2014

The Impact Of Diet On Goat Rumen Microbes And Innate Immune Gene Expression

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Animal Sciences

Major: Animal Health

Major Professor: Dr. Mulumebet Worku

Greensboro, North Carolina

2014

The Graduate School
North Carolina Agricultural and Technical State University
This is to certify that the Master's Thesis of

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

Greensboro, North Carolina
2014

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Biographical Sketch

Ahmed Abdalla was born in Khartoum, Sudan on January 19, 1982. He received his high school diploma from Alfaisal secondary school, Khamis Mushait, KSA. He graduated from University of Khartoum, Faculty of Veterinary science with a Bachelors degree in 2004. After his graduation he worked for central veterinary research laboratory, Soba, Khartoum, Sudan.

Mr. Abdalla joined North Carolina Agricultural and Technical State University as a graduate student in the Department of Animal Sciences in the spring of 2010. He has worked and helped in ongoing research projects in the department under supervision of Dr Mulumebet Worku. Mr. Abdalla has assisted in teaching the lab section of the course Techniques in Biotechnology. He has also worked with high school, undergraduate and graduate students in training on research techniques. His work was presented at national and international conferences including The Association of Research Directors (ARD) 16th Biennial Research Symposium, Atlanta, GA, USA and the ADSA ASAS Joint Annual Meetings, Phoenix, AZ and abstract have been published.

Dedication

I would like to dedicate this project to my family and my uncle Mr. Adil Ibrahim who was my motivation and offered all possible support for my success after Allah. Also I would like to thank all my friends and the Department of Animal Sciences at North Carolina Agricultural and Technical State University. I hope this study will be helpful to producers in the goat industry.

Acknowledgements

I would like to thank God for the guidance, blessing and strength that have allowed me to progress through life and achieve this research. I am grateful to my dad (may God have mercy on his soul) and my mom Mrs. Awatif Ibrahim, my wife Dr Maie Osman, my son, my sisters and all my family for being a strong motivation. I would like to thank Dr. Mulumebet Worku for her guidance, support and her patience through my study. I am grateful to Dr Niki Whitely who provided me with samples and valuable information to conduct my research. Next I would like to thank Mr. Hamid Ismail who helped me performing laboratory techniques and statistical analysis. I would also like to thank some of the graduate students and research assistants Ms. Jamie Williams, Ms. Joi Jackson, Mr. Randall Merriot and Dr. Kwaku Gyenai for helping me with all possible support. Finally, I would like to thank the members of my thesis committee Dr. Ralph Noble, Dr. Salam Ibrahim and Dr. Abraham Woldeghebriel. I would also like to thank the Evans Allen program and the Agricultural research program at NCATSU for their support and offering a great chance to achieve my career goals.

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List of Abbreviations

AE	elution buffer
APS	Ammonium persulphate
ASL	Buffer Stool lyses buffer
AW1	wash buffer 1
AW2	wash buffer 2
BLAST	basic local alignment search tool
bp	base pair
ºC	Degrees Celsius
C	Carbon
cDNA	Complementary DNA
CD14	Cluster of differentiation 14
CGA	Community genome array
CT	Condensed tannins
Cq	Cycle quantity
DNA	Deoxyribonucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence *in situ* hybridization
GIN	Gastrointestinal Nematodes
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour

HT	Hydrolysable tannins
IL-1α	Interleukin-1α
IL-8	Interleukin 8
IL-2Rα	Interleukin 2 receptor α
IFN-γ	Interferon gamma
ICAM-1	Intercellular Adhesion Molecule 1
IP-10	IFN-inducible protein-10
IFNr	Interferon production regulator
KD	Kilodalton
LBP	LPS-binding protein
LPS	Lipopolysaccharide
μg	Microgram
μl	Microliter
MHC I	Class I major histocompatability complex
mRNA	messenger RNA
NK cells	natural killer cells
nM	nano-Mole
N	Nitrogen
ng	nanogram
OD	Optical Density
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PAMPS	Pathogen Associated Molecular Patterns

pg/ml	Pico-gram per microliter
PRR	Pattern Recognition Receptors
RNase	Ribonuclease
RNA	Ribonucleic Acid
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
SSU	rRNA small subunit ribosomal RNA
SL	*Sericea lespedeza*
TAE	Tris-acetate-EDTA
TH1 Cells	T Helper 1 Cells
TNF-α	Tumor Necrosis Factor alpha
TLR-4	Toll like Receptor 4
TEMED	Tetramethylethylenediamine
V	volt
WDATCP	Wisconsin Department of Agriculture, Trade and Consumer Protection
16S rDNA	Sixteen Small Subunit of Ribosomal DNA
16S rRNA	Sixteen Subunit of Ribosomal RNA

Abstract

Gastrointestinal nematodes in goats have developed resistance to chemical anthelmintics, resulting in the need for alternative control strategies. One approach is incorporation of *Sericea Lespedeza* (SL) as high quality forage that suppresses these parasites in goats due to its high condensed tannin (CT) content. However, little is known about its effect on rumen microorganisms and innate immunity in goats. A diverse collection of microorganisms is found in the goat rumen and *Bifidobacteria* is one of the important organisms in the immunity, yet its presence in the goat rumen is not fully studied.

The objective of this study was to evaluate the impact of a diet containing SL on goat rumen microorganisms, *Bifidobacteria* and on the goat's innate immune gene expression. Microbial DNA was isolated from rumen samples using the QIAamp DNA kit (Qiagen, USA) to compare rumen microbes and *Bifidobacteria* in control group and group that received SL in diet. The isolated microbial DNA was amplified using 16S rDNA universal bacteria and *Bifidobacterium* genus specific primers to compare between goats that received SL in diet and control groups by PCR/ DGGE analysis. Blood was collected for immune markers and pro-inflammatory cytokines profiling, as well as total serum protein analysis. Serum was extracted and used for evaluation of secretion of pro-inflammatory cytokines (TNFa, IFNr, GCSF, GMCSF, IL-1a, IL-8, IP-10 and RANTES) using a commercial Enzyme-linked immunosorbent assay kit.

Analysis of DGGE results showed different band patterns between control and animals that received SL diet. A 580-bp *Bifidobacterium* specific band was observed in control group and no band was detected in goats fed a diet containing SL ($P < 0.002$). Gene expression profiling of innate immunity markers (CD-14, TLR-2, TLR-4, IL-10, IL-8, IL-2, INF-γ, and TNF-α) were found to be higher in treatment animals compared to controls. *Sericea Lespedeza* diet affected

the secretion of pro-inflammatory cytokines by increasing their level in serum ($p < 0.0002$), except for IL-8, and RANTES in goats fed SL in the diet were decreased ($p < 0.0001$). This suggests that tannins from SL affect the goat's innate immune response and rumen microbes.

CHAPTER 1

Introduction

Studies indicate that nematodes of goat represent a great hazard for goat health and production in hot humid areas such as southeastern United States (Sahlu et al., 2009). Internal parasites pose the greatest challenge to goat production in humid areas largely due to anthelmintic resistance. *Haemonchus contortus* is a common pathogenic nematodes that spread in humid climates of the US and worldwide. The extensive use of anthelmintics to control nematode infection over the past years, resulted in a significant level of anthelmintic resistance (Burke et al., 2011). Alternative control strategies including immunity enhancement via nutrition, vaccination, pasture management , and genetic resistance are required (Sahlu et al., 2009). Tannin containing plants like SL may give a possible alternative to control nematode infection (Burke et al., 2011). Feeding SL hay to goats significantly reduced fecal egg count (FEG) and increased packed cell volume (PCV), however little is known about the effect of SL diet on rumen microorganisms and innate immunity (Shaik et al., 2006) (Burke et al., 2011). Tannin in SL can cause fluctuations in the rumen microbes, which found to play significant role in animal health (Kamra, 2005; Pitman, 2006).

The innate immune system is composed of physical, chemical and microbiological barriers that include the normal rumen microorganisms. A diverse collection of microorganisms has been shown to be exist in the goat rumen (Beutler & Hoffmann, 2004). *Bifidobacteria* is an important organism in the immunity of the gastrointestinal tract and its presence in the goat rumen is not fully studied. *Bifidobacteria* populations have been detected in the gastrointestinal tract, where they can provide health promoting benefits to the host (Volkova, 2006). Plants containing tannin are able to modulate immune response by the effect on immune cells like T

cells resulting in changes in the expression of innate immunity markers and cytokines secretion (Graff et al., 2009). The objective of the current study was to explore the effect of a diet containing SL on rumen microbial population especially *Bifidobacteria* and on innate immunity marker gene expression.

CHAPTER 2

Literature Review

2.1 **Goats**

Goats belong to the Bovidae family within the suborder of ruminants including chevrotain, deer, elk, caribou, moose, giraffe, okapi, and antelope. Along with suborder of camel, swine, and hippopotamuses, goats make up the even-toed hoofed animals of Artiodactyla, which evolved 20 million years ago, and are classified as herbivorous. 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 characterized by the presence of rumen, which is the site of initial digestion of the animals. The rumen forms the first chamber in the alimentary canal of ruminant animals. Ruminant animals have a four chambered stomach the rumen, reticulum, omasum and abomasums. The rumen serves as the primary site for microbial fermentation of ingested feed. After being swallowed, bolus travels down the esophagus and is deposited in the rumen and contractions of the reticulorumen propel and mix the recently ingested feed into the rumen mat (Cronjé, 2000). The material is a thick mass of digesta, consisting of partially degraded, long, fibrous material. Most material in the mat has been recently ingested, and as such, has considerable fermentable substrate remaining. Digestion in the reticulorumen is a complex process and occurs through fermentation by microbes in the reticulorumen. The reticulorumen is one of the few organs present in animals in which digestion of cellulose and other recalcitrant carbohydrates can proceed to any appreciable degree (Cronjé, 2000).

Goat industry is growing in the United States, especially with population growth and demographic changes lead to higher demand for goat production (Maxey et al., 1996). Goat

industry in the United States has undergone excessive changes in the last two decades (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 elimination and the increasingly diverse ethnicity of the American public (Sahlu, 2009).

Goats are hardy animals in general but, they are still susceptible to a number of diseases. Among the conditions affecting goats are internal parasites, respiratory diseases such as pneumonia, foot rot, pregnancy toxemia and feed toxicity. Goats are also susceptible to various viral and bacterial diseases, such as foot and mouth disease, caseous lymphadenitis, pink eye, and mastitis. They can transmit a number of zoonotic diseases to people, such as brucelosis, tuberculosis, Q-fever, and rabies (Smith, 1994).

Some studies about the goat industry have focused on emergence of parasite resistance to chemical anthelmentics as a major obstacle to the goat industry and food safety (Thomas H Terrill et al., 2001). Gastrointestinal nematodes have developed resistance to chemical anthelmintics thus 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, the presence of residual antibiotics in foods of animal origin and the increasing interest for organic production has led to the search for other kinds of disease preventive strategies.

2.2 Internal Parasites and Use of Plants as Anthelmintic

Parasitic infection can cause excessive economic loss for producers through deterioration of the herd health and loss of production (Miller & Craig, 1996). Helminth infections are a main obstacle for loss of production in livestock, particularly those owned by the poor worldwide (Githiori, Athanasiadou, & Thamsborg, 2006). Traditionally a number of plant remedies deemed

suitable for each parasitic disease. Seeds or the foliage of plants such as garlic, onion, mint, walnuts, dill, or parsley have been used to treat animals that suffer from gastrointestinal parasitism, while cucumber and pumpkin seeds have been associated with the expulsion of tapeworms from the gastrointestinal tract (Githiori et al., 2006). A group of plants with denoted anthelmintic properties have also been included in the British pharmacopoeia (Githiori et al., 2006). Example of these plants included, oil of chenopodium that derives from *Chenopodium ambrosioides*, was used for many years in the UK to treat nematode parasite infections (Strongylus, Parascaris and Ascaris). Several controlled studies, both in vitro and in vivo was in some cases supported (Hördegen, Hertzberg, Heilmann, Langhans, & Maurer, 2003) (Githiori et al., 2006), while other were rejected such as (Githiori, 2004). A number of reports indicated the nutritional effects of feeding CT containing forages such as SL, primarily because of their influence on protein metabolism in animals (Shaik et al., 2006) (Thomas H Terrill et al., 2001) (T. Terrill, Windham, Hoveland, & Amos, 1989).

The most commonly used strategies in controlling gastrointestinal nematodes infection in the US is frequent treatment with anthelmintic drugs, but this strategy has led to greatly increased prevalence of anthelmintic resistance in goats (Mortensen et al., 2003) (Shaik et al., 2006), and resistance has been reported to drugs within all three major classes of anthelmintics (Thomas H Terrill et al., 2001). Those studies further emphasized the need for alternatives of anthelnintics like SL.

Moist and warm pastures are optimum for nematodes like *Haemonchus* worms and the parasite can spread quickly among the herd resulting in great losses(Miller & Craig, 1996). Control of *Haemonchus contortus* for example, is important for healthy goat farming and production. Use of SL with feed to control internal parasites and especially Haemonchus was

proposed by (T. H. Terrill et al., 2007). The need for understanding of different effects that SL supplementation induces in goats from health perspective is necessary.

2.3 *Haemonchus contortus*

Haemonchus are gastrointestinal nematodes of small ruminants that cause anemia, emaciation, edema and gastric disturbance. *Haemonchus contortus* is a pathogenic gastrointestinal nematode (GIN) parasite infecting small ruminants. It is a blood-feeding, abomasal worm, and growing animals are the most susceptible to infection. Adults can also be susceptible under heavy infection conditions.

Primarily affecting small ruminant production in subtropical and tropical areas, the geographic range of *H. contortus* is worldwide, and it can be found in temperate areas (Waller, 2006). As a highly pathogenic blood-feeder, this parasite is a major challenge for goat production in southeastern USA, with the rapidly increasing demand for grass-fed or organically produced livestock, and the growing popularity of specialty meat-type goat (South African Boer, New Zealand Kiko), increased the chances of *H. contortus* infection in goats in this region. The generally warm, moist environmental conditions that favor year-round pasture growth in southeastern region of the USA are also ideal for development and survival of the free-living stages (eggs and larvae) of gastrointestinal nematodes (GIN), particularly *Haemonchus contortus*, the ‘barbers pole’ worm (Maxey et al. 1996).

This parasite causes a major problem in the goat industry in that region especially with the development of chemical anthelmintic resistance which necessitates the use of plants alternatives (Githiori et al., 2006).

2.4 *Sericea lespedeza*

One of the early methods to cure diseases was the use of plant extract (Waller et al., 2001). Research and development of plant alternatives as a cheaper and more sustainable control and new management programs work together for better economics and animal health benefits (Worku, Franco, & Miller, 2009). The method used involved use of fresh or dried plants; plant parts or extracts; and plants or their extracts in combination with other compounds such as copper (Athanasiadou, Githiori, & Kyriazakis, 2007). Research to support ethnobotanic information on alternative anthelmintics and should iclude measurements of animal performance, immunity and behavior (Athanasiadou et al., 2007).

Sericea Lespedeza is native to the continent of Asia and can be grown in various areas that include pasture, rangelands, roadsides, eroded slopes, ditches, fence rows, and prairies. Wherever it is grown, there must not be heavy shade, since it is intolerant to shade. Because it can adapt to acidic soils with low fertility, it has potential for being a very useful low-input forage (Burke et al., 2011) (Puchala, Min, Goetsch, & Sahlu, 2005). It is also drought and insect tolerant as well as highly rich in crude protein. In spite of these positive qualities of SL, it is often classified as a weed in some states. In the Midwest it is considered a noxious weed because it is considered unpalatable to grazing beef cattle (Shaik et al., 2006) (Burke et al., 2011).

Sericea Lespedeza is a legume containing CT and resists drought, planted widely throughout the southern USA as a grazing, hay, soil restoration and conservation crop. Condensed tannins are types of secondary compounds found in some plants and are classified based on their chemical structure, they are found tremendously in leaves of plants from grasses and legumes (Mueller-Harvey, 2006). *Sericea Lespedeza* has been a major focus of current condensed tannin research, where grazing or feeding (dry products such as hay and pellets) has

been shown to reduce FEG, reduce worm burdens and, in some cases, negatively affect larval development and survival in the feces (Burke et al., 2011). Consuming plants high in tannins have been found to be also effective in reducing internal parasites in herbivorous animals (Min et al., 2005). The mode of action of the CT is not fully understood but, it has been speculated that while in the abomasum, SL builds a complex around the vulva of adult *H. contortus* females (Burke et al., 2011) (Paolini et al., 2003). This would have an effect on the eggs being laid and passed out in the feces. This may be why consumption of SL (and other CT containing plants) has led to reduced fecundity of female worms (Burke et al., 2011).

2.5 Tannins

Tannins are long-chain polyphenolic secondary compounds that can bind with protein and other macromolecules in aqueous solutions (Frutos, Hervás, Giráldez García, & Mantecón, 2004). The high affinity of tannins for proteins lies in the formers' great number of phenolic groups (Frutos et al., 2004). Scientific reports showed that tannins have traditionally been divided into two groups: the condensed and the hydrolysable tannins. Hydrolysable tannins (HT) are made up of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids (mainly gallic and hexahydroxydiphenic acid). The CT, or proanthocyanidins, are non branched polymers of flavonoids units, and usually have a higher molecular weight than the HT (1000-20000 Da compared to 500-3000 Da) (Frutos et al., 2004; McLeod, 1974; Mueller-Harvey & Caygill, 1999). It is known that in the living plant cells, both HT and CT molecules are isolated within the cell in vaculoles, and believed to be only released into the cytoplasm when cell damage or death occurs. It is now recognized that CT may occur in either a 'free (soluble)' or a bound form to either protein or cell-wall carbohydrate, and that only the soluble CT depresses in vitro protein and fiber digestibility (Brooker, 2001). Researchers described condensed tannins as

flaven-3-oligomers carrying varying degrees of oxidation on the A and C rings of each monomer and that the chirality of C-4, where linkage of monomers occurs, and C-3 open up the possibilities for considerable structural variability (Brooker, 2001).

The major interesting feature about tannins is its complexation with protein (Brooker, 2001). Scientific references indicated that the very obvious precipitation that occurred when tannins and proteins were mixed and the critical nature of the formation of stable complexes resistant to microbial degradation in the leather industry has lead to a view that complexation is irreversible. While this is true under some circumstances, it is far from being always the case (Brooker, 2001). There has been ample evidence that the interaction of tannin and protein is an event with a very variable outcome and that complexation without precipitation or with reversible precipitation is not infrequent (Brooker, 2001). Studies on tannins chemistry illustrated that particular tannin is now known to exhibit different affinities for different proteins and the extent of that variation is considerable. Proteins with an open structure and those rich in the amino acid proline appear to have a particularly high complexation coefficient while glycoproteins, globular proteins and those of low molecular weight have low affinities. The high proline content of the salivary proteins of some mammalian herbivores has attracted attention as a possible pre-digestion process for the elimination of anti-nutritive tannins(Austin, Suchar, Robbins, & Hagerman, 1989; Brooker, 2001).

Tannins interfere in nutrition of ruminants through prevention of digestion and absorption of protein until it reaches the abomasum and favorably yield more high quality protein (Brooker, 2001) (Barry & McNabb, 1999). Studies indicated that the major benefit of tannins in feed has been thought to be the protection of plant proteins from digestion in the rumen and their subsequent release as protein available for digestion and utilization by the ruminant (Brooker,

2001). Studies with *Lotus* spp of varying CT content (2.2% and 5.5%) have confirmed that tannins do protect dietary proteins from digestion in the rumen, increase the flux of essential amino acids (EAA) to small intestine, and at low CT concentrations, increase the apparent absorption of EAA in the intestines (Brooker, 2001; Waghorn, Jones, Shelton, & McNabb, 1990). Some studies also, suggested that CT in temperate forages can be used to improve the efficiency of nitrogen (N) digestion and to increase the productivity of grazing animals (Barry & McNabb, 1999). In one of the studies performed to test the effect of cassava leaves, containing CT, total serum protein were found to be increased in serum as goats receive higher level of cassava leaves in diet (Oni et al., 2012).

The tannins of numerous plant species help to control certain internal parasites of animals, for example the nematode *Trichostrongylus colubriformis.* It is speculated that the positive effect on the host animal might be associated with a direct negative effect on the parasites themselves plus an indirect effect in the form of increased availability and digestive utilization of protein (Frutos et al., 2004). It is also well documented that CT help in bloat prevention in ruminants (Frutos et al., 2004). Literature showed that when these animals graze on leguminous plants containing CT (for example *Onobrychis viciifolia*) there were unquestionable benefits in the prevention of bloat (Frutos et al., 2004; McMahon et al., 2000).

The HTs are reported to be usually highly toxic to non-ruminants, but less toxic to ruminants because they may be degraded by either acid or enzymatic hydrolysis in the rumen, and absorbed phenolics excreted in urine as glucuronides (Brooker, 2001). The HTs toxicity has been found usually associated with rates of ingestion which exceed the rumen capacity for degradation. Absorbed HT may cause liver and kidney necrosis, jaundice, photo-sensitization and death in severe cases (Brooker, 2001). The toxic effects of CT are less well understood, but

generally binding to plant proteins and cell wall carbohydrates decreases the digestibility of usually protein and sometimes fiber (Brooker, 2001) (Van Soest, Conklin, & Horvath, 1987). Nevertheless, recent scientific findings indicated that feeding ruminants forages containing CT such as calliandra, *Acacia* and

Mangifera sp leaves, contained high (6–9%) levels of CT while keeping total tannin content in the diet relatively low with the highest (1.2%), there was no toxicity due to tannins (Tangendjaja & Wina, 1999) (Brooker, 2001).

Tannins can also impair the digestive process by complexing with secreted enzymes. Moreover, tannins have anti-nutritive effects through their association with their ability to combine with dietary proteins, polymers such as cellulose, hemicellulose and pectin, and minerals thus retarding their digestion (McSweeney, Palmer, McNeill, & Krause, 2001). Depending on their chemical nature and concentration in feedstuffs, tannins may be valuable to ruminants due to positive effects such as protein sparing action and anthelmintic activity (Singh, Chaudhary, Agarwal, & Kamra, 2011) (Mahmood et al., 2007).

Condensed tannin have also been shown as a suppressive substance for rumen bacterial growth and/or alter their metabolism (Rojas et al., 2006). Several examples in the literature included, animals fed on tannin rich *Calliandra calothyrsus*, showed considerable reduction in the population of *Ruminococcus* spp. and *Fibrobacter* spp. (Singh et al., 2011).

2.6 Rumen Microbiology

The inhabitants of the rumen microbial ecosystem, a complex consortium of different microbial groups living in symbiotic relationship with the host, act synergistically for the bioconversion of lignocellulosic feeds into volatile fatty acids which serve as a source of energy for the animals (Kamra, 2005). The microbial ecosystem of the rumen is well studied in

domesticated animals like cattle, sheep and goats (Kamra, 2005). This microbial population includes members that belong to the three domains of life: Eubacteria (bacteria), Archaea (methanogens), and Eukarya (protozoa and fungi) (Maxey et al., 1996).

Bacteria constitute the most significant member of the microbial population based on cell mass (>50%), number (10^{10} to 10^{11}/g of contents), small subunit ribosomal RNA content and contribution to ruminal fermentation (Maxey et al., 1996). Based on evolutionary lineage and molecular characteristics, methanogens are placed in a new domain, called Archaea (meaning antiquity; Nagaraja, 2012).

There are two types of protozoa in the rumen, flagellated and ciliated. The flagellated protozoa do not exceed 10^3 per g of contents and their contribution to ruminal fermentation is insignificant. Ciliated protozoa constitute a significant portion of the microbial cell mass (range from 0 to 50%) and include a variety of morphological types that are broadly grouped under holotrichid and entodiniomorphid ciliates (Maxey et al., 1996).

Fungi in the rumen are characterized by a specialized, two-stage life cycle. There is the zoosporic stage consisting of actively motile, flagellated spores that attach to feed particles, germinate, and develop into vegetative stage consisting of mycelia structure. Because of the two-stage life cycle and the ability of the mycelial structures to grow extensively on feed particles, it is not possible to quantify fungal cell mass in the rumen. However, based on some indirect estimates, fungi are believed to account for about 10% of the microbial mass (Maxey et al., 1996). The rumen also has bacteriophages (viruses that infect bacteria) that were first recognized by electron microscopic observations of ruminal contents. As many as 10^{11} phage particles have been counted per g of ruminal contents and more than 125 different morphological types have been described according to the scientific reports (Maxey et al., 1996).

The rumen harbors various types of bacteria which are active in degradation of legnocellulosic components of the feed, (D. N. Kamra, 2005). The microbial ecology of the rumen can be modulated by environmental factors such as the type of nutrition. In current study we focused on the effects of SL diet with high CT content on rumen microorganisms, yet several research works indicated that one important adaptation of ruminal metabolism to counter anti-nutritive effects of forage tannins may involve the microbial degradation of these compounds. This seems logical since simple plant phenolics undergo varying degrees of transformation and degradation by microorganisms during passage through the gastrointestinal tract of ruminants (McSweeney et al., 2001).

Bifidobacteria as an example, proliferate in the rumen especially when the animals are fed carbohydrate rations (Trovatelli & Matteuzzi, 1976). Presence of *Bifidobacteria* in the rumen of calves fed different rations was studied to obtain the effect of these different rations on *Bifidobacteria* (Trovatelli & Matteuzzi, 1976). Despite the important role of *Bifidobacterium* in animal health and food microbiology, yet its presence and role as probiotic bacteria in goat rumen is poorly studied. Scientific reports indicated that tannins are generally regarded as inhibitory to the growth of microorganisms but the mechanisms involved are poorly understood. One of the suggested explanations of tannin inhibitory effect is its ability to complex with bacteria through reactivity of polyphenolic group with the cell wall of bacteria and the secreted extracellular enzymes. Either one of these interaction is likely to inhibit the transport of nutrients into the cell and retard the growth of the organism (Bell, Etchells, SINGLETON, & SMART, 1965). Some of the methods explained effects of tannins on rumen microorganisms like *Fibrobacter succinogenes* , based on cell associated and extracellular endoglucanase activity which inhibited by CT (100–400 ug/ml) from *Lotus corniculatus* under in-vitro conditions

(McSweeney et al., 2001) (Bae, McAllister, Yanke, Cheng, & Muir, 1993). Growth of proteolytic bacteria (*Butyrivibrio fibrisolvens*, *Ruminobacter amylophilus* and *S. bovis*) was reduced by condensed tannins but a strain of *Prevotella ruminicola* was tolerant of CT (<600 ug/ml) from *Onobrychris viciifolia* (McSweeney et al., 2001) (Jones, McAllister, Muir, & Cheng, 1994). Other studies indicated that fiber degrading ability of rumen fungi may be less sensitive to the inhibitory effects of condensed tannins compared with cellulolytic bacteria (McSweeney et al., 2001). Through this review, the significance of rumen microbes is clearly established and in our study we tried to better understand the effect of CT from SL diet on rumen microorganisms.

2.7 Bifidobacteria

Bifidobacterium is a genus of gram-positive, non motile, often branched anaerobic bacteria inhabiting the gastrointestinal tract and vagina (Schell MA, 2002). *Bifidobacteria* are one of the major genera of bacteria that make up the gut flora. *Bifidobacteria* aid in digestion, associated with a lower incidence of allergies (Björkstén B, october 2001) and used as probiotics. Before the 1960s, *Bifidobacterium* species were collectively referred to as "*Lactobacillus bifidus*" (Schell MA, 2002). *Bifidobacteria* exert a range of beneficial health effects, including the regulation of intestinal microbial homeostasis, the inhibition of pathogens and harmful bacteria that colonize and/or infect the gut mucosa, the modulation of local and systemic immune responses, the repression of procarcinogenic enzymatic activities within the microbiota, the production of vitamins, and the bioconversion of a number of dietary compounds into bioactive molecules (Mayo, 2010). Therefore, these bacteria play an important role in the modulation of gastrointestinal tract, antagonism against pathogenic microbes and maintain the intestinal mucosal barrier. Some bacterial strains possess remarkable anticarcinogenic and

anticholesteolemic effect, produce a range of metabolic substrates which are subsequently utilized by the host and are able to stimulate the immune system (Volkova, 2006). *Bifidobacterium* species are also known to be used as a probiotic which is believed to induce health promoting effects in humans and animals. They favorably alter the intestinal microflora balance (Collado & Sanz, 2007) , inhibit the growth of harmful bacteria (Lahtinen et al., 2009) , promote good digestion (Salazar, Gueimonde, Hernandez-Barranco, Ruas-Madiedo, & de los Reyes-Gavilan, 2008), boost immune function, and increase resistance to infection. Some studies indicated that, the presence and species of *Bifidobacteria* in goat rumen and the true extent of their distribution I n the gut is unknown because most of rumen organisms cannot be cultured ex vivo, and their culture takes a long time and many steps to determine the specific bacterial species (Collado & Sanz, 2007; Shi et al., 2007; Volkova, 2006).

One of the researches shed the light on *Bifidobacteria* distribution in the rumen of ruminants a study was conducted to determine the numbers and species of *Bifidobacteria* presence in the rumen of calves fed high-roughage and high-concentrate diets. With the roughage ration the *Bifidobacteria* were not detectable in a 10^{-3} dilution, whereas with the concentrate ration their number was high, usually in the order of 10^{8} to 10^{9}/ml of rumen fluid. Some *Bifidobacteria* detected by using hybridization tests, included *Bifidobacterium ruminale*, *Bifidobacterium globosum*, and an apparently new species (Trovatelli & Matteuzzi, 1976). However, the presence and role of *Bifidobacteria* in goat rumen is not clearly established (Havenaar, 1992; Volkova, 2006).

Bifidobacteria strains that survive and grow under rumen-like conditions, resist gastric acidity and the presence of lysozyme, bile salts and pancreatic enzymes are poorly studied (Draksler, Gonzáles, & Oliver, 2004). However there is interest in the use of *Bifidobacteria* as a

probiotic in goat feed supplement. Moreover, isolation techniques lack the ability and precision to detect *Bifidobacteria* in goat rumen (Volkova, 2006). Thus, traditional culture-based microbiology techniques are not precise enough to study *Bifidobacteria* in a diverse ecosystem like the rumen. Recently, culture based and non culture based analyses of the bacterial diversity revealed that Bifidobacterial DNA has been detected in goat fecal samples (Lamendella, Domingo, Kelty, & Oerther, 2008) but further studies are needed. In this study we hypothesize that *Bifidobacteria* strains can be detected in goat rumen using the Polymerase Chain Reaction (PCR) and can be sequenced later to identify their specific phylogentic information.

2.8 Use of Molecular Techniques in Rumen Microbiology

The identification and enumeration of a specific species of bacterium in the rumen ecosystem is difficult due to the large number of biochemical tests to be performed and lack of sensitivity and specificity of the techniques. In addition, a very large proportion of rumen microbes (like in any other ecosystem) are non culturable, but live in the rumen. The large number of non culturable bacteria in the rumen may due to the fact that the number of total viable bacteria in a particular medium is much lower than its microscopic count. Moreover, single culture medium which can support growth of all the culturable bacteria of rumen is unavailable. Therefore, whatever information is available on the culturable bacteria of the rumen is incomplete. The classification of rumen bacteria based on phenotypic characteristics and biochemical tests is not sufficient to study the diversity among the culturable organisms. In addition, with the development and application of a variety of cultivation-independent, molecular techniques, it has become clear that cultivation-based methods have only identified approximately 10 to 20% or less of the total microbial population harbored in the rumen (Maxey

et al., 1996;(Hördegen et al., 2003). Therefore, improved techniques are needed for quantifying specific microbes in this complex ecosystem.

Developments in molecular biology had provided new approaches. Literature described nucleic acid based techniques primarily through the use of the RNA sequence associated with the small subunit ribosome, 16S rRNA for bacteria and methanogens, 18S rRNA for protozoa and fungi, and analyses of genes (metagenomics and genomics) and gene expressions (transcriptomics) have been employed to understand the structure and function of the microbial community of the rumen (Maxey et al., 1996).

Variations in the numbers of rumen bacteria can be studied easily by using different oligonucleotide DNA probes, homologous to some regions of bacterial sixteen subunit of ribosomal ribonucleic acid (16S rRNA) (Koizumi et al., 2002). Some papers indicated the use of universal probe to estimate total microbial mass and a species-specific probe can be used to determine relative proportions of total ribosomal ribonucleic acid (rRNA) of a species of a particular bacterium (rumen microbes and *Bifidobacteria* in this review)(Briesacher et al., 1992). Scientific literature revealed that the ratio of sixteen subunit of ribosomal ribonucleic acid (16S rRNA) to the total cellular RNA does not change over the growth curve, thus 16S rRNA can be used as a measure of total bacterial population in the rumen (Briesacher et al., 1992). Specific DNA probes can also be used to enumerate a single bacterial strain in a mixed population if the concentration of this particular bacterium exceeds 10 million cells per ml of the rumen content (Attwood, Lockington, Xue, & Brooker, 1988). Several studies indicated that nucleic acid probes can be used to identify specific catabolic functions (Sayler et al., 2001), and resolve population differences using (Githiori et al., 2006). Molecular techniques are more robust and quicker way to investigate rumen microbes under different treatment. Microarray

technology as an example, is becoming popular because up to 10 000 probes can be immobilized on a glass support, making high-throughput processing possible when a large number of targets or samples are being evaluated(Githiori et al., 2006). In their study they describe the development and application of a community genome array (CGA) approach based on the immobilization of whole rumen bacterial chromosomes (the probes) on a nylon support. CGAs were applied to rumen samples obtained from sheep that had been consuming tannin-containing diets consisting of acacia angustissima to assess the effects of this plant on rumen microbial populations. Their studies were also validated with Northern analysis of key microbial species (Githiori et al., 2006). Scientific studies showed that PCR primers have been designed and revalidated for the detection of 13 species of rumen bacteria and these used with real-time PCR for quantification of bacteria in the rumen microbial ecosystem (Tajima et al., 2001).

Some researchers included molecular schemes based on ribosomal RNA and DNA (16S/18S rRNA/rDNA) used to analyze rumen microbial ecology. The analysis based on 16S/18S rRNA/rDNA of a microbial community relies on the techniques of nucleic acid amplification, cloning and sequencing to acquire data on 16S/18S rRNA/rDNA sequences directly from rumen samples or medium. Sequence comparison serves to place the novel species in the existing phylogenetic classification if the new 16S rDNA similarity is less than 95% (Amann, Ludwig, & Schleifer, 1995) equal to 97% (Deng, Xi, Mao, & Wanapat, 2008) or 98% (Deng et al., 2008; Tajima et al., 2001) by online similarity search using the basic local alignment search tool (BLAST) (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2004; Madden, Tatusov, & Zhang, 1996) and Ribosomal Database Project (RDP) (Mao, 2007). Although 97% similarity is frequently defined the classification threshold in most articles.

Hybridizations using phylogenetic-group-specific probes are often used as a prelude to direct the amplification/cloning/sequencing studies(Mackie et al., 2003). Probes designed to specifically hybridize to the sequence can be used to detect or quantify cell abundance and activity, study spatial distribution, monitor enrichment and isolate newly unidentified community members (Amann et al., 1995; Kane, Poulsen, & Stahl, 1993). Universal probes are used to quantify the amount of rRNA contributed by all populations present in samples (Zheng, Alm, Stahl, & Raskin, 1996). Absolute or relative abundance of a given sequence or organism can much more reliably be determined by fluorescence *in situ* hybridization (FISH) or blot hybridization such as Southern and Northern, respectively(Dehority, Tirabasso, & Grifo, 1989).

In the present study, universal bacterial PCR primers of the 16S rDNA were used in DGGE analysis and gel electrophoresis to monitor the changes on rumen microbes and *Bifidobacteria*.

2.9 Innate Immunity

The immune system consist of several parts each plays specific role to protect the host against pathogenic agents. Immunity is divided into two major categories, innate immunity and adaptive immunity, yet these categories overlap (Provenza & Villalba, 2010).

The innate immune system considered to be the first line of defense (Dietrich, 2002), works through immune mechanisms and cells that perform their role in nonspecific manner and have no memory. The innate and adaptive immune systems work together as integrated system. Innate immune systems provide immediate protection against infection, and are found in plant and animal (Alberts, 2002). The innate immune system is divided into three functions: Barrier function, Phagocytosis, and inflammation. These functions are mediated by a network of innate immune cells. Interfaces between the host and the environment, such as the skin, mucosa, and

intestine, highlight the interconnectedness of these three functions (J. S. Holderness, 2012). Innate and adaptive immune systems are regulated by a complex network of chemical signals, including enzymes, immunoglobulins and cytokines (Provenza & Villalba, 2010). Throughout these barriers are cells containing receptors to detect pathogens and induce immune programs to destroy them, either by altering the environment or recruiting other cell mediators to do so. These barriers also regulate inflammatory responses since overt inflammation destroys the integrity of this barrier and exposes the host to the external environment (J. S. Holderness, 2012). At the gut mucosal level, the innate immune response not only provides the first line of defense against pathogenic microorganisms but also provides the biological signals that instruct the adaptive immune system to elicit a response. Noncommensal and probiotic bacteria are also able to induce a gut mucosal immuneresponse (Perdigo´n, 2001). Toll-like receptor (TLR) signaling by commensal bacteria plays an essential role in maintaining mucosal immunity (Imler & Hoffmann, 2001).

To maintain these functions, cells of the innate immune system must sense damage and/or the presence of pathogen. This sensing of the external environment is mediated by a large number of receptors called Pattern Recognition Receptors (PRR), which allow the host immune system to rapidly control disease and tissue damage by recruiting appropriate cells to sites of inflammation (J. S. Holderness, 2012). Of particular interest to early innate responses is the γδ T cell. Early in infection, many immune responses are regulated by the γδ T cell's ability to respond to host pathogen associated molecular patterns (PAMPs), and thus eliciting appropriate responses (J. S. Holderness, 2012).

Many plant products are taken as nutritional supplements for their ability to activate innate immune responses and there are numerous examples of this type of activity. Several

studies showed herbal products have been used as effective treatments for millennia and their use is increasing rapidly, yet in many cases their safety and specific mechanisms of action are not well defined (Graff et al., 2009). Those herbal products like Yamoa™ derived from *Funtumia elastica* tree, can induce immune response through plants compounds that affected innate lymphocytes, especially γδ T cells (Graff et al., 2009). Our study is an attempt to shed the light on SL effect on innate immune response through monitoring several innate immunity markers. γδ T cells have the capacity to rapidly mediate a large array of effector activities and are recognized as an important cell of the innate immune system. γδ T cells are potent cytolytic cells, and produce an array of cytokines that enhance the activities of macrophages, neutrophils, and other lymphocytes. γδ T cells can also present antigen, induce or suppress inflammation, and are important to the health of epithelial cell monolayers (Graff et al., 2009). Scientific literature explained that some herbal products can induce transitional activation of γδ T cells, antigen-independent priming, that is characterized by increased expression of a few cytokines, such as interleukin 8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF), cell surface proteins, such as interleukin 2 receptor α (IL-2Rα, CD25), and increased responsiveness to downstream stimuli (Graff et al., 2009).

A common effect described for plant polyphenols is their ability to impact the immune system; however, these responses are highly variable (J. S. Holderness, 2012). Tannins are phoenolic plant compound found in SL, in this research change in immune profile of goat is anticipated based on these available literatures. There are numbers of structures that polyphenols can form and the wide array of either inflammatory or anti-inflammatory responses that polyphenols can generate (J. S. Holderness, 2012). One of the identified inhibitory effects of

anti-inflammatory polyphenol EGCG on the MyD88 pathway, which involved in activation of TLRs (J. S. Holderness, 2012).

Several types of chemicals are able to modulate immune response have been identified in mammals, including proteins such as interferon gamma (IFN-γ) and granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (GMCSF) (Schepetkin & Quinn, 2006). Besides these signaling compounds from the immune system, some carbohydrates isolated and purified from microorganisms have immune stimulating properties such as lipopolysaccharide (LPS). Scientific research revealed that some plants compounds like polysaccharides derived from Acai berry are able to activate human γδ T cells and monocytes and consequently, helped in protection against *F. tularensis* and *B. pseudomallei* (Hördegen et al., 2003). Their study concluded that the protective effect of Acai berry primarily due to augmentation of NK cell IFN-γ secretion. The present study prospective focus on evaluation of innate immune markers through comparing gene expression profiles of control group and SL treated group.

2.9.1 Toll like receptors (TLRs). Literature denoted that for innate immune system to function, it must first recognize changes to its environment (J. S. Holderness, 2012). This is done, in large part, by the expression of PRR that sense common patterns associated with disease or cell damage. Originally, the TLR were discovered in drosophila and its deletion from the fly resulted in spontaneous colonization with commensal microbes. According to scientific research TLRs represent a mechanism for the innate immune system to deliver a rapid response to a changing environment (J. S. Holderness, 2012). Ten TLRs have been identified recognize many molecular patterns from gram-positive bacteria, gram-negative bacteria, viruses, and other parasites (J. S. Holderness, 2012). Cytokines associated with TLR activation include TNFα, IL-6, and IL-1β, as well as chemokines, such as IL-8 (J. S. Holderness, 2012).

In addition, TLR are membrane-anchored proteins which are variably expressed by different cell types in the intestinal tract. They can recognize different microbial structures and activate a number of innate host defenses. These microbial sensors are type I transmembrane receptors with extracellular leucine-rich repeats and an intracellular signaling domain known as the TIR domain (Cerenius et al., 2010).

Liopopolysaccharide (LPS) is a cell wall component of Gram positive bacteria and strong immunostimulant. Toll like receptor 4 (TLR-4) is essential for recognition of LPS. For LPS recognition complex formation of TLR-4, MD2 protein and Cluster of differentiation 14 (CD14) on various cells, such as macrophages and denderitic cells, is necessary (Maxey et al., 1996). LPS is associated with an accessory protein, LPS-binding protein (LBP) in serum, which converts oligometic micelles of LPS to monomer for delivery to CD14, which is a glycosylphosphatidylinositol (GPI)- anchored high affinity membrane protein. Cluster of differentiation 14 (CD14) attract LPS for binding to the TLR-4 /MD2 complex (Takeda, Kaisho, & Akira, 2003).

Toll like receptor 2 (TLR-2) recognizes various bacterial components, such as lipoprotein/lipopeptide and peptidoglycan from Gram negative and Gram positive bacteria, and lipoteichoic acid from Gram positive bacteria, a phenol soluble modium from Staphylococcus aurus, and glycolipids from Treponema maltophilum (Takeda et al., 2003). TLR-2 is also reported to recognize LPS from non entero bacteria (Takeda et al., 2003).

Toll like receptors recognize a vast assortment of PAMPS. This provides a mechanism for the innate immune system to deliver a rapid response to a changing environment. In our experiment gene expression profiles for TLR-4, TLR-2 and CD14 were compared between the experimental groups to elucidate changes in the gene expression profile of those markers.

2.9.2 Cytokines. The immune response involves lymphoid cells, inflammatory cells, and hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins collectively designated cytokines to denote their role in cell-to-cell communication (Fitzgerald, 2001).

Many cytokines are referred to as interleukins, a name indicating that they are secreted by some leukocytes and act upon other leukocytes. Interleukin 10 (IL-10) was originally described as a cytokine produced by T helper 2 cells (TH2); however, it was realized that macrophages were greater producers and to lesser extent dendritic cells. It is an 18 KD protein that was initially called cytokine synthesis inhibitory factor because it inhibits cytokine synthesis by TH1 cell subset and by activated macrophages and dendritic cells. The surprise is that the same cells produce IL-10, activated macrophages, are the major target of its inhibitory activities (Elgert, 2009).

IL-10 is the prototype cytokine for a family of cytokines with sequence homology and similar helical structure IL-10. Interleukin 10 (IL-10) and IL-10 related molecules regulate inflammatory response (Elgert, 2009).

Interleukin-2 (IL-2) originally was considered to be strictly a growth factor for activated T cells. However, IL-2 is also different molecule that promotes T- cell activity and B cell activity (Growth and j-chain synthesis). IL-2 also induces secretion of INF-γ and IL-4 by T-cells. IL-2 synergize with IL-12 to increase cytotoxicity by natural killer cells (NK cells) and lymphokine-activated killer cells (LAK cells; Fitzgerald, 2001).

Interferon gamma (IFN-γ) was originally discovered because of its ability to induce cells to block or inhibit the replication of a wide variety of viruses. Antiviral activity is a property it shares with IFN-α and IFN-β. However, unlike these other interferons, IFN-γ plays a central role

in many immunoregulatory processes, including the regulation of mononuclear phagocytes, B-cell switching to certain IgG classes, and the support or inhibition of the development of T helper (TH-cell) subsets. Interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), play a central role in the development of chronic inflammation. TH1 cells, NK cells, and TC cells release IFN-γ, while activated macrophages secrete TNF-α. Activation of macrophages by IFN-α promotes increased transcription of the TNF-α gene and increases the stability of TNF-α mRNA. Both effects result in increased TNF-α production. Tumor necrosis factor alpha (TNF-α) acts synergistically with IFN-γ to initiate a chronic inflammatory response. Both cytokines together induce much greater increases in Intercellular Adhesion Molecule 1(ICAM-1), E-selectin, and class I major histocompatability complex (MHC I) molecules than either cytokine alone. The increase in intercellular adhesion molecules facilitates the recruitment of large numbers of cells in a chronic inflammatory response (Thomas J. Kindt, 2007).

Interleukin 1alpha (IL-1α) is an important regulatory cytokine, which usually released after an injury. One unique function of IL-1α is that it can localize to the nucleus where it alters transcription. Also, due to its nuclear localization, IL-1α is released during necrosis/apoptosis and, thus, functions as a sensor for tissue damage (J. S. Holderness, 2012).

Interferon-gamma (IFN-gamma)-inducible protein-10 (IP-10), a member of the C-X-C sub-family of chemokines, is known to be produced by monocytes, lymphocytes, keratinocytes and endothelial cells in response to IFN-gamma (Cassatella et al., 1997).

Interleukin 8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are associated with transitional activation state of γδ T cells (antigen-independent priming) which increase responsiveness to downstream stimuli by γδ T cells (Graff et al., 2009). Some studies indicated that whereas monocyte/macrophages are very sensitive to bacterial PAMPs, priming of γδ T cells requires a much higher concentration of PAMPs, and results in a much

more subtle priming response (Graff et al., 2009). Interestingly, it is been proven that plants polyphenole tannin is effective in increasing the potency of γδ T cell and responsiveness (Graff et al., 2009; J. Holderness et al., 2007). Accordingly, our study proposes that SL treatment can increase expression of those cytokine and help in γδ T cell responsiveness.

The literature explained the effect of tannin on innate immunity performed in human, accordingly modulation of innate immunity is anticipated as a result of feeding SL containing diet to goat.

The objective of this study was to test the effect of feeding SL containing diet to goats, on rumen microbes and expression of selected genes associated with innate immunity. The experiment was performed using molecular technique to explore changes in microbial population in goat rumen with specific elucidation on *Bifidobacteria* as an important organism in immunity of host. Innate immunity was explored through gene expression study of the important TLRs and interleukins, comparison of serum inflammatory cytokines and total protein.

CHAPTER 3

Material and Methods

3.1 Animals

In a previous study, Boer and Spanish goats were treated with SL in an experiment to determine the impact of supplementing SL in three concentrations in diet (SL free, 50% SL and 75% SL) a loose or in pellet diet on gastrointestinal nematodes (GIN) in small ruminants (Burke et al., 2011). Samples were collected from 16 goats at the NC A&T State University project Dr Niki Whitley PI. The group included control (n = 7) and treatment of 50% SL (n = 4), and treatment of 75% SL (n = 5). At the end of the study rumen, blood, and fecal samples were collected in the abattoir under sterile conditions using clean sterile glass jars, Pax gene and serum tubes and stored at -20 °C to study the effect of the treatment on rumen microorganisms, focusing on *Bifidobacterium*, and on innate immunity of the host. Rumen content was collected to study the effect of the treatment on rumen microbes and the presence of *Bifidobacterium*. Blood samples were collected to evaluate the impact of SL treatment on immune marker expression and information about experimental design can be found in appendix A. Institutional animal care and use approval was obtained under reference number 09-0270-09-A4R.

3.2 Rumen Microbes

Rumen microbes were studied to indicate the effect of SL diet on rumen microorganisms in term of microbial profiles and detection of Bifidobacteria in rumen samples and establish comparison between the control and treatment group. Rumen content was collected from all 16 goats, control (n = 7) and treatment of 50% SL (n = 4), and treatment of 75% SL (n = 5) in the abattoir under sterile conditions using clean sterile glass jars and stored at -20 °C. Frozen rumen samples were thawed at room temperature and aliquots of 10 ml rumen content was transferred

to a clean 30 ml tubes using clean new graduated pipettes for each sample. Samples were homogenized using bio-homogenizer, and centrifuged at 6500 × g using Eppendorf 5810 rotor for 30 minutes, then the pellets were collected and used for further step. The homogenization step is to ensure isolation of total microbial DNA from rumen content from liquid and particles associated microbes.

3.2.1 Extraction of Microbial DNA. Total microbial DNA was isolated from rumen contents using QIAmp DNA stool kit (QIAGEN Sciences, USA). First, after thawing the samples at room temperature, 200 ml of rumen sample was obtained using 1000 µl pipette and clean pipette tip. Stool lyses buffer (ASL Buffer; QIAGEN Sciences, USA) was added to the sample (1.4 ml), then vortexed until homogenized. Samples were then moved to water bath at 95 °C for 5 minutes, vortexed for 15 seconds and centrifuged for 1 minute. Supernatants were transferred to a new 2 ml tubes (1.2 ml), then one inhibitor adsorption tablet (inhibitEX tablet, QIAGEN Sciences, USA) was added to each tube, vortexed and incubated for 1 minute at room temperature. Then samples were centrifuged for 3 minutes in 14,000 × g, supernatants were collected in a new 1.5 ml tubes and the pellets were discarded. Proteinase K (15 µl) were added to a new 1.5 ml tube then 200 µl of the supernatants were added to the 1.5 ml tube containing proteinase K. After that, 200 µl of the lyses buffer (AL buffer, QIAGEN Sciences,USA) was added to each tube, vortexed for 15 seconds, and then transferred to 95 °C water bath for 10 minutes. Ethanol 100 % was added to the samples (200 µl), transferred to spin columns and centrifuged for 1 minute at full speed. The spin columns were then transferred to a new 2 ml tubes and 500 µl of wash buffer 1 (AW1 buffer, QIAGEN Sciences, USA) was added to the columns and centrifuged for 1 minute at full speed. The washing step was repeated again using washing buffer 2 (AW2 buffer, QIAGEN Sciences, USA) and centrifuged for 3 minutes at full

speed. The columns were then removed to a new 2 ml tube and 200 µl of DNA elution buffer (AE) buffer is added to each and then centrifuged for 1 minute at full speed to collect isolated DNA.

3.2.2 DNA Concentration and Purity. Concentration and purity for the isolated DNA were determined using Nanodrop spectrophotometry (NanoDrop Spectrophotometer ND-1000). Measurement was performed by taking one microliter of isolated DNA and evaluated at Optical Density (OD) of 260 nm/230 nm for purity and 260 nm/280 nm to evaluate DNA concentration.

3.2.3 Rumen Microbial DNA. Isolated total microbial DNA was used in PCR, the mixtures contained 125 ng of genomic DNA as a template for each animal's rumen content. The following primers were used for PCR amplification across the 16S rDNA variable (V3) region: forward,5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACG GGAGGCAGCAG-3'; reverse, 5'-ATTACCGCGGCTGCTGG-3' (Shi et al., 2007; Skillman, Evans, Strömpl, & Joblin, 2006). Polymerase chain reaction was conducted using Super Taq Plus Polymerase kit (Ambion, USA). PCR for this experiment was performed using the following PCR program: one cycle (94°C for 5 min), 25 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) and one cycle (72°C for 7 min), (Shi et al., 2007). Aliquots of 5 µl were examined by electrophoresis on 2% agarose gel (w/v) stained with 1 µg/ml Ethidium Bromide, following the same procedure in (3.2.6) to verify the sizes and amounts of the PCR products.

3.2.4 Comparison of Rumen Microbial DNA. Amplified DNA samples were ran in Denaturing Gradient Gel Electrophoresis (DGGE) experiment to observe the presence of anticipated difference in bands pattern across the gel. Denaturing gradient gel electrophoresis was performed on the DCode Universal Mutation Detection system (16-cm system; Bio-Rad, UK). Isolated DNA from rumen samples of goats treated with SL (n = 5) and control goats

treated with 0% SL (n = 6) were used as a representative samples for DGGE analysis. Denaturing gradient gel electrophoresis in parallel gradient gel ranging from 40% to 60% (10% acrylamide) was ran at 130 V, 60 °C for 5 h. Briefly, parallel gradient gel casting started with the preparation of low (40%) and high (60%) density solutions as described in Table1. Ammonium persulphate was prepared by weighing 0.1 g APS into 1.5 ml microcentrifuge tube, and then 1 ml of distilled de-ionized water was added and vortexed until APS completely dissolved. Using delivery system parallel gel was casted by loading the syringes with low and high solution after adding 14.4 µl N, N, N', N' Tetramethylethylenediamine (TEMED) and 144 µl APS. Total of 7 liter of 1X TAE buffer was prepared by heating distilled water in the Decode system tank to 55 °C, then 140 50X TAE buffer was added to bring the total volume of the buffer to 7 liter. After that the gel loaded in the Decode system and ran at 130 V, 60 °C for 5 h. Gels were then stained with Ethidium Bromide solution 1 µg/ml for 35 minutes, washed two times in sterile distilled water, and visualized using gel transluminator (Gel Doc 2000, BIORAD).

Table 1

Solutions Used in the Preparation of 40% and 60% Denaturant Solutions for DGGE

Solution	0% denaturant solution	60% denaturant solution
Urea	8.4 g	12.6 g
40% Acrylamide/Bis (37.5:1)	10 ml	10 ml
TAE 50X	1 ml	1 ml
Formamide	6 ml	12 ml
Distilled Water	Enough to bring the solution to 50 ml	Enough to bring the solution to 50 ml

3.2.5 Detection of *Bifidobacterium*. *Bifidobacterium* genus specific primers shown in table 2 was used to investigate the presence of *Bifidobacterium* genome in the rumen contents of goats (*n* = 16), those primers were designed on the basis of 16S rDNA sequences (Matsuki, Watanabe, & Tanaka, 2002). Treatment groups include, (*n* = 4) received 50% SL diet, (*n* = 5) received 75 % SL diet and control group (*n* = 7) received 0% SL diet. See (Table 2) for sequences of used primers and product size.

Table 2

Bifidobacterium Genus Specific Primers Set: Bif164-PCR (Forward) and Bif662-PCR (Reverse)

Target	Name of primers	Sequence	Product size pb	Reference
Bifidobacterium	Bif164-PCR	GGGTGGTAATGCCGGATG	523	(Matsuki et al., 2002)
	Bif662-PCR	CCACCGTTACACCGGGAA		

The PCR reaction included 250 nanogram (ng) of the isolated DNA to be used as a template, 5 µl 10x PCR buffer, 2.5 µl deoxynucleotide triphosphates (dNTPs), 0.5 µl each primer and 1 µl Taq polymerase in 25 µl reaction, all procedure was performed on ice. The PCR program used was one cycle of 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 4 minutes, then 1 cycle of 72°C for 6 minutes using (MWG-BIOTECH primus 96) thermocycler. Tubes containing PCR products were then stored at 4°C.

Electrophoresis was conducted to visualize the amplified sequence of 16S rDNA to determine the presence of *Bifidobacterium* species in the rumen samples. Agarose gel 2% (w/v) was prepared by adding 1 gm of agrose (LE, analytical grade agarose, Promega Corporation, USA) to 50 ml 1x TAE buffer then heated using microwave until completely dissolved. Gel was casted on the electrophoresis set and transferred to electrophoresis box, and covered with 1x

Tris-acetate-EDTA (TAE) buffer. Amplicons were prepared in 1.5 ml Eppendorf tubes by adding 5 µl amplicon, 5 µl distilled de-ionized water and 2 µl loading dye (Blue/Orange 6X, Promega, USA) then mixed by vortexing, briefly centrifuged, and loaded into the gel using 10 µl gel loading pipette. Molecular marker of ten DNA fragments with sizes 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 base pair (McMahon et al.), (EZ Load 100 bp Molecular Ruler, Bio-Rad Laboratories, Inc., USA) was used to determine the size of amplified sequences. Electrophoresis was run at 150 volts for 20 minutes and stained using Ethidium Bromide solution 1 µg/ml for 10 minutes and washed for 5 minutes using distilled de-ionized water. The gels were viewed using gel transluminator (Gel Doc 2000, BIORAD).

3.3 Blood

Blood samples were collected by jugular venipuncture. Blood samples for serum extraction were collected in non-treated tubes in day 28 to evaluate immune cytokines and serum protein levels. Concomitantly, 2.5 ml of jugular whole blood was collected using PAXgene Blood RNA tubes for RNA isolation.

3.3.1 Isolation of RNA from goat blood. Total RNA from white blood cells was extracted using PAXgene tubes (PreAnalytiX Gmb, Feldbachstrasse, Switzerland). Blood collected in PAXgene Blood RNA tube was used for the purification of intracellular RNA from whole blood samples using ZR Whole-Blood RNA Miniprep kit. Tubes were kept at -20 °C before transfer to 4 °C for 24 hours, and then left at room temperature for 2 hours. Tubes were centrifuged (Eppendorf, centrifuge 5810 R) at 4000×g for 10 minutes. The mixture was transferred to Zymo-Spin IIIC Column in a collection tube (ZR Whole-Blood RNA Miniprep kit, ZYMO Research corp., USA) in 600 µl aliquots using five columns per sample and centrifuged at 14,000 × g for 2 minutes (Eppendorf 5402). Pre-Wash buffer (400 µl) was added to the

columns and centrifuged at 12,000 × g for 30 seconds. Then, wash buffer was added (400 µl) to the column and centrifuged at 12,000 × g for 30 seconds. The Zymo-Spin IIIC Columns were transferred into RNase free tubes. RNA recovery buffer (100 µl) was added to Zymo-Spin IIIC Columns in RNase free tubes and centrifuged at 12,000 × g for 30 seconds. Ethanol 100% (100 µl) was added to the flow through in the RNase free tubes and mixed well by pipetting. The mixture was transferred after that to Zymo-Spin IC Columns (ZR Whole-Blood RNA Miniprep kit, ZYMO Research corp.) in a collection tube and centrifuged at 12,000 × g for 30 seconds. Ribonucleic acid (Salazar et al.) Prep Buffer (ZR Whole-Blood RNA Miniprep kit, ZYMO Research corp.) was added (400 µl) to the columns and centrifuged at 12,000 × g for one minute, and then the flow through was discarded. Ribonucleic acid wash buffer (800 µl) was added to the columns and centrifuged at 12,000 × g for 30 seconds, and the flow through was discarded. The Zymo-Spin IC Columns were then centrifuged in an empty collection tubes at 12,000 × g for two minutes. The columns were then removed carefully and transferred into 1.5 ml RNase free tubes. The elution of RNA was performed after that by adding 6 µl of DNase/RNase free water (ZR Whole-Blood RNA Miniprep kit, ZYMO Research corp.) directly to the column matrix and centrifuged at 10,000 × g for 30 seconds to elute RNA.

3.3.1.1 Real time polymerase chain reaction. Isolated total RNA was used to produce complementary DNA (cDNA) from messenger RNA (mRNA) and used in reverse transcription using RETROscript Kit (Ambion, USA) following the manufacturer's instruction. Briefly, using RNase free PCR tubes RNA ≈ 1µg, 2µl oligo, and 12µl nuclease free water were added for each sample, then mixed by spinning tubes briefly. Tubes heated after that to 85 °C for 3 minutes using thermocycler (MWG-BIOTECH primus 96). Tubes moved to ice after that and 2 µl 10X RT buffer, 4µl dNTPs mix, 1µl RNase inhibitor, 1µl reagent (MMLV-RT), then mixed briefly

by spinning and incubated at 44 °C for one hour then 95 °C for 10 minutes. Then isolated cDNA was used in real time PCR to analyze gene expression in the three groups 0%, 50% and 75% SL.

3.3.1.2 Gene expression profiles of innate immunity markers. The produced cDNA were then used as a template in duplicates for quantitative real time PCR (qPCR) using SsoAdvanced SYBR Green Supermix kit (BIORAD Laboratories, USA) following the manufacturer's instruction to evaluate gene expression of immune markers. Primers for the immune markers; Cluster of Differentiation 14 (CD-14), Toll Like Receptor-2 (TLR-2), Toll Like Receptor-4 (TLR-4), Interlukin-10 (IL-10), Interlukin-8 (IL-8), Interlukin-2 (IL-2), Interferon-γ (INF-γ), and Tumor Necrosis Factor-α (TNF-α) was used in the quantitative real time PCR. Reaction was set by adding SsoAdvanced SYBR Green Supermix (10 µl), forward and reverse primers (250 nano-Mole nM), cDNA (100 ng), and DNase/RNase free water to 20 µl. Real time PCR result was then analyzed to compare gene expression between control and treatment groups using Glyceraldehydede 3-phosphate dehydrogenase (GAPDH) as the reference gene. The obtained cycle quantities (Cq) of cDNA was used in Livak equation to obtain fold change for each genes using means obtained from 12 samples of control and animals received SL in diet. Livak methods analyze gene expression change through calculating the difference in the amount of RNA in each samples based on Cq for each sample and using internal control gene as a normalizer (Livak, 2001).

3.3.2 Enzyme-linked immunosorbent assay of pro-inflammatory cytokines. Extracted serum from experimental animals was examined using the Human inflammation ELISA Strip (Signosis, Inc., USA) following manufacturer's instruction, for profiling of pro-inflammation cytokines. Signosis's Inflammation ELISA Strip kit allows quantitative profiling

and measuring of 8 cytokines; Tumor necrosis factor (TNFα), interferon production regulator (IFNr), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GMCSF), interleukin-1α (IL-1α), interleukin-8 (IL-8), IFN-inducible protein-10 (IP-10), and Regulated on Activation Normal T Cell Expressed and Secreted (RANTES). Standards and samples were poured (100 µl) into pre-coated wells, and then incubated for 1 hour at room temperature with gentle shaking. Wells were aspirated, and washed by adding 200 µl of 1X assay wash buffer then repeated three times. After that, 100 µl diluted biotin-labeled antibody mixture was added to each well and incubated for one hour at room temperature with gentle shaking. The aspiration /wash process were repeated, and the 100 µl of substrate was added to each well and incubated for 30 minutes. Stop solution 50 µl was added to each well and the OD was determined with microplate reader at 450 nm. The observed readings were used to produce standard curve to obtain average concentration of the samples in Pico-gram per microliter (pg/ml). Means of triplicate observations obtained and analyzed using analysis of Analysis of variance (Schell MA).

3.3.3 Total serum protein evaluation. Total serum protein concentration was evaluated using the Pierce bicinchonninic assay (Fisher Scientific, Rockford, USA) following manufacturer's instruction. Pooled samples of extracted serum from treated and control goats were used and read using spectrophotometer to obtain concentration of protein. Working reagents and protein standards solutions were prepared accordingly using test tubes and glass beaker. The test tube procedure was used in this protocol, where 0.1 ml of each standard and unknown sample replicates into an appropriately labeled test tube. Working reagent 2.0 ml was added to each tube and mixed well. Tubes were covered and incubated at 37 °C for 30 minutes, cooled to room temperature and OD was measured using spectrophotometer set to 562

nanometer (Oni et al.). Standard curve was obtained by plotting the means blank corrected 562 nm measurement for each BCA standards against its concentration in µg/ml.

3.4 Statistical Analysis

Concentrations of pro-inflammatory cytokines, total serum protein, as well as concentration and purity of DNA were determined using a standard curve and Microsoft Excel to generate means. Statistical Analysis Software System (SAS Institute, Cary, NC) frequency procedure was used to analyze gels for *Bifidobacteria* detection. Analysis of variance (Schell MA) was used to evaluate differences between treatment and control group for ELISA of pro-inflammatory cytokines, total serum protein, cDNA concentration and real time PCR. Means were considered significant at the 5% level of probability.

CHAPTER 4

Results and Discussion

4.1 Concentration and Purity of Isolated DNA

The concentration and purity reported from three groups of animals treated with 0%, 50% and 75% SL in the diet (Figure 1). Means for concentration of isolated DNA were calculated for the three groups showed no significant difference ($p < 0.5215$). Concentration of isolated DNA was the highest in treatment group of SL 75% and control group with mean concentration of 150.11ng/µl and 109.89 ng/µl, respectively. The lowest mean concentration was obtained from treatment group received 50 % SL diet with mean concentration of 80.88 ng/µl. The mean purity was 1.83, 1.915, and 1.86 for DNA isolated from 0%, 50%, and 75% SL diet, respectively, indicating adequately pure DNA for downstream PCR application.

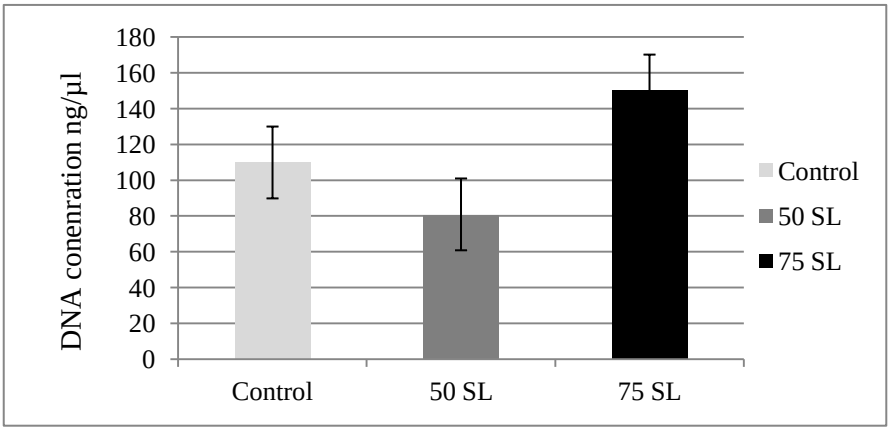

Figure 1. Mean concentration of isolated microbial DNA from control group (0%), 50%, and 75% SL in diet (Mean ± SD).

4.2 Comparison of Rumen Microbial DNA

Amplification of 16S rDNA gene was performed using PCR as described in 3.2.4. After running gel electrophoresis for amplification products specific bands of 200 bp were observed on gel viewer, insuring primers specificity for target sequence of 16S rDNA gene (see Figure 2).

The step is important to insure that all samples used in the experiment contain the amplified sequence necessary for establishment of legitimate comparison between treatment and control group in denaturing gel. Figure 2 negative control showed no bands, while selected amplified samples (839, 820, 848, 850 and 807) showed a clear 200 bp bands.

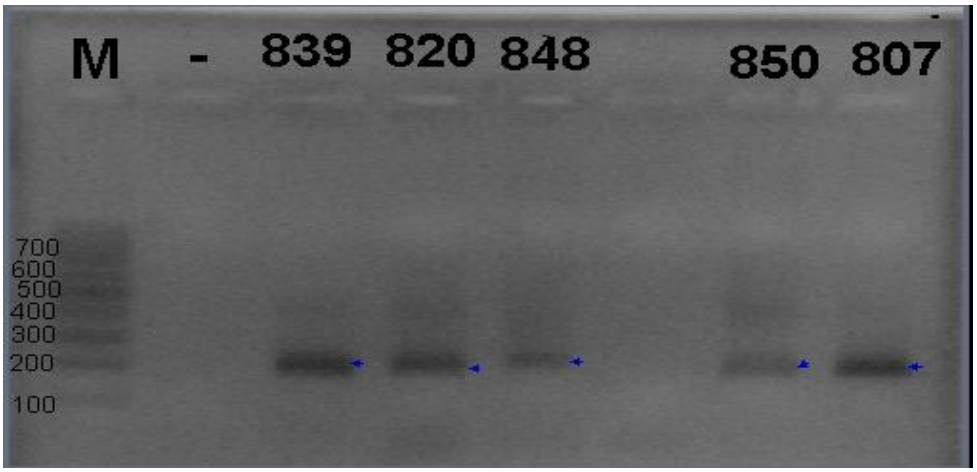

Figure 2. Amplified sequence of microbial 16S rDNA gene. M = marker; - = negative control

The amplified sequences were then used in a denaturing gradient gel electrophoreses DGGE experiment was performed to detect changes in microbial population. The result from DGGE experiment showed similar pattern in both control group and treatment groups. Several maintained specific bands in control group were absent in SL 75% and SL 50% (see Figure 3). Control and treatment groups showed conserved bands indicated in Figure 3 as (1, 2, and 3). Those bands may represent tannin tolerant microorganisms as they continue to appear under treatment condition as in lanes 50% and 75%. Bands indicated as (4, 5, and 6) in control groups lanes shown as 0%, were not observed in treatment groups lanes 75% and 50%. Those bands may represent tannin intolerant microorganisms since their specific bands did not consistently appear on the gel. Animals treated with 0% SL maintained similar bands patterns indicated by numbers (1, 2, 3, 4, 5, and 6), while those specific bands indicated by numbers (4, 5, and 6) were absent in 75% and 50 % SL treated animals (see Figure 3).

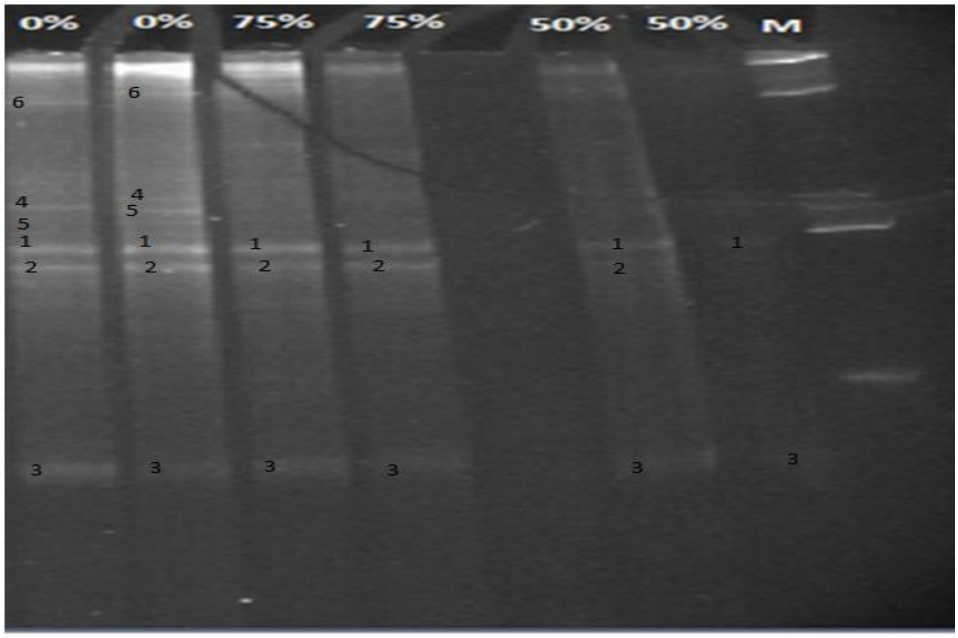

Figure 3. Denaturing gradient gel electrophoresis of amplified rumen microbial DNA. Numbers 1-6 indicate specific microbial bands showed in lanes 0% (Control group), 50% (50% SL diet), and 75% (75%SL diet).

Denaturing gradient gel electrophoresis results suggest a change in the total rumen microbial populations after SL treatment. Six bands were observed in controls (0%) and only three clear bands are observed in groups received SL in diet (50% and 75%). The role of rumen microbes extend from its significance in fermentation of plant nutrient and immunity, to environmental effects through rumen methanogen which has been identified as the single largest source of anthropogenic methane (Cheng, Mao, Liu, & Zhu, 2009; D. Kamra, 2005). Thus rumen microbial populations shift will not only affect host nutrition and immunity, but would reflect on environment (Mathison et al., 1998) (Cheng et al., 2009), in our study the results obtained through DGGE analysis suggest that feeding SL diet had influenced rumen microbial population. Similar studies using this approach were conducted to investigate rumen methanogenic microbes to help reduce methane emission (Cheng et al., 2009). Investigation on

pair of primers for DGGE analysis of ruminal methanogenic Archaea and to detect the archaeal communities in the rumen of goat (Cheng et al., 2009). There work concluded that a specific used primers pair was suitable for investigating methanogenic archaeal community in the rumen, and they were able to precisely determine the dominant methanogenic Archaea in the rumen of goat. Such studies have opened the door to widely investigate molecular diversity of rumen methanogenic Archea using PCR-DGGE. Likewise, this study can provide a solid ground for more exploration of molecular diversity of rumen *Bifidobacteria* and its distribution through the gastrointestinal tract of goat. The influence of SL treatment on rumen microbial population is strongly expected since SL hay considered to be a high tannin containing plant (Shaik et al., 2006). Several studies indicated highly significant effect of tannin from plant on rumen microbes by suppressing and/or alteration of their metabolism (Rojas et al., 2006). Several authors have reported that tannins prevent or at least interfere with the attachment of rumen microorganisms to plant cell walls, and it is well known that such attachment is essential for degradation of plant cells to occur (Frutos et al., 2004). Tannins might have a direct effect on ruminal microorganisms, e.g., by altering the permeability of their membranes, yet this effect varies based on susceptibility of rumen microorganisms tolerance to tannins (Frutos et al., 2004).

4.3 Detection of Bifidobacterium

Following the procedure mentioned in (3.2.6) amplified sequences of 16S rDNA gene were obtained from control animals (820, 824, 846, 850, 848, 839 and 810) and showed *Bifisobacteria* specific bands of ≈ 523 bp except for one goat (810) which failed to show any specific band (see Figures 4a, 4b, and 4d). On the other hand animals fed 50 % SL diet (816, 836, 803 and 807) and animals fed 75% SL diet (828, 852, 814, 809 and 827) none of the

treatment groups animals exhibited observable *Bifidobacteria* specific bands (see Figures 4a, 4b, 4c, and 4d).

The effect of SL treatment groups on *Bifidobacteria* detection in rumen fluid was found to be significant ($p < 0.002$) using frequency procedure and Chi square test. Six animals (820, 824, 846, 850, 848, 839) out of the seven control animals of 0% SL showed *Bifidobacteria* genus specific band of 523 bp, all treatment groups 50% SL ($n = 4$) and 75 % ($n = 5$) did not show any specific bands.

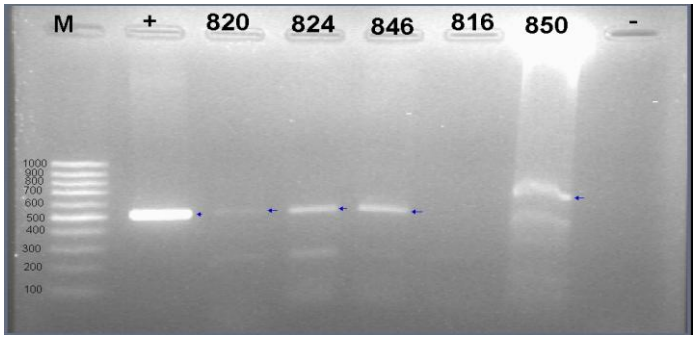

Figure 4a. Amplified 16S rDNA sequence of *Bifidobacteria*. M = marker, + = positive control, 820 = control animal (0% SL), 824 = control animal (0% SL), 846 = control animal (0% SL), 816 = treatment group (50% SL), 850 = control animal (0% SL), - = negative control.

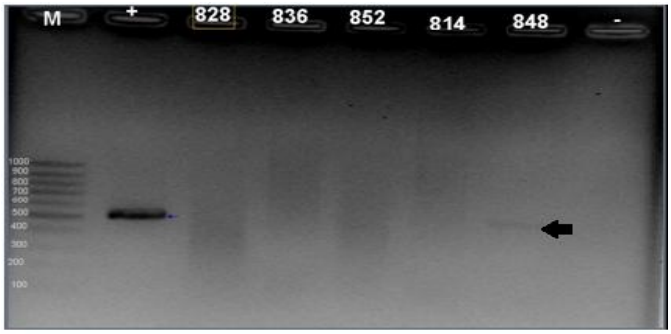

Figure 4b. Amplified 16S rDNA sequence of *Bifidobacteria*, M = marker, + = positive control, 828 = treatment group (75% SL), 836 = treatment group (50% SL), 852 = treatment group (75% SL), 814 = treatment group (75% SL), 848 = control animal (0% SL), - = negative control.

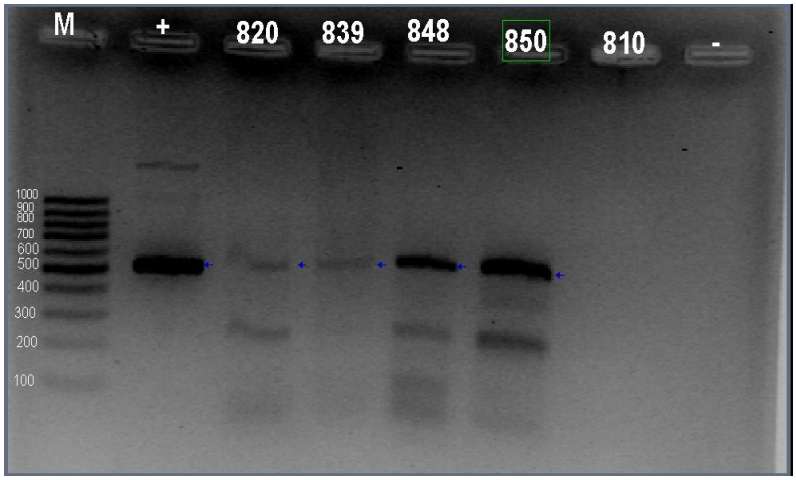

Figure 4c. Amplified 16S rDNA sequence of *Bifidobacteria*. M = marker, + = positive control, 820 = control animal (0% SL), 839 = control animal (0% SL), 848 = control animal (0% SL), 850 = control animal (0% SL), 810 = control animal (0% SL), - = negative control.

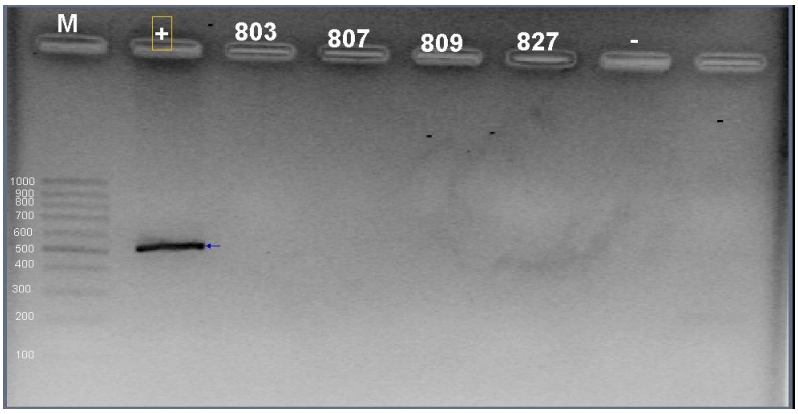

Figure 4d. Amplified 16S rDNA sequence of *Bifidobacteria*. M = marker, + = positive control, 803 = treatment group (50% SL), 807 = treatment group (50% SL), 809 = treatment group (75% SL), 827 = treatment group (75% SL), - = negative control.

Interestingly, control animals showed consistent specific bands of *Bifidobactria* in comparison to animals receiving SL in diet indicating a highly significant change in *Bifidobactria* population ($p < 0.002$). Detection of *Bifidobacteria* in the gastrointestinal tract of ruminants was investigated (Volkova, 2006) and its beneficial effect on innate immunity also has been studied (Mayo, 2010). Results from this research indicated a possible alteration in innate

immunity in goat gastrointestinal tract and a shift in *Bifidobactria* population after consumption of SL diet, but more research work is still needed to clarify how *Bifidobactria* population change in response to SL treatment. One important outcome is that molecular approach used to investigate *Bifidobactria* was adopted in several ecological studies and even rumen microbes studies (Volkova, 2006) (Lamendella et al., 2008), but our study novelty comes in successfully using molecular techniques to identify *Bifidobactria* in goat rumen.

4.3.1 Real time polymerase chain reaction. The quality of isolated cDNA from the reverse transcription of collected RNA was determined following manufacturer instruction as explained in (3.3.1 and 3.3.1.1). Means of isolated cDNA concentration found to be 1587.109 ng/µl in control group compare to 1631.142 ng/µl group received SL in diet (see Figure 5). Differences between observed means of cDNA concentration obtained were not significant using ANOVA procedure ($p < 0.8148$). Information about quantity and quality of isolated cDNA can be found in appendix B.

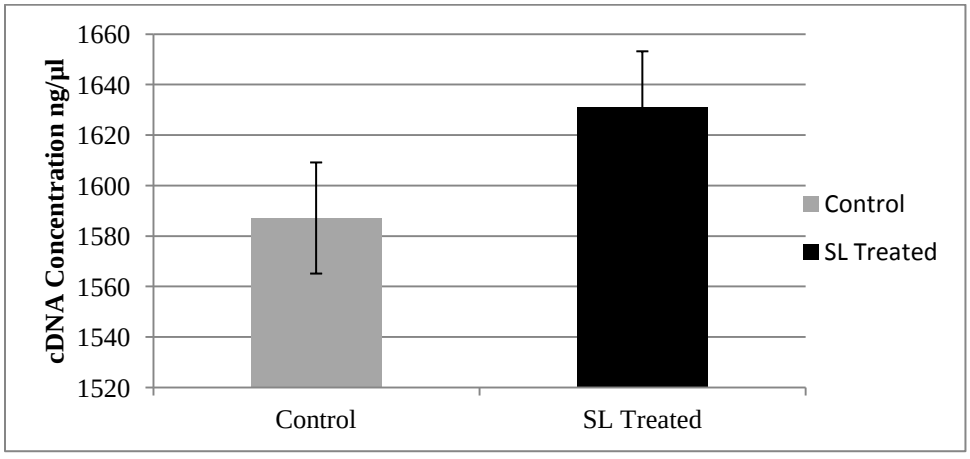

Figure 5. Mean concentration of isolated cDNA (Mean ± SD).

4.3.2 Gene expression profiles of innate immunity markers. The aim behind performance of gene expression studied here is to explore possible systemic effect of SL diet on important genes of innate immunity. Expression of eight immune markers (CD-14, TLR-2,

TLR-4, IL-10, IL-8, IL-2, INF-γ, and TNF-α) was analyzed using Livak method (also known as the $2^{-\Delta\Delta Cq}$ method) to compare gene expression profile between treatment and control animals. To determine the relative expression of target genes in the test samples and calibrator samples using reference gene (GAPDH) as the normalizer, the expression levels of both the target and the reference genes were determined using RT-qPCR as shown in (3.3.1.2). Determined cycle quantity (Cq) values were used in Livak method to obtain fold change of each gene to evaluate changes in gene expression of innate immunity markers (see Figure 6).

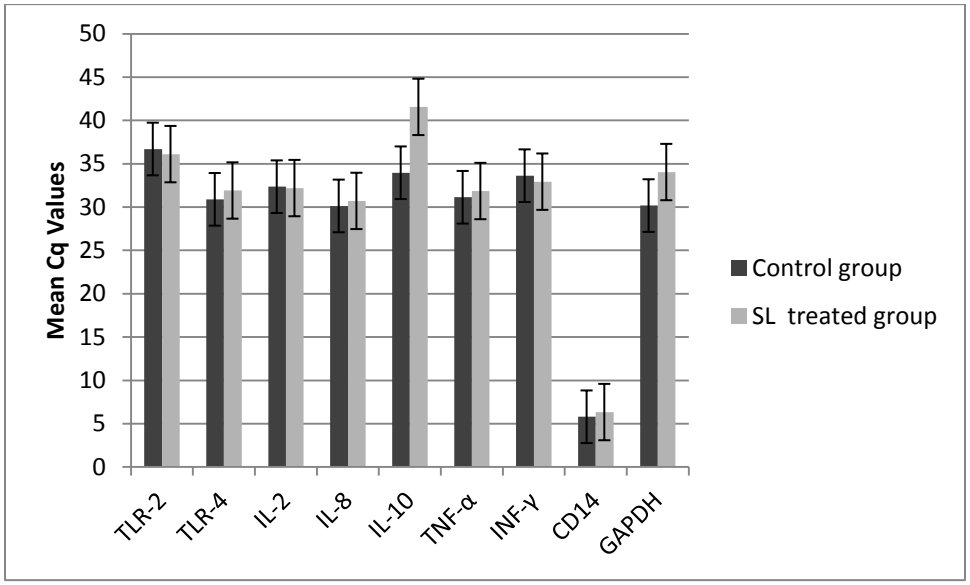

Figure 6. Means Cq values obtained from RT-qPCR (Mean ± SD).

Using Livak method (Livak, 2001), TLR-2 gene expression profile exhibited high fold change of 22, meaning that SL treated group expressed TLR-2 at 22 higher level compare to control (see Table 3). This immune marker plays several important immune functions including response to gram-positive bacteria, bacterial peptides, and yeast zymosan (Kurt-Jones et al., 2002). Interferon-gamma (INF-γ) had expressed the highest fold change level of 23 in treatment group compare control group. The two genes TLR-2 and INF-γ had shown high fold change compare to the other innate immunity markers. These findings suggest stimulation of γδ T cells

and NK cells as a result of SL treatment. Figure 5 shows mean cycle quantity values of amplified cDNA for the test genes, while Table 3 shows the calculated fold change for each of the genes.

Comparison of fold change level for TLR-4 and CD14 had showed increase in the expression of those genes in levels of 7 and 10 respectively (see Table 3). It is been discussed in (2.10.1) the importance of those genes in recognizing PAMPs by immune cells and stimulation of innate immune response. Moreover, our results revealed an increase in expression of IL-2 by 16 fold in treatment compare to control group (see Table 3). Interleukin -2 (IL-2) is important in activation of T cells and B cells. Another cytokine work to increase innate immunity through synergistic effect with INF-γ and it interfere in initiation of chronic inflammation is tumor necrosis factor alpha (TNF-α). The increase fold change for TNF-α was 8 in treatment compare to control. These findings indicate beneficial effect of SL by increasing expression of these immune markers.

Table 3

Fold Change in Expressed Genes Based on Livak Method

Gene	TLR-2	TLR-4	IL-2	IL-8	IL-10	TNF-α	INF-γ	CD14
Fold Change	22	7	16	9	0.075	8	23	10

Interleukin 10 (IL-10) secretion exhibited the lowest increase in gene expression by fold change of 0.075363 (see Table 3). This cytokine is important in regulation of inflammatory response and known for its inhibitory effect on macrophage activation as explained in (2.10.2). Our results revealed that IL-8 expression had increased by 9 fold in treatment group in compare to control group (see Table 3). Interleukin 8 (IL-8) is known to be associated with boosting

responsiveness of γδ T cells, and this action can result from tannin treatment according to previous studies (J. Holderness et al., 2007).

Scientific literature indicated that phenolic plant compound like tannin can modulate immune response through two distinct pathways induced by polyphenols. These include the anti-inflammatory polyphenol and the oligomeric procyanidins (J. S. Holderness, 2012). Those studies also indicated existence of plant polyphenols capable of modulating TLR-mediated signals. Different studies have also shown the influence of rumen microbes and *Bifidobacteria* on the immune response (Collado & Sanz, 2007; Ebaid & Hassanein, 2007). Our data from RT-qPCR showed a fold change gene expression of immune markers in all examined genes, supporting the suggested effect of SL treatment on innate immunity.

4.4 Enzyme-linked Immunosorbent Assay of Pro-inflammatory Cytokines

Cytokines TNF-α, IFNr, G-CSF, GM-CSF, IL-1a and IP-10 expressed highly significant increase after treatment ($p < 0.0002$), while IL-8 and RANTES showed also highly significant decrease after treatment ($p < 0.0001$; see Figure 7).

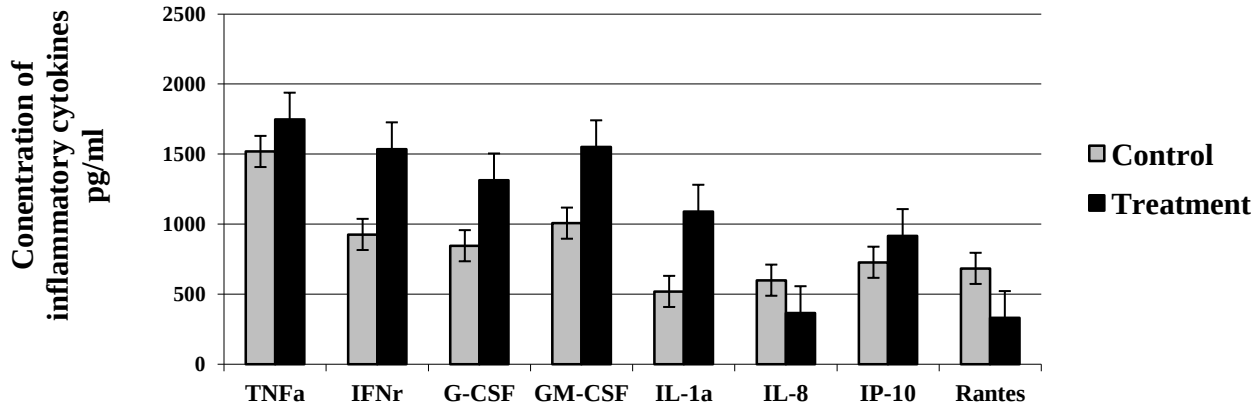

Figure 7. Means concentration of inflammatory cytokines (Mean ± SD).

Increased production of the cytokines activates macrophages, stimulating these cells to increase microbicidal activity, up-regulate the level of class II MHC, and secrete other cytokines such as IL-12, which induces TH cells to differentiate into the TH1 subset. Interferon gamma (IFN-γ) secretion by T helper 1 (TH1) cells also induces antibody-class switching to immunoglobulin G (Ventura et al.) classes that support phagocytosis and fixation of complement (Thomas J. Kindt, 2007). Interleukin 8 (IL-8) has suppressive effects on the macrophage and serves to further diminish the biologic consequences of TH1 activation (Thomas J. Kindt, 2007). Our results suggest a significant alteration in cytokines profile emphasize our hypothesis that using SL treatment has also significant effect on host innate immunity. Different studies have also shown the influence of rumen microbes and *Bifidobacteria* on the immune response (Collado & Sanz, 2007; Ebaid & Hassanein, 2007). Modulating inflammatory cytokines may return to changes in rumen microbes as a result of SL treatment.

4.5 Quantification of Total Serum Protein

Serum total protein is an important immunological and health parameter. Total serum protein can be affected with type of nutrition that animal receive. Effect of feeding tannin containing plant leaves were found to help increase serum total protein (Oni et al., 2012). However our results showed slight decrease in serum total protein (147.2333 µg/µl) in treatment group compares to (170.8006 µg/µl) control group (see Figure 8).

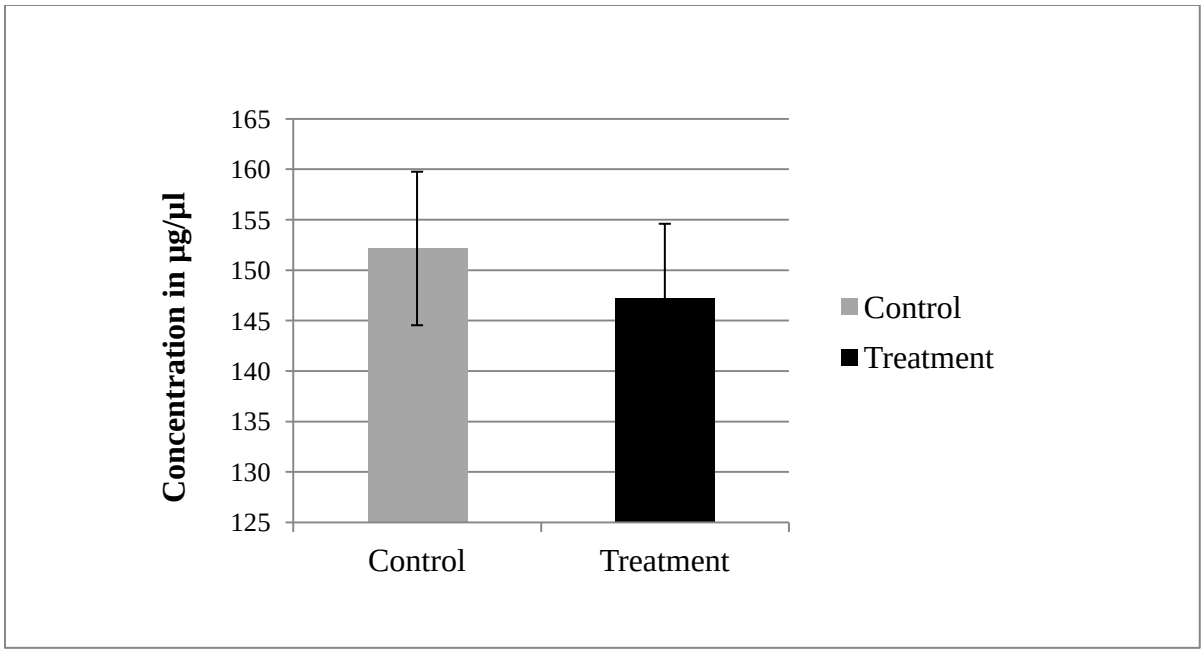

Figure 8. Means concentration of total serum protein (Mean ± SD).

CHAPTER 5

Summary and Future Research

The use of SL alters both rumen microbial population and selected markers of goat innate immunity. Amplified sequence of 16S rDNA gene between treatment and control groups as shown in DGGE and *Bifidobactria* were significantly different. Enzyme-linked immunosorbent assay of pro-inflammatory cytokines showed differential effect.

This study explored possible effect of feeding SL diet on goat rumen microbial population and *Bifidobacteria* which was established through our results. The effect of SL diet on innate immunity status was also altered based on the changes in innate immune markers and cytokines profiles. The study suggests an effect of SL treatment on immune status, but thorough investigation of the specific overall immune outcome is beyond the scope of this study and further research is needed to clarify how tannin from SL diet would alter the overall innate immune status with different SL concentration.

CHAPTER 6

Conclusion

Dietary tannins from SL may affect the goat's innate immune response and the composition of rumen microorganisms. Comparison of rumen microbial profile revealed a change in bands pattern which indicate change in rumen microbial composition which may have negative effect on nutrition and immunity of the animals. Gene expression results indicated a fold increase in gene expression of immune markers in all examined genes, supporting the hypothesis that an effect of SL treatment on innate immunity is possible. Obtained results from ELISA indicated different inflammatory cytokines response. These findings might indicate favorable SL diet effect in term of immune response but further research is needed as the complexity of different immune pathways lead us to expect some adverse outcomes.

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Appendix A

Animals used in the study were reared in North Carolina Agricultural and Technical State University farm. Initially thirty Boer Spanish cross goats (n = 30) were used in the study, both males (n = 15) and females (n = 15). The goats were divided into three groups, each group received dosage of SL at different concentration level for a period of four weeks. Goats were assigned to three levels of SL treatment in alfalfa pellet 0%, 50% and 75% for a period of four weeks. Animals were naturally infected with nematodes through pasture grazing (Burke et al., 2011).

Appendix B

Isolated cDNA purity and concentration showed in ng/µl

Animal ID	cDNA concentration ng/µl	Purity
35	1931.7	1.7
36	1662.0	1.6
27	1934.1	1.7
12	1751.5	1.7
30	1958.8	1.7
31	1981.3	1.64
26	1486.6	1.7
14	2208.3	1.6
15	925.8	1.5
32	1276.7	1.7
37	1575.6	1.6
25	1324.9	1.6
29	1776.6	1.7
13	2014.2	1.7
23	2138.8	1.63
4	1896.0	1.7
10	1722.1	1.7
20	1433.6	1.64
1	1689.9	1.58
2	956.8	2.08
22	471.0	1.7
33	1092.0	1.6
21	1823.6	1.6

Appendix C

Results of immune cytokines ELISA

cytokine	Non-treated *pg*/ml	Treated *pg*/ml	Non-treated *pg*/ml	Treated *pg*/ml	Non-treated *pg*/ml	Treated *pg*/ml
TNFa	1522.5	1772.55	1514.39	1723.5	1518.445	1748.025
IFNr	927.2	1542.6	925.4	1529.9	926.3	1536.25
GCSF	840.9	1318.05	850.8	1309.9	845.85	1313.975
GMCSF	1009	1561.7	1004.4	1539.8	1006.7	1550.75
IL-1a	514.5	1093.3	524.5	1088.1	519.5	1090.7
IL-8	593.6	364.5	605.8	369.1	599.7	366.8
IP-10	716.3	917.2	739.01	917.2	727.655	917.2
Rantes	674.5	332.7	693.6	331.8	684.05	332.25