are primarily associated with the protection of epithelial surfaces. There are indications that $\gamma\delta$ T cells can mediate cytotoxicity with variable involvement of MHC. These cells also may play a role in infectious diseases and therefore provide an important line of defense against bacterial diseases. The fact that the percentages of these cells decrease significantly in the mammary gland during times of increased susceptibility to disease suggests that these lymphocytes may constitute an essential line of defense against mastitis-causing pathogens (Aitken et al., 2011).

The primary role of B cells is to produce antibodies or immunoglobulins against invading pathogens. Following pathogen recognition, B cells internalize, process, and present antigen in the context of MHC molecules to Th cells. The Th cells become activated and secrete certain cytokines including IL2, which in

turn induce proliferation and differentiation of the B cells into antibody-producing plasma cells or memory B cells.

Macrophages are the predominant cell type found in the milk and tissues of healthy, involuted, and lactating mammary glands. Whereas these cells have a role in early nonspecific defense as phagocytes and regulators of inflammation, macrophages also play a key role in antigen processing and presentation. Antigens from ingested bacteria are processed within macrophages and appear on the membrane in association with MHC molecules. When a naïve Th cell encounters antigen complexed with MHC molecules on macrophages, it can proliferate and differentiate into a memory cell or cytokine-producing effector cell as described above.

Adaptive Humoral Defenses

Antibodies function as the soluble effectors of specific or humoral immune responses (Table 2). Antigen-activated B cells proliferate and differentiate into antibody-secreting plasma cells. Antibodies in lacteal secretions are synthesized locally or are selectively transported from the blood stream. Four classes of immunoglobulins are known to influence mammary gland defense against mastitis-causing bacteria and include IgG₁, IgG₂, IgA, and IgM. In healthy glands, the concentration of immunoglobulin is low during lactation but slowly increases during the nonlactating periods and reaches peak concentrations during colostragenesis. High concentrations of immunoglobulin also occur in

the mammary gland during inflammation. The concentration of immunoglobulin in the gland depends upon the degree of permeability of secretory tissue and the number of Ig-producing cells that are present in the mammary gland (Aitken et al., 2011).

MODULATING MAMMARY IMMUNE MECHANISMS TO MASTITIS PATHOGENS

Although the mammary gland is equipped with a diverse and highly interactive network of cellular and humoral defense mechanisms, there are certain times in the cow's lactation cycle when the immune system is compromised, resulting in higher rates of new IML. Indeed, research suggests that the bovine immune system is especially compromised during the periparturient period, which explains why fresh cows are susceptible to so many different health disorders, including mastitis. The development of innovative strategies that can enhance otherwise impaired mammary gland defense mechanisms during periods of increased disease susceptibility could have a major effect on the incidence of mastitis.

Role of Vaccination in Mastitis Control

The purpose of vaccination against mastitis-causing bacteria is to stimulate the cow's immune system to protect it against subsequent infection. For example, vaccination may increase circulating antibodies or a cell-mediated immune response directed against certain mastitis pathogens to prevent or limit bacterial growth after invasion. The resulting enhanced immunity may also minimize pathogen damage to milk-producing tissues, modify the inflammatory response, and promote tissue repair. Progress has been made in the effort to develop vaccines for preventing both environmental and contagious mastitis. Commercial mastitis vaccines are currently available for *E. coli* and *Staph. aureus*, and several experimental vaccines based on these 2 pathogens have been the focus of the pharmaceutical industry and academic institutions for many years. Far less information is available on mycoplasma vaccines, and no streptococcal vaccines are commercially available.

Coliform Vaccines

The most success has been realized with the gram-negative common core vaccines, and the National Animal Health Monitoring System (NAHMS) estimates that coliform mastitis vaccines are used on one-third of all US dairy farms. Bacterins (killed or attenuated bacterial preparations) formulated against the coliform

genera (e.g., *Escherichia*, *Klebsiella*, *Enterobacter*) have been developed because the proportion of mastitis caused by these environmental pathogens has increased in many herds. This may be due to the trend for low SCC milk, an increase in cow susceptibility to coliform mastitis, and higher density housing, which increases exposure to environmental pathogens. In addition, common herd health practices such as teat dipping and antibiotic therapy are not effective in controlling coliform mastitis primarily because of the continuous exposure to these pathogens in the cow's environment (Smith et al., 1985). A high proportion of clinical cases occurs within the first 3 mo of lactation, mainly during the first 2 wk, causing marked losses in milk production (\$100 to \$200 per case).

Control of coliform mastitis has been made possible through the development of mutant gram-negative bacteria. Vaccines used to combat gram-negative pathogens focus on using the mutant gram-negative core antigen, which lacks the chains that protect the lipopolysaccharides of gram-negative pathogens. This characteristic is important because the antibodies produced by the vaccinated animals are specific to the exposed lipopolysaccharides of all gram-negative organisms, whether they are of the genus *Escherichia*, *Klebsiella*, or *Enterobacter*. Thus, such vaccines stimulate the production of antibodies against common core antigens in the bacterial cell wall that are cross-protective against a wide variety of gram-negative microorganisms.

Enviracor J-5 Coliform Vaccine. One such vaccine product is the *E. coli* Enviracor J-5 bacterin administered subcutaneously at drying off, 30 d later, and again within 14 d of calving (Enviracor J-5, Zoetis, Florham Park, NJ). Following initial observations that cattle with low naturally occurring serum titers against *E. coli* J5 experienced a 5-fold increase in clinical coliform mastitis, researchers in California (González et al., 1989) investigated the efficacy of immunization in reducing the incidence of clinical disease in a vaccine field study. Vaccinated animals received subcutaneous injections at drying off, 28 d later, and within 14 d of calving. Results showed that, during the subsequent 100 d of lactation, the incidence of clinical cases of coliform mastitis in vaccinates was 2.57% compared with 12.77% in the controls, a reduction of 80%. This same vaccine was subsequently evaluated in Ohio (Hogan et al., 1992), where vaccinations were given subcutaneously at drying off, 30 d later, and 2 d after calving. Compared with controls, vaccinated cows exhibited fewer bacteria in milk and lower rectal temperatures following infusion of *E. coli* 30 d into lactation. In addition, milk production and DMI were depressed to a greater extent in controls than in vaccinates. Blood and milk antibody titers were higher in vaccinated than in

control cows. It was concluded that vaccination with the *E. coli* J5 bacterin did not prevent infections but did reduce severity of clinical signs following intramammary challenge with a wild *E. coli* strain. This bacterin was also found efficacious in reducing the occurrence of IMI and clinical signs of mastitis in first lactation heifers (Hogan et al., 1999).

A partial budget analysis of on-farm implementation of an *E. coli* J5 vaccination program conducted in 1991 demonstrated that use of the vaccine on all cows in a herd was profitable when incidence of clinical coliform mastitis exceeded 1% (DeGraves and Fetrow, 1991). Using such a program would yield a \$57 profit per cow per lactation.

J-Vac Coliform Vaccine. Another gram-negative vaccine based on the *Escherichia coli* mutant strain is the J-Vac, manufactured by Merial Ltd. (Athens, GA). Studies on this bacterin indicate that it is approximately 60% effective in reducing expression of clinical coliform mastitis. In addition, J-Vac reduces the depression in milk production normally encountered with endotoxemia. Following label instructions, this vaccine is administered subcutaneously in the neck at drying off and 2 to 4 wk later. The injection regimen is followed after each lactation to provide adequate antibody levels during the periparturient period and during early lactation to help provide protection against clinical coliform infections.

Endovac-Bovi Coliform Vaccine. Another USDA-licensed coliform vaccine is a bacterin-toxoid formulated from an Re-17 mutant of *Salmonella typhimurium* (Endovac-Bovi; Immvac Inc., Columbia, MO). It works similarly to vaccines formulated with the *E. coli* J5 in stimulating protection against common gram-negative core antigens. In addition, the toxoid component is believed to stimulate immune cells in the cow's body to enhance antibody production to *Salmonella typhimurium* Re-17. A field trial to test this vaccine in Arizona utilized cows immunized intramuscularly at dry-off and again 2 to 3 wk prepartum and compared them with unvaccinated controls. Data collected over the first 5 mo of lactation showed a 42% reduction in clinical cases of coliform mastitis in vaccinates compared with controls, and a 67% reduction in repeat episodes. In addition, the mortality rate for cows with clinical coliform mastitis was 61% lower in vaccinated cows. The protection afforded by this vaccination program was thought to be due to enhanced opsonization and phagocytosis of neutrophils.

***Staph. aureus* Vaccines**

Early efficacy studies on the only commercial *Staph. aureus* vaccine in the United States (Lysigin, Boehringer-

Ingelheim Vetmedica Inc., St. Joseph, MO) suggest that it will increase the spontaneous cure rate against *Staph. aureus* IMI and lower SCC, but it does not prevent new IMI in adult cows (Pankey et al., 1985). Research conducted over the past 15 yr has demonstrated that several experimental *Staph. aureus* vaccines, as well as one commercial vaccine, can reduce the new infection rate in dairy heifers. For example, a *Staph. aureus* vaccine formulated to stimulate pseudocapsule and α -toxin antibodies was evaluated in heifers in New York State (Sears et al., 1990). At 4 and 2 wk before calving, heifers were given subcutaneous injections into the supramammary lymph node of the mammary gland, and after calving, heifers were challenged with *Staph. aureus*. Vaccinates demonstrated a 52% reduction in the development of new IMI after challenge. In addition, 64% of IMI in control cows became chronic compared with only 12% in vaccinates. Subsequently, a field trial in Norway (Nordhaug et al., 1994) evaluating a *Staph. aureus* vaccine demonstrated that vaccinated heifers injected subcutaneously in the area of the supramammary lymph node of the mammary gland at 8 and 2 wk before calving showed a 46% reduction in new IMI during the subsequent lactation compared with controls. Antibody titers to *Staph. aureus* pseudocapsule and α -toxin were markedly elevated in the serum of vaccinates, and these titers remained significantly higher in serum and milk during the entire lactation compared with those of unvaccinated controls. In Argentina, a vaccine formulation was evaluated in bred heifers vaccinated intramuscularly at 8 and 4 wk and 1 and 5 wk postpartum (Giraud et al., 1997). This immunization program demonstrated that the frequency of new *Staph. aureus* IMI was reduced from 18.8% in controls to 6.4% for vaccinates, for an overall reduction of 66%.

In view of the above studies showing success of experimental vaccines in heifers, researchers in Louisiana evaluated the commercial *Staph. aureus* vaccine Lysigin in young dairy animals (Nickerson et al., 1999). At 6 mo of age, 35 Jersey heifers in a research herd were vaccinated per label instructions intramuscularly, 14 d later, and at 6-mo intervals until calving. Results demonstrated that the rate of new IMI at freshening was reduced 44.7% and SCC reduced 50% in vaccinates compared with controls. Serum samples demonstrated that anti-staphylococcal antibody titers remained higher in vaccinated heifers compared with controls throughout the study.

Subsequently, Middleton et al. (2006) compared 2 experimental *Staph. aureus* formulations with Lysigin in heifers. Animals were vaccinated twice, 28 d apart, during late gestation and, after calving, they were challenged by intramammary infusion with *Staph. aureus*

in early lactation. All quarters became infected with *Staph. aureus*, and at the end of the study, there were no differences in *Staph. aureus* clearance rates, SCC, or milk yields. The authors suggested that milk may contain insufficient vaccine-induced opsonizing antibody to effectively prevent infection following the *Staph. aureus* challenge.

In contrast to the work of Middleton et al. (2006), Nickerson et al. (2009) found beneficial results in using Lysigin to manage *Staph. aureus* mastitis in heifers. Using 106 Holstein heifers 6 to 18 mo of age in a commercial herd, 53 animals were vaccinated intramuscularly using a 5-mL dose, and 53 served as unvaccinated controls; 14 d later and at 6-mo intervals thereafter through calving, the vaccinated group was boosted with Lysigin. Vaccine efficacy data showed that the percentage of heifers with *Staph. aureus* IMI at freshening was lower in vaccinates (13.3%) compared with controls (34.0%)—a reduction of 60.9%. Also, SCC collected during the first week of lactation were lower in vaccinates compared with controls (287,000 vs. 522,000/mL).

Thus, use of experimental and commercially available *Staph. aureus* vaccines may be used to prevent new infections when used in heifers. Efficacy has been shown to range between 44 to 66%, and this prevention strategy may represent a major control mechanism for managing *Staph. aureus* in the future, especially as new antigens and adjuvants are added to vaccine preparations. The NAHMS estimates that *Staph. aureus* vaccines are used on 5 to 7% of US dairy farms.

Mycoplasma Vaccines

A commercially available *Mycoplasma bovis* bacterin (Mycomune, Bioimmune Inc., Scottsdale, AZ) was developed that is injected subcutaneously by giving 2 doses at 2- to 4-wk intervals during the prepartum period and the third dose 2 to 3 wk prepartum, which claims to reduce the incidence and severity of mycoplasma mastitis. In a California trial conducted in 2002, serum antibody titers were shown to increase 4-fold and milk titers 10-fold compared with controls after the third vaccination given 2 to 3 wk prepartum. Vaccination was shown to prevent the new IMI rate after experimental challenge with *M. bovis* in early lactation, minimize culling, and reduce mycoplasma-positive bulk tank cultures; milk production was maintained in vaccinates but decreased markedly in controls. To date, no peer-reviewed studies are available, so efficacies of mycoplasma vaccine attempts have not been established. One problem is that the surface antigens of mycoplasma organisms are highly variable due to the diversified family of lipoproteins (variable surface proteins) that can change over time in response to host or environmental conditions.

This makes immunization to a specific strain or clone difficult because of rapid diversification of antigens, and the practice is not recommended. The NAHMS estimates that mycoplasma mastitis vaccines are used on 1 to 2% of US dairy farms.

Strep. uberis Vaccines

There are currently no commercially available vaccines directed against the environmental streptococci, such as *Strep. uberis*, despite its prevalence during the prepartum period (Smith et al., 1985). However, several commercial and academic institutions have been examining the development of such vaccines.

Dietary Supplementation

The nutritional status of dairy cattle, especially during the periparturient period, is directly related to the efficiency of the immune response (Sordillo, 2016). Nutritional requirements of cows fluctuate throughout the lactation cycle, and any mismanagement of the diet will adversely affect immunity and disease resistance. Meeting the nutritional requirements of the periparturient cow is especially challenging because of the increased metabolic demands associated with the onset of copious milk synthesis and secretion following calving. Consequently, early lactation cows experience severe negative energy and protein balances, which are directly related to immune dysfunction. Although the interaction between nutrient metabolism and immunity is complex and not completely understood, the need for a balanced supply of dietary micronutrients (vitamins and trace minerals) is essential in optimizing immune responses and increasing disease resistance at calving. In fact, dairy cattle with overt deficiencies in certain vitamins and minerals during this time exhibit increased incidence and severity of mastitis.

Most of the current information on micronutrients and their immunoregulatory properties in controlling mastitis focuses on selenium (Se), vitamin E, vitamin A, copper, and zinc. Of all of these micronutrients, vitamin E and Se are the best characterized with respect to their role in supporting immune responses that can influence mastitis susceptibility. For example, supplementation of cows with vitamin E and Se improves the functional capabilities of bovine neutrophils compared with neutrophils isolated from cows with marginal or overt deficiencies. Supplementing cows with vitamin E and Se during late gestation effectively reduces the incidence and severity of clinical mastitis while also preventing the establishment of new IMI in early lactation. Additionally, vitamin A, copper, and zinc have significant immunoregulatory functions that can opti-

mize immune responses needed for improved mastitis resistance (Sordillo, 2016). A common mechanism by which most of these micronutrients function is through their antioxidant properties and protecting immune cell populations from the toxic effects of ROS generated by presence of IMI. The primary function of antioxidants is to reduce oxidative damage to cellular macromolecules such as membrane phospholipids. During inflammation, neutrophils and macrophage produce large quantities of ROS, which kill invading bacteria. In order for ROS not to cause damage to the cow's own cells, it is important that adequate amounts of antioxidants be provided by the diet so that they can be incorporated into mammary gland tissues and immune cell populations. The key to ensuring that dairy cattle consume adequate amounts of these micronutrients is by direct herd testing to detect any nutrient deficiencies. Supplementing cows' diets with recommended doses of vitamins and minerals is a practical means of enhancing mammary gland immunity and reducing the incidence of mastitis.

Immunostimulants

The development of strategies to optimize host immunity during times of increased disease susceptibility by using biological response modifiers (immunostimulants or immunomodulators) has been explored extensively. Cytokines are immunomodulators that have been investigated for their capacity to bolster immune responses and reduce or prevent IMI. Administering the recombinant form of these immunomodulators during times when normal cytokine expression is compromised seems to be a logical approach to enhance disease resistance. The recombinant cytokines studied to date include IL1, IL2, granulocyte-colony stimulating factor (G-CSF), and IFN- γ , which have been shown to enhance the functional capabilities of cells involved in both innate and adaptive immunity. For example, IFN- γ , G-CSF, and IL-1 were found effective in increasing neutrophil migration to the mammary gland and enhancing bactericidal activity. Both IFN- γ and IL2 also were able of enhancing T cell responses to specific antigen, suggesting that these cytokines may be effective mastitis vaccine adjuvants. As with any intervention strategy, however, the key to effective translation of experimental findings to practical on-farm application is to clearly define the threshold between therapeutic and toxic doses of these potent immunomodulators (Sordillo and Streicher, 2002).

CONCLUSIONS, IMPLICATIONS, AND THE FUTURE

Dairy cattle are especially susceptible to mastitis during certain stages of the lactation cycle. The fre-

quency of new IMI is greatest during the early dry period, lower in the fully involuted mammary gland, and dramatically increases during the periparturient period. Changes in the incidence of IMI with respect to lactation stage are directly related to changes in the composition, magnitude, and efficiency of the mammary gland defense system. The development of innovative immune modulation strategies that can enhance an otherwise impaired response during periods of increased susceptibility to disease could have a major effect on the incidence of mastitis. The challenge that confronts researchers now is to gain a better understanding of the complex interactions between the pathogenesis of bacteria, host responses needed to eliminate the pathogens from the mammary gland, and methods to enhance the immune potential of these factors before disease is established.

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