Contemplating the Energetic Consequences of Bovine Mastitis

Benjamin D. Enger[1]
Department of Animal Sciences
The Ohio State University

Abstract

Mastitis is a common and expensive disease in the dairy industry that significantly reduces the quality and quantity of milk produced by the dairy cow. The reduction in the amount of milk produced is resultant of a multitude of factors, but the purpose of this article is to reflect upon how the activated immune system may consume specific substrates necessary for milk synthesis. The 3 key immune cells implicated in mastitis, the neutrophil, macrophage, and lymphocyte, have marked demands for substrates to carry out their immune related functions. For instance, both the neutrophil and macrophage phagocytose bacteria and kill the bacteria by the generation of reactive oxygen species. Generation of these reactive oxygen species is a largely glycolytic process. Additionally, amino acids are used by some lymphocytes for the synthesis and production of antibodies. Uniquely, in the instance of mastitis, these active immune cells are specially located in direct competition with the secretory mammary cells, which would compete for the substrates that would be used for milk synthesis.

Introduction

Mastitis remains the most common and expensive disease in the US and global dairy industries. The economic losses that result from mastitis are consequence of: 1) reduced milk production and quality, 2) increased labor, veterinary costs, and drug usage, 3) discarding abnormal milk and antibiotic laden milk, and 4) prematurely culling affected animals. Although the losses of mastitis are a consequence of many factors, the greatest financial loss is due to reduced milk production in affected animals (Blosser, 1979). Milk yield loss in response to mastitis has been recognized to occur for decades, but seldom is the question asked: *Why does this occur?* The answer to this question has many answers and many of which are interconnected and not independent of one another. The objective of this article is to consider and speculate how mastitis reduces milk production. Given the nutritional emphasis of the attendees of the Tri-State Dairy Nutrition Conference, a specific focus of this article will be the key substrates that are necessary for milk synthesis and contemplate what happens to these substrates during a mastitis event.

Factors Influencing Milk Production

The mechanisms that affect milk production should be briefly reviewed and appreciated if we are to build upon this concept and reflect how mastitis affects milk production. On the farm, numerous management and genetic decisions are dissected, scrutinized, and implemented with the goal of increasing milk production per cow. It is easy to be overwhelmed

[1]Contact at: 143 Gerlaugh Hall, 1680 Madison Avenue, Wooster, OH 44691, (330) 263-3801, Email: enger.5@osu.edu.

by the mountain of decisions that the dairy owner, herdsman, veterinarian, and nutritionist must make to achieve this goal. An incomplete listing of some of the key factors known to affect milk production include days in milk, nutrition status and energy balance, milking frequency, parity, breed, heat stress, metabolic diseases, and mastitis. Cutting through this thick fog of interweaving and associated elements, it can be simplified and recognized that only 2 factors actually determine a cow's milk production. Milk production is solely dictated by the number of milk secreting cells in the gland and the average rate at which these cells synthesize and secrete milk components. Yes, much simpler. All management practices used to improve milk yield affect one, or both of these central elements, and anything that would affect one or both of these key elements would ultimately affect milk production, for better or worse.

Mammary cell number

The idea that the number of mammary cells secreting milk would influence the amount of milk produced is nothing new and has been examined for decades. If differences in the number of cells in a lactating mammary gland were profound enough, differences in udder size would be observed. Make no mistake, this method of appraising an udder's productive capacity based on size is extremely unreliable and crude. It is being used here strictly for illustrative purposes. Perhaps a relevant and striking example would be the comparison of udders from beef and dairy cattle. In general, the udders of beef cows are smaller and far less productive than their dairy counterparts. Keys et al. (1989) made such a comparison and examined the udders of 10 Holstein and 10 Hereford heifers during their first gestation and at 49 days in milk. Animals were randomly selected for euthanasia at 150, 180, and 260 days of gestation and at 49 days in milk. At euthanasia, the udders were removed and examined. Researchers quantified the total amount of DNA in the collected udders. Total mammary DNA was used as a proxy for the number of cells in the gland because DNA is constant among cells and allows for comparisons to be made on the number of cells between glands. Overall, it was observed that the amount of total mammary DNA increased as gestation progressed, indicating growth of the udder for both the beef and dairy breeds. Even though both udders grew, the amount of total mammary DNA was starkly different between breeds. The Holstein udders had anywhere from 2 to 4.7 times the amount of total mammary DNA than the Hereford's during the sampled time points. Many studies have sought to define the relationship between mammary cell number and milk production and have been summarized by Davis (2017). Overall, the described relationship between milk yield and mammary cell number vary considerably from study to study. Some have defined a relationship as high as $r = 0.69$, assuming the udder is healthy (Davis, 2017), to a complete lack of a relationship (Knight, 2000). Needless to say, mammary cell number does not explain milk yield entirely but would indeed influence the amount of milk produced.

Mammary cell activity

The other part of the milk production equation is mammary cell activity. A profound example of how mammary cell activity affects milk yield can be inferred from a study by Capuco et al. (1997). In this study, researchers studied the dry period and sought to understand why it is so integral for the next lactation's performance. Two treatment groups were used. The first was a group of 13 multiparous cows that were dried-off 60 days prior to expected calving and represented the "typical" dry period. The other treatment included 13 multiparous cows that were not dried-off and continuously milked during this time. During the 60-day period,

cows were selected and euthanized in groups of 3 or 4 per treatment group at 53, 35, 20, and 7 days before expected calving. At euthanasia, the udder was removed and used for analysis. When the entire udder was ground and analyzed to measure the amount of DNA as a means to gauge the number of cells in the glands, there was no difference between these 2 treatments. This indicates that the traditional dry period does not affect the number of cells that would be in the gland at the next lactation rather than if the animal was milked continuously. This observation is peculiar as it is well documented that cows that have a dry period produce considerably more milk than cows that do not (Swanson, 1965; Schlamberger et al., 2010) or experience a dry period that is inadequate in length (Sanders, 1928). When Capuco et al. (1997) examined these mammary tissues further to understand changes within the gland, it was observed that there was more cell death and proliferation in the non-lactating cow mammary glands, indicating removal and replacement of cells. The researchers concluded that the dry period facilitates "turnover" and replacement of damaged and senescent secretory mammary epithelial cells. This turnover is expected to allow these cells to be more active during the next lactation and are thought to be the reason why having a dry period before the ensuing lactation results in greater milk production rather than continuous milking.

Mastitis

With a clearer understanding of what dictates milk production, let us come back to mastitis. *What is mastitis?* Mastitis is simply inflammation of the mammary gland: masto- from the Greek meaning breast and *-itis* from the Latin meaning inflammation. Inflammation in the bovine mammary gland can develop for many reasons, but the predominant reason is an intramammary infection (**IMI**). Most IMI are a result of bacteria entering the mammary gland via passage through the teat streak canal, proliferating, and establishing an infection. The inflammation that is present during an IMI originates solely from the bovine and is her response to the IMI. Almost counter intuitively, this inflammatory response is beneficial from a biological standpoint because it serves as a means to eliminate the pathogen while also removing any damaged cells and tissues in the mammary gland. This is important as successful removal of all these elements would not only clear the infection but also prevent tissue necrosis that would exacerbate the inflammatory cascade and cause further tissue damage.

Reflecting on this biological discussion of what is inflammation is of limited help because it is not all that definitive or measurable. This is why the dairy industry, in large, measures mammary inflammation by quantifying the number of cells in milk. These cells are more commonly referred to as somatic cells. The concentration of these cells in a milliliter of milk is referred to as the somatic cell count (**SCC**). Although simple, this measure provides great utility. An increase in the SCC is indicative of an increase in the number of immune cells in the mammary gland. This is because immune cells are recruited to the mammary gland to address an invading pathogen during an IMI. Logically, an increase in the number of immune cells in the gland would indicate that there is an active immune response occurring and inflammation is present. Indeed, quantifying the number of somatic cells in milk has occurred for over a century (Campbell, 1909; Prescott and Breed, 1910). A center point of this effort has been to understand the relationship between the SCC and the presence of bacteria in milk, indicating an IMI (Campbell, 1909; Cherrington et al., 1933). Many SCC thresholds have been presented and discussed over the years on what SCC value should be used as a cutoff to indicate an IMI.

This conversation becomes easily complexed when the nuances and intricacies of this concept are recognized. For instance, should the SCC cutoff be determined for a composite sample of all 4 quarters that has been collected throughout the entire milking, or should it instead be a foremilk sample collected from a single quarter? Each one would likely require its own cut-off. Additionally, mastitis pathogens differentially affect the SCC and using a single threshold may not apply for all pathogens; this could result in misclassification of a gland's infection status. With this brief acknowledgment of this complex system, there will not be a detailed discussion here but instead the reader is referred to Schepers et al. (1997), Jashari et al. (2016), and Petzer et al. (2017) where a more detailed discussion can be found. Instead, let us simply appreciate that SCC is used as a gauge for mammary inflammation, and a higher SCC would be grossly indicative of greater inflammation. Indeed, it is well described that there is a negative relationship between increasing SCC and milk production (Table 1).

Types of immune cells

The neutrophil, macrophage, and lymphocyte are the 3 core immune cells that comprise the SCC. Importantly, these cells have different functions when it comes to responding and clearing an IMI. The neutrophil is the primary immune cell that is initially recruited to an IMI and is part of the innate immune system. These cells are the "first responders" and seek to identify and neutralize pathogens while, at the same time, recruit other immune cells to the site of infection or inflammation. This is achieved by neutrophils producing chemical messages that "attract" and "communicate" with other immune cells. Neutrophils seek to neutralize/kill bacteria by either phagocytosis, producing and releasing cytotoxic granules into the immediate environment and/or forming extracellular nets to "tangle" and trap bacteria (Amulic et al., 2012). An example of a bovine neutrophil that has phagocytosed several staphylococci is depicted in Figure 1 (Panel A). Killing internalized bacteria is of paramount importance so that bacteria do not freely proliferate inside the cell. When the neutrophil "grabs" the bacteria, it releases some reactive oxygen species to begin killing the bacteria (Paape et al., 2002). Examples of a few reactive oxygen species would include hydrogen peroxide, superoxide, and hydroxyl radicals. The bacteria that are bound to the neutrophil's cell membrane are subsequently internalized by the pseudopodia of the neutrophil and are continuously subject to reactive oxygen species (Paape et al., 2002). Surrounding mammary tissues may be damaged by the reactive oxygen species during the process of binding and internalizing bacteria. The result of this can lead to further increases in the inflammatory status of that tissue by having neighboring mammalian cells produce and release more chemical to attract more immune cells. Neutrophils are short lived as the typical half-life of a neutrophil in blood is 8.9 hours and only remain in mammary tissues for 1 to 2 days after migrating from the blood (Paape et al., 2002). Because of the neutrophils' short life, continuous recruitment into the gland is necessary to maintain a sustained immune response.

Macrophages are also part of the innate immune system, but they have different functions than the neutrophil. An example macrophage is depicted in Figure 1 (panel B), and it can be easily appreciated that these cells are rather large. Macrophages are primarily regarded as tissue resident immune cells that serve as sentinels to detect pathogens while also assisting in "directing" any initiated immune response. Macrophages are not short lived like neutrophils but can persist in tissues for months (van Furth, 1968). Similar to neutrophils, they can phagocytose bacteria and also produce

chemical messages to attract other immune cells to infected/inflamed tissues. Importantly with macrophages' phagocytosis of bacteria, macrophages can present bacterial contents and parts to other immune cells to stimulate an adaptive immune response. This allows an adaptive immune response to be generated for the specific infectious agent.

Grossly stated, B and T cells comprise the lymphocytes and are part of the adaptive immune system; an example lymphocyte from a mammary gland is presented in Figure 1 (panel B). These cells are specifically recruited for precise tasks after careful generation and selection. For the B cell, a primary purpose is to produce antibodies that assist with the immune response. These antibodies are made of amino acids and are designed to either opsonize bacteria, which labels the bacteria for phagocytosis, or they may be used to thoroughly coat the invading pathogens so the pathogen cannot bind to mammary tissues. Together, these mechanisms contribute to removing the pathogen from the udder. T cells, on the other hand, do not synthesize and secrete antibodies; instead, they perform several other functions. T cells can help direct the immune response by regulating the production of chemical messages that would influence how many immune cells might be recruited to the site of inflammation. The T cells can also help stimulate and activate B cells. This is achieved by the B and T cell interacting and directing how the B cell should develop. Additionally, T cells can identify bacterial infected cells and direct them to undergo controlled cellular death to contain the infection's spread.

Metabolic Demands of the Immune System

While a large number of various immune cells and their respective functions have been reviewed, it is most important to recognize that all the cellular processes associated with these functions and mechanisms consume energy, some to a great magnitude. For instance, neutrophils and macrophages that phagocytose bacteria require energy and substrates for not only "chasing down" and ingesting the bacteria but also producing the reactive oxygen species necessary for killing the bacteria. For the neutrophil, glucose is a significant metabolite that is used for energy during these processes. As discussed by Paape et al. (2002), glycogen granules are present in the cytoplasm of the neutrophil and comprise 20% of the cell's dry matter components. This is significant given glycogen is merely repeat glucose molecules. Glycogen can be broken-down via glycogenolysis and the resulting individual glucose monomers can be used for the generation of ATP via glycolysis. Indeed, the neutrophil is largely categorized as a glycolytic cell (Kramer et al., 2014) and is recognized to uptake glucose from the surrounding environment, as well as use the intracellular glycogen stores during phagocytosis (Borregaard and Herlin, 1982). This is important as it is largely recognized that glucose is not overly abundant in the lactating ruminant and a large proportion of this glucose is used in the synthase of lactose. Lactose is regarded as the chief osmoregulator of milk and considerably influences milk yield. Indeed, milk yield is dramatically reduced when lactose synthesis is impeded (Stacey et al., 1995). It is, therefore, logical to expect that if glucose were instead utilized by the immune system rather than lactose synthesis, milk production would be reduced.

Systemic immune response

Kvidera et al. (2017) recently investigated the effects of the activation of the immune system on glucose utilization at the whole animal level. Researchers utilized 18 lactating dairy cows and divided them amongst

3 treatment groups. The 3 treatment groups were a control group receiving no treatment, a lipopolysaccharide (**LPS**) treatment group that received a single intravenous bolus of LPS, and the third was another LPS treatment group that received continuous glucose administration to maintain blood glucose concentration. As to be expected, LPS administration elicited an increase in the concentration of various acute phase proteins in the blood, signifying that an immune response was generated. Blood glucose levels spiked immediately after the LPS bolus infusion, and then sharply decreased to their lowest point at approximately 3 hours after LPS administration. The LPS cows receiving glucose infusion had their blood glucose concentrations "rescued" to pre-infusion baseline levels by 4 hours post LPS challenge; these blood glucose levels were similar to the control cows for the remainder of the 12-hour study. In contrast, LPS cows that did not receive glucose remained hypoglycemic after the initial spike and were consistently lower that the other treatments. Kvidera et al. (2017) concluded that the LPS induced immune system activation consumed a considerable amount of glucose because of the immune system's activation. Overall, the researchers estimated that during their 12-hour experiment, the immune system consumed greater than a kilogram of glucose. The authors explicitly emphasize the fact that this calculation is significantly underestimated.

Localized immune response

Let us turn back to mastitis and appreciate that during a mastitis event, there is an activated immune response at the local level of the mammary gland. This activated immune response includes the previously discussed neutrophils, macrophages, lymphocytes, and all their associated cellular functions. An example of mammary tissues from an uninfected and *Staphylococcus aureus* infected bovine mammary gland are presented in Figure 2 (panels A and B). The stark increase in the number of immune cells that can be present in inflamed tissues is striking as immune cells can be observed in both the luminal space and tissues of the mammary gland. The result of this localization is that the active immune cells are placed in the same locale as mammary cells seeking to uptake glucose for lactose and milk synthesis. With the increased understanding of glucose utilization of the immune system as demonstrated by Kvidera et al. (2017), I would expect a similar phenomenon to occur, but at the localized level of the mammary gland. Because the neutrophil is recognized to be central to the initial immune response during mastitis and comprises the largest percentage of the SCC, I would expect these cells to utilize a significant amount of glucose in these mammary tissues. This would reduce the amount of glucose available for lactose synthesis.

Briefly mentioned earlier, amino acids play a significant role as a substrate required for the synthesis of antibodies in B cells, but no studies were identified that quantify the metabolic and amino acid requirements for bovine antibody synthesis. As such, no definitive statement can be made on how antibody synthesis at the local level of the mammary gland might affect milk protein synthesis. It is, however, well appreciated that a considerable presence of plasma cells (a type of B cell that produces antibodies) is found in bovine mammary gland tissues (Enger et al., 2018) and that certain types of plasma cells become more prevalent during an IMI (Nickerson and Heald, 1982). It could be speculated that if the demand of these activated plasma cells is significant enough to consume a large amount of amino acids, some of which being essential, milk protein synthesis would likely be reduced. The fact that the concentration of the key whey proteins, α-lactalbumin and β-lactalbumin, and total casein proteins are

reduced during subclinical mastitis may support this notion (Ishikawa et al., 1982; Pyorala, 2003).

Lastly, pictured in Figure 3 are mammary tissues that were collected from uninfected and *Staphylococcus aureus* infected mammary tissues. These tissues were examined in a previous study that sought to understand how mastitis affects the proliferation of the cells in the mammary gland (Enger et al., 2019). A key focus was to examine the epithelial cells that would be responsible for milk synthesis and determine if mastitis would affect the number of these cells that were proliferating. Interestingly, a greater number of cells in the stroma compartment of *Staphylococcus aureus* infected tissues were observed to be positive for proliferation when compared to tissues from uninfected mammary glands. The existence of these proliferating cells is associated with the fact that these tissues contained greater infiltration of immune cells. The majority of these proliferating cells were putatively classified as immune cells, more of the lymphocyte and macrophage nature given their nuclear shape. The significance of this observation suggest that immune cells are going to this location and then receiving signals to grow and divide. Cellular proliferation would require substrates from the surrounding environment. Admittedly, it cannot be determined here if these cells are proliferating in the mammary gland itself or traveling to other immune related tissues. Yet, the fact that these cells are positive for the proliferation marker indicates that these cells would indeed be growing and initiating specific cellular processes, which require energy, to divide.

Conclusion

It is well established that mastitis negatively affects milk production. The energetic/substrate demands of a mastitis event have been discussed and it can be appreciated that there are undeniably energy requirements for an activated immune system. In the instance of mastitis, the activation of the immune cells is focused at the local level of the mammary gland, which places these cells in direct competition with the mammary cells that would be synthesizing and secreting milk components. This competition is likely to redirect the same nutrients that would be used for milk secretion and synthesis to the activated immune cells in the gland. As such, it is important to recognize that nutrients being fed to the cow to support milk production may be instead being utilized by the immune system to address a preventable disease. This emphasizes the importance of preventing mastitis and limiting its prevalence and incidence as the consequences of mastitis are likely to negate any intended improvements in milk yield that are nutritionally driven.

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Table 1. Milk yield losses associated with milk SCC and SCC linear score.[1]

SCC (cells/mL)	SCC Linear Score	Predicted milk yield (lb/day)	Cumulative milk yield loss (lb/day)
12,500	0	64.2	0
25,000	1	62.9	1.3
50,000	2	61.6	2.6
100,000	3	60.3	3.9
200,000	4	59.2	5
400,000	5	57.6	6.6
800,000	6	55.9	8.3
1,600,000	7	54.1	10.1
3,200,000	8	51.9	12.3
6,400,000	9	49.5	14.7

[1]Table adapted from Akers and Nickerson (2011) who adapted and utilized data from Jones et al. (1984).

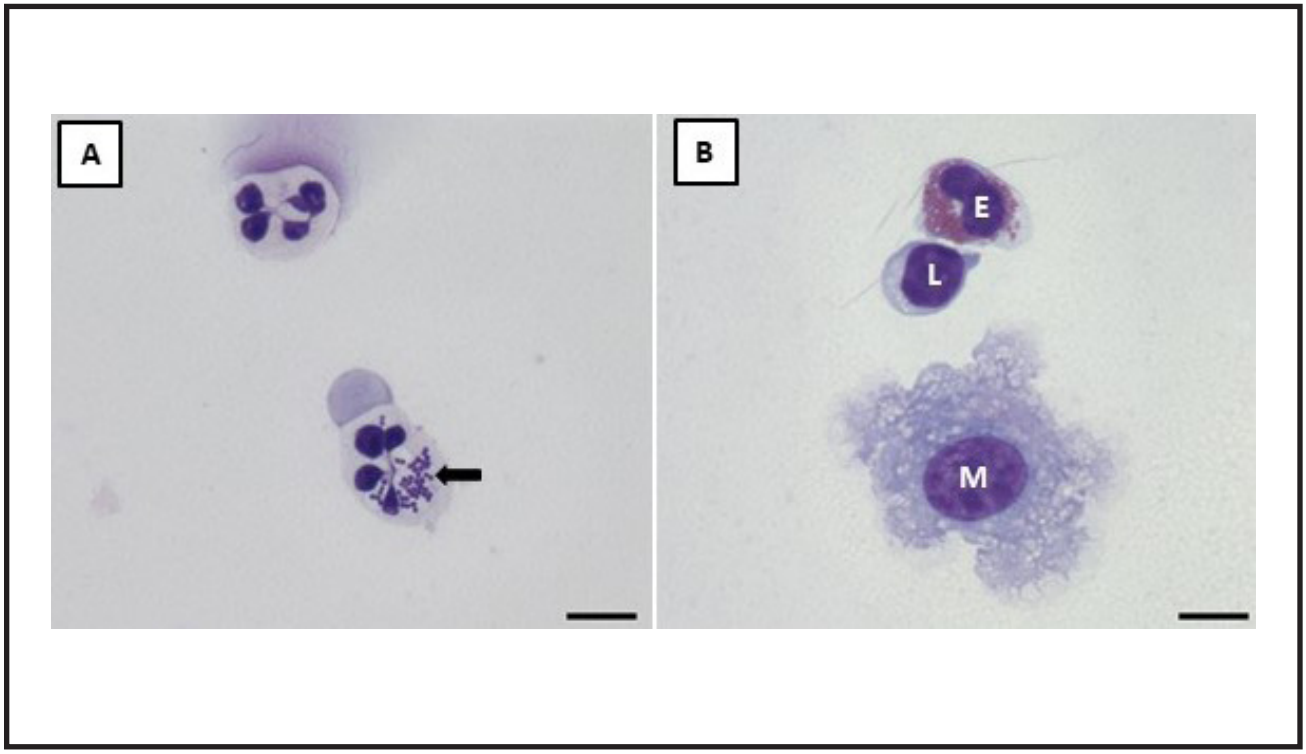

Figure 1. Somatic cells collected from bovine mammary glands stained with Wright–Giemsa stain are presented. Neutrophils (n = 2) are shown in panel A with the lower neutrophil containing intracellular *Staphylococcus aureus* (arrow). Panel B depicts a macrophage (M) a lymphocyte (L) and an eosinophil (E). Images are from Enger et al. (2018). Scale bars = 10 µm.

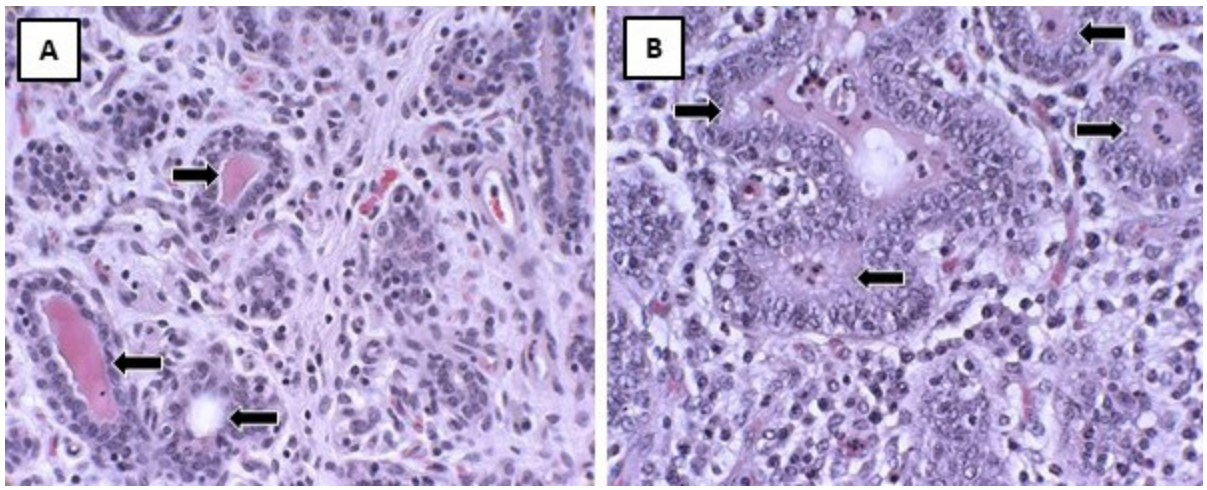

Figure 2. Bovine mammary tissues collected from an uninfected (panel A) and a *Staphylococcus aureus* infected mammary gland (panel B) are presented. No immune cells are present in the luminal space (arrows) of the uninfected mammary tissues but immune cells are abundant in the lumens of *Staphylococcus aureus* infected glands. A considerable increase in the number of immune cells in the stromal compartment of the *Staphylococcus aureus* gland compared to the uninfected gland is evident. Unpublished images from Enger et al. (2018).

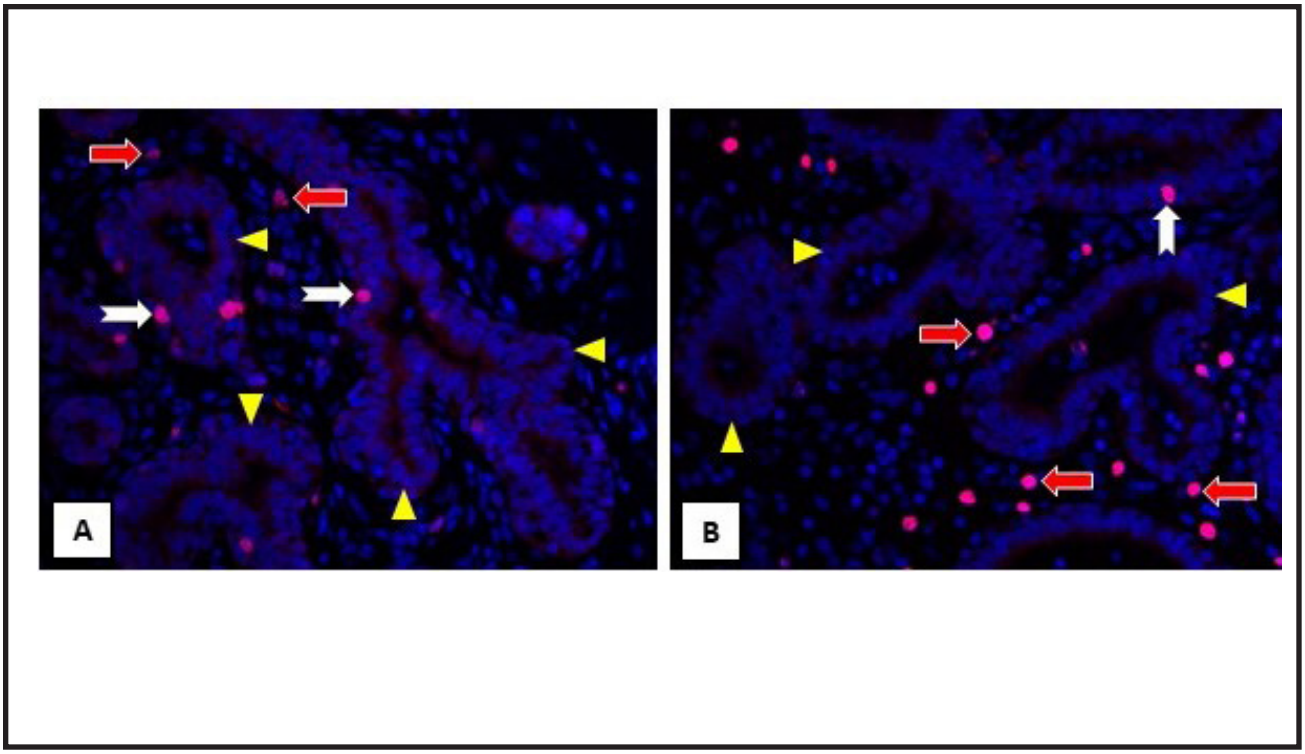

Figure 3. Florescent labeling of proliferating cells (Red) was conducted in uninfected (panel A) and a *Staphylococcus aureus* infected (panel B) mammary tissues. Blue objects are nuclei. Epithelial structures are identified with yellow triangles and proliferating cells in the epithelium are identified by notched white arrows. More proliferating cells were observed in the stromal compartment (red arrows) of *Staphylococcus aureus* infected mammary tissues than non-infected and these cells were putatively identified as being immune cells. Note that immune cells are abundant in the lumen of the *Staphylococcus aureus* mammary tissues, indicating a marked degree of immune cell infiltration of these tissues. Unpublished images from Enger et al. (2019)