CHAPTER III
INFLAMMATORY IMMUNE RESPONSES IN EXPERIMENTALLY INDUCED MASTITIS IN LACTATING MICE
<table>
<thead>
<tr>
<th>Sl. No</th>
<th>TITLE</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>INTRODUCTION</td>
<td>125</td>
</tr>
<tr>
<td>3.2</td>
<td>MATERIALS AND METHODS</td>
<td>129</td>
</tr>
<tr>
<td>3.3</td>
<td>OBSERVATIONS</td>
<td>135</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Effect of mastitis induced infection on the inflammatory cytokines of mammary gland of mice.</td>
<td>135</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Effect of mastitis induced infection on myeloperoxidase activity of mammary gland of mice.</td>
<td>136</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Effect of mastitis induced infection on N-acetyl-β-d-glucosaminidase activity of mammary gland of mice.</td>
<td>136</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Effect of mastitis induced infection on haemogram of mice.</td>
<td>136</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Effect of mastitis induced infection on lactation hormones of mice.</td>
<td>139</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Effect of mastitis on histopathological changes of inguinal lymph node of mammary gland in experimentally induced lactating mice.</td>
<td>141</td>
</tr>
<tr>
<td>3.4</td>
<td>DISCUSSION</td>
<td>142</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Effect of mastitis induced infection on the inflammatory cytokines of mammary gland of mice.</td>
<td>142</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Effect of mastitis induced infection on myeloperoxidase activity of mammary gland of mice.</td>
<td>145</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Effect of mastitis induced infection on N-acetyl-β-d-glucosaminidase activity of mammary gland of mice.</td>
<td>146</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Effect of mastitis induced infection on haemogram of mice.</td>
<td>147</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Effect of mastitis induced infection on lactation hormones of mice.</td>
<td>148</td>
</tr>
<tr>
<td>3.4.6</td>
<td>Effect of mastitis on histopathological changes of inguinal lymph node of mammary gland in experimentally induced</td>
<td>152</td>
</tr>
</tbody>
</table>
lactating mice.

3.5 SUMMARY AND CONCLUSION 154
3.1 INTRODUCTION

In the inflammatory response the mammary gland immune system is activated to eliminate the pathogen. This defense mechanism includes anatomical, cellular and soluble factors that act in coordination and are crucial to modulation of mammary gland resistance and susceptibility to infection (Oviedo Boyso et al. 2007). Once bacteria successfully penetrate the teat end opening, it is the efficiency of these defense mechanisms that determines the resistance of the mammary gland to new intramammary infection (IMI). The mammary gland is protected by a variety of defense mechanisms, which can be separated into two distinct categories: innate immunity and specific immunity. Innate immunity, also known as nonspecific responsiveness, is the predominant defense during the early stages of infection. Nonspecific responses are activated quickly at the site of infection by numerous stimuli. Nonspecific or innate responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer (NK) cells, and by certain soluble factors (Sordillo, 2005).

Lactation is considered the final phase of the mammalian reproductive cycle, and the mammary gland provides milk for nourishment and disease resistance to the newborn. However, the cellular and soluble immune components associated with mammary tissues and secretion also can play an important role in protecting the gland from infectious diseases, such as mastitis. The mammary gland is a complex organ that provides neonatal offspring with milk for nourishment and disease resistance. Specific and innate immune factors associated with mammary gland tissues and secretion also play a vital role in protecting the gland from infectious disease (Sordillo and Streicher, 2002).
The cells of the immune response interact with each other by direct contact and with the help of functional different proteins with cell-regulatory functions, called cytokines. Cytokines are a diverse group of nonenzymic proteins, which affect diverse target cell population as biologic response modifiers. Although every nucleated cell type can produce cytokines, most lineages express only a subset of cytokine genes (Akira and Kishimoto, 1997; Fitzpatric and Kelso, 1998).

Cytokines are generally not produced constitutively, and the stimuli that can trigger synthesis vary with the cell type and its differentiation or activation state and determine which cytokines are produced by the cell (Arai et al., 1990 and Abbas et al., 1997). Cytokines produced from the activated immune cells, monokines, interferons and interleukines, modulate the inflammation and the immune response by regulating the growth, differentiation and ability for migration of immune cells. After antigen stimulation, certain combinations of the produced cytokines have influence over the type and magnitudes of the immune response that appears (Mchugh et al., 1995; Abbas et al., 1996).

Cytokines play an important role in inflammatory responses (Arai et al., 1990, Bonfield et al., 1995). They are involved in the initiation and development of inflammation. These cytokines initiate, amplify, and perpetuate the inflammatory response in mastitis (De and Mukherjee, 2009). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) are the cytokines involved in the inflammatory process of mastitis (Persson et al., 2003). Among these cytokines, TNF-α, the earliest and primary endogenous mediator of an inflammatory reaction, can elicit the inflammatory cascade, cause damage to the vascular endothelial cells and induce alveolar
epithelial cells to produce other cellular factors and chemotactic factors, such as IL-6 (Giebelen et al., 2007). IL-1β also can stimulate the production of several secondary cytokines that amplify inflammation, such as IL-6. IL-6, produced by macrophages, is one of the most common inflammatory cytokines (Martin, 1999; Hodge et al., 2005). IL-6 is involved in acute septic shock during mastitis caused by pathogens (Bannerman et al., 2004). These cytokines can stimulate vascular endothelial cells to induce vascular endothelial intercellular adhesion molecule expression and up-regulation, which results in endothelial cell and leukocyte adhesion, leukocyte migration, granulocyte degranulation, capillary permeability increase, and migration to inflammatory positions, leading to mammary damage (Zheng et al., 2006).

Myeloperoxidase (MPO), a hydrogen peroxide (H$_2$O$_2$) oxidoreductase, is specifically found in mammalian granulocytic leukocytes, including polymorphonuclear leukocytes (PMNs), monocytes, basophils, and eosinophils (Schultz et al., 1965), and it contributes to the bactericidal capabilities of these cells. N-acetyl-β-d-glucosaminidase (NAGase) is an intracellular, lysosomal enzyme which is released into milk from neutrophils during phagocytosis and cell lysis, and to some degree, damaged epithelial cells (Kitchen et al., 1984). NAGase is released from damaged mammary epithelial cells (Kitchen et al., 1980). However, a small fraction of milk NAGase can also come from leukocytes (Capuco et al., 1986).

Stresses due to pregnancy, parturition and lactation stimulate the production of a variety of stress hormones that can have major effects on the immune response (Sordillo, 2005). At the onset of pregnancy the endocrine system undergoes dramatic changes. The growth of the mammary gland is stimulated by growth hormone and prolactin,
adrenocortical steroids, oestrogens and progesterone, and that of the gastrointestinal (GI) tract by gastrin, CCK and secretin. The onset of lactation is accompanied by increases in the blood volume, cardiac output, mammary blood flow and blood flow through the GI-tract and liver, aiming to provide the udder with nutrients and hormones for regulation of milk synthesis. Food intake and distribution of nutrients to the mammary gland are partially regulated by hormones as well as the repartitioning of nutrients away from body stores towards the udder. Besides central mechanisms, local mechanisms within the mammary gland regulate initiation of lactation, maintenance, regulation of blood flow and mammary gland cell apoptosis (Svennersten-Sjaunja and Olsson, 2005). Milk production is controlled by the lactogenic hormones prolactin and GH during lactogenesis and galactopoiesis. Both prolactin and GH are essential for the transition from a proliferative to a lactating mammary gland although GH dominates over prolactin during galactopoiesis in ruminants (Flint, 1997), unlike the situation in rodents and humans.

Therefore, to understand the inflammatory immune response of the intramammary infection induced by bacterial pathogens. The present investigation was aimed to evaluate the inflammatory immune responses of mastitis induced by *S. aureus*, *E. coli* and *B. subtilis* inoculum’s in mice by estimating the inflammatory cytokine levels, inflammatory enzyme activities, haemogram, hormone levels and histopathology of inguinal lymph node of mammary gland after 24 h of infection in lactating mice.
3.2 MATERIALS AND METHODS

3.2.1 Inoculum preparation

The intramammary inocula preparation was based on the method described by Brouillette et al., (2004) with minor modifications. S. aureus, E. coli and B. subtilis were isolated from a clinical case of bovine mastitis and grown overnight at 37°C in the Tryptic soy broth medium to reach mid-exponential growth. The actual number of colony forming unit (CFU) injected was confirmed by spreading the inoculum on to an agar plate and counting the colonies after overnight incubation. Bacterial concentration of the culture was determined using a standard curve plotting CFU as a function of the absorbance at 600 nm, the cultures were further serially diluted and suspended in phosphate buffer saline (PBS) to the desired number of CFU/ml.

3.2.2 Animals

Laboratory bred adult female Swiss albino mice were used in the experiments. Mice aged 90-120 days old weighing between 30-32 g was used. They were housed in separate polypropylene cages containing sterile paddy husk as bedding material. The mice were provided with standard mice pellet diet “Gold Mohar” (Krish Scientist’s Shopee, Bangalore) and water ad libitum. The mice were maintained under normal day/night schedule (12 L: 12 D) at room temperature 25 ± 2°C.

3.2.3 Mouse mastitis model of infection

The intramammary inoculation technique was based on the method described by Brouillette and Malouin (2005). The lactating mice used for inducing intramammary
infection were of 12-14th day of the parturition weighing 36-38 g. The pups were removed 1-2 h before bacterial inoculation of mammary glands and a mixture of ketamine/xylazine at 87 and 13mg/kg of weight, respectively, was used for anesthesia of the lactating mice. A 1ml syringe with 28 gauge blunt needle was used to inoculate both L4 (on the left) and R4 (on the right) abdominal mammary glands. The study was approved by the Institutional Animal Ethical Committee, Department of Biotechnology and Microbiology, Karnataka University, Dharwad, India. CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) (Animal House Registration No. 639/02/a/ CPCSEA) guidelines were followed for maintenance and use of the experimental animals.

3.2.4 Infection profile

Lactating mice were divided into five groups of 4 mice each. Group I were injected with PBS (PBS vehicle serves as control), group II were inoculated with S. aureus inoculum doses of $2.6 \times 10^3$ CFU, group III mice were inoculated with E. coli inoculum doses of $2.6 \times 10^3$ CFU and group IV mice were inoculated with B. subtilis inoculum doses of $2.6 \times 10^3$ CFU suspended in PBS (100μl/gland) were inoculated through R4 and L4 teat canals of mice respectively. After 24 h of infection mice were euthanized, the mammary gland tissues were dissected out and collected in PBS and stored at $-20^\circ$C for cytokines assay, myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase) enzyme activities and histopathology of inguinal lymph node. Blood serum collected was assayed for haemogram and hormone analysis.
3.2.5 Preparation of mammary tissue and serum

The frozen mammary tissues were thawed, weighed and homogenized with sterile physiological saline (1:6, W/V) on ice and then centrifuged at 2000 g for 40 min at 4 °C according to the method of Zhu et al., (2004) with minor modifications. Lipid was removed and the supernatant was collected. The supernatant was centrifuged again at 2000 g for 20 min at 4 °C to remove any remaining lipid. The supernatant was collected and stored at −20 °C until analyzed. Its protein concentration was determined using the Bradford method and the results were expressed as mg/mL protein. Serum was separated by centrifugation (2000 g, 15 min) and stored at −20°C until analyzed.

3.2.5.1 Cytokines assays of mammary tissue

The tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were quantified by enzyme-linked immunosorbent assay (ELISA) kits (KRISHGEN BioSystems, Mumbai, India) following the manufacturer's protocol as given below for each assay.

Principle: Enzyme-linked immunosorbent assay, commonly known as ELISA (or EIA), is similar in principle to radioimmunoassay (RIA) but depends on an enzyme rather than a radioactive label. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate.

All the reagents were brought to room temperature. An aliquot of 100μl/well of standards and samples were added to the microtiter plate coated with TNF-α, IL-1β and IL-6 separately. The standard concentrations for TNF-α (2000 pg/ml), IL-1β and IL-6
(4000 pg/ml) used were diluted to six fold serial dilutions according to their concentrations (pg/ml). The plate was sealed and incubated at room temperature for 2 h. The plate was aspirated and washed four times with wash buffer and residual buffer was blotted. An aliquot of 100 µl of diluted detection antibody was added to each well, plate was sealed and incubated at room temperature for 2 h. Plates were again washed four times with wash buffer. An aliquot of 100µl of diluted Streptavidin-HRP solution was added to each well, plate was sealed and incubated at room temperature for 30 mins. Again the plates were washed four times, for the final wash; wells were soaked in wash buffer for 30 seconds to 1 mins for each wash. Further, an aliquot of 100µl of freshly mixed TMB Substrate solution was added and incubated in the dark for 15 mins. Positive wells turned bluish in color. The reaction was stopped by adding 100µl of stop solution to each well the positive wells turned blue to yellow. Absorbance was read at 450 nm within 30 minutes of stopping reaction and the concentration levels were expressed in pg/ml.

3.2.5.2 Myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase)

MPO and NAGase levels were also determined using a commercial kit purchased from the KRISHGEN BioSystems, Mumbai, India. MPO activity in homogenates of mammary tissue was evaluated following the manufacturer's protocols. Briefly, tissues were thawed immediately before the assay was conducted and homogenized in 10% (W/V) 20 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 10 min at 4 °C. The pellet, resuspended with sonication in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, was further disrupted by three freeze–thaw cycles. The samples or sera were then centrifuged at 10,000 g for 5
min at 4 °C and the supernatant was collected for the assay. MPO activity was assessed by mixing the sample with TMB chromogen substrate solution and incubating for 3 min. The reaction was terminated by the addition of 0.18 M H₂SO₄. The absorbance at a wavelength of 460 nm was determined for the resulting mixture. Horseradish peroxidase was used as a standard and the results were expressed as U/g protein or U/L. For NAGase, the optical density (OD) of paranitrophenol released during the reaction (at 37°C) of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide substrate by the NAGase contained in the analyzed samples was measured at a wavelength of 400 nm.

3.2.5.3 Haemogram

Blood samples were collected by cardiac puncture technique under sodium pentobarbital anesthesia (40 mg/kg) in sodium heparin coated tubes. The hematological parameters viz. hemoglobin level, RBC count, various types of WBCs count and platelet count were analyzed by Swelab Alfa Mindray 5640 Cell Counter.

3.2.5.4 Hormone Assay

The blood collected in dry tubes were allowed to stand for 10 min at room temperature to clot and then centrifuged at 3000 rpm for 10 min for serum separation. The supernatant (serum) was then tipped off into separate vial and subsequently subjected for the assessment of Prolactin, Glucocorticoids, Growth hormone, Progesterone and Estradiol levels by Fully Automated Bidirectionally Interfaced Chemi Luminescent Immuno Assay (CLIA).

Principle: The CLIA Kit is based on a solid phase enzyme-linked immunosorbent assay. The assay system utilizes one anti-hormone antibody for solid phase (microtiter wells)
immobilization and another mouse monoclonal anti-hormone antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the test sample molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 60 mins incubation at room temperature, the wells are washed with wash buffer to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in a Luminometers. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of hormone in the sample. By reference to a series of respective hormone standards assayed in the same way, the concentration of hormone in the unknown sample is quantified.

All the reagents were brought to room temperature. The desired numbers of coated wells in the holder were dispensed with 50 µl of standards, samples, and controls into appropriate wells. An enzyme conjugate reagent of 100 µl was added to each well, thoroughly mixed for 30 sec and incubated at room temperature (18-25 °C) for about 60 minutes. The microtiter wells were rinsed and flicked 5 times with washing buffer. The wells were blotted sharply onto absorbent paper to remove residual water droplets. An aliquot of 100 µl Chemiluminescence substrate solution was dispensed into each well and gently mixed for 5 sec. Absorbance was read with a chemiluminescence microwell reader and using the mean absorbance value for each sample the corresponding concentration of hormone in ng/ml from the standard curve was determined.

3.2.6 Statistical analysis

Statistical significance between the control and experimental data were subjected to one way analysis of variance (ANOVA) together with post-hoc Dunnett's test (P<0.05).
3.4 DISCUSSION

3.4.1 Effect of mastitis induced infection on the inflammatory cytokines of mammary gland of mice.

Cytokines are a diverse group of non enzymic proteins, which affect diverse target cell population as biologic response modifiers. Although every nucleated cell type can produce cytokines, most lineages express only a subset of cytokine genes (Akira and Kishimoto, 1997; Fitzpatrick and Kelso, 1998). Cytokines are chemicals released by cells that allow them to communicate with each other (Yan et al., 2011).

TNF-α, IL-1β, and IL-6 are cytokines involved in the inflammatory process of mastitis (Persson et al., 2003). These cytokines initiate, amplify, and perpetuate the inflammatory response in mastitis (De and Mukherjee, 2009). TNF-α, IL-1β, and IL-6 can stimulate vascular endothelial cells to induce vascular endothelial intercellular adhesion molecule expression and up-regulation, which results in endothelial cell and leukocyte adhesion, leukocyte migration, granulocyte degranulation, capillary permeability increase, and migration to inflammatory positions, leading to mammary damage (Zheng et al., 2006).

TNF-α

TNF-α is defined as an “early” cytokine. The proinflammatory cytokines act as indicators of inflammation (Vincent et al., 2002 and Li et al., 2010). TNF-α, the earliest and primary endogenous mediator of an inflammatory reaction, can elicit the inflammatory cascade, cause damage to the vascular endothelial cells and induce alveolar epithelial cells to produce other cellular factors and chemotactic factors, such as IL-6 (Giebelen et al., 2007).
In the present study, results indicated that there was a significant increase in the TNF-α concentration in mammary gland tissue of mice in the induced groups may be due to the infiltration of PMN into the mammary gland increased the tissue damage thereby increasing the cytokine TNF-α concentration affecting the immunity of mice. Inflammatory immune response mediated by both intrinsic virulence factors of the bacterial pathogen (Hornef et al., 2002) and the rapidity and nature of the immune response of the cow to the pathogen (Burvenich et al., 2003). Similar results were reported by the earlier investigators suggesting increased concentration of TNF-α is the earliest and primary endogenous mediator of the process of an inflammatory reaction (Chu et al., 2010). Significant expression of TNF-α has been shown in many different types of inflammatory processes, including mastitis (Schmitz et al., 2004; Guo et al., 2013). Therefore, significant increase in the TNF-α concentration of mammary gland of mice was due to the inflammation elicited by induction of mastitis by intramammary infection of S. aureus, E. coli and B. subtilis.

**IL-1β**

IL-1β plays an important role in the regulation of host immune responses against pathogens (Verdrengh et al., 2004). IL-1β also can stimulate the production of several secondary cytokines that amplify inflammation, such as IL-6 (Martin, 1999).

In the present study, the results revealed that there was a significant increase in the IL-1β concentration in mammary gland tissue of mice in the induced groups may be due to the soluble factors, virulence factors released after the intramammary infection by the pathogens. Similar results were reported suggesting IL-1β mediates both local and
systemic inflammatory responses when there is increase in IL-1β concentration (Miller et al., 2007; Guo et al., 2013). Significant expression of IL-1β as inflammatory response in mastitis induced mice (Li et al., 2013). However, significant increase in the IL-1β concentration of mammary gland of mice was due to the inflammation reaction ensued by induction of intramammary infection of S. aureus, E. coli and B. subtilis.

IL-6

IL-6, produced by macrophages, is one of the most common inflammatory cytokines (Hodge et al., 2005). IL-6 is an important cytokine mediator released in many aspects of the immunological and inflammatory responses (Panesar et al., 1999).

In the present study, the observations showed that there was a significant increase in the IL-6 concentration in mammary gland tissue of mice in the induced groups may be due to chemotactic factors released by leukocyte migration, granulocyte degranulation, capillary permeability increase, and migration to inflammatory positions, leading to mammary damage. Similar results were reported suggesting IL-6 is involved in acute septic shock during mastitis caused by pathogens (Bannerman et al., 2004). Significant expression of IL-6 has been shown in many different types of inflammatory processes, including mastitis whenever there is expression of TNF-α and IL-1β (Chen et al., 2013). Consequently, significant increase in the IL-6 concentration of mammary gland of mice was due to the inflammation elicited by induction of mastitis by intramammary infection of S. aureus, E. coli and B. subtilis.
3.4.2 Effect of mastitis induced infection on myeloperoxidase activity of mammary gland of mice.

Myeloperoxidase (MPO), a hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) oxidoreductase, is specifically found in mammalian granulocytic leukocytes, including polymorphonuclear leukocytes (PMNs), monocytes, basophils, and eosinophils (Schultz et al., 1965), and it contributes to the bactericidal capabilities of these cells. MPO activity has been widely accepted as an enzyme marker to measure and quantitate the PMN content in a variety of tissues (Mullane et al., 1985). MPO plays an important role in neutrophil antimicrobial responses (Klebanoff, 2005).

In the present study, the observations showed that there was a significant increase in the MPO activity in mammary gland tissue of mice in the induced groups may be due to increased polymorphonuclear leukocytes (PMNs), including lymphocytes, monocytes, eosinophils and basophils, which contributes to the bactericidal capabilities as a defense mechanism. But uncontrolled increased activity may damage the tissue and increase the consequences of the infection. MPO activity, a marker of neutrophil influx into tissue, is directly proportional to the number of neutrophils in the tissue (Krawisz et al., 1984). Similar results were reported suggesting increase soluble factors, chemokines and chemotactic factors will increase the MPO activity due to inflammatory reaction (Li et al., 2013). Consequently, significant increase in the MPO activity in mammary gland tissue gland of mice was the inflammatory immune response elicited after induction of mastitis by intramammary infection of \textit{S. aureus}, \textit{E. coli} and \textit{B. subtilis}.
3.4.3 Effect of mastitis induced infection on N-acetyl-β-d-glucoseaminidase activity of mammary gland of mice.

N-acetyl-β-d-glucoseaminidase (NAGase) is an intracellular, lysosomal enzyme which is released into milk from neutrophils during phagocytosis and cell lysis (Kitchen et al., 1976). Gulcosaminidase damage epithelial cells and gets secreted in large quantities in the mammary gland in response to mastitis (Kitchen et al., 1984). NAGase is released from damaged mammary epithelial cells (Kitchen et al., 1980). However, a small fraction of milk NAGase can also come from leukocytes (Capuco et al., 1986).

In the present study, the observations showed that there was a significant increase in the NAGase activity in mammary gland tissue of mice in the induced groups may be due to the toxins released by pathogens and phagocytosis action of neutrophils and other immunological factors elevate the NAGase activity. PMNs which release oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-b-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) (Viguier et al., 2009). Our results concur with the results of Kitchen et al., (1976), who reported that gulcosaminidase was a good marker for subclinical mastitis. Urech et al., (1999) also found that glucosaminidase levels could be used to identify cows with mastitis infection. Similar results were reported suggesting NAGase has been found to originate also from white blood cells that increase in milk as a result of inflammation in mastitis (Person et al., 2007; Gu et al., 2009). Consequently, significant increase in the NAGase activity in mammary gland tissue gland of mice was the inflammatory immune response elicited after induction of mastitis by intramammary infection of *S. aureus, E. coli* and *B. subtilis*. 

146
3.4.4 Effect of mastitis induced infection on haemogram of mice.

The cells that circulate in the bloodstream are generally divided into three types: white blood cells (leukocytes), red blood cells (erythrocytes), and platelets (thrombocytes). Abnormally high or low counts may indicate the presence of many forms of disease, and hence blood counts and hemoglobin level are amongst the most commonly performed haemogram tests during a bacterial infection.

In the present study, the observations showed that there was a significant decrease in hemoglobin level, RBC and platelet count of mice in induced groups. But the total WBC count and neutrophils, lymphocytes, eosinophils, monocytes and basophils were significantly increased in the induced groups, which might be due to severe bacterial infection, decrease in the hemoglobin level due to decreased RBC and platelet count and increased WBC count to counteract the bacterial infection in the circulatory blood. Similar results were reported suggesting increase of white blood cells as defense mechanism to engulf the pathogens by phagocytosis the virulence factors released also hamper the haemogram of mice (Notebaert et al., 2008).

Similarly Mona et al., (2008) studies in Egypt suggests that there was decrease in the hemoglobin percentage and RBC and increase in the WBC number of the buffalo suffering from intramammary infection by bacteria when compared with that of healthy buffalo. Likewise, Cebra et al., (1996) have reported similar changes in the haemogram level of the cows with bovine mastitis compared to healthy cows. Correspondingly the Mona et al., (2010) have also reported comparably changes of haemogram parameter of the Friesian cows. Consequently, significant decrease in the hemoglobin level, RBC and platelet count and increased WBC count activity in serum of mice was due to the
inflammation elicited by induction of mastitis by intramammary infection of *S. aureus*, *E. coli* and *B. subtilis*.

### 3.4.5 Effect of mastitis induced infection on lactation hormones of mice.

Metabolic hormones, growth factors, and prolactin are all necessary for normal development of the mammary gland with some special importance for the sex steroid hormones (Lamote *et al.*, 2004). The growth of the mammary gland is stimulated by growth hormone and prolactin, adrenocortical steroids, estrogens and progesterone. The onset of lactation is accompanied by increases in the blood volume, cardiac output, mammary blood flow and blood flow through the GI-tract and liver, aiming to provide the udder with nutrients and hormones for regulation of milk synthesis. Food intake and distribution of nutrients to the mammary gland are partially regulated by hormones as well as the repartitioning of nutrients away from body stores towards the udder. Besides central mechanisms, local mechanisms within the mammary gland regulate initiation of lactation, maintenance, regulation of blood flow and mammary gland cell apoptosis (Svennersten-Sjaunja and Olsson, 2005).

**Prolactin**

As the name indicates, prolactin (PRL) is known as the hormone of lactation. In ruminants, it provides the primary stimulus for lactogenesis (Akers, 1985). A role for PRL in the onset of lactation was indicated by a peak in concentrations of the hormone in circulation immediately prior to parturition (Ingalls *et al.*, 1973). Indeed, PRL is released during milking or suckling, indicating a role for the hormone in the maintenance of milk production (Koprowski and Tucker, 1973b; Akers, 1985). Prolactin has been shown to
maintain both the structural integrity and the functional activity of the mammary epithelium during lactation in rodents (Tucker, 1969; Flint and Gardner, 1994).

In the present study, the observations showed that there was decrease in the PRL level of mice in the induced groups, which may be due to cytokines produced from the activated immune cells, monokines, interferons and interleukines, modulate the inflammation and the immune response by regulating the prolactin hormone. After antigen stimulation, certain combinations of the produced cytokines have influence over the hormone type and magnitudes of the hormonal response that appears (Akers, 2002). Similar results were reported suggesting endotoxins and cytokines induce fever and alter neurotransmitter activity in the brain and hormone secretion by the pituitary and other endocrine glands during mastitis (Burvenich et al., 1999).

Glucocorticoids

Cortisol is the predominant endogenous glucocorticoid in cattle, and, as mentioned previously, its major function is differentiation of the lobule-alveolar system, to enhance the action of prolactin in stimulating differentiation of the epithelium and milk protein gene expression in the mammary gland and uptake of glucose by the mammary gland during lactogenesis (Akers, 2002).

In the present study, the observations showed that there was decrease in the glucocorticoids level of mice in the induced groups may be due to acute mastitis-induced secretion of inflammatory mediators affect the release of hypothalamic corticotrophin-releasing hormone, which activates the adrenal axis. Both local and systemic inflammatory responses affect the glucocorticoids level (Miller et al., 2007). Similar
results were reported suggesting increased soluble factors, chemokines and chemotactic factors will decrease the glucocorticoids due to inflammatory reaction (Li et al., 2013).

**Growth hormone**

Growth hormone (GH) is widely known for its galactopoietic effect in lactating dairy cattle. GH is important in milk production and mammary growth for ruminant lactation (Accorsi et al., 2002). In ruminants, the action of GH on the mammary gland is mediated mainly by the insulin-like growth factor (IGF) signaling axis (Etherton, 2004).

In the present study, the observations showed that there was decrease in the GH level of mice in the induced groups may be due to release of IGF in blood and milk, which stimulate the immune response by releasing inflammatory mediators and cause GH to decrease during mastitis. Expression of GH receptor in the mammary stroma is critical for normal mammary development, supporting the concept that the action of GH on the mammary epithelium is indirect and may be mediated by locally-produced IGF from the stroma (Kelly et al., 2002). Similar results were reported suggesting in rodents that the GH functions to enhance the secretion of PRL to maintain the lactation and production of milk in the alveoli (Accorsi et al., 2002; Wall and McFadden, 2012).

**Progesterone**

Progesterone is an ovarian hormone, synergized with estrogen to induce lobule-alveolar growth and support mammogenesis during pregnancy in cattle coincided with increased secretion of both estrogen and progesterone (Randel and Erb, 1971). Progesterone induces DNA synthesis at the end buds and along the walls of the mammary ducts (Bresciani, 1968). Throughout gestation, proliferation of the mammary epithelium
is dependent on progesterone and during lactation its secretion is decreased (Svennersten-Sjauanja and Olsson, 2005).

In the present study, the observations showed that there was decrease in the progesterone level of mice in the induced groups is due to inflammatory reaction induced by the pathogens, increase the immune cells in the blood flow with the toxins and other soluble factors which hamper the secretion of progesterone. However, during lactation progesterone secretion is decreased but when the pathogens infect the mammary gland, its expression decreases even more due to circulating immune cells in the blood (Wall and McFadden, 2012). Similar results were reported suggesting that the decreased level of progesterone level of mice in the induced groups during bacterial mastitis was due to inflammatory immune response (Akers, 2002).

**Estradiol**

Estradiol, one among the estrogen hormone is secreted by the ovary, as well as the placenta of pregnant animals, and this hormone is mainly involved in the growth and development of the mammary gland during puberty and pregnancy (Erb, 1977; Schams et al., 1984; Tucker, 1985). Prior to parturition, estrogen is one of the first hormones to increase in circulation, indicating a role for estrogen in lactogenesis (Akers, 2002). Estrogen also stimulates the anterior pituitary gland to secrete PRL, and it increases the expression of PRL receptors in the mammary epithelium (Tucker, 2000).

In the present study, the observations showed that there was decrease in the estradiol level of mice in the induced groups may be due to may be due to the toxins released by pathogens and phagocytosis action of neutrophils and other immunological
factors. Inflammatory immune cell infiltration stimulates the pituitary gland and decreases the level of estradiol (Sordillo, 2005). Similar results were reported suggesting endotoxins and cytokines induce fever and alter neurotransmitter activity in the brain and hormone secretion by the pituitary and other endocrine glands during mastitis (Burvenich et al., 1999).

3.4.6 Effect of mastitis on histopathological changes of inguinal lymph node of mammary gland in experimentally induced lactating mice.

Lymph nodes are small, oval lymphoid organs ranging in diameter from 1 to 25 mm. Each lymph node is covered by a capsule of dense connective tissue. A lymph node functions like a kitchen water filter, purifying lymph before it reaches the venous circulation. As lymph flows through a lymph node, at least 99 percent of the antigens in the lymph are removed. Antigens removed in this way are then processed by the macrophages and "presented" to nearby lymphocytes. A minor injury commonly produces a slight enlargement of the nodes along the lymphatic vessels draining the region. This symptom, often called "swollen glands," typically indicates inflammation or infection of peripheral structures.

Histopathological evidences of inguinal lymph node showed that increased flow of lymphocytes and neutrophils in S. aureus, E. coli and B. subtilis induced mastitis which may be due to mammary gland tissue damage caused by PMN migration leading to the damage of lymph node due to the bacterial infection, which act by rapidly dividing in the host by inhibiting the phagocytosis by PMN cells. Mastitis leads to losses in mammary function are directly related to disruption of alveolar cell integrity, sloughing
of cells, induced apoptosis, and increased appearance of poorly-differentiated cells (Akers and Nickerson, 2011). Similar results were reported suggesting the presence of functional PMN is crucial to the host defense against bacterial pathogens but neutrophils may promote tissue injury and disturb mammary function, via reactive oxygen metabolite generation (Paape et al., 2003). Consequently, histopathological evidence of inguinal lymph node of mouse was the inflammatory immune response elicited after induction of mastitis by *S. aureus*, *E. coli* and *B. subtilis*. 
3.5 SUMMARY AND CONCLUSIONS

The objective of the present investigation was to assess inflammatory immune responses of mastitis induced by *S. aureus, E. coli* and *B. subtilis* inoculum's in mice by estimating the inflammatory cytokine levels, inflammatory enzyme activities, haemogram, hormone levels and histopathology of inguinal lymph node of mammary gland after 24 h of infection in lactating mice.

- The results showed that there was a significant increase in the inflammatory cytokine concentrations of TNF-α, IL-1β and IL-6 in mammary gland tissue of mice in the induced groups compared with that of control.

- Relatedly, there was a significant increase in the inflammatory enzyme activities of MPO and NAGase in mammary gland tissue of mice in the induced groups compared with that of control.

- The haemogram results indicated that there was a significant decrease in hemoglobin level, RBC and platelet count of mice in induced groups compared with that of control. But the total WBC count and neutrophils, lymphocytes, eosinophils, monocytes and basophils were significantly increased in the induced groups compared with that of control.

- The lactating hormone results revealed that there was decrease in the prolactin, glucocorticoids, growth hormone, progesterone and estradiol levels of mice in the induced groups compared with that of control.
• The histopathological changes of inguinal lymph node of mammary gland showed increased number of neutrophils and lymphocytes in the induced groups compared with that of control.

Therefore, the results indicate that inflammatory cytokines and enzymes are produced and released by immune cells at the infection site following activation by mediators elicited inflammatory immune response. Thus, the results of the present study revealed that the experimentally induced mastitis in lactating mice leads to detrimental inflammatory effects on the mammary gland tissue and immunity of mice.
Table 1. Effect of mastitis induced infection on the inflammatory cytokines of mammary gland of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inoculum (CFU)</th>
<th>Cytokines assay (ng/ml protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>IL-1β</td>
</tr>
<tr>
<td>I</td>
<td>Control</td>
<td>2.86±0.46</td>
<td>3.29±0.32</td>
</tr>
<tr>
<td>II</td>
<td>S. aureus</td>
<td>13.25±0.61*</td>
<td>14.38±0.47*</td>
</tr>
<tr>
<td>III</td>
<td>E. coli</td>
<td>12.43±0.58*</td>
<td>11.86±0.53*</td>
</tr>
<tr>
<td>IV</td>
<td>B. subtilis</td>
<td>10.67±0.84*</td>
<td>9.37±0.64*</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 5 animals  *Significant P ≤ 0.05 vs Control

Table 2. Effect of mastitis induced infection on myeloperoxidase activity of mammary gland of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inoculum (CFU)</th>
<th>MPO (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>2.96±0.16</td>
</tr>
<tr>
<td>II</td>
<td>S. aureus</td>
<td>10.35±0.27*</td>
</tr>
<tr>
<td>III</td>
<td>E. coli</td>
<td>8.43±0.38*</td>
</tr>
<tr>
<td>IV</td>
<td>B. subtilis</td>
<td>6.52±0.23*</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 5 animals  *Significant P ≤ 0.05 vs Control
Table 3. Effect of mastitis induced infection on N-acetyl-β-d-glucoseaminidase activity of mammary gland of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inoculum (CFU)</th>
<th>NAGase (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>20.26±0.26</td>
</tr>
<tr>
<td>II</td>
<td><em>S. aureus</em></td>
<td>53.38±0.33*</td>
</tr>
<tr>
<td>III</td>
<td><em>E. coli</em></td>
<td>47.43±0.25*</td>
</tr>
<tr>
<td>IV</td>
<td><em>B. subtilis</em></td>
<td>35.51±0.37*</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 5 animals *Significant P < 0.05 vs Control

Table 4. Effect of mastitis induced infection on haemogram of mice.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Inoculum (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.82±0.36</td>
</tr>
<tr>
<td>RBC (×10⁶/mm³)</td>
<td>8.86±0.24</td>
</tr>
<tr>
<td>WBC (×10³/mm³)</td>
<td>14.83±0.68</td>
</tr>
<tr>
<td>Platelets (×10³/mm³)</td>
<td>240.56±1.52</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>22.42±0.64</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>86.21±0.41</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>2.32±0.06</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>1.41±0.02</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.18±0.013</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 5 animals *Significant P ≤ 0.05 vs Control
### Table 5. Effect of mastitis induced infection on lactation hormones.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inoculum (CFU)</th>
<th>Hormone assay (ng/ml)</th>
<th>Prolactin</th>
<th>Glucocorticoids</th>
<th>Growth Hormone</th>
<th>Progesterone</th>
<th>Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td></td>
<td>21.2±0.43</td>
<td>12.5±0.032</td>
<td>5.8±0.002</td>
<td>8.5±0.045</td>
<td>0.252±0.001</td>
</tr>
<tr>
<td>II</td>
<td><em>S. aureus</em></td>
<td></td>
<td>15.4±0.35*</td>
<td>8.3±0.054*</td>
<td>3.4±0.008*</td>
<td>4.4±0.062*</td>
<td>0.234±0.004*</td>
</tr>
<tr>
<td>III</td>
<td><em>E. coli</em></td>
<td></td>
<td>16.3±0.32*</td>
<td>9.4±0.045*</td>
<td>3.8±0.005*</td>
<td>6.2±0.038*</td>
<td>0.242±0.003*</td>
</tr>
<tr>
<td>IV</td>
<td><em>B. subtilis</em></td>
<td></td>
<td>18.6±0.28</td>
<td>10.2±0.061</td>
<td>4.4±0.006</td>
<td>7.4±0.034</td>
<td>0.248±0.002</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 5 animals  *Significant P ≤ 0.05 vs Control
Graph 1. Effect of mastitis induced infection on the inflammatory cytokines of mammary gland of mice.

Graph 2. Effect of mastitis induced infection on myeloperoxidase activity of mammary gland of mice.
Graph 3. Effect of mastitis induced infection on N-acetyl-β-d-glucosaminidase activity of mammary gland of mice.

Graph 4. Effect of mastitis induced infection on haemogram of mice.
Graph 5. Effect of mastitis induced infection on haemogram of mice

- Neutrophil
- Lymphocytes
- Eosinophil
- Monocytes
- Basophil

WBC'S

- Control
- S. aureus
- E. coli
- B. subtilis

*Significant

Graph 6. Effect of mastitis induced infection on lactation hormones of mice.

- Prolactin
- Glucocorticoids
- Growth Hormone
- Progesterone
- Estradiol

Lactation Hormones

- Control
- S. aureus
- E. coli
- B. subtilis

*Significant