2015

Ex Vivo Effects Of Water Extracts Of Sericea Lespedeza On Cow, Sheep And Goat Blood

Emmanuel K. Asiamah
North Carolina Agricultural and Technical State University

Ex Vivo Effects of Water Extracts of Sericea Lespedeza on Cow, Sheep and Goat Blood

Emmanuel K Asiamah

North Carolina A&T State University

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Animal Sciences

Major: Integrated Animal Health

Major Professor: Dr. Mulumebet Worku

Greensboro, North Carolina

2015

The Graduate School
North Carolina Agricultural and Technical State University

This is to certify that the Master's Thesis of

Emmanuel Kwaku Asiamah

has met the thesis requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2015

Approved by:

Dr Mulumebet Worku Major Professor	Dr Osei Yeboah Committee Member
Dr Chastity English Committee Member	Dr Ralph Noble Department Chair

Dr. Sanjiv Sarin
Dean, The Graduate School

Biographical Sketch

Emmanuel Asiamah holds a Bachelor's degree in Agriculture (Crop and Soil Science) from the Kwame Nkrumah University of Science and Technology and currently pursuing MSc. (Integrated Animal Health systems) at North Carolina A&T State University. He was a graduate research assistant in the Mushroom Biology and Fungal Biotechnology Lab in the Department of Natural Resources and Environmental Design from August 2012 to December 2013. Emmanuel has attended and presented at both national and international conferences. These include a presentation of an outstanding poster on Temperature Requirements for Mycelia Growth in Ganoderma spp, of African origin at the 34th Annual Mid-Atlantic States Mycology Conference at Beltsville, Maryland and two oral presentations on *Ex vivo* of Effects Serecia Lespedeza and Transcription of TOLL-like receptor 2 (TLR2) in Bovine Blood in Response to Microbial and Plant Derived Molecular Patterns at the American Society of Animal Science Southern Section conference in Atlanta, GA. In addition he was also selected to be a member of the North Carolina A & T State University Chapter of Gamma Sigma Delta, The Honor Society of Agriculture. Mr. Asiamah also served as student representative of the School of Agriculture and Environmental Science on the North Carolina A&T Chapter of the Graduate Student Advisory Council. He holds a Six Sigma Green Belt. Emmanuel's current research compares the association of Wnt 5a, Wnt 3a, TLR 4, TLR2, and TNF alpha activation in cattle sheep and goats. Hence his transition from the Department of Agriculture and Natural Resources to the Department of Animal Sciences in the final year of his Master's program. He was also selected to be a member of the North Carolina A & T State University Chapter of Gamma Sigma Delta, He has become competent in isolation of neutrophils, proteins and RNA from blood samples.

The United States Department of Agriculture-Evans Allen Program in the School of Agriculture and Environmental Sciences at North Carolina A & T State University, provided funding for his research study.

Dedication

This thesis work is dedicated to Almighty God for giving me life, strength and guidance to make all this possible. This work is also dedicated to my entire family, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve. Also, to Dr. Osei Agyemang Yeboah who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having him in my life.

Acknowledgments

I would never have been able to finish my thesis without God, the guidance of my committee members, help from friends, and support from my family.

First and foremost, I give thanks to the Almighty God for His never ending grace and mercies that have enabled me to get this far.

I would like to express my deepest gratitude to my advisor, Dr. Millie Worku, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. I would like to thank Dr. Osei Yeboah, who has let me experience higher education beyond the classrooms, patiently corrected my behavior, corrected my writing and financially supported my research. He has been a mentor, a father and a friend. Special thanks goes to Dr. Chastity English who was willing to participate in my final defense committee at the last moment and also corrected my writing.

I would also like to thank Mr. Hamid Ismail for helping with the statistical analysis and making sure I had all the supplies I needed for my research. Next, I would like to thank Dr.Andrea Byers for her guidance during the SDS PAGE analysis. I also thank Ms. Veronica Carmichael for her support throughout my stay in the Department of Animal Sciences and always being willing to help.

I would like to thank Kingsley Ekwemalor and Sarah Adjei-Fremah who as good friends, were always willing to help and give their best suggestions. It would have been a lonely lab without them. Many thanks to all the students in Dr Worku's lab; Noel Facey, Caleb Huffman, Daija Dowe, Evelyn White and Chris Tart for helping with sampling, and also the laboratory analysis that followed.

Special thanks also go to Mr. Gary Summers and Corey Burgess of the North Carolina A & T State University school farm for helping me collect blood samples from the Animals. My research would not have been possible without their help.

Finally I would also like to thank my parents, siblings, uncles, aunties and cousins. They were always supporting me and encouraging me with their best wishes and fervent prayers.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ΔΔ C	Change in cycle threshold
16S rDNA	Sixteen Small Subunit of Ribosomal DNA
16S rRNA	Sixteen Subunit of Ribosomal
APS	Ammonium persulphate
AW1	wash buffer 1
AW2	wash buffer 2
bBp	base pair
BCA	Bicinchoninic Acid Assay
Bp	base pair
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
Cq	Cycle quantity
CT	Condensed tannins
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
GIN	Gastrointestinal Nematodes
h	hour
HT	Hydrolysable tannins

IFNr	Interferon production regulator
IFN-γ	Interferon gamma
IL-1α	Interleukin-1α
IL-2Rα	Interleukin 2 receptor α
IL-8	Interleukin 8
IP-10	IFN-inducible protein-10
KD	Kilodalton
LBP	LPS-binding protein
LN	Lipolysaccharide and Nystatin combined
LPS	Lipopolysaccharide
LS	Lipolysaccharide and Serecia Lespedeza combined,
mRNA	messenger RNA
ng	nanogram
NK cells	natural killer cells
nM	nano-Mole
NS	Nystatin and Serecia Lespedeza combined
NYS	Nystatin
ºC	Degrees Celsius
OD	Optical Density
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

pg /ml	Pico-gram per microliter
PGN	Peptidoglycan
PN	Peptidoglycan and Nystatin combined
PRR	Pattern Recognition Receptors
PS	Peptidoglycan and Sericea Lepedeza combined
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT- PCR	Real time Polymerase chain reaction
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SL	Serecia Lepedeza
SSU rRNA	small subunit ribosomal RNA
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TH1	Cells T Helper 1 Cells
TLR-2	Toll like Receptor 2
TLR-4	Toll like Receptor 4
TNF-α	Tumor Necrosis Factor alpha
V	volt
WNT	Wingless homolog gene
α	Alpha
β	Beta
µg	Microgram

µl	Microliter

Abstract

This study was conducted to evaluate the effect of a water extract of Sericea Lespedeza (SL), the lipid raft inhibitor NYS and known pathogen associated molecular patterns (LPS and PGN) on gene activation in ruminant (cow, sheep and goat) blood. Sericea Lespedeza, a high tannin containing legume has been shown to be a useful component of feed for control of gastro intestinal nematodes in ruminants. Blood was collected from four adult female Holstein Friesian cows (N=4), Spanish x Boer goats (N=4) and St Croix sheep (N=4) and incubated with 100 ng /mL^{-1} of SL in the presence and absence of LPS, PGN or NYS. Whole blood was also incubated with 100 ng mL^{-1} ng/ml of LPS, PGN or NYS without addition of SL. Phosphate-buffered saline (PBS) served as negative control. Total protein concentration, migration pattern, secretion of WNT5a, TLR2 and prostaglandin E2 alpha in plasma was also determined. Total RNA was isolated from cells using Trizol. Samples with RNA integrity number >7 were used for cDNA synthesis. Transcription of TLR2, TLR4, WNT, FZD, β catenin, TNFα and GAPDH was evaluated in cow, sheep and goat blood. The effect of Nystatin on expression of 84 genes on the cow WNT signaling pathway and human innate and adaptive immunity arrays was assessed in cow blood using real time PCR. Fold change in transcript abundance was calculated using the Livak method. Agarose gel electrophoresis and ethidium bromide staining was used to visualize amplicons. TLR2 and FZD were up regulated in response to PAMPS. Species specific gene expression and modulation by PAMPS was observed. Water extracts of SL reduced PGE2a in sheep and cows. Transcription and translation of genes involved in innate and adaptive immunity and the WNT signaling pathway in ruminant blood is responsive to diverse PAMPS and can be modulated by SL.

CHAPTER 1

Introduction

Animal agriculture is a major contributor of the United States economy. In 2013 alone, total farm cash receipts exceeded $390 billion, including $171.7 billion in cash receipts from livestock and its related products (JEC 2013). Livestock contributes to about 13% of the energy to the world's diet. An estimated 77 million tons of plant protein are fed to livestock to produce 58 million tons of animal protein each year (Steinfeld et al., 2006). Animal-source foods, such us cattle, goat and sheep products are good sources of high quality protein and micronutrients that are essential for normal development and good health.

Sustainable animal production requires that disease causing parasites and pathogens be effectively controlled. Failure to do so would result in poor growth rates, ailments and death in animal populations. The United States as well as other countries spend billions of dollars on pathogen eradication and disease management programs (Bradley, 2002; Zhao and Lacasse, 2008). However, diseases caused by opportunistic environmental parasites and pathogens are still the major constraints to optimum animal production. Most animal diseases are caused by bacteria (gram positive and gram negative), viruses, protozoa internal parasites such as gastrointestinal nematodes (GIN) and other external parasites. Among the animal diseases which affect the profitability of rearing animals, mastitis, an inflammation of the mammary gland, is considered to be one of the expensive diseases in terms of production losses (Bardhan et al. 2013). Economic losses caused by mastitis in the U.S. are around $200 per cow per year (WHO 2013). Gastrointestinal parasites, especially *Haemonchus contortus* is also of great concern impacting goat and sheep production systems all over the world (Terrill et al., 2007). At present goat and sheep rearing face greater threat due to anthelmintic resistances worldwide (Mortensen

et al., 2003) due to frequent use of anthelmintic. Infection by parasites and microbial pathogens trigger an inflammatory response which results in the elimination of the infectious agents (Majno et al 2003). Bacterial infections are mostly caused by gram negative and gram positive bacteria such as *Eschericia .Coli*, *Stapylococcus aureus* and *Stretomyces noursei.*

Elimination of pathogens depends on how efficient the immune system of the host coordinates the delivery of blood components (plasma and leukocytes) to the site of infection or injury (Boermans et al., 2009). Improper orchestration of the immune response to both bacteria and gastrointestinal infections may lead to sepsis (Werners et al., 2005).Sepsis is a condition caused by an overwhelming immune response to infection. The sequencing of the cattle, sheep and goat genomes have led to an increased understanding of the genetics underlying the immune response mechanism. Innate immunity especially, plays a critical role in host defense against infection since it is the first line of defense.

The innate immune response is triggered by Toll-like receptors (TLRs) (Barton et al 2008).Toll-like receptors are pattern recognition receptors(PRRs) that recognize highly conserved structural motifs known as pathogen-associated microbial pattern(PAMPs) The TLR family comprises a total of 13 genes. Ten TLR genes have been identified in human, pig, mouse, cattle and sheep (Chang et al., 2006, Chang et al., 2009, Werling and Coffey, 2007 and McGuire et al., 2006). Raja et al (2011) have also sequenced and characterized toll-like receptor genes 1–10 in goat. Gram-positive peptidoglycan (PGN) and gram-negative lipopolysaccharide (LPS) bacteria, designated pathogen associated molecular patterns (PAMPs), are recognized by the innate immune system by the TLRs (Adib-Conquy and Cavaillon, 2007). Specifically, LPS is a ligand for TLR-4 and PGN is a ligand for TLR-2. Understanding the innate immune mechanism and signaling mediated through TLRs may provide more understanding into the disease

resistance of cattle, sheep, and goats, as well as aid in drug designing and animal selection in breeding programs. Economic losses in the animal industry from diseases result in reduced reproductive efficiency, decreased production of meat, milk, wool and costs of treatment of the animal.

Control programs based on antibiotics are failing to control diseases because of the increased prevalence of parasites and pathogens to resistance to antibiotics (Pomroy et al., 2002). Due to the shortcomings of the traditional methods for controlling diseases, new avenues have been pursued. Food safety, animal welfare and public health concerns have also fueled the interest of plant-based based alternatives. In vitro studies have shown that forages containing condensed tannins (CT) have anthelmintic and antibacterial effects and potentially could be used to control GIN and bacterial infections (Molan et al., 2000; Min et al., 2008). Sericea lespedeza (SL), also known as *Lespedeza cuneate* is one of the high tannin containing legumes that are being studied extensively for its possible health boosting benefits. It has been shown to be a useful component of feed for control of gastro intestinal nematodes in small ruminants (Min et al 2008).

This study was structured to generate new knowledge to enhance the cattle, sheep and goat industry by exploring and identifying genetic immune markers to improve host resistance against diseases. This study was conducted to evaluate the effect of a water extract of Sericea Lespedeza (SL), the lipid raft inhibitor NYS and known pathogen associated molecular patterns (LPS and PGN) on gene activation in ruminant (cow, sheep and goat) blood.

CHAPTER 2

Literature Review

2.1 Ruminants – Goats, Sheep and Cattle

2.1.1 Goat Production

Goats are one of the earliest domesticated species. They are ruminants that have great economic importance, and have been used for their milk, meat, hair, and hide over much of the world (Coffey, 2004). Goats are able to adapt to almost any climate and convert forage into quality meat products .Goats are able to feed on otherwise non-production vegetation such as shrubs and rangeland (Solaiman, 2007). This makes them attractive alternatives over other livestock to small farms with little capital investment to start a farm (Fehr et al., 2004). Goat production has increased in the U.S in the past decade (Sahlu, 2009). The number of meat goat rose from 415,000 to 2.5 million between 1987 to 2008 due to several factors like the introduction of Boer goats and the increase in foreign-born ethnic groups, migrating to the U.S. with a preference for goat meat and products (Solaiman, 2007). Moreover, goat meat is a wholesome and lean meat and it can be a beneficial meat-substitute for people seeking healthier diet alternatives (Solaiman, 2007). Even though goats are hardy animals by nature, they are still susceptible to a number of diseases. Among the conditions affecting goats are gastrointestinal parasites, respiratory diseases such as pneumonia. Goats are also susceptible to various viral and bacterial diseases, such as foot and mouth disease, caseous lymphadenitis, pink eye, and mastitis.

The development of resistance to anthelminthic by gastrointestinal nematodes has increased. Therefore there is a need for alternative control strategies such as the use of SL as a high-quality, low input forage that suppresses gastro-intestinal parasites in goats (Fleming et al.,

2006). Moreover, the development of drug resistance by bacteria in foods of animal origin also led to the search for other kinds of disease preventive strategies.

2.1.2 Sheep production

Sheep are among the most efficient of all the domestic animals. They have been used by man for thousands for their wool, others primarily for food. Over the last 2 decades, the number of sheep in this US has been cut in half. The number has been declining since the late 1940s. Both products – lamb meat and wool – have seen declining consumption in the U.S (Zygoyiannis, 2006). Human-made fibers (nylon, rayon and polyester) have become more prevalent and inexpensive. Because of this, people are wearing less and less wool. The introduction of artificial fibers is reducing the significance of the main purpose of sheep production for wool.

This decreased demand for wool has also led to the reduction in sheep meat production. The decline in sheep production can also be attributed to advancements in poultry production, low prices for sheep products and high prices of cultivated forages (Morris, 2009). In spite of this, however, sheep production is still practiced in the mountain and pacific regions as well as the southern plains of the United States (Jones, 2004).

Sheep just like goats can also adapt to extreme environmental conditions. Sheep are very agile and graze easily in the most rugged of mountain terrain, especially where cattle choose not to feed. Furthermore, some sheep breeds are well suited to survive on sparse desert range that would not be used otherwise. Sheep therefore have the ability to convert the natural forage of these extreme habitats into protein for human uses. Sheep, like their goat counterparts also are also susceptible to pathogens and parasites (Hepworth et al., 2009). However, breeds like the St.

Croix sheep have also been reported to be parasite-resistant in many studies and are useful in co-grazing practices to control parasitic infections (Zajac, 2002).

2.1.3 Dairy cow production

America's dairy industry is an important contributor to the overall nation's economy estimated at $140 billion in economic output. The average U.S. cow produces an average of 7 gallons per day. That's nearly an average of 2,500 gallons a year. Annually, U.S. dairy farms produce almost 196 billion pounds of milk (USDA, 2013). Bovine mastitis is considered economically unbearable and complicated disease in dairy animals worldwide (Wyder et al., 2011). Staphylococcus aureus is the most common etiological pathogen of contagious bovine mastitis and it has the potential to develop resistance to almost all the antimicrobial agents (Barkema et al., 2009).

2.2 Microbial pathogens and Pathogen Associated Molecular patterns

Some carbohydrates isolated and purified from microorganisms have immune-stimulating properties. Unlike T and B cells, phagocytic cells of the innate immune system do not recognize every possible antigen. Instead, they recognize a few highly conserved structures called "pathogen-associated molecular patterns" (PAMP), present in many different microorganism, and interact with receptors on the surface of immune cells (Janeway et al., 2005). Innate immune recognition is based on the detection of invariant molecular signatures through called pathogen associated molecular patterns.

The most important PAMPs are conserved cell-surface structures like lipopeptides (LPN), peptidoglycans (PGN) and lipopolysaccharides (LPS) which are unique to bacteria. In addition, PAMPs might be group-specific such as type III secretion peptides (Nurnberger et al., 2004). LPS are major parts of Gram-negative bacteria cell surfaces, composed of a hydrophobic

lipid A, a covalently linked non-repetitive core oligosaccharide, divided into inner and outer core, and the O-antigen of oligosaccharide-repeating units (Meyer et al., 2001) They are mostly recognized via their lipid anchor by a family of PRRs, named Toll-like receptors (TLRs). LPS is a well-known inducer of a strong pro-inflammatory cytokine response and is the best characterized TLR ligand of microbial origin. TLR4-mediated LPS recognition plays a critical role in the detection of Gram-negative bacteria (Medzhitov et al 2007)**.**

2.2.1 The immune response

Innate and adaptive immunity are the two arms of the vertebrate immune system that cooperate to protect the host against microbial infections. Innate immunity is the first line of host defense against invading microorganisms. Unlike adaptive immunity, it is immediately active and does not require a previous encounter with the infective agent. Innate immune cells residing in tissues, such as macrophages, fibroblasts, mast cells, and dendritic cells, as well as circulating leukocytes, including monocytes and neutrophils, recognize pathogen invasion or cell damage with intracellular or surface-expressed pattern recognition receptors (PRRs). These receptors detect, either directly or indirectly, pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells. Activated PRRs then oligomerize initiate signaling cascades that trigger the release of factors that promote recruitment of leukocytes to the region.

The cells involved are called white blood cells or leukocytes. They are divided into two groups based on their morphology namely, granulocytes and agranulocytes. Eosinophils, neutrophils and basophils are granulocytes whereas are lymphocytes (T and B cells) and macrophages are agranulocytes.

2.2.2 Toll like receptors

Toll-like receptors (TLRs) constitute a novel protein family involved in innate immunity and respond to a wide spectrum of microorganisms, including fungi, bacteria, viruses, and protozoa. The Toll protein first discovered in Drosophila, was shown to be essential for determining the dorsal ventral patterning during embryogenesis (Morisato and Anderson ,1995) and an early form of the innate immune system TLRs are evolutionarily conserved proteins (the oldest TLR identified so far is expressed in *Caenorhabditis elegans.* TLR 4are the main pathogen recognition receptors on macrophages .In order to bind to the cell wall components of the Gram negative bacteria, it requires another protein MD 2 to bind to its cleft and another receptor CD14. CD 14 on its own is a receptor that initiates phagocytosis on a particular pathogen. The transcription factor NF-kB is a pivotal regulator of the inducible expression of key proinflammatory mediators that contribute to an immune response. NF-kB is a hetero- or homodimeric transcription factor which binds to the promoter of a wide range of different target genes. NF-kB dimers are kept inactive through sequestering in the cytoplasm via binding to IkB proteins, which mask their nuclear localization signal and prevent their nuclear translocation. TLR signaling cascades lead to the phosphorylation of IkB, which targets this protein for ubiquitination and proteasomal degradation, leading to the release of NF-kB dimers. Inducible phosphorylation of IkB is mediated primarily by the IkB kinase complex, a large multisubunit complex consisting of at least two catalytic subunits and a regulatory subunit. Induction of NF-kB-dependent gene expression is central to the development of a strong proinflammatory response. Many of the genes activated by NF-kB are themselves upstream activators of NF-kB, further amplifying the host defense response to microbial challenge.

Proinflammatory gene expression by TLRs is also regulated by activation of mitogen activated protein kinases, leading to the phosphorylation of multiple proteins, including several transcription factors. Blumenthal et al (2006) performed a microarray-based gene-expression screen with human macrophages infected with mycobacteria or conserved bacterial structures. *M. tuberculosis*, the causative organism of tuberculosis, critically depends on TLR2 and TLR4 to induce secretion of the proinflammatory cytokines tumor necrosis factor alpha and interleukin. They found mRNA for *WNT5A*, a homolog of Wingless in *Drosophila* species, to be consistently up-regulated in response to all stimuli.

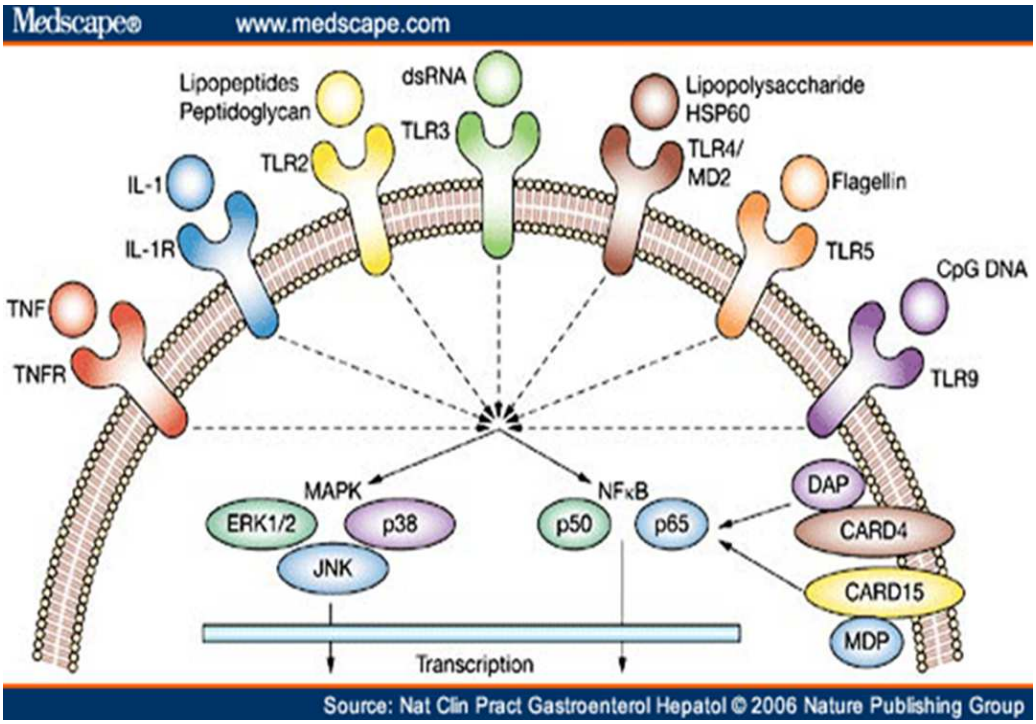


Figure 1 Toll like receptor pathways

2.2.3 Wingless homolog gene signaling pathway

The Wnt pathway is an evolutionary conserved pathway present in all humans, mice, worms and lime molds. It plays a key role in embryogenesis, cell differentiation, apoptosis, motility and polarization of cells. Abnormal regulation of Wnt signaling may lead to malformation, cancer or degenerative disease since it is involved in this diversity of essential functions (Coudreuse and Korswagen, 2007). Recent studies have shown that mutations in genes encoding ß-catenin or other Wnt pathway components have been identified in certain inflammatory bowel diseases (Lee et al., 2008). There are 19 Wnt proteins and 10 Fzd receptors identified in humans, whose function, regulation, and interaction in different cellular processes are currently an active area of research. Wnt homologs induce at least three distinct signaling pathways namely, the Wnt/beta-Catenin, the Wnt/Planar Cell Polarity (PCP) and the Wnt/Ca^{2+} signaling pathway. MacDonald et al. (2009), Habas and Dawid (2005) and Wallingford and Habas (2005) have reviewed this pathway thoroughly. Activation of the Wnt/beta-Catenin pathway requires co-receptors Lipoprotein Receptor-Related Protein (LRP)-family and leads to an inhibition of the Glycogen Synthase Kinase 3 beta (GSK3beta). This leads to an accumulation of beta-Catenin in the cell, it then translocate into the nucleus, where it drives the expression of T-Cell Factor (TCF)/Lymphoid Enhancer-Binding Factor (LEF) transcription factor-dependent genes. ß-Catenin however is not detectable in the cytoplasm or nucleus in unstimulated cells because free ß-catenin levels is controlled by the destruction complex. Since Wnt signally pathway is involved in homeostatic activities in stimulated cells, it is hypothesized that they may be involved in inflammatory regulation.

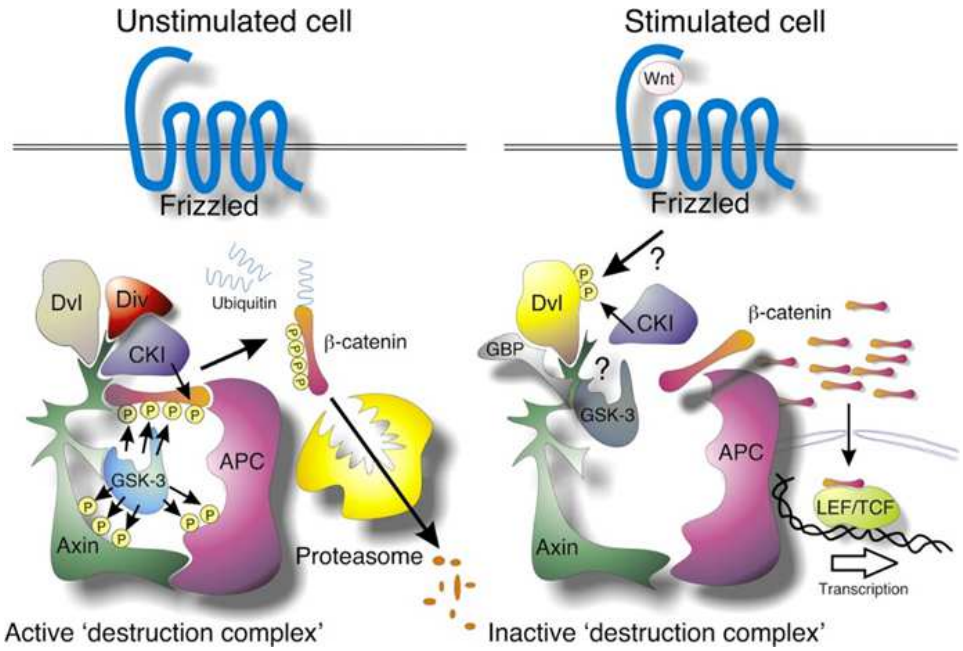


Figure 2 Wnt Signalling pathway

2.2.4 Cytokines

Cytokines are regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to make disease worse (proinflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory) (Dinarello, 2000). They are originally called lymphokines and monokines to indicate their cellular sources, it became clear that the term "cytokine" is the best description, since nearly all nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them.

There are 18 cytokines with the name interleukin (IL). Tumor necrosis factor (TNF) are also cytoknies that retained its original biological description. Another way to look at some cytokines is their role in infection and/or inflammation. Cytokines that promote are called proinflammatory cytokines, whereas other cytokines that suppress the activity of proinflammatory cytokines are called anti-inflammatory cytokines. For example, IL-4, IL-10, and IL-13 are potent activators of B lymphocytes. However, IL-4, IL-10, and IL-13 are also

potent anti-inflammatory agents. They are anti-inflammatory cytokines due to their ability to suppress genes for proinflammatory cytokines such as IL-1, TNF, and the chemokines (Elgert, 2009).

2.2.5 Prostaglandin (PGE2)

Prostanoids are metabolites of arachidonic acid through the cyclooxygenase (COX) pathway. They are the best known lipid mediators that contribute to inflammatory pain (Kawabati, 2011). Among the prostanoids, prostaglandin E2 (PGE2) is involved in numerous processes that lead to the classical signs of inflammation: pain, swelling and redness. The production of PGE2 is controlled by the cyclooxygenase (COX) enzymes, which are the targets of non-steroidal anti-inflammatory drugs (NSAIDs) (Ricciotti and Fitzgerald, 2011).), the most frequently used analgesics, reduce production of PGE2 by inhibiting COX-1 and/or COX-2, and thereby suppress inflammatory pain (Kawabati, 2011)

2.3 Some economically important microbial diseases in animals

2.3.1 Mastitis

Mastitis is an inflammation of the mammary gland resulting from bacterial infections. Staphylococci in particular is the common pathogen of contagious bovine mastitis. The reservoir of this bacteria is generally in the udder or teat. This disease is primarily of importance in dairy sheep and goats (Barillet, 2007). Pathogens colonize mammary gland spread by milking machines, milkers and from animal to animal. *Staphylococcus aureus, Streptococcus agalactiae Corynebacterium bovis* and other *Streptococcus spp.* & *Staphylococcus spp.Mycoplasma spp*. can also cause the disease.

2.3.2 Footrot

Footrot is the major cause of lameness in farm animals. It is a bacterial disease caused by Dichelobacter (Bacteroides) nodosus (D. nodosus). It is considered to be one of the most important animal welfare problems. Footrot is highly contagious, being easily transmitted from animal to animal via pasture, bedding or handling pens. It can even be spread by animals that do not show clinical signs of disease. This disease is a major cause of economic loss and currently it is estimated to have economic costs to the UK industry of £31M per annum (Bishop, 2005).

2.3.3 Tuberculosis

Tuberculosis in animals is very common in cattle and other bovids for which the disease is generally referred to as bovine tuberculosis. *Mycobacterium bovis*, a member of the Mycobacterium tuberculosis complex (Smith et al., 2006) is the major causative agent of bovine tuberculosis. It is a disease of high economic relevance within the context of livestock farming as it directly affects animal productivity and also influences international trade of animal products.

Aside its high economic losses, animal tuberculosis bears a zoonotic potential and is therefore of public health concern (Cosivi et al., 1998 and Renwick et al., 2007).

2.4 Parasitism

Parasitism in ruminants is a substantial problem plaguing farmers across the United States especially the south eastern part. Animals that are overburdened with parasites can be hindered in their reproductive performance, experience reduced growth rates, and become less productive overall, whether their purpose be meat, fiber, or milk (Knox, 2006). Among the parasitic diseases, endoparasites are of greatest importance in ruminants. Common parasites of sheep and goat include roundworms, coccidia, tapeworms, and liver flukes. The younger sheep and goats especially are more susceptible to parasites due to their lack of a fully developed immune system (Patra, 2007).Gastrointestinal parasite infection is the most important limiting factor of goat and sheep productivity (Terrill et al., 2007). Haemonchus contortus cause the most severe damage in goats and sheep populations (Miller, 2006).

2.4.1 Haemonchus Contortus

Haemonchus contortus, a nematode parasite found in small ruminants, is of economic importance to the sheep and goat industry worldwide (Troell et al., 2005). The blood sucking parasite *Haemonchus contortus* which is found in the abomasum of the sheep and goat causes significant blood loss. Each worm removes 0.05 ml blood per day so that sheep with a 500 *H. contortus* may loss about 250 ml per day resulting in decrease in erythrocytes, lymphocytes, hemoglobin, PCV, body weight and wool growth (Gadahi et al., 2009). Outbreaks are most severe in warm, humid climates. An optimum temperature of between 50° and 80° celsius is conducive for larval growth. Haemonchus has a life cycle lasting approximately four weeks. The larvae travel to the abomasum of the animal when ingested by the animal. They burrow into the

mucosa and develop into true adults in 21 days (Fitch, 2006). The adult female is capable of depositing 5,000 to 10,000 eggs per day through hosts' feces (Sarf, 2006). Endoparasites result in huge economic losses.

2.5 Control of diseases

2.5.1 Antibiotics

The potential benefits associated with the use of antibiotics in food animals includes, treatment of disease, improvement of feed efficiency and the improvement of carcass quality (Andreasen et al., 2005). The use of feed-based antimicrobials has consistently been shown to benefit livestock production, increasing the ability of farms to maintain profitable margins (Phillips et al., 2004). This lowers manure output and thus the effects of animal wastes on the environment thereby lowering animal pathogen loads (Ebner and Mathew, 2000; Rattanatabtimtong et al., 2005). Many foodborne pathogens are not easily controlled in livestock by vaccines. These organisms have a commensal association with their food animal hosts, making eradication difficult, if not impossible. Antibiotic-based feed or water additives may be a practical approach to limiting foodborne transfer of these organisms (Phillips et al., 2004).

2.5.2 Anthelmintics for Gastrointestinal Control

Fundamentally, the control of parasitic infections has been based on the well implemented use of anthelmintic (Hounzangbe-Adote et al., 2005a). Anthelmintics were developed primarily to get rid of an existing infection rather than prevent infection. There has been an increase in resistance to numerous anthelmintics in nematode populations despite its strategic use to treat infections (Hounzangbe-Adote et al., 2005a). The exponential growth of resistance to anthelmintics is becoming a global problem. There are three major classes of anthelmintics used against gastrointestinal nematodes. These are benzimidazoles, avermectins

and imidothiazoles. Scientific studies have demonstrated nematode resistance to drugs in all three classes of anthelmintics (Zajac and Gipson, 2000; Terrill et al., 2001; Mortensen et al., 2003).

The resistance of nematodes to anthelmintics has made the prevention of Haemonchosis vulnerable. As a result of this growing resistance problem, alternate venues of controlling *H. contortus* infection are being pursued. Also, the cost of anthelmintics, especially in developing countries is another reason for different methods of control to be explored (Hounzangbe-Adote et al., 2005a).

2.5.3 Breeding for Resistance

Natural disease resistance refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization (Hurt, 1958). Disease resistance is a particularly important attribute of livestock in low-input livestock production systems in the developing world (Bishop et al., 2002). The animal genome always influences and determines susceptibility to bacterial and other pathogenic diseases as well.

Breeding of genetically resistant animals against gastrointestinal nematodes, which may prove to be a more effective means of control as well as, reduce dependence on anthelmintics. Resistance to infectious diseases is often the critical determinant of the sustainability of such systems, and improving resistance is perceived as a primary target for genetic improvement program. Breeding for resistance against gastrointestinal nematodes could be beneficial in terms of cost of anthelmintics and in reducing the number of worms toward production (Dominik, 2005).Another important factor affecting the prospect of genetically resistant animals is that the variability of resistance within breeds, such as that found in *H. contortus* (Stear et al., 1994), may possibly equal to that of the variability within breeds themselves if traits that prove to be resistant

against nematode infection were used more frequently in breeding programs, the livestock industry would benefit immensely (Dominik, 2005).

2.5.4 Vaccines

Development of vaccines against gastrointestinal nematodes is one of the most researched control methods today. As of yet, no vaccine has been made commercially available to producers (Newton et al., 2003). There are two classes of experimental vaccines. These are hidden and natural antigens (Newton et al., 2003). Since H. *contortus* is a blood feeding nematode, hidden antigens, also referred to as concealed or covert antigens are successful against them. This is because "high levels of antibody to the injected antigen are ingested with the blood meal" (Newton et al., 2003). Natural antigens however can be used against both blood-feeding and non-blood-feeding nematodes (Newton et al., 2003). Natural antigens are advantageous because the immune response is boosted due to exposure by field conditions (Smith et al., 2006). Hidden antigens have also shown immune response memory to challenge infections (Smith et al., 2006).

2.5.5 Pasture Sanitation

The pairing of pasture management and anthelmintic use has been another venue used to control nematodes, but has many shortcomings. For example, 'dose and move' and rotational grazing is becoming less feasible, due to the limited land size of many small holder farmers and the communal land ownership of many pastoralists (Githiori et al., 2003). Due to the limitation and decreasing availability of land, use of ethno veterinary medicine is on the rise (Hammond et al., 1997; Dano and Bogh, 1999). This method has been effective at controlling nematode parasites, but "places heavy genetic selection pressure for resistance on nematode populations" (Kaplan et al., 2004). This occurs because the drug is being administered to all animals, exposing

this drug to all worms, leaving an unexposed environment of eggs and larvae called refugia (Kaplan et al., 2004).

There are many different factors affecting transmission of nematodes from grazing, such as effects due to climate and habitat destruction. Climatic issues affecting the transmission cycle are temperature, rain, humidity, barometric pressure, sunlight, cloud cover and wind (Stromberg, 1997). Habitat destruction is comprised of birds, insects, fungi, and wild mammals that have the resources to reduce number of larvae on pasture (Stromberg, 1997). Both factors vary both seasonally and annually (Stromberg, 1997). Because the variables involved in the calculation process are difficult, in terms of measuring (climate, larval development and behavior), it is hard to determine with complete accuracy the amount of larvae on pasture (Stromberg, 1997).

2.5.6 Plant based anti infectives

Control programs based on anthelmintics are failing to control GIN because of the increased prevalence of GIN resistance to anthelmintics (Pomroy et al., 2002). Thus, alternative GIN control strategies need to be developed. Plant based anthelmintic have also been explored for use in the elimination of GIN with such extracts like: Garlic (*Allium sativum*), Neem (*Azadirachta india*,), Wormwood (*Artemisia absinthium*) and Tobacco (*Nictiana tobacum*) (Worku, Franco and Baldwin, 2009).

Successful production of small ruminants requires that gastrointestinal nematodes (GIN) be effectively controlled. Failure to do so will result in poor growth rates, ill-thrift and death. *In vitro* studies have shown that forages containing condensed tannins (CT) have anthelmintic effects and potentially could be used to control GIN (Molan et al., 2000). Recently, there has been much interest in feeding plants containing condensed tannins (CT) to animals to reduce and the effects of infection with gastrointestinal nematodes. In addition, many antibacterial-

promoting effects have been reported for plant tannins and their investigation is now increasingly relevant (Chung et al., 1998).

2.5.7 Condensed Tannins

Interest in alternative methods to control gastrointestinal nematodes of ruminants has increased in response to the development of anthelmintic resistance in populations of parasitic nematodes (Jackson and Coop, 2000). Among the alternative methods is the possible use of tanniferous plants. Tannins are usually classified either hydrolysable tannins (HT) or CT (proanthocyanidins) based on their molecular structure. Condensed tannins are the most widely distributed (Butter et al., 2000) .Condensed tannins are oligomers or polymers of flavonoid units linked by carbon-carbon bonds which are not susceptible to cleavage by hydrolysis (Waghorn and McNabb, 2003). They are called condensed tannins because of their condensed chemical structure.

In sheep, (Athanasiadou et al., 2001) condensed tannins are capable of reducing parasite infection. This is dependent upon the source of the condensed tannins used as well as the amount of condensed tannins required to attain the desired effect (Waghorn et al., 2003). In several studies, sericea lespedza, condensed tannin containing forage, has been used to decrease parasitism (lower total worm burdens, fecal egg counts, and larval development) in goats (Min et al., 2004, 2005; Shaik et al., 2004, 2006). Similar results were obtained using quebracho extracts except no difference in worm number was noted (Paolini, 2003). These studies give promise to condensed tannins as a future control method against gastrointestinal nematodes. Min et al (2008) also reported some antimicrobial activities of SL.

2.6 Sericea Lespedeza

Sericea lespedeza is a legume planted widely throughout the southern USA as a grazing, hay, and soil restoration and conservation crop. It is a perennial, warm season forage (Powell et al., 2003), brought to the U.S from its native Asia. SL; *Lespedeza cuneata*; contains 15.2% of CT. the adaptability of SL to acidic soils with low fertility, it enhanced its potential of being a useful low-input forage for the southern United States (Puchala et al., 2005). Sericea lespedeza is drought tolerant (Ball et al., 1991) and has high concentration of protein (Gamble et al., 1996). In spite of these attributes, it is often classified as a weed and some states are trying to rid of them. It was originally purposed to fight against soil erosion, improve soil and act as a habitat and cover for wildlife. It is now used as a forage plant as well. Sericea lespedeza is capable of growing in a variety of areas, such as pastures, roadsides, rangelands, prairies, and eroded slopes, but is intolerant of shade. Notwithstanding the negatives surrounding this forage, there are many positives, such as its ability to improve production and animal health.

2.7 Nystatin

The Gram-positive bacteria of the genus *Streptomyces* are the main producers Nystatin (Hopwood 1997). Nystatin disrupts lipid rafts by binding to cholesterol within the plasma membrane without extracting it. This antifungal agent has been used widely to demonstrate the involvement of lipid rafts in biological processes (Smart et al 2002)

2.7.1 Lipid rafts

Lipid rafts are small platforms, composed of sphingolipids and cholesterol content within cell membranes. These assemblies are fluid but more ordered and tightly packed than the surrounding bilayer. They are evolutionarily conserved structures that play a role in a number of

signaling processes involving receptors expressed by a variety of cell types (Brown and London, 2000).

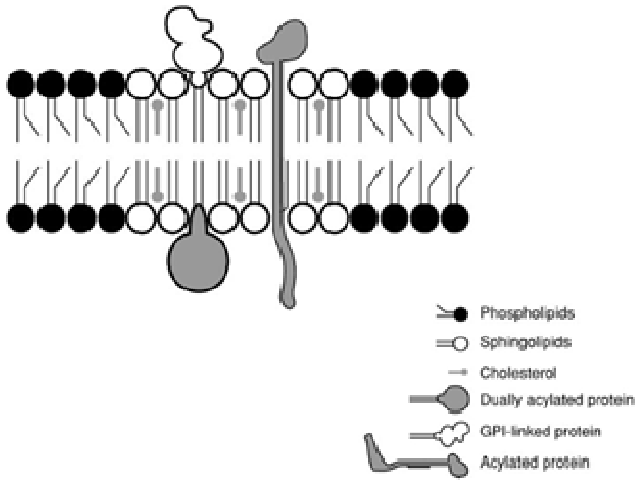


Figure 3 Cross section of the Lipid raft

CHAPTER 3

Methodology

3.1 Animals

Four clinically healthy adult female Holstein Friesian cows, four adult female Spanish x Boer goats and four adult female St Croix sheep from North Carolina A&T State University Animal farm were used in the study. None of the animals used exhibited any evidence of disease or received medications during the 4 week period prior to blood sampling. All protocols for handling of the animals were approved by the Institute of Animal Care and Use Committee (IAUCUC).

3.2 Housing and Diet

The cows were housed the NCA & T dairy farm. They are raised on a pasture based system of dairy farming. The goats and sheep were housed at the NCA & T unit in the night and were put out to graze on the pasture during the day. The goats and sheep were also provided with 17% pellet feed. Water was also available 24 hours *ad libitum* to all the animals.

3.3 Blood Sampling

Whole blood was collected aseptically from the jugular vein of the animals into vaccutainer tubes containing 1ml of the anti-coagulant acid citrate dextrose (Macdonald et al, 2006). The tubes were placed on ice immediately after collection. The samples were transported to the laboratory.

3.4 Total White Blood Cell and Cell viability

Cell Viability was assessed using the Trypan blue dye exclusion method using TC10 cell counting instrument (Biorad). Samples of whole blood were diluted 1: 100 in saline. Ten (10) µl of diluted whole blood and 10µl of trypan blue were combined in a 1.5 ml test tube. The mixture

was pipetted up and down for five times to mix the cells and the dye. Ten (10) µl of the mixture was loaded in one of two chambers of the counting slide. The slide was then inserted in to the TC10 cell counter for automatic cell counting. Cell counting was done in duplicates and an average was taken. Cell viability was expressed as a percentage of [(total viable and non- viable cells/total cells)] on the TC 10 cell counter (Bio-Rad).

White Blood Cell Differential counts were conducted on whole blood treated with all ten treatments and PBS control using Wright Staining procedure. A thin smear of blood was made on a glass slide and left to dry at room temperature overnight. The air dried slide was dipped in Wright's stain for10 seconds. Excess stain was washed of the stained slide with deionized water. The slide was then air dried before reading under a light microscope (Carolina Biologicals). Smears were read under oil immersion for cell counts. The differential cells were counted up to 100 for numerical representation of various cells present in the blood sample.

3.5 Preparation of Bacterial PAMPS

One Hundred (100) ng/mL each of Escherichia Coli derived Lipopolysaccharide (LPS) (Sigma-Aldrich St. Louis, MO), Staphylococcus aureus derived Peptidoglycan (PGN) (Sigma-Aldrich St. Louis, MO or Nystatin (NYS) (St Louis, MO) was prepared.

3.6 Preparation of Serecia Lespedeza (SL) Extract

Fifty 50 g of SL powdered leaves obtained from Dr. Nikki White of NCA&T was put in 500 ml of PBS and stirred for 30 minutes. After 30 minutes, the entire content in the volumetric flask was filtered through a filter paper. The filtrate was again passed through a 0.2 micron filter to obtain a fine filtrate free of particles and other microbes. BCA assay was done on the filtrate and a standard curve was used to obtain 100ng/ml.

3.6.1 Stimulation of Whole Blood

One mL of blood from each animal was incubated with100 ng/mL each of *Escherichia Coli* derived Lipopolysaccharide (LPS) (Sigma-Aldrich St. Louis, MO), *Staphylococcus aureus* derived Peptidoglycan (PGN) (Sigma-Aldrich St. Louis, MO or Nystatin (NYS) (St Louis, MO) and water extract of Serecia Lespedeza (SL) (Dr. Nikki White) either individually or in pairwise combinations to assess the expression of select genes and other inflammation indicators.

Samples were incubated at 37ºC, with 85% humidity and 5% CO_2 for 30 minutes. Samples incubated with Phosphate Buffer Saline (PBS) served as negative control. All the reagents used were prepared with (diethyl pyrocarbonate (DEPC) -treated endotoxin free water. At the end of the incubation period cells were spun down at 1700 x g at 4ºC for 5 minutes. Supernatants were collected and stored at -80C to measure total protein concentration, prostaglandin E2α levels. Trizol was added to cell pellets and stored for RNA isolation.

3.7 Gene Expression Profiling

3.7.1 Isolation of RNA

Both treated and control samples underwent RNA extraction following incubation. This was done according to Ambion manufacturer's instruction. The appropriate precautions were used to avoid RNase contamination throughout in the entire procedure. Half (0.5) mL of 100% of isopropanol was added to the each tube containing the homogenized cell pellets in the TRIzol® Reagent and incubated at room temperature for 10 minutes. It was then centrifuged at 12,000× g for 10 minutes at 4°C. Supernatant was removed from the tube leaving only the RNA pellet. The pellet was washed with 1 mL of 75% ethanol. The sample was vortexed briefly, and centrifuged at 7500×g for 5 minutes at 4°C. Supernatant was discarded and washed. RNA pellet was air

dried for 10 minutes. The RNA pellet was suspended in 30 µl RNase free water by passing the solution up and down several times through a pipette tip. RNA was stored at –80°C.

3.7.2 RNA concentration and purity

The RNA concentration in nanogram per microliter (ng/µl) and purity were assessed using a Nanodrop Spectrometer ND 1000(Thermo Scientific Inc., Waltham, MA). The optical densities were read at 260nm.

3.7.3 Quality and Integrity of RNA

Total RNA was pipetted into an RNA 6000 Nano LabChip® (Agilent) and RNA integrity was determined using Agilent® Bioanalyzer. Manufacturer's protocol was followed. (See appendix B). Complimentary DNA (cDNA) synthesis was performed with 500ng/µl RNA (purity 260/280= 1.8, RIN = 7).

3.7.4 cDNA Synthesis

Both Qiagen and Retroscript kits were used to make cDNA for the real time Polymerase chain reaction (PCR) and the wingless homolog and human innate and adaptive pathway arrays.

3.7.4.1 Qiagen. Genomic DNA elimination mix for each RNA was prepared sample according to manufacturer's protocol (See Appendix A). Genomic DNA elimination mix was incubated for 5 min at 42°C, and placed on ice for at 1 min. Reverse-transcription mix was prepared mix according manufacturers protocol (See Appendix A).

Ten (10) µl of reverse-transcription mix was added to each tube containing 10 µl genomic DNA elimination mix and mixed gently. It was incubated at 42°C for exactly 15 min and 95°C for 5 min to stop the reaction. Ninety (91) µl of RNase-free water was added to each reaction and

mixed by pipetting up and down several times. Reactions were placed on ice The RT² SYBR Green Mastermix was spun for 15s to bring the contents to the bottom of the tube.

Note: RT² SYBR Green Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions were prepared at room temperature (25°C). PCR components mix was prepared in a loading reservoir using the RT² Profiler PCR Array format, as described in Appendix A.

This mixture was aliquoted into the wells of the RT² Profiler PCR Array. PCR is performed and finally relative expression is determined using data from the real-time cycler and the ΔΔCT method.

3.7.4.2 Ambion Retroscript cDNA synthesis procedure. Three (3) µl of total RNA, 2 µl of oligodT and 7 µl of RNase free water was added to a PCR tube. It was mixed, spun briefly and heated at 75^{o}C in a thermocycler. The remainder of the real time components was then added. (2 µl of 10X RT buffer, 4 µl of dNTP mix, 1 µl of RNase inhibitor and 1 µl of MMLV RT). It was then incubated and at 42^{o}C for 1 hour and 92^{o}C for 10 minutes in the thermocycler. The cDNA obtained was then stored at -20 ^{o}C for PCR.

3.8 Real Time PCR

3.8.1 Primers

Primers were sequenced commercially specifically for TLR2, TLR4, WNT5A, Frizzled, TNFα (MWG, Biotech Huntsville AL) (Table 1). The House keeping gene GAPDH was used as an internal control and for normalization. Fold change in transcript abundance was calculated using the Livak method with samples maintained in PBS serving as controls (Livak and Schmittgen 2001).

Table 1

Sequences of Primers used

Gene	*Primer*	*Sequence 5'>3'*	*Reference*
Wnt5a	Forward	*GGGAATTAAGAATGCAAAATGCCAAT*	Zeisberg et al., 2007
	Reverse	*AAAATCTAGAACAACACCAATGAAA*	
Frizzled	Forward	*ATCGGAATTCTACCCAGGCGACCAATA*	Zeisberg et al., 2007
	Reverse	*ATCGAAGCTTAGAGACAAGGAACCA*	
βcatenin	Forward	*CGTTTAGCAGAGCCTTCCCAGAT*	Zeisberg et al., 2007
	Reverse	*CCAGCCAGACGTACAGCCATTT*	
TLR2	Forward	*GTTGGAGGCCGGTGGCAACA*	Montes et al., (2006).
	Reverse	*TGTTGCCACCGGCCTCCAAC*	
TLR4	Forward	*AGGCAGCCATAACTTCTCCA*	Montes et al., (2006).
	Reverse	*ATCATTGAAGCTCAGATCTAAAT*	
TNFα	Forward	*CTGCACTTCGGGGTAATCGG*	Leutenegger et al., 2000
	Reverse	*CAGGGCGATGATCCCAAAGTA*	
GAPDH	*Forward*	*GTCTTCACCACCATGGAG*	Olah et al., (2006)
	Reverse	*CTCCATGGTGGTGAAGAC*	

3.8.2 Agarose gels electrophoresis to detect amplified genes

Amplified PCR products were run on 0.8 % agarose gel using 9µl of amplified cDNA and 1 µl of six times loading dye (Thermo Scientific) at 100 volts for 30 minutes. Gels were stained with 1µl/ml ethidium bromide, washed and visualized using Bio- rad gel documentation system (Bio-Rad Laboratories, Hercules, CA).

3.8.3 Evaluation of the effect of exposure to Nystatin on expression of genes in the WNT Signaling Pathway

The Cow WNT Signaling Pathway RT² Profiler PCR (Qiagen) was used in profiling the expression of 84 genes related to WNT-mediated signal transduction. WNT signaling comprises of the canonical pathway, planar cell polarity (PCP) and a calcium ion-dependent pathways (non-canonical). The WNT array used contains signaling ligands and receptors as well as other downstream signaling molecules for all three pathways. WNT signaling regulators as well as downstream target genes are also included.

The Human Innate & Adaptive Immune Responses RT² Profiler™ PCR Array (Qiagen) was used in profiling the expression of 84 genes involved in the host response to bacterial infection and sepsis. The genes included genes related to the IL-1R and Toll-like Receptor (TLR) Signaling Pathways including IL-1R and TLR genes involved in the detection of pathogens profiled by the array. Genes involved in the detection of bacteria, acute-phase response, complement activation, the inflammatory response, and the antibacterial humoral response are also represented on this array.

Samples were prepared as described *in 3.5.4a* above. Total RNA from Nystatin and PBS treated cow blood (N=4) were converted to cDNA using oligodT primers (Qiagen) and added to qPCR master mix and aliquoted across the PCR arrays in duplicates. Amplification was accomplished on a Bio-Rad CFX Connect real time PCR instrument (BioRad).

3.9 Evaluation of the concentration of total plasma protein

Total protein concentration in plasma harvested from control (PBS) and treated whole blood was measured using the bicinchoninic acid assay (BCA) following the manufacturer's instructions (Thermo Scientific™ Pierce). Briefly twenty (25) µL of each standard (Albumin

BSA) or unknown sample was pipetted into a 96 microplate well in 3 replicates. (*See appendix for standard preparation*). Two hundred (200) µL of the Wash Reagent (WR) was added to each well and plate was mixed thoroughly on a plate shaker for 30 seconds. It was then covered and incubated at 37°C for 30 minutes. The plate was cooled and brought to room temperature. The absorbance was measured at 562nm in a plate reader (BioTek). The average 562nm absorbance measurement of the Blank standard replicates was subtracted from the 562nm measurements of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average Blank corrected 562nm measurement for each BSA standard versus its concentration in µg/mL. The standard curve was then used to determine the protein concentration of each unknown sample.

3.9.1 Evaluation of prostaglandin (PGE2α) secreted in plasma

Prostaglandin (PGE2α) concentration in plasma from control (PBS) and treated whole blood was evaluated using a commercial Enzyme-linked immunosorbent assay (Cayman) following the manufacturer's instructions. (See *appendix B for standard and buffer preparations*).One Hundred (100) µl of EIA Buffer was added to NSB wells. Fifty (50) µl of EIA Buffer was also added to the B_0 wells. Fifty (50) µl from tube #8 was added to both of the lowest standard wells (S8). Fifty (50) µl from tube #7 was also added to each of the next two standard wells (S7). The procedure was continued until all the standards were aliquoted. The same pipette tip should was used to aliquot all the standards. Fifty (50) µl of sample was added per well. Each sample should be assayed was assayed in triplicate**s.** Fifty (50) µl of AChE Tracer was added to each well except the TA and the Blk wells. Fifty (50) µl was added to each well except the TA, the NSB, and the Blk wells. The plate was covered with a plastic film and incubated over night at 4°C. Wells were emptied and rinsed five times with Wash Buffer. Two hundred (200) µl of

Ellman's Reagent was added to each well. Five (5) µl of tracer was added to the TA well. The plate was covered with plastic film and placed on an orbital shaker equipped with a large, flat cover for 90 minutes to develop in the dark. The bottom of the plate was wiped with a clean tissue to remove fingerprints, dirt, etc. The plate cover was removed. The plate was inserted into a micro plate reader (BioTek) read at 410nm. The statistical software SAS (SAS institute Cary), was used to plot %B/B0 for standards S1-S8 versus PGE2 concentration using a logit transformation.

3.10 Determination of Wnt5a and TLRs levels using ELISA

Levels of Wnt5a and TLR 4 were determined in plasma aliquots (100 µl) by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Cusabio) for human Wnt5a, and human TLR 4 according to the manufacturer's instructions.

3.11 SDS-PAGE

A mini gel apparatus (Bio-Rad's Mini- PROTEAN® 3 cell was assembled. The discontinuous SDS-PAGE was done using 0.75-mm thick slab gels with a 4.5% stacking gel and 12.5% separating gel. Monomer gel solution was poured into glass gel cassettes. Deionized water was added slowly over the monomer solution to ensure that the top of the gel was even. After 30 minutes, the water was discarded and running gel overlay was added on the gel. The gels polymerized overnight at room temperature. The gel overlay was then decanted. A 4.5% stacking gel solution was prepared and pipetted onto the 12.5% separating gel and a comb was inserted into the stacking gel. The stacking gel was allowed to polymerize for 30 minutes. The comb was removed after polymerization was complete. Plasma from control and treated samples as well as markers were carefully loaded in to the appropriate wells. Electrophoresis was carried out with a current of 50 watts for approximately 1 to 2 hours using the Bio-Rad® A mini gel apparatus

(Bio-Rad's Mini- PROTEAN® 3 cell Criterion Gel System and the Ephortec® 1000 volt power supply unit.

3.11.1 Silver Staining.

Slab gels were stained using the Tsai and Frasch's method (1982). All steps were performed at room temperature. Powder-free gloves were used throughout the silver staining procedure. Gels were transferred from gel cassettes into staining trays filled with fixative (40% ethanol (ETOH), 5% acetic acid, 55% deionized water) and placed on a shaker overnight. After the overnight fixing step, the fixative was discarded and a freshly prepared oxidizer solution (0.7g oxidizer, 100ml of fixative) was added. The gels were placed on the shaker for 5 minutes. The gels were washed in deionized water for 15 minutes. This step was repeated twice. The gels were incubated for 30 minutes in a silver stain prepared fresh and followed by three 10 minute washes. The gels were developed in a solution containing citric acid (50mg), 37% formaldehyde (0.5ml) and 1 liter DH_2O. The gel was visualized on a Gel doc XR (Biorad).

3.11.2 Western blot analysis.

Separated on a one dimensional gel were transferred on to 0.22µ nitrocellulose membrane. Air bubbles were removed. The nitrocellulose membrane and gel sandwich was placed in a moist chamber and incubated for 20 minutes at 70˚C. Through diffusion blotting, proteins were transferred from the gels onto 0.22um nitrocellulose. The membrane was blocked for 1 hour in 3% bovine serum albumin (BSA) (Kirkegaarde and Perry Laboratories (KPL, MD). Affinity purified specific mouse antibodies to [Wnt5a (Fisher) at a 1:1000 dilution] in antibody buffer was poured over the membrane. The membrane was incubated with the antibody at room temperature (RT) for 2 hours, followed by washing with KP wash buffer.

The second antibody, HRP labeled goat anti-mouse was added and incubated at RT for I hour. The addition of 1% tween 20 was added to the diluted KP wash to reduce background staining. Proteins were detected using a tetramethlybenzidine substrate system (KPL, MD). The blots were rinsed in deionized water to stop the color development.

3.12 Data Analysis

Data are represented as means for purity and concentration of RNA. For white blood cell differential cell counts and TC 10 counts, means of treatment groups were compared with one-way Analysis of variance (ANOVA). When the ANOVA showed that there were significant differences between the groups, Least squares means was used to identify the sources of these differences. $P \leq 0.05$ was considered statistically significant.

Concentrations of total plasma protein, WNT5A, PGE2 and TLR4 ELISAs were determined using a standard curve. A repeated measure analysis (proc glm) was performed to identify main effects of animals and treatments, as well as their interactions.

Real time PCR data was analyzed using the Livaks method. Housekeeping gene GAPDH and samples treated with PBS were used to determine the $\Delta\Delta Ct$. Where $\Delta Ct = (\text{Target genes}_{treat} - GAPDH_{treat}) - \Delta Ct\ (\text{Target genes}_{PBS} - GAPDH_{PBS})$.

Fold change = $2(-\Delta\Delta Ct)$ (Livak and Schmittgen 2001)

CHAPTER 4

Results and Discussion

4.1 Total Cell Count and Viability in Whole Blood.

The average cell concentrations for cows, sheep and goats before treatment was 1.01 x 10^6, 8.07x 10^7 and 8.92 x 10^6 cells/ml respectively. The results indicated that treatment had an effect on cell viability. Species variation was also observed. Table 2 below shows percentage viable cells after treatment in the three animal species. (Initial viable cells were considered to be 100%).

Table 2

Percentage cell viability after whole blood incubation with various treatments

	Treatments										
	L	P	S	N	LP	LS	LN	PS	PN	NS	PBS
Animal											
Cow	93	75	87	98	83	91	97	90	83	83	97
Sheep	70	71	68	85	73	63	84	74	71	71	80
Goat	60	66	70	75	70	94	67	79	83	80	66

In cow blood, samples incubated with peptidoglycan had the lowest viability of 75%. The rest ranged from 83 – 97%. Including the control.

In sheep blood, samples incubated with Serecia Lespedeza had the lowest viability of 68%. The highest was found in samples incubated in Nystatin (85%).

In goat blood, samples incubated with Lipopolysaccharide had the lowest viability (60%). The highest was found in samples incubated with Lipopolysaccharide and Serecia in combination (94%).

The results show that there is variation in how various species respond to SL and different bacteria cell wall stimuli. Viability is dependent on the type of stimuli and animal species. Cow had the highest viable cells after treatment. There is no literature so far on variation in cell viability in ruminant species after PAMP stimulation.

4.1.1 White blood cell (WBC) differential count

4.1.1.2 Sheep. Average lymphocyte population was 70% in all samples. Samples incubated with NYS/SL had a monocyte population mean greater than the control (3.5%). the rest were not different from the control samples. Basophil 0.94 and Eosinophil 0.87. Neutrophil population was 31%. There was no change in population after incubation ($p>0.05$).

4.1.1.3 Cow. Lymphocyte population was lower in treated samples compared to the control ($p<0.0001$). Average lymphocyte population in all treatments was 70% whereas the population was 90% in control samples. Monocytes in PBS ranged from 0-2% where by monocytes in treatments ranged from (1- 10%) in almost all the samples. Lymphocytes population in samples incubated with LPN, SL and PGN/SL were significantly greater than the control ($p<0.0001$). The rest were not. Neutrophil concentration increased in all samples compared to the control ($p<0.001$). Concentration increased from 7 – 56% in treated samples. Mean Basophil and Eosinophil populations in both control and treated samples was 0.84 and 0.87 percent respectively. There were no treatment effect on Basophil and Eosinophil population.

4.1.1.4 Goat. Goat lymphocytes were the same in both treated and control samples (62.5%). Samples treated with SL had the highest population of monocytes (9%) at the 0.05 level of significance. The rest were not different from the controls (4%). Neutrophil population was not significantly changed. Average of 29.5%. Basophils and Eosinophils also averaged 1.127%.ans 0.94% respectively.

The principal function of leukocytes is to defend the animal against invading microorganisms by phagocytosis or the release of cytokines into the blood stream, thus contributing to cellular inflammatory responses. (Luster, 2005). The variation in change of the number of leukocytes in different species is an indication of differences in general inflammatory

response to infection between species. Lymphocytes and Neutrophil populations were observed to be higher than the rest of the leukocytes in all three ruminants. There was no significant change in their population compared to the normal leukocytes levels in ruminants (Khan and Line 2010).

Both control and treated samples were within the normal white blood population range for all the animals. Hence treatment did not have an effect on WBC.

4.2 Concentrations and Purity of Total RNA

The concentration and purity of RNA extracted from whole blood was measured to evaluate the treatment effect on mRNA transcription.

Concentration and purity levels were not affected by treatment. There was no species variation observed as well. (*Table 3).*

Table 3

RNA purity and concentration in controls

Animal	RNA purity	RNA Concentration(ng/ul)
Goat	1.80	3572.5
Sheep	1.80	3446.03
Cow	1.80	3444.7

There were no significant differences in RNA concentrations between the treatments in all species ($p>0.05$). Also, there was no significant difference in RNA purity either. RNA purity depends on the technique employed in the extraction. A value of ~2.0 is generally accepted as "pure" for RNA. All RNA samples averaged 1.7.

4.2.1 RNA integrity

RIN values ranged from 3- 7 in all species. Good RNA with RIN value of 7 was used for subsequent Real time experiments (Figure 4).

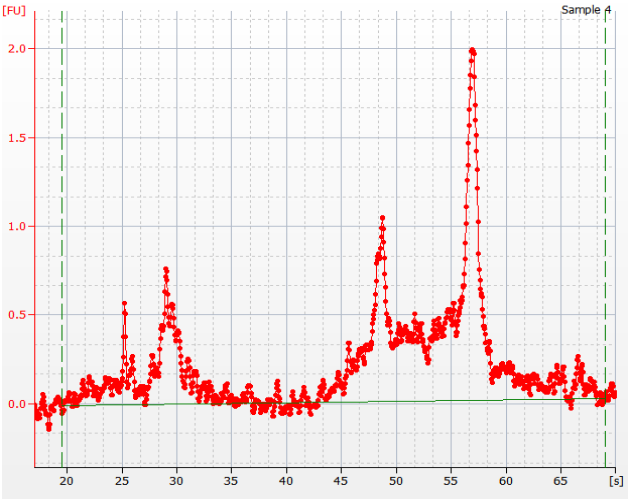
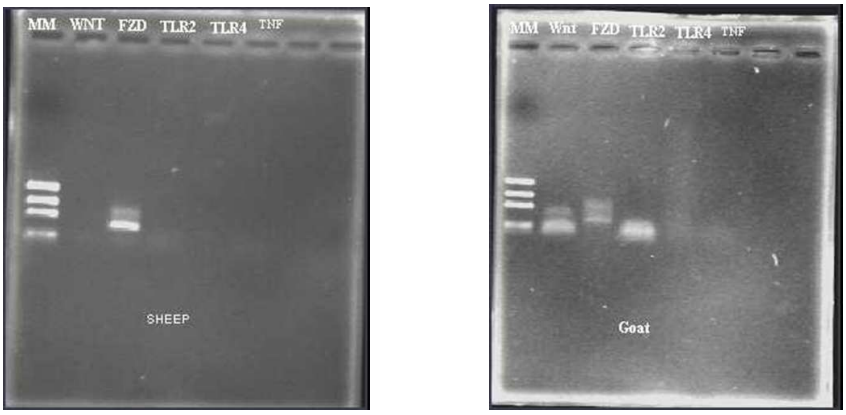


Figure 4 Graph showing RNA with an RIN value of 7

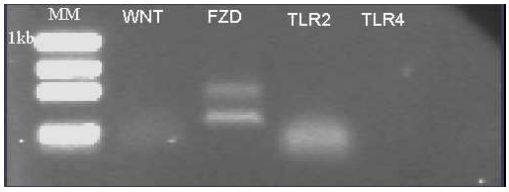
4.3 Detection of Amplified products

GAPDH was expressed in all three animals (Goats N=4, Sheep N=, Cow N=4). Thus, all three species used in the study had the genes that code for GAPDH. GAPDH is a housekeeping gene. No fold changes in gene expression of immune markers WNT5A, TLR 4 and TNF alpha were observed. However, all genes except for TNF alpha were observed to be expressed when the RT PCR products of PBS were visualized on a 0.8% agarose gel (Figures 5). Thus, even though the genes were present in all 3 species, the treatment did not increase their levels. This could be that in vitro stimulation of whole blood with 100ng/ml of LPS, PGN, NYS and SL was not adequate for TLR 4, WNT5A and TNF alpha up regulation. In addition, no fold change can be attributed to transcription inhibiting factors. Figure 5 shows visualization of the presence of WNT5A, Frizzled, TLR2 , TLR4 and TNFα on a 0.8% agarose gel using 9 µl of amplified

cDNA and 1 µl of six times loading dye. The primers used were specific to bovine and ovine and caprine genes.



a. Sheep *b.Goat*



c.Cow

Figure 5 Detection of Amplified WNT5A,FZD, TLR2,TLR 4and TNF alpha in untreated blood

4.3.1 Fold change in TLR 2 expression

Cow samples exposed to individual treatment (LPS, PGN, NYS or SL) had lower transcript levels (0.3, 0.5, 0.4 and 0.6 folds) respectively compared to samples that were incubated with mixtures of treatments. The highest transcript levels were observed when samples were exposed to PGN and NYS together (2.5 fold). Exposure to SL in combination with PGN was also associated with increased transcript levels (1.5 fold).

Similarly, high fold changes (≥ 2) were observed in sheep samples exposed to PGN, PGN/ NYS (2.4 and 2.2 fold) respectively. But the highest gene fold was observed in samples

exposed to LPS and SL together. However, samples exposed to individual treatment (LPS, NYS or SL) had lower transcription levels (0.1, 0.1, and 0.1 folds) respectively compared to samples that were incubated with mixtures of treatments.

In Goat blood, the highest fold change in the sample was observed in samples exposed to LPS/NYS PGN/NYS, NYS, and (7.3, 6.9 and 6). It was observed from this study that goats responded more to Nystatin and other immunomodulators co-stimulated with Nystatin

Scientific literature indicates that plant compounds like tannins and other polyphenols can modulate immune response through pathways .These include the anti-inflammatory polyphenol and the oligomeric procyanidins (Holderness, 2012). Polyphenols produced by plants exhibit antifungal and anti-inflammatory properties (Leiro et al., 2004) .Those studies also indicated existence of plant polyphenols capable of modulating TLR-mediated signals. The data from the RTqPCR experiment showed a fold change in gene expression of TLR 2 in SL treated samples, supporting the suggested effect of tannins, in this case that of Serecia Lespedeza on innate immunity.

Nystatin is a lipid raft inhibitor derived from the bacterium *Streptomyces noursei.* Razonable et al (2005) and Sau et al (2003) found TLR2 to recognize *Streptomyces nodosus*-derived antifungal polyene drug amphotericin B and linked it to its proinflammatory activities. Since Nystatin also has a similar action to amphoterene B they hypothesized that Nystatin could serve as PAMP that induce cell activation through TLR, which in turn may activate signaling pathways that result in secretion of cytokines that mediate biologic effects. Razonable et al (2009) again reported that Nystatin induces cytokine secretion in TLR2-expressing but not TLR2-deficient cells. In this study, it was observed in all 3 species that Nystatin induced

transcription of TLR 2 which may in turn activate the induction of other cytokines. Hence, Nystatin may possess some proinflammatory characteristics (Table 4).

Table 4

Fold changes of TLR2 expression

TREATMENT	Cow	Sheep	Goat
Lipopolysaccharide (LPS)	0.6	0.1	0.0
Serecia Lespedeza (SL)	0.5	0.1	1.7
Peptidoglycan (PGN)	0.4	2.4*	1.3
Nystatin (NYS)	1.4	0.1	6.0*
PGN /SL	1.5	0.6	4.1*
LPS/ SL	0.5	1.8	4.8*
LPS/PGN	0.4	2.7*	0
LPS/NYS	1.2	1.9*	7.3*
PGN/NYS	2.5*	2.2*	6.9*
NYS/SL	0.3	1.1	0.7

*= significant, Fold changes ≥ 2 is considered significant

4.3.2 Fold change in Frizzled Receptor

In Cow blood the highest fold change was observed when Peptidoglycan was co-stimulated with SL (13.52). That was the only significant fold change (≥ 2). The rest were low (<1). In goat blood however, significant fold changes were observed when PGN was co-stimulated with SL (4), LPS/PGN (2) and LPS/SL (2). In sheep samples stimulated with LPS and SL showed the highest fold change (2.7). Followed by SL alone (2.4).In all 3 animals, it was observed that samples co-incubated with water extract of SL showed increase in fold change.

Frizzled is a receptor for Wnt which is involved in homeostasis. While TLRs promote inflammation, Wnt signaling pathway ensures order by homeostasis. Secondary metabolites derived from natural products including bioactive compounds such as tannins, and phenols reported to inhibit inflammation (Olszanecki et al., 2002). We assume that in this case, frizzled receptor is upregulated in the SL samples to expedite the Wnt signaling pathway to keep inflammation levels in check.

Hence SL may possess some anti-inflammatory properties. To our knowledge, up regulation of frizzled receptor by SL in ruminant blood has not been reported. The expression of Frizzled receptor after stimulation with SL in ruminant blood is a novel discovery that need to be explored. The relevance of the frizzled receptor to homeostasis needs to be studied.

Table 5

Fold changes of Frizzled receptor expression

TREATMENTS	Cow	Goat	Sheep
LPS	0.22	0	0.0
PGN	0.0	1	0.1
SL	0.97	1	2.4
NYS	0.03	0	0.1
PGN/SL	13.52*	4*	0.6
LPS/PGN	0.08	2*	1.8
LPS/SL	0.74	2*	2.7*
LPS/NYS	4.52*	4*	1.9*
PGN/NYS	0.21	1	2.2*
NYS/SL	0.0	1	1.1

*= significant, Fold changes ≥ 2 is considered significant

4.3.3 Effect of Nystatin exposure on expression of Cow samples

4.3.3.1 Wnt signaling genes in cow blood. All 84 genes were expressed on the array. Below are the upregulated genes. Upregulation means more highly expressed compared to the reference gene (GAPDH) whereas down-regulated means lower expression compared to the reference gene (GAPDH).

Table 6

Up regulated genes on the Wnt array

Gene no	***Gene name***	***Fold change***	***Gene function***
B02	DAB2	6.4	Cell Growth & Proliferation
B08	FBXW11	2.0	WNT Signaling Negative Regulation
C04	FZD3	2.1	Wnt/ Canonical
H05	YWHAZ	5.6	House keeping
G07	WNT9A	3.1	WNT/Ca+2
F04	WISP1	2.5	Wnt-1 Inducible Signaling Pathway
H04	TBP	2.5	TATA-box binding factor

There were no down regulation genes detected in the results of the Wnt signaling pathway assay. Genes that code for cell growth and proliferation, both the canonical pathway and non-canonical pathway were upregulated. (Table 7) Interestingly, the house keeping gene YWHAZ was also upregulated. YWHAZ proteins are a group of highly conserved proteins that are involved in many vital cellular processes such as metabolism, signal transduction, apoptosis and cell cycle regulation. They also enhance the activity of many proteins with proliferative and/or survival functions, such as Raf kinases, and antagonize the activity of proteins that promote cell death and senescence. (Anderson et al., 2009). TBP is a gene that binds to the core

promoter to position the polymerase properly. It may participate in basal transcription. WISP1 is also a downstream regulator in the Wnt/Frizzled-signaling pathway. It is associated with cell survival.

FBXW11, a Wnt signaling negative regulator was also upregulated. Negative regulatory genes decrease the rate, frequency, or extent of the Wnt signaling pathway through beta-catenin. This in turn causes a change in the transcription of target genes (Cenciarelli et al, 1999). This implies that the lipid raft inhibitor, Nystatin modulates the Wnt signaling in cow whole blood .Nystatin is a lipid raft inhibitor which is produced by the bacteria *Streptomyces noursei* in the immediate environment cows. To my knowledge, this is the first time cow whole blood samples treated with Nystatin has been found to up regulate those genes (Qiagen).

4.3.3.2 *Human innate and adaptive pathway genes in cow blood*

Table 7

Up regulated genes in the human innate and adaptive PCR array

Gene name	Fold changes	Gene function
MAPK1	81	Innate Immunity
MAPK8	31	Innate Immunity
TLR 7	27	Defense Response to Viruses
IL 10	3.7	Innate immunity
CD 40	6	Defense Response to Bacteria
MX 1	7.1	Antiviral response
TBX 21	2.0	Transcription factor

Table 8

Down regulated genes on the human innate and adaptive array

Gene name	**Gene function**
APCS	Innate immunity
IFNA1	Adaptive immunity(cytokine)

Genes involved in both innate and adaptive response were upregulated. This includes receptors that respond to both bacteria and viruses (CD 40 and TLR 7, MX1 respectively). This was an interesting observation since Nystatin is an antifungal agent. MAP Kinase activities were also highly upregulated. MAPK It is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. Since the receptors in the array were upregulated, we can say that MAPK should also be upregulated in order to transmit signals to the nucleus to ensure homeostasis. Increasing activity of MAPKs and their involvement in the regulation of the synthesis of inflammation mediators at the transcription level, make them potential targets for novel anti-inflammatory therapeutics.TBX21 which is also a transcription T-box genes encode transcription factors involved in the regulation of developmental processes.

However two genes on the array were down regulated. APCS has been demonstrated that this protein binds to apoptotic cells at an early stage, which raises the possibility that it is involved in dealing with apoptotic cells in vivo. IFNA1 is a protein whose genes by this gene are

produced by macrophages and has antiviral activity. It may be down regulated since all the Nystatin is an antifungal agent derived from bacteria.

From the results of this study, Nystatin modulates immune response in cow blood by gene activation that may in turn cause some down regulation of other genes.

4.4 Total plasma protein concentration

Total plasma protein concentration was measured using the bicinchoninic acid assay (BCA). BCA assay is an effective method of detecting the increase in protein in blood plasma. Increase in protein levels in blood plasma are markers of inflammation. The total plasma protein results gives evidence of inflammation in the blood after stimuli. Protein levels were high in plasma of samples exposed to PGN, NYS, PGN/NYS and SL ($p<0.05$).

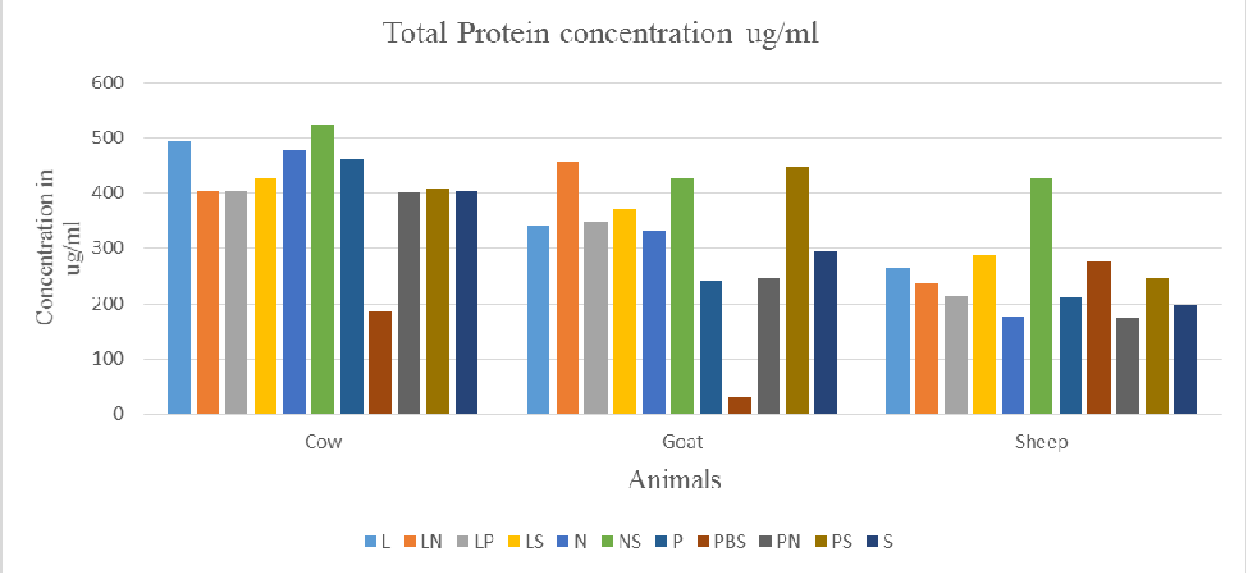


Figure 6 Total protein concentration in cow sheep and goat plasma

4.4.1 Total Protein Concentration in Cow blood

All cow blood samples incubated with the various treatments were significantly different from the ones incubated with PBS (control). Samples incubated with Nystatin and SL in combination had the highest protein level (522µg/ml) followed by LPS alone (495 µg/ml). The

rest were not significantly different from each other at the 0.05 significance level. The average concentration of the rest of the samples was 428 µg/ml.

4.4.1.1 SDS PAGE for cow, sheep and goat blood plasma

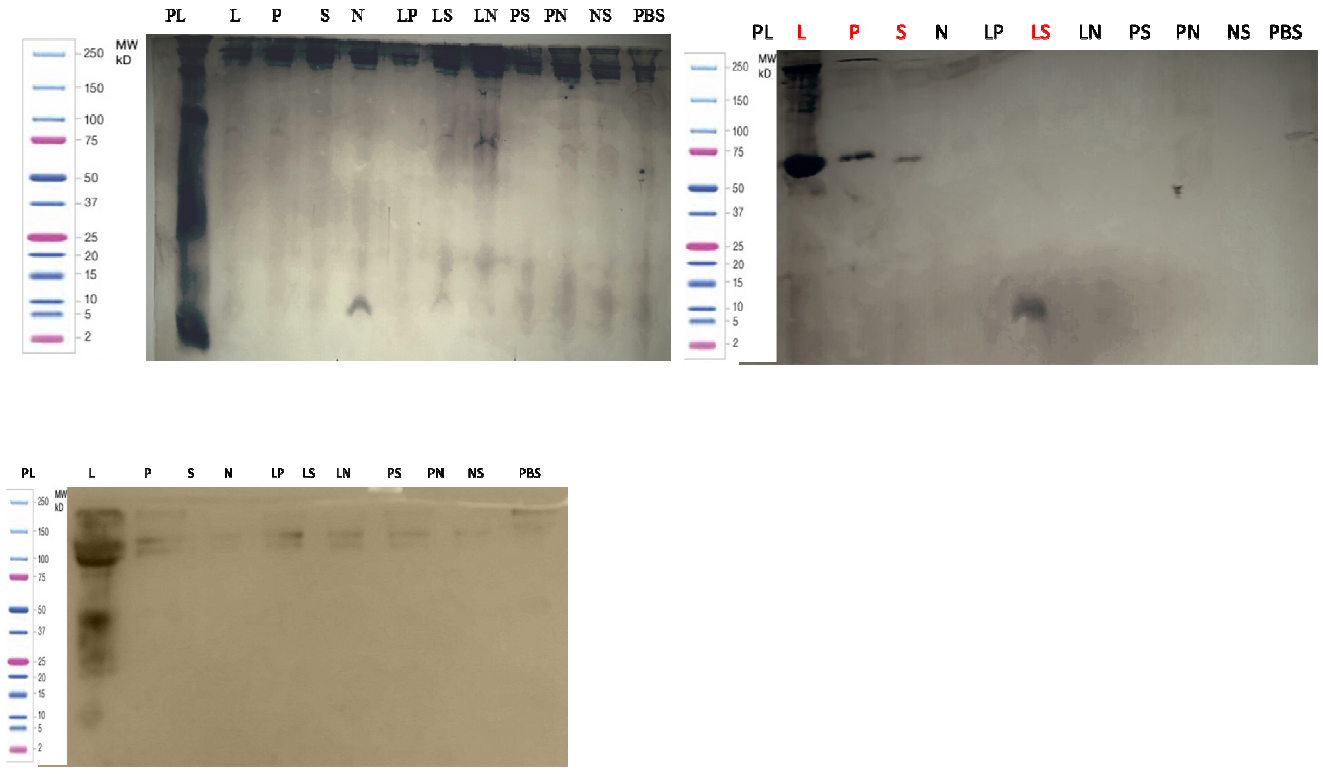


Figure 7 SDS page gel for cow, sheep and goat

Top left Cow, Top right Sheep, and Bottom left Goat

The SDS PAGE gel images shows high band intensity around 250kD in all samples. Other low intensity bands were observed in all treatments. This indicates that there were different molecular weights of proteins present in all the samples.

4.4.1.1.1. Detection of Wnt 5a with Western blotting. Wnt5a was not detected using the nitrocellulose membrane after western blotting analysis in all samples either. This may be due to an antibody specific problem. There is the need for optimization of the protocol in future analysis.

4.4.2 Total Protein Concentration in Goat blood

In goat blood also, total protein concentration in all samples were significantly higher than that of the control (PBS) at the significant level of 0.05. The highest concentration was recorded in samples incubated with LPS/NYS, PGN/ SL and NYS/SL However, there was no significant difference between the 3 samples. The average concentration of the three was 443.3 µg/ml. Samples incubated with PS and PGN however recorded the lowest concentrations with an average of 242.6 µg/ml.

4.4.3 Total protein concentration of Sheep

In sheep blood also, the highest total protein concentration was recorded in samples incubated with Nystatin and SL combined (427.5 µg/ml). Interestingly, total protein concentration in samples incubated with LPS, LPS/SL, PGN /SL, LPS/NYS were not significantly different from blood incubated with PBS (258.7 µg/ml and 276 µg/ml respectively). Samples incubated with Nystatin alone, however was lower than the control. Inflammation causes extra protein to be released from the site of inflammation and circulates in the bloodstream.

4.5 Prostaglandin levels (PGE2)

The concentration of Prostaglandin E2A alpha was determined using a commercial ELISA as described in the previous chapter (Cayman Biologicals). Data were analyzed as

recommended for commercial assays. Overall, goat had the lowest PGE2 levels among the 3 ruminants. (Figure 8).

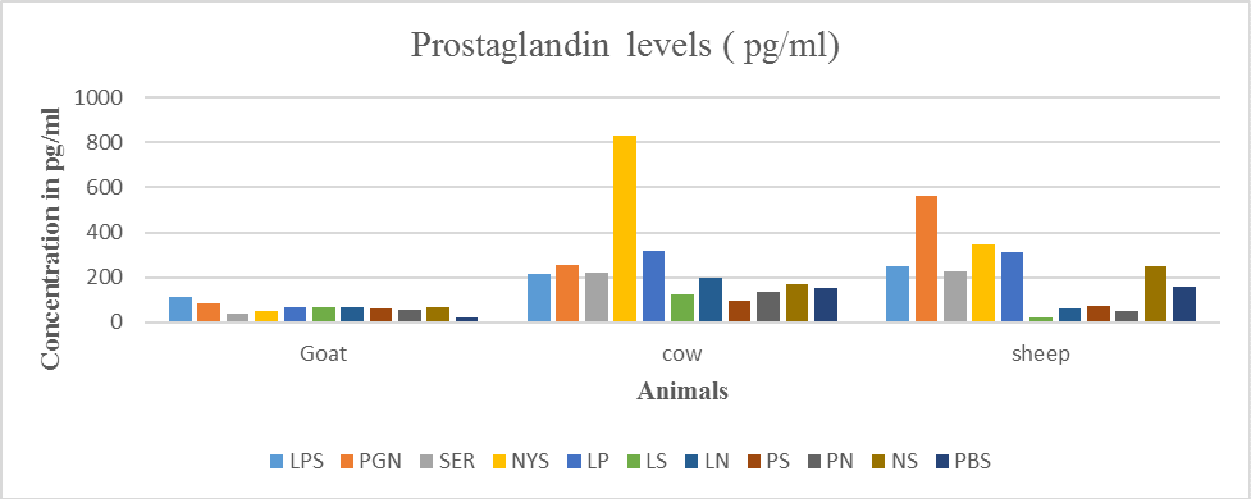


Figure 8 Prostaglandin (PGE2) levels in cow sheep and goat blood plasma

4.5.1 PGE 2 concentration in Goats blood

In goat blood, LPS had the highest mean of 110 pg/ml, followed by PGN (86 pg/ml). However, when blood was incubated with LPS/ SL, prostaglandin concentration dropped to 67.6. Similarly, when blood was incubated with PGN/SL, prostaglandin levels dropped 62 pg/ml. Prostaglandin levels in samples incubated with Nystatin on the contrary increased from 51 pg/ml to 69 pg/ml when it was co-incubated with SL. All treatments were significantly different from the control (PBS) which has 24.7 pg/ml of prostaglandin.

PAMPS cause different effect on inflammatory mediator secretion – LPS and PGN can be modulated with SL not NYS (Figure 8)

4.5.2 Prostaglandin Concentration Sheep blood

Samples incubated with PGN alone, LPS alone and LPS/PGN had the most prostaglandin concentration levels of 565 pg/ml, 349 pg/ml, 313 pg/ml and 251 pg/ml respectively. Contrary to

what happened in goat blood, Prostaglandin levels decreased when Nystatin was paired with SL. Prostaglandin levels also decreased significantly when LPS and PGN were both paired with SL. That is, LPS/SL (22.9 pg/ml) and PGN/SL (71 pg/ml) respectively ($p < 0.001$).

4.5.3 Prostaglandin Concentration in cow blood

In cow blood, the highest prostaglandin level was found in samples incubated with Nystatin (830 pg /ml. Followed by samples incubated with LPS/PGN and PGN. 313 and 254 respectively. However, prostaglandin levels dropped significantly when Nystatin, LPS and PGN were paired with SL. That is, 171 123 and 94 pg/ml ($p < 0.001$). This was similar to what happened in sheep blood. From the results, treatment and species interaction effect was significant ($p<.0001$)

Almost all cell types, when exposed to LPS, Peptidoglycan or other stimuli , activate NF- κ B and other transcription factors, that leads to the expression of inflammatory genes, such as COX-2, inflammatory cytokines and chemokines. Therefore all agents that can suppress these transcription factors have the potential of inhibiting the expression of COX-2 thereby reducing prostaglandin production (Gerhäuser et al., 2003). From the study, it was shown that SL was effective in reducing inflammatory effect of LPS and PGN in all three by species reducing the PGE levels.

However, Nystatin alone ncreased prostaglandin levels in cow which is a novel discovery and needs more investigation. This could be due to the binding of the lipid raft inhibitor to cholesterol in the lipid raft thereby degrading its integrity through hole formation in cells. Cellular components are then released into the blood stream which then triggers inflammatory response that may lead to increase in prostaglandin formation (Wong et al., 2009). Endogenous molecules are also capable of inducing inflammation. Although prostaglandin

released in high amounts may cause tissue damage and other related inflammation problems, it stimulates and regulates a wide array of immune functions, thus playing an important role in the host defense system. Although all the ruminants responded to the stimuli, variation in their responses to each of the PAMPS was clear. This could be due to the differences in their genetic makeup and their extent of susceptibility and resistance or tolerance to certain stimuli. (Figure 8)

4.6 Concentration of WNT5a and TLR4 and Enzyme -linked immunosorbent assay (ELISA)

Undetectable levels of Wnt5a and TLR4 (concentrations <<0.001) were observed in all samples compared to the negative control. Plasma samples need to be concentrated by protein concentrators in future research.

CHAPTER 5

Conclusion and Future Research

Exposure of ruminant blood to diverse PAMPS modulates gene expression. The result suggests that the immune response of ruminants is modulated by co-exposure with microbes as it occurs in mixed infections in pasture. Transcription of TLR2 and Frizzled receptors in cow, goat and sheep blood is variably responsive to different bacterial PAMPS. SL, which is a high tannin containing legume increased gene transcription in ruminant blood. The lipid raft inhibiting function of NYS may contribute to TLR2 transcription in cows, sheep and goats. Transcription of genes involved in innate and adaptive immunity and the WNT signaling pathways in ruminant blood is responsive to diverse PAMPS, impacted by inhibition of lipid rafts, and can be modulated by water extracts of SL. The expression of wingless homolog pathway genes in ruminant blood in response to SL and other PAMPs need to be explored since it may be important in homeostasis and inflammation. Expression of wingless homolog genes in ruminant blood has not been reported yet.

The effect of SL on TLR2 and Frizzled expression in response to microbial products may offer an avenue for the exploitation of plant-derived tannins to regulate inflammatory response and enhance the cow, goat and sheep innate response.

References

Adib-Conquy, M., & Cavaillon, J. M. (2007). Stress molecules in sepsis and systemic inflammatory response syndrome. *FEBS letters*, *581*(19), 3723-3733.

Andreasen, C. B., Spickler, A. R., & Jones, B. E. (2005). Swedish antimicrobial regulations and their impact on food animal production. *Journal of the American Veterinary Medical Association*, *227*(1), 41-45.

Athanasiadou, S., Kyriazakis, I., Jackson, F., & Coop, R. L. (2000). Consequences of long-term feeding with condensed tannins on sheep parasitised with Trichostrongylus colubriformis. *International Journal for Parasitology*, *30*(9), 1025-1033.

Ball, D.H., Hovelan, C.S., Lacefield, G.D. (1991) Southern forages. The Potash and Phosphate Institute and the Foundation for Agronomic Research, Atlanta

Barkema, H. W., Green, M. J., Bradley, A. J., & Zadoks, R. N. (2009). Invited review: The role of contagious disease in udder health. *Journal of dairy science*, *92*(10), 4717-4729.

Bishop, S. C., & Morris, C. A. (2007). Genetics of disease resistance in sheep and goats. *Small Ruminant Research*, *70*(1), 48-59.

Blumenthal, A., Ehlers, S., Lauber, J., Buer, J., Lange, C., Goldmann, T., & Reiling, N. (2006). The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory

responses of human mononuclear cells induced by microbial stimulation. *Blood*, *108*(3), 965-973.

Bradley, A. J. (2002). Bovine mastitis: an evolving disease. *The Veterinary Journal*, *164*(2), 116-128.

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., & Pagano, M. (1999). Identification of a family of human F-box proteins. *Current biology*, *9*(20), 1177-S3.

Coffey, L., Margo Hale, and Ann Wells. (2004). Goats: *Sustainable Production Overview*. National Sustainable Agriculture Information Service.

Cosivi, O., Grange, J. M., Daborn, C. J., Raviglione, M. C., Fujikura, T., Cousins, D., & Meslin, F. X. (1998). Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. *Emerging infectious diseases*, *4*(1), 59.

Coudreuse, D., & Korswagen, H. C. (2007). The making of Wnt: new insights into Wnt maturation, sorting and secretion. *Development*, *134*(1), 3-12.

Danø, A. R., & Bøgh, H. O. (1999). Use of herbal medicine against helminths in livestock: renaissance of an old tradition. *World Animal Review (FAO) Revue Mondiale de Zootechnie (FAO) Revista Mundial de Zootecnia (FAO)*.

Dominik, S. (2005). Quantitative trait loci for internal nematode resistance in sheep: a review. *Genetics Selection Evolution*, *37*(Suppl. 1), S83-S96.

Ebner, P. D., & Mathew, A. G. (2000). Effects of antibiotic regimens on the fecal shedding patterns of pigs infected with Salmonella Typhimurium. *Journal of Food Protection®*, *63*(6), 709-714.

Gadahi, J. A., Arshed, M. J., Ali, Q., Javaid, S. B., & Shah, S. I. (2009). Prevalence of gastrointestinal parasites of sheep and goat in and around Rawalpindi and Islamabad, Pakistan. *Veterinary World*, *2*(2), 51-53.

Githiori, J. B., Höglund, J., Waller, P. J., & Baker, R. L. (2003). The anthelmintic efficacy of the plant, Albizia anthelmintica, against the nematode parasites Haemonchus contortus of sheep and Heligmosomoides polygyrus of mice. *Veterinary Parasitology*, *116*(1), 23-34.

Hopwood, D. A. (1997). Genetic contributions to understanding polyketide synthases. *Chemical reviews*, *97*(7), 2465-2498.

Hounzangbe-Adote, M. S., Paolini, V., Fouraste, I., Moutairou, K., & Hoste, H. (2005). In vitro effects of four tropical plants on three life-cycle stages of the parasitic nematode, Haemonchus contortus. *Research in Veterinary Science*, *78*(2), 155-160.

Jackson, F., & Coop, R. L. (2000). The development of anthelmintic resistance in sheep nematodes. *Parasitology*, *120*(07), 95-107.

Leutenegger, C. M., Alluwaimi, A. M., Smith, W. L., Perani, L., & Cullor, J. S. (2000). Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan® polymerase chain reaction. *Veterinary Immunology and Immunopathology*, *77*(3), 275-287.

Kaplan, R. M. (2004). Drug resistance in nematodes of veterinary importance: a status report. *Trends in parasitology*, *20*(10), 477-481.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.*, *20*, 781-810.

McDonald, S. L., & Silver, A. (2009). The opposing roles of Wnt-5a in cancer. British journal of cancer, 101(2), 209-214.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature*, *449*(7164), 819-826.

Miller, J. E., Burke, J. M., Terrill, T. H., & Kearney, M. T. (2011). A comparison of two integrated approaches of controlling nematode parasites in small ruminants. *Veterinary parasitology*, *178*(3), 300-310.

Min, B. R., & Hart, S. P. (2003). Tannins for suppression of internal parasites. *J. Anim. Sci*, *81*(Suppl 2), E102-E109.

Min, B. R., Hart, S. P., Miller, D., Tomita, G. M., Loetz, E., & Sahlu, T. (2005). The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. *Veterinary parasitology*, *130*(1), 105-113.

Molan, A. L., Alexander, R. A., Brookes, I. M., McNabb, W. C., & Peterson, S. W. (2000). Effects of an extract from sulla (Hedysarum coronarium) containing condensed tannins on the migration of three sheep gastrointestinal nematodes in vitro. In *Proceedings of the New Zealand Society of Animal Production* (Vol. 60, pp. 21-25).

Molan, A. L., Waghorn, G. C., Min, B. R., & McNabb, W. C. (2000). The effect of condensed tannins from seven herbages on Trichostrongylus colubriformis larval migration in vitro. *Folia parasitologica*, *47*(1), 39-44.

Morisalo, D., & Anderson, K. V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. *Annual review of genetics*, *29*(1), 371-399.

Oláh, J., Tőkési, N., Vincze, O., Horváth, I., Lehotzky, A., Erdei, A., & Ovádi, J. (2006). Interaction of TPPP/p25 protein with glyceraldehyde-3-phosphate dehydrogenase and their co-localization in Lewy bodies. *FEBS letters*, *580*(25), 5807-5814.

Nürnberger, T., Brunner, F., Kemmerling, B., & Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological reviews*, *198*(1), 249-266.

Paolini, V., Bergeaud, J. P., Grisez, C., Prevot, F., Dorchies, P., & Hoste, H. (2003). Effects of condensed tannins on goats experimentally infected with Haemonchus contortus. *Veterinary parasitology*, *113*(3), 253-261.

Patra, A. K. (2007). Nutritional management in organic livestock farming for improved ruminant health and production-An overview. *Livest. Res. Rural Dev*, *19*(3), 41.

Perry, B., & Sones, K. (2009). Global livestock disease dynamics over the last quarter century: drivers, impacts and implications. *Rome, Italy: FAO. (Background paper for the SOFA 2009)*.

Puchala, R., Min, B. R., Goetsch, A. L., & Sahlu, T. (2005). The effect of a condensed tannin-containing forage on methane emission by goats. *Journal of Animal Science*, *83*(1), 182-186.

Raja, A., Vignesh, A. R., Mary, B. A., Tirumurugaan, K. G., Raj, G. D., Kataria, R., ... & Kumanan, K. (2011). Sequence analysis of Toll-like receptor genes 1–10 of goat (Capra hircus). *Veterinary immunology and immunopathology*, *140*(3), 252-258.

Rattanatabtimtong, S., Mathew, A., Chattin, S., Jarboe, E., & Clift, R. (2005, January). Effects of in-feed anti-salmonella egg yolk antibodies on shedding and antibiotic resistance of bacteria in swine. In *JOURNAL OF DAIRY SCIENCE* (Vol. 88, pp. 270-270). 1111 N DUNLAP AVE, SAVOY, IL 61874 USA: AMER DAIRY SCIENCE ASSOC.

Razonable, R. R., Henault, M., Lee, L. N., Laethem, C., Johnston, P. A., Watson, H. L., & Paya, C. V. (2005). Secretion of proinflammatory cytokines and chemokines during amphotericin B exposure is mediated by coactivation of toll-like receptors 1 and 2. *Antimicrobial agents and chemotherapy*, *49*(4), 1617-1621.

Renwick, A. R., White, P. C. L., & Bengis, R. G. (2007). Bovine tuberculosis in southern African wildlife: a multi-species host–pathogen system. *Epidemiology and Infection*, *135*(04), 529-540.

Sahlu, T., Dawson, L. J., Gipson, T. A., Hart, S. P., Merkel, R. C., Puchala, R., & Goetsch, A. L. (2009). ASAS Centennial Paper: Impact of animal science research on United States goat production and predictions for the future. *Journal of animal science*, *87*(1), 400-418.

Sau, K., Mambula, S. S., Latz, E., Henneke, P., Golenbock, D. T., & Levitz, S. M. (2003). The antifungal drug amphotericin B promotes inflammatory cytokine release by a Toll-like receptor-and CD14-dependent mechanism. *Journal of Biological Chemistry*, *278*(39), 37561-37568.

Sen, M., Lauterbach, K., El-Gabalawy, H., Firestein, G. S., Corr, M., & Carson, D. A. (2000). Expression and function of wingless and frizzled homologs in rheumatoid arthritis. *Proceedings of the National Academy of Sciences*, *97*(6), 2791-2796.

Shaik, S. A., Terrill, T. H., Miller, J. E., Kouakou, B., Kannan, G., Kaplan, R. M., & Mosjidis, J. A. (2006). Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infection in goats. *Veterinary Parasitology*, *139*(1), 150-157.

Shaik, S. A., Terrill, T. H., Miller, J. E., Kouakou, B., Kannan, G., Kallu, R. K., & Mosjidis, J. A. (2004). Effects of feeding sericea lespedeza hay to goats infected with Haemonchus contortus. *South African Journal of Animal Science*, *34*(Supplement 1), 248-250.

Smart, E. J., & Anderson, R. G. (2001). Alterations in membrane cholesterol that affect structure and function of caveolae. *Methods in enzymology*, *353*, 131-139.

Solaiman, S. (2007). Assessment of the meat goat industry and future outlook for US small farms. *Tuskegee University. Retrieved December*, *1*, 2008.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., & Haan, C. D. (2006). *Livestock's long shadow: environmental issues and options*. Food and Agriculture Organization of the United Nations (FAO).

Terrill, T. H., Mosjidis, J. A., Moore, D. A., Shaik, S. A., Miller, J. E., Burke, J. M., ... & Wolfe, R. (2007). Effect of pelleting on efficacy of sericea lespedeza hay as a natural dewormer in goats. *Veterinary parasitology*, *146*(1), 117-122

Terrill, T. H., Miller, J. E., Burke, J. M., Mosjidis, J. A., & Kaplan, R. M. (2012). Experiences with integrated concepts for the control of Haemonchus contortus in sheep and goats in the United States. *Veterinary parasitology*, *186*(1), 28-37.

Waghorn, G. C., & McNabb, W. C. (2003). Consequences of plant phenolic compounds for productivity and health of ruminants. *Proceedings of the Nutrition Society*, *62*(02), 383-392.

Wong, S. W., Kwon, M. J., Choi, A. M., Kim, H. P., Nakahira, K., & Hwang, D. H. (2009). Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *Journal of Biological Chemistry*, *284*(40), 27384-27392.

Worku, M., Franco, R., & Baldwin, K. (2009). Efficacy of garlic as an anthelmintic in adult Boer goats.

Worku, M., Franco, R., & Miller, J. H. (2010). Evaluation of the activity of plant extracts in Boer goats. *American Journal of Animal and Veterinary Sciences*, *4*(4), 72.

Zajac, A. (2002). Controlling goat parasites—Is it a losing battle. *Goat Connection. http://goatconnection. com/articles/publish/article_112. shtml*.

Zajac, A. M. (2006). Gastrointestinal nematodes of small ruminants: life cycle, anthelmintics, and diagnosis. *Veterinary Clinics of North America: Food Animal Practice*, *22*(3), 529-541.

Zeisberg, E. M., Potenta, S., Xie, L., Zeisberg, M., & Kalluri, R. (2007). Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer research*, *67*(21), 10123-10128.

Zhao, X., & Lacasse, P. (2008). Mammary tissue damage during bovine mastitis: causes and control. *Journal of animal science*, *86*(1), 57.

Zygoyiannis, D. (2006). Sheep production in the world and in Greece. *Small ruminant research*, *62*(1), 143-147.

Appendix A

Reagents

l. Acid Citrate dextrose

For 500ml:

22 g trisodium citrate (anhydrous)

8g citric acid

25g dextrose

Bring up to 500 mls with sterile water autoclave and store at4^{o}C. This should be used in

The following proportion 1 ml ACD: 9mI of blood

2. 1.7% Agarose Gels

0.86g

50m1 0.5 X TAE buffer

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

3. DEPC-treated water

1 ml of DEPC to I liter of distilled mixed vigorously and let stand

4. 2.7% saline

27gNaC1

Bring volume up to l liter With DEPC-treated water, autoclave and store at 4'C.

5. 0.0132 M Phosphate Buffer Saline (PBS), pH 7.4

For PMN

Potassium Monobasic, K H_2PO_4

Weight 136.09

13609 x 0.0132 = 1.796g/L

Potassium Phosphate Dibasic

Formula weight 174.183

174.183 x 0.0132=2.299 g/L

Make 2 liters of K_2HP0_4 and 1 liter ofKH_2P0_4in 0.85% saline to make 1500 ml of buffer at a time. Use the KH2P04 to bring pH down to 7.4.

6. 0.85% saline

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

7. Wash Buffer 0.05% Tween20 in PBS, pH7.4

2223g Potassium Phosphate Dibasic, K2HP04

5.62g potassium Phosphate Monobasic, KH2P04

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 of Tween 20 to 1 liter of PBS, mix thoroughly and store at CC.

Appendix B

Protocols

Wright's Staining Procedure

Differential cell counts were by applying a thin smear of whole blood collected from jugular vein of the animals. Differential cell count on whole blood was conducted as followed:

1. Place a drop of whole blood toward the frosted edge of glass slide.

2. Take a glass slide and slide it against blood droplet and push second slide at a 30^0 angle and let dry at room temperature

3 Dip air dried slide in wright's stain for approximately 10 seconds.

4. Decolorize strained slide with deionized water for 10 seconds to wash off excess stain.

5 Rinse slide in deionized water, if for additional 10 seconds and air dry slide prior to reading.

6. Smears were under oil immersion microspore for cell counts.

Differential cells were counted up to 100 for numerical representation of cells 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

Tsai and Frasch Silver Stain

1. Transfer gels from gel cassettes into staining trays filled with. Handle the gels with powder-free gloves.
2. Place on a shaker overnight to fix the samples. Use Powder-free gloves throughout the silver staining procedure. Perform all steps at room temperature.
3. After the overnight fixing step, discard the fixative and add the oxidizer solution. Make fresh daily. Place the gel on the shaker for 5 minutes.
4. Wash the gel in DH_2O for 15 minutes. Repeat this step twice.
5. Incubate the gel 30 minutes in a silver stain. Prepare fresh and follow with three 10 minute washes.
6. Develop the gel in developer solution.

Solutions

1. Fixative

Ethanol	400 ml
Acetic acid	50 ml
DH_2O	550 ml

2. Oxidizer (Prepare fresh daily)

Solution (1)	100 ml
Periodic acid	0.7 g

3. Silver Stain (Prepare fresh daily)

DH_2O	115 ml
NaOH (0.4 g/ 100ml DH_2O)	28.0 ml
*AgNO3 (1 g/5ml)	5.0 ml
*Conc NH_4OH	2.0 ml

*Add last and simultaneously

4. Developer

Citric acid	50 mg
37% Formaldehyde	0.5 ml
DH_2O	1 liter

5. Stop Solution (10% Acetic acid)

Acidic acid	100 ml
DH_2O	900 ml

Western Blot Analysis using Kierkegaard and Perry Reagents

1. Nitrocellulose was cut to gel size and soaked in DH2O.

2. Gel and nitrocellulose sandwich was prepared by placing the nitrocellulose on top of the gel and removing all air bubbles and placing in moist chamber.

Or

Electroblotting using Semiphor Blotter: Soakprecut membrane, blotting paper (Sigma) in Towbin buffer. Prepare sandwich (2 pieces of blotting paper, 0.2 u nitrocellulose membrane, and 2 pieces of blotting paper).

3. Proteins were transferred to the membrane for 2 hours @ 100 mA.

4. The membrane was removed from gel gently and placed in a staining container.

5. The membranes were blocked in KP Milk block for one hour.

6. The membrane was washed for 5 minutes in DH2O, follow with two 5 minutes washes in 1X KP wash.

7. Membrane was probed overnight in first antibody [Wnt5a (Fisher) at a 1:1000 dilution].

8. Repeat step 6.

9. Probe membrane with second antibody Goat anti-Rabbit (BioRad at 1:3000 dilution) for 2 hours.

10. Repeat step 6.

11. Detect specific proteins using a tetramethylbenzidine membrane substrate kit (KP).

Genomic DNA elimination mix

Component	**Amount**
RNA	25 ng–5 µg
Buffer GE	2 µl
RNase-free water	Variable
Total volume	10 µl

Reverse-transcription mix

Reverse-transcription mix Component	**Volume for 2 reactions**
5x Buffer BC3	8 µl
Control P2	2 µl
RE3 Reverse Transcriptase Mix	4 µl
RNase-free water	6 µl
Total volume	20 µl

PCR components mix

Array format:	**96-well plate**
2x RT² SYBR Green Mastermix	1350 µl

cDNA synthesis reaction	102 µl
RNase-free water	1248 µl

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

• Measure 1.25 g Agarose powder and add it to a 500 ml flask

• Add 125 ml TAE Buffer to the flask. (The total gel volume well vary depending on the size of the casting tray)

• Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).

• Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.

• Seal the ends of the casting tray with two layers of tape.

• Place the combs in the gel casting tray.

• Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).

• Carefully pull out the combs and remove the tape.

• Place the gel in the electrophoresis chamber.

• Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

• Add 6 µl of 6X Sample Loading Buffer to each 25 l PCR reaction

• Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.

• Carefully pipette 20 l of each sample/Sample Loading Buffer mixture into separate wells in the gel.

• Pipette 10 µl of the DNA ladder standard into at least one well of each row on the gel.

Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

Running the gel

• Place the lid on the gel box, connecting the electrodes.

• Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red")

• Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – it should not exceed 5 volts/ cm between electrodes!

• Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.

• Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).

• Let the power run until the blue dye approaches the end of the gel.

• Turn off the power.

• Disconnect the wires from the power supply.

• Remove the lid of the electrophoresis chamber.

• Using gloves, carefully remove the tray and gel.

BCA assay

Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	**Volume of Diluent (µL)**	**Volume and Source of BSA (µL)**	**Final BSA Concentration (µg/mL)**
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	700	100 of Stock	250
B	400	400 of vial A dilution	125
C	450	300 of vial B dilution	50
D	400	400 of vial C dilution	25
E	400	100 of vial D dilution	5
F	400	0	0 = Blank

Prostaglandin

Prostaglandin E 2

EIA Standard

Reconstitute the contents of the PGE 2 EIA Standard (Item No. 414014) with 1.0 ml of EIA Buffer. The concentration of this solution (the bulk standard) will be 10 ng/ml. Stored at 4°C; this standard will be stable for up to 4 weeks.

NOTE: If assaying culture medium samples that have not been diluted with EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

To prepare the standard for use in EIA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl EIA Buffer to tube #1 and 500 µl EIA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 1 ng/ml (1,000 pg/ml). Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove

500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

Appendix C

Effect of Nystatin exposure on expression of Cow samples

Table C1

Cow Wnt5a RT2 profiler array results

Gene Symbol	**PBS**	**NYS**	**Fold changes**
AES	21.49	21.55	0.3
APC	21.51	21.36	0.2
AXIN1	21.27	21.26	0.3
AXIN2	36.63	31.21	0.0
BCL9	38.33	31.23	0.0
BTRC	37.17	30.91	0.0
CCND1	30.64	30.19	0.2
CCND2	29.40	29.18	0.2
CSNK1A1	31.32	32.88	0.8
CSNK2A1	29.93	31.14	0.6
CTBP1	30.21	30.51	0.3
CTNNB1	30.55	31.07	0.4
DAAM1	33.42	34.52	0.6
DAB2	32.44	37.04	6.4
DIXDC1	30.51	30.45	0.3

DKK1	30.15	31.52	0.7
DKK3	30.38	30.61	0.3
DVL1	29.66	28.85	0.1
DVL2	30.50	31.01	0.4
FBXW11	31.42	34.39	2.0
FBXW4	29.66	30.32	0.4
FGF4	33.51	33.19	0.2
FOSL1	30.27	30.39	0.3
FOXN1	29.64	30.13	0.4
FRAT1	30.01	29.84	0.2
FRZB	30.21	30.33	0.3
FZD1	29.72	30.06	0.3
FZD3	30.56	33.56	2.1
FZD4	29.80	30.14	0.3
FZD5	29.62	29.70	0.3
FZD6	30.10	30.07	0.3
FZD7	30.35	32.32	1.0
FZD8	30.04	30.33	0.3
FZD9	30.36	30.70	0.3
GSK3A	30.60	31.04	0.4
GSK3B	29.86	29.94	0.3
JUN	30.13	30.55	0.3

KREMEN1	30.33	31.80	0.7
LEF1	31.20	31.91	0.4
LRP5	29.74	29.77	0.3
LRP6	29.86	30.21	0.3
MAPK8	29.86	30.03	0.3
MMP7	29.46	29.45	0.3
MYC	30.04	29.62	0.2
NFATC1	29.37	29.20	0.2
NKD1	29.50	29.27	0.2
NLK	31.78	34.19	1.4
PITX2	29.25	29.23	0.3
PORCN	30.12	29.73	0.2
PPARD	30.16	29.99	0.2
PRICKLE1	30.49	31.63	0.6
PYGO1	29.63	30.22	0.4
RHOA	29.20	28.90	0.2
RHOU	30.24	30.21	0.3
RUVBL1	29.72	29.45	0.2
SFRP1	30.22	30.32	0.3
SFRP4	30.05	30.37	0.3
SOX17	30.61	30.45	0.2
TCF7	30.43	30.47	0.3

TCF7L1	30.05	29.94	0.2
TLE1	30.25	30.48	0.3
VANGL2	30.49	30.94	0.4
WIF1	31.01	32.11	0.6
WISP1	30.41	33.66	2.5
WNT1	32.48	32.26	0.2
WNT10A	30.16	29.84	0.2
WNT11	29.99	30.86	0.5
WNT16	29.23	29.81	0.4
WNT2	30.04	29.48	0.2
WNT2B	29.78	30.10	0.3
WNT3	30.47	31.74	0.6
WNT3A	29.85	29.65	0.2
WNT5A	31.82	31.96	0.3
WNT5B	30.12	30.20	0.3
WNT6	29.47	30.89	0.7
WNT7A	30.30	31.41	0.6
WNT7B	31.14	32.35	0.6
WNT8A	32.28	33.39	0.6
WNT9A	32.08	35.66	3.1
CBY1	29.50	29.29	0.2
DKK2	29.57	29.35	0.2

DKK4	30.31	30.65	0.3
KREMEN2	30.11	30.87	0.4
MAP2K7	30.24	29.66	0.2
ACTB	30.57	31.08	0.4
GAPDH	29.14	29.90	0.0
HPRT1	29.89	30.42	0.4
TBP	31.19	34.42	2.5
YWHAZ	32.40	36.81	5.6
BGDC	29.13	30.34	0.6
RTC	30.80	30.30	0.2
RTC	30.15	29.96	0.2
RTC	29.74	31.09	0.7
PPC	29.97	30.36	0.3
PPC	30.48	29.53	0.1
PPC	31.38	30.89	0.2

Table C2

Human innate and adaptive RT2 profiler results

Position	Gene Symbol	PBS	NYS	Fold change
A01	APCS	38.09		
A02	C3			
A03	CASP1			
A04	CCL2			
A05	CCL5		39.02	
A06	CCR4		36.36	
A07	CCR5		33.18	
A08	CCR6			
A09	CCR8		40.64	
A10	CD14		38.76	
A11	CD4		35.39	
A12	CD40	39.71	37.15	6
B01	CD40LG		35.12	
B02	CD80			
B03	CD86			
B04	CD8A	39.73		
B05	CRP			

B06	CSF2		39.52	
B07	CXCL10			
B08	CXCR3		37.69	
B09	DDX58		37.53	
B10	FASLG		36.27	
B11	FOXP3			
B12	GATA3		34.74	
C01	HLA-A	37.92	39.03	0.5
C02	HLA-E	36.31		
C03	ICAM1		39.98	
C04	IFNA1	36.96		
C05	IFNAR1	38.73	40.51	0.3
C06	IFNB1		39.72	
C07	IFNG			
C08	IFNGR1		29.25	
C09	IL10	37.61	35.72	3.7
C10	IL13		33.29	
C11	IL17A		40.58	
C12	IL18		32.37	
D01	IL1A		30.18	
D02	IL1B		39.20	
D03	IL1R1		39.69	

D04	IL2			
D05	IL23A			
D06	IL4		36.41	
D07	IL5		39.06	
D08	IL6		39.46	
D09	IL8		35.00	
D10	IRAK1		36.48	
D11	IRF3		39.14	
D12	IRF7			
E01	ITGAM		35.98	
E02	JAK2			
E03	LY96			
E04	LYZ			
E05	MAPK1	39.70	30.02	818.3
E06	MAPK8	37.97	29.39	382.8
E07	MBL2			
E08	MPO		40.20	
E09	MX1	38.76	35.93	7.1
E10	MYD88		38.08	
E11	NFKB1	37.41	38.26	0.6
E12	NFKBIA		37.12	
F01	NLRP3	36.09	38.67	0.2

F02	NOD1	39.38	38.77	1.5
F03	NOD2	33.85	36.10	0.2
F04	RAG1	34.96		
F05	RORC		29.27	
F06	SLC11A1	36.40	31.74	25.4
F07	STAT1		40.41	
F08	STAT3		32.64	
F09	STAT4		39.42	
F10	STAT6	39.18	39.09	1.1
F11	TBX21	38.03	37.03	2.0
F12	TICAM1		38.39	
G01	TLR1	32.60	39.09	0.0
G02	TLR2			
G03	TLR3		39.18	
G04	TLR4	36.29		
G05	TLR5	37.19		
G06	TLR6		33.98	
G07	TLR7	38.97	34.24	26.6
G08	TLR8		32.32	
G09	TLR9	39.02	40.30	0.4
G10	TNF	37.63		
G11	TRAF6		35.53	

G12	TYK2	38.78	40.44	0.3
H01	ACTB		29.03	
H02	B2M		40.64	
H03	GAPDH		30.56	
H04	HPRT1			
H05	RPLP0		37.61	
H06	HGDC		40.68	
H07	RTC		30.92	
H08	RTC		30.67	
H09	RTC		30.51	
H10	PPC	21.16	20.87	
H11	PPC	21.15	21.22	
H12	PPC	21.22	21.38	

Table C3

Summary of gene expression

	GAPDH	**TLR2**	**TLR4**	**TNFα**	**WNT5A**	**FZD**	**B CATENIN**
Treatment							
PBS	+	+	-	-	+	+	-
LPS	+	+	-	-	+	+	-
PGN	+	+	-	-	+	+	-

NYS	+	+	-	-	+	+	-
SL	+	+	-	-	+	+	-
LPS/PGN	+	+	-	-	+	+	-
LPS/NYS	+	+	-	-	+	+	-
LPS/SL	+	+	-	-	+	+	-
PGN/NYS	+	+	-	-	+	+	-
PGN/SL	+	+	-	-	+	+	-
NYS/SL	+	+	-	-	+	+	-

+ indicates gene detection, - indicates no gene detection.