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In Vitro Modulation Of Gene Expression In Bovine Blood Neutrophils

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In Vitro Modulation of Gene Expression in Bovine Blood Neutrophils

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Biographical Sketch

Ashley R. Turner was born in Gastonia, North Carolina on October 5, 1977. She graduated from North Carolina A&T State University in 1999 with a BS in Laboratory Animal Science. After graduation, she pursued a career in the pharmaceutical industry conducting cardiovascular and renal disease studies.

In 2009, she returned as a graduate student to pursue a Master's degree in Animal Health under the mentorship of Dr. Mulumebet Worku. She has presented her research findings at the biennial meeting of the Association of 1890 Research Directors (ARD) in Atlanta, GA and at the 2011 Gamma Sigma Delta Showcase of Excellence in Research Graduate Student Paper Competition where she received the second place awarded at North Carolina A & T State University in Greensboro, NC. Her current research involves studying the *in vitro* modulation of gene expression in bovine neutrophils. The United States Department of Agriculture-Evans Allen Program in the School of Agriculture and Environmental Sciences at NC A&T State University, provided funds to support this research study.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACD	Acid Citrate Dextrose
bp	base pairs
C	Celsius
CD14	Cluster of Differentiation Antigen 14
cDNA	complementary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
DEPC	Diethyl pyrocarbonate
DHIA	Dairy Herd Improvement Association
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GPI	Glycosyl-phosphatidylinositol
HRP	Horseradish peroxidase
IFN-γ	Interferon gamma
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IRAK	Interleukin 1 receptor-associated kinase
KH_2PO_4	Potassium Phosphate Monobasic
K_2HPO_4	Potasssium Phosphate Dibasic
LPS	Lipopolysaccaride
mRNA	messenger Ribonucleic Acid
MWT	Molecular Weight

MYD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor kappa B
NaCl	Sodium Chloride
NaN_3	Sodium Azide
NTC	No Template Control
Nramp1	Natural resistance associated macrophage protein 1
No.	Number
NYS	Nystatin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptors
Q	Quebracho
QC	Quebracho Control
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SCC	Somatic cell count
TIR	Toll/interluekin 1 receptor
TLR	Toll like Receptor
TLR2	Toll like Receptor 2

TLR4	Toll like Receptor 4
Tm	Melting temperature
TMB	3,3,5,5-tetramethylbenzidine
TNF-α	Tumor Necrosis Factor- alpha

Abstract

Concerns for animal welfare, food safety, and security fuel interest in understanding modulators of innate immunity. The accumulation of neutrophils and activation of toll-like receptors (TLRs) at infection sites are critical events of innate host defense. The objectives of this study were to evaluate the effects of Lipopolysaccaride (LPS), Peptidoglycan (PGN), Nystatin (NYS), and Quebracho (Q) extract on bovine neutrophils activation in relation to expression of genes encoding *TLR2*, *TLR4*, natural resistance-associated macrophage protein 1 (*Nramp1*), and the cytokines *TNF-α* and *IL-10*. Genes in genomic DNA were detected from blood samples, except IL-10. Neutrophils were isolated from four Holstein Friesian cows and cultured with LPS (100ng), PGN (1μg), NYS (2500U), or ethanol (62%); Q (1.5X) or maintained in PBS at the following time intervals: 0, 15, 30 or 60 minutes. Total RNA was extracted and concentration, purity and time of incubation, had an effect on RNA concentration, producing highly significant results ($p<0.0001$). Specific primers were used to amplify *Nramp1*, *TLR2*, *TLR4*, *TNF-α* and *IL-10* mRNA using a reverse transcriptase polymerase chain reaction (RT-PCR). Neutrophils suspended in PBS showed basal expression of *TLR 2, 4*, and *TNF-α*. Exposure of neutrophils to LPS resulted in induction of *Nramp1*. Peptidoglycan induced gene expression of *TLR 2* at 60 minutes. Nystatin treatment induced *TLR 2* expression but suppressed *Nramp1* at 60 minutes. Gene modulation with ethanol was seen with *TLR 2* and *Nramp1*. Quebracho suppressed *NRAMP1* gene expression after 30 and 60 minutes. ELISA results did not show a treatment effect, but demonstrated a significant decrease in TNF-α secretion compared to PBS and/or ethanol ($p <0.05$), after 30 minutes of incubation. This study demonstrated modulation of genes expressed by treatment with bacterial components and immunomodulators in bovine neutrophils *in vitro.*

CHAPTER 1

Introduction

Infections by the bacteria *E. coli* and *S. aureus* are two major causes of acute and chronic mastitis, respectively (Smith & Hogan, 1993; Sutra & Poutrel, 1994). Inflammation caused by bacterial infections is a protective mechanism used by the host to eliminate disease-causing pathogens from the body. Elimination of pathogens is often dependent on an efficient innate immune system in the host, induction of chemokines, such as IL-8 to attract neutrophils to the site of infection, and involves other proinflammatory cytokines (e.g. IL-1β and TNF-α) (Mount, Karrow, Caswell, Boermans, & Leslie, 2009).

Neutrophils are one of the mediators of the host innate immune system. They are among the first to encounter pathogens. Pathogens, like gram-negative and gram-positive bacteria, carry pathogen associated molecular patterns (PAMPs) (Akira & Sato, 2003; Razonable, Henault, Watson, & Paya, 2005) which are identified by pattern recognition receptors (PRRs) that can be found on the membrane of immune cells (i.e. macrophages, neutrophils, and epithelial cells) and tissues (i.e. lung, skin and spleen) (Tirumurugaan et al., 2010). Pathogen associated molecular patterns activate cell signaling and secretion of proinflammatory cytokines (D. D. Bannerman et al., 2004; Mount, et al., 2009). Lipopolysaccaride (LPS) and peptidoglycan (PGN) are specific PAMPs that are recognized by toll-like receptors,(TLRs).

Bacterial cell well components induce TLRs expression. For example, TLR2 is normally activated by peptidoglycan; while TLR4 has been known to respond to lipopolysaccaride (D. D. Bannerman, et al., 2004; Fan, Frey, & Malik, 2003). Activation of these toll-like receptors causes secretion of the proinflammatory cytokine TNF-α (Oak, Mandrekar, Catalano, Kodys, & Szabo, 2006).

Pharmacological drugs, like nystatin, which is a lipid raft inhibitor, have recently been shown to utilize PAMPs to induce TLR2 expression and secretion of TNF-α, during host-pathogen interaction (Razonable, et al., 2005). Lipid or membrane rafts functional responsibility range from viral and toxin entry to cell signaling (Pike, 2006). Lipid rafts are portals for pathogens, such as *E coli* strains, which bind to (glycerophospholipids) GPI-anchored protein CD48, as an infection strategy into the host cell (Fantini, Garmy, Mahfoud, & Yahi, 2002).

It is important to note how pathogens exploit normal cellular function to survive within the host, albeit using lipid rafts or sequestering essential metals for microbial replication. The uptake of essential metals allows pathogens the ability to survive in the host after phagocytosis. Interactions between host and pathogen determine the expression of natural resistance associated macrophage protein 1 (Nramp1), a divalent metal transporter that can limit the bacterium's ability to replicate (Forbes & Gros, 2001) in the phagosome. The presence or absence of Nramp1 relates to resistance or susceptibility to bacterial infections (Gomes & Appelberg, 1998). This gene has polymorphisms in humans, porcine and mice, which implicate Nramp1 regulates the host's innate immunity against pathogens (Ables, Nishibori, Kanemaki, & Watanabe, 2002; G. Zhang, Wu, Ross, Minton, & Blecha, 2000).

Concerns for animal welfare, food safety and security fuel interest in the use of new plant alternatives, such as based extracts, to modulate innate immunity. Antibiotics are used to prevent further spread of diseases, but have some drawbacks, particularly, in food production settings (Sutra & Poutrel, 1994). Currently, the use of antibiotics to treat mastitis in cows is the main protocol in farming production, but the frequency of extra label use of antibiotics has resulted in bacterial resistance (Raymond, Wohrle, & Call, 2006). Evidence has pointed toward tannin-rich forage plants, like quebracho as a possible alternative to antibiotics in ruminants

(Athanasiadou, Kyriazakis, Jackson, & Coop, 2001). Quebracho, a South American plant, is known for its antibacterial promoting effects. The studies showed that the use of tannins might help in reducing the use of antibiotics for treating bacterial infections while boosting the immune system of ruminants to help prevent further diseases caused by pathogens. To date, *in vivo* studies have been conducted to investigate the use of quebracho in ruminants, primarily in sheep and goat (Athanasiadou, 2001;Brunet, 2008;Villalba, 2010).

The objective of this study was to use bovine neutrophils stimulated with components of bacterial pathogens (LPS derived *E.coli* and PGN derived *S. aureus*), and immunomodulators, such as Nystatin, and a plant extract (quebracho) to determine the expression of *Toll like receptors 2* and *4*, *Nramp1*, *TNF-α*, and *IL-10* genes. It is hypothesized that specific pathogens and immunomodulators will elicit distinctive patterns of gene expression, associated with the neutrophil's role in host innate immune response.

CHAPTER 2

Literature Review

2.1 Role of Neutrophils in Dairy Cow Innate Immunity

The innate immune system is comprised of various cell types such as neutrophils, monocytes/macrophages, mast cell, basophils and eosinophils as well as some non-immune cells, such as kerantinocytes and hepatocytes (Kasten, Muenzer, & Caldwell, 2010a; Preiss et al., 2008). Phagocytic cells have the ability to express cytokines and chemokines. Neutrophils, also known as polymorphonuclear cells (PMN) (Sohn et al., 2007), are the first responders involved in innate immunity at the site of infection, when pathogens are present. They are classified as polymorphonuclear cells because of the multilobed shape of their nucleus, when compared to lymphocytes and monocytes. Their mechanisms in responding to pathogens have been studied for both physiologic and pathologic activities (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000b). Neutrophils' phagocytic properties aid in host defense against disease causing pathogens, but also contribute to the inflammatory pathology of most diseases; if they go unregulated (Seely, Pascual, & Christou, 2003). Neutrophil recruitment process consists of migration from the blood to the extravascular tissues, recognition of pathogens and necrotic tissues, and removal of the pathogen from the host. In livestock, the most notable disease associated with neutrophil's pathological state is mastitis (Paape, Miller, & Ziv, 1991).

Mastitis is inflammation of the mammary gland affecting milk producing animals and is usually associated with microorganisms derived from bacteria such as: *Escherichia coli*, *Bacillus* spp., and *Streptococcus* spp., which are the most common species found in environmental mastitis (Schroeder, 2010). Previous studies have shown that diverse forms of LPS derived from *E.coli* can impact gene expression (Worku & Morris, 2009). Other gram negative and gram

positive bacteria that are common pathogens in cattle are *Mycobacterium tuberculosis*, *Salmonella typhi*, and *Campylobacter fetus*, which live in soil and water and can be transmitted to cattle through grazing or drinking devices. Once the bacteria is in the body this provides an optimal condition for replication of the pathogen resulting in disease.

Health conditions of dairy cows can be compromised by both environmental (*E.coli*) and contagious (*S.aureus*) mastitis (National Mastitis Council, 2011), two forms of mastitis that progress differently, but provide the same results upon infection. According to the Dairy Herd Improvement Association (DHIA), a scoring system is used to assess subclinical mastitis in cows. This system runs on a 10-point scale (0 to 9), with zero indicating no infection and nine highly infected. The primary indication is an elevated somatic cell count- SCC (neutrophils and epithelial cells) in milk that can be attributed to the innate immune system reacting to the invasion of bacteria in the mammary. In addition to bacterial infections leading to elevated SCC; trauma and injury are other pathological states that can cause elevated SCC in cows (Geishauser, Querengasser, Nitschke, & Sorbiraj, 1999).

Bacterial infections pose a food safety problem to human health particularly, regarding unpasteurized or improperly pasteurized milk because many pathogens or toxins can be transferred from infected quarters directly to humans. Such an invasion of microorganisms can also translate to decreased milk yield and milk composition (National Mastitis Council, 2011). According to the National Mastitis Council (2011), SCC in dairy herds averages 228,000 cells/ml in 2011 (National Mastitis Council, 2011). The current legal limit is 750,000 cells/ml for Grade A producers in many states, except California where, the legal limit is 400,000 cells/ml (National Milk Producers Federation, 2011). The type or extent of mastitis is usually indicated based on these numbers and appearance of milk as being either subclinical or clinical.

Subclinical mastitis is classified as "hidden," caused by *S. aureus* that can lead to chronic infection of the mammary gland, due to the pathogen's ability to cause false negatives and produce no visual signs in milk production; subclinical mastitis is detected upon SCC monitoring. Clinical mastitis is usually more apparent, with the appearance of flakes, clots, and watery consistency of milk. Sometimes caused by *E. coli* this infection is typically acute infections, because it is easily detected and is treated more efficiently (National Mastitis Council, 2011)

Septicemia or septic shock is generally caused by a skin or mucosal wound that has undergone a bacterial infection that can be minute and difficult to detect. Septic shock or toxic shock syndrome is a multisystemic disease characterized by rapid onset of fever, hypotension, and multi-organ failure, which often leads to death (Chenier, Leclere, Messier, & Fecteau, 2008). Neutrophil recruitment is the key feature in both septic shock and mastitis, due to the inflammatory response associated with these diseases. Pathogenic states usually start as an acute inflammatory response initiating neutrophils (PMN) to sites of infection. If the acute inflammation persists it can progress to chronic inflammation, which is characterized by the presence of macrophages (Robbins, Cotran, & Kumar, 2010) and neutrophils.

2.1.1 Neutrophils and mastitis. The release of secreted proteins called cytokines; makes it possible for leukocytes to be recruited from the blood to the site of infection or injury. Leukocytes migrate across the endothelium after sensing chemoattractants. They use several adhesion molecules to bind themselves to the site of injury or where they are needed. Selectins are cell adhesion molecules that are regulated by cytokines and their ligands and consist of three types: L-selectin expressed on neutrophils, P-selectin expressed on platelets and endothelium, and E-selectin expressed on endothelium (Robbins, Cotran, et al., 2010). Neutrophils

constitutively express L-selectin on the tips of their microvilli, but also express ligands for P- and E-selectins (Doyle et al., 1997). The ability to firmly bind to the endothelial surface is contributed by integrins, a family of heterodimeric leukocyte surface proteins with a high affinity for the endothelial surface (Monfardini et al., 2002). This can be triggered by various agonists such as LPS (bacterial), IL-8 (chemoattractants), as well as cytokines and growth factors (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000a).

A high number of neutrophils present in milk are an indication of a bacterial infection in the mammary gland. Several types of pathogens such as *S. aureus, E. coli,* and *S. uberis* (Leigh, Egan, Ward, Field, & Coffey, 2010) can alter the overall health of cows and cause SCC in milk to be elevated. Neutrophils predominate the site of infection within the first 6 to 24 hours and are replaced by monocytes in 24 and 48 hours (Robbins, Cotran, et al., 2010). Once neutrophils have been activated their life span is short-lived (24 to 48 hours) they undergo apoptosis (Seely, et al., 2003).

2.1.2 Recognition, phagocytosis and antimicrobial capacity of neutrophils. The recognition of microbes by neutrophils and other innate immune cells, prevents microbes from evading host defense mechanisms. Neutrophil recognition of bacterial pathogens opsonized by IgG antibodies, allows Fc receptors to present microbes for phagocytosis. Alterations in these receptors in cattle impede neutrophils' ability to destroy pathogens (Worku, Paape, Di Carlo, Kehrli, & Marquardt, 1995).

Bacterial infections activate several signaling pathways (i.e kinases, transcription factors, lipid membranes, and cytokines) that interact with pathogens through domains on the mammalian membrane (Riethmuller, Riehle, Grassme, & Gulbins, 2006). Leukocytes recognize microbial structures based on pathogen associated molecular patterns (PAMPs). They also

recognize molecules secreted from injured or necrotic cells, called danger associated molecular patterns (DAMPs) (Robbins, Kumar, & Cotran, 2010). The cellular recognition receptors called, pattern recognition receptors (PRR) seek out and aid in destroying pathogens. Following recognition, activation is signaled by pro-inflammatory cytokines such as: TNF-α (tumor necrosis factor-alpha), Interleukin-8 (chemokine), and growth factors (G-CSF and GM-CSF) (Douglas D. Bannerman, Chockalingam, Paape, & Hope, 2005; Witko-Sarsat, et al., 2000b).

Only when the host's normal physiological state is disrupted, does neutrophil activation result in phagocytosis, antimicrobial killing of pathogens by superoxides, and release of degradative enzymes. Upon phagocytosis neutrophils engulf invading microorganisms and form phagosomes that secrete reactive oxygen species (ROS). Superoxide anions, hydrogen peroxide, and hydroxyl radicals are major reactive oxygen species that are produced in neutrophils. The physiological function of these ROS in neutrophils and other leukocytes is to modulate expression of cytokines, adhesion molecules and chemokines, as part of the inflammatory response. To aid in destroying the phagocytized microbe; neutrophils once activated by pathogens undergo a short lifespan, this activation can cause tissue damage, due to neutrophils radical behavior, if left unregulated (Sohn, et al., 2007). The regulation of neutrophil destructive nature can be attributed to antioxidant mechanisms, such as superoxide dismutases, lactoferrin, ferritin, and transferrin, which are all transport proteins that minimize the level of ROS formation.

Neutrophil derived microbicidal molecules are packed in granules that are released upon cell activation. The release of soluble antimicrobial proteins destroys invading microorganism such as: gram-negative and gram-positive bacteria as well as fungal infections. These include primary granules (azurophilic) which are characterized by defensins, myeloperoxidase, and

bactericidal/permeability increasing protein (BPI); secondary granules (specific) such as lactoferrin which has bactericidal and fungicidal activity in neutrophil the ability to bind to iron; tertiary granules (cathepsin) that are proteases breaking apart other proteins.

2.2 Role of Nramp1 in Neutrophils

Natural resistance associated macrophage protein 1 (Nramp1) is an iron transporter protein known to induce susceptibility or resistance to disease, depending on the substitution of an amino acid (glutamic acid or aspartic acid) on the 169 position (Ables, et al., 2002). It is expressed exclusively in macrophages and neutrophils and has a membrane protein that shares structural characteristics with ion channels and transporters. Its function and mechanism of action in relation to bovine neutrophils remains unknown (Worku & Morris, 2009). Divalent metals (Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+}) are cofactors for many enzymatic reactions in all life forms (Forbes & Gros, 2001). Microbe survival in the host is essential for replication of the microorganisms' species and in that survival it requires iron, which is not readily available. This divalent metal ion is just as essential to the host as it is to pathogens (Gomes & Appelberg, 1998). Microbe reliance on the host for nutrients, as well as optimal living conditions is paramount to its survival.

Studies have demonstrated that Nramp1's role in antimicrobial activity is regulated by polymorphonuclear (PMN) leukocytes (Canonne-Hergaux et al., 2002). In humans, neutrophils are the site of Nramp1 messenger RNA expression, which was discovered through subcellular fractionation using immunoblotting with granule-specific markers, indicating tertiary granules (gelantinase) present in Nramp1. Studies of fungus (*Candida albicans*) containing phagosomes formed in neutrophils indicated that Nramp1 is recruited from the tertiary granules to the

phagosomal membrane upon phagocytosis; thus, providing evidence for the role of Nramp1 as an antimicrobial defense of human neutrophils (Canonne-Hergaux, et al., 2002)

The mechanism of action for Nramp1 is debatable as a phagosomal membrane (Forbes & Gros, 2001). Some researchers have documented that following phagocytosis by macrophages; pathogens are bound to the membrane of phagosomes resulting in Nramp1 recruitment (Forbes & Gros, 2003). While other studies have demonstrated Nramp l presence in neutrophils play an important role in antimicrobial properties (Canonne-Hergaux, et al., 2002).

Bacteria and fungi synthesize siderophores (a high affinity compound) that captures iron from the host protein-ferritin or transferrin (Ratledge & Dover, 2000). It is suggested that the addition of iron, which enters the phagosome by phagocytic cells via Nramp1 is the reason some bacterial or fungal infections persist once phagocytized by neutrophils (G. Zhang, et al., 2000). Activated Nramp1 produces reactive oxygen species, such as hydroxyl groups and superoxide anions, through peroxide, perhaps even nitric oxide by way of the Fenton and Haber/Weiss reactions (Forbes & Gros, 2003).

2.3 Infections and Inflammation

Most bacterial infections are produced from either gram-negative or gram-positive species. Lipopolysaccaride (LPS) and peptidoglycan (PGN) are usually associated with gram negative and gram-positive bacteria, respectively. Both types of bacteria are known to induce neutrophil diapedesis and cause acute inflammation in the host (Robbins, Cotran, et al., 2010). With mastitis, the infection is localized to the mammary gland, and with septic shock or blood poisoning the bacterium is secreted in the lumen.

Lipopolysaccaride (Figure 1), is found in the outer membrane of gram-negative bacteria, and acts as an endotoxin to elicit strong immune responses in animals. LPS protects the

membrane from certain chemical attacks, as well as provide structural integrity to the cell wall, by binding to the CD14/TLR4/MyD88 receptor complex on phagocytic cells, LPS will induce pro-inflammatory cytokines in various cells, especially macrophages (Akira & Sato, 2003).

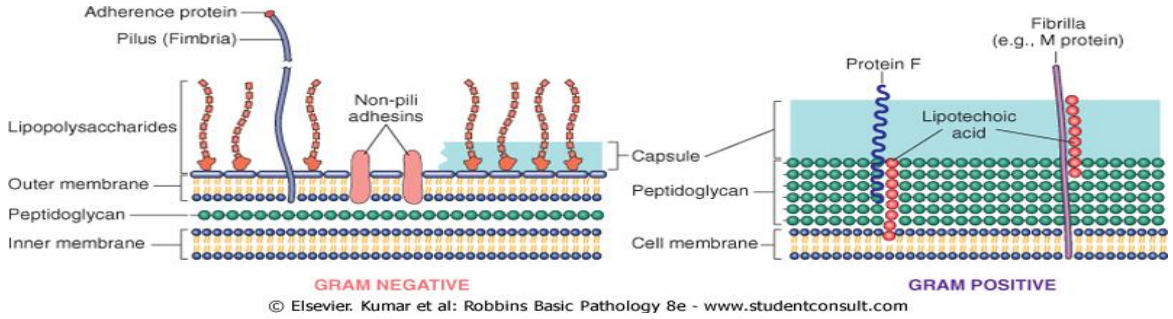


Figure 1. Illustration of the cell wall of gram-negative and gram-positive bacteria**.**

Gram-negative bacteria, such as *Escherichia coli* have cell walls composed of LPS, which is not found in gram-positive bacteria. The outer membrane contains the lipopolysaccharide, known as endotoxin and consists of a lipid portion called lipid A embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface (Ulevitch & Tobias, 1999). A thin layer of peptidoglycan is present between the inner and outer membrane of gram-negative bacteria (Figure 1.) to prevent osmotic lysis of the cell, as well as aiding bacteria in evading phagocytosis (Robbins, Cotran, et al., 2010).

Gram-positive bacteria such as *Staphyloccocus aureus* are composed of a thick peptidoglycan layer, which has lipoteichoic acid embedded through the cell membrane (Figure 1). This structural layer functions in the same manner as LPS, in providing strength to the bacterial cell wall (Robbins, Cotran, et al., 2010). The significance of the cell wall whether derived from gram-negative or gram-positive bacteria, serves the same purpose with protecting

against phagocytosis. Mastitis associated with gram-positive bacteria is usually classified as contagious mastitis, and acts slower than gram-negative mastitis causing pathogens (Schroeder, 2010). The latent ability of gram positive bacteria, contributes to its subclinical/ chronic appearance in cows.

2.4 Toll-Like Receptors

In mammals, toll like receptors (TLRs), named after the *Drosophila* protein Toll and other cytoplasmic receptors (Tirumurugaan, et al., 2010; Dirk Werling, Piercy, & Coffey, 2006) can recognize bacteria, fungi, parasites and viruses to elicit an inflammatory response and induce the production of various mediators. Toll like receptors are proteins that recognize microbes once they have breached the natural barrier of the organism such as the skin or intestinal tract mucosa, or teat end, and activate immune cells. The number of known TLRs range from 10 to 13 (Menzies & Ingham, 2006; Mucha, Bhide, Chakurkar, Novak, & Mikula, 2009; Tirumurugaan, et al., 2010) in mammals. Toll like receptors differ from one another based on their expression pattern, target gene expression, and ligand specificities, with 10 TLRs for bovine (Menzies & Ingham, 2006). Research has shown that TLRs can be found in neutrophils, macrophages and dendritic cells, as well as epithelial cells (Tirumurugaan, et al., 2010; Vahanan et al., 2008; Worku & Morris, 2009).

The specificity of each TLR is different; however, all contain the same leucin-rich repeat extracellular domain and a common intracellular domain to the IL-1R type intracellular signaling domain, called the Toll/IL-1R (Dirk Werling, et al., 2006).

2.4.1 Toll-like Receptor 4. Toll-like receptor 4 is a well-known microbial attachment to both gram negative and positive bacteria and has enabled a considerable amount of research. Toll like receptor 4 is related to destruction of pathogens by the host cells, such as neutrophils,

macrophages, and dendritic cells (Dirk Werling & Jungi, 2003). Toll like receptor 4 is part of a large family of transmembrane proteins and is linked particularly to bacteria with the lipopolysaccaride component (Akashi et al., 2000). The expression of TLR 4 has been seen in goat, water buffaloes, and bovine neutrophils (Tirumurugaan, et al., 2010; Vahanan, et al., 2008), bovine macrophages and dendritic cells (D. Werling, Hope, Howard, & Jungi, 2004), and human lymphocytes (Blomkalns et al., 2011). The receptor binds to ligands such as LPS and lipoteichioc acid; and has been known to react with PGN in gram positive bacteria (Ulevitch & Tobias, 1999).

2.4.2 Toll-like Receptor 2. It has been well documented that TLR 2 is associated with recognition of a wide array microbial products such as peptidoglycan (PGN) and lipoteichoic acid, which are associated with gram positive bacteria (Hadley, Wang, Foster, Thiemermann, & Hinds, 2005b). Toll like receptor 2 expression can be induced by PGN derived from *Staphylococcus aureus* as well as gram negative bacteria such as LPS derived from *Escherichia coli* (Opal & Esmon, 2003). Many of these studies investigated the interactions of monocytic cell lines and gram negative bacteria for priming effects of PGN and LPS signaling (Hadley, et al., 2005b).

The TLR 2 signaling pathway shares similarities with IL-1/TLR4 signaling (Oak, et al., 2006). Toll like receptor 2 dependent NF-κB activation requires the Toll/interleukin 1 receptor (Tirumurugaan, et al.) domain, which is the epitope for the interleukin-1 (IL-1) receptor-associated kinase complex (TIR-MyD88-IRAK) that induces TNF receptor associated factor 6 (TRAF6). The role of TLR 2 in the innate immune system is to detect pathogens and host material that is released during injury. Activation of the nuclear factor kappa β (NF-κβ) pathway leads to the production of antimicrobial mediators (Opal & Esmon, 2003).

2.5 Cytokines

Cellular reaction to inflammation caused by mastitis results in releasing of cytokines. Inflammation can be acute (rapid or short-lived) or chronic (long-lasting); various microbes or trauma can stimulate this. Here we focus on acute inflammation and the primary leukocyte (neutrophils) for orchestrating innate immunity responses to infection.

There are two categories of cytokines: proinflammatory that promote inflammation and anti-inflammatory, which suppress inflammation. Neutrophils are first to appear at the site of infection and release pro-inflammatory cytokines such as TNF-α, IL-8, and IL-1β (Dinarello, 2000). Tumor necrosis factor- alpha TNF-α, is the primary cytokine present in systemic inflammation and responsible for regulating leukocytes; and initiating fever (Paape, Rautiainen, Lilius, Malstrom, & Elsasser, 2002). Studies focusing primarily on milk samples and leukocyte (neutrophil and lymphocyte) population changes after bacterial infection, reported that TNF-α concentrations increased upon *E. coli* intramammary challenge, compared to *S. aureus* (Douglas D. Bannerman, et al., 2005; D. D. Bannerman, et al., 2004). This cytokine has been well documented to undergo upregulation of gene expression using various bacterial derived LPS (D. D. Bannerman, et al., 2004) and PGN (Chenier, et al., 2008), as well as Nystatin (Razonable, et al., 2005).

The role of IL-10 (Interleukin 10), during inflammation has been studied amongst various inducers ranging from bacterial infections to parasitic infections (Couper, Blount, & Riley, 2008). Different types of cells can produce IL-10, most notably macrophages. Many have debated whether neutrophils have the ability to produce certain cytokines such as IL-10, as an antagonist to many proinflammatory cytokines, such as TNF-α, IL-1β and IL-4 (Paape, Duenas, Wettemann, & Douglass, 2000; Tomita, Wang, Paape, Poultrel, & Rainard, 2000). The amount

of IL-10 production by neutrophils is little in comparison with monocytes/macrophages (Reglier, Arce-Vicioso, Fay, Gougerot-Pocidalo, & Chollet-Martin, 1998). However, IL-10 has been documented as an important cytokine to regulate inflammation in sepsis using mouse neutrophils (Kasten, Muenzer, & Caldwell, 2010b). Others have reported that high concentrations of IL-10 could be induced by pathogens as a means of survival; to prevent pathogen clearance (Couper, et al., 2008).

2.6 Nystatin

Nystatin is an antifungal agent-lipid raft inhibitor derived from a gram positive bacteria, *Streptomyces noursei* (Brown, Hazen, & Mason, 1953). The importance of a lipid raft inhibitor lies with its ability to inhibit host- bacterial pathogen interactions (Riethmuller, et al., 2006). Bacterial invasion of the host is normally associated with caveolae endocytosis or lipid rafts that have been found in many phagocytes (i.e neutrophils) (Shin & Abraham, 2001). Bacterial pathogens that are known to use alternative means to infect the innate immune system of the host are: *E. coli* (Duncan, Li, Shin, Carson, & Abraham, 2004), *C. jejuni* (Wooldridge, Williams, & Ketley, 1996), and *P. aeruginosa* (Grassme et al., 2003); and information regarding neutrophils is very scarce. One study highlights nystatin's ability to induce inflammatory cytokines such as IL-1β, IL-8 and TNF-α, by binding to or activating TLR2 (Razonable, et al., 2005). Studies have shown that changes in lipid raft composition rearranges membrane proteins (Riethmuller, et al., 2006) which may be similar to altered Fc receptors that impede bacterial binding to neutrophils (Zaas, Duncan, Rae Wright, & Abraham, 2005). Literature reports have suggested that lipid or membrane rafts are associated with cell signaling and can facilitate receptor clustering, such as with TLR 2 and 4 (Oak, et al., 2006).

Nystatin's effect on fungal (Volpon & Lancelin, 2002) , bacterial, and parasitic pathogens can be attributed to the extraction and destruction of cholesterol from lipid rafts (Riethmuller, et al., 2006). This causes the contents in the cell to leak out and the cell dies, which then sends a cascade of signaling to death receptors (Razonable, et al., 2005). Animal and fungal cells contain sterols- cholesterol and ergosterol, respectively, nystatin has a lower affinity for cholesterol (Volpon & Lancelin, 2002), which allows for some disruption of cellular signal transduction (Fernandes et al., 2006). Nystatin has been researched for treatment of mastitis caused by fungal infections (Kitamura, Anri, Fuse, Seo, & Itakura, 1990). A complete understanding of Nystatin's mechanistic effects on neutrophils is still unknown.

2.7 Quebracho

Quebracho is a South American plant that has been noted to have anti-microbial (Peters, Komaragiri, Paape, & Douglass, 2000) and anti-viral (Lupini, Cecchinato, Scagliarini, Graziani, & Catelli, 2009) activities. Quebracho is a condensed tannin derived from polymeric flavanoids that are comprised of carbohydrates and proteins (Hagerman et al., 1981). Condensed tannins have been under recent study, due to their ability to reduce fecal egg counts (*Ostertagia ostertagi*, *Hemonchus contortus*, and *Nematodirus filicollis)*, increase weight gain, and milk secretion (Villalba, Provenza, Hall, & Lisonbee, 2010). Quebracho has been documented to decrease the viability of L_3 (third stage larval) in parasites in sheep (Athanasiadou, et al., 2001).

Studies have shown that consumption of tannin rich plants such as *Sericea lespedeza*, sainfoin, and sulla have reduced the number of fecal eggs and host resilience to helminthes when given in hay and silage (Brunet, Jackson, & Hoste, 2008). Some variations have been documented that are based on the host, amount of tannin content, parasite species, and level of infection (Min et al., 2005). The use of Quebracho has been identified as tannin forage capable

of reducing helminthes populations in the host (Virginie Paolini, Audrey Frayssines, France De La Farge, Philippe Dorchies, & Herve Hoste, 2003).

Many studies have been conducted using tannin-rich plants on small ruminants from an *in vivo* perspective, using sainfoin (*Onobrychis viciifolla*) extract on the exsheathment of infective larvae, species of *Haemonchus contortus* and *Teladorsagia circumcincta*, which were reduced after 3 hours of treatment. Confirmation was made by the addition of polyvinyl polypyrrolidone, an inhibitor of tannins by the lack of exsheathment of infected larvae in the control group (Brunet, Jackson, et al., 2008).

Other researchers investigated the prospect of using tannin-rich plants as a means to combat parasitic infections and these studies were solely looking at the fecal egg count (Chenier, et al.) or exshealthment of larvae in many small ruminants, along with the use of certain tannin-richs plants to improve host resilience (Brunet et al., 2008). Studies in dairy cows investigated the influence quebracho condensed tannin extract had on fermentation and lactational performance (Dschaak, et al., 2011). Benchaar and colleagues studied the effects of quebracho extract on digestion, ruminal fermentation characteristics, protozoal populations, and milk production in ruminally cannulated lactating cows (Benchaar, et al., 2008). Unknown to researchers is the effect Quebracho, a plant based tannin, has on induction of TNF-α, using bovine neutrophils. Many studies have investigated fecal egg count or larval survival as a cause of inflammation in small ruminants (Athanasiadou, et al., 2001; Brunet, Jackson, et al., 2008). Other studies have investigated quebracho's anti-viral effects on poultry enteric viral infections (Lupini, et al., 2009). This study investigated the impact of LPS, PGN, Nystatin, and Quebracho on gene (*TLR 2, TLR 4, NRAMP1, TNF-α and IL-10*) modulation in using bovine neutrophils.

Chapter 3

Materials and Methods

3.1 Animals

Four clinically healthy Holstein-Freisian cows from the North Carolina Agricultural and Technical State University dairy farm were selected for this study. The University Animal Care and Use Committee approved the protocol. The cows were not under any medical or feeding treatment during the period of this study.

3.1.1 Blood Collection. Thirty milliliters of blood was collected from the jugular vein of each cow into 15-ml BD Vacutainer® tubes (BD Diagnostics, Franklin Lakes,NJ). All tubes contained ~1.0 ml of anti-coagulant acid citrate dextrose (Macdonald, Li, Su, & Pike, 2006). Samples were placed on ice immediately following collection. Blood samples collected from the jugular were used for evaluation of packed cell volume (PCV), DNA and RNA isolation, and blood smears.

3.1.2 Packed Cell Volume (PCV) and Somatic Cell Count (SCC). Packed cell volume was performed using whole blood collected from four cows in this study. The presence or absence of anemia or dehydration was determined by packed cell volume measuring red blood cells in a hematocrit. Whole blood collected into ACD tubes filled heparinized capillary tubes three quarters of the length of the capillary tube. Capillary tubes were in triplicate for each cow (N=4) in this study. Excess blood was removed and one end of the tube was sealed with Cha-seal (Chase Instruments, Corp; Rockwood, TN). Tubes were placed in a microhemtocrit centrifuge (Damon/IEC Division, Needham, MA) and spun for 5 minutes. After centrifugation, tubes were removed and read using a microhematocrit reader (Damon/IEC Division, Needham, MA). Somatic cell count (SCC) was recorded according to Dairy Herd Improvement (DHI)

records indicating somatic cell scores ranging from 0 to 9, based on the amount of white blood cells (WBC) present in the milk. Cows used in this study had recorded scores ranging from 0 to 4, indicating that they were mastitis-free.

3.1.3 Isolation of genomic DNA. Isolation of DNA was performed using FTA® cards (Whatman, Piscataway, NJ) from whole blood samples. Briefly using a 3mm Harris Uni-Core device (Whatman Cat. No. WB100039), one-3mm (1/8") a sample disc was removed from the center of FTA Elute card and transferred into a 1.5 ml microcentrifuge tube, 500 µl of sterile water was added to tubes and immediately pulse vortexed 3 times, for a total of 5 seconds. Using a pipette tip, the disc was removed from the wash and gently squeezed against the side of the tube and immediately transferred to a 0.5 ml microcentrifuge or PCR tube containing 30 µl of sterile water. The discs were completely immersed in water by briefly centrifuging the tube for 10 seconds at 700 x *g*. The tube was then transferred to a water bath at 95°C for 30 minutes. Sample solution was pulse vortexed 15 times during the incubation period. At the end of the incubation period, tubes were removed from the water bath and pulse vortexed approximately 60 times then centrifuged for 30 seconds at 700 x *g*, to separate the matrix from the eluate, which contains purified DNA. Using a sterile pipette tip FTA Elute matrix disc was removed and discarded.

3.1.4 Isolation of Neutrophils. Neutrophils were isolated using the modified Carlson and Kaneko method (Carlson & Kaneko, 1973), by hypotonic lysis of red blood cells (RBC). Briefly, blood samples from each cow were individually pooled into 50 ml polypropylene conical tubes and maintained on ice, gently inverted three times, and centrifuged (20 minutes at 1800 x *g* at 4°C) in a swing bucket rotor (Eppendorf Model 5810R centrifuge, Hauppauge, NY). Red blood cells were removed from each tube with a disposable pipette, down to 5 ml. Tubes

were gently inverted to resuspend polymononuclear cells and remaining red blood cells. Approximately 20 ml of ice cold DEPC-water was added to tubes and inverted for ~45 seconds to lyse red blood cells. To restore isotonicity, 10 ml of ice cold 2.7% saline was added and gently inverted to mix. Tubes were placed on ice periodically to keep cells cold. Suspensions were centrifuged for 2 minutes at 700 x *g* at 4°C and supernatant was removed. Cells were gently resuspended by adding ten millilters of 0.0132 M phosphate buffered saline (PBS) (pH 7.4) to the cell pellet. The process was repeated 2-3 times until a white neutrophil (PMN) enriched pellet clear of all RBCwas obtained. Cells were resuspended in PBS at a volume of 5 or 10 ml depending on the pellet size.

3.2 Viability and Purity of Neutrophils

Viability of neutrophils was checked by trypan blue dye exclusion method. Isolated PMN were mixed with trypan blue (Sigma-Aldrich cat#T8154) at a 1:2 dilution rate. Cell suspensions in trypan blue dye were placed in a microfuge tube. Approximately 10µl of the mixture was loaded into each side of the hemacytometer chambers. Cells were viewed under 100x magnification and viable and non-viable cells were counted. Cell counts were done in duplicate and an average was taken; cell viability was expressed as a percentage of total viable and non-viable cells. After cell viability and the concentration of PMN was determined. A white blood cell (WBC) differential cell count was determined using Wright's stain as described in the appendix to determine neutrophil's purity. Cells were adjusted to 10^7 cells per ml. All reagents were prepared in diethyl pyrocarbonate (DEPC)-treated lipopolysaccaride (LPS) free milli-Q water (Millipore, Billerica, MA).

3.3 Stimulation of Neutrophils

Cells from each of four cows were maintained in PBS at pH 7.4 (Sigma-Aldrich, St. Louis, MO), *Eschericha coli* derived lipopolysaccaride, *Staphylococcus aureus* derived peptidoglycan, Nystatin, Quebracho, and 62% ethanol (Sigma-Aldrich) to assess the expression of select genes and cytokines by neutrophils. Cells suspended (10^7) in 1 ml of PBS were placed into 2.0 ml RNase-free tubes and treated with either 1 ml of 200 ng Lipopolysaccharides from *E.coli* (0111:B4 Cat. No.L3012, Sigma-Aldrich, St. Louis, MO); 2500U Nystatin (Cat. No.N1638, Sigma-Aldrich, St. Louis, MO); 2 µg Peptidoglycan S*taphylococcus aureus* (Cat. No. 77140, Sigma-Aldrich, St. Louis, MO) or *Aspidosperma* quebracho-blanco (3X, Herb Pharm, Williams, OR). Aliquots of cells suspended in 1 ml PBS or 62% ethanol were used as negative controls. Peptidoglycan was used as a positive control in this study. Cells were incubated at the following timepoints: 0, 15 minutes, 30 minutes, or 1 hour at 37°C with ~85% humidity and 5% CO_2. After incubation, tubes were placed on ice and samples centrifuged at 1700 x *g* at 4°C for ~ 5 minutes to pellet cells. The supernatant was collected and stored at-70°C to detect TNF-α cytokine secretion. Pellets were washed with PBS 2X by centrifuging 1700 x *g* at 4°C for 2 minutes. Cells were then used for RNA isolation

3.3.1 RNA Isolation. All samples from both unstimulated and stimulated bovine neutrophils underwent RNA extraction according to Ambion manufacturer's instruction. One ml of TRIzol reagent (Invitrogen Life Technologies Corp, Carlsbad, CA) was sadded to each microcentrifuge tube for RNA isolation. All samples were checked for RNA purity and concentration using a Nanodrop Spectrophotometer 1000 (Thermo Scientific Inc, Waltham, MA).

3.3.2 Evaluation of nucleic acid (DNA and RNA) Concentration and Purity.

Concentration and purity of DNA and RNA were assessed using a Nanodrop Spectrometer 1000 (Thermo Scientific Inc, Waltham, MA). Optical densities were read at A260/280 for nucleic acid purity. Concentrations were measured as nanograms per microliter (ng/µl).

3.4 Primers

All primers were synthesized by MWG Biotech Inc (Huntsville,AL). The following genes were evaluated in isolated genomic DNA and RNA isolated from neutrophils. Primers for *TLR 2*, *TLR 4* (Menzies & Ingham, 2006), *NRAMP1* (Ables, et al., 2002), *TNF-α* (Cludts, Cleuter, Kettmann, Burny, & Droogmans, 1993), *IL-10* and *GAPDH* (housekeeping gene) (Vieira et al., 1991) were used (Table 1).

3.4.1 Multiplex PCR to Detect Genes in Genomic DNA. The Multiplex PCR kit (Qiagen) was used according to manufacturer's instructions. A 50µl reaction was made in 0.2 ml PCR tubes using 50 ng of DNA.

Table 1

Primer sequences and PCR product size

Gene	Primer sequence	Expected Primer Length (bp)
GAPDH	Forward:5'GTCTTCACCACCATGGAG 3' Reverse: 3'CTCCATGGTGGTGAAGAC 5'	198
TLR 2	Forward:5'GTTGGAGGCCGGTGGCAACA 3' Reverse: 3'TGTTGCCACCGGCCTCCAAC5'	501
TLR 4	Forward:5'AACCACCTCTCCACCTTGATACTG 3' Reverse: 3'CCAGAAAGACCTTGAATACAGG5'	410
NRAMP1	Forward:5'CATGAAGCCAACTGCCAAGG3' Reverse:3'GAAGCCTGCAAGATGACCAACA5'	433
TNF-α	Forward:5'CTGCACTTCGGGGTAATCGG3' Reverse:3'CAGGGCGATGATCCCAAAGTA5'	549
IL-10	Forward:5'GCCAAGCCTTGTCTGAGATGATC3' Reverse:3' CTCCCTGGTTTCTCTTCCTAAGA3'	800

Multiplex components consisted of 25µl PCR master mix, 2µM each primer, and RNase-free water. The conditions for PCR reactions were as follows: 95°C for 15 minutes (activation), 94°C for 30 seconds, 60°C annealing temperature and 30 cycles for specific genes; 72°C for 90 seconds, and 72°C for 10 minutes in a thermocycler (MWG Biotech, Foster City, CA).

3.4.2 Evaluation of gene expression using reverse transcriptase polymerase chain reaction (RT-PCR). The Qiagen One-Step RT-PCR kit (Qiagen,Valencia,CA) was used to detect transcription of selected genes in neutrophils exposed to different compounds. A 25-µl reaction was made in a 0.2 ml PCR tube using 1000 ng of RNA, 5 µl buffer, 1µl dNTP , 1µl Enyzme Mix, 0.6 µM primer and RNase-free water. Amplified products were stored at ~4°C for immediate analysis or ~20°C for future analysis of RT-PCR products.

3.4.3 Detection of amplified genes. Amplified products were run on a 1.7% agarose gel with PCR markers (Promega, Madison, WI) for approximately 25 minutes. Molecular weight markers were used to identify molecular weights of genes based on published size (Table 2). Amplification of *gapdh*, a housekeeping gene was used as a loading control. Gels were stained with 1µg/ml ethidium bromide, washed and visualized using a Bio-Rad© Gel documentation system (Bio-Rad Laboratories, Hercules, CA).

3.5 Evaluation of Cytokine TNF-α Secreted in Supernatants from Neutrophils

Secretion of TNF-α was measured using cell supernatant collected and stored at -70˚C. The concentration of TNF-α was determined using a DuoSet© Bovine TNF-α sandwich ELISA kit (R&D Systems, Minneapolis, MN). A flat bottom 96 well plate (Nalge Nunc International) was coated overnight with 100 µl per well of goat anti-bovine TNF-α capture antibody at a working dilution of 0.8 µg/ml. Following overnight incubation, each well was aspirated and washed with wash buffer (400 µl) three times by hand. The plate was inverted onto a towel after

the final wash to ensure the removal of excess liquid. Plates were blocked with 300 µl of blocking buffer and incubated at room temperature for 2hours. Blocking buffer was then aspirated and washed with wash buffer three times. Cell supernatant samples and TNF-α standard were added at 50 µl per well in triplicate and incubated for 2 hours at room temperature. Standards ranged from 0 pg/ml to 1pg/ml. Detection antibody (100 µl) containing 72 µg/ml biotinylated goat anti-bovine TNF-α was added to each well and incubated for 2 hours at room temperature. A working dilution of 1:200 of Streptavidin-HRP (100 µl) was added to each well and incubated for 20 minutes at room temperature. Plates were aspirated and washed as described before with wash buffer three times and inverted on a towel to remove excess liquid. A 100 µl of TMB substrate solution (ThermoScientific, Rockford, IL) was added to each well and incubated for 20 minutes at room temperature, out of direct light. Afterwards, 50 µl of stop solution (2N H_2SO_4) was added to each well. Optical density was read at an absorbance of 450 nm using a MR600 micro plate reader (Dynatech Laboratories Inc., Chantilly, VA). A standard curve was generated to identify optical density of unknown samples. Unknown sample concentrations were quantified by interpolating the optical density from the standard curve and GraphPad Prism (GraphPad Software, LaJolla, CA) generated the line of best fit.

3.6 Statistical Analysis

Statistical analyses were performed using SAS 9.1 (SAS Institute Inc. Cary, NC, USA). A repeated measurement analysis (Proc GLM) was performed to identify main effects of time and treatment, as well as their interactions. Least squares mean was used for paired comparison to differentiate significance between treatments and control. A probability of $P < 0.05$ was chosen as the level of significance. Calculations are presented as mean ± SD (standard deviation) for purity and concentration of DNA. Mean neutrophil viability and PCV data were

calculated using Microsoft Excel. Gel data was tabulated to summarize the presence and absence of the expected molecular weight bands.

Chapter 4

Results and Discussion

4.1 Packed Cell Volume (PCV) and Somatic Cell Counts (SCC)

Somatic cell scores for all four cows are represented in Table 2. The test is a useful way to detect mastitis. Somatic cells present in milk are white blood cells, most importantly; neutrophils. According to the Dairy Herd Improvement Association (DHIA) a somatic cell count below 750,000 cells per ml, represents the legal federal limit for good quality milk (National Federation of Milk Producers, 2011). Overall, SCC levels for all cows tested in this study were within acceptable range for mastitis-free status as seen in Tables 2 and 3.

The presence or absence of anemia or dehydration was determined by evaluating the packed cell volume measuring red blood cells in a hematocrit. A normal packed cell volume for the bovine is between 24-46% (Epperson, Hoblet, Smith, Hogan, & Todhunter, 1993). An increase in this percentage is an indication of dehydration and a decrease is associated with anemia. In Table 2, the mean PCV of 28.5% (N=4) calculated for animals studied showed no indication of anemia or dehydration. Thus animals used in this study were mastitis-free and not anemic (Epperson, et al., 1993) based on these criteria.

Table 2

Mean somatic cell score and packed cell volume

Animal No.	**Somatic Cell Score (SCC)**	**Packed Cell Volume (PCV %)**
1788	3.7 ± 0.3	31.0 ± 0.1
2965	2.3 ± 0.3	29.0 ± 0.2
3000	0.0 ± 0.0	27.0 ± 0.9
3001	1.0 ± 0.0	27.0 ± 0.3
Mean	1.7 ± 0.2	28.5 ± 0.4

Table 3

Relationship between somatic cell score and somatic cell count

Somatic Cell Score	Approximate Range (cells/ml)
0	0 to 17,000
1	18,000 to 34,000
2	35,000 to 70,000
3	71,000 to 140,000
4	141,000 to 282,000
5	283,000 to 565,000
6	566,000 to 1,130,000
7	1,131,000 to 2,262,000
8	2,263,000 to 4,525,000
9	4,256,000 +

(Source: National Mastitis Council, 2011)

4.2 Viability and Purity of Isolated Neutrophils

The average viability and purity of isolated neutrophils after isolation from whole blood samples was 98.0 and 96.4%, respectively. Bovine neutrophil purity levels averaged ~96% as determined by Wright's stain method for all the samples (N=4). Contaminating leukocytes were lymphocytes and monocytes (Table 4). Cell membrane impairment was determined by trypan blue exclusion, this method allows one to see the extent of cell membrane impairment by the amount of blue dye the neutrophil has taken up. Cell viability determined by trypan blue exclusion was 98%.

Table 4

Differential cell count after neutrophil isolation

Animal No.	Neutrophil	Lymphocyte	Monocyte
1788	97.0 ± 0.6	3.0 ± 0.3	0.0 ± 0.0
2965	96.0 ± 0.6	2.0 ± 0.3	2.0 ± 0.5
3000	94.3 ± 0.9	5.0 ± 0.3	1.0 ± 0.1
3001	98.3 ± 0.3	2.0 ± 0.6	0.0 ± 0.0
Mean	96.4 ± 0.6	3.0 ± 0.7	0.7 ± 0.1

4.3 Concentrations and Purity of DNA Isolation

The Whatman FTA Elute card was used to isolate DNA from whole blood. Once DNA was extracted, it was measured using a nanodrop spectrophotometer for concentration and purity of DNA. The absorbance for nucleic acid measurements was read at 260 nm; the reported wavelength reading was A260/280, with a value of 1.8 indicating a good sample quality. All DNA samples from cows (N=4) studied averaged a reading of 1.8 and an average concentration of 0.9 µg of DNA present (Table 5). Samples of DNA from all four cows were used for *TLR 2, TLR 4, NRAMP1, TNF-α* and *IL-10* detection in genomic DNA.

Table 5

DNA purity and concentration

Animal No.	DNA Purity (A260/280)	DNA Concentration (µg)
1788	1.5 ± 0.04	1.7 ± 0.4
2965	1.8 ± 0.05	0.8 ± 0.1
3000	2.2 ± 0.01	0.6 ± 0.1
3001	1.8 ± 0.02	0.5 ± 0.0
Mean	1.8 ± 0.03	0.9 ± 0.2

In Figure 2, the presence of *GAPDH, TLR 2, TLR 4, TNF-á* and *IL*-10 were visualized on a 1.7% agarose gel using 50 ng of DNA, and stained with 1µg ethidium bromide. All genes were present in genomic DNA, as represented in Figure 2. Gene expression for interleukin 10 was not represented at the expected 800 bp marker but was seen at 200 bp. Primers used were specific to bovine genes except for IL-10, which was based on a goat specific sequence (Ables et al., 2002; Cludts et al., 1993 ; Menzies et al., 2006; Vieira et al., 1991) Expected molecular weights (MWT) for genes are: *GAPDH* (198bp), *TLR 2* (501bp), *TLR 4* (410bp), *NRAMP1* (433bp), *TNF-α* (549bp), and *IL-10* (800bp). Thus all cows used in this study had the genes for *GAPDH, TLR 2 TLR 4, IL-10* and *NRAMP1*.

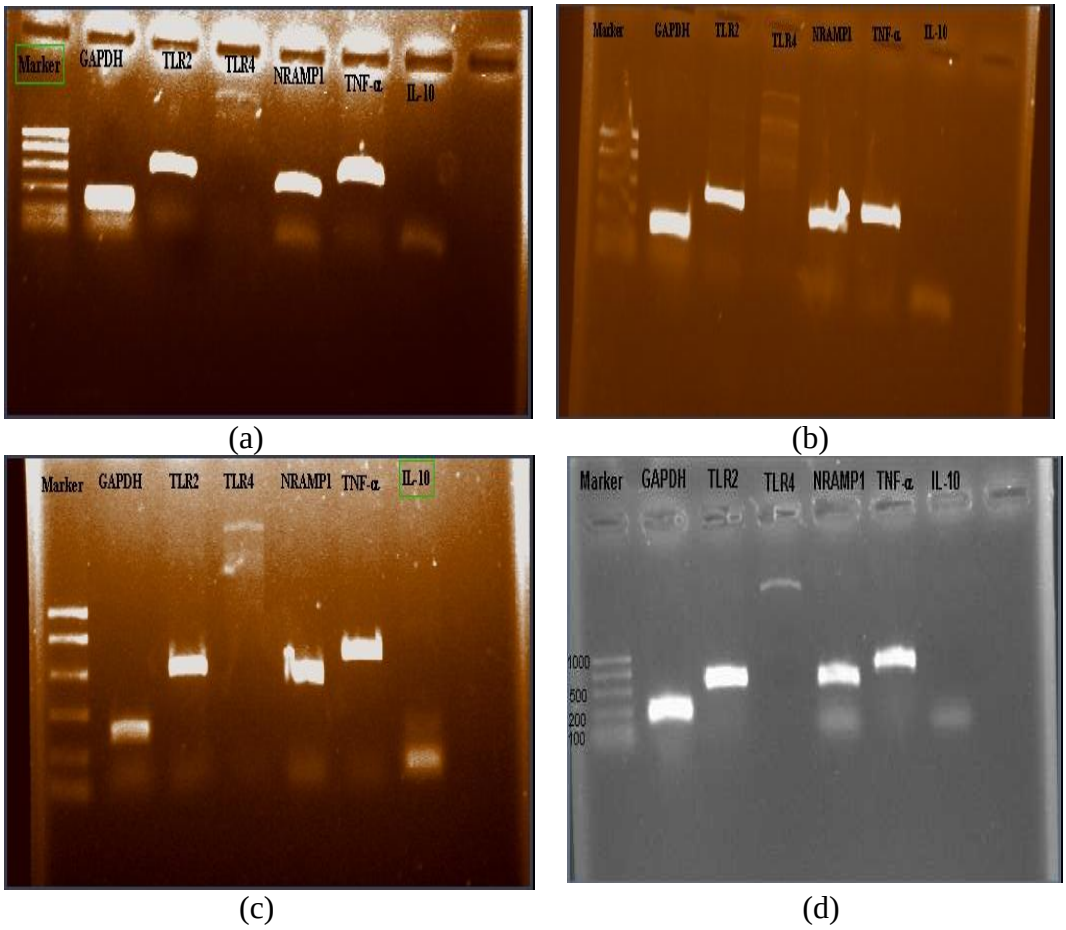


Figure 2. Genomic DNA from cow number (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

4.4 Concentration and Purity of Isolated RNA

The concentration and purity of isolated RNA was measured from bovine neutrophils to determine the treatment effect on mRNA transcription. Neutrophils isolated from whole blood samples suspended in PBS or 62% ethanol (quebracho control), LPS, PGN, nystatin, or quebracho. Neutrophils suspended in PBS were used as negative controls to determine a baseline gene expression for bovine neutrophils. Ethanol was used as a control for quebracho, due to the manufacturer's (Covenant Health, Brevard, NC) use of 62% of ethanol as an extraction solvent in the preparation of the quebracho extract. This was used to differentiate

between the effects of gene expression patterns by ethanol and quebracho. Extraction of total RNA using TRIzol© reagent and quantification and purity of RNA was performed using BioRad nanodrop spectrometer. For comparison of samples with controls, the hash (#) and asterisk (*) signs indicate highly significant ($P<0.0001$) or significant ($p<0.05$), respectively, for RNA purity and concentration in Figures 3 and 4. A lack of either one indicates no significance was seen for both RNA purity and concentration amongst treatment groups.

Statistical analysis indicated that incubation time had an effect on RNA concentration, showing highly significant results ($p<0.0001$) and an interaction between time and group ($p=0.0013$) using the least squared mean of all four cows. In Figure 3, increased highly significant results ($p<0.0001$) was seen for PGN and ethanol, while increased significant results ($p<0.05$) was seen for all other treatment groups compared to control seen at 15 minutes. We see a slightly lower significant level ($p<0.05$) for ethanol compared to control at 30 minutes. Quebracho increased RNA concentration from 15 to 30 minutes, but still displayed an increase in significance ($p<0.05$) compared to control. Lower RNA concentrations are measured at 60 minutes for all treatment groups. Nystatin and ethanol produced increased significant results ($p<0.05$), while quebracho produced increased significance ($p<0.0001$) against the control group. The only requirement for RNA concentration is that the reading from the spectrophotometer represents positive numbers, indicating the presence of RNA, while a negative number, indicate the lack of RNA presence in the sample (Hogan et al., 1993). In this study, time and treatment can change the RNA concentration of cells exposed to compounds. This is an indicator of overall cell activation due to time of incubation or concentration of treatment. Overall, these results indicated RNA transcription is increased in the presence of immunomodulators.

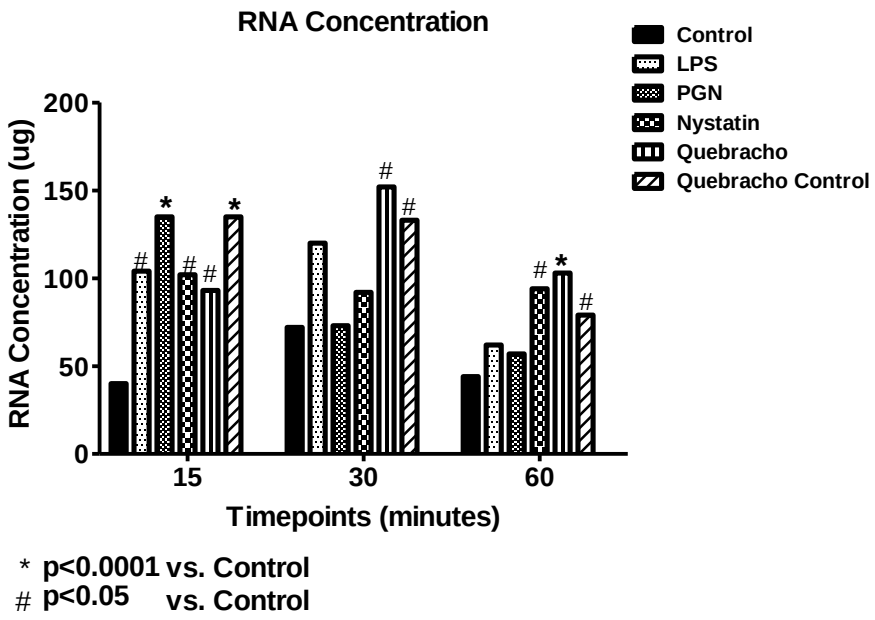


Figure 3. Concentration of RNA extracted from bovine neutrophils at different timepoints.

No significant difference was observed on RNA purity, in relation to the effect of time ($p>0.05$) in Figure 4. However, RNA purity significance ($p<0.001$) is seen for interaction between time and group using SAS analysis. Increased RNA purity was highly significant ($p<0.0001$) at 15 minutes for all treatment groups compared to control. Total RNA purity for LPS and PGN showed decreased significance ($p<0.05$) after cell exposure of 30 and 60 minutes, respectively, in relation to control. In the present study, quebracho and ethanol decreased significance ($p<0.05$) at both 30 and 60 minutes

Using an absorbance reading is the most common method to determine purity of DNA and RNA samples. Nucleic acids absorb light at a wavelength of 260 nm, so the absorbance or optical density of DNA and RNA are often reported at this wavelength. For RNA, a pure sample will have an A260/A280 value of ~2.0. The purity of an RNA at a value of 2.0 is optimal to

avoid protein contamination in the sample. These results indicated RNA transcription is increased in the presence of immunomodulators at the earlier timepoint of 15 minutes.

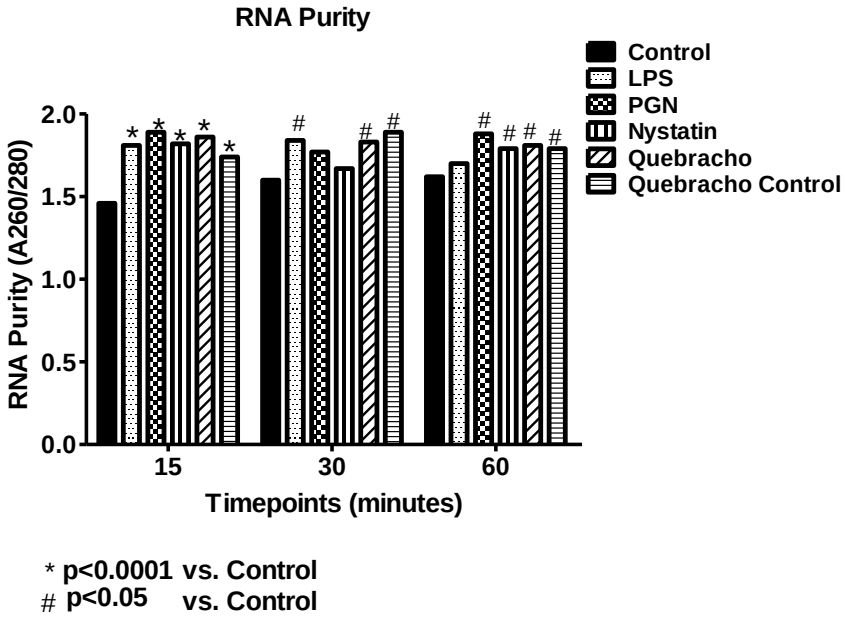


Figure 4. Purity of RNA extracted from bovine neutrophils at different timepoints.

4.5 Gene Expression in Bovine Neutrophils Suspended in PBS

Bovine neutrophils were suspended in PBS to determine a baseline for gene expression when compared to other treatment groups in this study. Neutrophils were isolated and incubated at 0, 15, 30, and 60 minutes in PBS and were evaluated for mRNA transcript by RT-PCR. Gene expression was observed for *GAPDH*, a loading control, in all animals (N=4). Expression of *TLR 2* and *NRAMP1* was seen for animal 1788 at 0 minutes in Figure 5. Animals 2965 and 3000 showed gene expression for *TLR 4* and *TNF-α* at 0 minutes. While animal 2965 showed expression of *NRAMP1* at 0 minutes. No gene expression was seen for *IL-10* (N=4) for unstimulated cells at 0 minutes. Here the study showed *TNF-α* gene expression for animals

(2965 and 3000) in untreated neutrophils, which could be an example of polymorphism amongst same breeds of cattle.

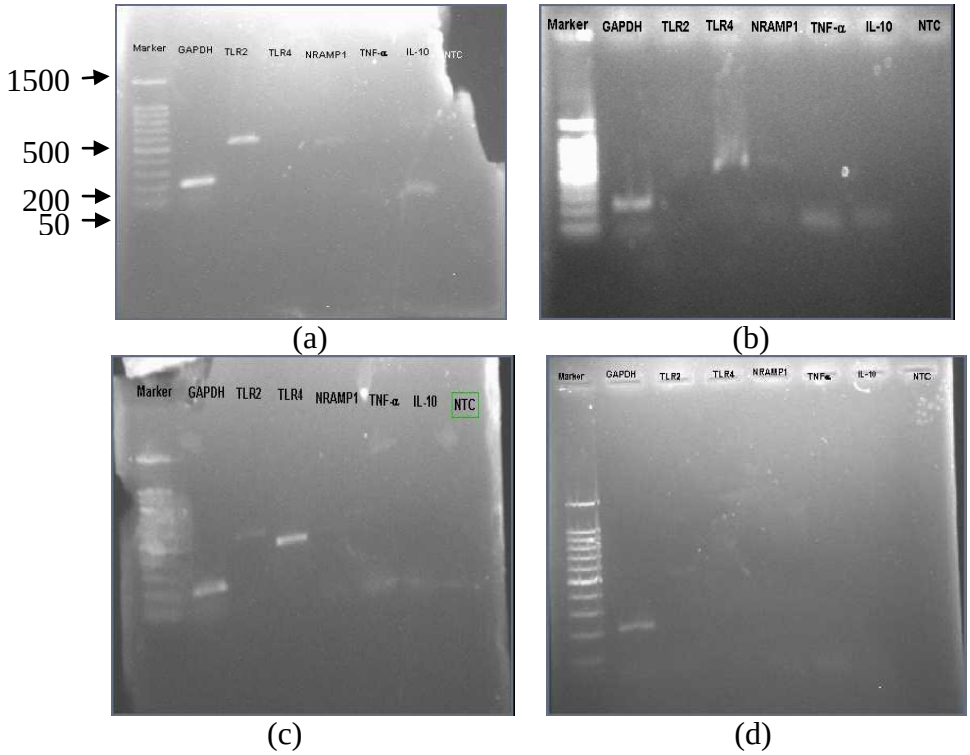


Figure 5. Gene expression in untreated bovine neutrophils incubated at 37°C in 5% CO_2 and 85% humidity for 0 minutes; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

In Figure 6, *TLR 4* gene expression was seen for animals 1788 and 3001 while animal 3001 expressed *TLR 2* compared to the zero timepoint. There was no change in gene expression for animals 2965 and 3000 from zero to 15-minute timepoint, except with *TNF-α*. Only one animal (3000) displayed *Nramp1* at the 15 minute timepoint. There was no gene expression for *TNF-α* seen after 15-minutes exposure time interval in Figure 5. Comparing Figures 4 and 5, toll-like receptor-2 expression was only seen in animal 3001 at 15-minute time interval, indicating possible cow variability.

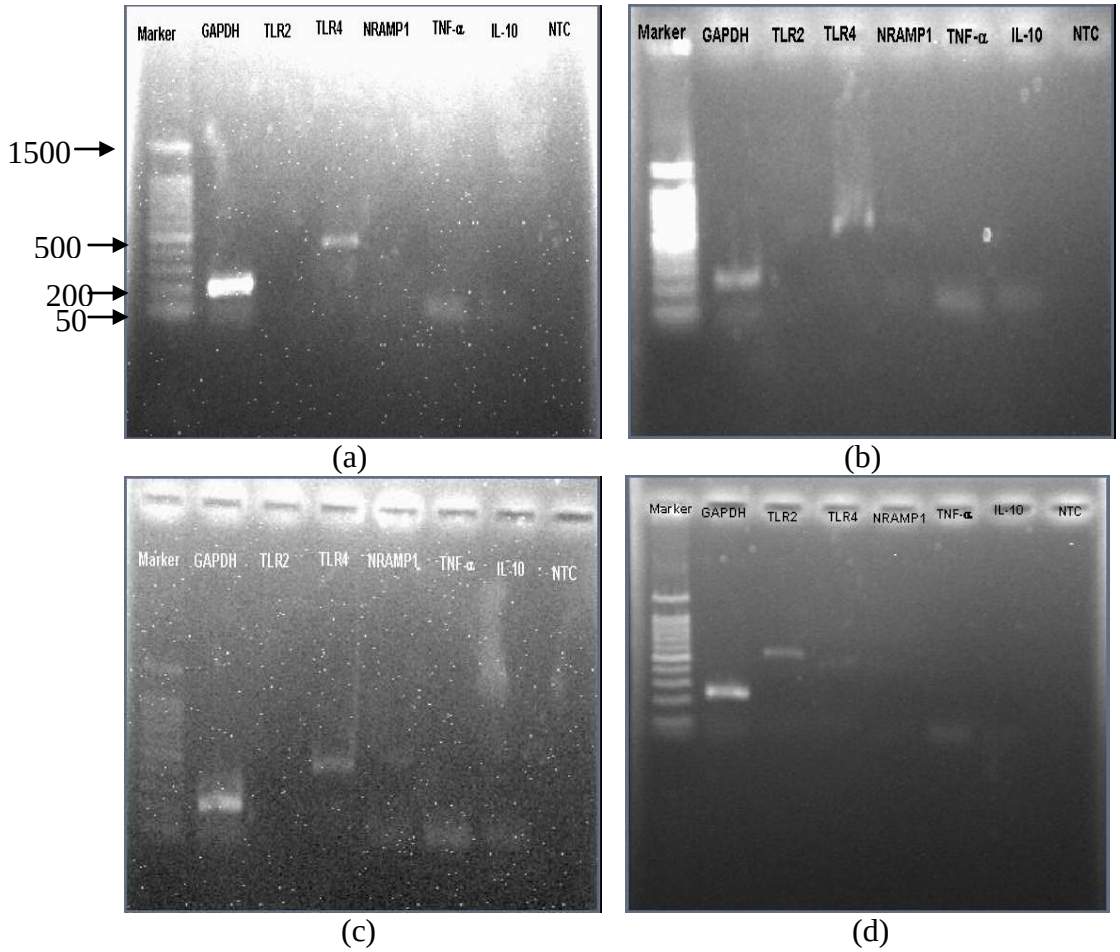


Figure 6. Gene expression in untreated bovine neutrophils incubated at 37°C in 5% CO_2 and 85% humidity for 15 minutes; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

No change in gene expression for *TLR 4* was seen for half of the animals, except for animal 1788 and 3001 as illustrated in Figure 5. The lack of gene expression for animal 3001 at 0 time interval could be due to gene variability for this cow, compared to the others. Only two animals remained unchanged for *TLR 4* expression after 15 minutes of incubation in PBS, indicating a stress-induced gene expression trend (Wang et al.), or some other stimuli to induce expression of the *TLR 4* transcript in Figure 5. Transcription of *TNF*-α was not seen at the mRNA level after 15 minutes of incubation in PBS. The lack of amplification of *TNF*-α

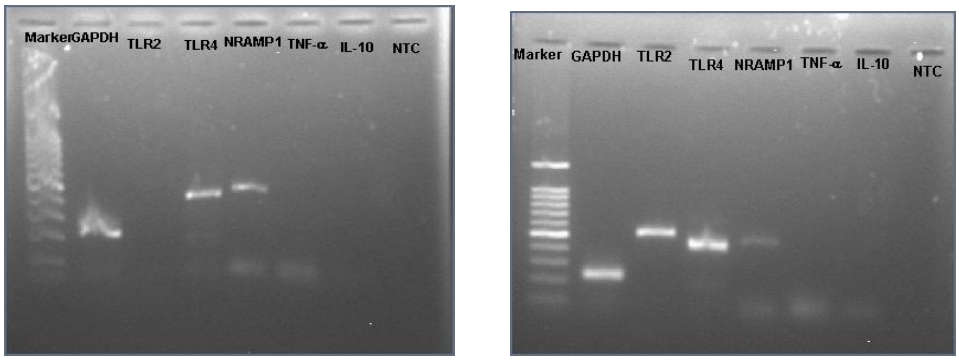
transcript amongst the 15-minute group could be attributed to the cell's adaptation to its environment or individual genetic characteristics.

Gene expression variability was seen for *TLR 2,* and *TLR 4* in untreated neutrophils, at the 30-minute time interval (Figure 7). Transcription for *TLR 2* was seen in two out of four animals at the 30 minute time interval, and *TLR 4* expressions was observed in only one animal (#3001) in the same treatment group. *Nramp1* was transcribed in three animals, but not seen for animal 3001. No expression was seen for *TNF-*α for all four animals. Results with unstimulated bovine neutrophils demonstrate that time and cow genetics could play a synergistic role on transcription of selected genes.

Nramp1, a divalent transporter, has been linked to host susceptibility or resistance to pathogens in bovine, murine, and porcine (Ables, et al., 2002). The gene for Nramp1 is expressed mainly in macrophages, spleen, and lungs (Feng et al., 1996).

(a) (b)

Figure 7. Gene expression in untreated bovine neutrophils incubated at 37°C in 5% CO_2 and 85% humidity for 30 minutes; cows used (a) 1788, (b) 2965, (c) 3000 and (d) 3001.

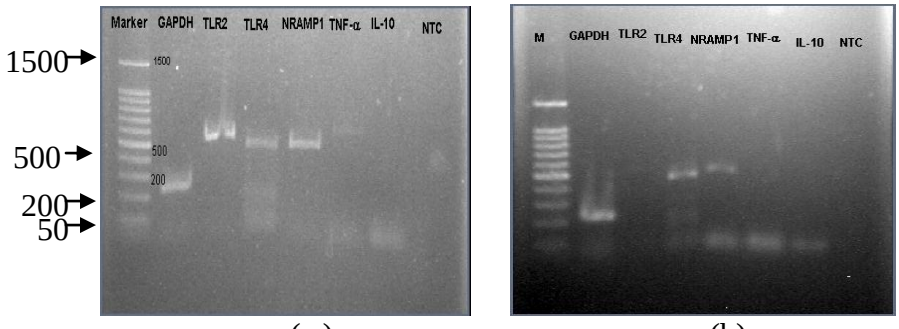


(c) (d)

Figure 7. (cont).

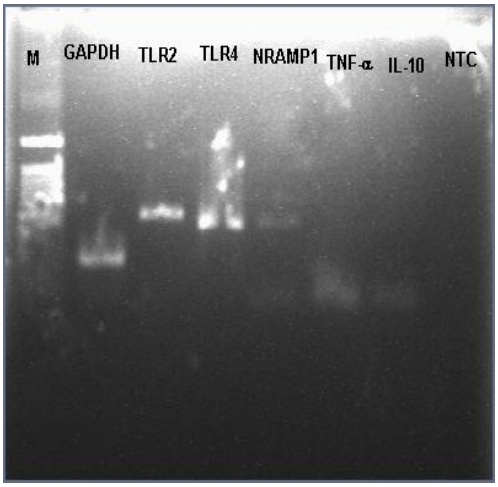
Previous studies were done (Worku & Morris, 2009) as well as the current study have shown that bovine neutrophils are capable of expressing the *NRAMP1* gene. The mechanism of gene expression is unclear for Nramp1 induction in mice and porcine; however, it is known that LPS produces induction in the same animals (G. Zhang, et al., 2000).

At the 1-hour interval (Figure 8), *TLR 2* was not expressed when compared to zero timepoint PBS. A time effect was seen with a down regulation of *TLR 2* in unstimulated bovine neutrophils (N=1). However, there appeared to be an expression of *TLR 4* in unstimulated bovine neutrophils (N=2). Cow variability was not seen for the one-hour timepoint when comparing transcription of selected genes of unstimulated cells.



(a) (b)

Figure 8. Gene expression in untreated bovine neutrophils incubated at 37°C in 5% CO_2 and 85% humidity for 60 minutes; cows used (a) 1788, (b) 2965, (c) 3000 and (d) 3001.



(c)

(d)

Figure 8. (cont).

All four animals tested did not express *TNF*-α at the one-hour timepoint. Toll-like receptor 4 was expressed in only half of the animals tested at the 60-minute time interval, this could indicate a time dependent gene expression for *TLR 4* and induction of Nramp1 in neutrophils. The differences in gene expression can be possibly due to cow variation linked to polymorphisms that affect gene expression and resistance and susceptibility to disease as previously proven in bovine and buffaloes (Ables, et al., 2002).

4.6 Expression of TLR2, TLR4, Nramp1, TNF-α , and IL-10 in neutrophils treated with *Eschericha coli* derived lipopolysaccharide.

Lipopolysaccaride is a component of gram-negative bacteria. It produces a different response by the innate immune system compared to gram-negative bacteria (Ulevitch & Tobias, 1999). To evaluate transcription of *TLR 2, TLR 4, NRAMP1, TNF*-α, and *IL-10* at the mRNA level, bovine neutrophils collected from four cows were treated with *E.coli* derived lipopolysaccharide (100 ng) and incubated at 15, 30 and 60 minutes. A previous study demonstrated that *NRAMP1* could be expressed at the 30-minute timepoint for PMN treated with

LPS at 10ng (Worku & Morris, 2009). In this study, we investigated the effects of LPS (100ng) on bovine PMN at 15, 30 and 60-minute timepoints (Figures 9, 10, and 11) for expression of *TLR 2 TLR 4, NRAMP1, TNF-*α, and *IL-10*. There was no animal-to-animal variation seen at the mRNA level for selected genes in Figure 9.

Modulation of gene expression in bovine neutrophils stimulated with LPS for 15 minutes in Figure 9, shows expression of *TLR 2* in 3 out of four animals tested. Expression of *NRAMP1* was seen in all animals tested when compared to the 15-minute time interval for PBS. No change was seen for *TLR 4, TNF-α,* or *IL-10.*

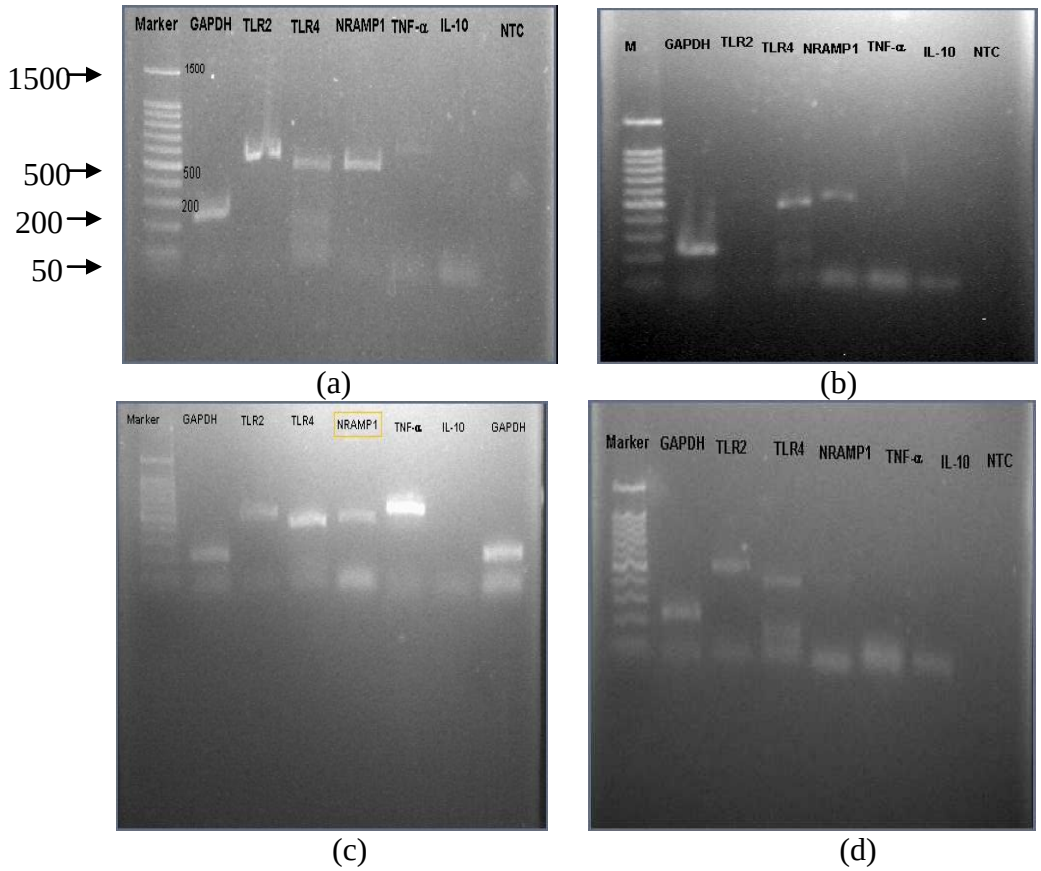


Figure 9. Gene expression in bovine neutrophils treated with 100 ng of E.coli derived lipopolysaccaride serotype 0111:B4 for 15 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

A treatment effect was seen for LPS stimulated neutrophils, particularly with transcription of *TLR 2, TLR 4,* and *NRAMP1* seen in Figure 10 compared to PBS. In Figure 10, neutrophils stimulated with LPS, produced a more consistent pattern of gene expression at the 30 minute time interval, in comparison with untreated bovine neutrophils at the same time point. Therefore we concluded that gene modulation was produced in LPS stimulated bovine neutrophils.

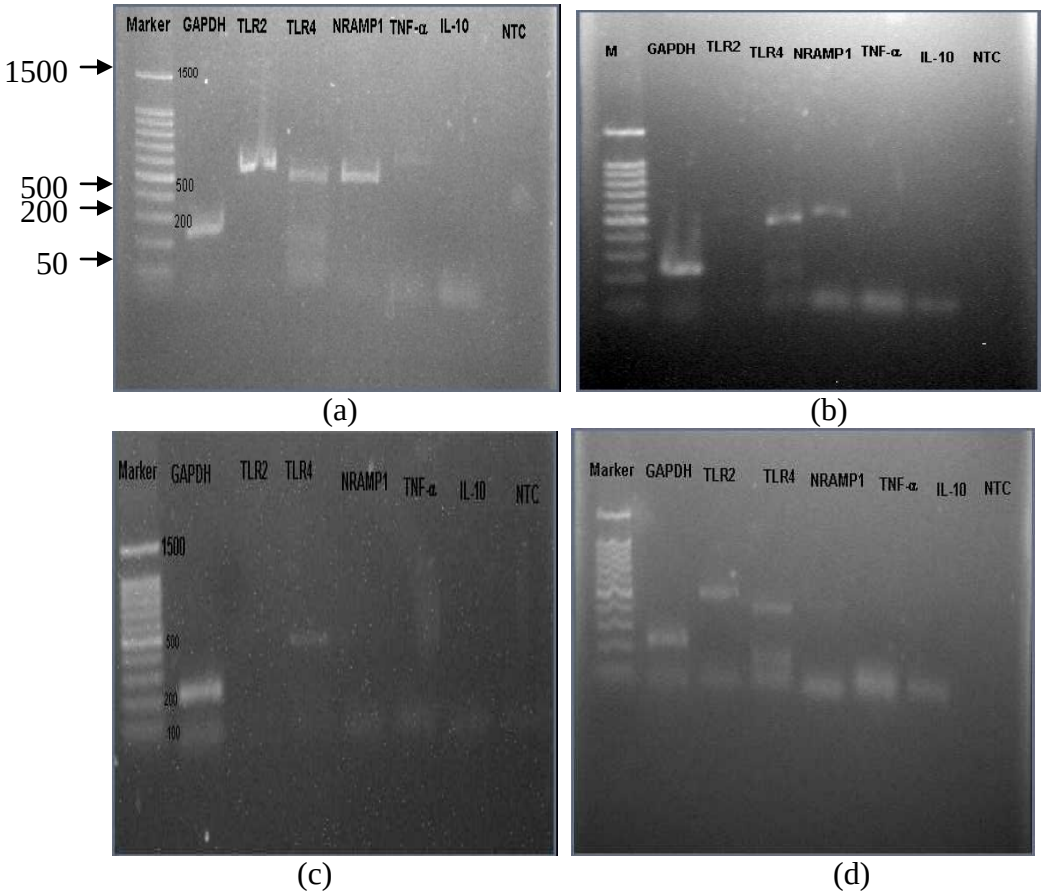


Figure 10. Gene expression in bovine neutrophils treated with 100 ng of E.coli derived lipopolysaccride serotype 0111:B4 for 30 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b)2965, (c) 3000, and (d) 3001.

To measure gene expression in bovine neutrophils treated with LPS (100ng), cells were incubated for 60 minutes at 37°C, 5% CO_2 and 85% humidity. In Figure 11, extraction of RNA from bovine neutrophils resulted in transcription of *TLR 2* in all animals tested (N=4). Animal variation was seen for *Nramp1* gene expression in 2 out of 4 animals and only one (animal 3000) expressed *TNF-α* when compared to PBS. No animals tested expressed *IL-10* at the 60-minute time interval (N=4) at the mRNA level. Tumor necrosis factor- alpha gene expression was has been reported in milk samples stimulated with LPS stimulated from 16 to 32 hours (D. D. Bannerman, et al., 2004; Shuster, Kehrli, Rainard, & Paape, 1997). Here, bovine neutrophils stimulated with LPS elicited transcription of *TLR 2* and *TNF-α*. While TLR2 gene expression is seen early in Figure 8, its activation has been noted in papers associated with LPS extracted from *Porphyromonas gingivalis* or *Leptospira interrogans* (Teixeira, Almeida, & Gazzinelli, 2002).

Expression of *Nramp1* is associated with leukocytes, but with greater abundance in macrophages as demonstrated in Zhang's study there is a time dependent induction of *Nramp1* by LPS (G. Zhang, et al., 2000). In this study, *Nramp1* was seen at earlier time intervals with neutrophils in PBS and when stimulated with LPS (100ng). Studies have looked at bovine neutrophils and various immunodulators for short time intervals, in relation to the innate immune system (Worku & Morris, 2009). A previous study investigated the effect of different forms of LPS on bovine neutrophils in certain genes (*TLR4, Nramp1*, and *CD14*) and cytokines (IL-8, IL-1β, and TNF-α) under earlier time intervals. It is known, that LPS induces proinflammatory cytokines such as TNF- alpha and IL-1β in a dose dependent manner in bovine neutrophils (Worku & Morris, 2009).

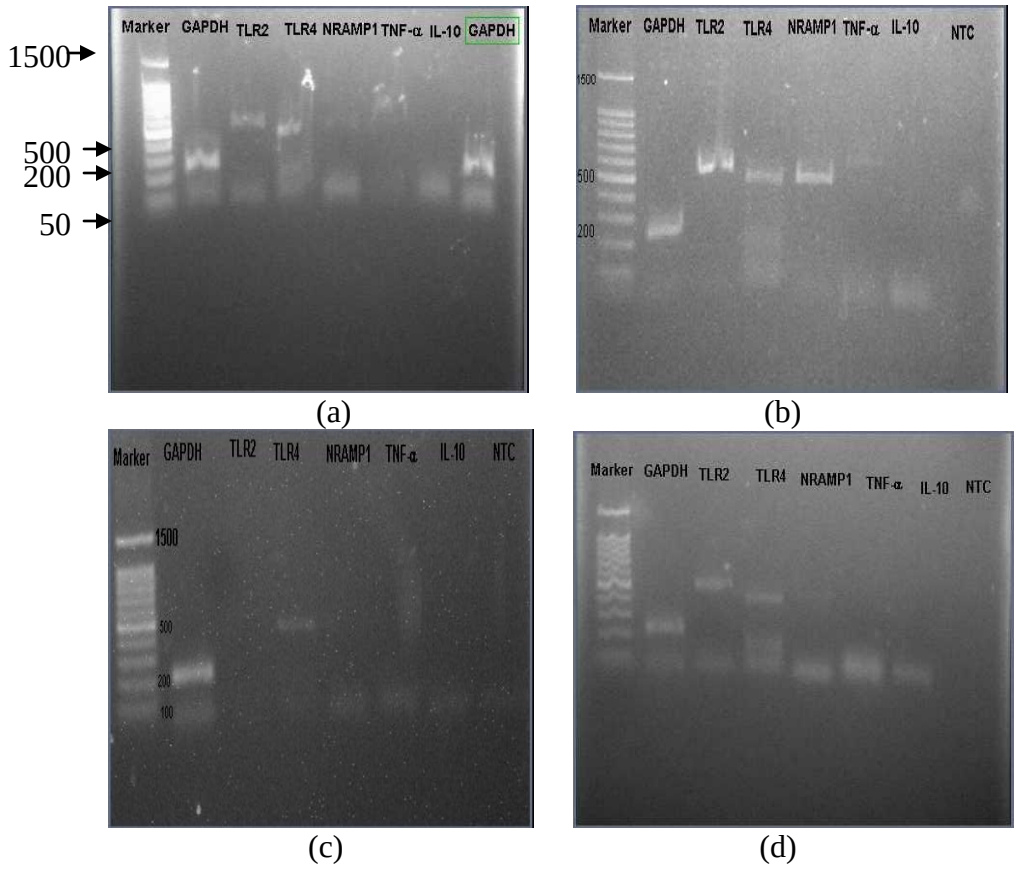


Figure 11. Gene expression in bovine neutrophils treated with 100 ng of E.coli derived lipopolysaccride serotype 0111:B4 for 60 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 30001.

4.7 Expression of TLR2, TLR4, Nramp1, TNF-α and IL-10, in neutrophils treated with *Staphylococcus aureus* derived peptidoglycan.

Peptidoglycan is a component of the cell wall of gram-positive bacteria. The delayed effect of *S. aureus* derived peptidoglycan, maybe relevant to patterns of gene expression in cases of subclinical mastitis and TNF-α cytokine production (Riollet, Rainard, & Poutrel, 2000), in milk.

To evaluate gene expression of bovine neutrophils stimulated with peptidoglycan (PGN) Polymorphonuclear cells were incubated for 15 minutes with PGN and RNA was extracted to

determine transcription of *TLR 2*, *TLR 4*, *Nramp1*, *TNF-α*, and *IL-10*. Results showed that *S. aureus* derived peptidoglycan (1µg) modulated expression of *TLR 2* (N=1) at the 15 minute time interval (Figure 12). Transcription of *TLR 4*, *Nramp1*, *TNF-α* and *IL-10* was not observed in all of the animals tested (N=4) at this timepoint (Figure 11) compared to PBS.

Cow variation was not detected at the 15 minute time interval. The expression of *TLR 2* and *4* has been documented for neutrophils (Fan, et al., 2003; Kurt-Jones et al., 2002; Sabroe et al., 2003; Tsuda et al., 2004), in murine and humans for stimulation with PGN at earlier time intervals. This study appears to be the first to investigate the stimulation of *TLR 2* in bovine neutrophils stimulated with PGN at time intervals under one hour. Although there was no change detected for *TLR 2* at 15 minutes when compared to PBS. We noticed a constant expression of *TLR 4* in all animals tested, particularly with control samples; which could be due to stress endured by the animals from restraint during blood collection. However, the lack of change in gene modulation does eliminate possible contamination during culturing of bovine neutrophils.

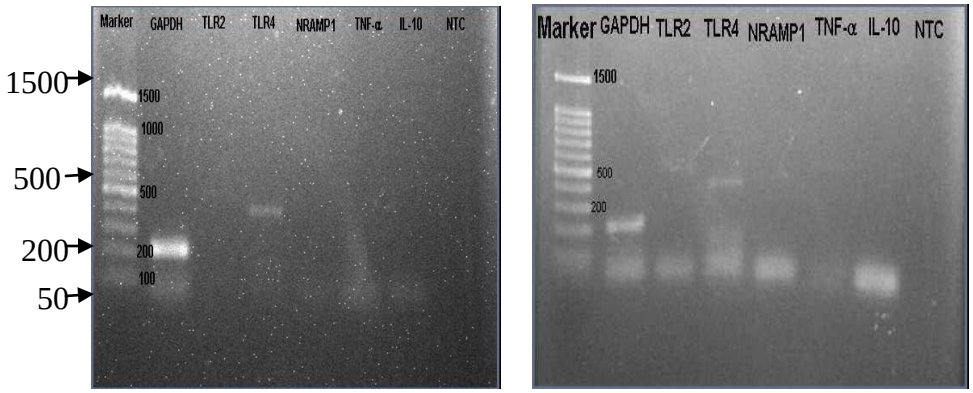
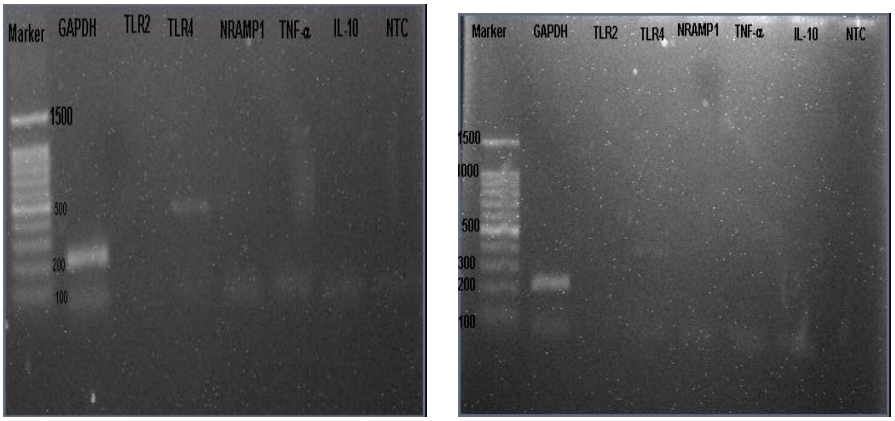


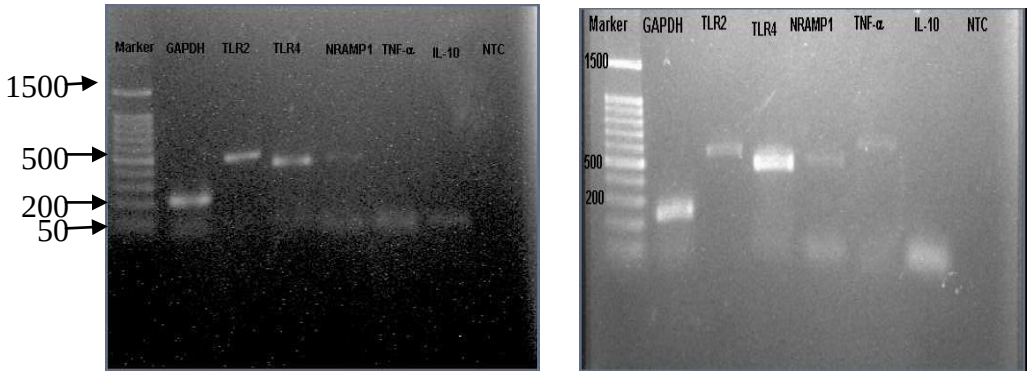
Figure 12. Gene expression in bovine neutrophils stimulated with 1µg of *S.aureus* derived peptidoglycan and incubated for 15 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.



(c) (d)

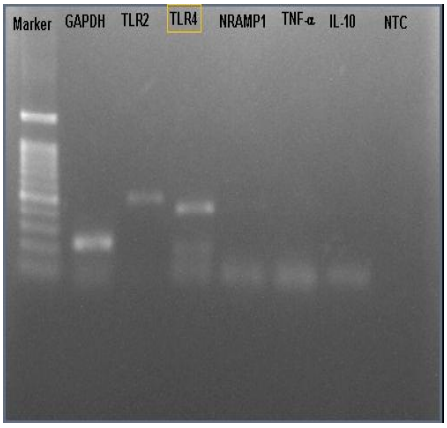
Figure 12. (cont).

Gene expression was seen for *TLR 2* (animal 3000) and *TLR 4* (animals 1788 and 2965); while *NRAMP1* (animal 3000) lacked gene expression at the 30-minute time interval in bovine neutrophils (Figure 13) when compared to PBS. Here animal variation is observed, due to the effect of genetics. This may be a contributing (PGN) to the slow acting effect of *S.aureus* in bovine neutrophils (Teixeira, et al., 2002). *Nramp1* expression in animals 1788 and 2965 remained unchanged compared to PBS for the 30 minute timepoint.

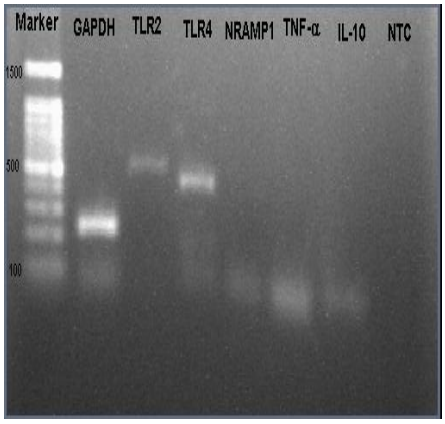


(a) (b)

Figure 13. Gene expression in bovine neutrophils treated with 1µg of *S.aureus* derived peptidoglycan at 30-minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.



(c)



(d)

Figure 13. (cont).

Nramp1 is less abundant in neutrophils, as opposed to macrophages (G. Zhang, et al., 2000). In the current study, we have demonstrated that *Nramp1* expression was not modified by PGN in 3 out of 4 animals; however, gene expression in neutrophils can be seen as early as 30 minutes when compared to PBS at the same time. Peptidoglycan induction of *TLR 2* remains consistent with published literature (Hadley, et al., 2005b); however, here we see induction of *TLR 4*, usually associated with LPS (gram-negative bacteria) that presents more questioning regarding, *TLR4'*s specificity to LPS.

To evaluate bovine neutrophils stimulated with PGN (1µg) after 60 minutes of incubation at 37°C, 5%CO_2 and 85% humidity. Cells were processed for RNA extraction to determine transcription of *TLR 2, TLR 4, Nramp1, TNF-α,* and *IL-10*. Figure 14 results showed gene expression of *TLR 2* (N=4) and *Nramp1* (N=3) at 60 minutes of incubation with PGN compared to PBS. Expression of *TNF*-α was not seen at the 60-minute time interval for all animals tested (N=4). When compared to PBS at the same time interval *TLR 4* (N=4) remained unchanged, as well as *Nramp1* (animal 3001).

Similar results have been reported on the regulation of human monocyte surface expression of *CD 14*, *TLR 2* and *4* in whole blood (Hadley, et al., 2005b). The results of this study are in agreements with Hadley, et al. (2005) indicating that *TLR 4* responses to stimulation by PGN over a time range of 0 to 24 hours (Hadley, et al., 2005b). These results show that stimulation of neutrophils with PGN induces expression of both *TLR 2* and *TLR 4* at 60 minutes in bovine neutrophils.

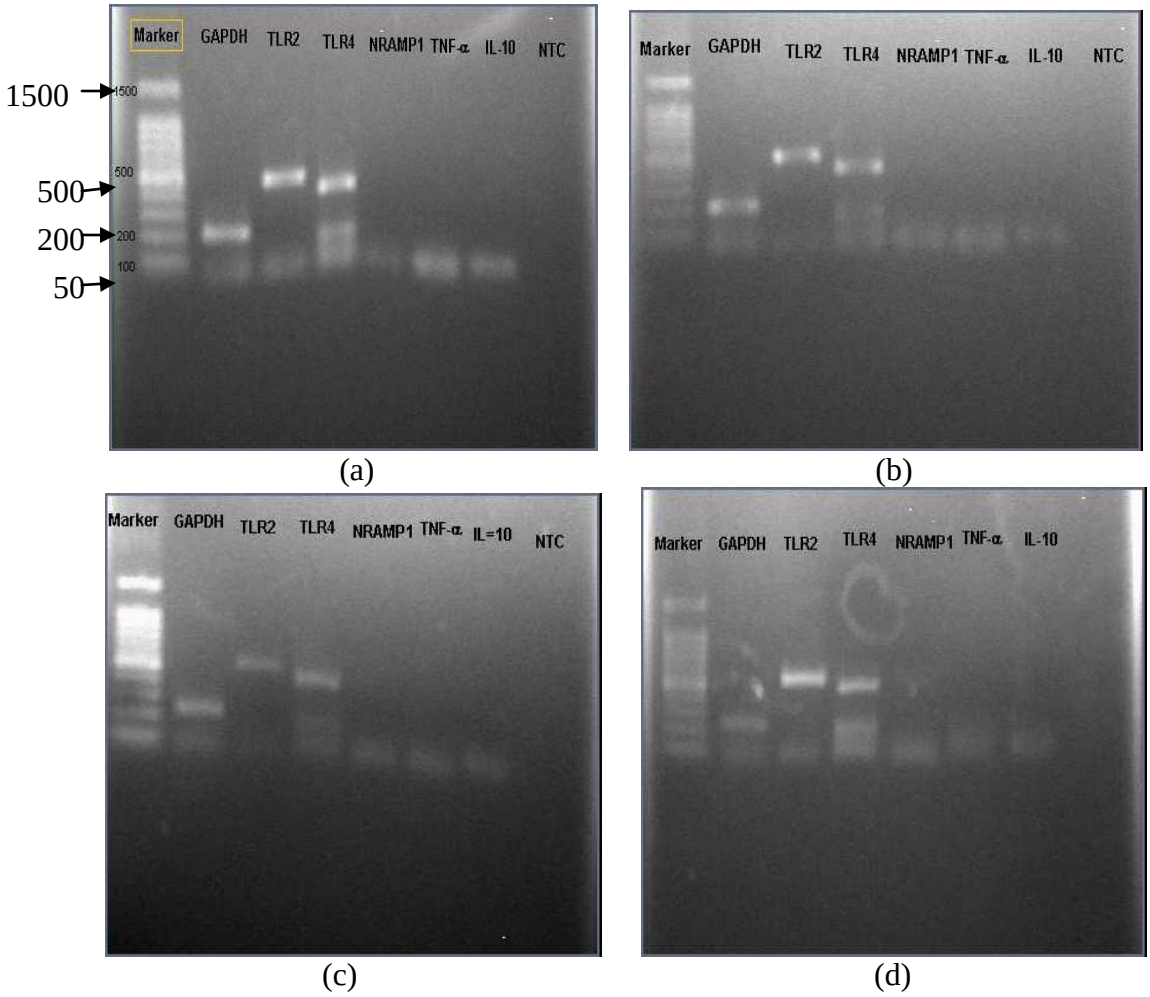


Figure 14. Gene expression in bovine neutrophils treated with 1µg of *S.aureus* derived peptidoglycan for 60 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c)3000, and (d) 3001.

This study demonstrated that PGN exposure results in *TLR 2* and *Nramp1* gene expression when compared to untreated cells at the same time point. Some researchers have debated that PGN, only modulates *TLR 2*, while others claimed activation of both *TLR 2* and *4* when cells are exposed to PGN (Akira & Sato, 2003; Hadley, et al., 2005b; Wang, et al.; Wang et al., 2011; Dirk Werling, et al., 2006). Thus PGN can serve as a positive control for *TLR 2* activation.

Literature search for such an interaction of *TLR 4* and PGN resulted in a study using human monocytes stimulated with LPS alone, PGN alone, and LPS plus PGN. In all three treatments, there was a transient *TLR 4* expressions on human monocytes after 1 to 3 hours. (Hadley, Wang, Foster, Thiemermann, & Hinds, 2005a).

4.8 Expression of TLR2, TLR4, Nramp1, TNF-α and IL-10, in Neutrophils treated with Nystatin.

Gene expression of neutrophils stimulated with Nystatin, an antifungal agent, known as a lipid raft inhibitor induced *TLR4* expression. To evaluate the effect of Nystatin on bovine neutrophils, cells were stimulated at 15, 30 and 60 minute timepoints in the presence of 5% CO_2, 37°C, and 85% humidity. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on all RNA samples.

In this study, two out of four cows used expressed *Nramp1* gene expression (N=2) in bovine neutrophils stimulated with Nystatin (2500U) compared to expression in untreated neutrophils at the 15-minute time interval (Figure 15). Expression of *TLR 2* was seen in only three animals (N=3), two animals expressed Nramp1 and one out of 3 animals expressed *TLR 4* at the 15-minute time interval compared to PBS. The lack of *TNF-α* gene expression in this study could be due to the amount and time effect of Nystatin used to stimulate neutrophils. A

study using TLR2 expressing cells and TLR deficient cells demonstrated secretion of IL-1β, IL-8, and TNF-α after 24 hours and 5μg/ml of treatment with Nystatin (Razonable, et al., 2005). In this study, expression was not seen possibly, due to time of incubation and treatment concentration differences.

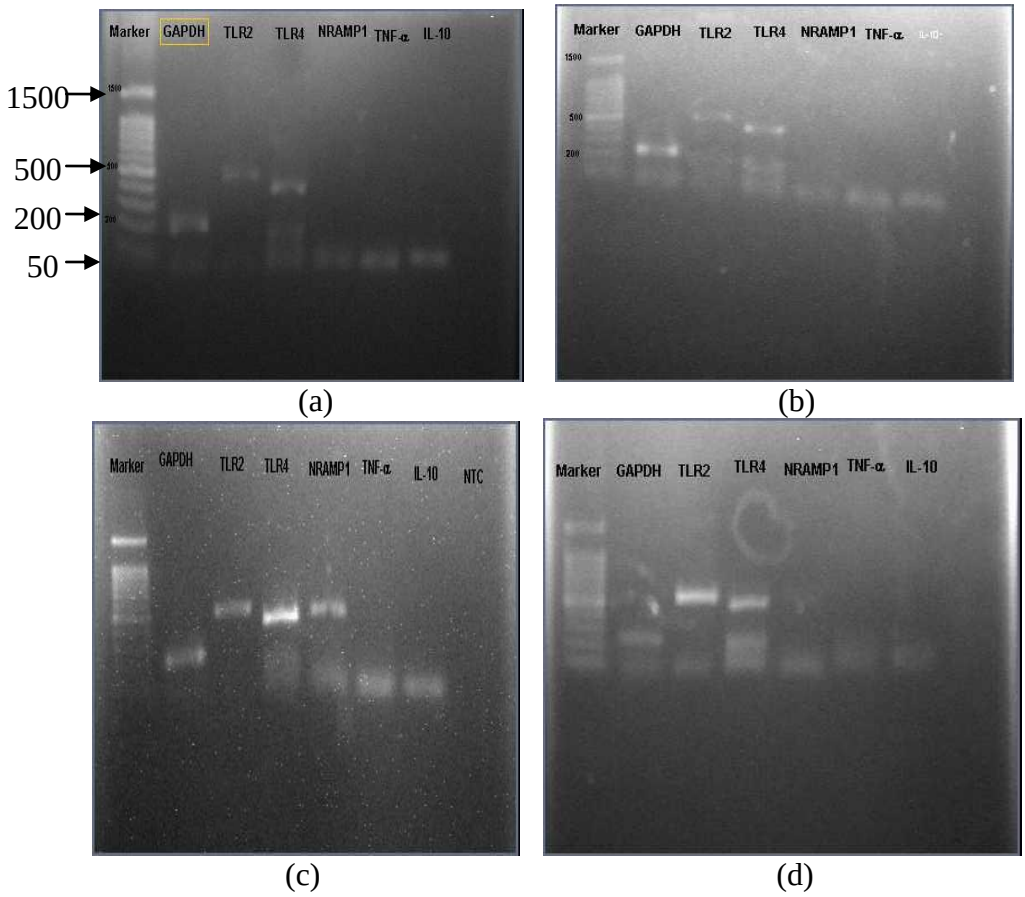


Figure 15. Gene expression in bovine neutrophils stimulated with Nystatin (2500U) at 15 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

Cells were immediately placed in PGN after isolation, RNA extraction was performed, and mRNA gene expression was visualized using a 1.7% agarose gel. Extracted RNA was used in RT-PCR to identify gene expression of *TLR 2* (501bp), *TLR 4* (410bp) and *Nramp1*(433bp).

At the 30 minute (Figure 16) timepoint *TLR 2* (N=1) and *TLR 4* (N=2) were expressed, however, no expression for *Nramp1* (N=3) was observed for neutrophils stimulated with Nystatin compared to PBS.

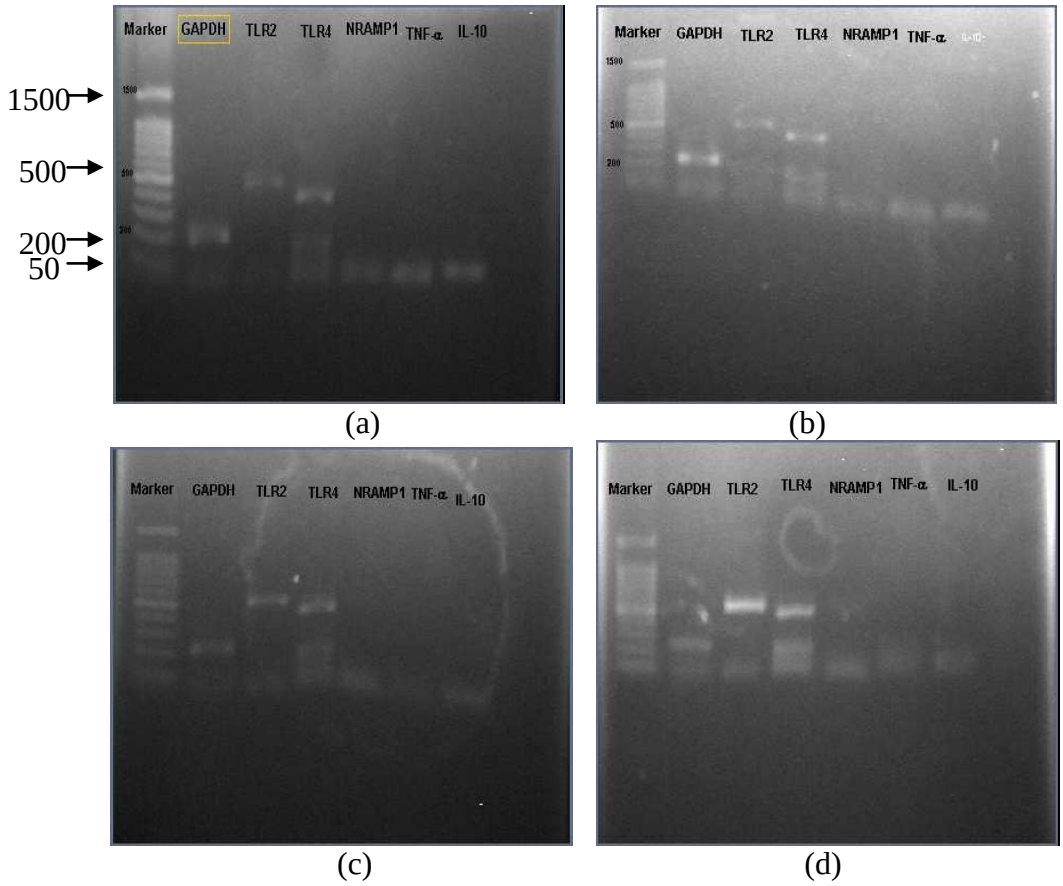


Figure 16. Gene expression in bovine neutrophils treated with 2500U of Nystatin for 30 minutes at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000, and (d) 3001.

Comparing Nystatin and PBS at the 30 minute time interval, there appears to be no change or alteration in *TLR 2* expression, except with animal 3000. Expression of *TLR 4* was seen in animals 1788 and 2965. However, the consistency is seen with Nystatin expressing both *TLR 2* and *4* in all animals, whereas PBS produced a variegated pattern amongst cows in this study.

Results for bovine neutrophils stimulated with NYS at 60 minutes induced expression of *TLR 2* in all animals compared to PBS in Figure 17. Expression of *TLR4*, *TNF-α*, and *IL-10* (N=4) in all animals remained unchanged in PBS of the same time interval. There was a lack of gene expression for Nramp1 when comparing PBS and NYS treated samples at 60 minutes. Expression of *TNF-α* and *IL-10* was not seen at the 60-minute time interval in response to nystatin. These results should be validated by more studies.

Biological membranes consisting of cholesterol, sphingolipids, and other phospholipids, are mainly associated with lipid rafts (Fantini, et al., 2002). Nystatin's weak affinity to cholesterol in animal cells is enough to destroy these membranes (Fantini, et al., 2002). Lipid rafts are composed of cholesterol, and glycosphingolipids; intracellular pathogens use these rafts to evade phagocytosis by leukocytes (Pike, 2006). Evidence has shown that bacterial, viral, and parasitic pathogens utilize endocytosis of lipid rafts as a way to enter the host (Zaas, et al., 2005). The destruction of these membranes and added toxicity produces an inflammatory affect to animal cells (Hac-Wydro, Kapusta, Jagoda, Wydro, & Dynarowicz-Latka, 2007; Ramanathan, Minton, Ross, & Blecha, 2004).

In the study conducted by Razonable and colleagues, it was proposed that Nystatin is a PAMP and activates secretion of TNF-α in TLR2 expressing cells (Razonable, et al., 2005). However, in the current study, Nystatin maybe acting as a PAMP by activation of *TLR 2* and *TLR 4* transcription; due to the lack of *TNF-α* expression, perhaps due to either time or dose dependent limitations. Recognition of nystatin by TLRs maybe attributed to the homologous nature of TLR and its ability to bind with MyD88 alone with CD14, which is utilitzed by many TLRs (Robbins, Cotran, et al., 2010; Tirumurugaan, et al., 2010; Dirk Werling & Jungi, 2003). CD14, an adaptor molecule, has a GPI-linked protein, that resides in lipid rafts and mediates cell

signaling by interacting with TLR2 and TLR4 (Szabo, Dolganiuc, Dai, & Pruett, 2007). Changes in receptor expression have been associated with disruption of lipid rafts impacting signaling through TLR and secretion of cytokines (Pike, 2006)

For neutrophils suspended in PBS at the 60 minute time interval, Nramp1 was expressed (Figure 7), however, expression was not seen in Figure 17 using bovine neutrophils., possibly due to earlier time intervals or lower dose concentration used in this study. Nystatin treatment can disrupt lipid rafts and may impact bacterial-host interaction contributing to evasion of host response mechanisms by pathogens (D. D. Bannerman, et al., 2004; Fantini, et al., 2002).

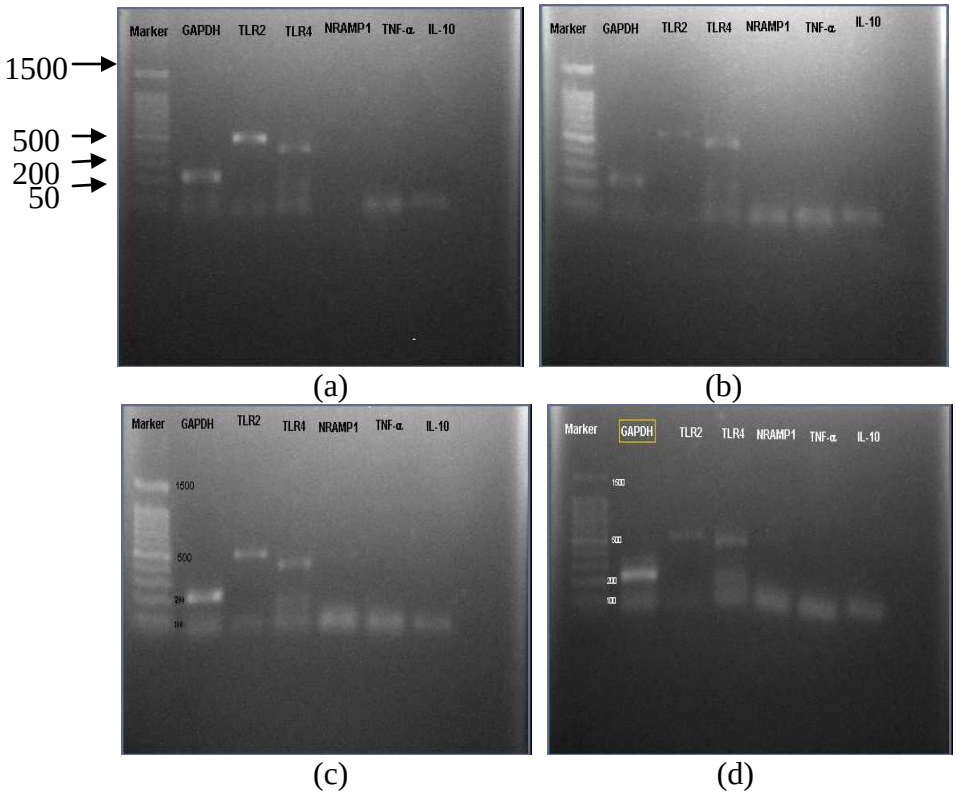
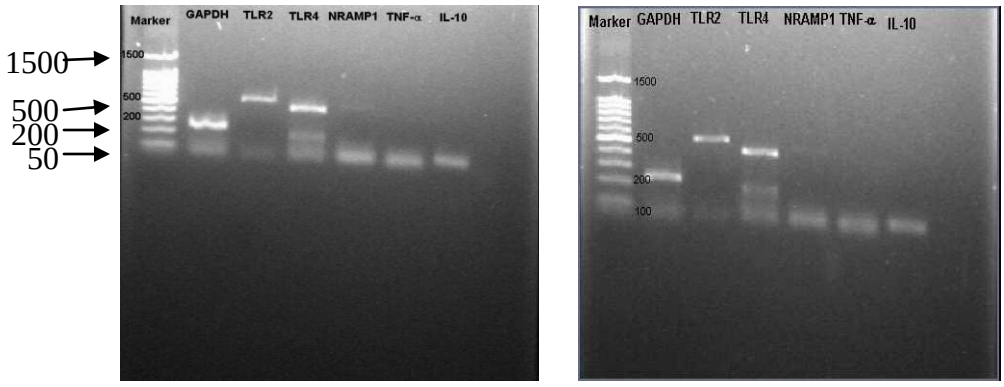


Figure 17. Gene expression in bovine neutrophils treated with Nystatin (2500U) at 60 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a) 1788, (b) 2965, (c) 3000 and (d) 3001.

4.9 Expression of TLR2, TLR4, Nramp1, TNF-α and IL-10, in Neutrophils treated with 62% Ethanol (Quebracho Control).

Bovine neutrophils exposed to 62% ethanol expressed *TLR 2*, and *TLR 4* at 15, 30, and 60-minute timepoints at the RNA level in Figures 18, 19, and 20 compared to PBS. The following genes were not expressed under these conditions: *Nramp1*, *TNF-α,* and *IL-10* at the RNA level. Since ethanol was used in the extraction process of quebracho, we investigated transcription caused by ethanol for comparison against quebracho treated neutrophils and PBS. To determine the effects of stimulation with ethanol, bovine neutrophils were incubated at 15, 30, and 60 minutes at 37°C and RNA extracted from treated cells then amplified. Incubation in PBS induced *TLR 4*, while ethanol induced expression of *TLR 2* in neutrophils. The induction of *TLR 2* in Figure 19, is similar to what is observed for the same two cows effects of Nystatin in neutrophils at 30 minute time intervals. Animals 2965 and 3000 were not used to supply limits.

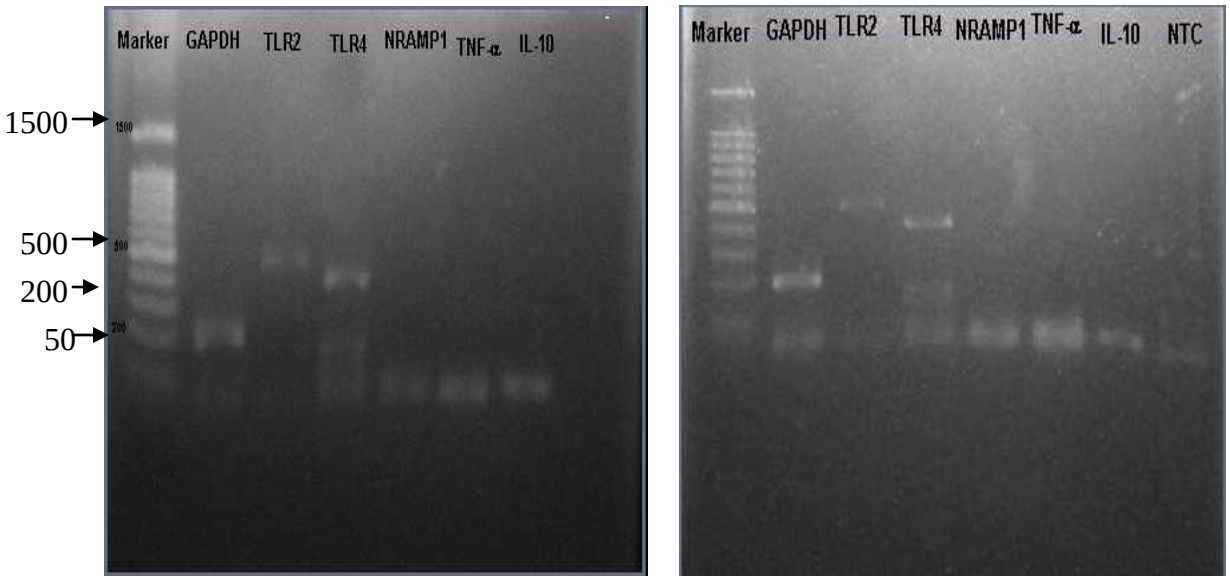


(a) (b)

Figure 18. Gene expression in bovine neutrophils treated with 62% Ethanol at 15 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

Although the mechanism of ethanol's activity is undefined, many researchers have speculated that like Nystatin, ethanol alters lipid rafts and inhibits TLR signaling (Szabo, et al.,

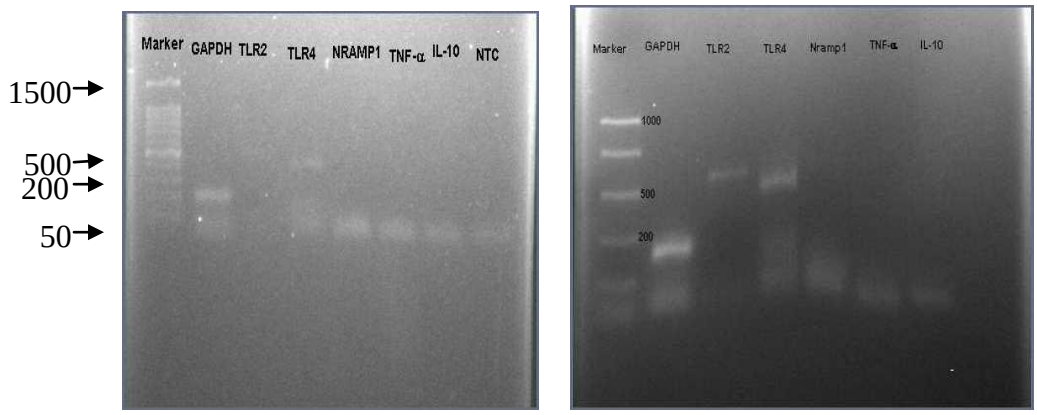
2007). It has been documented that in low doses, ethanol reduced proinflammatory cytokine synthesis of TNF-α against response to pathogenic stimuli (Nelson, Bagby, Bainton, & Summer, 1989). Bacterial pathogens use this same mechanism to alter lipid rafts to enter the host to induce bacterial infections (Zaas, et al., 2005).



(a) (b)

Figure 19. Gene expression in bovine neutrophils treated with 62% Ethanol at 30 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

Expression of *TLR 4* remained unchanged at the 60-minute time interval compared to PBS; however, cow variation is detected in animal 3001, as gene expression can be seen for *TLR 2* in Figure 20. Expression for *Nramp1* was not detected at the same time interval in Figure 20 compared to PBS. To my knowledge, no studies have been conducted investigating gene expression by bovine neutrophils stimulated with ethanol, therefore; more studies are needed to explain the similar effects seen between ethanol and PBS.



(a) (b)

Figure 20. Gene expressio in bovine neutrophils treated with 62% Ethanol at 60 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

4.10 Expression of TLR2, TLR4, Nramp1, TNF-α and IL-10, treated with Quebracho.

Quebracho treatment modulated gene expression in bovine neutrophils. Cow variation was seen at this time interval for stimulation with quebracho extract. No gene expression was observed for *TLR 2* and *TLR4* in one animal. Most published data investigates its use as an alternative to control gastrointestinal parasites in sheep and goats. Many studies in cows were conducted for the effects of condensed tannin extract from quebracho trees to reduce methane emissions from cattle (Beauchemin, McGinn, Martinez, & McAllister, 2007), the effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows (Dschaak et al., 2011), as well as digestion, ruminal fermentation, protozoal populations, and milk production from dairy cows fed quebracho condensed tannins (Benchaar, McAllister, & Chouinard, 2008). Little information is available about the mechanism and cell signaling actions of quebracho *in vitro* in bovine neutrophils.

One study investigated the population of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* ; and measuring pathophysiological effects of inflammatory cells

(mast cells, globular leukocytes, and eosinophils) in goats (V. Paolini, A. Frayssines, F. De La Farge, P. Dorchies, & H. Hoste, 2003). Results for Paolini's study showed that there was a significant effect of Quebracho on mast cells in the small intestine of goats. This is relevant to this study due to the use of ruminants (i.e. cows) and the effect of quebracho on innate immune cells. Treatment with quebracho compared to ethanol and PBS showed no expression of *TLR 2* at the 15-minute time interval (Figure 21). Transcription of TNF-α was seen for animal 1788 at 15 minute timepoint when compared to ethanol and PBS.

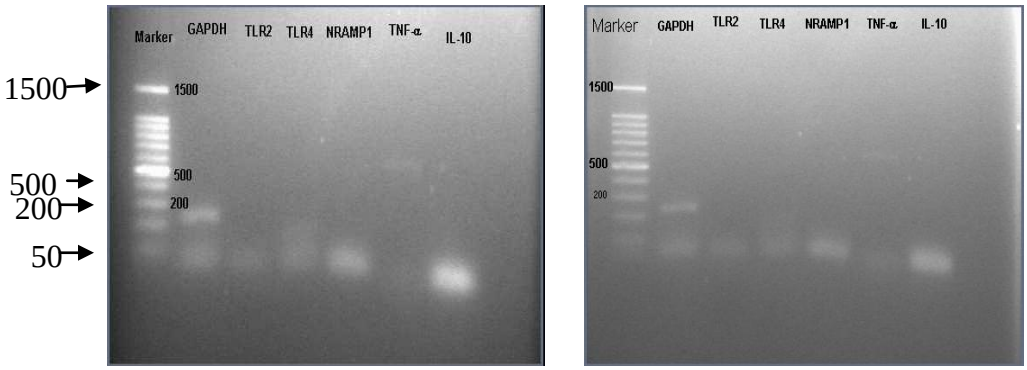


Figure 21. Gene expression in bovine neutrophils treated with Quebracho (1.5x) at 15 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

Bovine neutrophils stimulated with quebracho extract (1.5x) for 30 minutes incubation showed a change in *TLR2* (animal 1788) gene expression compared to PBS in Figure 22. The use of 1.5X concentration of quebracho was randomly chosen, due to lack of information pertaining to *in vitro* studies using quebracho. Quebracho extract is commercially available at 3X concentration, however, due to its small amount. The product was diluted to 1.5X for this study and 1 ml aliquots were added to cells. No gene expression was seen for *TNF-α,* and *IL-10* (N=2) in animals tested at the 30-minute time interval when compared to both ethanol and PBS.

However, animal 1788 did show a lack of gene expression for Nramp1 when compared to PBS. Also, animal 1788, did not express *TLR 4* when compared to PBS, but did express *TLR 4* when compared to ethanol. Animal 3001 did show expression of *TLR 4* when compared to both ethanol and PBS. The evaluation of quebracho at 30 minutes allowed some transcription of *TLR 2* (Figure 22) to be induced. The lack of gene expression for *TNF-α* proves some insight into quebracho's ability to suppress proinflammatory production. However, more studies would need to be conducted to gain a better understanding of quebracho's role in innate immunity regarding neutrophils due to the use of two animals used in this study.

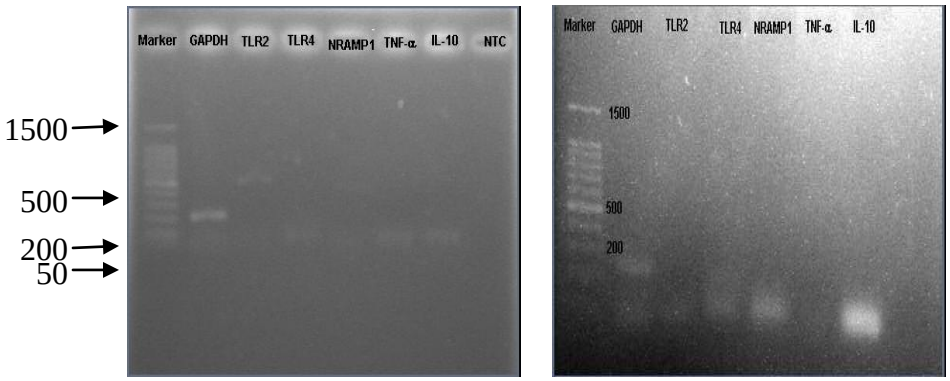
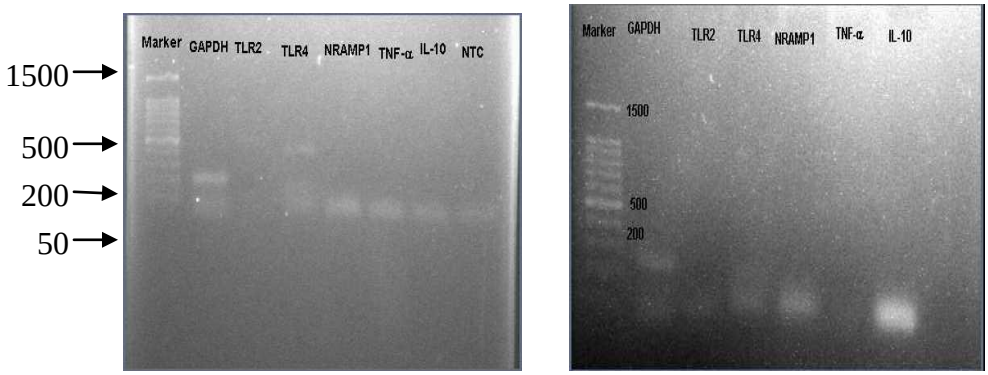


Figure 22. Gene expression in bovine neutrophils treated with Quebracho (1.5x) at 30 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

Stimulation of bovine neutrophils with quebracho extract for one hour resulted in no gene expression of *TLR 4* (animal 3001) and *Nramp1*. Only change detected for *TNF-α,* was at the one-hour time interval, but no change in *IL-10* for animals tested (N=2) in Figure 23 compared to PBS and earlier time points. It is possible that quebracho's effect on bovine neutrophils at the 60-minute time interval could be caused by a longer incubation period. Comparing quebracho and ethanol at the 60-minute time interval remain unchanged for gene

expression, except with animal 3001 for *TLR 2*. It appears the one hour time interval has an effect on gene expression, similar to ethanol for *TLR 4* and *Nramp1* indicating an effect of ethanol. Studies on alcohol consumption have shown Szabo's study using humans and animal cells treated with ethanol, suppressed innate immunity and inflammation (Szabo, et al., 2007). It is unclear whether quebracho or ethanol is responsible for gene suppression at the 60-minute time interval in control or treated samples.



(a) (b)

Figure 23. Gene expression in bovine neutrophils treated with Quebracho (1.5x) at 60 minute time interval at 37°C in 5% CO_2 and 85% humidity; cows used (a)1788, (b) 2965, (c) 3000, and (d) 3001.

Table 6 provides an overall summary of gene expression in bovine neutrophils stimulated with various immunomodulators. Transcription of *TLR 2, TLR 4* and *TNF-α* was seen in unstimulated neutrophils at zero hour timepoint. No transcription was seen for *Nramp1* or *IL-10* in the animals tested at the same time interval. Unstimulated bovine neutrophils showed transcribed genes for *TLR2, TLR4* and *Nramp1* for both 15 and 30-minute time intervals. However, one-hour incubation, unstimulated neutrophils transcribed for *TLR 4* and *Nramp1*. GAPDH was expressed constitutively in all samples, however, transcription of *IL-10* was not

seen in all treatment groups tested, this may be due to primer sequence specificity; and therefore, other primers should be tested.

Table 6

Summary of Gene Expression

0 minutes								**15 minutes**						
	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10		GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10
Treatment													
PBS	+	+	+	-	+	-		+	+	+	+	-	-
LPS	NC	NC	NC	NC	NC	NC		+	+	+	+	+	-
PGN	NC	NC	NC	NC	NC	NC		+	-	+	-	-	-
Nystatin	NC	NC	NC	NC	NC	NC		+	+	+	+	+	-
62% Ethanol	NC	NC	NC	NC	NC	NC		+	+	+	+	-	-
Quebracho	NC	NC	NC	NC	NC	NC		+	-	-	-	-	-
30 minutes								**1 hour**					
	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10		GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10
Treatment													
PBS	+	+	+	+	-	-		+	-	+	+	-	-
LPS	+	+	+	+	+	-		+	+	+	+	+	-
PGN	+	+	+	+	+	-		+	+	+	+	-	-
Nystatin	+	+	+	-	-	-		+	+	+	-	-	-
62% Ethanol	+	+	+	-	-	-		+	+	+	-	-	-
Quebracho	+	-	-	-	-	-		+	-	+	-	-	-

4.11 Effect of treatment on TNF-α secretion from bovine neutrophils

The detection of proinflammatory cytokine TNF-α is one key indication of inflammation that aids in host defense against pathogens. To investigate the effects of LPS, PGN, Nystatin, ethanol, and quebracho treatment of secretion of the cytokine TNF-α from neutrophils, cell

supernatant extracted from bovine neutrophils stimulated with phosphate buffered saline, lipopolysaccaride, peptidoglycan, Nystatin , 62% ethanol, and quebracho were pooled and used in an ELISA. This assay was used to measure translation of TNF-α at the 30-minute time interval for all treatment groups and PBS. A standard curve was generated ranging from 0 to 1000 ng/ml to measure concentration of the cytokine in cell supernatant samples in relation to standards. Effects of PGN and LPS on cytokine secretion are known and can be used as positive controls for a gram negative and gram positive effect (Mount, et al., 2009). All treatment samples were normalized by subtracting PBS from LPS, PGN, Nystatin, and ethanol was subtracted from quebracho to represent negative TNF-α concentrations in Figure 24. Pooled supernatants from four cows from all treatment groups did not show a treatment effect, but demonstrated a significant decrease in TNF-α secretion compared to PBS and/or ethanol ($p < 0.05$), after 30 minutes of incubation in Figure 24.

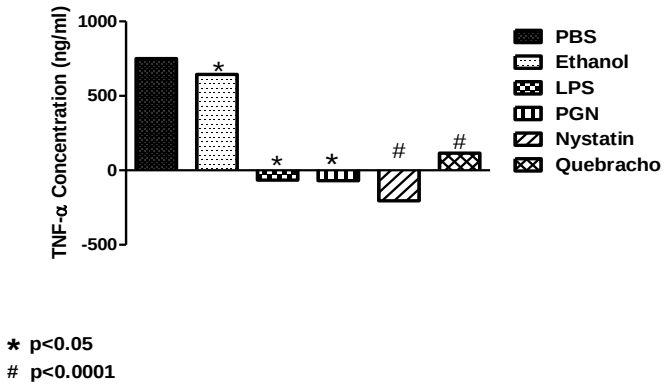


Figure 24. ELISA results of TNF-α cytokine secretion by bovine neutrophils treated with various immunomodulators.

The TNF-α cytokine has been detected in milk samples after intramammary infections with both *E. coli* and *S. aureus* (D. D. Bannerman, et al., 2004), bovine neutrophils stimulated with LPS (Gilbert, Poutrel, & Sutra, 1994), and in *TLR 2*- expressing human monocyte-derived

THP1 cells using Nystatin (Razonable, et al., 2005). In the present study, there was more TNF-α secretion in the PBS and ethanol treated samples compared to samples treated with LPS, PGN, Nystatin, or quebracho. We could rule out contamination of control samples by treatment groups, due to samples being separately incubated and the same stock of PBS was used to dilute LPS, PGN and Nystatin.

The decrease in measured TNF-α did not produce a treatment effect for bovine cell supernatants stimulated with compounds over baseline. Although, this same trend is not seen in a previous study investigating bovine neutrophils stimulated with higher concentrations of LPS ranging from 1μg to 100μg and longer incubation times (Gilbert, et al., 1994), we can infer that increasing these parameters could provide the same desired effects in bovine neutrophils.

Bovine neutrophils stimulated with PGN demonstrated in the current study had similar effects of TNF-α level to LPS, but lower cytokine secretion than PBS in Figure 23. Other studies that investigated *S. aureus* intramammary infection showed that TNF-α was not up regulated in milk and increased in production after 24 hrs (Riollet, et al., 2000). This could be an indication that increased TNF-α response may require longer incubation times of peptidoglycan to have an effect on bovine neutrophils.

In this study, concentration of TNF-α in cell supernatant after stimulation of bovine neutrophils with Nystatin did not produce similar effects to Razonable's research, which showed TNF-α production over unstimulated cells (Razonable, et al., 2005); although, there were differences between this study and Razonable's, which used monocytes, lower concentration of nystatin and an incubation time of 24 hours. However, comparing Nystatin with Quebracho did produce highly negative significance ($p<0.0001$) for TNF-α secretion. It is possible that differences in these factors could have contributed to the effect seen here in this study.

From this study it is clear that TNF-α cytokine can be produced with stimulation of ethanol, but the cytokine was seen expressed over ethanol when subtracted from background of quebracho. It has been documented that acute ethanol exposure in human neutrophils can alter TNF-α production with ethanol in a concentration dependent manner, with amounts ranging from 0.1% above and 0.3 - 1.5% below basal levels after 24hrs of culturing human neutrophils (Taïeb et al., 2002). In addition, another study identified the effects of acute ethanol on the inhibition of PMN migration to inflammation sites (P. Zhang et al., 2002). Thus ethanol in various concentrations using neutrophils and exposure times can alter TNF-α secretion. Further studies using more animals may be needed to better understand the effects produced by quebracho in the production of TNF-α over ethanol. A study using RAW 264 cells (mouse leukemic monocytes) were treated with ginger extract, soluble and insoluble ethanol for 18 hrs and supernatant was assayed for TNF-α production. Cells treated with ginger extract secreted more TNF-α over both types of ethanol, and concluded that active molecules from ginger may not be inhibiting production of TNF-α (Ueda, Ippoushi, & Takeuchi, 2010). None of the treatments resulted in secretion of TNF-α in this study above that observed in controls. More studies will need to be conducted, however, the concern here is that LPS, NYS and PGN also inhibited TNF-α production and quebracho caused transcription of TNF-α.

CHAPTER 5

Conclusion

The findings of this study will provide a better understanding of the neutrophil's role in innate immunity. Transcription of *TLR2*, *TLR4*, *Nramp1*, and *TNF-α* mRNA was observed in bovine neutrophils treated with PBS, LPS, PGN, Nystatin, quebracho, or ethanol at various time intervals. Gene expression was seen as early as 15 minutes at the transcription level in all treatment groups and with PBS. Ligand-induced stimulation of *TLR 2, 4* and *Nramp1* was observed. This study, allows some insight to the use of bacterial agents, such as LPS, PGN, and Nystatin for modulation of gene expression in neutrophils.

Further studies are recommended to evaluate the impact of modulators on TNF-α secretion and the effect on the innate immune system. Variation was observed between cows in gene expression. The research supports the importance of genetic resistance and susceptibility, in relation to breed selection. Selective breeding could provide optimal results for the resistance of common disease affecting the dairy industry.

Future studies are recommended to broaden the understanding of the neutrophil's role in innate immunity and to investigate whether other livestock, such as sheep and goats are capable of producing similar results as the bovine and the impact of genetic variability within the same breeds. Reagents specific for bovine and LPS free could correct any deficiencies that were seen in this study. Such studies are needed to provide a better understanding of the neutrophils role in innate immunity for breed selection. The findings will help to understanding the mechanism of bacterial infections associated with mastitis, primarily in the dairy industry to aid farmers in selective breeding management practices to aid in developing more disease resistant livestock.

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Appendix A

Reagents

1. Acid Citrate dextrose

   For 500ml:
   22g trisodium citrate (anhydrous)
   8g citric acid
   25g dextrose

   Bring up to 500 mls with sterile water, autoclave and store at4°C. This should be used in the following proportion: 1ml ACD: 9ml blood.

2. 1.7% Agarose Gels

   0.86g agarose
   50ml 0.5X TAE buffer

   Agarose was dissolved in 50mls of 0.5X TAE buffer in microwave until no particles were present.

3. DEPC-treated Water

   1ml of DEPC was added to 1 liter of distilled water, mixed vigorously and let stand overnight than autoclave.

4. 2.7% Saline

   27g NaCl

   Bring volume up to 1 liter with DEPC-treated water, autoclave and store at 4°C.

5. 0.0132 M Phosphate Buffered Saline (PBS), pH7.4

   For PMN Isolation Procedure

   - Potassium Monobasic, KH_2PO_4
     Formula Weight = 136.09
     136.09 x 0.0132 = 1.796 g/L

   - Potassium Phosphate Dibasic, K_2HPO_4
     Formula Weight = 174.183
     174.183 x 0.0132 = 2.299 g/L

Make 2 liters of K_2HPO_4 and 1 liter of KH_2PO_4 in 0.85% saline to make 1500 ml of buffer at a time. Use the KH_2PO_4 to bring pH down to 7.4.

6. 0.85% Saline

   8.5g NaCl into 800 ml of DEPC treated water, mix on stirplate until completely dissolved, than add remaining DEPC treated water to 1000 ml.

7. Wash Buffer 0.05% Tween 20 in PBS, pH 7.4

   2223g Potassium Phosphate Dibasic, K_2HPO_4
   5.62g Potassium Phosphate Monobasic, KH_2PO_4
   11.68g Sodium Chloride

   Dissolve all contents into 2 liters of DEPC treated water and adjust pH. Add 0.5 ml of Tween 20. Autoclave and store at 4˚C.

8. Blocking Buffer 5% Tween 20 in PBS with 0.05% NaN_3

   5g Tween 20
   100 ml of PBS
   50 ul Sodium azide

   Dissolve all contents thoroughly and store at 4˚C.

9. Reagent Diluent 5% Tween20 in PBS, pH 7.2

   Mix 50 mls of Tween 20 to 1 liter of PBS, mix thoroughly and store at 4˚C.

Appendix B

Protocols

Wright's Staining Procedure

Differential cell counts were assessed by applying a thin smear of whole blood collected from the jugular vein of the cow. Neutrophils isolated as per the procedure in materials and methods section were resuspended in PBS. Differential cell counts before and after neutrophil isolation were conducted as followed:

1. Place a drop of blood or isolated neutrophil suspension toward the frosted edge of the glass slide.
2. Take a second glass slide and slide it up against the blood or neutrophil suspension droplet and push the second slide at a 30º angle and let dry at room temperature.
3. Dip air dried slide in wright's stain for approximately 10 seconds.
4. Decolorize stained slide with deionized water for 10 seconds to wash off excess stain.
5. Rinse slide in deionized water, if necessary for additional 10 seconds and air dry slide prior to reading.
6. Smears were observed under oil immersion microscope for cell counts.

Differential cells were counted up to 100 for numerical representation of various cells present in whole blood sample. The total number of isolated neutrophils present out of 100 cells was counted based on cellular morphology and staining characteristics and the value was used to obtain purity and concentration of total isolated neutrophil population.

Cell viability

To aid in determining the cell viability and concentration of cells in a suspension, the use of a vital dye, trypan blue solution (Sigma-Aldrich, St. Louis, MO cat.#T8154), to determine

viable vs. nonviable cells (the dye is excluded from viable cells). Note: Always wear protective clothing, gloves, and eyewear.

1. Obtain a clean hemacytometer with cover slip for the procedure.
2. Obtain a uniform suspension of isolated neutrophils. Pipette the cell suspension up and down gently in the 50 ml conical tube 5-7 times.
3. Prepare a 1:2 dilution of the cell suspension in trypan blue. Approximately 20µl of isolated PMN is needed to load 10µl onto each chamber of the hemacytometer.
4. Take ~15 µl of isolated PMN from the 50 ml conical tube containing isolated PMN and place the cell suspension into the 1.5 ml RNase-free microcentrifuge tube.
5. Add 15µl of trypan blue solution to the 1.5 ml microcentrifuge tube combining the cell suspension and gently swirl the contents.
6. Pipette up and down several times to ensure a uniform cell suspension and place on ice.
7. Load both sides of the counting chambers with 10 µl of cell suspension and place a coverslip over the counting chambers.
8. Determine the number of cells (total and viable) in the cell suspension by viewing cells under a microscope at 100x magnification.
9. Count the cells in all four x $1mm^2$ areas of the chamber.
10. Calculate the cell concentration by using the following formula for total (or viable) cells. Read both sides of the chamber and average both sides.

    Total (or viable) cells in $4mm^2$ / 4 = cells per mm^2

    Cells per mm^2 / Dilution Factor (DF) = cells/10^{-4} ml x 10^4 = Cells/ml

    Cells/ml x total volume of cell suspension = Total (or viable) cells recovered

    Total (or viable) cells recovered x cell purity of PMN Wright's stain = number of

total (or viable) PMN

11. Clean the hemacytometer as soon as possible after use. Clean with dilute bleach solution followed by 70% isopropanol. Air dry hemacytometer and dispose ot trypan blue contaminated article in biohazard waste.

Qiagen© Multiplex PCR

For a 50µl reaction:

Component	Volume per reaction	Final Concentration
2x Qiagen Multiplex PCR Master Mix	25 µl	3mM $MgCl_2$
10x Primer Mix, 2µM each primer Mix	5µl	0.2µM
RNase-free water	Variable	N/A
DNA Template	Variable	≤ 1µg DNA/50µl
Total Volume	50 µl	

CR Thermal cycler conditions

Steps	Time or Cycles	Temperature
Initiation	15 minutes	95º C
Denaturation	30 seconds	94ºC
Annealing	40 seconds	57-63ºC
Extension	90 seconds	72ºC
Number of Cycles	33 cycles	N/A
Final Extension	10 minutes	72ºC

Procedure

1. Thaw template DNA, primer solutions, dNTP Mix, 2x Qiagen Multiplex PCR Master Mix, and RNase-free water. Mix thoroughly before use.
2. Prepare a reaction mix according to the 50µl reaction table.
3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.
4. Add template DNA to individual PCR tubes containing reaction mix.

5. Program thermal cycler according to program outlined above for conditions.
6. Place the PCR tubes in the thermal cycler and start the cycling program.

Qiagen© OneStep RT-PCR

For a 50µl reaction:

Component	Volume per reaction	Final Concentration
5x Qiagen OneStep RT-PCR Buffer	10 µl	1x
Forward Primer	3 µl	0.6µM
Reverse Primer	3 µl	0.6µM
DNTP Mix (containing 10mM of each dNTP)	2µl	400µM of each dNTP
Qiagen OneStep RT-PCR Enzyme Mix	2.0 µl	N/A
RNase-free water	Variable	N/A
RNA Template	Variable	1pg – 2 µg/reaction
Total Volume	50 µl	N/A

RT-PCR Thermal cycler conditions

Steps	Time or Cycles	Temperature
Reverse transcription	30 minutes	50º C
Initiation	15 minutes	95ºC
Denaturation	1 minute	94˚C
Annealing	1 minute	50-68ºC
Extension	1 minute	72˚C
Number of cycles	33	N/A
Final Extension	10 minutes	72º C

Procedure

1. Thaw template RNA, primer solutions, dNTP Mix, 5x Qiagen OneStep RT-PCR Buffer, and RNase-free water, and place them on ice.
2. Prepare a master mix according to the 50µl reaction table.
3. Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.
4. Add template RNA to individual PCR tubes.
5. Program thermal cycler according to program outlined above for conditions.

6. Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached 50˚C. Then place the PCR tubes in the thermal cycler.

Procedure for TNF-α ELISA

1. A flat bottom 96 well plate (Nalge Nunc International) coated overnight with 100 µl per well of goat anti-bovine TNF-α capture antibody at a working dilution of 0.8 µg/ml.
2. Plates were washed with wash buffer containing 0.05% Tween 20 in PBS (400µl) three times.
3. Plates were blocked with 300µl of blocking buffer containing 5% Tween 20 in PBS with 0.05% NaN_3 and incubated at room temperature for ~2hours.
4. Afterwards, plate was aspirated and washed with wash buffer three times.
5. Samples and standard was added at 50µl per well in triplicate and incubated for 2 hours at room temperature.
6. Generation of a standard curve was created to identify optical density of unknown samples standards ranged from 0 pg/ml to 1pg/ml.
7. Plates were washed again three times with wash buffer.
8. Detection antibody (100µl) containing 72µg/ml biotinylated goat anti-bovine TNF-α was added to each well and incubated for 2 hours at room temperature.
9. Working dilution of 1:200 of Streptavidin-HRP (100µl) was added to each well and incubated for 20 minutes at room temperature.
10. Plates were aspirated and washed as before with wash buffer three times.
11. A 100µl of substrate solution containing 1:1 mixture of Color Reagent A and B (R&D systems) was added to each well and incubated for 20 minutes at room temperature, out of direct light.

12. Stopping solution (2N $H_2 SO_4$) of 50µl was added to each well.
13. Optical density was read at an absorbance of 450nm using a MR600 micro plate reader (Dynatech Laboratories Inc., Chantilly, VA).

Appendix C
Summary of Gene Expression by Cow

PBS 0 hour

Animal Numbers	1788	2965	3000	3001
GAPDH	+	+	+	+
TLR2	+	-	-	-
TLR4	-	+	+	-
Nramp1	+	-	-	-
TNF-α	-	-	-	-
IL-10	-	-	-	-

15 minutes

Animal Numbers	1788						2965						3000						3001					
	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10
Treatment																								
PBS	+	-	+	-	-	-	+	-	+	+	-	-	+	+	+	-	-	-	+	-	+	-	-	-
LPS	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	-	-	+	+	+	+	-	-
PGN	+	-	+	-	-	-	+	-	+	-	-	-	+	-	+	-	-	-	+	-	+	-	-	-
Nystatin	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-
62% Ethanol	+	+	+	+	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	+	+	+	-	-
Quebracho	+	-	-	-	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	+	+	-	-	-

NC= Not Conducted

30 minutes

Animal Numbers	1788						2965						3000						3001					
	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10
Treatment																								
PBS	+	+	-	+	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	-	-	-
LPS	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-
PGN	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-
Nystatin	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-
62% Ethanol	+	+	+	-	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	+	+	-	-	-
Quebracho	+	+	-	-	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	+	+	-	-	-

60 minutes

Animal Numbers	1788						2965						3000						3001					
	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10	GAPDH	TLR2	TLR4	Nramp1	TNF-α	IL-10
Treatment																								
PBS	+	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	-	-
LPS	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	-	-
PGN	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-
Nystatin	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-
62% Ethanol	+	+	+	-	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	+	+	-	-	-
Quebracho	+	+	+	-	-	-	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	+	-	-	-	-	-

NC=Not Conducted