

**Doctoral Thesis**

**Studies on Mastitis Caused by Translocated-  
Bacterial Components in Ruminants**

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**Graduate School of Biosphere Science  
Hiroshima University**

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# Chapter 1

## General Introduction

Milk is secretion obtained from healthy animals, such as cows, goats, sheep, and buffalo. Milk and dairy products are an integral part of human nutrition since they carry biological value nutrients that are highly significant for several biochemical and physiological functions. The range of milk production worldwide varies significantly among countries, depending on dietary habits, milk processing technologies available, market demand, and social and cultural circumstances. The per capita consumption of milk and milk products is higher in developed countries.

In Japan, the per capita milk consumption ranges from 30 to 150 kg/capita/year (FAO, 2020), with 7,290 million tons of milk production and 729,500 lactating cows in 2019 (Ministry of Agriculture, Forestry and Fisheries (MAFF), 2020). According to MAFF, this milk production has dropped 15% since 1998, while the decline in dairy cows number has been less precipitous due to increasing herd sizes which reaches 88.8 head per farm in 2019 (up to 5% from the previous year). Meanwhile, the number of dairy farming households nationwide has also dropped by almost 60% in the last 20 years. The reasons for the decline include a harsh working environment that allows a few days off and a lack of willing successors. Furthermore, the increase in milk production is often associated with a greater risk of certain diseases. Milk secretion in the dairy cow has a high metabolic priority and is maintained at the cost of reproductive and metabolic processes. As a result, diseases may develop (Fleischer et al., 2001). The prevalence of clinical mastitis, peracute mastitis, metabolic disorders, and prepartum disorders in dairy cows in the temperate zone of Japan has been raised to 28.0, 13.3, 3.7, and 4.0%, respectively (Fukushima et al., 2020).

Importance of good dairy farming practice is required to ensure the quality of milk under generally acceptable conditions, whereas animal health is one of the areas that needed good practice. Mastitis is of considerable interest because its occurrence harms animal well-being and farm profitability. For decades, researchers worked to identify mechanisms of infection, define the clinical and subclinical states of the disease, discover appropriate screening tests, identify pathogen-specific characteristics, and develop effective milking procedures to develop successful control programs. Even tremendous significant advances in mastitis control have been made during the last century, mastitis remains an important subject of future research because of its complexity.



## **Mastitis in Ruminants**

Mastitis is broadly defined as the inflammation of the mammary gland. It is usually referred to an intramammary inflammatory reaction caused by an infectious agent, primarily bacteria, but also mycoplasma, fungi, and algae (Zadoks et al., 2011; Zhao & Lacasse, 2008). In the 1950s, at least 99% of mastitis cases were caused by *Streptococcus agalactiae*, streptococci, staphylococci, and bacillary mastitis (Murphy, 1956), but today, mastitis pathogens are broadly classified as contagious and environmental pathogens (Rysanek et al., 2009). The main contagious microorganisms are *Staphylococcus aureus* (*S. aureus*) and streptococcus species, which adapt to the environment of the mammary gland, which can be potentially be spread from cow to cow during milking. On the other hand, *Streptococcus uberis*, *Enterococcus* spp., coliforms, *Arcanobacterium pyogenes*, and coagulase-negative staphylococci (CNS) are considered as environmental pathogens which mainly be transferred from the contaminated environment to the mammary gland (Bogni et al., 2011; Reyher et al., 2012).

Mastitis negatively affects the physical-chemical characteristics, composition, and milk yield due to the inflammatory process and the damage of epithelial cells as well as changes in the somatic cells or microorganisms in the infected mammary gland (Ogola et al., 2007). Thus, mastitis is the most devastating disease condition in terms of economic losses occurring throughout the world (Kumar et al., 2010). Moreover, besides economic matters and animal health, mastitis poses a threat to human health since it may be responsible for zoonoses and food toxin infections (Blum et al., 2008; Fernandes et al., 2011). Nowadays, mastitis also reduces reproductive efficiency and causes infertility in dairy animals (Kumar et al., 2017; Wolfenson et al., 2015). Mastitis is known to increase the interval from calving to first service and the number of services per conception. The release of cytokines during the inflammatory process may lead to a failure in Gonadotropin-Releasing Hormone release in the preovulatory period (Hockett et al., 2005; Schrick et al., 2001). Mastitis is also reported to be related to a metabolic disease such as hypocalcemia (Reinhardt et al., 2011), ketosis (Uyarlar et al., 2018), and coincides with metritis (Jánosi et al., 2003) and endometritis (Bacha & Regassa, 2010).

There are two main forms of mastitis; clinical and subclinical mastitis. Clinical mastitis is characterized by sudden onset, swelling, and redness of the udder, pain, and reduced and altered milk secretion from the affected quarters. The milk may have clots, flakes, or watery in consistency and accompanied by fever, depression, and anorexia (Bachaya et al., 2011; Khan & Khan, 2006). In subclinical mastitis, there are no visible abnormalities of the milk or udder except when using diagnostic tools (Hashemi et al., 2011; Radostits et al., 2007). Subclinical

mastitis was first defined by the combination of somatic cell count (SCC) and some pathogenic bacteria threshold along with the development of faster and automated methods to enumerate somatic cells in the 1960s (Paape et al., 1965). Nowadays, subclinical mastitis is important since it is 15 to 40 times more prevalent than the clinical form, usually precedes the clinical form, is of longer duration of infection, difficult to detect, adversely affects milk quality and production, and constitutes a reservoir of microorganisms that lead to infection of other animals within the herd (Seegers et al., 2003).

Mammary gland inflammation and pathogen presence are used to detect mastitis. Inflammation can be detected based on SCC, electrolytes, enzymatic markers, or acute phase proteins (Pyörälä et al., 2011; Viguiet et al., 2009). SCC can be used to monitor the status of subclinical mastitis in herds or individual cows which is an important component of assessing milk quality, hygiene, and mastitis control (Sharma et al., 2011). The SCC of healthy quarters is usually below 100,000 cells/mL in dairy cows (Leitner et al., 2003). The high SCC is not only related to the presence of microorganisms in the udder but also the type of microbes that could affect the SCC in milk (Costa et al., 2019). In some cases, high SCC is detected in milk samples without the presence of microorganisms and it does not indicate that the gland is healthy since around 15-35% of clinical mastitis cases are culture-negative (Barkema et al., 1998; Hisaeda et al., 2016; Kawai et al., 1999; Miltenburg et al., 1996). Conversely, cows with low SCC can also hide mastitis pathogens (Katsande et al., 2013). Therefore, the bacterial culture is necessary to accurately diagnose the disease.

Due to the predominance of bacterial causes in mastitis, the use of antimicrobial agents in the treatment and control continues to be an important subject of investigation. Antibiotic therapy is the main strategy for mastitis treatment, nowadays. However, besides the cost-benefit of the treatment, the emergence of resistance to antibiotics has become a critical issue. The overuse and misuse of antibiotics in the dairy farm may represent a serious problem, not only for resistance matter but also for the potency of resistant bacteria entrance in the food chain (White & McDermott, 2001). Some alternative treatment methods showed great promise for future treatment of mastitis through *in vitro* studies, such as bacteriophages, vaccines, nanoparticles, cytokines, and natural compounds from plants, animals, and bacteria. However, there is no contemporary alternative available for *in vivo* administration (Gomes & Henriques, 2016). Besides the use of antibiotics, the overall udder health status must be improved during the dry period by reducing the number of new infections (Krömker & Leimbach, 2017).

## **Anatomy of the mammary gland**

The mammary gland is the organ that produces milk and is specially organized for optimal function in milk synthesis and ejection. In cows and other ruminants, the udder is divided into two distinct halves, separated by the medial or median suspensory ligaments. There are no evident gross anatomical barriers between the front and rear glands (quarters) on either side of the udder, but each udder half is nearly independent and has its vascular system, nerve supply, and suspensory apparatus. The surface epidermis of the udder is composed of stratified squamous epithelium and is covered with fine hair; conversely, the teats are hairless. The teat skin is thin and devoid of sebaceous glands (Nickerson & Akers, 2011).

The secretory tissue is located in the distal regions of the udder relative to the position of the teats and gland cistern. It is composed of cells grouped into lobules, which are themselves divided into lobes. The secretory tissue is drained by a network of ducts, which open into a cistern. In ruminants, milk is stored in the cistern and the alveolar lumen before being discharged through the teat during milking or suckling (Frandsen, 1986).

The mammary gland is a complex organ of various tissue and cell types. Epithelial cells and fibroblasts are predominant components of mammary parenchyma and stroma, respectively (Connor et al., 2007). The alveoli are the functional units of the mammary gland. They consist of a layer of polarized mammary epithelial cells (MECs). At their apical pole, these cells lead to the alveolar lumen, which contains secreted milk. At their basal pole, they directly interact with contractile myoepithelial cells and with stromal tissue, which is composed of fibroblasts, adipocytes, and lymph and blood vessels. These allow the input of nutrients required for milk synthesis. The neurohypophysial hormone oxytocin causes the myoepithelial cells to contract, forcing newly synthesized milk into the lumen of the alveoli (Nickerson & Akers, 2011).

## **Mammary gland immune system**

The mammary gland is a complex organ of various tissue and cell types that are protected by a variety of defense mechanisms, separated into two categories: innate immunity and adaptive immunity, which interact closely to protect against mastitis microorganisms (Sordillo et al., 1997). The innate immune system is the first line of defense against pathogens once they have penetrated the physical barrier of the teat canal and it evolves into a highly effective host defense (Rainard & Riollet, 2006). In contrast, the adaptive immune system responds more robustly to threats to which it has previously been exposed, however, it is slow to respond to novel threats (Tizard, 2018).

## Teat skin and teat canal defense (anatomical defense)

Infections causing mastitis enters through the teat canal. For this reason, the teat end is considered to be the first line of defense against pathogens. Teat defenses act by preventing entry of pathogens into the udder, which consist of intact skin that provides a hostile environment for bacterial multiplication, teat canal closure mechanisms that reduce the risk of entry between milking, and bacteria adherent to the keratin in the teat canal is flushed out at the next milking (Blowey & Edmondson, 2010).

The teat canal is lined with keratin, a waxy material that is derived from squamous epithelium. This structure enables the trapping of invading bacteria, thus hindering their migration into the gland cistern because of its antimicrobial agents containing (Smolenski et al., 2015). The skin epithelium has a similar morphology to that of the teat canal and also has a host-defense function (Elias, 2007). For example, S100 proteins have been identified in normal mammalian skin, and S100A7 in bovine skin is highly effective in killing *Escherichia coli* and is moderately effective against other bacterial species (Regenhard et al., 2010). Because of the vital importance of the teat canal to prevent mastitis, it is clear that any damage to the teat end, such as hyperkeratosis, physical trauma, milking machine damage, and excessive dilatation of the canal, will compromise the defense mechanisms (Blowey & Edmondson, 2010).

## **Innate immune system**

The innate immune system predominates in the early stage of infection and is mediated by several innate immune components including macrophages, neutrophils, natural killer (NK) cells, and cytokines (Akira & Takeda, 2004). It consists of many diverse subsystems, the most important one is called inflammation, which is a process whereby defensive cells and antimicrobial molecules converge on sites of microbial invasion and tissue damage (Tizard, 2018). It is initiated when specific pattern-recognition receptors (PRRs) on the surface or within host cells bind to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), released from damaged cells. Different PRRs react with specific PAMPs or DAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct antipathogen responses (Akira et al., 2006).

## Pattern-recognition receptors

In the mammary gland, PRRs are expressed in milk leukocytes and epithelial cells lining the mammary gland (Strandberg et al., 2005). One of the common PRRs is the toll-like

receptor (TLR) family, with 10 types identified in cattle (Menzies & Ingham, 2006). Some TLRs are located on cell surfaces where they can bind extracellular PAMPs such as bacteria and fungi, while others are found inside cells, where they can bind intracellular PAMPs such as viruses (Tizard, 2018). TLRs are expressed on various immune cells, including macrophages, dendritic cells, B cells, T cells, and non-immune cells such as fibroblasts and epithelial cells. In the mammary gland, these 10 TLRs are detected in tissue from the alveolar, ductal, gland cistern, and teat canal from infected and healthy quarters (Whelehan et al., 2011).

Each TLR detects distinct PAMPs derived from bacteria, viruses, mycobacteria, fungi, and parasites (Akira et al., 2006). When a PAMP binds to its corresponding TLR, signals are passed to the cell, resulting in multiprotein signaling complexes form, signal transduction cascades are initiated, and proinflammatory molecules are produced by the cell (Tizard, 2018). For example, lipopolysaccharide (LPS) from gram-negative bacteria can stimulate the TLR4 through binding with CD14 (Sohn et al., 2007). LPS binds to the LPS-binding protein (LBP) present in serum then this LPS-LBP complex is subsequently recognized by CD14. LPS stimulation is then followed by the interaction between CD14 and TLR4 in the membrane and induces the synthesis and release of pro-inflammatory cytokines (Paape et al., 2002). In this case, TLR4 may use an adaptor protein called MyD88 to activate the transcription factors, nuclear factor kappa-B (NF- $\kappa$ B). NF- $\kappa$ B activates the genes for 3 proteins, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- $\alpha$ ) transcription (Tizard, 2018).

Different TLR triggers the production of different cytokine mixtures, and different PAMP triggers distinctly different responses even within a single cell type. For example, the expression of three proinflammatory cytokines (IL-1, TNF- $\alpha$ , and IL-6) was quickly and strongly induced by the *Escherichia coli* (*E. coli*) challenge in mammary epithelial cells. In contrast, the *S. aureus* challenge did not induce the expression of IL-1 and TNF- $\alpha$  (Günther et al., 2011).

Pathogen recognition and the ability to increase a pro-inflammatory response, are the two most critical components of host innate immunity (Rainard & Riollet, 2006). A wide variety of components related to the innate immune responses have been identified in milk, including cellular defense components, such as leukocytes, and components contributing to humoral defense, such as complement system (CS), immune-modulating factors (pro- and anti-inflammatory cytokines), lactoferrin (LF), transferrin (TF), lysozyme (LZ), and components of the lactoperoxidase (LPO)/myeloperoxidase (MPO) systems, and other components such as oligosaccharides, gangliosides, reactive oxygen species (ROS), acute phase proteins (APPs),

ribonucleases, and a wide range of antimicrobial peptides (AMPs) and proteins (Alnakip et al., 2014).

### Cellular defense

Pathogens that enter the mammary gland through the teat end must then avoid the antibacterial activities of the mammary gland to establish disease. There is a variety of different types of cells in normal milk, and their activities play a vital role in the establishment of infection (Blowey & Edmondson, 2010). The total number of cells can be counted and expressed as the SCC. The milk somatic cells include 75% leukocytes, such as neutrophils, macrophages, lymphocytes, and 25% epithelial cells, and are known to be the major defense components of the mammary gland against infection (Paape et al., 2002; Sharma et al., 2011).

Neutrophils in the bloodstream are simply carried along by the flow. However, in inflamed tissues, these fast-moving cells slow down, bind to blood vessel walls, and emigrate into the tissues (Tizard, 2018). In the case of mastitis, number of neutrophils is increased and they can represent a large cellular portion in mastitic milk, up to 92% in cells of bovine milk (Paape et al., 1979). Once bacteria are attached to the neutrophil surface, they will be ingested and killed by the release of potent oxidants such as hydrogen peroxide, superoxide, and hydroxyl radicals from neutrophils (Paape et al., 2002). In addition to their phagocytic abilities, neutrophils are the source of small antimicrobial peptides, which can kill the pathogens of mastitis, such as cathelicidin-2 (Zhang et al., 2014a), and under the influence of bacterial products such as lipopolysaccharide, neutrophils produce many different cytokines such as IL-1, TNF- $\alpha$ , IL-6, IL-8 and transforming growth factor-  $\beta$  (TGF- $\beta$ ) (Tizard, 2018).

Neutrophils respond and ingest invading pathogens rapidly, but they are incapable of sustained phagocytic effort because of their short-living and limited energy. In contrast, macrophages move slower but are highly effective phagocytes, and they are generally the predominant cell type in healthy cow milk. Both macrophages and neutrophils have an essential role in phagocytosing microbial cells (Burvenich et al., 2003). Macrophages express many different PRRs to detect and respond to invading bacteria and viruses as well as tissue damage. They also produce many cytokines, the most important of these are IL-1, IL-6, IL-12, IL-18, and TNF- $\alpha$ , and also chemokines, such as IL-8, that recruit and attract neutrophils (Tizard, 2018). Besides detecting and killing pathogens and producing cytokines, macrophages also contribute directly to the repair of damaged tissues by removing dead, dying, and damaged cells and assist the healing process (Peiser et al., 2002; Wynn et al., 2013).

## Humoral defense

The activity of immune system cells is activated when PAMPs or DAMPs bind to their PRRs. As a result, they synthesize and secrete molecules that trigger inflammation, inhibit microbial growth, and initiate the first steps in adaptive immune response (Tizard, 2018). The cells of the immune system can synthesize and secrete hundreds of different proteins that control the immune response, which is called cytokines. At present, a variety of cytokines, such as interleukins (IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-12), colony-stimulating factors (CSF), interferon-gamma (IFN- $\gamma$ ), and TNF- $\alpha$  have been found in the healthy and infected mammary gland (Alluwaimi, 2004). When exposed to infectious agents, immune system cell signaling pathways activate the genes that result in the synthesis and secretion of three major cytokines, called TNF- $\alpha$ , IL-1, and IL-6. They also secrete a mixture of small chemotactic proteins called chemokines (Tizard, 2018).

**Tumor Necrosis Factor-  $\alpha$**  is primarily produced by macrophages in response to TLR stimulation. TNF- $\alpha$  can also be produced by endothelial cells, T cells, B cells, and fibroblasts. TNF- $\alpha$  is an essential mediator of inflammation, in combination with IL-1. It triggers changes in small blood vessels, thus a local increase in TNF- $\alpha$  causes the classic signs of inflammation, including heat, swelling, pain, and redness (Tizard, 2018). TNF- $\alpha$  plays an important role in mediating immune-inflammatory responses such as mastitis and endotoxic shock (Ohtsuka et al., 2001; Wenz et al., 2010). The role of TNF- $\alpha$  in the pathogenesis of mastitis caused by *E. coli* has been studied intensively. Besides initiating the host's early immune response, TNF- $\alpha$  seems to have a critical role in promoting the adherence, migration, attraction, and activation of neutrophils (Lee et al., 2003). Furthermore, TNF- $\alpha$  facilitates the transition from innate to adaptive immunity.

**Interleukin-1** is a member of a large family of cytokines that regulate the innate immune response. The most important of these are IL-1 $\alpha$  and IL-1 $\beta$ . Like TNF- $\alpha$ , IL-1 $\beta$  acts on nearby cells to initiate and amplify inflammation (Tizard, 2018). Although monocytes and macrophages are the main sources of IL-1 $\beta$ , it is also released by NK cells, B cells, dendritic cells, fibroblasts, and epithelial cells (Duque & Descoteaux, 2014). During the inflammatory response, IL-1 $\beta$  regulates the expression of adhesins by endothelial cells and neutrophil chemotaxis (Zhang & Issekutz, 2002). The dynamic production of IL-1 $\beta$  has been studied widely in *E. coli* and endotoxin-induced mastitis (Riollet et al., 2000; Strandberg et al., 2005). It has been documented that *E. coli* stimulation on mammary epithelial cells causes earlier and higher upregulation of IL-1 $\beta$  than *S. aureus* stimulation (Griesbeck-Zilch et al., 2008; Günther et al., 2011; Riollet et al., 2000).

**Interleukin-6** is a glycoprotein produced by macrophages, T cells, and mast cells, and its production is triggered by bacterial endotoxins, as well as by IL-1 $\beta$  and TNF- $\alpha$ . It promotes some aspects of inflammation, especially in response to tissue damage and severe infections, and it is a major mediator of the acute-phase reaction and septic shock (Tizard, 2018). IL-6 is involved in acute septic shock during mastitis caused by *E. coli* and *S. aureus*. It facilitates the exchange of neutrophils for monocytes in the mammary gland and is the main regulatory cytokines of acute-phase protein synthesis in hepatocytes (Ohtsuka et al., 2001; Ślebodziński et al., 2002).

**Chemokines** are a family of at least 50 small proteins which coordinate the migration of leukocytes and dictate the course of many inflammatory and immune response. They are produced by macrophages and mast cells and classified into four subfamilies based on their amino acid sequences (Tizard, 2018). One of the most important chemokines is CXCL8 (also called Interleukin-8), which is produced by macrophages and other cell types, such as epithelial cells, airway smooth muscle cells, and endothelial cells (Message & Johnston, 2004), including mammary gland epithelial and endothelial cells (McClenahan et al., 2006). IL-8 has the primary function of inducing chemotaxis to target cells, primarily neutrophils, to promote their migration toward the site of infection (Raman et al., 2011), and it is often found in acute diseases, such as mastitis, caused by *E. coli*, as well as in the chronic inflammatory phase of subclinical dry period mastitis (McClenahan et al., 2006; Watanabe et al., 2012).

**Antimicrobial molecules** are produced by the immune system cells and are associated with defense functions in concert with cellular defense; each system modifies the effector functions of the others. Antimicrobial molecules consisting of peptides, lysozyme, and complement (Tizard, 2018). In the mammary gland, non-specific antimicrobial molecules work independently and in concert with cellular factors to protect the mammary gland. These molecules include antimicrobial peptides, complement, lactoferrin, lysozyme, and lactoperoxidase (Isobe, 2017; Sordillo et al., 1997). Antimicrobial peptides are widely known and are produced in concentrated sites where microbes are most likely to be encountered.  $\beta$ -defensins, cathelicidins, and S100 proteins are intensively studied in the mammary gland.

Lingual antimicrobial peptide (LAP), first isolated from the inflamed bovine tongue, is one of the  $\beta$ -defensins and it is expressed in the epithelial cells that line various tissues, including epithelial cells of alveoli and milk duct in the mammary gland. The expression of LAP messenger RNA was reported in the mammary gland of cows, related to milk somatic cells and induced by mastitis (Isobe et al., 2009; Kawai et al., 2013; Swanson et al., 2004)



Cathelicidins are a major component of the neutrophil secondary granule and an increased level of these proteins is observed in a range of inflammatory conditions (Zanetti, 2004). Bovine cathelicidins were shown to have antimicrobial activity against bacteria obtained from bovine mastitic milk, including *E. coli*, *Klebsiella pneumoniae*, *S. epidermidis*, and *Prototheca* spp. (Tomasinsig et al., 2010). Later, several research indicated the potential role of cathelicidin as a mastitis marker in the cow as its level increased in milk following intramammary infection and highly correlated with SCC (Addis et al., 2016; Smolenski et al., 2011; Zhang et al., 2014a).

S100 proteins are a subfamily of EF-hand calcium-binding proteins that have a dimeric structure and can form oligomers (Nelson & Chazin, 1998). Several S100 proteins have been shown to play central roles in the innate immune response to infection by pathogenic organisms. Of the 25 different S100 genes, only S100A7, S100A8, S100A9, and S100A12 have been identified as innate immune modulators (Kozlyuk et al., 2019). S100A7, also known as psoriasin is a Ca-binding protein that exhibits antimicrobial activity against *E. coli* in humans (Gläser et al., 2005) and bovine (Regenhard et al., 2009). In bovines, S100 proteins are concentrated in the cornified layer of teat canal lining (Smolenski et al., 2015).

The complement system consists of a complex mixture of enzymes, regulatory proteins, and receptors that is essential for innate antimicrobial immunity. This system is activated by the presence of either PAMPs or by antigen-bound antibodies. Once activated, complement components, especially C3, the third component, bind irreversibly to bacteria and initiate bacterial killing (Tizard, 2018). C3 is synthesized locally in the mammary gland (Rainard, 2003) and the increase of C3 mRNA expression was induced by *S. aureus* and *E. coli* in mammary epithelial cells (Griesbeck-Zilch et al., 2008). Furthermore, the highest concentrations of complement are observed in mastitic milk, presumably due to the mobilization of complement components by transudation from the blood (Rainard, 2003).

Lactoferrin (LF), is an iron-binding glycoprotein, mainly produced by the secretory epithelium and leukocytes in the mammary gland (Molenaar et al., 1996). LF plays a role in mammary gland immunity through several pathways. It exerts its bacteriostatic effect by competing with bacteria for iron and makes it unavailable for bacterial growth and it also enhances the neutrophil respiratory burst (Tizard, 2018). Among mastitis-causing bacteria, *E. coli* and *S. aureus* are the most susceptible, whereas streptococci are more resistant (Chaneton et al., 2008). The concentration of LF in milk was reported to be associated with milk SCC and subclinical mastitis in cows (Hagiwara et al., 2003).

Lysozyme is one of the components of antimicrobial systems in milk. It cleaves the bond between N-acetyl-muramic acid and N-acetylglucosamine and destroys cell wall peptidoglycans in Gram-positive bacteria (Tizard, 2018). However, milk lysozyme alone is not a significant component of the mammary gland defense, and it must synergize with antibodies, complement, and LF (Rainard & Riollet, 2006).

Lactoperoxidase (LPO) system is a natural antibacterial system that has been adopted for protecting raw milk. The system consists of LPO, thiocyanate ( $\text{SCN}^-$ ), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The antibacterial effect of the system in milk is based on the oxidation of  $\text{SCN}^-$  ions catalyzed by the LPO enzyme to bacteriostatic products such as  $\text{OSCN}^-$ , in the presence of  $\text{H}_2\text{O}_2$  (Tizard, 2018). Bovine milk contains LPO and  $\text{SCN}^-$  but not normally  $\text{H}_2\text{O}_2$ . LPO is found in the mammary, salivary, and lachrymal glands of mammals and their secretions (Wolfson & Sumner, 1993). In milk, LPO is synthesized and released from mammary alveolar epithelial cells (Harada et al., 1973) and leukocytes (Shin et al., 2001).  $\text{SCN}^-$  is widely distributed in animal tissues and secretions, and in bovine milk, its concentration reflects blood serum levels and varies with breed, species, udder health, and feed (Kussendrager & Van Hooijdonk, 2000).  $\text{H}_2\text{O}_2$  is the third component of the LPO system and it may be generated endogenously, by neutrophils in the process of phagocytosis. Alternatively, many lactobacilli, lactococci, and streptococci produce sufficient  $\text{H}_2\text{O}_2$  under aerobic conditions to activate the LPO system (Wolfson & Sumner, 1993). LPO system in the mammary gland is dependent on the plane of nutrition, thus the function of the lactoperoxidase-thiocyanate-hydrogen peroxide system may be limited (Sordillo et al., 1997).

### **Adaptive immune system**

Although the innate immune system is crucial to the control of microorganism proliferation and the eradication of pathogens, it is tightly interrelated with adaptive immunity, which is fundamental to establishing a characteristic memory component that provides complete resistance to infection. The adaptive immune system recognizes specific determinants of a pathogen mainly through the antibodies molecules, macrophages, and several lymphoid populations, which subsequently facilitate selective elimination (Sordillo & Streicher, 2002).

During an adaptive immune response, molecules from invading organisms are captured, processed, and presented to the cells of the immune system. Once, the presented molecules bind to the cell surface receptors, they trigger a powerful immune response and the immune system can remember, makes some improvements, and, by adapting, responds even more

effectively when it encounters these organisms again (Tizard, 2018). In the mammary gland, both innate and adaptive immunity must be highly connected and coordinated to provide optimal protection from mastitis.

The adaptive immune system consists of two major branches; one is directed against extracellular, and the other is directed against intracellular invaders. Both branches depend upon the use of specialized white blood cells called lymphocytes. There are two major lymphocyte populations, B cells, and T cells. Immunity to extracellular invaders is mainly the function of B cells. This B-cell-mediated immune response is also known as a humoral immune response. On the other hand, immunity to intracellular invaders is the function of T-cells. This type of response is therefore called the cell-mediated immune response or cellular immune response (Tizard, 2018).

#### Humoral defense

Immunoglobulins (Igs) are the most important specific humoral factors in the adaptive immune system, and they are present in colostrum and milk (Stelwagen et al., 2009). Igs account for up to 70-80% of the total protein content in colostrum (20-150 g/L) to transfer passive immunity to newborns, whereas in the milk they account for only 1-2% of total protein (0.5-1 g/L) (Korhonen et al., 2000). The mammary gland plays an active role in regulating the levels of different Igs present in colostrum and milk, although the mammary epithelium itself does not synthesize Igs.

There are four classes of Igs that play dominant roles in mammary gland defense against pathogens; IgG1, IgG2, IgM, and IgA. The concentration of each Ig class in mammary secretion varies depending on the stage of lactation and infection status of the mammary gland. IgG and IgM act as opsonins and facilitate phagocytosis by neutrophils and macrophages, while IgA plays roles in toxin neutralization and bacterial agglutination (Paape et al., 2000). The majority of IgG are blood-derived and transported into mammary secretions between epithelial cells. IgA is secreted from B cells located in connective tissues and through epithelial cells (Hurley & Theil, 2011). During inflammatory conditions, the Ig content of both milk and colostrum increased (Stelwagen et al., 2009).

#### Cellular defense

The adaptive immune system involves two types of cells, lymphocytes, and antigen-presenting cells. The body mainly generates three specialized cell families, dendritic cells, macrophages as the antigen-presenting cells. Antigen-presenting cells are attracted by

microbial products and tissue damage and are activated by the same stimuli that trigger inflammation. Antigens from ingested bacteria are processed within macrophages and appear on the membrane in association with major histocompatibility complex (MHC) class II antigens. These MHC class II antigens are polymorphic membrane molecules that are required by lymphocytes for the recognition of foreign antigens (Tizard, 2018).

Lymphocytes are the cells responsible for adaptive immune responses and the defense of the body. Two types of lymphocytes regulate adaptive immunity, T cells that are responsible for cellular immune responses, and B cells that are responsible for antibody production. T cells can be further divided into  $\alpha/\beta$  T lymphocytes, which include CD4<sup>+</sup> (T-helper) lymphocytes and CD8<sup>+</sup> (T-cytotoxic or T-suppressor) lymphocytes, and  $\gamma/\delta$  T lymphocytes. CD4<sup>+</sup> T cells are very diverse and it differentiates into 4 major subpopulations called helper 1 (Th1), helper 2 (Th2), helper 17 (Th17), and regulatory (Treg) cells. The T-helper lymphocytes produce cytokines in response to the recognition of an antigen-MHC complex on the macrophage, and each T helper cell is distinguished by the mixture of cytokines that they secrete.

CD4<sup>+</sup> cell plays a vital role in the immune response to mastitis in dairy animals (Cao et al., 2012; Zhao & Lacasse, 2008), and an invasion of activated CD4<sup>+</sup> T cells in the udder is a typical characteristic of intramammary infection (He et al., 2011). CD4<sup>+</sup> cells produce a variety of immunoregulatory cytokines following antigen-recognition with MCH class II molecules; and are being memory cells following antigen-recognition (Sordillo & Streicher, 2002). Th1 cells produce IL-2, interferon (IFN)- $\gamma$ , and TNF- $\alpha$ . They promote cellular immune response such as the delayed hypersensitivity reaction and macrophage activation. These Th1-associated cytokines also inhibit Th2 immune response. Activated Th2 cells secrete IL-4, IL-5, IL-9, and IL-13. These cytokines stimulate B cell proliferation and immunoglobulin secretion but tend to suppress the cellular immune response. Th17 cells produce a mixture of cytokines, namely IL-17, IL-21, and IL-22. The two major functions of these cells are they mediate inflammation and they are potent B-cell helpers. Treg cells produce suppressive cytokines such as IL-10 and IL-35, which then suppress the response of both Th1 and Th2 cells and prevent inappropriate T cell activation in the absence of antigens (Tizard, 2018).

On the other hand, CD8<sup>+</sup> cells can exert either cytotoxic or suppressor functions. In coordination with major histocompatibility complex (MHC) class I molecules, CD8<sup>+</sup> recognizes and eliminates altered self-cells through antigen presentation. CD8<sup>+</sup> cells were found predominantly in milk during the early hours of bovine mastitis infected with *E. coli* (Mehrzhad et al., 2008), while its activation during bacterial intramammary infection, such as *S.*

*aureus* can suppress important host immune responses and predispose to a chronic pattern of infection (Park et al., 1993).

$\gamma\delta$  T lymphocytes play a role in antibacterial immunity and may provide a unique barrier function for the mucosal microenvironment against bacterial pathogens because their functions are primarily associated with the protection of epithelial surfaces. The  $\gamma\delta$  T lymphocytes preferentially migrate to epithelial surfaces and do not circulate extensively (Allison & Havran, 1991). Relative to blood, both humans and ruminants express greater levels of  $\gamma\delta$  T lymphocytes in mammary secretions and mammary parenchyma (Machugh et al., 1997).

### **Mammary gland immune response to *E. coli* LPS**

The clinical manifestations of mastitis may vary to different microorganisms that infect the mammary gland. One of the most important environmental bacteria that cause mastitis is *E. coli* (Barkema et al., 1998; Gao et al., 2017; Verbeke et al., 2014). Even though bovine mastitis caused by *E. coli* had a fast recovery rate, in extreme cases, it can lead to severe systemic clinical symptoms like sepsis concurrent with fever (Günther et al., 2011). And occasionally, it can result in subclinical and persistent pathology (Dogan et al., 2006). The outcome and severity of *E. coli* mastitis were mainly attributed to environmental factors and the innate immune response reacting to LPS as PAMPs. Intramammary infusion of LPS induced the same local signs as observed during *E. coli* mastitis (Burvenich et al., 2003).

LPS is the major outer membrane component of Gram-negative bacteria, composed of three distinct regions: lipid A, the core oligosaccharide, and the O-antigen polysaccharide and it is the most important bacterial virulence factor in coliform mastitis. When LPS is present in the udder, they encounter host cells, particularly, the MEC that play an important role in the innate immunity of the mammary gland.

The MEC responds by secreting chemokines (such as IL-8 and CCL20) and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) to recruit neutrophils in the mammary gland (Ohtsuka et al., 2001; Strandberg et al., 2005). The upregulation of these cytokines is mediated by LPS interaction with accessory molecules. The binding of LPS by TLR4 is greatly improved by CD14, a membrane-associated receptor found on monocytes and neutrophils. The binding of LPS to membrane CD14 (mCD14) is facilitated by an acute phase protein called LPS-binding protein (LBP), and the mCD14-LPS-LBP complex is then recognized by TLR4. Transmembrane signaling is mediated by TLR4, then cascades of activation are set in motion, leading to activation of nuclear factors such as NF- $\kappa$ B and activated protein-1 (AP-1). This

signaling pathway resulted in the expression of several pro-inflammatory cytokines and chemokines (Guha & Mackman, 2001).

The severity of the clinical course of intramammary infection by LPS has been linked to the challenging dose and cow factors (Burvenich et al., 2003; Jacobsen et al., 2004). Not only pro-inflammatory cytokines and chemokines, but LPS was also reported to affect the concentration of acute-phase proteins and plasma and milk metabolomes (Bannerman et al., 2003; Jacobsen et al., 2004; Johnzon et al., 2018). Furthermore, systemic exposure to LPS has been linked to several diseases in bovine, such as ruminal acidosis, fatty liver, coliform mastitis, retained placenta, metritis and endometritis, laminitis, displaced abomasum, milk fever, and downer syndrome (Ametaj et al., 2010; Boosman et al., 1991; Mao et al., 2013; Moyes et al., 2014; Ohtsuka et al., 2001; Williams et al., 2008a; Zebeli et al., 2011).

### **Aim of the study**

While different bacterial species have been identified as causative agents of mastitis, no bacteria proliferate in many clinical mastitis cases after enrichment for bacterial growth. Previous research reported that around 15-35% of clinical mastitis cases are culture-negative (Barkema et al., 1998; Hisaeda et al., 2016; Kawai et al., 1999; Miltenburg et al., 1996; Tolosa et al., 2015; Vasquez et al., 2019; Wedderkopp, 1997). The reasons for no growth are nonbacterial causes such as virus and injury (Tolosa et al., 2015; Wellenberg et al., 2002), low concentration of bacteria below the culture detection threshold (Kuehn et al., 2013; Vasquez et al., 2019), and no viable bacteria with the continuous inflammatory response or bacterial clearance during milk preservation period (Hisaeda et al., 2016; Koshiishi et al., 2017).

Interestingly, bacteria and LPS can translocate across the mucosal tissues, including the gastrointestinal tract (Emmanuel et al., 2007; Naaber et al., 2000), mammary gland (Dosogne et al., 2002; Wenz et al., 2001), and uterus (Credille et al., 2014; Mateus et al., 2003), into the systemic circulation. Even though, the mechanisms by which endotoxin translocates into systemic circulation are not fully understood (Eckel & Ametaj, 2020), the translocation of bacteria and bacterial components might be a potential cause of inflammation in an organ through the endogenous pathway.

Mammary gland inflammation in natural infection cases has been reported to be related to many other diseases. For instance, cows suffering from subclinical endometritis at weeks 4 and 8 postpartum had a high association with subclinical mastitis, with odds ratios of 4.5 and 3.6, respectively (Bacha & Regassa, 2010). Some research has also demonstrated that LPS derived from rumen increased the inflammatory genes expression and markers of the mammary

gland (Hu et al., 2020; Jin et al., 2016; Zebeli & Ametaj, 2009; Zhang et al., 2016), and milk metabolites such as casein and fat (Zebeli & Ametaj, 2009; Zhang et al., 2016). Furthermore, the rumen microbiota dysbiosis has been discovered as an important factor inducing mastitis in cows (Hu et al., 2020). However, direct evidence that LPS can translocate through blood circulation between organs has not been elucidated.

The goal of this study was to determine whether mastitis can be caused by bacterial components such as lipopolysaccharide through the endogenous pathway. The study in Chapter 2 aimed to assess the possible association between mammary gland inflammatory condition with the occurrence of endometritis, one of the reproductive diseases in dairy cows. The incidence of endometritis was examined related to the SCC in milk. The concentration of acute-phase proteins haptoglobin (Hp) and LBP and inflammatory parameters such as IL-1 $\beta$ , TNF $\alpha$ , IL-8 in milk were examined to determine the systemic and mammary gland inflammation.

The study in Chapter 3 aimed to determine the effect of intrauterine infusion of LPS on the mammary gland inflammatory response in the goat. LPS was infused into the uterus and the translocation of LPS to the mammary gland was examined by immunohistochemistry, while the inflammatory response was evaluated by measuring the SCC, milk yield, blood leukocyte count (BLC), the concentration of pro-inflammatory cytokines, chemokines, LBP, LF, S100A8, and LPO activity.

The study in Chapter 4 aimed to examine whether the translocated-LPS from the uterus can induce the inflammatory response in the mammary gland in the dexamethasone-treated (immunosuppressive) goat. The translocation of LPS was determined by immunohistochemistry, and the inflammatory response was evaluated by measuring SCC, milk yield, BLC, the concentration of pro-inflammatory cytokines, chemokines, LBP, serum amyloid A (SAA), LF, and S100A8.

The purpose of the study in Chapter 5 was to elucidate the movement of particles entering the uterus using carbon black ink infusion to clarify that the molecules can be translocated from the uterus to the mammary gland. The carbon black ink was infused into the uterus and the presence of carbon particles was examined histologically in tissues such as the mammary gland, liver, lung, spleen, and kidney.

A general discussion of the overall results was described in Chapter 6 to enlighten the possible translocation of LPS from the uterus to the mammary gland, and factors affecting the inflammatory response on it. Chapter 7 described a summary of the overall study.

## **Chapter 2**

### **Association of Endometritis with the Mammary Gland Inflammatory Condition in Dairy Cows**

#### **Introduction**

Reproduction is the key process supporting dairy production, as poor reproductive performance can lead to involuntary culling of dairy cows, resulting in substantial economic losses (LeBlanc, 2008). Low reproductive performance appears to be influenced by high disease incidence during the transition or periparturient period, which encompasses the period from 3 weeks prepartum to 3 weeks postpartum (Ingvartsen & Moyes, 2013). The transition period is considered to be the most critical period for dairy cows, as the endocrine and physiological changes occurring during parturition negatively affect the immune function (Goff & Horst, 1997).

The precise causes of impaired immune function in periparturient cows are unclear, but the necessary energy for lactation appears to exert an immunosuppressive effect beyond those associated with parturition itself (Kimura et al., 1999). Meanwhile, cows with greater negative energy balance supposed to have more pronounced impairment of at least some immune functions (Hammon et al., 2006), and the extent of this condition is generally related to the incidence and severity of metabolic disorders and infections (Grummer et al., 2004). In this case, clinical reproductive diseases can cause not only short-term problems such as milk yield loss (Wittrock et al., 2011) but also have long-term harmful effects on fertility. Moreover, cows that suffer periparturient diseases are more susceptible to develop other diseases (Ribeiro et al., 2013). Thus, a healthy passage through the periparturient period is essential for optimal reproductive efficiency in dairy cows.

Bacterial infection is common in the uterus of dairy cows during the early postpartum period; however, most cows eliminate these bacteria within the first 2 weeks after calving (LeBlanc et al., 2002). Persistent bacterial colonization in the uterus can lead to subsequent infections, with puerperal metritis, clinical endometritis, pyometra, and subclinical endometritis being the most common infections (Földi et al., 2006). Clinical endometritis is the most common periparturient disease, accounting for approximately 15% of the total disease prevalence (Ribeiro et al., 2013). This disease is characterized by the presence of purulent (>50% pus) or mucopurulent (50% pus and 50% mucus) uterine exudate in the vagina, 21 days or more after parturition, and it is not accompanied by any systemic signs (LeBlanc et al., 2002).



*Trueperella pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica* are the most frequently isolated bacteria from the bovine uterine lumen and endometritis-affected cows and are often associated with endometrial lesions (Williams et al., 2005). In addition to bacteria, the vaginal discharge of cows was reported to contain high concentrations of LPS, which are membrane components of Gram-negative bacteria, 1-2 days after calving (Dohmen et al., 2000). LPS derived from uterine inflammation, both during natural and induced endometritis, can increase the LPS concentration in the peripheral blood, leading to systemic inflammation (Williams et al., 2008b). Furthermore, LPSs have been reported to translocate from the uterus to the mammary gland via the bloodstream (Dosogne et al., 2002; Wenz et al., 2001), although there is no direct evidence reported.

Based on the evidence that LPS can translocate from the uterus to the circulation, this study hypothesizes that LPS can also translocate from the uterus to the mammary gland through the bloodstream. Therefore, this study was undertaken to determine the association of endometritis with mammary gland inflammation in dairy cows.

## **Materials and Methods**

### **Animals**

A total of 61 Holstein-Friesian dairy cows (bodyweight 600-750 kg, parity 1-6, 30-50 days postpartum) were included in this study. The experimental cows were housed at the experimental farm of Hiroshima University. Cows were milked twice per day at 08:00 and 15:00. Sampling was performed from June 2017 to July 2020. This study was approved and conducted in accordance with the guidelines of the Hiroshima University Animal Research Committee.

### **Identification of cows with endometritis**

Endometritis was identified by clinical examination, through vaginal inspection of vulval discharge with a speculum. For each cow, a sterilized stainless-steel speculum (40 × 5 cm) was used. To minimize contamination of the vagina, the tails of the cows were held, and the vulva and perineum were sprayed with alcohol and cleaned with dry paper towels before the tool was inserted. Every tool was moisturized with 20 mL of sterile saline solution (0.9%) before the examination.

The speculum was inserted into the vagina far enough to enable visualization of the external cervical bone and the lateral, dorsal, and ventral walls of the vagina. A flashlight was used to provide illumination. Discharge visible in the vagina was classified according to the

method of Williams et al. (2005) on a scale from 0 to 3. Clear or translucent mucus was equivalent to a score of 0 for healthy cows. A score of 1 described mucus containing flecks of white or off-white pus. A score of 2 described discharge containing less than 50% white or off-white mucopurulent material. In a score of 3, the discharge was composed of more than 50% white or yellow pus or was sanguineous. Endometritis was diagnosed as being all scores.

### **Blood and milk sampling**

Blood and milk samples were collected once for each cow at the time of the clinical examination. Blood (5 mL) was collected from the coccygeal vein into heparin-containing-vacuum tubes. Blood samples were then centrifuged at  $1,900 \times g$  for 10 min at 4 °C, and plasma was stored at -20 °C for enzyme immunoassay. Milk samples were collected by hand aseptically from active quarters of the mammary gland. Milk SCC was measured by DeLaval Cell Counter (DeLaval International AB, Tumba, Sweden). Milk samples were then centrifuged at  $1,900 \times g$  for 5 min at 4 °C. Milk fat was separated and skim milk was stored at -20 °C for enzyme immunoassay.

### **Enzyme immunoassay**

Competitive enzyme immunoassays were performed for measuring IL-1 $\beta$ , TNF $\alpha$ , and IL-8 concentrations in milk, while the concentration of Hp and LBP in plasma was measured by the sandwich method. Antibodies that target IL-1 $\beta$ , TNF $\alpha$ , and IL-8 each consisting of 12, 12, and 13 amino acids (GEERDNKIPVA, AEAKPWYEPIYQ, and AVLSRMSTELRCQ), were produced in rabbits by Scrum Inc. (Tokyo, Japan) purified using a HiTrap™ Protein G high-performance affinity column (GE Healthcare, Princeton, NJ, USA), following the manufacturer's protocol. All peptides were conjugated with horseradish peroxidase using the peroxidase-labeling kit-SH (Dojindo Molecular Technologies, Kumamoto, Japan).

The measurement of IL-1 $\beta$ , TNF $\alpha$ , and IL-8 in milk was carried out with a competitive method. A 96-well microplate was coated with 100  $\mu$ L of goat anti-rabbit IgG antibody (10  $\mu$ g/mL) as a secondary antibody diluted in carbonate buffer (a mixture of 0.05 M Na<sub>2</sub>CO<sub>3</sub> and 0.05 M NaHCO<sub>3</sub> diluted in ddH<sub>2</sub>O and adjusted to pH 9.7). For IL-8 measurement, the plate was directly coated with 100  $\mu$ L of rabbit anti-bovine IL-8 antibody (1  $\mu$ g/mL) diluted in carbonate buffer. The plate was then incubated at 20°C for 2 h. After incubation, the plate was washed with phosphate buffer saline (a mixture of 0.01 M of Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O, 0.01 M of KCl, 0.006 M of KH<sub>2</sub>PO<sub>4</sub>, and 0.55 M of NaCl) added with 0.02% of Tween detergent (PBST) to remove the excess antibody. The plate was then incubated for 30 min 20°C with 150  $\mu$ L of

assay buffer to block the nonspecific binding of assay reactants to the surface of the well. The assay buffer contained 0.05 M Boric acid adjusted at pH 7.8, 0.1% of Bovine Serum Albumin, and 0.05% Proclin 300. After blocking, 100  $\mu$ L of the rabbit anti-bovine IL-1 $\beta$  or TNF $\alpha$  antibodies (0.2  $\mu$ g/mL and 4  $\mu$ g/mL, respectively) were added to the wells and incubated for 2 h at 20°C. Fifty microliters (50  $\mu$ L) of standard and sample (samples dilution  $\times$ 1,000 for IL-1 $\beta$ , TNF $\alpha$ , and  $\times$ 50 for IL-8) were added to each plate in duplicate and triplicate wells, respectively, after washing the plate twice with PBST. Standard and samples were incubated for 3 h at 20°C. For IL-8 measurement, standard and sample were incubated with 50  $\mu$ L of horseradish peroxidase (HRP) conjugated IL-8 and incubated for 4 h at 20°C. Antibody, standard, and sample were diluted with the assay buffer. Fifty microliters (50  $\mu$ L) of HRP conjugated IL-1 $\beta$  and TNF $\alpha$  were added to the well without washing and incubated for 1 h at 20°C. One hundred and fifty microliters (150  $\mu$ L) of tetramethylbenzidine substrate solution (TMB with 0.1% hydrogen peroxide-urea) was added after washing the plate 3 times and incubated for 30 min at 20°C. Finally, the enzymatic reaction was stopped by adding 30  $\mu$ L of stop solution 2 N H<sub>2</sub>SO<sub>4</sub>. The optical density was measured using a microplate reader (Multiskan FC Microplate type 357, Thermo Fisher Scientific Co. Ltd., Tokyo, Japan) with a wavelength of 450 nm.

The measurement of Hp and LBP was conducted using the sandwich method. One hundred microliters (100  $\mu$ L) of the capture antibody (goat anti-bovine Hp IgG, GTX77180, 16 ng/mL), was coated in the 96-well microplate and incubated at 20°C for 2 h. The plate was washed twice with PBST, blotted dry, and blocked with 150  $\mu$ L of assay buffer for 30 min at 20°C. After blocking, the plate was then incubated for 2 h at 20°C with 100  $\mu$ L of standard and sample (sample dilution  $\times$ 30) in assay buffer. The plate was washed thrice, blotted dry, and incubated with 100  $\mu$ L of the detection antibody (rabbit anti-bovine Hp IgG, 20 ng/mL) for 2 h at 20°C. The plate was washed thrice and incubated for 1 h at 20°C with HRP-labeled anti-rabbit IgG, diluted 1: 200 in assay buffer. After washing the plate thrice and blotting it dry, 150  $\mu$ L of TMB substrate solution was added and incubated for 30 min at 20°C. At the end of the 30 min, 30  $\mu$ L of stop solution 2 N H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The absorbance was then read at 450 nm using the microplate reader.

The measurement of LBP was carried out with a streptavidin-biotin detection system. A 96-well microplate (H-type) was coated with 100  $\mu$ L of mouse anti-human LBP antibody (Hycult Biotech Inc., Wayne, PA, USA, 50 ng/mL) as capture antibody and incubated at 20°C for 3 h. The plate was washed thrice with PBST, blotted dry, and incubated with Tris Buffer Saline (TBS, a mixture of 50 mM of Tris adjusted at pH 7.8, 0.14 M NaCl, and 0.05% of

Tween) for 30 min at 20°C. After blocking, the plate was incubated with 100 µL of standard and sample (sample dilution ×50) in TBS at 20°C for 3 h. The plate was washed thrice with PBST and then incubated with 100 µL of biotinylated rabbit anti-bovine LBP antibody (Cloud Clone Corp. Katy, TX, USA, 50 ng/mL) as detection antibody for 2 h at 20°C. The plate was washed thrice with PBST and incubated with 100 µL of Streptavidin-HRP (R&D Systems Inc., Minneapolis, MN, USA) for 30 min at 20°C to bind biotin and produce enzymatic activity necessary for substrate detection. Finally, the plate was washed thrice with PBST, blotted dry, and incubated with 150 µL of TMB substrate solution for 30 min at 20°C. After 30 min, 30 µL of stop solution 2 N H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The absorbance was then read at 450 nm using the microplate reader.

Sample dilutions (1,000 times for IL-1β and TNFα, 50 times for IL-8 and LBP, and 30 times for Hp) yielding optical density readings in the linear portion of the appropriate standard curve were used to quantify the level of each protein. Curve fit statistics software (SkanIt RE for Multiskan GO 3.2, Thermo Fisher Scientific Co. Ltd., Tokyo, Japan) was used to plot a four-parameter logistic curve to fit the standard and aggregate the results of the test sample.

### **Statistical analysis**

Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). One-way ANOVA followed by Tukey's test was used to compare milk SCC (average and maximum values); milk IL-1β, TNFα, and IL-8 concentrations; and plasma Hp and LBP concentrations of cows with endometritis and healthy cows. The chi-squared ( $\chi^2$ ) test was used to compare the number of cows (percentage) with different SCC levels (low, moderate, high) among the two tested groups (endometritis and healthy).

### **Results**

Of the 61 cows included in this study, endometritis was detected in 12 cows. Following the criteria proposed by Williams et al. (2005), based on the characteristics of vaginal discharge, 9 cows were assigned a score of 1, and 3 cows were assigned a score of 2. The remaining cows (n = 49) were healthy, i.e., without endometritis. The SCC parameters included average and maximum SCCs for all quarters of each cow. The average and maximum SCC were not significantly different between groups (Fig. 1A).

For a more detailed examination of the association between the health condition and SCC, the SCCs were categorized as low (<300 × 10<sup>3</sup> cells/ml), moderate (300-1,000 × 10<sup>3</sup> cells/ml), or high (>1,000 × 10<sup>3</sup> cells/ml). The percentage of cows in each group (healthy and

endometritis) was analyzed based on the SCC category. Based on the average SCC values, the percentage of cows within each SCC category (low, moderate, and high) was not remarkably different among the healthy and endometritis (Fig. 1B). However, based on the maximum SCC values, the percentage of cows with endometritis was significantly lower than that of healthy cows in the low-SCC category ( $P = 0.015$ , Fig. 1C).

The concentrations of IL-1 $\beta$  and TNF- $\alpha$  in milk were not significantly different between groups (Fig. 2A and B), whereas, the concentration of IL-8 in milk was significantly lower in cows with endometritis than in healthy cows ( $P = 0.035$ , Fig. 2C). Conversely, cows with endometritis presented the highest LBP and lowest Hp concentrations, whereas no significant difference was detected among the tested groups (Fig. 3).

## Discussion

Several studies have reported the relationship between mastitis and reproductive performance in dairy cows, including longer intervals to conception, a higher probability of embryonic loss and abortion, and failure to conceive after the first service (Santos et al., 2004; Schrick et al., 2001; Wolfenson et al., 2015). However, little is known about the relationship between mastitis and other reproductive diseases. In a previous study, subclinical endometritis was found to be directly associated with subclinical mastitis in dairy cows both 4 and 8 weeks postpartum. This association might be attributed to the translocation of bacteria or bacterial products (Bacha & Regassa, 2010).

In the present study, of the 61 cows, 12 cows were diagnosed with endometritis. The prevalence of clinical endometritis in Japanese dairy herds has previously been reported. Two previous studies reported a clinical endometritis prevalence of 23.6% and 32.6%, within 60 days postpartum (Gautam et al., 2010; Gautam et al., 2009), whereas another study reported a prevalence of 26.6% from 21 to 60 days postpartum (Okawa et al., 2017). The prevalence of endometritis in the present study was recorded as 20.3%, which is lower than the previously reported values.

Somatic cell count was divided into average and maximum SCC. The average SCC was determined to represent the SCC of cows in each group (healthy and endometritis) while the maximum SCC was determined to represent the mastitis status of each individual cow since mastitis usually occurred in at least one-quarter of the mammary gland and do not affect the other quarters. The average and maximum SCC of cows with endometritis were not significantly different from those of healthy cows. However, when the maximum SCC was

classified as  $<300$ ,  $300-1,000$ , or  $>1,000 \times 10^3$  cells/ml, the percentage of cows with endometritis with the maximum SCC  $< 300 \times 10^3$  cells/ml was significantly lower than that of healthy cows. This result indicated that cows with endometritis had higher SCCs than healthy cows. However, the percentage of cows with endometritis with average SCCs  $< 300 \times 10^3$  cells/ml was not significantly different from that of healthy cows. Mastitis generally occurs in only one udder; therefore, the average SCC of the 4 udders does not reflect the occurrence of mastitis in any one udder. This explains why only the maximum SCC values in the low-SCC category were significantly different between the groups. Collectively, these results suggest a link between endometritis and mastitis; however, the causal relationship remains unknown.

Bacha and Regassa (2010) reported that cows suffering from subclinical endometritis at weeks 4 and 8 postpartum had a high association with subclinical mastitis, indicated that LPS translocates from the uterus to the mammary gland. However, there is no information that LPS was translocated from the mammary gland to the uterus in this study.

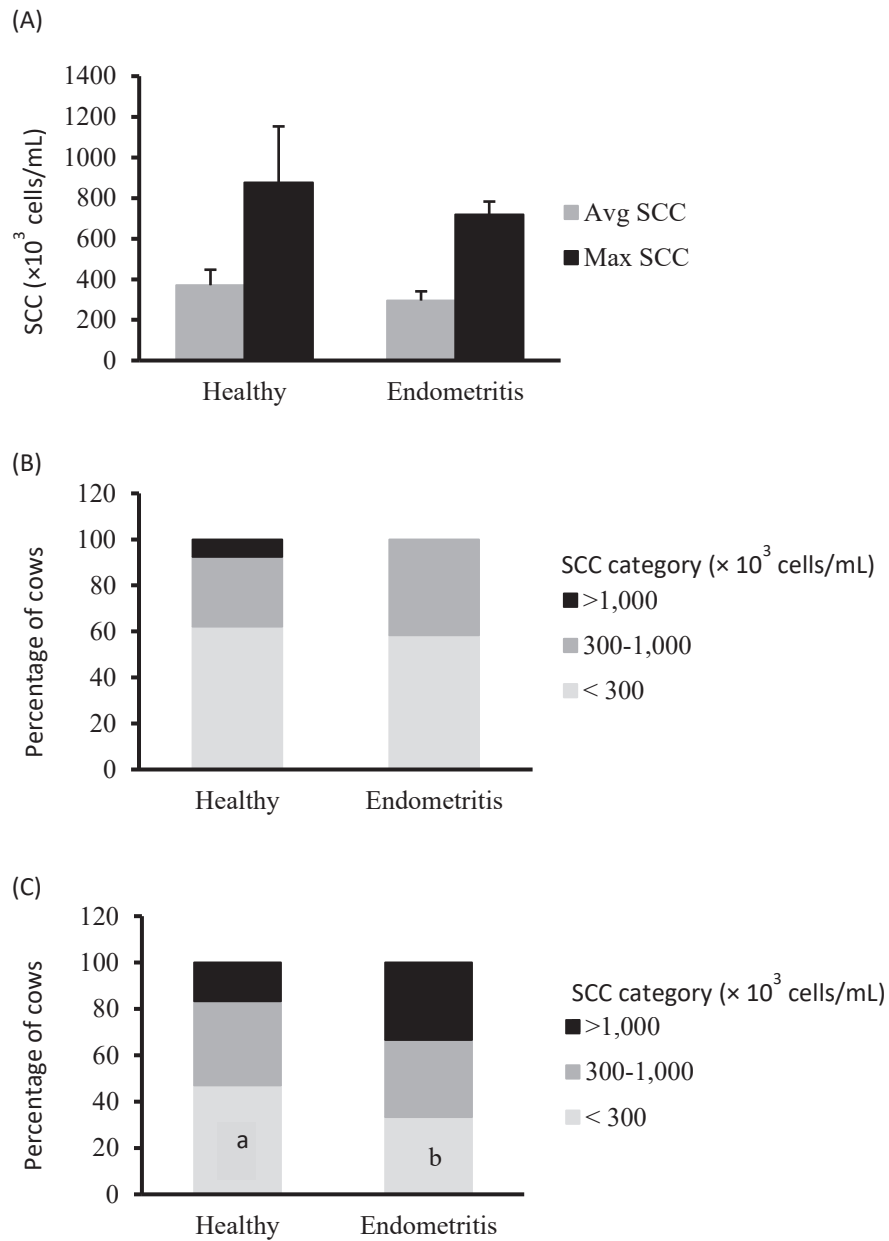
Milk IL-8 concentration of cows with endometritis was significantly lower than that of healthy cows. IL-8 is a chemokine that recruits leukocytes to the mammary gland. Numerous leukocytes are recruited to the udder in cows with endometritis. The reason for the low IL-8 concentration in the milk of cows with endometritis is not clear, although other chemokines, such as members of the C-C motif ligand (CCL) family, may be involved.

Plasma Hp and LBP concentrations were lower and higher, respectively, in cows with endometritis than in healthy cows, albeit the difference was not significant. Acute-phase proteins (APP), such as Hp, are produced in the liver and act as systemic markers during infection and inflammation in some organs. LBP is a soluble acute-phase protein that binds to bacterial LPS to elicit an immune response by presenting the LPS to important cell surface pattern recognition receptors, i.e., cluster of differentiation 14 (CD14) and toll-like receptor 4 (TLR4) (Paape et al., 2002). These observations showed that endometritis induced no or only mild systemic inflammation in the present study.

In conclusion, these results suggest that high SCC might be related to the occurrence of endometritis in dairy cows, indicating that some bacterial components can be transferred between the uterus and mammary gland.

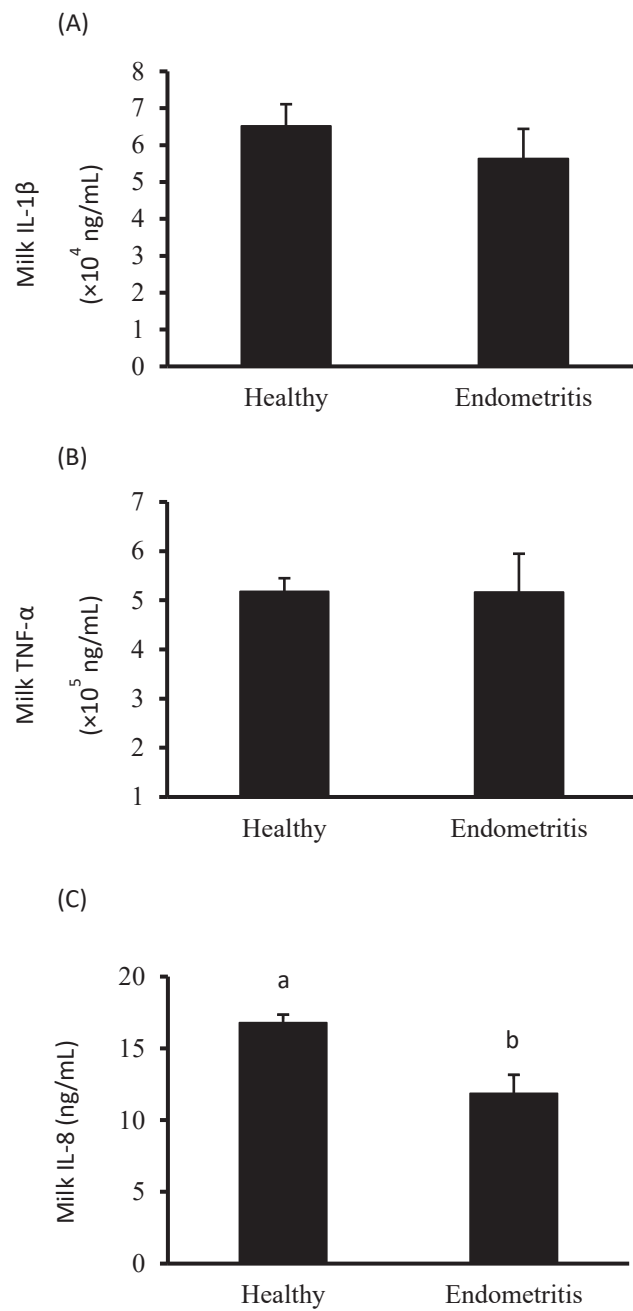
## Abstract

The occurrence of multiple metabolic and inflammatory diseases in dairy cows is higher during the periparturient period, which may be triggered by bacterial components, but not a viable bacterium. This study aimed to determine the association of endometritis with mastitis in dairy cows. Sixty-one Holstein dairy cows were clinically examined for endometritis approximately 30-50 days after calving. Blood and milk samples were collected for the determination of milk somatic cell count (SCC); milk interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and interleukin-8 (IL-8) concentrations; and plasma haptoglobin (Hp) and lipopolysaccharide-binding protein (LBP) concentrations. Of the 61 dairy cows included in this study, 12 were diagnosed with endometritis, whereas the remaining 49 cows were healthy. The average and maximum SCCs and plasma Hp and LBP concentrations were not significantly different between the healthy cows and those with endometritis. However, when the maximum SCC was classified as <300, 300-1,000, or >1,000  $\times 10^3$  cells/ml, the percentage of cows with the maximum SCC < 300  $\times 10^3$  cells/ml was significantly lower in the endometritis group than in the healthy group. These results suggested that high SCC might be related to the occurrence of endometritis, indicating that some bacterial components can be transferred between organs.

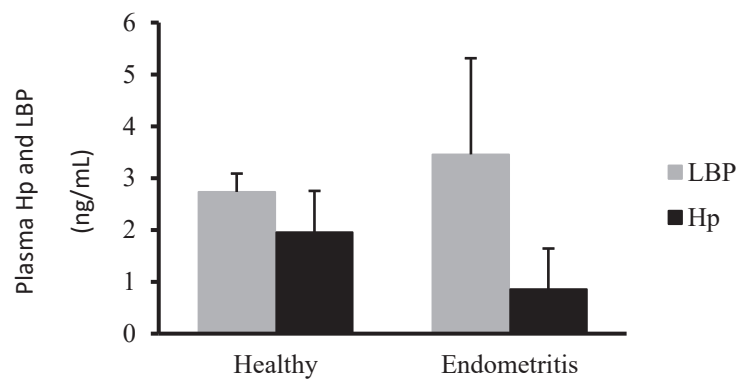


**Figure 1.** Average and maximum (A) milk somatic cell count (SCC) of post-partum dairy cows and percentage of cows with different levels of average (B) and maximum (C) SCC (low: <300, moderate: 300-1,000, high: >1,000  $\times 10^3$  cells/ml) in the two groups (healthy, n = 49; endometritis, n = 12). Values are expressed as mean  $\pm$  SEM. Values with different letters over the bars are significantly different ( $P < 0.05$ ).





**Figure 2.** Milk IL-1 $\beta$  (A), TNF $\alpha$  (B) and IL-8 (C) concentrations of postpartum dairy cows in the two groups (healthy, n = 49; endometritis, n = 12). Values are expressed as mean  $\pm$  SEM. Values with different letters over the bars are significantly different ( $P < 0.05$ ).



**Figure 3.** Plasma haptoglobin (Hp) and lipopolysaccharide-binding protein (LBP) concentrations of postpartum dairy cows in the two groups (healthy, n = 49; endometritis, n = 12). Values are expressed as mean  $\pm$  SEM.

## Chapter 3

### Effects of Intrauterine Infusion of Bacterial Lipopolysaccharides on the Mammary Gland Inflammatory Response in Goats

#### Introduction

Mastitis is one of the gravest diseases affecting dairy herds worldwide, in terms of animal health and economic importance, because it is often associated with reduced milk yield and the occasional, involuntary culling of affected animals. The annual incidence of clinical mastitis in dairy cows and small ruminants is generally lower than 5%, however, subclinical mastitis is 15-40 times more prevalent than clinical mastitis, although its prevalence varies among the countries (Bergonier & Berthelot, 2003; Leitner et al., 2004; Leitner et al., 2007; Seegers et al., 2003).

In sporadic cases of clinical mastitis in cows, sheep, and goats, *S. aureus* appears to be the most prevalent pathogen reported, while in subclinical mastitis, coagulase-negative staphylococci are frequently detected (Contreras et al., 2007; Dore et al., 2016; Zhao et al., 2014). Other pathogens, such as *Streptococcus* spp., Enterobacteriaceae spp., *Pseudomonas aeruginosa*, *Mannheimia haemolytica*, *Corynebacterium* spp., and fungi, can also cause mastitis in ruminants (Bergonier & Berthelot, 2003; Contreras et al., 2003; Gonzalo et al., 2004). However, in around 15-35% of clinical mastitis cases, no viable bacteria have grown after cultivation (Barkema et al., 1998a; Hisaeda et al., 2016a; Kawai et al., 1999; Miltenburg et al., 1996b; Wedderkopp, 1997).

The high percentage of culture-negative samples can be explained by a low concentration of pathogens in the mammary gland, uncommon pathogens, such as mycoplasma and viruses, or difficulties associated with culturing yeasts and mold (Wellenberg et al., 2002). Alternatively, pathogens might have been killed by the leukocytes and antimicrobial peptides during the preservation of milk, resulting in culture-negative (Hisaeda et al., 2016; Koshiishi et al., 2017) as milk contains several antimicrobial peptides that contribute to innate immune functions in the mammary gland (Isobe et al., 2009a, b, c; Isobe, 2017). However, these presumptions are still debatable because of the limited number of studies.

Previous research has demonstrated the possible relationship between mastitis and other diseases. Cows suffering from subclinical endometritis at weeks 4 and 8 postpartum had a high association with subclinical mastitis, with odds ratios of 4.5 and 3.6, respectively (Bacha & Regassa, 2010). Long-term feeding on a high-concentrate diet can cause rumen acidosis, which

is associated with the inflammatory and immune responses in the mammary glands of lactating cows (Hu et al., 2020; Jin et al., 2016; Zebeli & Ametaj, 2009; Zhang et al., 2016). These results strongly suggest that even bacterial components, in addition to completely viable bacteria, can induce inflammation in the mammary gland.

Postpartum uterine bacterial infections, such as metritis and endometritis, are common in dairy herds and can lead to enormous economic losses and infertility (LeBlanc et al., 2002). Gram-negative bacteria, such as *E. coli*, are the main cause of uterine inflammation, which is largely associated with bacterial LPS (Sheldon et al., 2002). LPS have been detected in the uterine fluid, plasma, and follicular fluid of cows with endometritis, suggesting that LPS can be translocated from the uterus to the ovary via the bloodstream (Dohmen et al., 2000; Herath et al., 2007; Magata et al., 2015; Mateus et al., 2003).

The previous chapter (Chapter 2) suggests the possible association between endometritis and mastitis. However, there is no direct evidence that LPS is translocated from the uterus to the mammary gland. Therefore, Chapter 3 aimed to determine the effects of intrauterine infusion of LPS on the mammary gland inflammatory response to clarify the possibility of bacterial components translocation from the uterus to the mammary gland.

After LPS reaches the mammary gland, inflammation may occur. At the same time, antimicrobial peptides (AMPs) are also upregulated in the mammary gland by LPS stimulation. Antimicrobial peptides are the components of the innate immune system that possess antimicrobial activity against a wide range of pathogens (Bahar & Ren, 2013). The expression and role of some AMPs were demonstrated in the mammary gland: such as lingual antimicrobial peptide (Isobe et al., 2009a, b; Swanson et al., 2004), lactoferrin (Huang et al., 2012; Kawai et al., 2013), psoriasin (Regenhard et al., 2010; Zhang et al., 2014a), and cathelicidins (Smolenski et al., 2011; Srisaikham et al., 2016; Zhang et al., 2014b), which play important roles in host mammary gland defense system.

S100A8 (Calgranulin A) is another family of AMPs, belonging to the S100 protein group, which exerts an antimicrobial activity by chelating several ions required for microbial growth (Corbin et al., 2008). This protein became an interesting research focus concerning its abundant concentration in the cytosol and calcium-binding capacity in phagocytes (Kerkhoff et al., 1998). In human and mouse, S100A8 and S100A9, are mainly expressed in neutrophils, together constituting approximately 45% of neutrophils in the cytosol (Edgeworth et al., 1991), one or both can be induced in other types of cells, such as monocytes or macrophages (Hsu et al., 2005; Xu et al., 2001), endothelial cells (McCormick et al., 2005), and keratinocytes (Eckert et al., 2004). Some in vitro experiments report calgranulin as one of the most upregulated genes

related to several inflammatory diseases and inflammation-associated cancers (Gebhardt et al., 2006; Srikrishna, 2011). Furthermore, the production and secretion of calgranulins were stimulated by various pro-inflammatory factors, such as IFN- $\gamma$  and TNF- $\alpha$  (Mork et al., 2003), IL-1 $\alpha$  (Hayashi et al., 2007), IL-10 (Hsu et al., 2005), while TGF- $\beta$  reduced it (Hayashi et al., 2007).

In ruminants, gene expression of S100A8 was detected in milk somatic cells in response to *Staphylococcus aureus* infection of mammary glands in goats (Cremonesi et al., 2012) and *Streptococcus uberis* infection in sheep (Addis et al., 2013), with a log-fold increase of 3.4 and 1.86 after the infection, respectively. S100A8 is one of the S100 proteins observed throughout the epithelial layer of the epidermis in which keratinocytes are the predominant cell type (Smolenski et al., 2015). However, the localization and function of S100A8 in the mammary gland is not well-known. Therefore, the preliminary experiment (Experiment 1) was undertaken before the main experiment (Experiment 2), to examine whether S100A8 can be used as a parameter of mammary gland inflammation.

## **Materials and Methods**

### **Experiment 1. Production of S100A8 and its upregulation by lipopolysaccharide stimulation**

#### **Animals**

Sixteen Tokara goats (bodyweight 20-25 kg, parity 1-4, mid-lactation stage, milk yield 300-800 mL/day) were used in this study. Goats were fed 0.6 kg of hay cubes and 0.2 kg of barley per day and had free access to water and a trace mineralized salt block. Diets were offered twice daily, at 08:00 and 15:00. Five goats were used for mammary gland tissues, milk somatic cells, and blood leukocytes collection for S100A8 mRNA expression by PCR. Three goats were used as udder models, unilateral LPS intramammary infused, and then sacrificed for tissue collection from both sides. The other 8 goats were intramammary infused with LPS and examined for S100A8 concentration in milk until 144 h after infusion. Only goats with low SCC ( $< 1,000 \times 10^3$  cells/mL) in milk were selected for the LPS challenge. Normal SCC in goat milk varies between  $270 \times 10^3$  and  $2,000 \times 10^3$  cells/mL (Paape et al., 2001), which is higher than in other species because goat milk secretion is apocrine (Souza et al., 2012). This study was conducted following the guidelines for animal experiments, Hiroshima University, Japan.

### **Collection of blood and milk samples**

Ten milliliters of blood was collected from the jugular vein of goats and transferred to an anticoagulated tube with heparin and centrifuged at  $1900 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Plasma was removed from the tube, buffy coat was transferred into a new tube, and ddH<sub>2</sub>O was added to rupture red blood cells, leaving leukocytes (Zhang et al., 2014b). Milk was collected from goat udder by hand-milking and centrifuged at  $1677 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Milk fat and skim milk were removed, leaving milk somatic cells (Nishikawa et al., 2018). Skim milk was stored for enzyme immunoassay, and a part of the cell pellet was added with PBS to resuspended for measurement of SCC with Countess<sup>®</sup> Automated Cell Counter (Life Technologies Japan Co., Ltd., Tokyo, Japan) following the previous method (Nishikawa et al., 2018). Blood leukocytes and milk somatic cells from uninfused goats were used for mRNA expression of S100A8 by PCR and protein localization by immunohistochemistry. To identify the S100A8 protein in milk somatic cells, the milk somatic cells pellet was homogenized in 1 mL of PBS for 1 min. After centrifugation, the resulting supernatant was used for enzyme immunoassay.

### **Collection of mammary gland tissues**

Mammary gland tissues, including the parenchyma and teat, were obtained within 20 min after euthanasia by exsanguination under sedation with Xylazine (Bayer HealthCare, Osaka, Japan) and anesthesia with Pentobarbital (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan). The tissues were frozen at  $-80^{\circ}\text{C}$  until the examination of mRNA expression of S100A8 by reverse-transcription-polymerase chain reaction (RT-PCR) or fixed in neutralized 10% (v/v) formalin in PBS for protein localization by immunohistochemistry.

### **Quantitative reverse-transcription (RT)-PCR analysis**

Total RNA of mammary gland tissues, blood leukocytes, and milk somatic cells were isolated with the Sepasol-RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan). One mL of Sepasol-RNA I Super G was added to the tube containing a piece of tissue, blood leukocytes, or milk somatic cells. The tube was homogenized and left at rest for 5 min, then 200  $\mu\text{L}$  of chloroform was added into it and mixed. The tube was left at rest for 3 min at room temperature and then centrifuged by  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The aqueous phase was transferred into a new tube carefully, and it was added by 500  $\mu\text{L}$  of 2-propanol, mixed, and kept at rest for 10 min. The tube was then centrifuged by  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  and the supernatant layer was discarded. One to 2 mL of 75% ethanol was added to the precipitation and stirred. The tube was centrifuged by  $12,000 \times g$  for 5 min at  $4^{\circ}\text{C}$  and the supernatant layer was removed.

The extracted total RNA samples were air-dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and kept at -80 °C until use.

The RNA samples were treated with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) on a PTC-100 programmable thermal controller (MJ Research, Waltham, MA, USA), programmed at 37°C for 45 min and 65°C for 10 min, as reported previously (Zhang et al., 2014a, b). The quality and quantity of the RNA were measured with the spectrophotometer Gene Quant pro serial 80-2114-98 (Biochrome Ltd., Cambridge, England). The quality of RNA was assessed by examining the RNA ratio (260/280). The ratio of RNA 260/280 was: mammary gland tissues ( $1.994 \pm 0.034$ ); teat tissues ( $2.096 \pm 0.026$ ); blood leukocytes ( $1.959 \pm 0.036$ ) and milk somatic cells ( $1.837 \pm 0.079$ ). The number of biological replicates was 5 for each sample.

Purified RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). Total RNA isolation and cDNA synthesis followed the manufacturer's instructions. The reaction mixture (10  $\mu$ L) consisted of 1  $\mu$ g of the total RNA, 2  $\mu$ L of 1  $\times$  RT buffer, 1  $\mu$ L of 1 mM dNTP mixture, 0.125  $\mu$ L of 20 U of RNase inhibitor, 0.25  $\mu$ L of 0.5  $\mu$ g of oligo (dt) 20, and 0.5  $\mu$ L of 50 U of ReverTra Ace. The reverse transcription was performed at 42°C for 30 min, followed by heat inactivation for 5 min at 99°C using the PTC-100 programmable thermal controller.

PCR amplification was carried out in a programmable thermal controller using a 20  $\mu$ L reaction mixture containing 0.5  $\mu$ M specific primers, 1  $\mu$ L aliquot of cDNA, 4  $\mu$ L of 1  $\times$  KAPATaq Extra Buffer, 2  $\mu$ L of 2 mM MgCl<sub>2</sub>, 0.6  $\mu$ L of 0.3 mM deoxyribonucleotide triphosphate mixture, and 0.1  $\mu$ L of 1.25 U KAPATaq Ex DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA). Target gene expression was examined by PCR using S100A8 goat-specific primers as 5'-AAGGGAATTACCACGCCGTC-3' for forward and 5'-TGTGAATGTCTTCATGGGCTGC-3' for reverse (accession No.: XM\_005677513). The same primer pairs described above were used for sequencing. Amplified PCR products were used for the direct sequencing method with the 3130xl Genetic Analyzer (Applied Biosystems, California, CA, USA) and confirmed to be identical to the goat S100A8 sequence reported in NCBI (<https://www.ncbi.nlm.nih.gov>), as described in Figure 4.

The PCR reaction was conducted under the following conditions: denaturation cycle at 94°C for 2 min, 35 cycles at 94°C for 15 s, 53°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 60 s. The expression of the  $\beta$ -actin gene was analyzed as an internal control for reaction efficiency. The primers for the  $\beta$ -actin gene were 5'-CATCACCATCGGCAATGAG-3' for forward and 5'-CCGTGTTGGCGTAGAGGTC-3' for

reverse (Zhang et al., 2014a, b). The PCR volume and reaction of  $\beta$ -actin were identical to the steps described above. The PCR products were separated electrophoretically on a 2% (wt/vol) agarose gel containing 1  $\mu$ g/mL ethidium bromide and were examined under UV illumination.

### **Immunohistochemistry**

Mammary gland tissues were fixed, dehydrated, and then embedded in paraffin in the usual manner. Sections of 3  $\mu$ m thick were air-dried on slides coated with aminopropyl triethoxysilane. After deparaffinization, antigen retrieval was performed by incubating sections with 20  $\mu$ g/mL Proteinase K for 30 min at 37°C. Sections were washed with PBS for 10 min and then incubated with the rabbit anti-S100A8 antibody (10  $\mu$ g/mL) in PBS for 3 h at 37°C. Sections were washed with PBS for 10 min. Immunoreaction products were identified using peroxidase-labeled goat anti-rabbit IgG antibody (MAX-PO-multi Histofine, Nichirei, Tokyo, Japan) for 1 h at 37°C, and then visualized by incubating the sections with the diaminobenzidine reaction mixture (a mixture of 3, 3'-diaminobenzidine-tetrahydrochloride with 0.05 M Tris buffer at pH 7.6 and 0.3% hydrogen peroxide). Sections were counterstained with hematoxylin, dehydrated, and covered. The normal rabbit IgG at 10  $\mu$ g/mL was used to replace the anti-S100A8 antibody for the negative control. S100A8 antibody was prepared in our laboratory by immunizing rabbits against synthetic S100A8 peptide consisting of 10 amino acids (CLEAHEIHKE).

Immunohistochemistry was also conducted to investigate the protein localization and define the number of positive cells of S100A8 in blood leukocytes and milk somatic cells from uninfused goats and milk somatic cells from infused goats. A total of 5 sample pairs (blood leukocytes and milk somatic cells) were taken from uninfused goats and 8 milk somatic cells samples from the infused goat at 0-144 h after LPS infusion. Cells were attached to slides and dried overnight. The slides were fixed with neutralized 10% formalin for 30 min. Cells were washed with PBS for 10 min and then incubated with the rabbit anti-S100A8 antibody (10  $\mu$ g/mL) in PBS for 3 h at 37°C. Sections were washed with PBS for 10 min. Immunoreaction products were identified using goat anti-rabbit IgG antibody conjugated to alkaline-phosphatase (SeraCare, Milford, MA, USA) for 1 h at 37°C, and then visualized by incubating the sections with ImmPACT Vector Red substrate kit (Vector Laboratories, Burlingame, CA, USA). Cells were counterstained with hematoxylin and then covered.



### **Image analysis for S100A8 frequencies**

For the S100A8-stained section, 5 images of blood leukocytes and milk somatic cells from uninfused goats and images of 8 milk somatic cells samples from infused goats were identified and imaged. Images were acquired with an image analysis software (NIS-Elements D Ver4.00.12, Nikon, Tokyo, Japan) affixed to a light microscope (Eclipse E400, Nikon, Tokyo, Japan) with a 40× objective. Acquired images were analyzed by counting the number of positively stained S100A8 cells in an area of  $7 \times 10^5 \mu\text{m}^2$  and only immune cells were included in the count total. Cells were simultaneously classified as being either positively or negatively stained for S100A8. This approach resulted in 100 cells being classified, and these data were used to calculate the percentages of S100A8-positive cells for each sample.

### **Intramammary infusion of LPS**

The concentration of S100A8 was measured in milk before and after the infusion of LPS in the mammary glands of eight goats. After milking completely, LPS from *Escherichia coli* O111: B4 (Wako Pure Chemical, Osaka, Japan) at a dose of 100  $\mu\text{g}$  dissolved in 5 mL of saline was infused into the mammary gland through teat canal, as described in previous researchers (Huang et al., 2012; Isobe, et al., 2009), while the contralateral gland was uninfused (control). Milk samples were collected from both glands by hand-milking before infusion (0 h) and at 12, 24, 48, 72, 96, 120, and 144 h after infusion and then centrifuged. Skim milk samples were stored at  $-20^\circ\text{C}$  and then used for measuring the S100A8 concentration by enzyme immunoassay.

### **Enzyme immunoassay**

A competitive enzyme immunoassay for S100A8 was performed using the S100A8 antibody as reported previously (Elgawish et al., 2018). A 96-well microplate was coated with 100  $\mu\text{L}$  of goat anti-rabbit IgG antibody (10  $\mu\text{g}/\text{mL}$ ) as a secondary antibody diluted in carbonate buffer and then incubated at  $20^\circ\text{C}$  for 2 h. After incubation, the plate was washed with PBST twice. The plate was then incubated for 30 min  $20^\circ\text{C}$  with 150  $\mu\text{L}$  of assay buffer. After blocking, 100  $\mu\text{L}$  of the rabbit anti-goat S100A8 IgG (0.5  $\mu\text{g}/\text{mL}$ ) was added to the wells and incubated for 2 h at  $20^\circ\text{C}$ . Standard and sample (50  $\mu\text{L}$ , sample dilution  $\times 10,000$ ) diluted with assay buffer were added to each plate in duplicate and triplicate wells, respectively, after washing the plate twice with PBST. Standard and samples were incubated for 3 h at  $20^\circ\text{C}$ . Fifty  $\mu\text{L}$  (50  $\mu\text{L}$ ) of S100A8-HRP conjugate was then added to the well without washing the plate and incubated for 30 min at  $20^\circ\text{C}$ . S100A8 peptide was conjugated with horseradish peroxidase

using the peroxidase-labeling kit-SH (Dojindo Molecular Technologies, Kumamoto, Japan). One hundred and fifty microliters (150  $\mu$ L) of TMB was added after washing the plate 3 times and incubated for 30 min at 20°C. Finally, the enzymatic reaction was stopped by adding 30  $\mu$ L of stop solution 2 N H<sub>2</sub>SO<sub>4</sub>. The optical density was measured using a microplate reader with a wavelength of 450 nm. Curve fit statistics software (SkanIt RE for Multiskan GO 3.2, Thermo Fisher Scientific Co. Ltd., Tokyo, Japan) was used to plot a four-parameter logistic curve to fit the standard and aggregate the results of the test sample.

### **Statistical analysis**

Statistical analysis was completed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). The differences in the percentage of S100A8-positive cells in blood leukocytes and milk somatic cells were checked by the paired-TTEST procedure. The percentage and the total number (percentage of S100A8-positive cells multiplied by the number of SCC) of S100A8-positive cells in milk after LPS infusion was reported as fold change, comparing to the baseline value at 0 h. The mean difference for each parameter was analyzed by one sample-TTEST procedure.

Continuous data (milk S100A8 concentration and SCC) were assessed for normality using the UNIVARIATE procedure. The log<sub>10</sub>-transformed value of the SCC (cells/mL of milk) was used for analysis to approximate normality. The repeated-measures analysis using the MIXED procedure of SAS was used to analyze continuous data. The repeated statement was used for repeated measurement analysis and treatment, time, and the interactions between treatment and time after infusions were included in the models as fixed effects. The structure of covariance (autoregressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria. A Tukey-Kramer adjustment test was performed to correct for multiple comparisons. The statistical significance of the contrast analysis was defined as  $P \leq 0.05$ .

## **Experiment 2. Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in goats**

### **Animals and LPS infusion**

Eighteen Tokara goats (bodyweight 20-25 kg, parity 1-4, mid-lactation stage, milk yield 300-800 mL/day) were used in this experiment. Goats were fed 0.6 kg of hay cubes and 0.2 kg of barley per day, with free access to water and a trace mineralized salt block. Feed was provided twice daily, at 08:00 and 15:00.

In this experiment, 8 goats were infused with 100 µg of LPS from *Escherichia coli* O111: B4 (Wako Pure Chemical) dissolved in 5 mL of saline, the other 8 were infused with 5 mL saline as control, and the remaining 2 were used for mammary gland tissue collection after LPS infusion. LPS infusion was carried out using a blunt end stainless steel infusion needle, inserted through the cervix to the uterus. Only goats with milk that had a low SCC ( $<1,000 \times 10^3$  cells/mL) were selected for this experiment. This experiment was approved by the Hiroshima University Animal Research Committee and was conducted following its guidelines.

### **Collection of blood and milk samples**

Blood and milk samples were collected before the infusion of saline or LPS (0 h) and at 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, and 144 h after infusion. Blood (5 mL) was collected from the jugular vein of goats and transferred to a tube containing heparin as an anticoagulant. The blood was then centrifuged at  $1,900 \times g$  for 10 min at 4 °C. Plasma was removed from the tube, and double-distilled water (ddH<sub>2</sub>O) was added to rupture the red blood cells, leaving only the leukocytes. Plasma was stored at -20 °C for enzyme immunoassay, and leukocytes were resuspended in PBS to measure the BLC.

Milk samples were collected by hand-milking and centrifuged at  $1,900 \times g$  for 5 min at 4 °C. The milk fat and skim milk were removed, leaving only the somatic cells of the milk (Nishikawa et al., 2018). Skim milk was stored at -20 °C for enzyme immunoassay, and cell pellets were resuspended in PBS for performing the SCC. BLC and SCC were conducted using a Countess® Automated Cell Counter (Life Technologies Japan Co. Ltd., Tokyo, Japan).

### **Enzyme immunoassay**

Enzyme immunoassay was conducted to measure the concentration of IL-1β, TNFα, IL-6, IL-8, IL-10, lipopolysaccharide-binding protein (LBP), lactoferrin (LF), and S100A8 in plasma and milk. Competitive enzyme immunoassays were performed for measuring the IL-1β, TNFα, and IL-8 concentrations, in both plasma and skim milk, IL-6 and S100A8 concentration in skim milk, and IL-10 concentration in plasma. The measurements of IL-1β, TNFα, and IL-8 concentrations were conducted following the method in Chapter 2 and the measurement of S100A8 was performed using the S100A8 antibody following the method in this chapter (Experiment 1).

The measurement of IL-6 and IL-10 concentrations were conducted the same as those of IL-1β and IL-8, respectively. Antibodies consisting of 11 and 13 amino acids (SKETLAENKL and RCHRFLPCENKSK), were produced in rabbits by Sigma® (St. Louis,

Missouri, USA) for IL-6 and IL-10 measurement, respectively. The antibodies were purified using a HiTrap™ Protein G high-performance affinity column (GE Healthcare, Princeton, NJ, USA), following the manufacturer's protocol. The peptide was conjugated with horseradish peroxidase using the peroxidase-labeling kit-SH (Dojindo Molecular Technologies, Kumamoto, Japan). Samples were diluted 1,000 times and 50 times for IL-6 and IL-10, respectively.

Lactoferrin and LPO measurements were performed as described in Kuwahara et al. (2017) and Isobe et al. (2011), respectively. For LF measurement, A 96-well microplate was coated with 100 µL of goat anti-rabbit IgG antibody (10 µg/mL) as a secondary antibody diluted in carbonate buffer and then incubated at 20°C for 2 h. After incubation, the plate was washed with PBST once. The plate was then incubated for 30 min 20°C with 150 µL of TBS buffer. After blocking, 50 µL of standard and sample diluted in TBS buffer (sample dilution ×5000) were added to each plate in triplicate wells, together with 50 µL of HRP-labeled goat LF and anti-bovine LF antibody (Life Laboratory Com., Yamagata, Japan). The plate was incubated for 4 h at 20°C. One hundred and fifty microliters (150 µL) of TMB was added after washing the plate 3 times and incubated for 30 min at 20°C. Finally, the enzymatic reaction was stopped by adding 30 µL of stop solution 2 N H<sub>2</sub>SO<sub>4</sub>. The optical density was measured using a microplate reader with a wavelength of 450 nm and analyzed.

To measure the milk LPO activity, defatted milk (10 µL) was mixed with TMB solution (200 µL) and incubated at 37°C for 30 min. After brief centrifugation at 6000 × g for 1 min, the optical density of the supernatant was measured with a wavelength of 655 nm using the microplate reader. LPO (Sigma, St. Louis, MO, USA) at a concentration of 0-10 U/mL was used as a standard (1 unit formed 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C).

Plasma LBP levels were determined at 0, 6, 12, and 24 h after infusion, using a commercially available LBP ELISA kit (Hycult Biotech, Frontstraat, PB Uden, Netherlands). Six plasma samples from the control and LPS groups were diluted and assayed according to the manufacturer's instructions. One hundred microliters (100 µL) of standard and sample diluted in dilution buffer (sample dilution ×50) was transferred to a pre-coated 12 microwell strips and incubated for 1 h at 20°C. The plate was washed 4 times with wash buffer and added by 100 µL of diluted biotinylated tracer and incubated for 1 h at 20°C. The plate was then washed 4 times and 100 µL of diluted streptavidin-peroxidase was added to it and incubated for 1 h at 20°C. One hundred microliters (100 µL) of TMB substrate was added after washing

the plate 4 times and incubated for 30 min at 20°C. Finally, the enzymatic reaction was stopped by adding 100 µL of stop solution. The optical density was measured as described above.

### **Collection of mammary gland tissues**

The deep area of the mammary gland tissues was collected from 2 goats. Tissues were obtained by biopsy, under sedation with Xylazine (Bayer HealthCare), and anesthesia with Pentobarbital (Somnopentyl; Kyoritsu Seiyaku Corporation). All biopsy instruments were autoclaved before use. Goats were milked completely before the biopsy to ensure that there was no milk left in their mammary glands. Mammary gland tissues were collected twice from one udder, 0 and 24 h after LPS infusion. The second biopsy was conducted at least 5 cm away from the first biopsy site, to avoid sampling from the same site and scar tissue. The incision was kept away from major blood vessels near the dorsal aspect and midline of the udder to prevent excessive bleeding. Mammary gland tissues were used to investigate the LPS localization by immunohistochemistry.

### **Immunohistochemistry**

Mammary gland tissues were fixed, dehydrated, embedded in paraffin, and then sectioned. Sections (3 µm thick) were air-dried on a slide coated with aminopropyl triethoxysilane. Immunohistochemistry was conducted in the same method as Experiment 1, except for the antibody. Sections were incubated overnight with the rabbit anti-LPS antibody (1 µg/mL, Hycult biotech) in PBS at 4 °C. The normal rabbit IgG at 1 µg/mL was used to replace the anti-LPS antibody for the negative control.

### **Statistical analyses**

Data were analyzed statistically using the SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). The continuous data were assessed for normality using the UNIVARIATE procedure. The repeated-measures analysis, using the MIXED procedure of SAS, was used to analyze the continuous data. The repeated statement was used for the repeated-measures analysis, and the model included the treatment, time, and interactions between the treatment and time. The baseline values were used as covariates for each analysis. The structure of the covariance (autoregressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria. A Tukey-Kramer adjustment test was performed to correct for multiple comparisons. The statistical significance of the contrast analysis was defined as  $P \leq 0.05$ .

## Results

### Experiment 1. Production of S100A8 and its upregulation by lipopolysaccharide stimulation

The S100A8 gene, as well as the  $\beta$ -actin gene, were expressed in the mammary gland parenchyma and teat of uninfused goats ( $n = 5$ ) (Fig. 5A). By immunohistochemistry, S100A8 protein was localized in the outermost layer of the teat skin and epidermis layer but not in the epithelium of alveoli in the uninfused gland (Fig. 6A and B). In the infused gland, S100A8 was not localized in the epithelial cells of alveoli but in the leukocytes infiltrated into the alveoli, ducts, and lamina propria of the teat skin (Fig. 6C and D). The negative control sections, incubated with normal rabbit IgG, did not exhibit any positive reaction (Fig. 6E and F). Some leukocytes collected from both blood and milk of uninfused goats were immuno-stained positively to S100A8 (Fig. 7A and B). Gene expression of S100A8 was also demonstrated in the blood leukocytes and milk somatic cells (Fig. 5B). Moreover, when milk somatic cells were homogenized, centrifuged, and the resulting supernatant was measured, S100A8 concentration was much higher than PBS without SCC (Fig. 8A).

When the percentage of S100A8-positive cell was significantly different between milk and blood (milk =  $96.2 \pm 1.0$ , blood =  $56.2 \pm 2.6\%$ ,  $P = 0.038$  Fig. 8B). The concentration of S100A8 in milk was measured before (0 h) and at 12, 24, 48, 72, 96, 120, and 144 h after infusion of LPS into the mammary gland. The mean concentration of S100A8 in milk was not affected during the entire experimental period by treatments ( $P = 0.332$ ), but it was affected by time after infusion ( $P < 0.001$ ) and an interaction between treatment and time ( $P < 0.001$ ). The mean concentration of S100A8 in milk was significantly higher at 72 h after the infusion of LPS in the LPS group than in the uninfused group. ( $P = 0.002$ ; Fig. 9A).

The mean SCC was altered by treatment ( $P < 0.001$ ), time after infusion ( $P < 0.001$ ), and interactions between treatment and infusion ( $P = 0.001$ ; Fig. 9B). The milk SCC started to increase at 12 h after the LPS challenge and persisted high until 144 h after infusion. The percentage of S100A8-positive cells in milk was not significantly different after intramammary infusion of LPS compared to 0 h ( $P = 0.522$ ), even though the total number of S100A8-positive cells increased significantly at 12-144 h than 0 h ( $P < 0.001$ ; Fig. 9C).

## **Experiment 2. Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in goats**

The mean BLC was affected by the treatment, time, and interaction between treatment and time (Fig. 10A). The mean BLC, at 2 and 72 h were significantly lower in the LPS group compared to those in the control group. The mean plasma LBP concentration was affected by only time (Fig. 10B). The mean plasma LBP concentrations at 12 and 24 h were significantly higher in the LPS group compared to that in the control group. In the plasma, the intrauterine infusion of LPS did not affect the concentrations of IL-1 $\beta$  (Fig. 11A), TNF $\alpha$  (Fig. 11B), IL-8 (Fig. 11C), and IL-10 (Fig. 11D), whereas, the mean concentrations of TNF $\alpha$  and IL-10 were affected by time ( $P < 0.001$  and  $P = 0.026$ , respectively).

The intrauterine infusion of LPS and the interaction between this treatment and time did not affect mean milk yield (Fig. 12A), SCC (Fig. 12B), LPO activity (Fig. 12C), LF concentration (Fig. 13A), and S100A8 concentration (Fig. 13B), however, these values were affected by time. Furthermore, in milk, the mean concentrations of IL-1 $\beta$  and IL-6 were affected by the treatment, time, and the interaction between treatment and time except for treatment in IL-1 $\beta$ . The mean concentration of IL-1 $\beta$  in milk was significantly different between the groups at 12 h (Fig. 14A), while the mean concentrations of IL-6 in milk at 6, 12, 24 and 48 h were significantly different between the groups (Fig. 14C). However, intrauterine infusion of LPS did not affect the mean milk concentrations of TNF $\alpha$  (Fig. 14B) or IL-8 (Fig. 14D).

LPS was not detected in the deep area of the mammary gland before LPS infusion (Fig. 15A), but it was detected in the connective tissue and interepithelial spaces of the alveoli of the mammary gland, 24 h after LPS infusion (Fig. 15B). The negative control sections, using normal rabbit IgG, did not show any positive reaction (Fig. 15C).

## **Discussion**

The study in this chapter hypothesized that intrauterine-infused LPS could be translocated to the mammary gland and induce a systemic inflammatory response in the mammary gland. Before this experiment 2, a preliminary experiment (Experiment 1) was carried out to examine whether S100A8, an antimicrobial peptide, can be used as a parameter of mammary gland inflammation. The S100A8 gene was expressed in the outermost layer of the teat skin and epidermis layer in the uninfused gland. Human skin epithelium is known to contain some antimicrobial proteins including lactoferrin, lysozyme, cathelicidin, RNase7,

S100A7, and several  $\beta$ -defensins, capable of suppressing bacterial growth and contributing to the skin host-defense function (Schauber & Gallo, 2008). In bovine and goat, the major localization site of the S100- protein family (S100A7, S100A8, S100A9, and S100A12) was observed throughout the epithelial layer of the epidermis in which keratinocytes are the predominant cell type (Smolenski et al., 2015; Zhang et al., 2014a).

The localization of S100A8 was also examined in the infused gland, and the results showed that S100A8 was not localized in the epithelial cells of alveoli but the leukocytes infiltrated into the alveoli, ducts, and lamina propria of the teat. Therefore, leukocytes from blood and milk somatic cells that contain leukocytes were isolated to precisely confirm the protein localization. Cells collected from both blood and milk of uninfused goats were immuno-stained positively to S100A8 and the concentration of S100A8 was higher in milk somatic cell-homogenized PBS than PBS alone. Together with the expression of S100A8 in the blood leukocytes and milk somatic cells, it is strongly suggested that S100A8 is produced by leukocytes. These results support previous research revealing that S100A8 was found to constitute approximately 45% of the total cytosolic of neutrophils (Edgeworth et al., 1991).

In the present results, the percentage of S100A8-positive cells was significantly higher in milk than in blood. Differences among leukocytes, including their morphology, viability, and enzyme activities, which are originated from blood and milk were extensively described in bovine species (Paape et al., 2003; Prin-Mathieu et al., 2002). These differences might cause a different number of S100A8-positive cells in milk and blood.

After LPS intramammary infusion, the milk SCC started to increase at 12 h after LPS challenge and persisted high until 144 h after infusion, while the increase of S100A8 concentration in milk was only observed at 72 h after infusion. Meanwhile, the percentage of S100A8-positive cells in milk was not significantly different after infusion even though the total number of S100A8-positive cells increased significantly. Taken together, it is demonstrated that S100A8 was not secreted immediately after LPS infusion, but by gradual secretion until 72 h, and that S100A8 production by leukocytes was likely associated with the increase of SCC.

Before the infusion of LPS into the mammary gland, the concentration of S100A8 in milk ranged from 13 to 43  $\mu\text{g/mL}$ . This result suggested that S100A8 might be constitutively produced and secreted spontaneously by somatic cells in milk. In the present experiment, only goats with low SCC were used. A large difference of S100A8 concentration among animals before LPS infusion was present and may reflect the different immune functions of goats.



S100A8 production is known to be stimulated by pro-inflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 $\beta$  in cultured keratinocytes (Mork et al., 2003) and anti-inflammatory cytokine IL-10 in macrophage (Xu et al., 2001). The secretion of several antimicrobial peptides in the mammary gland is regulated in different regions and time courses after bacterial infection (Isobe, 2017). The cathelicidin-2 in milk increased at 4 h after the intramammary infusion of LPS (Zhang et al., 2014b), while lactoferrin increased at 2 days after infusion (Huang et al., 2012). S100A8 may be secreted in a coordinated pattern with other AMPs to form the local defense against bacterial infection in the mammary gland.

In experiment 2, the systemic response to intrauterine LPS infusion was characterized by a significant decrease in BLC at 2 and 72 h after infusion, and the BLC in the LPS group remained lower than in the control group during the entire experimental period. This result supports the previous reports that suggested that LPS induced leukopenia, which could be attributed to a variety of causes, such as increased adhesiveness, which leads to an increased margination and sequestration of cells into the microvasculature (Jain et al., 1991; Paape et al., 2003; Shinozuka et al., 2018).

The mean concentration of LBP in the plasma was significantly higher in the LPS group compared to that in the control group at 12 and 24 h after infusion. The increase in the blood and milk LBP levels in cows has previously been reported to reach a maximal level within 24 h of intramammary infusion of LPS (Bannerman et al., 2003). The concentrations of LBP were also greater in the milk and blood of cows with naturally occurring mastitis than in those with healthy quarters (Zeng et al., 2009). LBP is a 58-60 kDa protein that catalyzes the transfer of bacterial LPS to CD14 (Schumann & Latz, 1999), which further facilitates LPS presentation to toll-like receptor 4 (TLR4), resulting in the activation of intracellular signaling pathways involved in the host's innate immune response (Schröder et al., 2003). The LBP concentration in the peripheral blood is an important indicator of systemic inflammation caused by circulating LPS. LPS translocation from the digestive tract into the bloodstream induced an increase in the plasma LBP concentration in a subacute ruminal acidosis experimental model (Khafipour et al., 2009b). These results suggested that an increase in the LBP was associated with an LPS challenge where the LPS from the uterus might enter the bloodstream.

Furthermore, LPS was not localized in the deep area of the mammary gland before intrauterine infusion of LPS, however, it was detected in the connective tissues and interepithelial spaces of the alveoli of the mammary gland 24 h after infusion. This suggests that LPS can be translocated from the uterus to the mammary gland through the bloodstream. The translocation of LPS from the digestive tract to the bloodstream is believed to be facilitated

by a high-concentrate diet, as demonstrated by previous studies (Bilal et al., 2016; Huo et al., 2013; Jin et al., 2016; Khafipour et al., 2009a; Liu et al., 2013). In the blood, LPS is bound mainly to LBP and ApoE of HDL/cholesterol, or the monocyte surface (Kitchens et al., 1999). As demonstrated previously, intravenously injected LPS can be found in monocytes and neutrophils in a blood smear (Mathison & Ulevitch, 1979), macrophages in the liver and spleen (Ge et al., 1994; Mathison & Ulevitch, 1979), and macrophages and intestinal epithelial cells in the small intestine (Ge et al., 2000).

The increased levels of circulating LPS would increase the concentrations of several pro-inflammatory cytokines that mediate the local and/or systemic response, such as IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  (Ohtsuka et al., 2001; Sohn et al., 2008). The examination of some pro- and anti-inflammatory cytokines in plasma was carried out to determine if the intrauterine infusion of LPS would induce changes in their concentrations. The result showed that only the TNF $\alpha$  concentration in the plasma tended to be higher at 1 h after infusion in the LPS group compared to the control group. In previous studies on uterine inflammation, mRNA expression of IL-1 $\beta$ , IL-8, and TNF- $\alpha$  in the endometrial cells (Fischer et al., 2010; Ghasemi et al., 2012), and the serum concentrations of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Kasimanickam et al., 2013) were significantly increased compared with uterus under non-inflammatory condition. This difference between our results and those of Kasimanickam et al. might be because of the different stimulations; LPS used in the present study was only a bacterial component, whereas viable bacteria were present in the uteri of dairy cows in their study.

In the present study, the concentrations of IL-1 $\beta$  and IL-6 in milk were significantly increased at 12 h and 6 to 48 h after intrauterine infusion of LPS, respectively. Pro-inflammatory cytokines IL-1 $\beta$  and IL-6 mediate the systemic response to Gram-negative mastitis and are upregulated by LPS (Guha & Mackman, 2001; Shuster et al., 1993). Therefore, the present findings suggest that intrauterine-infused LPS might cause the upregulation of some inflammatory components in the milk.

However, the milk SCC, milk yield, LF concentration, S100A8 concentration, TNF- $\alpha$  concentration, IL-8 concentration, and LPO activity did not show any significant changes. LF and LPO are two main proteins found in milk. LF, LPO, and S100A8 are secreted by leukocytes. IL-8 is a chemokine that recruits leukocytes from the blood to the tissues. In this study, the absence of an increase in the IL-8 concentration may prevent the increase of SCC, resulting in a constant LF and S100A8 concentration and LPO activity as these components are secreted by the leukocytes. Combined with the increase of IL-1 $\beta$  and IL-6 concentrations in milk, these

results suggested that the inflammatory response of the mammary gland was limited after intrauterine infusion of LPS.

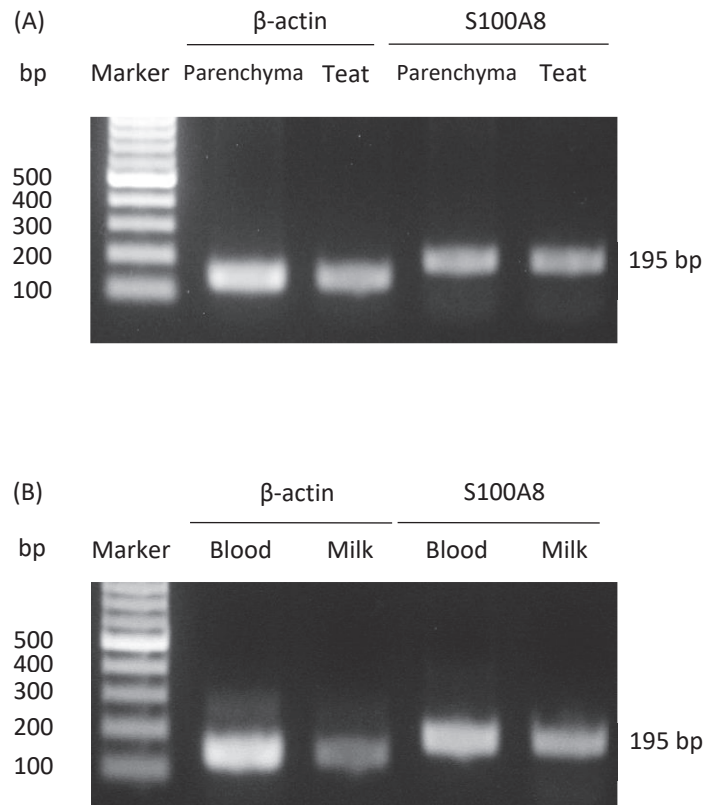
The results in this study suggest that intrauterine-infused LPS can be translocated to the mammary glands and upregulate some inflammatory components, however, the amount of LPS in the mammary gland after intrauterine-LPS infusion might be too low to induce symptoms of clinical mastitis. The translocation of LPS might be affected by some factors, such as the dose of LPS and the individual response to LPS to produce the inflammatory response in the mammary gland. Therefore, the effect of intrauterine infusion of LPS in different conditions is needed to be studied further.

## Abstract

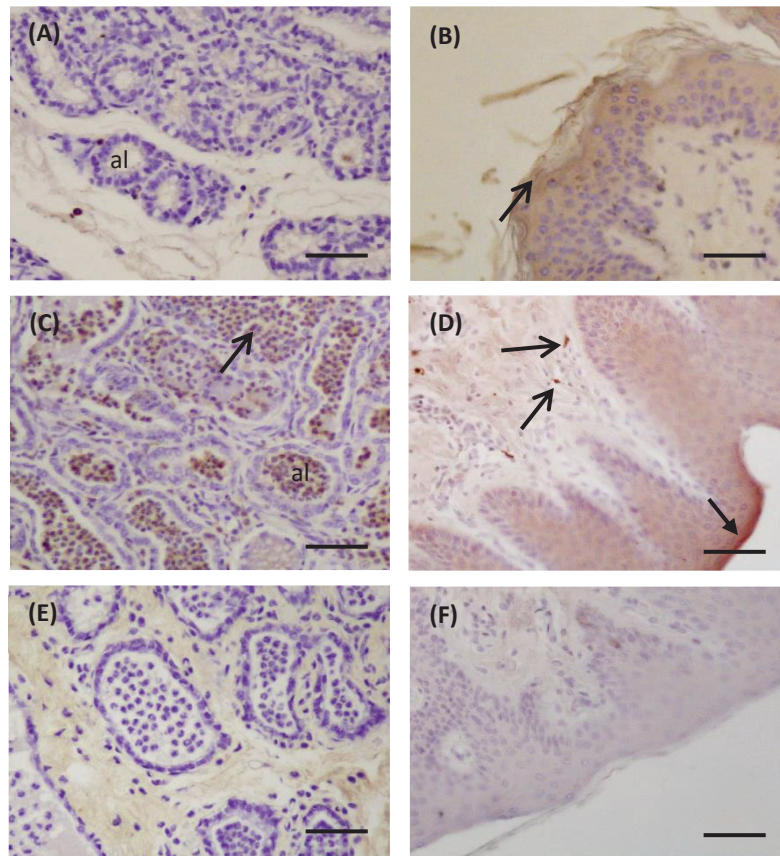
This study aimed to determine whether intrauterine-infused LPS could be translocated to the mammary gland and induce an inflammatory response in the mammary gland. The preliminary experiment (Experiment 1) was undertaken to elucidate the production of S100A8 in the mammary gland and its role in inflammation. S100A8 was immunolocalized in the outermost layer of the teat skin of udders with and without LPS infusion, whereas in the mammary gland, it was only immunolocalized in the leukocytes infiltrated in the alveoli after LPS infusion. S100A8 was also immunolocalized in the blood and milk leukocytes. The number of S100A8-positive cells in milk was higher than that in blood. The concentration of S100A8 in milk increased significantly at 72 h after intramammary infusion of LPS. In Experiment 2, sixteen goats were divided into two groups, subjected to intrauterine infusion of LPS, and two other goats were used for blood and milk samples collection. Blood and milk samples were collected before (0 h) and after the LPS challenge. When mammary gland tissues were immunostained with LPS, LPS was detected in the connective tissues and interepithelial spaces of the alveoli of the mammary glands 24 h after intrauterine infusion of LPS. In goats with the intrauterine infusion of LPS, the mean concentrations of IL-1 $\beta$  and IL-6 in milk were higher in the LPS group compared to that in the control group, whereas there were no changes in milk yield and SCC. LF, S100A8, TNF- $\alpha$ , IL-8 concentrations, and LPO activity were not different between LPS and the control group. In conclusion, LPS can translocate from the uterus to the mammary gland through the bloodstream and cause some inflammatory responses on it.

S100A8	gtgtaccacaactactccctgctgaaaggaattaccacgccgtctacagggatgacttg	420
sequence	-----ggaattaccacgccgtctacagggatgacttg	32
	*****	
S100A8	aagagactgtagagacagagtgtcctaagttttgaagaaaaggatgcagacacttg	480
sequence	aagagactgtagagacagagtgtcctaagttttgaagaaaaggatgcagacacttg	92
	*****	
S100A8	ttcaaagagttggacatcaatcaggatggggaattaacttcgaggagttcctcgtgctg	540
sequence	ttcaaagagttggacatcaatcaggatggggaattaacttcgaggagttcctcgtgctg	152
	*****	
S100A8	gtgataaaggtaggcctggcagcccatgaagacattcacaagaatagcagagctatcag	600
sequence	gtgataaaggtaggcctggcagcccatgaagacattcaca-----	192
	*****	

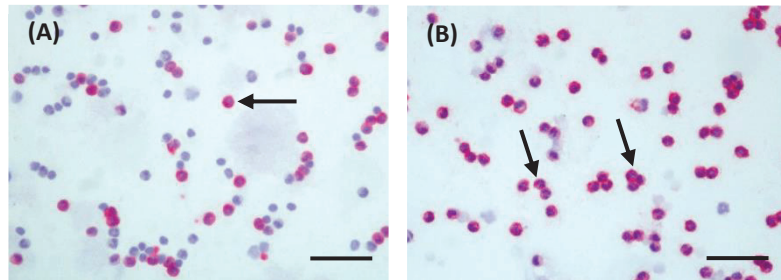
**Figure 4.** DNA sequences of S100A8 (XM\_005677513.3) and PCR product in the present study. Sequences were same, indicating that primers were attached in the S100A8 sequence correctly



**Figure 5.** Messenger RNA expression of S100A8 in parenchyma and teat of the mammary gland (A), blood leukocytes and milk somatic cells (B) (n=5). bp = base pair

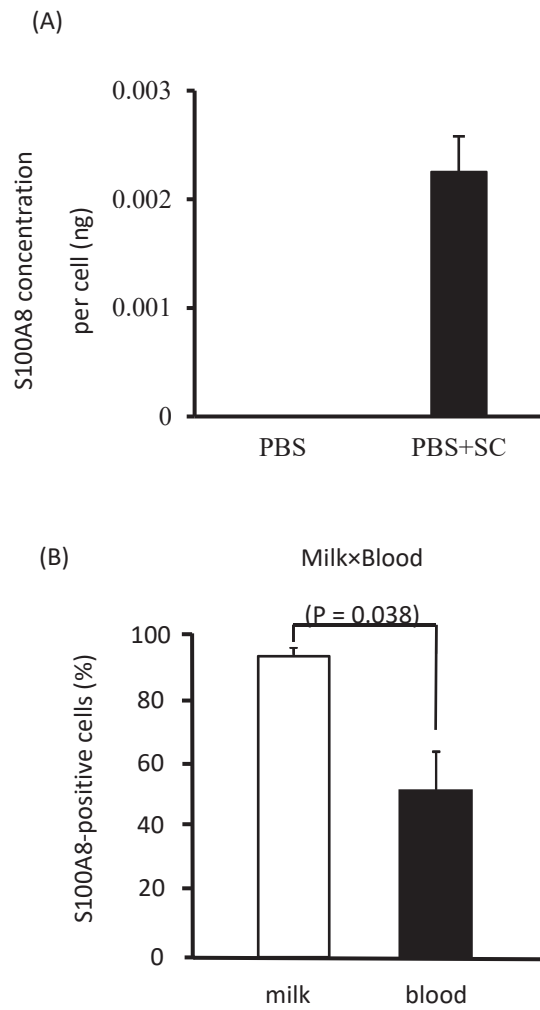


**Figure 6.** Representative photographs of mammary gland and teat immunostained with the S100A8 antibody (n=3). Alveoli and teat from uninfused (A and B) and LPS-infused mammary gland (C and D), respectively. E and F show a negative control in which the anti-S100A8 antibody was replaced with normal rabbit IgG. al = alveolus. Arrows show S100A8-positive reactions. Scale bars = 50  $\mu$ m.

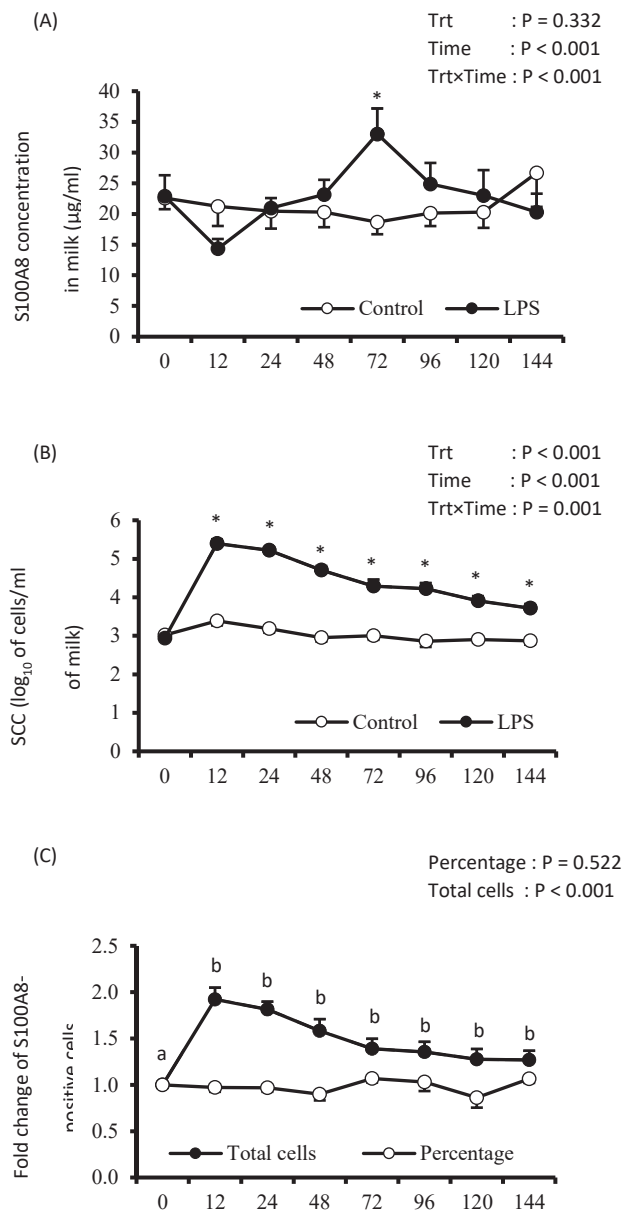


**Figure 7.** Representative photographs of leukocytes derived from blood (A) and milk (B) of uninfused goats. Arrows show S100A8-positive reactions. Scale bars = 50  $\mu$ m.



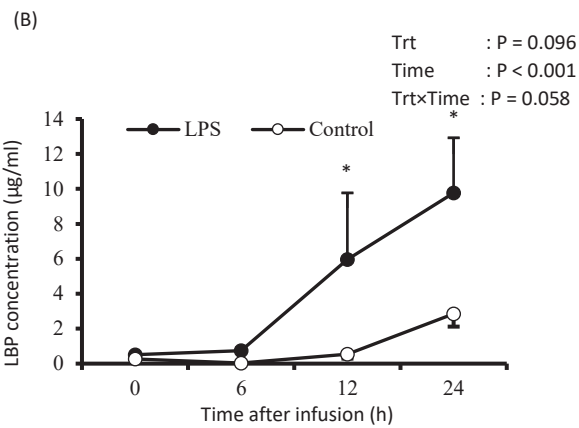
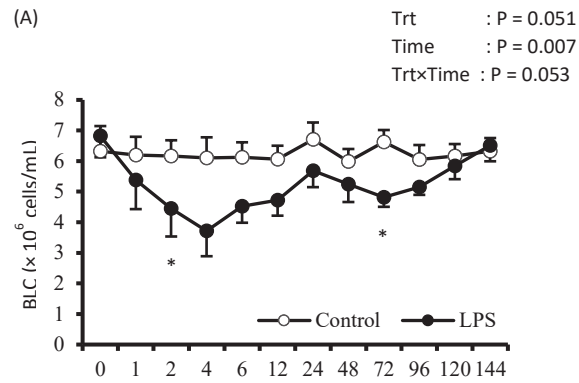


**Figure 8.** The concentration of S100A8 in PBS with and without homogenized milk somatic cells (A). Percentage of S100A8-positive cells in blood and milk (B) (n = 5, P = 0.038).

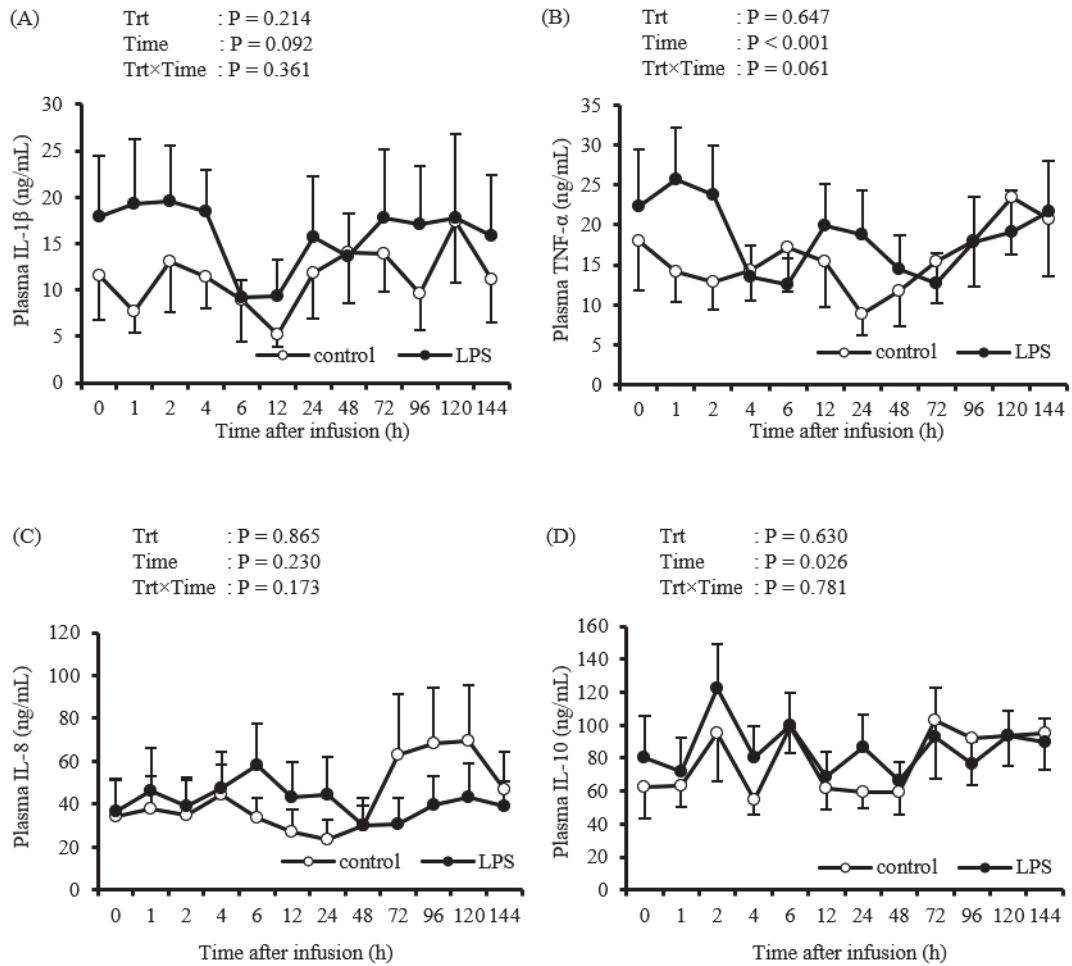


**Figure 9.** Changes in S100A8 concentration (A) and SCC (B) in milk of udders with and without LPS infusion. Changes in percentage and total cells of S00A8-positive cells with and without LPS infusion (C). Control = udders were uninfused; LPS = udders were infused with 100  $\mu\text{g}$  of LPS dissolved in 5 ml of saline ( $n = 8$ ). Results are reported as Mean  $\pm$  SEM. \* Significant difference between groups ( $P < 0.05$ ).

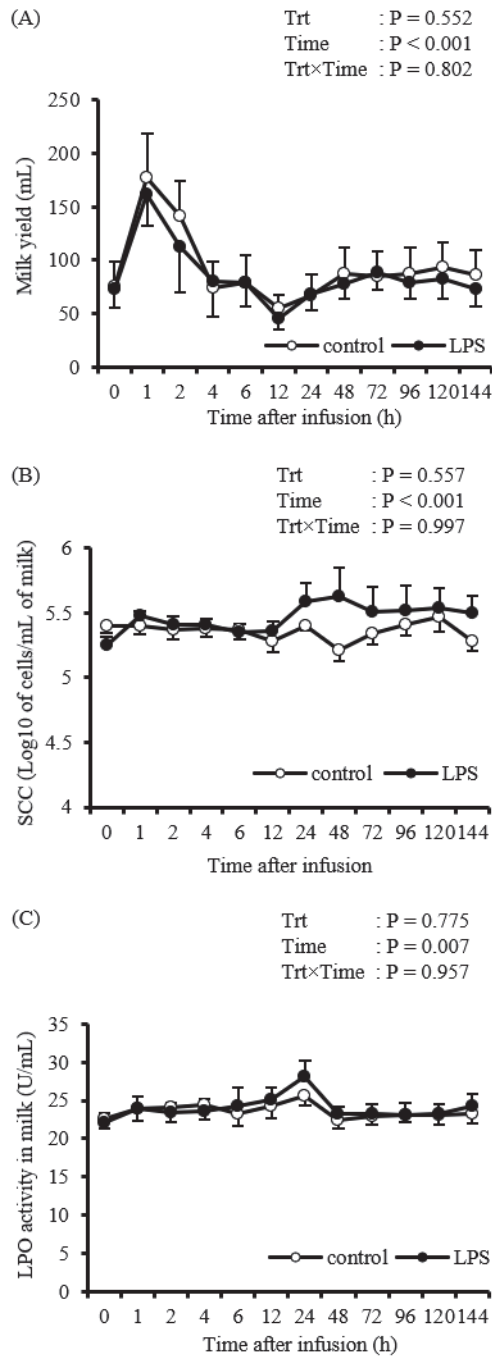
a, b: Different letters show significant difference between values in each time and 0 h.



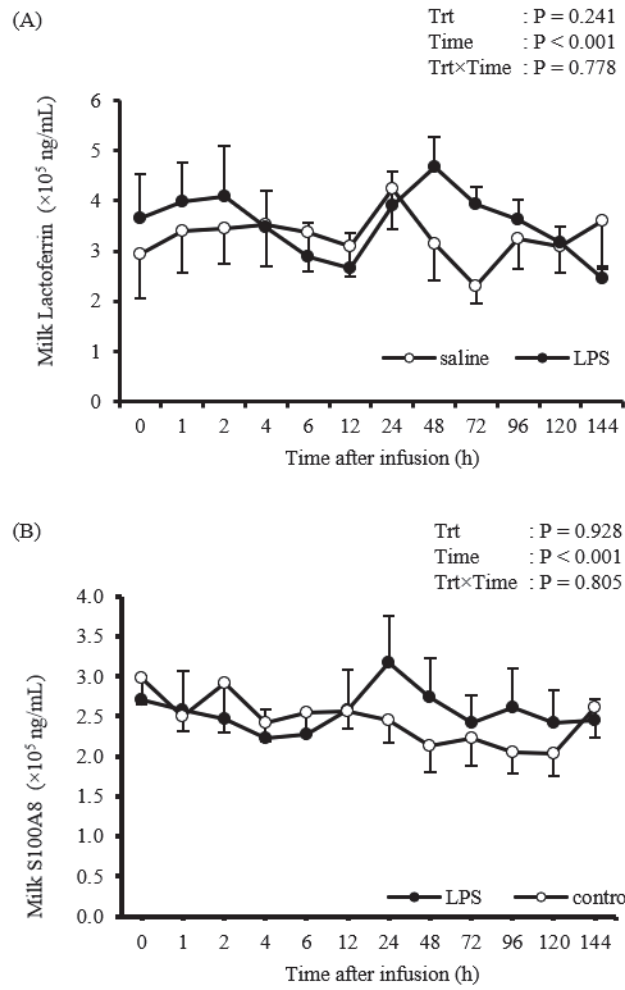
**Figure 10.** Changes in the blood leukocyte count (BLC, A) (control group, n = 8; LPS group n = 8) and plasma lipopolysaccharide binding protein (LBP; B) after intrauterine infusion of saline (control group, n = 6) and LPS (n = 6). Results are reported as mean ± SEM. \* Significant difference (P < 0.05) between the control and LPS groups



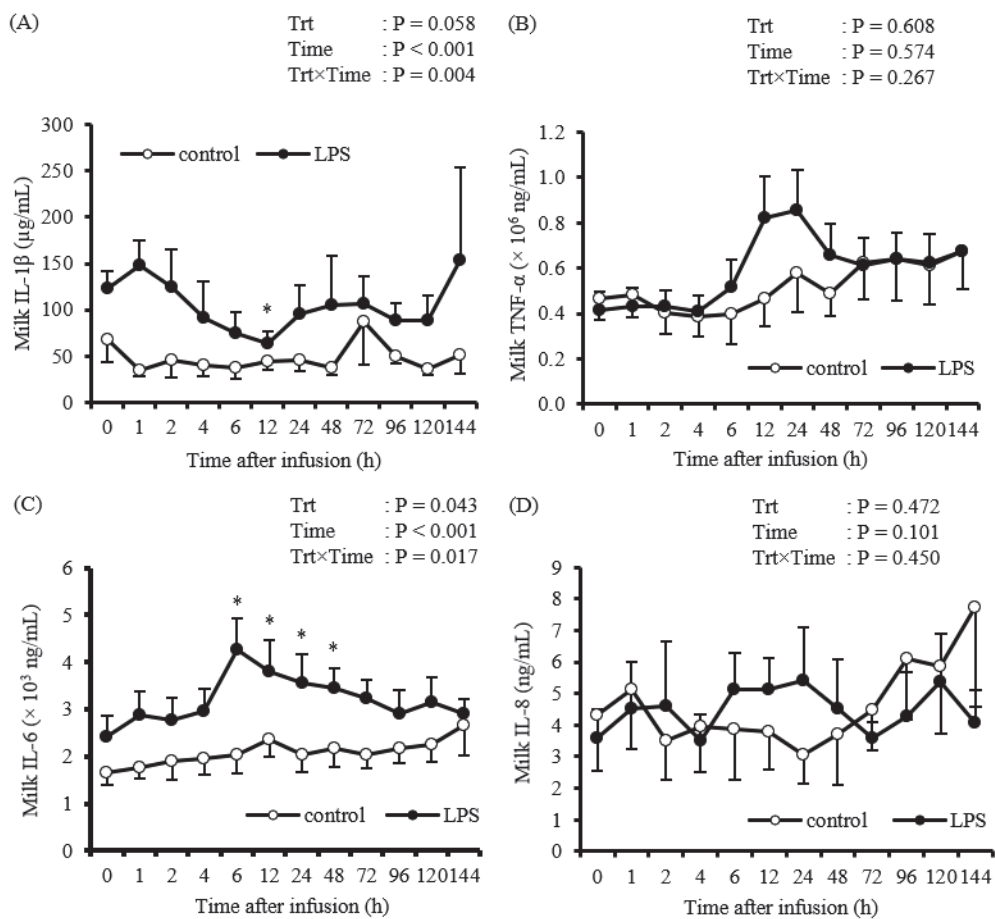
**Figure 11.** Plasma concentrations of IL-1 $\beta$  (A), TNF $\alpha$  (B), IL-8 (C), and IL-10 (D) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are reported as mean  $\pm$  SEM.



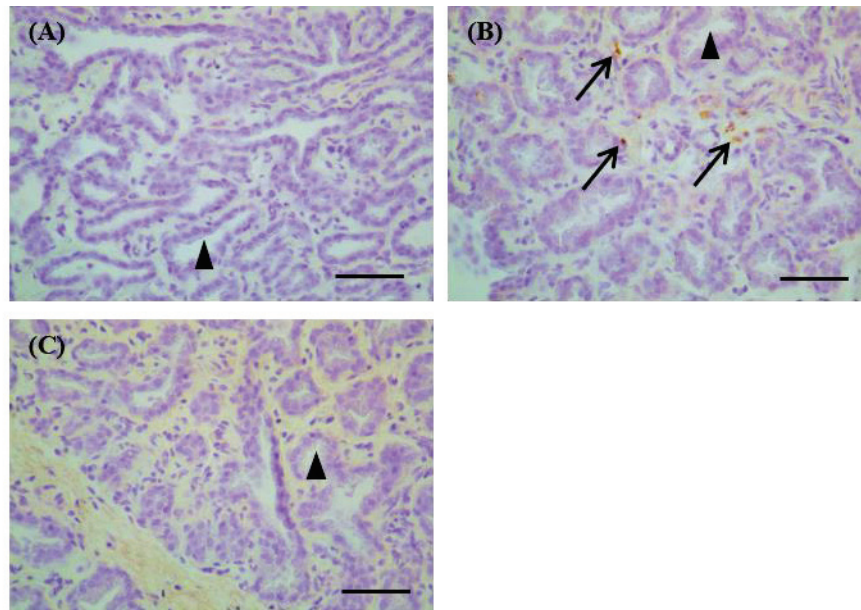
**Figure 12.** Milk yield (A), somatic cell count (SCC, B), and lactoperoxidase activity in the milk (LPO, C) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are reported as mean  $\pm$  SEM.



**Figure 13.** Milk concentration of lactoferrin (A) and S100A8 (B) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are reported as mean ± SEM.



**Figure 14.** Milk concentrations of IL-1 $\beta$  (A), TNF $\alpha$  (B), IL-6 (C), and IL-8 (D) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are reported as mean  $\pm$  SEM. \* Significant difference (P < 0.05) between the control and LPS groups



**Figure 15.** Representative photographs of the mammary gland immunostained with the LPS antibody before (A) and 24 h after LPS infusion (B) (n = 2). Negative control (C), in which the anti-LPS antibody was replaced with normal rabbit IgG. Arrows: LPS-positive reaction; arrowhead: alveoli. Scale bars = 50  $\mu$ m



## Chapter 4

### Effects of Intrauterine Infusion of Bacterial Lipopolysaccharides on the Mammary Gland Inflammatory Response in Dexamethasone-treated Goats

#### Introduction

When mastitic milk was cultured, causative pathogens were not detected in approximately 15-35% of clinical mastitis cases. The occurrence of subclinical endometritis in cows 4 and 8 weeks postpartum was strongly associated with subclinical mastitis, with an odds ratio of 4.5 and 3.6, respectively (Bacha & Regassa, 2010). Therefore, the effect of intrauterine infusion of lipopolysaccharides (LPS) on mammary gland inflammation was investigated in Chapter 2. Higher concentrations of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in milk along with LPS localization in the mammary gland after infusion suggested the possibility of bacterial LPS translocation from the uterus to the mammary gland via the bloodstream. However, the milk somatic cell count (SCC), and some cytokines were not significantly higher after intrauterine infusion of LPS, indicating limited inflammation of the mammary gland under normal conditions.

Under stress conditions, the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes are activated to maintain homeostasis in response to stress stimuli (Smith & Vale, 2006). Cortisol production is associated with immune suppression under chronic stress (Ju et al., 2014; Wang et al., 2011). The combined effects of multiple stressors, including high and low temperature, nutritional deficiency, parturition, the transition to the milking herd, and herd management during the periparturient period, can increase and prolong the magnitude of immunosuppression, further increasing animal susceptibility to disease (Trevisi et al., 2011). Based on the commonly-accepted theory that stressors suppress immune function and enhance disease susceptibility, this study hypothesizes that immunosuppression may enhance the effect of intrauterine-infused LPS on the mammary gland inflammatory response.

In this study, glucocorticoid was used as an immunosuppressant agent. Glucocorticoids possess strong immunosuppressive properties and are widely used in human and veterinary medicine (Diehl et al., 2017). Synthetic glucocorticoids, such as dexamethasone, bind exclusively to albumin, thus exhibiting higher bioavailability and longer biological half-life than cortisol (Melby, 1977). Glucocorticoids inhibit T-lymphocytes and antigen-presenting cells and induce the downregulation of proinflammatory cytokines (van Kooten et al., 2009).

Therefore, the present study was conducted to examine the intensity of mammary gland inflammation after intrauterine infusion of LPS in dexamethasone-treated goats.

## **Materials and Methods**

### **Animals and LPS infusion**

Eighteen Tokara goats (bodyweight 20-25 kg, parity 1-4, mid-lactation stage, milk yield 300-800 mL/day) were used in this study. Goats were provided with 0.6 kg of hay cubes and 0.2 kg of barley per day, with free access to water and a trace mineral salt block. Diets were offered twice daily, at 08:00 and 15:00.

Sixteen goats were selected for intrauterine infusion of saline or LPS without tissue collection, and 2 goats were selected for mammary gland tissue collection after LPS infusion. For intrauterine infusion of LPS, 16 goats were divided into the control and LPS groups (n = 8 each). All goats were intramuscularly injected with dexamethasone (Kyoritsu Seiyaku, Tokyo, Japan) daily for 5 days (0.1 mg/kg body weight/day), followed by an infusion of 5 mL of saline with or without 100 µg of LPS from *Escherichia coli* O111: B4 (Wako Pure Chemical, Osaka, Japan) through the cervix into the uterus. A single intramuscular administration of 0.07 mg/kg of dexamethasone in bovines was previously reported to decrease plasma cortisol, neutrophils L-selectin expression, and Reactive Oxygen Species in the uterus 30 h after treatment (König et al., 2006). Administration of dexamethasone daily for 5 consecutive days was conducted to provide a heavier immunosuppression condition as previously conducted by Jagtap et al. (2012) with a higher dose of dexamethasone.

Only goats with low milk SCC ( $<1,000 \times 10^3$  cells/mL) were included and no other infection was induced in this experiment. This study was approved by and conducted following the guidelines of the Hiroshima University Animal Research Committee (No. C 19-4).

### **Collection of blood and milk samples**

Blood and milk samples were collected daily from 16 goats during the 5 days of dexamethasone administration, before (0 h) and at 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, and 144 h after saline or LPS infusion. Blood (5 mL) was collected from the jugular vein of goats and transferred to a tube coated with the anticoagulant heparin. Blood samples were then centrifuged at  $1,900 \times g$  for 10 min at 4 °C. The separated plasma was collected, and double-distilled water (ddH<sub>2</sub>O) was added to the cell pellet to rupture the red blood cells while retaining the leukocytes. Plasma was stored at -20 °C for enzyme immunoassay, and leukocytes were resuspended in PBS for determining the blood leukocyte count (BLC).

Milk samples were centrifuged at  $1,900 \times g$  for 5 min at 4 °C. Milk fat and skim milk separated from the somatic cell pellet were collected (Nishikawa et al., 2018). Skim milk was stored at -20 °C for enzyme immunoassay, and cell pellets were resuspended in PBS for determining the SCC. BLC and SCC were measured using a Countess® Automated Cell Counter (Life Technologies Japan Co. Ltd., Tokyo, Japan).

### **Enzyme immunoassay**

Competitive enzyme immunoassays were performed for measuring IL-1 $\beta$ , TNF $\alpha$ , and IL-8 concentrations in both plasma and skim milk, S100A8 and lactoferrin concentrations in skim milk, and IL-10, LBP, and serum amyloid A (SAA) concentrations in plasma. The concentrations of IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-10, S100A8, LBP, and lactoferrin were determined following previously described methods in Chapters 2 and 3. For SAA measurement, an antibody was produced by immunizing rabbit with 20 amino acids (CREANYKGADKYFHARGNYD) and purified using a HiTrap™ Protein G high-performance affinity column (GE Healthcare, Princeton, NJ, USA), following the manufacturer's protocol. Competitive enzyme immunoassay protocol for SAA was the same as those for IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-10, and S100A8. The plasma sample was diluted 50 times for the assay.

### **Collection of mammary gland tissues**

The deep area of the mammary gland tissues was collected from two goats by biopsy, before and 24 h after LPS infusion. All biopsy instruments were autoclaved before use. Goats were milked completely before the biopsy to ensure that little milk remained in their mammary glands. Deep sedation and anesthesia were achieved by slow intravenous injection of xylazine (Bayer HealthCare, Osaka, Japan) and pentobarbital (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan). Mammary gland tissues were collected twice in total, at 0 and 24 h after LPS infusion, from one udder. The biopsy was performed as previously described in Chapter 3. LPS localization in the tissues was assessed by immunohistochemical analysis.

### **Immunohistochemistry**

Immunohistochemistry was performed as described in Chapter 3.

### **Statistical analysis**

Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Continuous data were assessed for normality using the UNIVARIATE

procedure. Repeated-measures analysis, using the MIXED procedure of SAS, was used to analyze the continuous data, and the model included the treatment, time, and interaction between treatment and time. The baseline value was used as a covariate for each analysis. The covariance structure (autoregressive, unstructured, or compound symmetry) was selected based on the Bayesian information criterion. The Tukey-Kramer adjustment test was used to correct for multiple comparisons. Results of the contrast analysis were considered statistically significant at  $P \leq 0.05$  and highly significant at  $P < 0.001$ .

## Results

The SCC was affected by the time and interaction between treatment and time (Fig. 16A). The SCC in the LPS group was significantly higher than that in the control group 24 h after intrauterine infusion of LPS. The mean BLC was also affected by the time and interaction between treatment and time (Fig. 16B), and it was significantly lower in the LPS group than in the control group at 4 and 6 h after intrauterine infusion of LPS. The mean milk yield was significantly affected by the treatment and time (Fig. 16C), and it was lower in the LPS group than in the control group at 6 and 24 h after LPS infusion.

Intrauterine infusion of LPS did not affect the mean plasma concentrations of IL-1 $\beta$  (Fig. 17A), TNF $\alpha$  (Fig. 17B), IL-8 (Fig. 17C), and IL-10 (Fig. 17D). However, their mean concentrations were affected by time. In contrast, the plasma LBP and SAA concentrations were significantly affected by the treatment (Fig. 18A and B). The concentrations of LBP and SAA in plasma were significantly higher at 24 and 48 h, respectively, in the LPS group than in the control group. The mean milk concentrations of IL-1 $\beta$  (Fig. 19A), TNF $\alpha$  (Fig. 19B), IL-8 (Fig. 19C), and lactoferrin (Fig. 20A) were affected only by time, whereas the mean milk S100A8 concentration was affected by the treatment (Fig. 20B). The mean concentrations of IL-1 $\beta$ , S100A8, and lactoferrin were significantly higher in the LPS group than in the control group 24 h after infusion.

LPS was not detected in the deep area of the mammary gland before LPS infusion (Fig. 21A), but it was detected in the connective tissue and inner spaces of alveoli 24 h after LPS infusion (Fig. 21B). The negative control sections, incubated with normal rabbit IgG, did not exhibit any positive staining (Fig. 21C).

## Discussion

The study in Chapter 3 reported that intrauterine-infused LPS can be translocated to the mammary gland under normal conditions, inducing an increase in IL-1 $\beta$  and IL-6 concentrations in milk, whereas the SCC remained unchanged. It is possible that translocation of LPS from the uterus to the mammary gland can induce heavier inflammation under immunosuppressive conditions than under normal conditions. Therefore, dexamethasone was used in the present study to induce an immunosuppressive condition.

Intrauterine infusion of LPS resulted in a sharp decrease in the milk yield at 6 and 24 h after infusion, and in the BLC at 4 and 6 h after infusion. This result was accompanied by anorexia and lethargy condition within 24 h after LPS infusion and it is consistent with that of our previous study in Chapter 3, where LPS infusion exerted a longer and more obvious effect on milk yield and BLC. In the present study, leukocytes might have been recruited to the uterus, i.e., the site of inflammation. However, intrauterine infusion of LPS did not significantly alter the plasma concentrations of IL-1 $\beta$ , TNF- $\alpha$ , IL-8, and IL-10. IL-8 is a chemokine recruiting leukocytes to the site of inflammation. Therefore, IL-8 may be one of the targets of dexamethasone, so that somatic cells, i.e., leukocytes, could not be recruited to the mammary gland.

Plasma IL-1 $\beta$ , TNF $\alpha$ , IL-8, and IL-10 concentrations were not significantly different between the LPS and control groups after intrauterine infusion of LPS. This may be due to the weak immune reaction induced, possibly because of the immunosuppressive effect of dexamethasone treatment. On the other hand, the concentration of plasma LBP was elevated 24 h after LPS infusion. The increase of LBP in plasma was the same condition as the result of the study in Chapter 3. It suggests that the increase of plasma LBP was associated with the LPS challenge where the LPS from the uterus might enter the bloodstream since LBP concentration in the peripheral blood is an important indicator of systemic inflammation caused by circulating LPS.

Furthermore, SAA concentration in plasma was significantly higher at 48 h after LPS infusion in the LPS group than in the control group. SAA is one of the major acute-phase proteins in ruminants, and the intense increase of SAA concentration is usually occurred shortly (24 h) after tissue injury (Sharifiyazdia, 2012). SAA was reported as a sensitive indicator of mastitis in dairy cows (Åkerstedt et al., 2007). This may explain the LPS-positive reaction observed in the mammary gland tissues 24 h after intrauterine LPS infusion. In Chapter 3, LPS was detected only in the connective tissue of the mammary gland under normal conditions.

However, an LPS-positive reaction was observed in the inner alveolar spaces of the mammary gland under dexamethasone treatment. This suggests that LPS can be more easily translocated via blood circulation under dexamethasone treatment than under normal conditions.

LPS could be translocated via the bloodstream because it could not be eliminated by macrophages or leukocytes under dexamethasone treatment. External administration or excessive release of corticosteroids *in vivo* typically induce leukocytosis, neutrophilia, lymphopenia, eosinopenia, basopenia, and monocytosis or monocytopenia, which suppresses the immune system (Tizard, 2018). Glucocorticoids, including dexamethasone, can inhibit several initial stages of the inflammatory response (Coutinho & Chapman, 2011). Glucocorticoids inhibit cytokine release from inflammatory and other cell types, mainly by suppressing the expression of inflammatory genes (Barnes, 1998). Glucocorticoids have been shown to inhibit the secretion of IL-1 $\beta$  from human monocytes and neutrophils (Amano et al., 1993; Langereis et al., 2011), of TNF- $\alpha$  from human monocytic cells and eosinophils (Steer et al., 2000; Uings et al., 2005), and of IL-8 from human airway epithelial cells and monocytic cell lines (Kwon et al., 1994; Tsaprouni et al., 2007). These results indicate that dexamethasone suppressed cytokine expression, resulting in a weak immune response, consequently allowing LPS translocation via the bloodstream.

Furthermore, milk SCC and IL-1 $\beta$ , S100A8, and lactoferrin concentrations were significantly higher in the LPS group than in the control group 24 h after infusion. This suggests that LPS translocation from the uterus to the mammary gland can induce inflammation in the latter. The effects of corticosteroids on immune response are variable and depend on the dose, physiological adaptation of the host, and the time of administration and evaluation post-drug administration (Pruett et al., 1987; Tizard, 2018). Injection of a high dose of dexamethasone for 5 consecutive days induced severe immunosuppression, marked by a decrease in total leukocyte and lymphocyte counts, which recovered to the normal state 24 h after the last injection (Jagtap et al., 2012). This suggests that the effect of dexamethasone administration lasted for 24 h after the last injection. Therefore, milk SCC and IL-1 $\beta$ , S100A8, and lactoferrin concentrations did not increase until 24 h after LPS infusion, i.e., until the removal of the dexamethasone effect. In conjunction, these results suggest that dexamethasone suppresses the immune reaction during the 24 h after LPS infusion, resulting in the translocation of more LPS to the mammary gland. After 24 h of LPS infusion, the translocated LPS initiates an immune reaction in the mammary glands, thereby increasing the SCC.

The concentration of IL-1 $\beta$  was higher in the LPS group than in the control group 24 h after intrauterine LPS infusion. IL-1 $\beta$  is a pro-inflammatory cytokine inducing inflammation.

Increased IL-1 $\beta$  levels subsequently increase the SCC in the mammary gland. Therefore, the SCC increased 24 h or later after LPS infusion. Milk concentrations of S100A8 and lactoferrin were significantly higher in the LPS group than in the control group 24 h after LPS infusion. S100A8 is present in neutrophils (Corbin et al., 2008), whereas lactoferrin is produced by epithelial cells and leukocytes (Baveye et al., 1999; Huang et al., 2012). Therefore, it is likely that leukocytes in milk may stimulate the production and secretion of S100A8 and lactoferrin into milk.

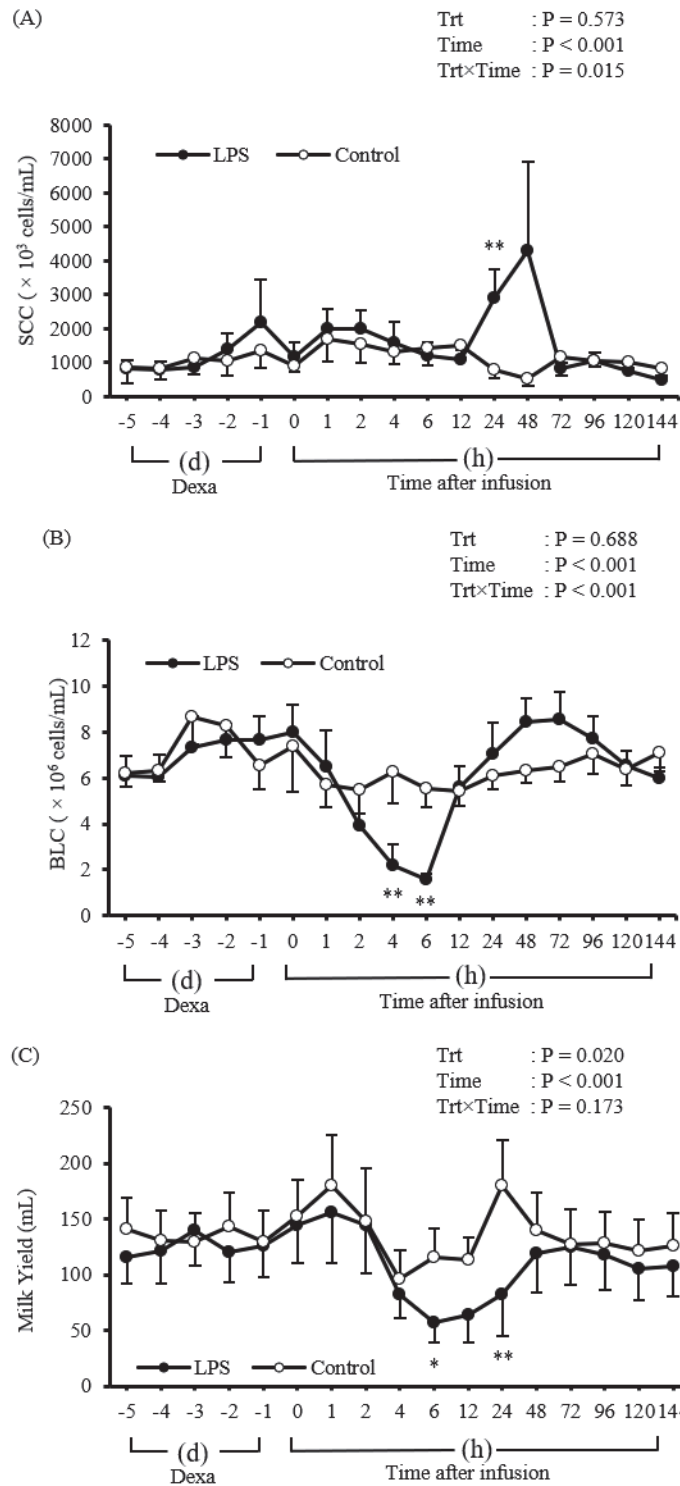
LPS was detected in the connective tissues and inner alveolar spaces of the mammary gland 24 h after infusion, whereas it was not detected before infusion. This result suggests that LPS can be translocated from the uterus to the mammary gland via the bloodstream. Previous studies have demonstrated the transfer of LPS from the digestive tract (Bilal et al., 2016; Huo et al., 2013; Jin et al., 2016; Khafipour et al., 2009a; Liu et al., 2013), uterus (Mateus et al., 2003), and the mammary gland (Dosogne et al., 2002; Hakogi et al., 1989) into the bloodstream. The mechanism of LPS translocation throughout the body remains poorly understood; however, LPS movement across the epithelium of various tissues and into the bloodstream can be likely attributed to paracellular and transcellular transport (Mani et al., 2012). Of these, the dominant transport mechanism of LPS in mammary epithelial cells remains unclear.

In conclusion, dexamethasone suppressed the immune system within 24 h of intrauterine infusion of LPS, resulting in LPS translocation from the uterus to the mammary gland. The reduction of the dexamethasone effect later than 24 h after its last injection, induced inflammation in the mammary gland, increasing milk SCC, cytokine, and antimicrobial peptides. This study concludes that dexamethasone administration before intrauterine infusion of LPS enhanced the translocation of LPS and induces a heavier inflammatory response in the mammary gland.

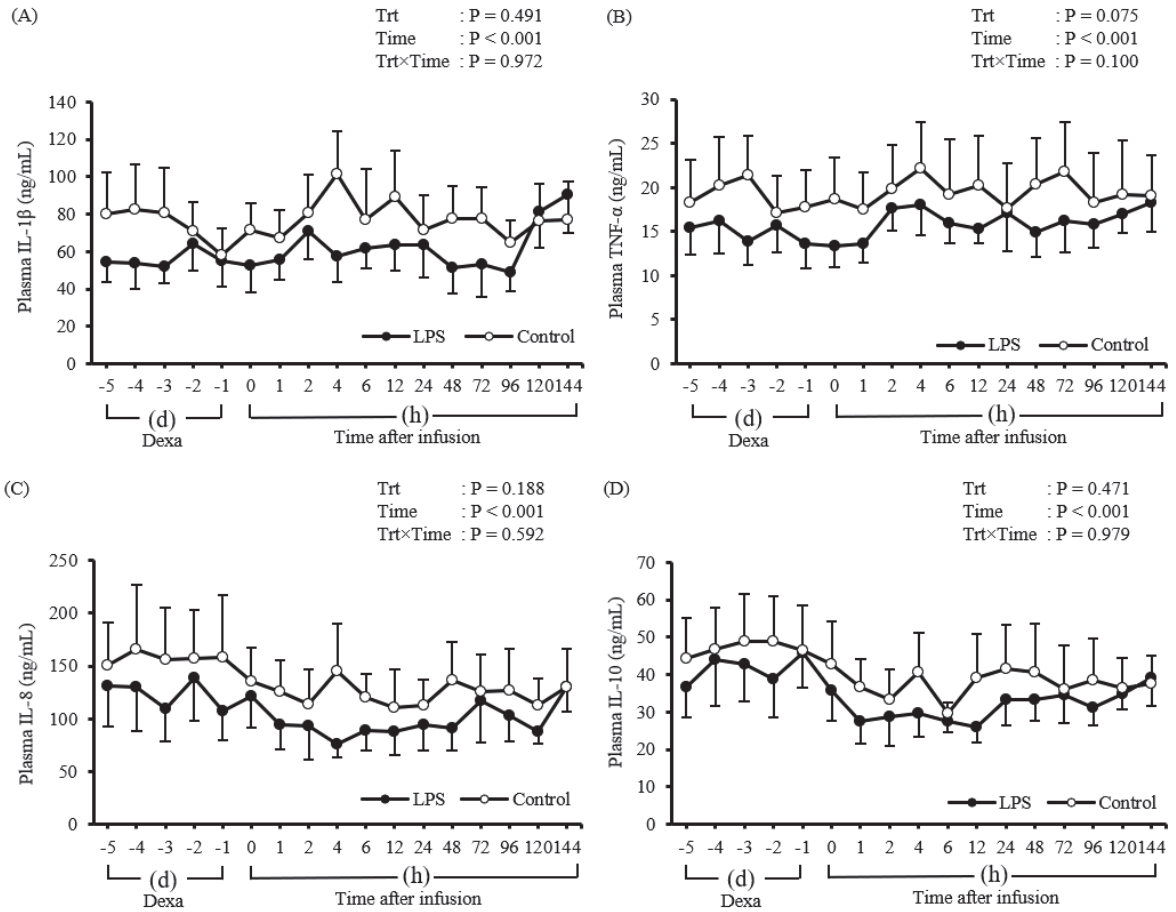
## Abstract

In Chapter 3, it was demonstrated that intrauterine-infused lipopolysaccharide (LPS) can be translocated to the mammary gland to induce weak inflammation. In this chapter, the aim was to determine if dexamethasone treatment facilitated the translocation of LPS from the uterus to the mammary gland to induce a heavy inflammatory response. Sixteen goats were divided into control and LPS groups, subjected to daily dexamethasone administration before saline or LPS infusion. Milk and blood samples were collected before and after LPS infusion to determine the milk yield and somatic cell count (SCC) and blood leukocyte count (BLC), cytokines, antimicrobial peptides, lipopolysaccharide-binding protein (LBP), and serum amyloid A (SAA) concentrations. Mammary gland tissues were collected from two goats before and 24 h after LPS infusion for immunohistochemical analysis of LPS. The mean SCC in the LPS group was significantly higher, whereas the milk yield was significantly lower than that in the control group after LPS infusion. The mean BLC in the LPS group was significantly lower than in the control group after LPS infusion. Furthermore, milk concentrations of IL-1 $\beta$ , S100A8, and lactoferrin were higher in the LPS group than in the control group after infusion. LPS was detected in the connective tissues and inner alveolar spaces of the mammary glands 24 h after LPS infusion. In conclusion, dexamethasone administration facilitated the translocation of intrauterine-infused LPS to the mammary gland, where it induced inflammatory response. Therefore, LPS translocated from other organs, such as the uterus, can induce heavy inflammation in the mammary gland under immunosuppressive conditions.

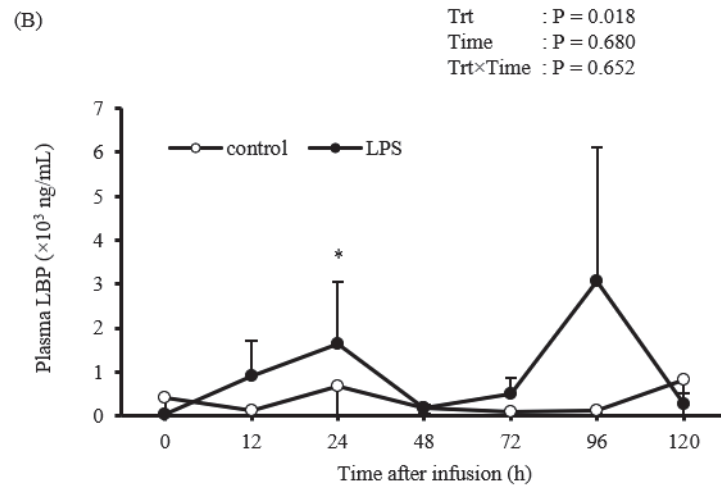
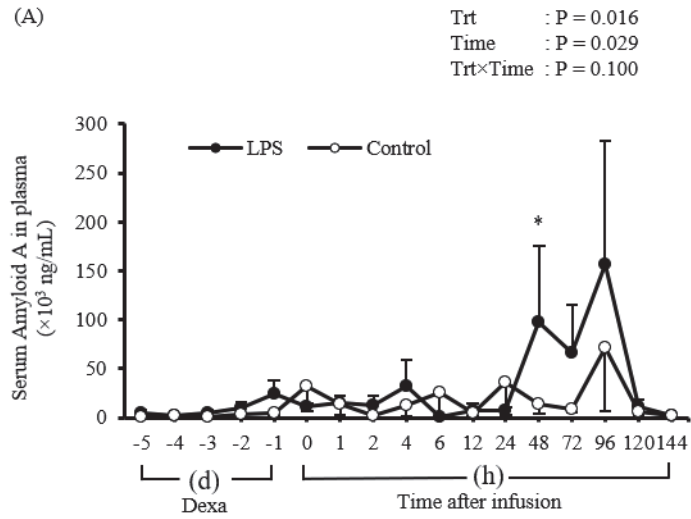




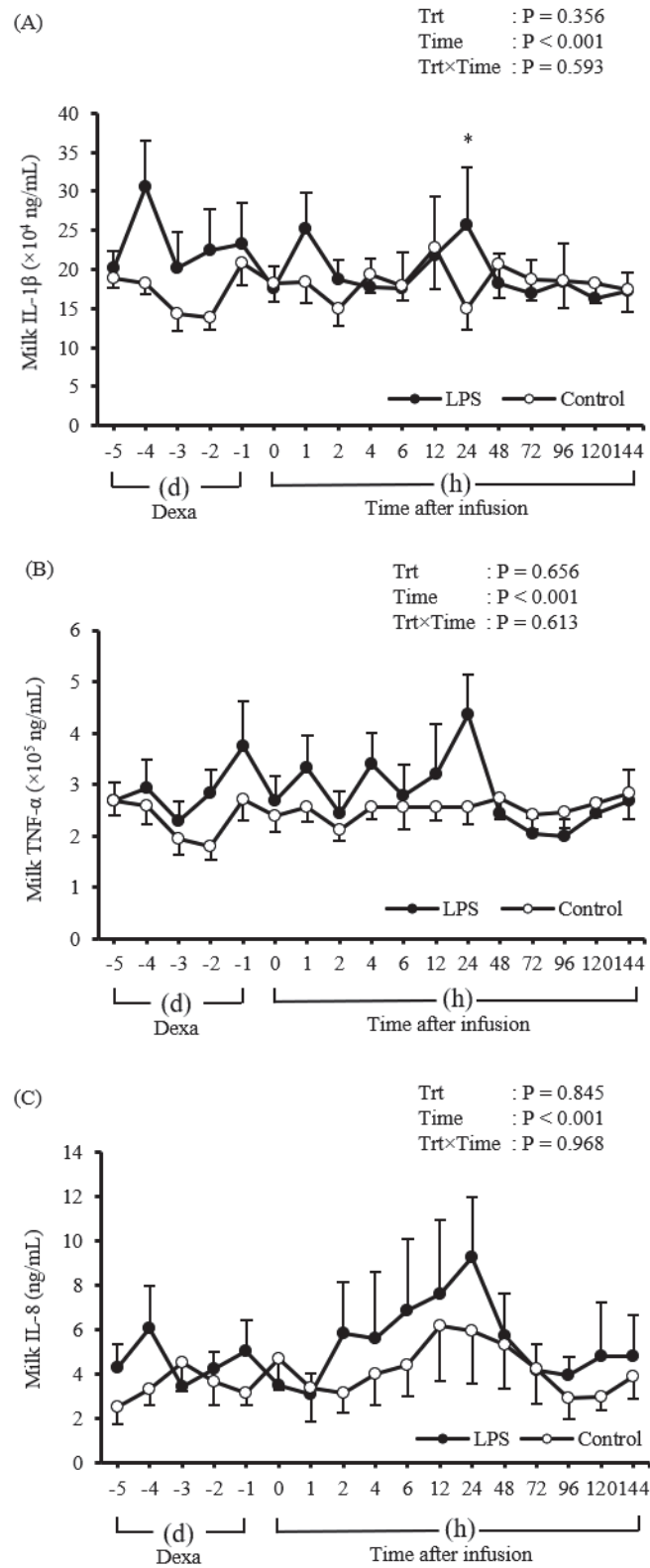
**Figure 16.** Milk somatic cell count (SCC; A), blood leukocyte count (BLC; B), and milk yield (C), after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are represented as mean  $\pm$  SEM. Difference between the control and LPS groups was significant (\* P < 0.05; \*\* P < 0.001).



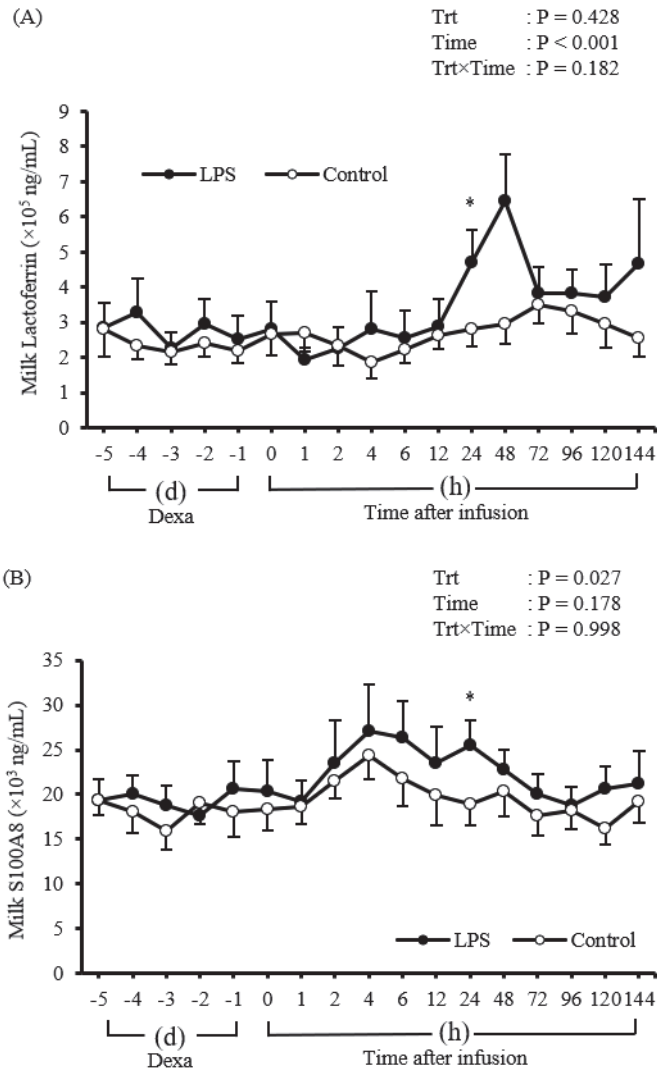
**Figure 17.** Plasma concentrations of IL-1 $\beta$  (A), TNF $\alpha$  (B), IL-8 (C), and IL-10 (D) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are represented as mean  $\pm$  SEM.



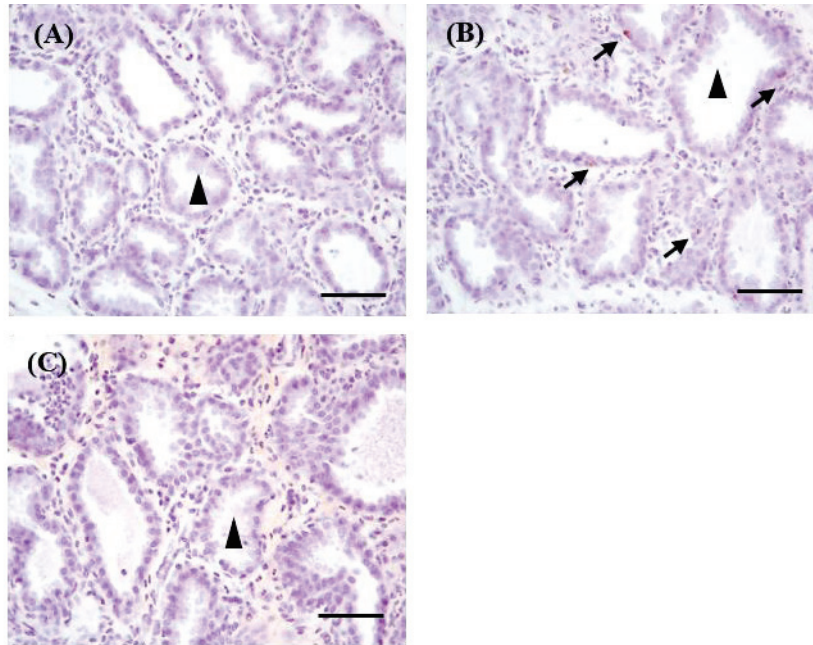
**Figure 18.** Plasma concentrations of serum amyloid A (A), and lipopolysaccharide binding protein (B) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are represented as mean  $\pm$  SEM. \* Significant difference between the control and LPS groups ( $P < 0.05$ )



**Figure 19.** Milk concentrations of IL-1 $\beta$  (A), TNF $\alpha$  (B), and IL-8 (C) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are represented as mean  $\pm$  SEM. \* Significant difference (P < 0.05) between the control and LPS groups



**Figure 20.** Milk concentrations of lactoferrin (A) and S100A8 (B) after intrauterine infusion of saline (control group, n = 8) and LPS (n = 8). Results are represented as mean  $\pm$  SEM. \* Significant difference (P < 0.05) between the control and LPS groups



**Figure 21.** Representative photographs of the mammary gland immunostained with the LPS antibody, before (A) and 24 h after LPS infusion (B) (n = 2). Negative control (C), in which the anti-LPS antibody was replaced with normal rabbit IgG. Arrows: LPS-positive reaction; arrowhead: alveoli. Scale bars = 50  $\mu$ m

## Chapter 5

### Translocation of Carbon Black Ink from the Uterus to Mammary Gland

#### Introduction

Lipopolysaccharide (LPS) is an important outer membrane component of Gram-negative bacteria, composed of (1) the lipid A, which is an endotoxin and the main virulence factor, (2) the core oligosaccharide which is a non-repeating oligosaccharide that is linked to the glucosamine of lipid A, and (3) the O-antigen, an extended polysaccharide that is attached to the core oligosaccharide as is in direct contact with the external environment (Raetz & Whitfield, 2002). LPS establishes a permeability barrier that protects the cell from the entry of toxic molecules such as antibiotic and bile salts (Nikaido, 2003), and it is the primary bacterial component encountered by the host immune system, often plays a major role in bacterial pathogenicity (Scott et al., 2017).

Several research have reported that LPS can translocate from the gastrointestinal tracts (Emmanuel et al., 2007; Khafipour et al., 2009a; Williams et al., 2013), uterus (Mateus et al., 2003), and mammary gland (Dosogne et al., 2002; Hakogi et al., 1989; Kobayashi et al., 2013) to the bloodstream. However, the results of these studies remain controversial because the body has developed compartmentalization to prevent high amounts of LPS from entering the bloodstream and the small size, biochemistry, and low concentration of LPS in hosts make them difficult to target in detection assays (Sakamuri et al., 2014).

There are some methods for detecting LPS in clinical samples. The immunohistochemical detection method might be ideal to recognize the translocated-LPS. In Chapters 3 and 4, LPS was detected in the mammary gland, whereas this cannot prove that LPS observed in the mammary gland come from the uterus. Infection may occur in other organs, thus its LPS may enter the mammary gland. Intravenous injection of LPS was conducted in rats. The immunohistochemical detection of LPS in several organs, such as the liver, kidney, spleen, lung, and aorta was conducted using the avidin-biotin complex (ABC) method. The result showed that LPS was only detected after administration of 1 mg/kg and 5 mg/kg of LPS, while the reactivity was decreased in the case of 100 µg/kg of LPS. Moreover, no positive reaction was detected with a dose of 10 µg/kg of LPS (Uragoh et al., 1988). Thus, it seems difficult to detect the movement of *in vivo* LPS by the immunohistochemical method. Therefore, this chapter aimed to confirm LPS translocation from the uterus to the mammary gland by elucidating the movement of carbon black ink infusion.

It has already been reported that amorphous carbon particles are generally well-tolerated by the body (Neugebauer et al., 1981). Carbon black is the most common amorphous carbon particles, with a molecular weight of 12.011 g/mol, that have been subjected extensively in biological experiments. Commercial carbon black may contain 88-99.5% carbon, 0.3-11% oxygen, 0.1-1% hydrogen, up to 1% inorganic material, small amounts of other organics, and traces of sulfur, depending upon the method of manufacture. All commercial carbon blacks consist of 1-100 µm sized agglomerates.

One of the most popular carbon black agents is India ink. Generally, black particles in ink have various particle sizes. They are mixed and dispersed from fine particles having a particle diameter of 0.05 µm to coarse particles of 0.5 µm. India ink is often used as a phagocytosis labeling agent or a cell differentiation tracer because it is easily administered, relatively non-toxic, and easily observed in cells that have taken it up. India ink has been widely used diagnostically for detecting cryptococcal meningitis (Cohen, 1984), endoscopic colonic tattooing (Lane et al., 1996), lymphatic mapping (Wood et al., 2001) with long-lasting stain and with relatively low risk of adverse reaction and toxicity. This study aimed to elucidate the movement of particles entering the uterus using carbon black ink infusion to clarify that the molecules can be translocated from the uterus to the mammary gland.

## **Materials and Methods**

### **Animals and carbon black ink infusion**

Four Tokara goats (bodyweight 20-25 kg, parity 1-4, mid-lactation stage, milk yield 300-800 mL/day) were used in this study. Goats were fed 0.6 kg of hay cubes and 0.2 kg of barley per day, with free access to water and a trace mineralized salt block. Feed was provided twice daily, at 08:00 and 15:00. Only goats with milk that had a low SCC ( $<1,000 \times 10^3$  cells/mL) were selected for this experiment.

Goats were assigned into 4 different treatments, based on the mastitis induction and the time of tissue collection. All goats were infused with 10 mL of carbon black ink into the uterus aseptically using the same method of LPS intrauterine infusion as previously described in Chapters 2-4. Goats 1 and 2 were infused with carbon black ink into the uterus and sacrificed 6 h and 24 h after infusion, respectively. Goat 3 and 4 was infused with 100 µg of LPS from *Escherichia coli* O111: B4 (Wako Pure Chemical, Osaka, Japan) dissolved in 5 mL of saline into one side of the mammary gland 16 h before carbon black ink intrauterine infusion and sacrificed 6 h and 24 h after carbon black ink infusion, respectively.



## **Collection of blood and tissues**

Blood was collected in a preliminary study using a goat, before (0 h) and after (2, 4, 6, 12, 24, and 48 h) intrauterine infusion of carbon black ink. Blood (5 mL) was collected from the jugular vein of the goat and transferred to a tube containing heparin as an anticoagulant. The blood was then centrifuged at  $1,900 \times g$  for 10 min at 4 °C. Plasma was discarded, and double-distilled (ddH<sub>2</sub>O) was added to rupture the red blood cells, leaving only the leukocytes. Leukocytes were attached to slides and dried overnight.

Tissues were collected within 20 min of euthanasia by exsanguination, under sedation with Xylazine (Bayer HealthCare, Osaka, Japan) and anesthesia with Pentobarbital (Somnopenyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan). Tissues collected from each goat were the body of the uterus, both sides of the mammary gland, liver, lung, spleen, and kidney. The tissues were fixed in neutralized 10% formalin in PBS for histological analysis.

## **Histology analysis**

Tissues were fixed, dehydrated, embedded in paraffin, and then sectioned. Sections (3 μm thick) were air-dried on a slide coated with aminopropyl triethoxysilane. After deparaffinization, slides were hydrated and stained with hematoxylin for 3 min. Slides were rinsed with water for 10 min and then stained with eosin solution for 1 min. After rinsed in the water, the slides were dehydrated and covered. Leukocytes sections were fixed with methanol for 10 min, rinsed with water, and stained with hematoxylin-eosin as described above. Sections were observed under a light microscope (Eclipse E400, Nikon, Tokyo, Japan). Each section was examined qualitatively for the presence of carbon particles from the carbon black ink infusion.

## **Results**

Carbon particles were found in leukocyte sections at 4 to 48 h after infusion (Fig. 22). Carbon particles were found in the uterus, liver, lung, kidney, spleen of Goat 1, 3, and 4. In addition, they were also found in the mammary gland of Goat 3. The carbon particles were found in the uterus as the site of infusion (Fig. 23). They were also found in the primary organs, such as the liver (Fig. 24) and the lung (Fig. 25). In the spleen and kidney, carbon particles were found in lower intensity (Fig. 26 and 27). Furthermore, carbon particles were only found in the mammary gland of goat 3 (Fig. 28), which was mastitis-induced 16 h before the intrauterine infusion of carbon black ink. This goat was then sacrificed 6 h after carbon infusion.

However, carbon particles were not found in all tissues of goat 2, which was intrauterine infused with carbon black ink and sacrificed 24 h later.

### **Discussion**

Carbon particles are usually assumed to be non-toxic and are readily identifiable by light and electron microscopy (Al-Ali et al., 1988). In vivo studies in chicken reported that basophils, neutrophils, and monocytes showed phagocytosis of dermally-implanted carbon particles (Dhodapkar et al., 1982). Furthermore, another study in hens reported that intravenous-injected carbon particles were phagocytosed by macrophages in the blood vessels and entered the theca interna of preovulatory follicles (Yoshimura & Okamoto, 1998). In this experiment, carbon particles might be spread through the bloodstream, since they were found in the leukocytes sections as soon as 4 h after infusion, peaked at 6-12 h, and gradually decrease 24 h after infusion. However, the results were inconsistent among goats. This finding was in accordance with a previous study that elucidates that polymorphonuclear ink cells increased rapidly after injection of India ink, reaching the maximum in 6 h after small doses and in 12 h after large doses, then disappearing gradually in several days (Nagao, 1920).

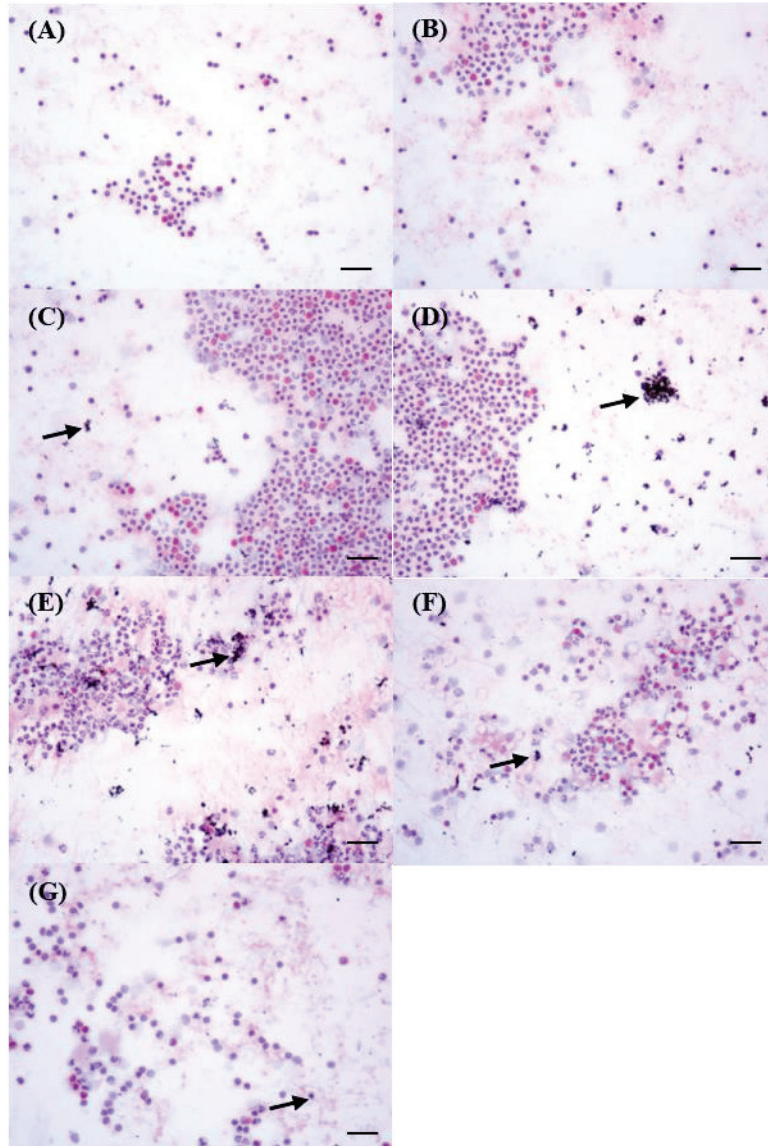
In the mammary gland, carbon particles were only found in Goat 3, which was induced by mastitis, then intrauterine infused with carbon black ink and sacrificed 6 h after infusion. In Goat 1 which was infused with carbon into the uterus and sacrificed 6 h after infusion, and Goat 4, which was induced by mastitis, then intrauterine infused with carbon black ink, and sacrificed 24 h after infusion, carbon particles were found in other tissues, but not in the mammary gland. However, carbon particles were not found in any tissues in Goat 2, which was infused with carbon into the uterus and sacrificed 24 h after infusion. The carbon particles infused into the uterus were presumed to move through the bloodstream, heart, lung, heart, and to other tissues such as the liver, kidney, spleen, and mammary gland. The accumulation of carbon particles was observed mainly in the lung. Among the liver, kidney, spleen, and mammary gland, the liver and spleen had more ink. This was in accordance with several studies in rabbits and chicken, which reported that carbon particles from India ink injection were primarily deposited in the endothelial cells of the liver, marrow, and spleen (Nagao, 1920; Ohata & Ito, 1986).

In Goat 3, which was induced by mastitis, then intrauterine infused with carbon black ink and sacrificed 6 h after infusion, carbon particles were detected in a small amount in the mammary gland. It seems that carbon particles found in the mammary gland of Goat 3 might

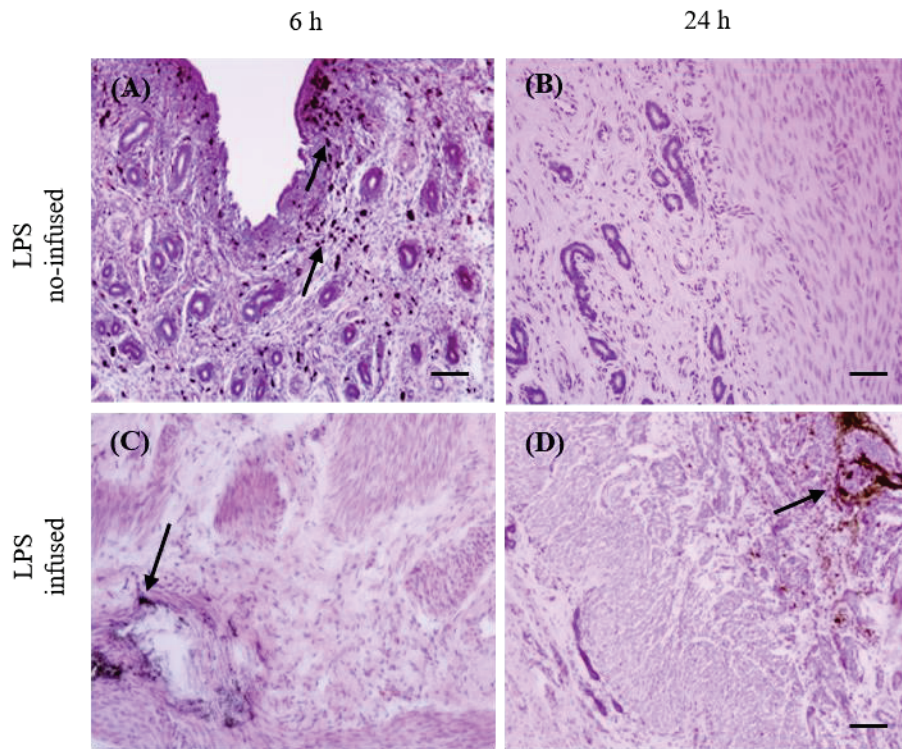
be related to the mastitis condition and time of tissue collection. When pathogens enter the mammary gland, the migration of immune cells will occur from the bloodstream to the site of inflammation. Prolonged and excessive inflammation may induce the damage and apoptosis of endothelial cells, characterized by the upregulated accumulation of leukocytes at the site of infection. When carbon particles are present in the bloodstream, they may also be recruited into the mammary tissues through the space between endothelial cells. However, carbon particles were not found in Goat 4 which was also induced by mastitis, then intrauterine infused with carbon black ink, and sacrificed 24 h after infusion. The difference between the results of Goat 3 and 4 might be related to the time of tissue collection. Carbon particles might be cleared from the mammary gland 24 h after infusion since carbon particles did not have receptors in the mammary gland. These results suggest it may be possible that bacterial components like LPS would be translocated from the uterus to the mammary gland.

## **Abstract**

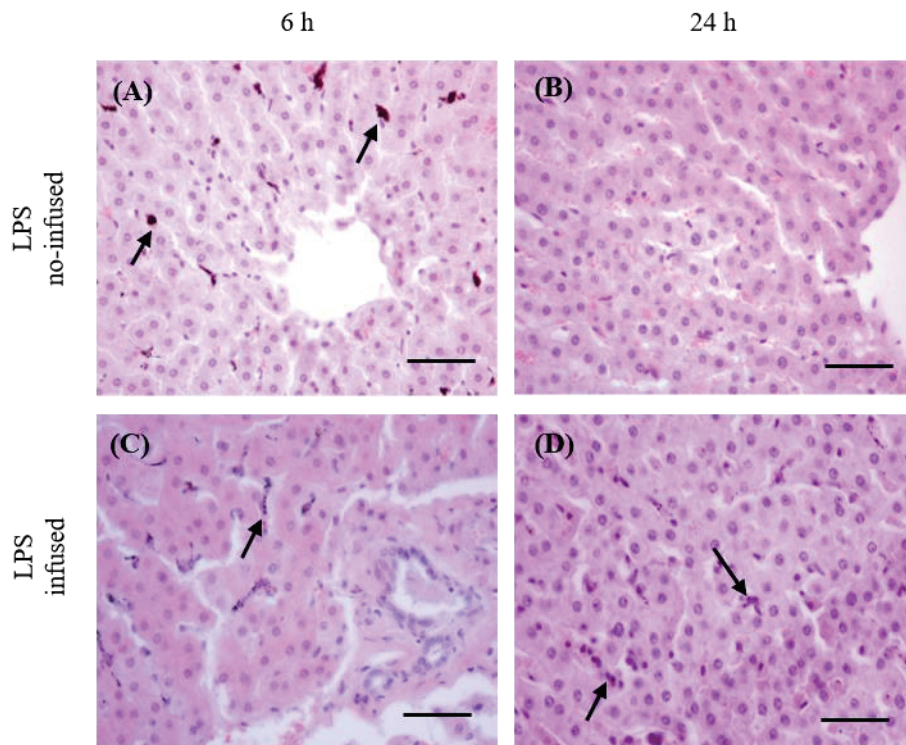
LPS was detected in the mammary gland after LPS intrauterine infusion in the previous chapter. However, there is no direct evidence that the intrauterine infused-LPS is translocated to the mammary gland. Therefore, to clarify this issue, this chapter determined whether intrauterine-infused carbon particles can translocate to the mammary gland. Four goats were assigned to 4 different treatments. Two goats were induced with mastitis 16 h before infused with carbon black ink into the uterus and sacrificed at 6 and 24 h after infusion. Two other goats were sacrificed at 6 and 24 h after intrauterine infused with carbon black ink. Carbon particles were found in the mammary gland of goat with mastitis-induced and sacrificed 6 h after carbon black ink infusion. Carbon particles were also found in all tissues (liver, lung, spleen, and kidney) except in the goat which was not induced with mastitis and sacrificed 24 h after infusion. These results indicate that carbon particles can translocate from the uterus to the mammary glands, suggesting the possible translocation of LPS from the uterus to the mammary gland.



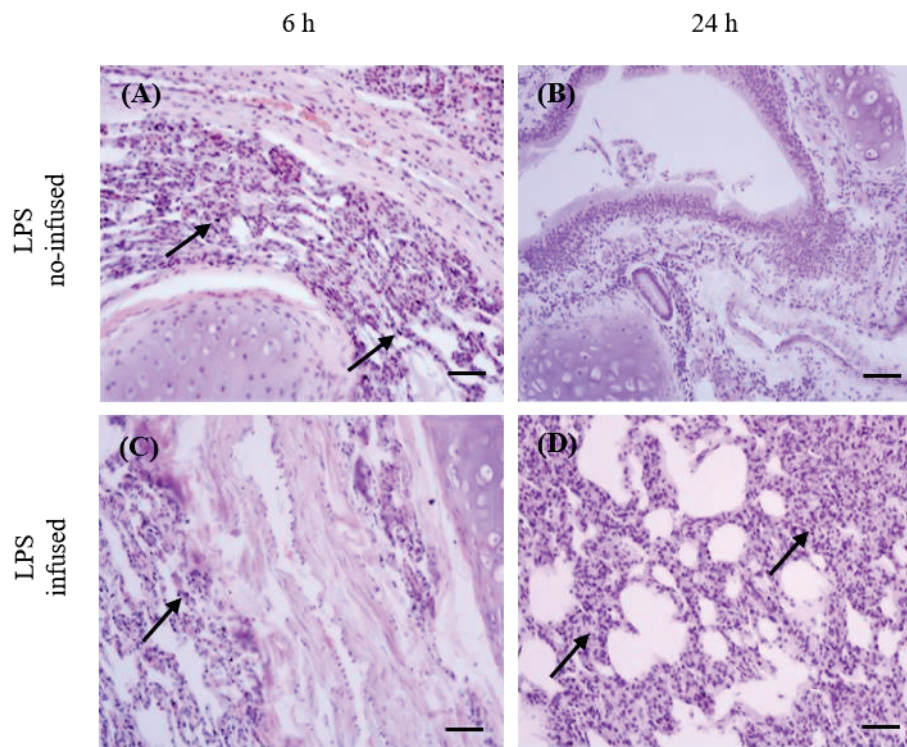
**Figure 22.** Representative photographs of the leukocyte sections 0, 2, 4, 6, 12, 24, and 48 h (A-G) h after intrauterine infusion of carbon black ink. HE staining. Arrows: carbon particles. Scale bars = 50  $\mu$ m



**Figure 23.** Representative photographs of the uterus after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m

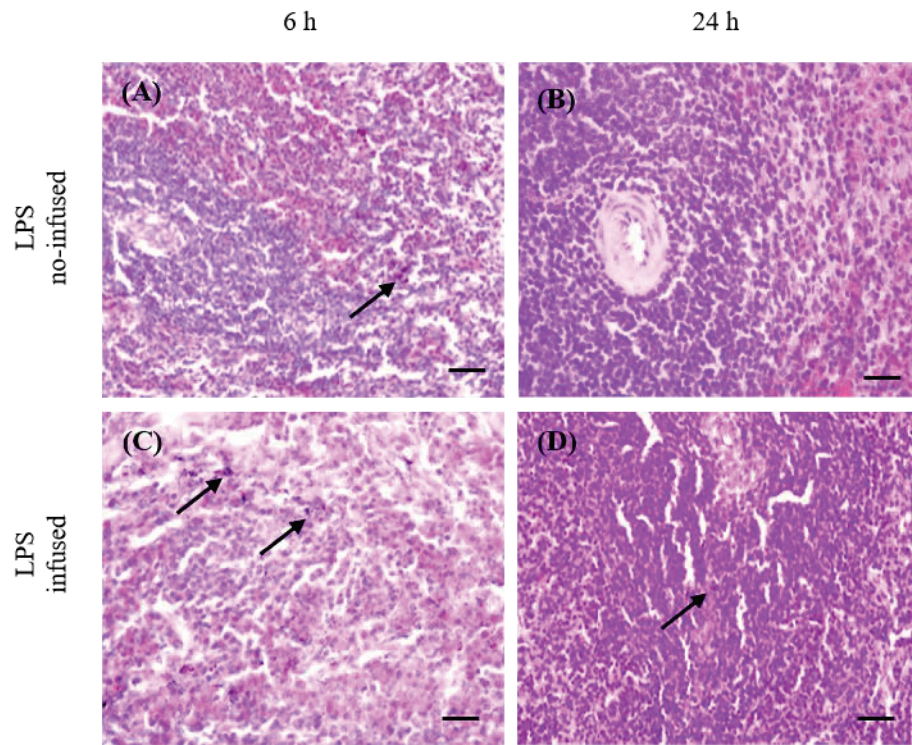


**Figure 24.** Representative photographs of the liver after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m

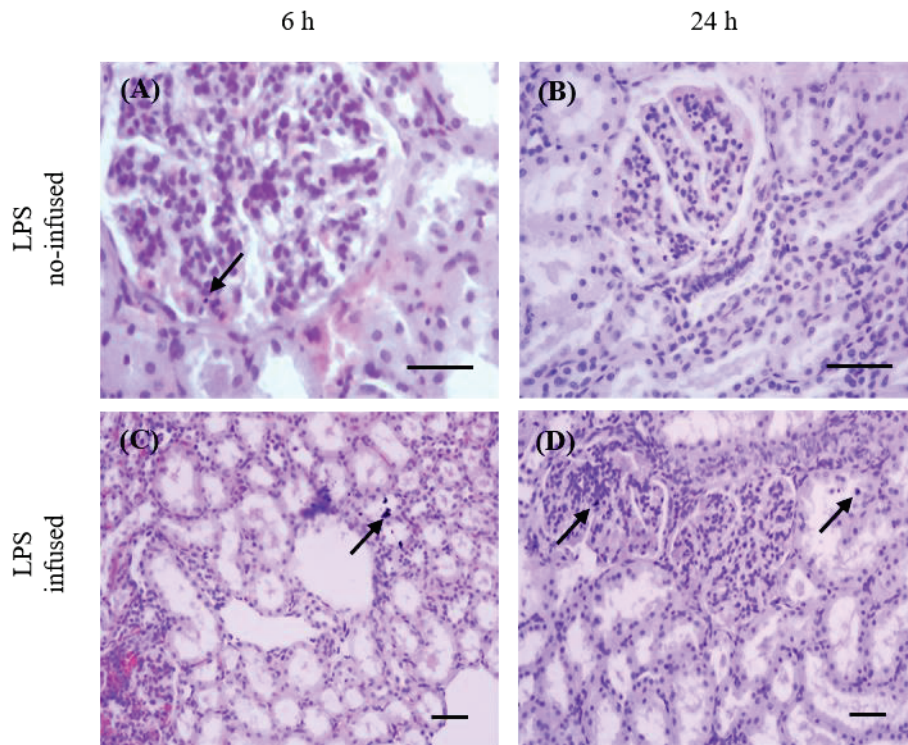


**Figure 25.** Representative photographs of the lung after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m

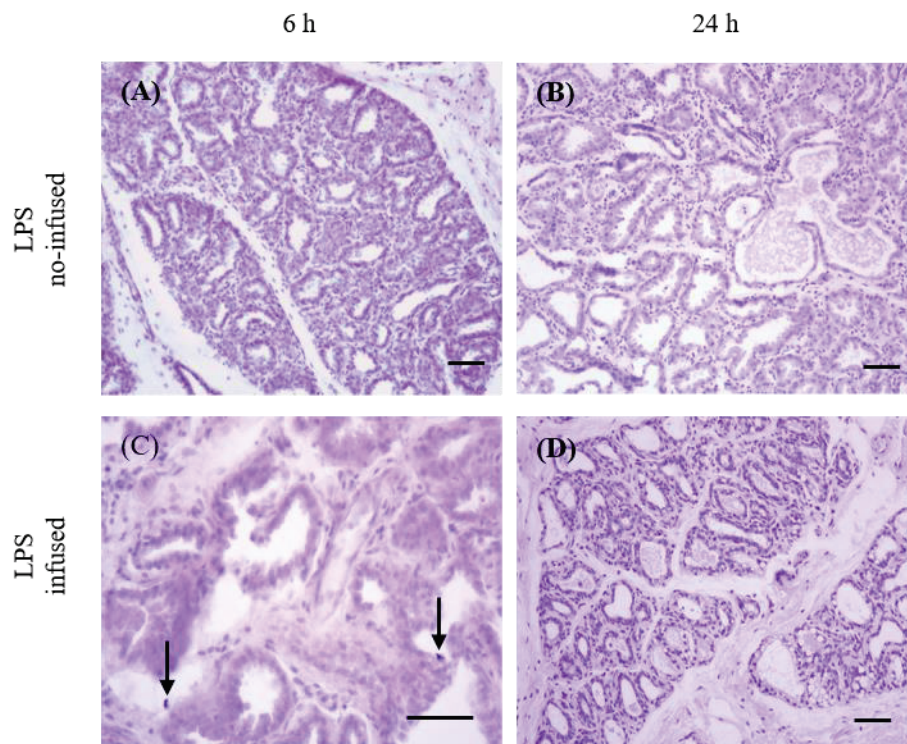




**Figure 26.** Representative photographs of the spleen after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m



**Figure 27.** Representative photographs of the kidney after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m



**Figure 28.** Representative photographs of the mammary gland after intrauterine infusion of carbon black ink. HE staining. (A) Goat 1, intrauterine infused with carbon black ink and sacrificed 6 h later; (B) Goat 2, intrauterine infused with carbon black ink and sacrificed 24 h later; (C) Goat 3, mastitis induced, intrauterine infused with carbon black ink and sacrificed 6 h later; (D) Goat 4, mastitis induced, intrauterine infused with carbon black ink and sacrificed 24 h later. Arrows: carbon particles. Scale bars = 50  $\mu$ m

## Chapter 6

### General Discussion

It is well-recognized that mastitis is the most frequent disease of dairy cows. Despite tremendous significant research in mastitis during the last century, it still produces detrimental effects on animal well-being and dairy farm profitability. Thus, it is essential to strengthen the detection and diagnosis of the disease, including the understanding of mastitis pathogens. For decades, *Streptococcus agalactiae* and *Staphylococcus aureus* were considered the most contagious pathogens, but in the early 1980s, the emerging importance of mastitis caused by opportunistic environmental organisms, such as coliforms, has been issued. This issue has been followed by the discovery of several gram-positive and gram-negative bacteria that originate primarily from environmental exposure. Furthermore, it has been found that not only live bacteria but also the bacterial component, such as lipopolysaccharide (LPS) can cause mastitis. LPS is now able to translocate within the body and its mechanism is poorly understood. Therefore, the current study focused on the possibility of mastitis caused by LPS originating from inflammation of other organs in the body and the host-sensing mechanism against endogenous LPS.

The incidence of mastitis has been studied intensively in the postpartum period in dairy cows. However, despite its effects on reproductive performance have been reported, the association between mastitis and reproductive diseases is still limited. Therefore, in Chapter 2, it was determined the relationship between endometritis with mastitis, represented by the milk SCC values in post-partum dairy cows. The average and maximum SCC were not significantly different among healthy and endometritis cows. But when the maximum SCC was classified as  $<300$ ,  $300-1,000$ , or  $>1,000 \times 10^3$  cells/ml, the percentage of cows with the maximum SCC  $< 300 \times 10^3$  cells/ml was significantly lower in the endometritis group than in the healthy group. These results suggest that high SCC might be related to the occurrence of endometritis in dairy cows, which indicates that some bacterial components may be transferred between organs. Previous studies reported that cows with metritis are also at risk of developing mastitis (Dervishi et al., 2016; Mikulková et al., 2020). However, the causal of this relationship is still undetermined.

In Chapter 3, the intrauterine infusion of LPS was conducted to determine the possible translocation of LPS from the uterus to the mammary gland and the translocated-LPS effects on the mammary gland inflammatory response under the normal physiological condition. LPS was detected in the connective tissues and interepithelial spaces of the alveoli of the mammary

glands 24 h after intrauterine infusion of LPS. In goats with the intrauterine infusion of LPS, the mean concentrations of IL-1 $\beta$  and IL-6 in milk were higher in the LPS group compared to that in the control group, whereas there were no changes in milk yield and SCC. These results suggest that LPS can translocate from the uterus to the mammary gland through the bloodstream and cause some inflammatory responses. However, SCC was not increased, which may be due to weak inflammation using healthy goat.

LPS has been also detected in the plasma of cows with metritis (Magata et al., 2015) and endometritis (Mateus et al., 2003). The mechanisms by which LPS translocate into the systemic circulation and other organs are not fully understood. In general, the translocation of LPS has been proposed in two main pathways, paracellular and transcellular. This mechanism has been proposed for intestinal tract-LPS translocation to the bloodstream. The paracellular transport of LPS occurs through tight junctions formed between intestinal epithelial cells, while the transcellular transport occurs using receptor-mediated endocytosis through the epithelial cells (Drewe et al., 2001; Hietbrink et al., 2009).

In the paracellular pathway, disruption of the tight junction protein complexes between epithelial cells decreases the barrier function, thus increasing paracellular permeability. In the intestinal tract, the permeability of the intestinal epithelium can be altered by many factors such as ruminal acidosis, increased LPS in the luminal side, metabolic and environmental stressors, and inflammation (Emmanuel et al., 2007; Mani et al., 2012). In the transcellular pathway, LPS may enter the circulation by moving across polarized cells while tight junctions remain intact. Further evidence suggests that this occurs via receptor-mediated endocytosis facilitated by TLR4, where other proteins such as MD2, and CD14 are necessary (Guo et al., 2013).

In the blood, LPS is bound mainly to high-density lipoprotein (HDL) and low-density lipoprotein (LDL), a process facilitated by LBP and soluble CD14 (sCD14) (Kitchens et al., 1999). LPS bound to lipoproteins will be redirected to the liver and degraded by hepatocytes or white adipose tissue. In a minority case, a high amount of LPS may cause septic shock or reach other tissues throughout the body. A weak immune system increases the chance of developing this serious complication as the immune response may not function properly. In the tissues, LPS may trigger the secretion of more pro-inflammatory cytokines from local macrophages that enter into the systemic circulation and contribute to an inflammatory state (Creely et al., 2007). In the study of Chapter 3, LPS in the bloodstream might not selectively be translocated to the mammary gland, but also into other organs. However, this study was only focused on the mammary gland inflammatory response, thus the effects of translocated-LPS to the other organs were unnoticed and needed to be studied further.

Meanwhile, a single intrauterine infusion of LPS might not seem reliable to stimulate a naturally occurring endometritis and lead to the translocation of LPS into the mammary gland. In natural cases, cows with high uterine pathogens had higher peripheral concentrations of acute-phase proteins, indicating the systemic inflammation occurrence (Williams et al., 2007). However, intrauterine infusion of a single dose of LPS has been reported to induce systemic effects (Peter et al., 1989), and repeated intrauterine infusion of LPS induced subclinical endometritis caused by the repeated transcervical passage rather than the LPS itself (Lüttgenau et al., 2016). Furthermore, the body can learn from experience, and the learning ability of immune cells may become less sensitive to LPS when they are constantly exposed to low levels of LPS, called LPS-tolerance (Seeley & Ghosh, 2016).

A single dose of LPS used in Chapter 3 induced a weak inflammation and goats were susceptible to LPS, a modification of cow factor seems more reliably to stimulate a heavier inflammation in the mammary gland. Thus, Chapter 4 was undertaken to examine the effect of intrauterine infusion of LPS on the inflammatory response of the mammary gland under the immunosuppressive condition using dexamethasone administration.

The mean SCC in the LPS group was significantly higher, whereas the milk yield was significantly lower than that in the control group after LPS infusion. Furthermore, milk concentrations of IL-1 $\beta$ , LBP, S100A8, and lactoferrin were higher in the LPS group than in the control group after infusion. LPS was detected in the connective tissues and inner alveolar spaces of the mammary glands 24 h after LPS infusion. It concluded that dexamethasone administration enhanced the translocation of intrauterine-infused LPS to the mammary gland, where it induced an inflammatory response on it. Therefore, LPS translocated from other organs, such as the uterus, can induce heavy inflammation in the mammary gland under immunosuppressive conditions.

The translocation of LPS in the study in Chapter 4 might be related to the immunosuppressive conditions. Dexamethasone is known as a potent immunosuppressive agent. It is well-known that during the immune suppression conditions, the proliferation of leukocytes is severely depressed. The ability to aggregate and phagocytosis of neutrophils, the cytotoxic activity of lymphocytes, as well as production of chemotactic cytokine IL-8 activating these leukocytes are usually reduced at this time. If the administration of dexamethasone suppressed the immune system, LPS had only a little chance to bound to the immune cells, it suggested that LPS might not be cleared immediately from the bloodstream and can easily translocate to other tissues in the body. In the study in Chapter 4, LPS was found in the mammary gland 24 h after intrauterine infusion of LPS along with the occurrence of

inflammation. It is suggested that dexamethasone suppressed the immune system, facilitated the translocation of LPS to the mammary gland, and enhanced the inflammatory response in the mammary gland.

Furthermore, in the previous report, chronic stress associated with the elevation of serum corticosterone was reported to decrease the epithelial tight junction proteins that were associated with an increase in epithelial paracellular permeability in the intestinal tract in the stress rat model (Zheng et al., 2013). It can be assumed that dexamethasone administration affected the permeability of mammary gland epithelial tight junction and increased the epithelial paracellular permeability, thus allowed LPS to enter easily into the mammary gland.

The results of the current study also revealed that the mammary gland is susceptible to LPS. LPS was immunolocalized in the mammary gland 24 h after infusion. At the same time, LPS induced an acute inflammation within 24 h with no significant changes in plasma inflammatory markers concentrations (Chapter 3 and 4). In milk, the concentration of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in Chapter 3 and IL-1 $\beta$ , antimicrobial peptides LF and S100A8 in Chapter 4 were significantly elevated 24 h after LPS intrauterine infusion. The administration of dexamethasone in Chapter 4 might play an important role in enhancing the inflammatory response of the mammary gland.

However, there is no direct evidence that LPS found in the mammary gland comes from the uterus. Thus, in Chapter 5, the intrauterine infusion of carbon black ink was conducted to elucidate the movement of particles. In Chapter 5, intrauterine-infused carbon particles were found in the mammary gland of goat with mastitis and sacrificed 6 h after carbon black ink infusion. Therefore, components like LPS can translocate from the uterus to the mammary glands.

Several studies have demonstrated that LPS weakens the blood-milk barrier after LPS infusion into the mammary gland. Kobayashi et al. (2013) reported that LPS weakens the blood-milk barrier as soon as 3 h after LPS intramammary infusion in lactating mice caused by compositional changes of claudins in alveolar epithelial tight junctions. While, another study showed that intramammary infusion of LPS disrupts the blood-milk barrier 3 h after infusion in dairy cows, as evidenced by an elevation of lactate concentration in blood and IgG concentration in the milk (Lehmann et al., 2013). These previous studies confirm the results of why carbon particles were only found in the mammary gland of goat with mastitis-induced. Mastitis-induced LPS might affect the alveolar epithelial tight junctions and allowed the translocation of carbon particles into the mammary gland.

From the study in Chapter 4, where intrauterine-infused LPS moved easily into the mammary gland following dexamethasone treatment and study in Chapter 5, where carbon particles were only found in the mammary gland with mastitis, the translocation of LPS from the uterus to the mammary gland seems to be influenced by some abnormal physiological conditions. Therefore, factors affecting the translocation of LPS throughout the body are required to be further examined.

In conclusion, the high SCC in the milk of dairy cows is related to the occurrence of endometritis in the postpartum period. In the goat model, the intrauterine infusion of LPS can translocate to the mammary gland through the bloodstream and induce the inflammatory response on it. Moreover, the heavier inflammatory response was revealed in the dexamethasone-treated goats. Evidence suggests that not only viable bacteria but also bacterial components such as LPS are involved in the occurrence of multiple diseases in dairy cows. In the dairy farms, the possibility of LPS translocation between organs urges veterinarians to consider the comprehensive health assessment rather than signs-based diagnosis. For decades, mastitis has been diagnosed based on the clinical signs, the increase of SCC in milk, and the presence of bacteria or other pathogens by culture. However, today, mastitis can be caused by bacterial components that are non-culturable, can be translocated from other organs in the body, and difficult to be detected and treated. Thus, the application of biological systems approaches, for instance, by determining the specific biomarkers of mastitis in dairy cows, including the evaluation of pro-and anti-inflammatory cytokines, acute-phase proteins, and antimicrobial peptides concentrations in plasma and milk, especially in the periparturient period and under the stress conditions, may improve the understanding of the etiology, pathomechanisms, and early diagnosis of mastitis in dairy cows. Furthermore, given that LPS can be translocated through mucosal membranes, developing new technologies such as mucosal vaccines is of utmost importance to prevent LPS-related diseases.



## Summary

Mastitis is one of the most important diseases in ruminants regarding the economic, animal health, and food hygiene perspectives. Knowledge of the causative pathogens is important to effectively control intramammary infections. Lipopolysaccharide (LPS), a Gram-negative bacterial component can translocate within the body and its presence can induce the inflammatory response, whereas the mechanism of its translocation to the mammary gland is poorly understood. This study aimed to determine whether mastitis can be caused by bacterial components (LPS) through the endogenous pathway.

### **1. Association of endometritis with the mammary gland inflammatory condition in dairy cows**

Multiple metabolic and inflammatory diseases such as endometritis and mastitis occur during the periparturient period in dairy cows. If the bacterial component can be transferred, there must be a close association between endometritis and mastitis. Thus, this study aimed to examine the possible association of endometritis with mammary gland inflammatory conditions in post-partum dairy cows. Sixty-one Holstein dairy cows were clinically examined for endometritis at 30-50 days after parturition. Blood and milk samples were collected for the measurement of Haptoglobin (Hp), and lipopolysaccharide-binding protein (LBP) in plasma, and somatic cell count (SCC), IL-1 $\beta$ , and TNF $\alpha$ , and IL-8 concentrations in milk. Of the 61 dairy cows, 49 cows were determined as healthy cows, while 12 cows were diagnosed with endometritis. The average and maximum SCCs were not significantly different between the healthy cows and those with endometritis. However, when the maximum SCC was classified as <300, 300-1,000, or >1,000  $\times 10^3$  cells/ml, the percentage of cows with the maximum SCC < 300  $\times 10^3$  cells/ml was significantly lower in the endometritis group than in the healthy group. These results suggested that high SCC might be related to the occurrence of endometritis in dairy cows, indicating that some bacterial components can be transferred between the uterus and mammary gland.

### **2. Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in goats**

This experiment aimed to determine the effects of LPS infusion on the mammary gland inflammatory response to clarify the possibility of bacterial components translocation from the uterus to the mammary gland. Sixteen goats were divided into two groups, subjected to intrauterine infusion of LPS or saline (control). The concentrations of IL-1 $\beta$

and IL-6 in milk were higher in the LPS group compared to that in the control group, whereas there were no changes in milk yield or SCC. In the immunohistochemistry, LPS was detected in the connective tissues and interepithelial spaces of the alveoli of the mammary glands at 24 h after intrauterine infusion of LPS. These results suggest that intrauterine-infused LPS can be translocated from the uterus to the mammary glands and caused some inflammatory responses.

### **3. Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in dexamethasone-treated goats**

The combined effects of multiple stressors, including high and low temperature, nutritional deficiency, parturition, the transition to the milking herd, and herd management during the periparturient period, can increase and prolong the magnitude of immunosuppression and increase animal susceptibility to disease. The previous study revealed that LPS can translocate from the uterus to the mammary gland and induce a weak inflammation, thus, this experiment aimed to determine if dexamethasone (immunosuppressive agent) treatment enhances the translocation of LPS from the uterus to the mammary gland to induce a heavy inflammatory response. Sixteen goats were divided into control and LPS infusion groups after daily dexamethasone administration for 5 days. Milk and blood samples were collected before and after LPS infusion. The mean SCC in the LPS group was significantly higher, whereas the milk yield was significantly lower than that in the control group after LPS infusion. Plasma lipopolysaccharide-binding protein (LBP) and serum amyloid A (SAA) concentrations were significantly higher in the LPS group than in the control group after LPS infusion. Furthermore, the LPS group has higher milk concentrations of IL-1 $\beta$ , S100A8, and lactoferrin than the control group after LPS infusion. In the immunohistochemistry, LPS was detected in the connective tissues and inner alveolar spaces of the mammary glands at 24 h after LPS infusion. These results suggest that dexamethasone administration enhanced the translocation of intrauterine-infused LPS to the mammary gland, where it induced a heavier inflammatory response than in normal physiological conditions.

### **4. Translocation of carbon black particles from the uterus to mammary gland**

In the previous experiment, LPS was detected in the mammary gland, whereas this cannot prove that LPS observed in the mammary gland comes from the uterus. Therefore, this study aimed to confirm LPS translocation from the uterus to the mammary gland using

carbon black ink. Four goats were assigned to 4 different treatments of carbon black ink intrauterine infusion. Two goats were intrauterine infused with carbon black ink and sacrificed at 6 and 24 h after carbon black ink infusion. Two other goats were induced with mastitis by LPS intramammary infusion, 16 h before infused with carbon black ink into the uterus and sacrificed at 6 and 24 h after carbon black ink infusion. Carbon particles were found in the mammary tissue of goat with mastitis and sacrificed 6 h after carbon black ink infusion. These results suggested that carbon particles can translocate from the uterus to the mammary gland through the bloodstream. However, the translocation of carbon particles to the mammary gland might be influenced by the inflammatory condition of the mammary gland.

## **5. Conclusion**

The high SCC in milk may be associated with the occurrence of endometritis in dairy cows. Besides live bacteria, a bacterial component such as LPS in the uterus can also cause the inflammation of the mammary gland through the bloodstream. The inflammatory response of the mammary gland caused by LPS translocated from the uterus was heavier in the immunosuppressive condition compared to normal physiological conditions in the animal. This knowledge is expected to be useful to understand the mechanism of LPS translocation in dairy cows and establish the control mechanism of mastitis.

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