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The Effect Of A Mushroom (Coriolus Versicolor) Based Probiotic On Innate Immunity In Goats Naturally Infected With Gastrointestinal Parasites

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The Effect of a Mushroom (*Coriolus versicolor)* Based Probiotic on Innate immunity in Goats Naturally Infected with Gastrointestinal Parasites

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Biographical Sketch

Kingsley Ekwemalor was born and raised in Owerri, Imo state in Eastern Nigeria. He completed his undergraduate studies at the University of Port Harcourt, Rivers State Nigeria where he received a Bachelor's of Science in Plant Biotechnology. Following graduation, he worked in Total Nigeria Plc. In 2012, he decided to pursue his goal of higher education and was admitted into the Integrated Animal Health Systems graduate program at North Carolina Agricultural and Technical State University under Dr. Mulumebet Worku in the Department of Animal Sciences. He worked in the Genomic Diversity and Animal Biotechnology Laboratory. Mr. Ekwemalor was privileged to represent the Department of Animal Sciences and North Carolina Agricultural and Technical State University at national and international conferences where he presented his current research techniques and findings. He is a member of the American Society of Animal Sciences, member of the Graduate School Advisory Council (GSAC) and also a member of Gamma Sigma Delta the honor society for Agriculture. He is supported by the Agricultural Research and Food Safety Education Program. He has worked with high school, undergraduate and graduate students at the University Farm, research and teaching laboratories. His current research project deals with the evaluation of a mushroom based probiotic on animal health, in particular the effect on gene expression.

Dedication

I dedicate this work to Almighty God who has given me grace and life and also to my family for their support throughout my program.

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List of Abbreviations

ACD	Acid Citrate Dextrose
BCA	Bicinchoninic Assay
ºC	Degrees Celsius
cDNA	Complementary DNA
CR	Complement Receptor
CV	*Coriolus versicolor*
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
Epg	Eggs Per Gram
FEC	Fecal Egg Count
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GI	Gastrointestinal
GCSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
IFNr	Interferon Production Regulator
IL	Interleukin
IP	Interferon gamma-induced protein
Kg	Kilogram
L1	First Stage Larvae
L2	Second Stage Larvae
L3	Third Stage Larvae

L4	Fourth Stage Larvae
µg	Microgram
µl	Microliter
min.	Minutes
ml	Milliliter
%	Percent
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PRR	Pattern Recognition Receptors
PSK	Polysaccharide Krestin
PSP	Polysaccharide Peptide
RANTES	Regulated upon Activation Normal T-cell Expressed and Presumably Secreted
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute sec Seconds
spp	Species
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-Alpha

Abstract

Gastrointestinal parasites such as the nematode *Haemonchus contortus* and protozoa Coccidia pose a serious threat to the global goat industry due to resistance of parasites to anthelmintic drugs. The objective of this study was to evaluate the effect of extracts of CorPet biomass, a mushroom (*Coriolus versicolor*) on gastrointestinal parasite infection and innate immunity in goats. Fifteen female SpanishXBoer goats were drenched with 10 ml of either a hot extract of CorPet (N=5), a cold extract (N=5), or with sterile water (control N=5) for eight weeks. Treatment groups were reversed after four weeks. Body weight, body condition and FAMACHA scores, fecal and blood samples were collected weekly. Blood was used for white blood cell differential counts, packed cell volume and isolation of total RNA. Fecal samples were used for microbial DNA isolation and enumeration of parasite eggs. The Nanodrop spectrophotometer was used to determine the concentration and purity of DNA and RNA. Neutrophils were isolated using differential centrifugation and hypotonic lysis of red cells from blood. RNA was isolated from neutrophils using Trizol and then converted to cDNA. The human Toll-like receptor signaling pathway array was used to profile the expression of 84 genes. Total plasma protein concentration was determined using the BCA assay and migration patterns on silver stained gels. Pro-inflammatory cytokine and Prostaglandin E2α secretion was determined by ELISA. Data were analyzed using SAS statistical analysis software ($P<0.05$). Mushrooms did not affect health indicators including fecal egg counts ($p>0.05$) and increased plasma proteins ($p<0.001$). Treatment decreased GMCSF and increased GCSF, and IFNr, secretion. Treatment modulated expression of goat neutrophil Toll-like receptor pathway genes. Eight TLR's were expressed on goat neutrophils. The highest fold change was observed for TLR6 and TIRAP. Modulation of these genes using CV mushroom probiotics as an oral has implication for health.

CHAPTER 1

Introduction

Growth in population is increasing thereby increasing the demand in food. By the year 2050, it is projected that the world population would be expected to reach 8.3 to 10.9 billion. This population growth has led to the increased demand of food. The world goat population has been on the increase during the last three decades and currently stands at almost 840 million herds (Simela et al., 2008). They predominate as the major livestock in the poorest parts of the world (Simela et al., 2008). To ensure that food does not become a privilege-commodity, scientists are exploring new approaches with sustainable resources and innovations within these essential agricultural sectors to accommodate for and stabilize supply in this era of a rising population (Godfray et al., 2010).

Ruminant products contribute to one of the major sources of food and protein consumed worldwide. Production of small ruminant is a growing industry in the U.S due to stable ethnic markets (NASS, 2009), rapidly increasing demand for grass-fed, or organically produced livestock (Green et al., 2003), and the growing popularity of specialty meat type goat breeds (Joshi et al., 2011). Meat goats do not require much supplemental feed and complicated infrastructure to allocate the animals (Coffey, 2002). According to the United States Department of Agriculture and the National Agricultural Statistics Service, three million herds of goats were accounted for in the United States in the 2010 sheep and goat report. The recent increase in goat production also results from the rising demand by increase numbers of ethnic groups into the U.S. who favor goat meat and goat products (Engle et al., 2000). This new trend has brought about new opportunities to grow and sustain goat production in the U.S. (Nadarajah, 2010).

Goat production, however, is negatively affected by several factors: respiratory diseases such as pneumonia, pregnancy toxemia and feed toxicity, including infectious and parasitic diseases. Goats are vulnerable to viral and bacterial diseases, such as foot and mouth disease, and mastitis. Among the latter, gastrointestinal infection remains one of the main constraints to dairy goat production (Rinaldi et al., 2007). Gastrointestinal nematodes or parasites (GIN) are an important limiting factor in goat production systems globally, and especially in humid climates (Terrill et al., 2007). There are many parasites that cause an array of health problems in sheep and goat production, but none more significant than *H. contortus* and Coccidia *Emiria* spp (Miller et al., 2006). Due to overuse and incorrect application of anthelmintics to control parasitic infection, the results have been increases in anthelmintic resistance weakening many drug programs to control infection (Miller, 2000). However, as the anthelmintic resistance of parasites to available products is increasing the need for improvement on current procedures for their control becomes a priority (Thamsborgh et al., 1999). Introduction of breeding schemes for resistance (Mandonnet et al., 2001), improved farm management practices and employing the FAMACHA© system (Zajac, 2002) are many of the alternate approaches used. Plant based anthelmintics have also been explored for use in the elimination of GI nematodes.

One potential alternative for control of GI nematode infection is to take advantage of the host's immune response in selection programs to increase the level of resistance in the population. The innate immune system is the primary responder to non-specific pathogens and is governed by phagocytic dendritic cells and macrophages. The responsibilities of the innate immune system include the ability to differentiate between self (host) and foreign pathogens (Akira, 2006). For many years, probiotics have been known to confer health benefits and may have the ability to modulate immune responses. This research evaluates the effect of oral

administration of CorPet powder, a mushroom powder, of *Coriolus versicolor* on the immune response on goat.

The findings of this study could possibly aid in the improvements of GI nematode control by identifying the genes involved in the innate immune response in goats. This study could also provide the knowledge of the effects of mushroom probiotics as an alternative for control of GI nematodes in goats to help improve strengthen GI nematode management practices in the United States meat goat industry as it continues to develop.

The objective of this study was to evaluate the effect of extracts of CorPet on gastrointestinal parasite infection and innate immunity in goats.

CHAPTER 2

Literature Review

2.1 Goat Production

The world goat population has been on the increase during the last three decades and currently stands at almost 840 million herds (Simela et al., 2008). Goats are a critical resource for milk and meat in many parts of the world. They predominate as the major livestock in the poorest parts of the world (Simela et al., 2008). Production of small ruminant is a growing industry in the U.S due to stable ethnic markets (NASS, 2009), rapidly increasing demand for grass-fed or organically produced livestock (Green et al., 2003), and the growing popularity of specialty meat-type goat breeds (Joshi et al., 2011). Goat production is an important industry in southeastern United States because of availability of forages and a rising market demand particularly for organically produced meat (Robinson, 2004). It is an attractive enterprise for farmers due to the relatively low cost of breeding stock, a high reproductive rate, requirement of minimal capital input as compared to other livestock species, and the ability of small ruminants to thrive on native pasture or brush land that is unsuitable for cropping or grazing by cattle (Schiere et al., 2002). Meat goats do not require much supplemental feed and complicated infrastructure to allocate the animals (Coffey, 2002) and their sturdiness and small size allows them to adapt to different environments (Engle et al., 2000).

The recent rise in goat production and products in the U.S. can be directly attributed to the effects of an expanding market aligned with the on-going increase in foreign-born ethnic groups migrating to the U.S. with a preference for goat meat and products (Solaiman, 2007). Additionally, goat meat is considered as lean meat and can be a beneficial meat-substitute for people seeking healthier diet alternatives (Solaiman, 2007). Due to the increased number of

consumers of goat products, growth in demand is projected to follow (Nadarajah, 2010). Although this new facet of agriculture is growing, it faces many challenges due to the lack of organization and structure in the goat market (Nadarajah, 2010). This growth still brings many opportunities to improve and sustain the goat market as the demand for goat meat and other byproducts increase. Improving selective genetic schemes and nutrition within marketed goat breeds will be an important factor to the growth and future of the United States goat industry, as structure is gained (Nadarajah, 2010).

2.1.1 Parasite Problem in Goat Production. Goats are a critical resource for milk and meat in many parts of the world, but 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, pink eye, and mastitis. Parasitic infection is recognized worldwide as a limiting factor in the production of goats. Infection with internal parasites and gastrointestinal nematodes is a major threat to the health and productivity of small ruminants (sheep and goats) in the United States, particularly in recently-weaned kids and lambs (Bowan, 2009).

Gastrointestinal (GI) nematode infections are common in grazing ruminants worldwide and still are one of the main constraints to ruminant production because they can depress food intake, tissue damage, reduction in skeletal growth and live-weight gain (Waller, 1997). Economic losses caused by GI nematodes are associated with decreased production and limited performance (Abebe et al., 2000). Combining knowledge of GI nematodes epizoology with modern anthelmintics has allowed great success on parasite control over the past decades. However, as the anthelmintic resistance of parasites to available products is increasing the need for improvement on current procedures for their control becomes a priority (Thamsborgh et al.,

1999). The need for controlling GI nematodes is of great importance in small ruminant production.

Coccidiosis is a disease caused by a protozoa *Eimeria* spp which naturally occurs in soils is a major concern in young animals whose immune system has not matured (Kommuru et al., 2014). The coccidia that are more pathogenic in goats are *E. arloingi*, *E. christenseni*, and *E. ovinoidalis* (Kahn et al., 2010). During weaning, goats encounter lowered immune function creating favorable conditions for an outbreak of coccidiosis that can rapidly spread in a herd of flock causing devastating consequences of diarrhea, dehydration, and even death (Bowan, 2009). The parasite causes harm to the host by destroying the mucosal lining of the abomasum depleting nutrient up-take, and leading to liver failure in dairy goats (Khan et al., 2010). Coccidiosis is of great economic importance because of the losses due to clinical disease (diarrhea) and subclinical infections, as well as poor weight gain in particular (Chartier et al., 2011).

In the United States and other countries around the world, *Haemonchus contortus* also known as the barber pole warm is the most important parasite from the strongylid nematodes. *H. contortus* is one of the most prolific nematodes. According to Mortensen et al., 2003, *H. contortus* account for 75-100% of the total fecal nematode egg output which is a blood sucking parasite that thrives in warm climates. Female *H. contortus* reach about three cm in length, making this species one of the largest of the strongylid nematodes of ruminants (Elsheikha et al., 2011). A female worm may produce thousands of eggs each day and larvae on pastures can accumulate rapidly during the grazing season in regions where this species predominates. The prepatent period (length of time elapsing between infection of the host and parasite maturity) of *H. contortus* is about 17 to 21 days (Levine, 1985). Adult worms are short lived surviving in the host for only a few months (Courtney et al., 1983). *H. contortus* has great pathogenic

potential because fourth-stage larvae and adults feed on host blood. Consequently, its effects on a flock or herd are often insidious because routine observation of animals may not reveal the extent of infection and owners may not recognize that disease is present until deaths occur. With favorable conditions for the survival and growth of the 'free-living' stages of *H. contortus*, during the warmer months of the year throughout much of the southern and eastern United States (warm and moist) kids and lambs grazing with their mothers can acquire a significant GI nematode infection which then increase at weaning due to increase exposure to the infective third stage (L_3) larvae and stress (Kommuru et al., 2014). The fourth and final stage (L4) of the *H. contortus* life cycle is the reproduction stage. After the larvae penetrate the abomasum (true stomach) there is molting for a fourth and a fifth time to transition into the adult phase. Males and females feeds off of the host blood in the abomasum and reproduce with the female laying 5,000 to 10,000 eggs per day passing through the feces to the external environment (Zajac, 2006). The total process of the *H. contortus* life cycle, from the L1 to the L4 stage, takes roughly three weeks (Luginbuhl, 2000).

2.1.2 Life Cycle of *Haemonchus contortus*. The life cycle of parasites starts with adult females laying eggs while residing in the abomasum. The eggs when laid are oval in shape and pass out of the animal in feces. At 26^0C and 100% humidity, the eggs take fourteen to seventeen hours to hatch and develop into L1 larvae stage. L1 larvae develop on pasture into L2, L3 and following ingestion by ruminant host, into the L4 stages before reaching the adult stage. After copulation, the adult females start laying eggs eighteen days after an oral infection with L3 and can be fully gravid by day 33. The L3 is the infective larval stage in which the host inadvertently ingests the parasite and it develops into an adult in the abomasum where they feed on blood (Nikolaou, 2006).

2.2 Innate Immune System

Goat production is negatively affected by several factors including infectious and parasitic diseases. Among the latter GI infection remains one of the main constraints to goat production (Rinaldi et al., 2007).

The main function of the immune system is to distinguish its own cells and tissues from other external cells and tissues in order to protect against infestation. The immune system has various mechanisms to eliminate external agents which include inactivation, lysis, agglutination, precipitation, or phagocytosis of foreign cells (Kahn et al., 2010). The animal immune system is composed of two related functional elements: the innate immune system and the adaptive immune systems (Hoffmann et al., 1999). Both function in coordination to protect against invading microorganisms (Medzhitov et al., 2002).

Innate immunity is the first line of defense against organisms; it acts in a non-specific way through anatomical barriers (skin, mucus membrane), secretions and other elements. The adaptive immune system is the second line of defense which responds slower than the innate immunity system. Innate immune defense plays a key role in affording protection (Siwicki et al., 1994). Unlike the innate immune system, the adaptive immune system has the ability to "memorize" infectious agents allowing the adaptive immune system to serve as a rapid response system if pathological agents are encountered again (Medzhitov et al., 2002). The innate immune system consists of natural killer cells, T-cell and B-cell, basophils eosinophils, monocytes, macrophages and polymorphonuclear neutrophils. These cells are called white blood cells or leukocytes and are also divided into two groups based on their morphology, granulocytes and agranulocytes. Granulocytes include eosinophils, neutrophils and basophils and agranulocytes are lymphocytes (T and B cells) and macrophages (Kaplan et al., 2004).

A differential white blood cell count is an important tool used to provide clinical diagnosis, monitoring of disease and blood disorders (Houwen, 2001). This system quantifies and differentiates white blood cells at one particular time giving insight to infection and if treatments are working (Houwen, 2001). White blood cell differentiation counts can be accomplished manually or by automated electronic systems (Krapp et al., 2002). The white blood cell percentages are determined by the counting of the ratio and morphology differentiation of the first 100 white blood cells observed (Khan et al., 2010).

Table 1.

White Blood Cell Differential Count Normal Value for Goats in Percentage (%). Adapted from Khan and Line, 2010

Neutrophils	Lymphocytes	Basophils	Monocytes	Eosinophils
30-48	50-70	0-1	0-8	1-8

2.3 Diagnosis of Gastrointestinal Parasites

The most common symptoms of goats experiencing parasitic burden depends heavily on the observation of clinical symptoms associated with parasitic infection especially with *H. contortus* and Coccidia. The major limitation is instituting a selective approach which identifies those animals requiring treatment. Anemia is the most detrimental effect of *H. contortus* infection which leads to death. The most common symptoms include high fecal egg count (FEC), weight loss and diarrhea. Phenotypic criteria can be used to indirectly estimate resistance to parasites such as fecal egg counts, white blood cell differential count, FAMACHA, packed-cell volume or immunological traits (Dominik, 2005). The McMaster egg counting technique is used to determine the levels of parasitic burden. Goats infected with H. *contortus* with a FEC greater

than 1000 epg indicate heavy infection while 500 epg signifies mild infection (Taylor, 2010). Oocyte counts of 5000 per gram or more in feces of host suspected of coccidia infection is indicative of clinical infection (Schoenian, 2011). It is also very important to evaluate hematocrit levels periodically to diagnose parasitic infection (Miller, 2000). This method is effective in assessing anemia probably caused by parasitism in sheep and goats (Khan et al., 2010).

The use of the FAMACHA© card system is a system that was developed in South Africa for the classification of animals into categories based on levels of anemia caused by gastrointestinal parasites (Reynecke et al., 2011). In this method, called FAMACHA©, the ocular mucous membranes of sheep and goats are classified by comparison with a laminated color chart bearing pictures of sheep conjunctivae classified into five categories ranging from the normal red, through pink to practically white in severe anemia. Since anemia is the primary pathologic effect from infection with *H. contortus*, this system can be an effective tool for identifying those animals that require treatment (Kaplan, 2004). The FAMACHA system is widely used and is accepted by sheep and goat producers in the southern U.S. (Burke et. al., 2007).

Figure 1. FAMACHA Anemia Guide (Kaplan et al., 2004).

2.4 Treatment

One potential alternative for control of GI nematode infection is to take advantage of the host's immune response in selection programs to increase the level of resistance in the population. Resilience is the ability to be productive despite infection. The traditional treatment

for infection with GI nematodes in weaned animals is the use of anthelimintics. Control of GI infections in goats relies almost exclusively on multiple and regular dosing of anthelmintics (Rinaldi and Cringoli, 2012). All of the approved available anthelmintics for goats in the United States fall into one of three major drug groups: the benzimidazoles, the cholinergic agonists, and the macrocyclic lactones (Zajac, 2006). Only Decoquinate and Monensis are FDA approved for the prevention of coccidiosis, but no drugs are approved for treatment of coccidiosis in goats (Kommuru, 2014). Anthelmintics therefore are needed but current ones are ineffective due to anthelmintics resistance especially in the humid tropics, where the free-living stages of parasitic nematodes such as *H. contortus* (Kaplan, 2004) meet adequate climatic conditions to develop during almost the entire year (Mahieu et al., 2007).

Since the advent in the 1960s of modern broad-spectrum anthelmintics with wide safety margins, producers have relied on dewormers for control of GI nematodes in the United States. In the southeastern United States where *H. contortus* causes substantial losses, frequent treatments were used (and recommended to producers) throughout the grazing season in suppressive anthelmintic programs that kept egg production and pasture contamination at low levels (Kaplan 2004). For most producers, parasite control continues to include treatments with anthelmintic drugs, but any veterinarian working with small ruminant producers must stress the importance of alternative methods of parasite control that can be used in combination with anthelmintics (Zajac, 2006).

Due to growing levels of resistance in these gastrointestinal nematodes to current drugs and the lack of interest in developing new drugs by companies, small ruminant producers are desperate for solutions (Zajac, 2002). Drug resistance by nematodes has resulted in the search for better treatment methods including the use of non-chemical alternatives. They include production

practices and the use of genetics based breeding schemes to contend with anthelmintic resistance of GI nematodes.

Plant based anthelmintic have also been explored for use in the elimination of GI nematodes with such extracts like: Garlic (*Allium sativum*) (Worku et al., 2009), neem (*Azadirachta* india), wormwood (Artemisia absinthium), and tobacco (*Nictiana tobacum*) (Worku et al., 2009). Other measures which have been used include reduction of stock density and the maximization of pasture to reduce parasite numbers as alternative management practices. Placing animals on a high nutritional plan was described by Zajac (2002), to aid in the development of an immune response allowing goats to develop some natural immunity against the parasites before using anthelmintics as a best management practice. Finally, substances able to modulate immune functions are also being incorporated in animal feed to stimulate host defense (Gallois et al., 2008, Hoste et al., 2008). Improvement in animal nutrition can also impact the immune response including gene response, protein synthesis, modification and degradation, metabolism, signal transduction and cellular proliferation (Afacan et al., 2012).

The understanding of protective mechanisms regarding the initial steps of the host's response to pest or parasite derived molecules that can correlate with resistance or susceptibility to pathogens needs to be explored for economically important pests of ruminants. This understanding will aid the design of immune-modulatory strategies to induce a change in the magnitude of immune or non-immune responses.

2.5 Mushroom

The term mushroom can be defined as 'a macro fungus with a distinctive fruiting body, large enough to be seen with the naked eye and to be picked by hand'(Chang, 1999). The number of species mushrooms on earth is estimated at 140,000, yet maybe only 10% (approximately

14,000 named species) are known (Wasser 2002), of which only about 650 species are reported to be of medicinal value (Minato, 2010). Mushrooms have been appreciated and consumed for their nutritional value and medicinal properties in several countries over the years and have been used not only as a source of food but medicinal resource as well (Wasser, 2002). Researchers have considered mushrooms as healthy food because they are good sources of vitamins, minerals, proteins, and carbohydrates apart from the low level of lipids and low caloric content (Wasser, 2002 and Kalac, 2009). Mushroom extracts have been reported to exhibit antioxidant properties (Tsai et al., 2009), which makes them excellent sources of β-glucans and are rich in many bioactive metabolites of high medicinal value such as lectins, polysaccharides, phenolic and polyphenolics, terpenoids and volatile organic compounds (Kalac. 2013). The low total fat content and high proportion of polyunsaturated fatty acids relative to the total fatty acids of mushrooms are considered significant contributors to the health value of mushrooms (Sanodiya et al., 2009). Mushroom extracts have been shown to exhibit immumomodulator, antitumor/anticancer, antibacterial and antiviral, antioxidant, and antihypoglycemic activities. Therefore, there has been an urge to use mushrooms as an alternative method to promote animal health.

2.5.1 Coriolus versicolor. *Coriolus versicolor* (*CV*) formerly known as *Trametes versicolor* is pore-bearing basidiomycetes. Its fan-shaped fruiting bodies grow in overlapping clusters on dead logs (Fig. 2). The top of it is zoned, usually in shades of brown, white, gray, and blue bands while the underside is white and bears minute pores (Hobbs, 2004). This edible mushroom is widely distributed all over the world in temperate zones of Asia, Europe and North America and may be the most common shelf fungus in the Northern Hemisphere (Cui et al., 2003).

Figure 2. C. versicolor mushroom (fruiting body) growing on tree trunk (Courtesy of Frank L. Hoffman).

2.5.2 Morphology. The morphological characteristics of *CV* fruiting body have been described (Cui et al., 2003) as follows: 3–5 cm across brackets that are semicircular, flattened, thin, and tough. Young brackets are flexible and usually occur in tiers and spread along branches. The upper surface is velvety and attractively marked with concentric zones of varying colors: brown, yellow, gray, greenish, or black. The margin is usually wavy. The mushroom has white spores that are oblong and cylindrical. In agitated submerged culture, the fruiting body and spores do not form, and the fungus grows as dispersed or pelleted mycelium. More than 120 strains of *CV* have been recorded.

The therapeutic potential of *CV* is gaining acceptance among patients worldwide (Lau et al., 2004). The active ingredients in *CV* are polysaccharides, in particular polysaccharide krestin (PSK, a protein-bound polysaccharide) and polysaccharide-peptide (PSP) (Chan and Yeung, 2006). They exert their therapeutic effects by modulating the host's immune response. Both preclinical and clinical evidences have demonstrated that extracts from *CV* display a wide array of biological activities, including stimulatory effects on the immune system and inhibition of

cancer growth (Zhou et al., 2007). The aqueous extract of *CV* was found to be effective in activating T lymphocytes, B lymphocytes, macrophages, natural killer (NK) cells, and lymphocyte-activated killer cells (Ng, 1998), as well as promoting the production of antibodies and various cytokines, such as IL-2 and IL-6, and tumor necrotic factor (TNF) *in vivo* (Yang et al., 1999). Oral administration of PSP to normal mice resulted in an elevated production of cytokines that can stimulate the proliferation of T cells and activation to cytotoxic T cells (Rowan et al., 2003). *CV* polysaccharopeptides can remedy intestinal disorders and are beneficial in the therapy of opportunistic microbial infections that suppress the immune response (Cui et al., 2003).

Studies have demonstrated that dietary immunomodulators such as β-glucan show beneficial results in a wide variety of animal species (Hahn et al., 2006). The immunostimulant and immunomodulatory effects of β-glucan polysaccharides result in the regeneration of the host's ability to fight infections (Rieder et al., 2013). The immunomodulatory process initiates when the β-glucan binds to cell surface receptors of macrophages, lymphocytes and neutrophils (Chen et al., 2007). β-glucan activates B-lymphocytes and macrophages through toll-like receptors, modulating the immune system and inducing the production of cytokines (Liao et al., 2004). These cytokines in turn activate adaptive immunity through the production of B cells for antibody production and stimulation of T cell differentiation to T helper (Th) 1 and Th2 cells, which mediate cell and humoral immunities, respectively (Bochers et al., 2008). It was also demonstrated that β-glucan may enhance the innate immune responses, including lymphocytes proliferation and antibody production (Gopalalakannan et al., 2010). An opportunity exist to understand the harness therapeutic potential of the use of mushroom extracts in animal health and production to ensure food security and meet the protein needs of the expanding world

population. Mushrooms seem to be a potential candidate for prebiotics as it contains carbohydrates like chitin, hemicellulose, *β*- and α-glucans, mannans, xylans and galactans. Prebiotics can be defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Gibson et al., 2004).

2.5.3 Mushrooms in Animal Health. The health benefits using mushrooms or their extracts in animals have been extensively reported. *CV* is considered as a strong potential candidate for drug development in treatment and suppression of DNA and RNA synthesis and enhances immune functions (Hsiehet al., 2002). Mushrooms and mushroom components have been reported to have a myriad of positive health benefits, mainly on the basis of in vitro and in vivo animal trials (Roupas et al., 2012). Mushrooms and mushroom components exert many of their positive effects on health via a balance of T helper cells, the induction of interferon-gamma and certain interleukins or NO-mediated mechanisms. Many of these immunomodulating effects are due to the polysaccharide content of mushrooms, either from β-glucan or from polysaccharide–protein complexes (Roupas et al., 2012).

2.5.4 Mechanism of Action. The best-known effect of β-glucan consists of the augmentation of phagocytosis of granulocytes, monocytes, macrophages and dendritic cells. In this regard, macrophages considered being the basic effector cells in host defense against bacteria, viruses, parasites and tumor cells, play the most important role (Vetvicka et al., 1996). This innate immunity is based on non-clonal receptors (pattern recognition receptors, PRRs), which recognize certain molecules on the surface of invading microorganisms and are collectively termed as pathogen-associated molecular patterns (PAMPs). The first step of β-glucan -macrophage interaction is binding to specific receptors (PRRs) present on a surface of

the macrophage cell. For β-glucan recognition, the macrophages keep several receptors at their disposal: TLR-2 (Toll-like receptor 2), dectin-1, CR3 (complement receptor 3), lactosylceramide and probably others. However, the interaction between β-glucan and CR3 receptor is established more effectively than at the other receptors. Binding to PRRs, such as toll-like receptor-4 (TLR4), dectin-1 and complement receptor 3 (CR3) by polysaccharides can activate immune responses by enhancing the secretion of TNF-alpha, IL-6 and other inflammatory cytokines. Pathogen recognition receptors also serve to bind ligands that prime immune responses (Ofodile, 2007). While there has been considerable research on how isolated β-glucan activates cytokine production through dectin-1 and CR3, there are few reports on the mechanisms of how traditionally used medicinal mushroom preparations that contain polysaccharides, interact with PRR (Price et al., 2010). However, there are no published reports on the effects of *Coriolus versicolor* mushroom extracts on PRR pathways. No work has been done on goats.

2.6 Toll-like Receptors

Animals live in a wide variety of microbe-rich environments and hence it is crucial to have a sensitive innate defense mechanism which relies in part by recognizing conserved molecules that are unique to some classes of potential pathogens (Tirumurugaan et al., 2010). It is very important to understand the innate immunity against microbial components and its critical role in host defense against infection. Toll-like receptors (TLRs) are a highly conserved group of proteins that have been identified in mammals (Medzhitov et al., 1997). Following the discovery of Toll-like receptors (TLRs) in the mid-1990s it has been clearly shown that pathogen recognition by the innate immune system is broadly specific, relying on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect relatively conserved components of pathogens referred to as pathogen associated molecular patterns (PAMPs)

(Janeway et al., 2002). The PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (Akira et al., 2006) which initiates a complex signaling cascade to activate a wide variety of transcription factors and inflammatory cytokines (Takeda et al., 2005).

The TLR family consists of ten receptors: TLR-1 through TLR-10, which are very important in the identification of microbes (Takeda et al., 2005). The coding regions within the sheep and goats TLR 1-10 genes have been sequenced and found to be conserved and highly similar in nucleotide composition (Raja et al., 2011). Tirumurugaan et al. (2010), used bovine specific primers to amplify goat TLR mRNA. Toll-like receptor 4 (TLR-4) recognizes lipopolysaccharide, which is associated with the protein in gram-positive bacteria membranes and is bound; pathogen recognition activates the innate immune response (Akira et al., 2004). Effect of toll-like receptors has not been studied.

2.7 Cytokines

The nature of an immune response is greatly influenced by the pattern of cytokines secreted during that response. Cytokines are small proteins that transmit information from one cell to another. The analysis of cytokines secreted by immune cells in response to infectious agents is crucial to understand pathogenesis and immunity. Most cells in the body produce cytokines during inflammatory processes which represent a large series of regulatory proteins of the immunologic system. Many cytokines are referred to as interleukins, a name indicating that they are secreted by some leukocytes and act upon other leukocytes. Two general patterns of cytokine secretion by such cells have been described: In the Th1 response cytokines initiate cell mediated reactions defined as the activation of macrophages to combat infectious pathogens by releasing IL-1, IL-2, IL-8, and IL-12 to activate inflammation (Raghupathy, 2001). In the Th2

response, T-helper cells activate B-cells; interleukins IL-4, IL-5, IL-6, IL-10 and IL-13 are released to counter infectious agents caused by extracellular organisms (Raghupathy, 2001).

CHAPTER 3

Materials and Methods

3.1 Animals

Fifteen female SpanishXBoer goats were selected from the herd at North Carolina Agricultural and Technical University (NCA&TSU) Small Ruminant Research Unit. All animals were ear tagged prior to the study. Animals were clinically healthy and not under any treatment prior to the study. It was approved by Institutional Animal Care and Use Committee (IACUC). Initial screening was carried out to determine body weight, body condition, packed cell volume and the level of coccidian oocyst and *H. Conturtus* egg in fecal sample.

3.2 Experimental Design

A complete randomized design was used in assigning treatment to each before screening. A two period crossover design was conducted (Park et al., 2010) to analyze all variables.

3.3 Housing

During the experiment the goats were housed in a barn with wood shavings at night and pasture during the day. Water and feed supply were provided ad libitum. The barn had good sanitation and good management.

3.4 Feeding

The animals were supplemented under balanced diet feed (Southern States Quality kid and Goat Feed) which contained a high-protein, high-energy mixture fortified with vitamins and minerals such as ammonium chloride, copper and zinc. The goats were fed two pounds of feed per animal once a day. Also the animals were fed with hay ad libitum.

3.5 Preparation of Mushroom Extracts

CorPet powder (*Coriolus versicolor)* was purchased from Mycology Research Labs Ltd (United Kingdom). Twenty-five (25) grams of CorPet was weighed out and stirred in 250 ml of sterile endotoxin free distilled water. Protein content for the mushroom extract was determined using the Pierce™ Bicinchoninic Acid (BCA) Assay kit (Rockford, IL) to determine the level of protein to be administered to the animals.

3.5.1 Extract Preparation. Heated extracts was prepared by first weighing 25 g of CorPet powder in 250 ml of distilled water and heated with stirring for 20 minutes. The extracts were left to cool and then stored at 40C. Cold extracts were prepared by weighing 25 g of CorPet powder in 250 ml of distilled water and stirred for 20 minutes and stored in 40C.

3.5.2 Drench Administration. Ten (10) ml of the extract was given to each goat on daily basis for 60 days. Ten (10) ml of distilled water was also given to the control group daily for 60 days. Treatments were reversed after the first 30 days in a crossover design.

3.6 Sample Collection

All samples were collected and evaluated once a week throughout the experiment.

3.6.1 Body weight. Goats were weighed on a portable scale in Kg before they were fed in the morning.

3.6.2 Body condition Scores. Body condition was determined by physically assessing the rib areas using firm pressure with the fingers and running fingers down the goat's spine from the shoulders to the tail head, and degree of fat cushion over these bones determines the score. Body condition refers to the fleshiness of the goat which is scored from 1 to 5, where 1 is very lean, 2 is lean, 3 is moderately good condition, 4 is fat, and 5 is obese (Mendizabel et al., 2010).

3.6.3 FAMACHA Eye Color Score. The FAMACHA chart is a parasite monitoring system used to correlate fecal egg count (parasitic load) with color of the ocular conjunctiva (Kaplan et al., 2004). Using this method, animals were classified into 5 categories according to eye mucus color; category number 1 = red, non-anemic; 2= red-pink, non-anemic; 3 = pink, mildly anemic; 4 =pink-white, anemic; 5 = white, severely anemic.

3.6.4 Collection of Fecal Samples. Fecal samples were collected by applying lubricant and inserting the index middle finger directly into the rectum wearing sterile gloves for each animal. Collected samples were placed in labeled clean zip-lock plastic bags to determine fecal egg count.

3.7 Fecal Egg Count

A modified McMaster technique was used for fecal egg count as described by Whitlock (Kaplan et al., 2004). Two (2) grams of feces were weighed and dissolved in 28 ml of saturated sodium chloride to cause parasites eggs to float to the top of the liquid. Pellets were broken down using a spatula. Ten (10) ml syringe was used to transfer aliquots of the solution into both McMaster slide chambers and examined under an Olympus B X40 microscope using 10X magnification according to the Paracount-EPG^{TM} Fecal Analysis Kit (Chalex Corporation, Wallowa, OR). The number of coccidia oocyst and *H. Conturtus* egg were counted in duplicate, the average was calculated, and then multiplied by 50 to get the eggs per gram (epg) of feces for each animal (Kaplan et al., 2004).

3.8 Isolation of Microbial DNA from Fecal Samples

The QIAamp $^{(R)}$ DNA isolation stool mini kit (50) (QIAGEN Sciences, Maryland) was used to isolate DNA from fecal samples as recommended by the manufacturer. Briefly 200 mg of fecal samples from each goat was weighed and transferred into a clean tube and placed on ice.

Two (2) ml of lysis buffer (ASL) buffer was then added to each tube. Tubes were vortexed continuously for I minute until the stool sample was thoroughly homogenized. The stool lysate of 1.6 ml was pipette into a labeled 2ml micro centrifuge tube. The suspension was heated for 5 minutes at 70^0C. The tubes were vortexed for 15 seconds and centrifuged at full speed for 1 minute to pellet stool particles. The supernantant (1.2ml) was pipetted into a new 2 ml micro centrifuge tube. One inhibitEX tablet was added to each sample and vortexed immediately and continuously for 1 minute at room temperature to allow inhibitors to adsorb to the inhibitEX matrix. Samples were then centrifuged at full speed for 3 minutes to pellet stool particles and inhibitors bound to inhibitEX matrix. The supernatant was pipetted into a new 1.5 ml micro centrifuge tube and the pellet was discarded.

The sample was centrifuged at full speed for 3 minutes. The supernatant (200 µl) was transferred into a 1.5 ml micro centrifuge tube containing 15 µl of proteinase K. AL buffer (200 µl) was added to each tube and vortexed for 15 seconds. Tubes were then incubated for 10 minutes at 70^0C. Ethanol (200 µl) (96%) was added to the lysate and mixed by vortexing. The lid of a new QIAamp spin column was labeled and placed in a 2 ml collection tube. The complete lysate was carefully transferred to the QIAamp spin column without moistening the rim. The cap was properly closed and centrifuged at full speed for 1 minute. The QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 µl of buffer AW1 was added. The cap was closed and centrifuged at full speed for 1 minute. The QIAamp spin column was placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded. Wash buffer AW2 (500 µl) was added and centrifuged at full speed for 3 minutes. The collection tube containing the filtrate was discarded. The QIAamp spin column was transferred to a new 2 ml

collection tube and centrifuged at full speed for 1 minute. The old collection tube with the filtrate was discarded. The QIAamp spin column was transferred into a new-labeled 1.5 ml microcentrifuge tube and then 200 µl of buffer AE was then pipetted directly onto the QIAamp membrane. The cap was closed and incubated at RT for 1 minute, and then centrifuged at full speed for 1 minute to elute DNA. The concentration (260 nm) and quality or purity (260/280 nm) of the isolated DNA samples were determined using the Nanodrop Spectrophotometer 1000 3.7.1 (Thermo Scientific Inc., MA).

3.9 Blood collection

Blood samples (15 ml) were collected from the junglar vein into 2.5 ml PAXgene blood RNA tubes (QIAGEN Inc, Valencia,CA) for extraction of RNA. The PAXgene tube contains a reagent that immediately stabilizes intracellular RNA for 3 days at room temperature (18-25^{0}C) and 5 days at 2-8^{0}C. Blood ten (10) ml was also collected into vacutainer tubes containing 0.9ml of Acid Citrate Dextrose for isolation of neutrophils. Blood was also used to determine packed cell volume and blood smears were prepared for total white blood cell differential counts.

3.10 Laboratory Analysis

3.10.1 Packed Cell Volume. Heaprinzed hematocrit micro-Capillary tubes (Drummond cat # 1-000-7500-HC/5) were used to collect blood in duplicate from tubes containing Ethylenediaminetetraacetic (BD, Franklin Lakes, NJ). Mico-capillary tubes were sealed with clay sealant (Fisher Scientific Inc. Pittsburg, PA) and placed in numbered duplicate sample slots and then centrifuged for 5 minutes at 14,000 rpm in an IEC MB Micro Hematocrit centrifuge (Damon/IEC Division, Needham, MA). The capillary tubes were then placed in micro capillary reader (BD, Franklin Lakes, NJ) to determine the PCV.

3.10.2 Differential White Blood Cell Count. A thin blood smear was prepared in duplicate by placing a drop of blood (approximately 4 mm in diameter) on one end of a micro slide. A spreader slide was used at 45^0 angle to disperse the blood over the slides length in order to space out cells far enough to be counted and differentiated. The slide was left to air dry at room temperature then stained with Sure Stain Wright CS-432 solution according to the manufacturer's instructions (Fisher Scientific Inc. Pittsburgh, PA). The slides were dipped in Wright stain for 10 seconds then in deionized water for 15 seconds, and finally rinsed by dipping in fresh deionized water for 10 seconds. The slides were left to dry at room temperature. White blood cell differential counts were carried out by placing slides under 100X magnification of Olympus B X40 microscope using mineral oil (Sigma-Aldrich St Louis, MO). Counting was performed using Fisher Five Button Lab Counter (Fisher Scientific Inc. Pittsburgh, PA). Slides were counted in duplicate to identify the percentage of lymphocytes, neutrophils, monocytes, eosinophils, and basophils.

3.11 Plasma Preparation

Whole blood in a 15 ml tube containing anticoagulant citrate dextrose (ACD) was centrifuged at 3600 rpm for 20 minutes to separate plasma from blood cells. Plasma, which was at the top surface, was collected using a pipette into a 10 ml tube and stored at -70^0C.

3.11.1 Bicinchoninic Acid Assay (BCA). Pierce™ BCA Assay kit (Rockford, IL) was used to determine the total protein concentration content from plasma isolated from whole blood. Protein standards were prepared (Appendix A).

Briefly 25 µl of each standard and unknown sample was pipetted into a 96-microplate well (Thermo Scientific™ Pierce™, Rockford, IL). Working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Two hundred (200) µl of the

WR was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered and incubated at 37^0C for 30 minutes. Plates were cooled to room temperature (RT). A standard curve was created using the protein standard samples at 0µm/ml, 25µm/ml, 125µm/ml, 250µm/ml, 500µm/ml, 750µm/ml, 1000µm/ml, 1500µm/ml and 2000µm/ml and the absorbance was measured at 562nm on a plate reader (BioTek Instruments Inc, Winooski VT). Unknown sample concentrations were quantified by interpolating the optical density from the standard curve and also the line of best fit.

3.11.2 Evaluation of the Secretion of Cytokines

3.11.2.1 Preparation of Protein standards. The human inflammation ELISA strip protein standards (Signosis Inc, Santa Clara, CA) was used to profile selected cytokines. Two hundred (200) µl of the diluent buffer was added to the well of the first strip of a 96 well plate, and then 100 µl of diluent buffer was added to the remaining wells of the strips according (See Appendix C). Protein standards were added to the first strip. A multi-channel pipette was used to mix the dilutions and transfer 100 µl to the next dilution wells. This transfer was done until the 5^{th} strip. No transfer was done to the 6^{th} strip.

3.11.2.2 Cytokines. The Human Inflammation ELISA kit (Signosis Inc, Santa Clara, CA) was used to determine pro-inflammatory cytokine amounts of eight cytokines: Tumor necrosis factor-alpha (TNF-α), interferon regulator (IFNr), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1a (IL-1a), interleukin-8 (IL-8), interferon inducible protein 10 (IP-10), and regulated on activation, normal T expressed and secreted (RANTES) in pooled blood plasma. The assay was done in triplicate according to the given protocol.

Briefly according to the manufacturer, seals of the 96 well plates were properly removed and then 100 µl of standard, control and treatment plasma samples were added into each well in triplicate and incubated for 1 hour at room temperature with gentle shaking. Following incubation each well was aspirated and washed by adding 200 µl of 1X wash buffer. This process was repeated three times for a total of three washes. The plate was inverted onto a towel after the final wash to ensure the removal of excess liquid. One hundred (100) µl of diluted biotin-labeled antibody mixture was added to each well and incubated for 1 hour with gentle shaking. Each well was aspirated and washed by adding 200 µl of 1X wash buffer. This process is repeated three times for a total of three washes. The plate was inverted onto a towel after the final wash to ensure the removal of excess liquid. Afterwards, 100 µl of diluted streptavidin-HRP conjugate was added to each well and incubated for 45 minutes at room temperature with gentle shaking. Each well was aspirated as before and washed by adding 200 µl of 1X wash buffer. This process was repeated three times for a total of three washes. The plate was inverted onto a towel after the final wash to ensure the removal of excess liquid. A 100 µl of substrate solution was added to each well and incubated for 25 minutes at room temperature, followed by the addition of 50 µl of stop solution into each well. There was a color change from blue to yellow. The optical density was read at an absorbance of 450 nm using a micro plate reader (BioTek Instruments Inc, Winooski VT). A standard curve was generated to identify optical density of unknown samples. Unknown sample concentrations were quantified by interpolating the optical density from the standard curve and also the line of best fit.

3.12 Sample Preparation

The plasma protein sample was mixed with an equal volume of 2X treatment buffer (Laemmli, 1970) and boiled for 2 minutes to denature the proteins.

3.12.1 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). A discontinuous SDS-PAGE was done using 0.75-mm thick slab gels with a 4.5% stacking gel and 12 % separating gel. Monomer gel solution was poured into glass gel cassettes. Deionized water was added slowly over the monomer solution to ensure that the top of the gel was even. After 30 minutes, the water was discarded and running gel overlay was added on the gel. The gels polymerized overnight at room temperature. The gel overlay was then decanted. A 4.5% stacking gel solution was prepared and pipetted onto the 12 % separating gel and a comb was inserted into the stacking gel. The stacking gel was allowed to polymerize for 30 minutes. The comb was removed after polymerization was complete. The samples were carefully loaded in the appropriate wells. Electrophoresis was carried out with a current of 100 watts for approximately 1 to 2 hours using the LKB Slab Gel System and the Ephortec® 1000 volt power supply unit. The gels were silver stained by the method of Tsai and Frasch (1982).

3.12.2 Silver Staining. Slab gels were stained using the method of Tsai and Frasch (1982). Powder-free gloves were used throughout the silver staining procedure. All steps were performed at room temperature. Gels were transferred from gel cassettes into staining trays filled with fixative (40% ethanol (ETOH), 5% acetic acid, and 55% deionized water) and placed on a shaker overnight. After the overnight fixing step, the fixative was discarded and a freshly prepared oxidizer solution (0.7g oxidizer, 100ml of fixative) was added. The gels were placed on the shaker for 5 minutes. The gels were washed in deionized water for 15 minutes. This step was repeated twice. The gels were incubated for 30 minutes in a silver stain (Appendix F) prepared fresh and followed by three 10 minute washes and developed in a solution containing citric acid (50mg), 37% formaldehyde (0.5ml) and 1 liter DH2O, and stored in 5% acetic acid.

3.13 Detection of Prostaglandin E2α

Prostaglandin supernatant of control (PBS) and treated whole blood was evaluated using commercial Enzyme-linked immunosorbent assay (Cayman) following manufacturer's instructions. (See appendix for standard and buffer preparations).

One Hundred (100) µl of EIA Buffer was added to NSB wells, fifty (50) µl of EIA Buffer was also added to the B0 wells. This was followed by the addition of Prostaglandin E2 EIA Standards to wells. Fifty (50) µl from tube #8 was added to both of the lowest standard wells (S8). Fifty (50) µl from tube #7 was also added to each of the next two standard wells (S7). The procedure was continued until all the standards were aliquoted. The same pipette tip was used to aliquot all the standards. This was followed by adding fifty (50) µl of plasma sample for each treatment into each well in triplicates. Fifty (50) µl of AChE Tracer was added to each well except the TA and the Blk wells. Fifty (50) µl Prostaglandin E2α Monoclonal Antibody was added to each well except the TA, the NSB, and the Blk wells. The plate was covered with a plastic film and incubated over night at 4°C. The plate was developed by empting and rinsing five times with Wash Buffer. Two hundred (200) µl of Ellman's Reagent was added to each well. Five (5) µl of tracer was added to the TA well. The plate was covered with plastic film and placed on an orbital shaker equipped with a large, flat cover for 90 minutes to develop in the dark. The plate was read by wiping with a clean tissue to remove fingerprints, dirt, etc. The plate cover was removed and inserted into a micro plate reader (BioTek) and read at wavelengths 405, 410, 415, 417 and 420 nm. SAS was used to plot %B/B0 for standards S1-S8 versus PGE2α concentration using a logit transformation.

3.14 Isolation of RNA from Goat peripheral Blood

Total RNA was extracted from blood collected in PaxGene RNA collection tubes (QIAGEN Inc, Valencia, CA) using the ZR whole-blood Total RNA kit (Zymo Research, Orange, CA), as recommended by the manufacturer. Briefly, tubes were centrifuged at 3600 rpm for 15 minutes at 4^0C. The supernatant was removed and 5 ml of dietylpyrocarbonate (DEPC) treated water was added, vortexed and centrifuged for 10 minutes. The supernatant was removed and 3 ml of blood RNA buffer was added and incubated for 10 minutes on ice. Six hundred (600) µl of the mixture was transferred into a column with a collection tube and centrifuged at 14,000 rpm for 2 minutes. The supernatant was transferred to a fresh column and 400 µl of RNA was buffer was added to the column and centrifuged at 12,000 rpm for 30 seconds, then transferred into an RNAase free tube. One hundred (100) µl of RNA recovery buffer was then added to the column and centrifuged at 12,000 rpm for 30 seconds. One hundred (100) µl of ethanol was then added to the column with a collection tube and centrifuged at 12,000 rpm for 30 seconds. Four hundred (400) µl of RNA prep buffer was then added to the column and centrifuged at 12,000 rpm for 1 minute. The flow through was discarded. Eight hundred (800) µl of RNA wash buffer was then added to the column and centrifuged at 13,000 rpm for 30 seconds. The flow through was discarded. This step was repeated twice. The column was then transferred into an RNase free tube. 10 µl of DNase free water was then added to the column and centrifuged at 10,000 rpm for 30 seconds. This step was repeated again and elute was incubated at 65^0C for 5 minutes which was now stored at 20^0C.

3.15 Neutrophil Isolation

Neutrophil were isolated from whole blood using the modified Carlson and Kaneko method (Carlson and Kineko, 1973). Briefly, whole Blood from each goat was placed into a 50

ml polypropylene conical tube, mixed, balanced and placed on ice. The blood was centrifuged on a swinging bucket rotor (Eppendorf Centrifuge 5810 R) at 3500 rpm (1800 g) at 4^0C for 20 minutes. Centrifugation leads to the separation of blood components into plasma, buffy coat and red blood cell (RBC) layer. Using a vacuum, plasma, buffy coat and 1/3 of the RBC layer of each tube was carefully aspirated. The caps were replaced and gently inverted to re-suspend neutrophil/red cell layer and immediately 20 ml of ice cold DEPC treated distilled water was added. Red blood cells were lysed by inverting and rotating the tube for 45 seconds. Ten (10) ml of 2.7% saline was added to restore isotonicity and the contents were mixed by rotating and inverting the tube. Cells were kept cold by returning to ice periodically followed by centrifuging at 2000 RPM (700 g) at 4^0C for 2 minutes and discarding the supernatant. The cells were resuspended in 10 ml of ice cold PBS (pH 7.4) to the pellet and then it was gently inverted and rotated manually. The whole procedure was repeated in other to obtain a clear white pellet. After the last addition of cold PBS, cells were placed on ice.

3.15.1 Determination of Total Cell Count and Viability of Neutrophils. Viability and total cell count of isolated neutrophils was determined by using the Trypan blue dye exclusion method. Isolated neutrophil were mixed with Trypan blue (Sigma-Aldrich cat #T8154) in a ratio of 1:1. 10 µl of the mixture was then mixed properly and placed on a TC10 ^{TM} system counting slide. The slide was then inserted into the TC10 ^{TM} system (Biorad) to count. The number of cells that had not taken up the dye was counted as live cells and the results were given in % of live (viable) cells. Total number of cells was also obtained.

3.16 Isolation of Total RNA from Neutrophils

Neutrophils were adjusted to a concentration of $1x10^7$ cells/ml using PBS and re-suspended in 1 ml of Tri-reagent (Molecular Research Centre, USA). The recommended protocol

was used. Briefly, cells were incubated at room temperature for 5 minutes. Two hundred (200) µl of chloroform was added to the tubes shaken manually for 15 seconds and incubated at room temperature for 15 minutes. This was followed by centrifugation at 1200 g (9,000 RPM in Eppendorf Model 5810R Centrifuge) at 4^0C for 15 minutes. The aqueous phase containing the RNA was transferred to a clean tube. To the aqueous phase, 500 µl of isopropanol was added and incubated at 4^0C for 90 minutes to precipitate RNA. Samples were then centrifuged at 12,000 g at 4^0C for 8 minutes. The supernatant was discarded. The RNA pellet was then washed with 1 ml of 95% ethanol. Ethanol was decanted and the pellet was allowed to dry for 15 minutes at room temperature. The dried RNA pellet was dissolved in 30 µl of sterile DEPC-treated water and then stored at -80^0C until further use.

3.16.1 RNA Concentration and Purity. The ND-1000 UV/VIS nanodrop (NanoDrop Technologies) spectrophotometer was used to determine the concentration and purity of isolated RNA from PaxGene tubes and neutrophils and DNA from fecal samples. RNase free water was used as a blank. One (1) µl of RNA was dropped on the nanodrop and the concentration and purity was determined. Optical densities were read at A260/280 for nucleic acid purity.

3.17 Preparation of cDNA from PMN RNA

Complementary DNA (cDNA) was synthesized using a RETRO script Kit (Ambion Inc., Austin, TX) by taking 2 µg of total RNA from each sample and adding 2 µl of Oligo (dT) (Ambion Inc., Austin, TX)). The final volume was adjusted to 12 µl by the addition of nuclease free water in a PCR reaction tube. The samples were vortexed, spun and placed into a thermocycler (MWG Biotech, Martinsried, Germany) then heated for 3 min at 85ºC, removed and placed on ice. Next, the remaining reverse transcription components were added to each sample, 2 µl 10X RT buffer, 4 µL dNTP mix, 1 µl RNase Inhibitor and 1 µl reverse transcriptase

(RT) (Ambion Inc., Austin, TX) totaling 20 μL in volume. The tubes were vortexed, spun down and placed into the thermocycler for incubation at 44ºC for 1 hr. to activate reverse transcription followed by incubation at 92ºC for 10 min to stop the action. Finally, the cDNA products were measured for purity and concentration using the Nanodrop spectrophotometer (Thermo Scientific Inc., Waltham, MA) as described above and stored at -20ºC.

3.18 Real Time-PCR

The Qiagen Human Toll-like Receptor RT-PCR kit (Qiagen, Valencia, CA) was used to detect transcription. Ninety-six-well plates containing gene-specific primer sets for 84 relevant TLR pathway genes, 5 housekeeping genes, and 2 negative controls were used. After performing thermal cycling (according to the manufacturer's protocol), real-time amplification data were gathered by using manufacturers supplied software.

3.19 The Livak Method

Gene expression was normalized to internal controls GAPDH (housekeeping genes) to determine the fold change in gene expression between test and control samples by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The C(t) for each gene from the different samples was standardized to the sample GAPDH value [ΔC(t) = gene C(t) − GAPDH C(t)], and this value was then compared using the ΔΔC(t) method to determine the fold change {fold change = [2^(−ΔΔCT)]}. The significance value for the fold change in each gene fold change was calculated as the difference in gene expression between test samples (hot and cold) and control sample (water). A positive value indicates gene upregulation, and a negative value indicates gene downregulation.

3.20 Statistical Analysis

A crossover analysis, repeated measures and ANOVA was performed on all variables using the statistical analysis software package SAS (Cody and Smith 1997). Variables included FAMACHA, body weight, body condition, FEC, PCV, differential white blood cell count, DNA concentration, protein concentration and purity. To examine significance of the treatment differences, the F value was calculated by comparing the computed F value with the tabular F at 5% level of significance or $P<0.05$. All treatment differences with computed F values greater than tabulated F values at 5% of significance were considered significant. Treatment differences with computed F value smaller or equal to the tabulated F value at 5% level of significance were considered not significant. A Pierson correlation and coefficient was used to determine correlation between variables.

CHAPTER 4

Results and Discussion

4.1 Body Weight

Initial weight of goats used in this experiment had an average weight of >52 kg across all groups. There was no significant difference between treatments and control ($p<0.1045$) at the end of the study. During the last 4 weeks, there was a time effect ($p<0.001$) and also correlation between body weight and lymphocyte cell count ($r=0.22$, $p<0.0028$). There was no effect of CorPet mushroom on body weight. The average mean weight for control was 66.1 kg (Fig. 3). However CorPet treatment did have an effect on body weight gain, over time, it resulted in a mean weight of 62.7 kg in the treated animals. Results obtained were within the range of a normal adult female Boer goat (Coffey, 2002). Goats used in this study were 3 weeks after weaning and an increase in weight was expected across all treatments. Diet and feed were kept constant during experimental period in other to rule out any source of variation.

Body weight is an important parameter in determining the health status of an animal over a certain period of time. A decline in weight can be associated with parasitic infection, high temperatures that stress the animal and also lack of appropriate nutrition. It is expected that animals keep a relatively consistent body weight during growth. Healthy normal adult female Boer goats have a body weight that ranges from 55 kg to 90 kg (Coffey, 2002). In general, animal studies have been previously reported in the literature and these showed that mushroom enriched diet had no effect on body weight (Handayani et al., 2012). Also a study that was carried out on the effect of *Pleurotus ostreatus* on the effect of weight gain by Daneshamand (2011), showed that supplementation of mushroom or probiotic had no effect on weight gain during the entire experiment.

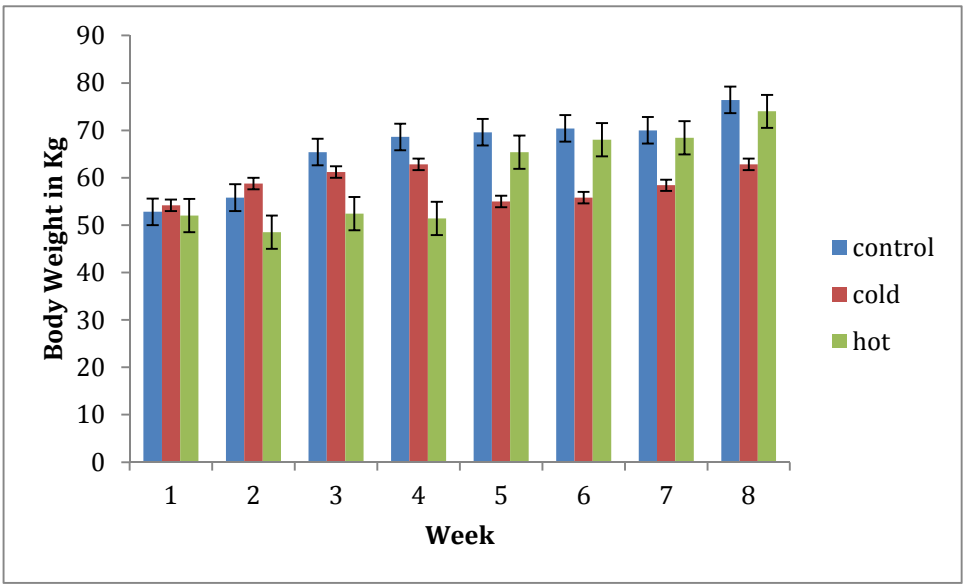

Figure 3. Effect of CorPet on Body Weight ($p>0.05$)

4.2 Body Condition

CorPet mushroom probiotic had no effect on body. There was no significant difference in body condition between animals in the control and treatment groups (Fig. 4). The body condition scores were in the range of 2 to 3 indicating a healthy animal condition (Mendizabel et al., 2011). CorPet mushroom probiotic had no effect on body condition. Results in this study shows that body condition scores could be used to identify goats which are anemic. Body condition scores is one of the tools goat farmers can use to achieve proper management of body reserves and has proved to be an effective and easy to use method (Mendizabal et al., 2011).

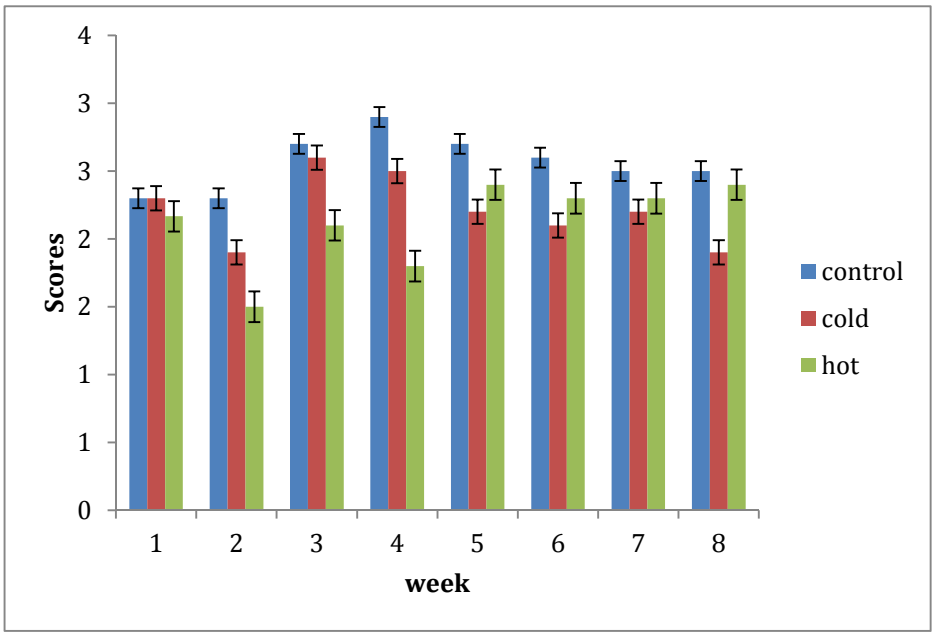

Figure 4. Effect of CorPet on Body Condition Scores ($p>0.05$).

4.3 FAMACHA

The FAMACHA scores were similar between control and treatment groups. There was no significant difference between treatments and control. There was no correlation between eye scores and PCV. The FAMACHA score results were in the range of red (FAMACHA = 1) to red-pink (FAMACHA = 2) indicating that anemia was generally not a major problem in the study population. This is an effective tool for identifying anemic goats (Kaplan et al, 2004).

The FAMACHA© method can be used as a safe and reliable approach to reducing pressure in goats on the selection for anthelmintics in relation to routine non-selective treatment for worm control (Kaplan et al., 2004). This tool could also be used to reduce cost and delay the development of anthelmintic resistance. It can also be used to improve the genetic resistance of individual herds or flocks (Bath et al., 2001).

4.4 Packed Cell Volume

There was no significant difference between treated and control animals. Ogbe et al., (2009) reported that there was no effect of aqueous mushroom extract of Ganoderma *lucidum* on PCV which correlates with the result obtained in this study. However was a time effect for the first four weeks ($p<0.05$) and the last four weeks ($p<0.002$) which may be associated with infection as the animals were on pasture. On a weekly base the treatment group had a lower PCV compared to control. The control group had an average PCV of 26% (Fig. %). A normal PCV for an adult goat ranges from 24 to 48% (Karki, 2008). PCV is an important tool in the diagnosis of parasitic infection (Vatta et al., 2002). Kaplan et al., (2004) reported a correlation between PCV and FEC but there was no correlation between FEC and PCV in this study. The level of infection may have been low and not caused anemic conditions.

The PCV obtained in this study was within the range of a healthy goat. Low PCV may be indicative of anemia, which is an undesirable condition that points to a compromised health status of the animal. It is also an

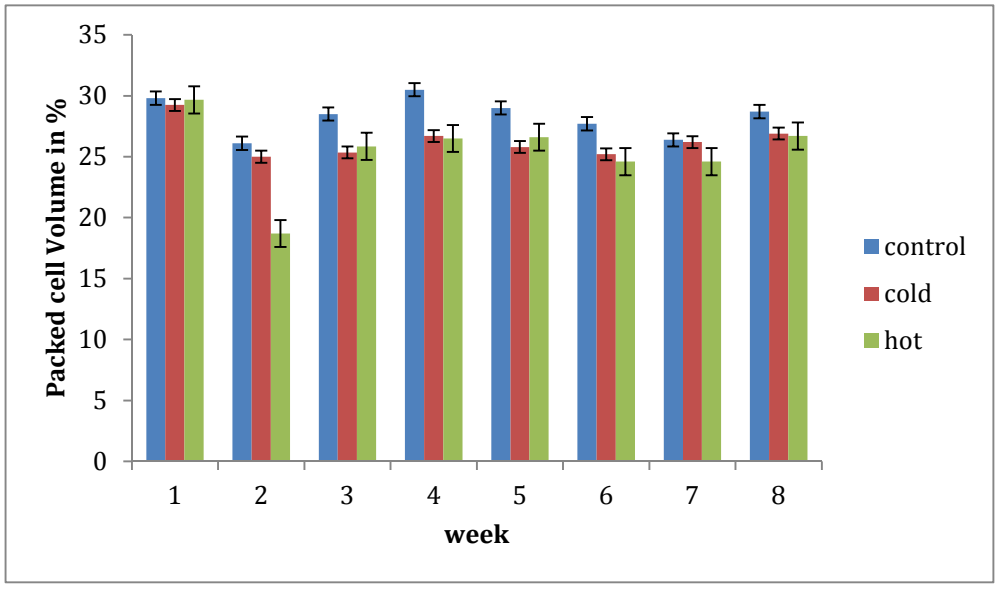

Figure 5. Effect of CorPet on PCV in Percentage.

4.5 Fecal Egg Count

4.5.1 Haemochus. *Haemonchus contortus* eggs and coccida oocytes were found in fecal samples. There was no significant difference between control and treatments. An average mean of 350 epg was obtained across treatments. However there was an observed trend of increase in *H. Contortus* egg count in the first 3 weeks compared to a decrease in the last 5 weeks with CorPet treatment (Fig. 6).

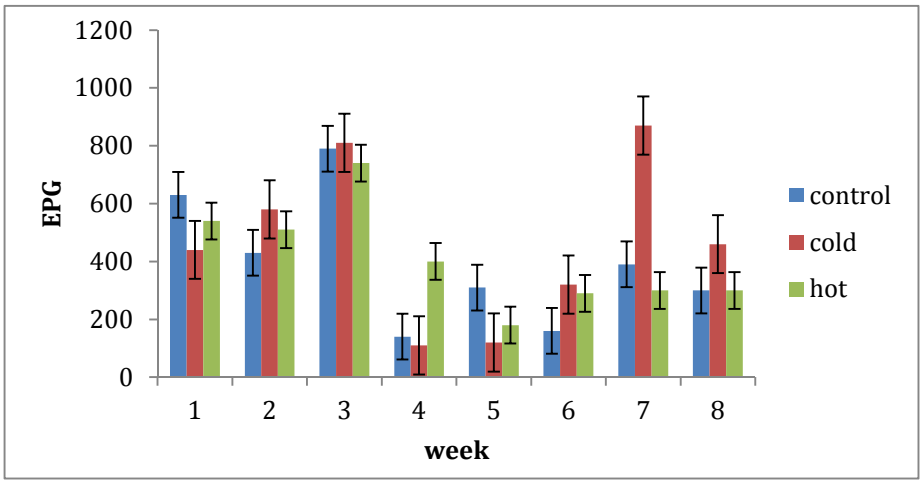

Figure 6. The Effect of CorPet Extracts on Haemochus Eggs Per Gram Feces.

4.5.2 Coccidia. There was no significant difference between treatments and control. There was an observable trend in the results obtained. At the beginning of the experiment there was a high oocyst count (Fig. 7). During the switch there was a decrease in oocyst count. There was no effect of CorPet extracts on coccidian oocyst count. Haemochus egg count and Coccidia oocyst count started off at a high rate but reduced after the first 4 weeks in treated and control animals. Cold extract treatment had fewer oocysts than the control in week 2, 3, 4, 5 and 7. Hot

extract treatment tended to increase; maybe extraction method had an effect.

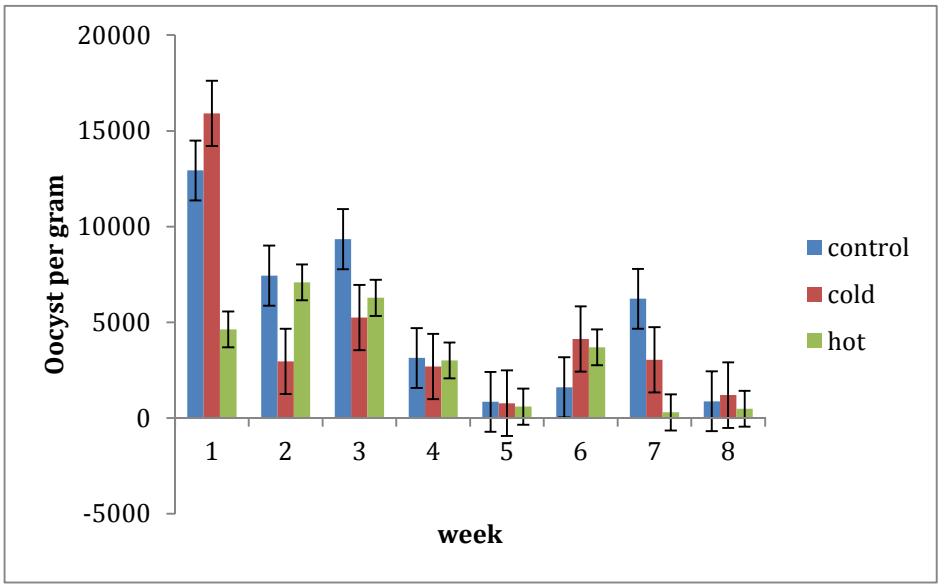

Figure 7. The Effect of CorPet mushroom Extracts on Coccidia Oocyst per Gram Feces.

Fecal egg count is a parameter widely used to determine parasitic infection in animals (Kaplan et al., 2004). Basically a high FEC is associated with an elevated presence of adult parasites in their productive stage in the hosts' digestive system (Kaplan et al., 2004). It is a tool to help a scientist to determine the presence of gastrointestinal nematode parasites and severity of it. Coccidiosis is one of the most important diseases of goats in the United States because of its effect on productivity, especially growing kids (Levine, 1985). Prevalence of *Eimeria* infection is common and contributes to production losses (Valentine et al., 2007). There are some reports demonstrating positive effects from feeding fungal myceliated grain to chickens (Willis et al., 2009) and these researchers have observed its potential to control coccidiosis. In another study, Ogbe et al. (2009) reported that the wild mushroom (Ganoderma lucidum) used to treat *E. tenella* infected broilers resulted in a reduction in the number of *E. tenella* oocysts shed in the faeces and led to improved weight gain.

Mushroom extracts can impact gastrointestinal nematode infections under certain conditions and therefore may eventually play a significant role in an integrated GIN management protocol for small ruminants. Bioactive compounds or polysaccharides are known to play vital roles in enhancing health; they block colonization of the intestine by pathogens, thereby improving their elimination from the body (Elmusharaf et al., 2006).

4.6 Microbial DNA in Fecal samples

The concentration and purity of the microbial DNA isolated from fecal samples is an indicator of the impact of diet on the microbial population in the rumen and impacts goat production and health. Samples from treatment and control groups were analyzed for week 0, 4 and 8. During the first week DNA concentration ranged from 14 to 19 ng/µl compared to the 8th week, which ranged from 5 to 16 ng/µl (Fig. 8). CorPet treatment reduced fecal DNA concentration from the observable trend.

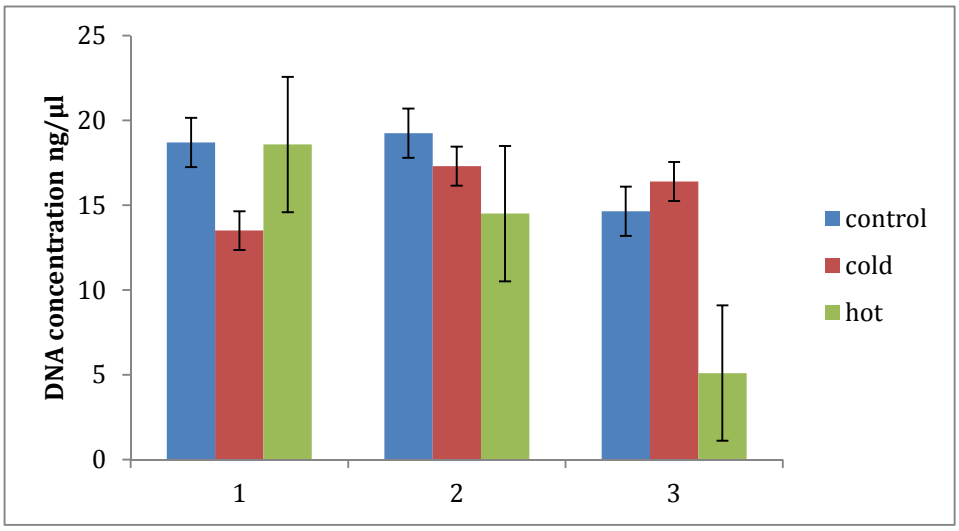

Figure 8. Concentration of Microbial DNA in fecal samples ng/µl for week 0(1), week 4(2) and week 8(3).

There are other results indicating the effect of mushroom on the microbial flora. Varshney et al., (2013) reported that when white button mushroom was added to the diet of mice,

it changed the composition of the normal flora which resulted in better control of inflammation and resolution of infection. This may also correlate to the result obtained in this study. Hot extract treatment tends to decrease microbial shedding.

4.7 White Blood Cell Differential Count

There was no significant difference in the percentages of neutrophils, lymphocytes, monocytes, basophils and eosinophils ($p<0.5222$) between treatments and control groups. There was a strong negative correlation between neutrophils and lymphocytes ($r=-0.94$, $p<0.0001$) as well as neutrophils and monocytes ($r=-0.19$, $p<0.0470$). Over time there was a significant increase in the last four weeks in the eosinophil count ($p<0.0001$). The higher production of eosinophils observed suggests a potential role in combating GI nematode infections (Balic et al., 2006). Over time there was a significant increase of lymphocyte count ($p<0.007$) and a decrease in neutrophil count ($p<0.001$) during the last four weeks (Fig. 9 & 10).

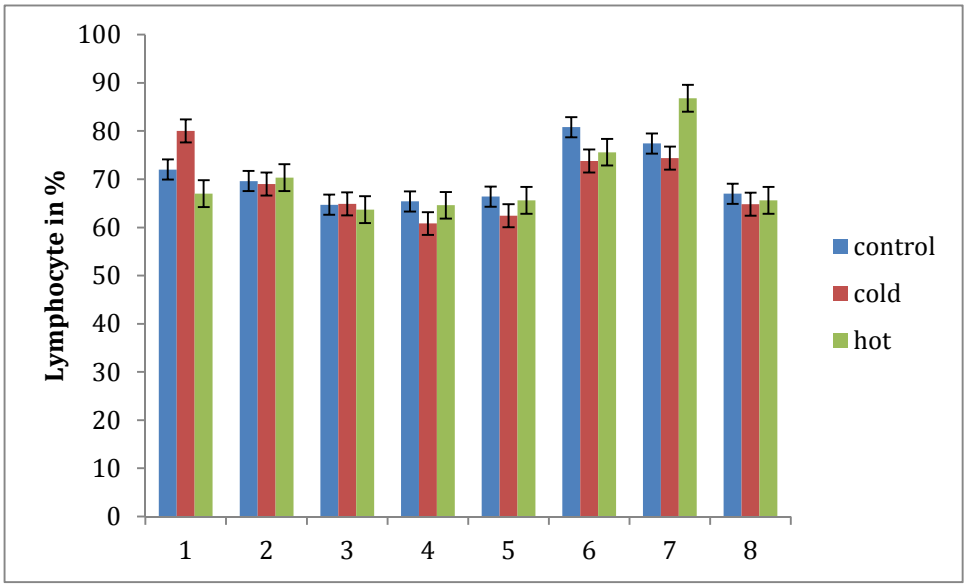

Figure 9. Effect of CorPet on Lympocyte Cell Count in Percentage.

The high lymphocyte counts in animals in this study might be attributed to stress and immune response to the environment which harbors various detectable and undetectable parasitic

organisms. The normal value for white blood cell differential count in goats are 30-48% neutrophils, 50-70% lymphocytes, 0-1% basophils, 0-8% monocytes and 1-8% eosinophils (Khan et al., 2010).

There was no effect of CorPet on differential white blood cell count. Stimulation of production of White blood cells (WBC) in an immunosuppressed animal model has been classified as an immunomodulatory effect (Kyakulaga et al., 2013). Some other reports have shown the positive effects of mushroom extracts on differential white blood cell count. Kyakulaga et al., (2013), reported that aqueous extracts of *Auricularia* sp and *Pleurotus* sp from Ugandan rain forests increased total and differential WBC counts in cyclophosphamide immunosuppressed Wistar rats.

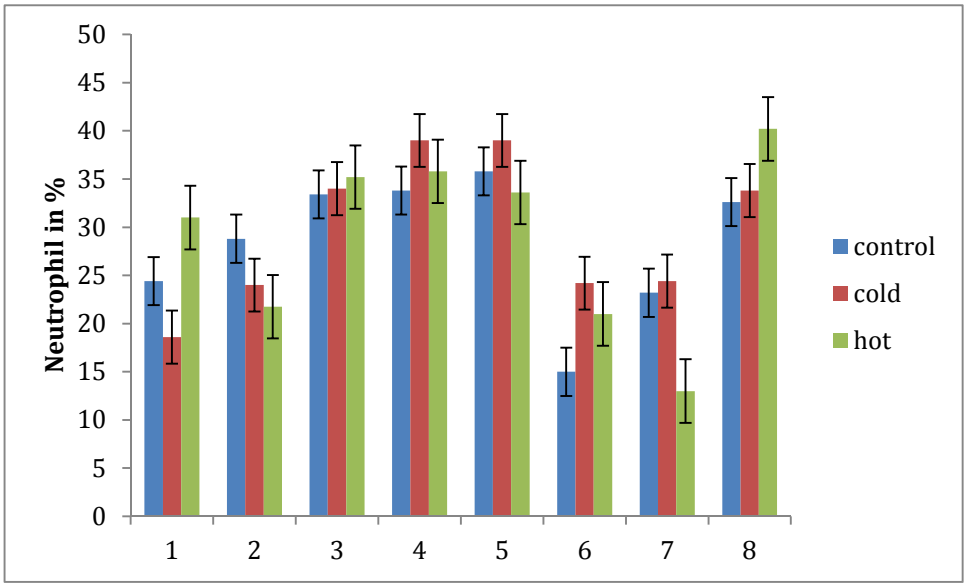

Figure 10. Effect of Corpet on Neutrophil Cell Count in Percentage.

The total white blood cell count is a tool to identify potential immune response to a parasitic challenge and parasite resistance (Kaplan et al, 2004). White Blood cell differential count normal value for goats in percentage are 34% neutrophils, 53 % lymphocytes, 1%

basophils, 5 % monocytes and 5% eosinophils (khan and Line, 2010). Observed differential cell count on the effect of Corpet treatment is shown in Table 2.

Table 2.

White blood differential cell count in percentage Averages (%) of Treatment Groups.

Week	Neutrophil	lymphocytes	Eosinophils	Basophils	Monocytes
0	31	67	1	1	0
4	33	64	1	2	0
8	36	62	1	1	0

A differential white blood cell count is an important tool used to provide clinical diagnosis, monitoring of disease and blood disorders (Houwen, 2001). This system quantifies and differentiates white blood cells at one particular time giving insight to infection and if treatments are working (Houwen, 2001).

4.8 Protein Concentration

There was a significant difference between treatments and control ($p<0.0001$). Over time there was an increase plasma protein concentration ($p<0.001$) for the first four weeks and a decrease in the last four weeks ($p<0.0001$). As observed during week 2 to 5, treatment had a high protein concentration compared to control (Fig 11). There was a positive correlation between plasma protein concentration and neutrophil cell count ($r=0.30$, $p=0.0013$). However, a significant negative correlation was observed between protein concentration and coccidia ($r=-0.26$, $p=0.0073$). The reduction in hematological parameters such as changes in plasma protein is common clinical symptom of gastrointestinal parasitism. Immunomodulation by probiotics may stimulate increase in plasma proteins (Johnson et al., 2009).

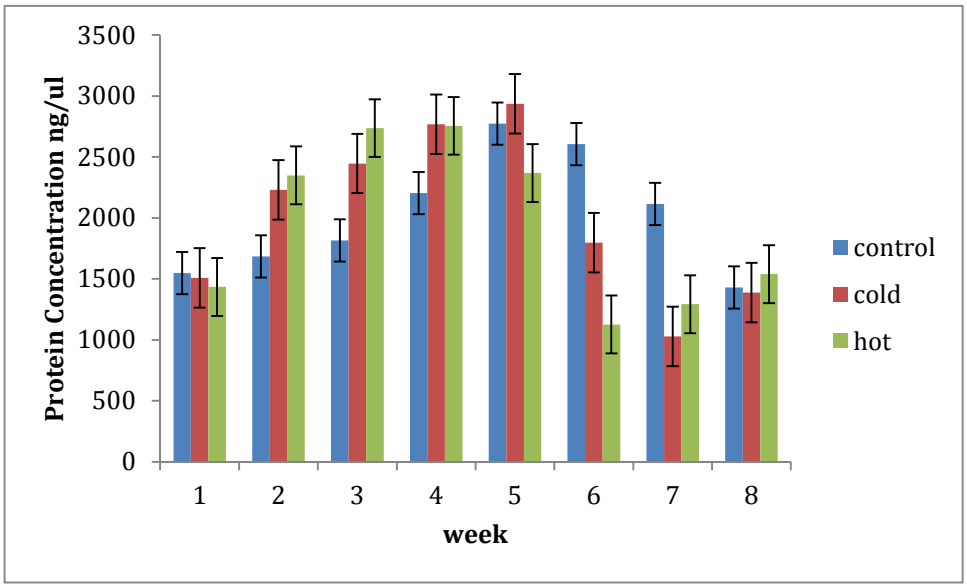

Figure 11. Effect of CorPet mushroom extracts on plasma protein concentration in ng/µl.

4.9 Plasma Protein Migration Pattern on SDS PAGE Gels

The SDS gel electrophoresis showed high band intensity among treatments. There was difference between treatment and control for week 0, 4 and 8. Protein bands were all the same across treatments (Fig. 12). Over time the density of bands observed increases and may be an indicator of the observed effect of CV on plasma protein concentration. Thus CV did not have adverse effect on cell proteins and white blood cell count.

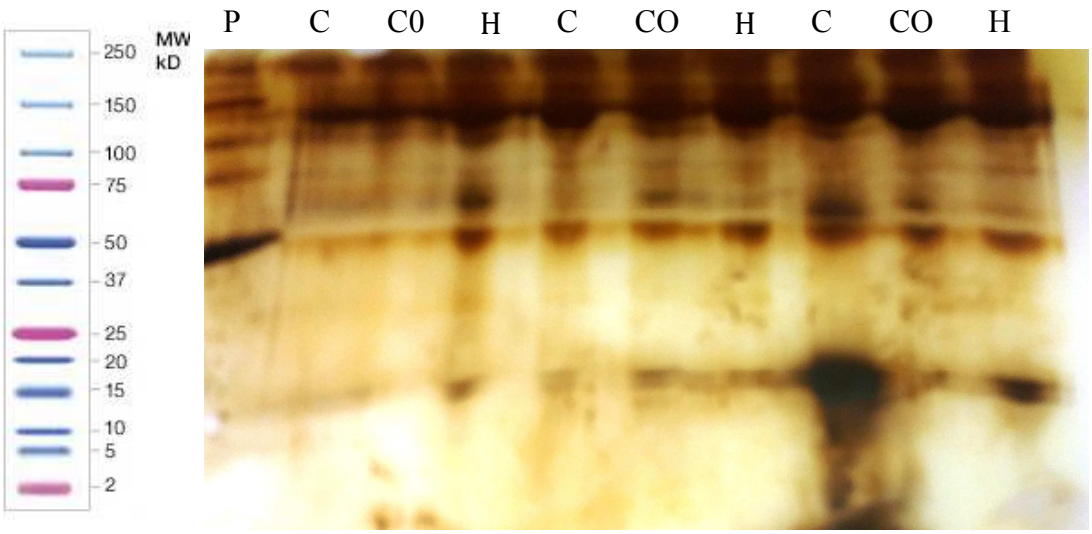

Figure 12. SDS Gel Electrophoresis. PM=marker, C1=control week 1, CO1=cold week1, H1= hot week 1, C4 control week 4, CO4=cold week 4, H4=hot week 4, C8=control week 8, CO8= cold week 8 and H=hot week 8.

4.10 Secretion of Proinflammatory Cytokines in Plasma

The release of proinflammatory cytokines is essential for host survival from infection, and is also required for the repair of tissue injury. Proinflammatory cytokine levels were measured in plasma from the treatment and control groups at week 0, 4 and 8 of the study. Comparing the production of Eight (8) proinflammatory cytokines, the CV extract induced detectable levels four (4) cytokines. TNF-α, IL-1a and IL-8 were not detected, IFNr, Rantes and GCSF increased while GM-CSF levels decreased from weeks 0 to 8. Lim (2011) reported that antiinflammatory response upon treatment with CV extract correlated with the reduced expression of TNF-α, IL-1β and IL-6 which corresponds to the low secretion of TNF-α obtained in this study. Yu et al (2009) also reported a decrease in levels of serum mucosal interleukin-2 (IL-2) and TNF-α in rat with oral ulceration of *Lentinus edodes.* Also Johnson et al., (2009) reported that In vivo study *of Agaricus blazei* reduced the levels of TNF-α by 84% and IL-2 by 46%. Batbayar et al., (2011) reported that β-glucan of Ganoderma lucidum induced the sereetion

of granulocyte colony-stimulating factor and regulated on activation, normal T expressed and secreted (RANTES) which correlates with results obtained in this study.

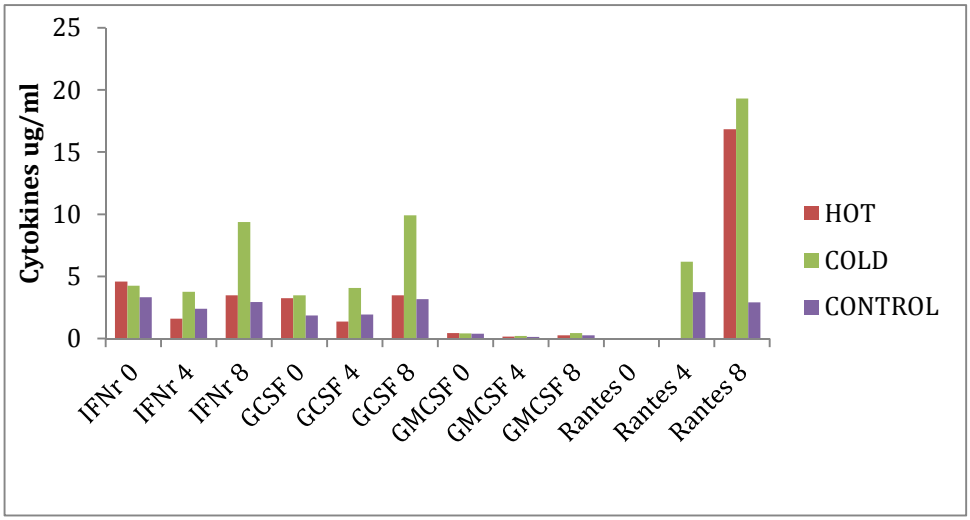

Figure 13. Effect of CorPet on the Secretion of Cytokines.

The immunomodulatory effects of mushroom are usually associated with the stimulation of the immune system by a variety of polysaccharides (Jedinak et al., 2011). β-glucan activates B-lymphocytes and macrophages through Toll-like receptors, modulating the immune system and inducing the production of cytokines (Liao et al., 2004). The antioxidant activity of *Coriolus versicolor* and the limited adsorption of its PSP and PSK across the intestinal mucosa to the blood have been reported. However in this study treatment with CV extracts by drenching stimulated cytokine secretion in different manner over time. GCSF and GMCSF may be associated with the release of granulocytes and mononuclear cells and associated with the observed increases in white blood differential counts for neutrophils and lymphocytes.

Coriolus versicolor has been reported to improve survival and immune function (*in vivo*) (Amberg et al., 2010). Infections with *Eimeria* have been observed to result in differential cytokine gene expression of IL-10 (Rothwell et al., 2004) and IL-8 in cattle (Coussens, 2004). In

this present study these cytokines were not secreted. Chang et al., (2009) reported that oral administration of Enoki mushroom displayed anti-tumor activity through activating both innate and adaptive immunity of the host to prime a cytotoxic immune response and IFN-γ played a key role in the anti-tumor efficacy. Seong et al., (2010) reported that when cells were stimulated with β-glucan the macrophage cells increased TNF-α expression, but in our study there was no secretion of TNF-α.

4.11 Secretion of Prostaglandin E2α

$PGE_2\alpha$ is an inflammatory mediator that secreted by various cells. In this study, over time there was a decrease of $PGE_2\alpha$ secretion from week 1 to week 8. Results in this study correlates with the study done by Jedinak et al., (2011) which reported that concentrate of oyster mushroom inhibited production of prostaglandin E2 through the down regulation of expression of COX-2.

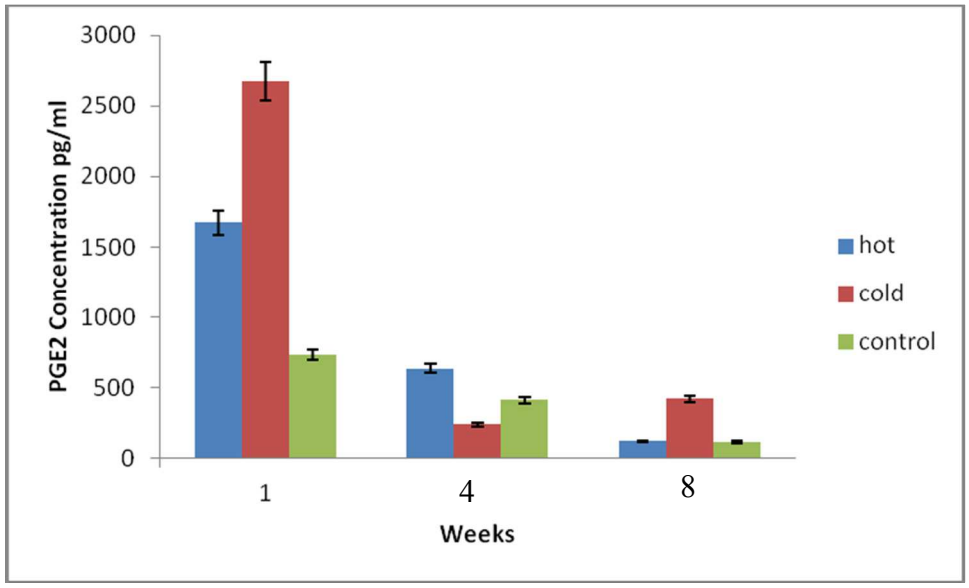

Figure 14. Effect of CorPet on the secretion of PGE2*α*.

4.12 RNA Concentration

There was no statistical significance between treatment and control ($p<0.42$). There was an observable trend of a decrease in RNA concentration in samples from treated goats compared to controls for the first 3 weeks and an increase in weeks 4 to 7 (Fig. 15). There was a time effect ($p<0.001$). Treatment increased RNA concentration in weeks 4 to 6.

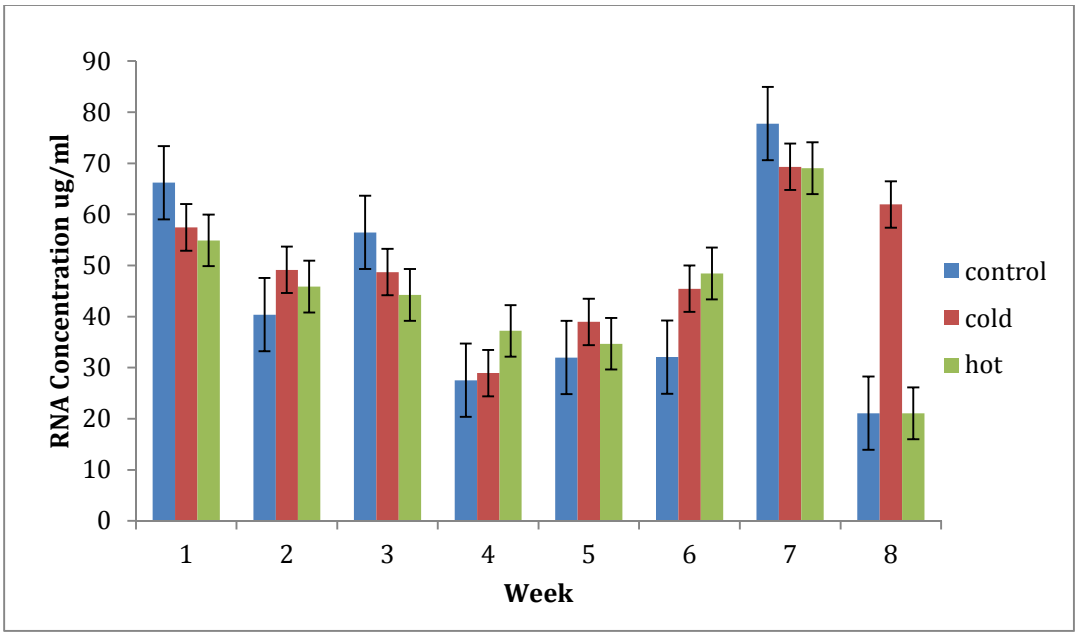

Figure 15. Effect of CorPet on RNA Concentration from whole Blood in PaXgene Tubes.

4.13 Effect of CorPet on Gene Expression

Innate immunity can be stimulated by the activation of pattern recognition receptors. At the beginning of the study the number of neutrophil genes detected in the Human Toll-like Receptor RT-PCR (Qiagen, Valencia, CA) array was similar for animals in the treatment groups (Table 3) (See gene list Appendix). Data was analyzed by subtracting the C(t) value from GAPDH. Also data was analyzed by using the C(t) values for each group as a control week 1. After 8 weeks, there was an effect of treatment on the regulation of genes. Thirty-seven (37) genes were up regulated and forty-seven (47) genes were down regulated for the hot treatment.

Twenty-eight (28) genes were up regulated, fifty-six (56) genes were unchanged for the cold treatment.

Table 3.

Number of Genes Expressed after 8 weeks.

	Control	Hot	Cold
Higher	38	26	21
Equal	20	11	10
Lower	26	47	53

These genes function as receptors, in signaling, adaptors and effectors of the toll-like receptor pathway. TAB1 and TIRAP (Appendix) had the highest fold change. At week 1 all Toll-like receptors except TLR3 and TLR7 were expressed. At week 8 TLR2 was unchanged and TLR7 was upregulated. Neutrophils express the majority of TLR family members, lacking only the intracellular receptors TLR3 and TLR7 (Prince et al., 2011) which correlates with the result obtained from this study. Ten Toll like receptors have been identified in goats and expression has been reported in different tissue (Tirumurugaan et al., 2010), however there are no reports in goat neutrophils. Bovine neutrophil have been reported to express TLR (Worku et al., 2009). The TLR1 family (TLR1, 2, 6 and 10) is involved in the recognition of gram-positive and gram-negative bacteria and heterodimers of TLR1 or TLR6 with TLR2 are crucial for the identification of several PAMPs (Kwong et al., 2011). TLRs recognize conserved pathogen-associated molecular patterns that are unique to microorganisms and are absent from higher eukaryotes (Han et al., 2003). Batbayar et al., (2011) reported that expression of TLR2, TLR4 and TLR6 was increased by β-glucan from *Ganoderma lucidum.* TLR2 signaling and TLR4 signaling

additionally require the bridging adapter Toll-interleukin- 1 receptor (TIR) domain containing adapter protein (TIRAP)/MyD88-adapter like (Mal) to link the receptors (Prince et al., 2011). The critical role of TIRAP in host defense against *Escherichia coli* has been reported (Jeyaseelan et al., 2005). This result correlates with our study.

Gene expression was analyzed for week 0 and week 8 (Table 4) was analyzed using the Livak method to determine the effect of treatment.

Table 4

Gene Expression for first week and week 8

	Week 1		Week 8	
	Hot	Cold	Hot	Cold
Up regulated	54	13	75	40
Unchanged	26	56	8	44
Down regulated	4	15	0	0

There was an increase in fold change for the hot treatment group for IL2, IL6, IL8, ECSIT, TAB1, TLR10 and TLR4 (Fig. 16). Mushroom supplementation increased intracellular interleukin (IL)-6 levels suggesting an enhancement of both innate and T cell-mediated immunity which leads to a more efficient surveillance and defense mechanism against microbial invasion (Ren et al., 2008b).

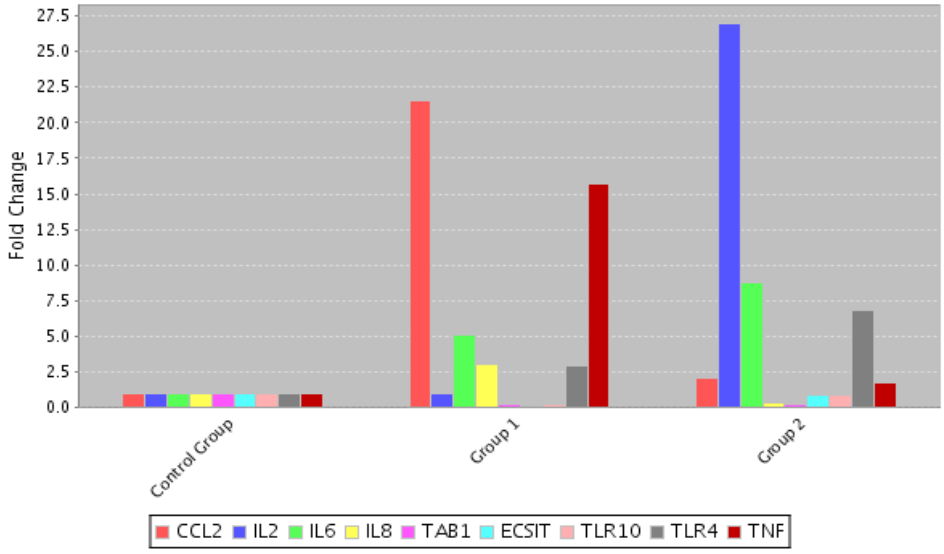

Figure 16. Expression change for a selected set of genes across all of the tested groups for week 1.Group1=Hot treatment, Group 2=Cold Treatment.

There was a decrease in fold change for CCL2 and TNF in the hot treatment. For the cold treatment group there was an increase in fold change for CCL2, IL8, TAB1, ECSIT, TLR4 and a decrease in IL2, IL6, TLR10 and TNF (Fig. 17).

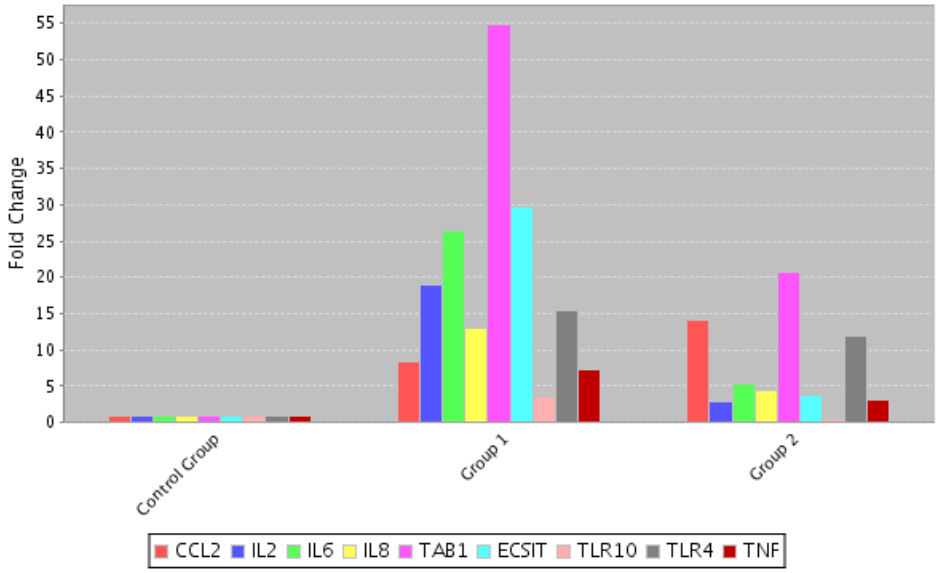

Figure 17. Expression change for a selected set of genes across all of the tested groups for week 1. Group1=Hot treatment, Group 2=Cold Treatment.

The decrease in fold change for TNF could correlate with the lack of secretion of TNF in the detection of cytokines. The fold change for TLR4, IL8, TAB1 and ECSIT was increased in both the hot and cold treatments. It has being reported that components of *Coriolus versicolor* stimulate T cell activation, induce IFN-γ and IL-2 production, reduce gene expression of cytokines (TNF-α, IL-1, IL-6, IL-8) (Cui et al., 2003) which is concordance with the lack of secretion of TNF-, IL-1a and IP-10 in plasma. Response to bacterial- and fungal-specific cell wall components through these receptors can be responsible for the initiation of not only the inflammatory response but also may direct the adaptive response through activation of specific T cells such as Th1, Th2 and T regulatory cell subsets (Price et al., 2010).

Thus in our study on goat neutrophils the administration of a probiotic containing CV modulated toll like receptors and molecules involved in downstream signaling. In light of the critical role of TLR in controlling bacterial infections the expression and modulation of these genes may be critical to goat health and modulation of innate immunity using mushroom based probiotics. The expression of 8 TLRs in goat neutrophils has implications to innate immunity in goat and efforts to modulate it through novel therapeutics.

CHAPTER 5

Conclusion and Future Research

Mushrooms and mushroom components have been reported to have a myriad of positive health benefits, mainly on the basis of *in vitro* and *in vivo* animal trials (Roupas et al., 2012). Although there has been relatively few direct intervention trials of mushroom consumption in animals, those that have been completed to date indicate that mushrooms and their extracts are generally well-tolerated. The results of this study show that mushroom extracts of *Coriolus versicolor* did not reduce gastrointestinal parasites in goats. The probiotics extracts of *CV* did not have an adverse effect on goat health and production. *CV* Extracts had an impact on protein concentration, cytokine levels and RNA. *CV* probiotics had an impact on gene expression targeting the toll-like pathway associated with innate immunity.

This study has identified gene and proteins that are responsive to mushroom supplementation and may serve as targets for future interventions to enhance goat immunity. Further studies using more samples are needed to assess the impact on microbial diversity, feed efficiency and on global gene expression.

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Appendix A

Reagents

1. Acid Citrate Dextrose
   For 500 ml
   22 g Trisodium citrate (Amhydrous)
   8 g Citric acid
   25 g Dextrose

   Bring up to 500 mls with sterile water
   Autoclave and store at 4^0C

2. DEPC-Treated Water
   1 ml of DEPC was added to 1 liter of distilled water, mixed vigorously and let stand overnight
   Then autoclave

3. 2.7% Saline
   27 g NaCl
   Bring volume up to 1 liter with DEPC

PCR Components Mix

2X RT2 SYBR Green Mastermix	1350 µl
cDNA	102 µl
RNase-free water	1248 µl
Total volume	**2700 µl**

PCR Thermal Cycling conditions

Cycles	Duration	Temperature
1	10 minutes	95^0C
40	15 seconds	95^0C
	1 minute	60^0C

Appendix B

Source: Pierce BCA Handbook

Preparation of Diluted Albumin (BSA) Standards

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

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

Appendix C

Source: Signosis Human Inflammation ELISA Strip Protein Standards Handbook

Reagent Preparation

Dilute the 5x Assay wash buffer to 1x buffer

40ml 5x Assay wash buffer

160ml ddH2O

Use serum-free conditioned media or original or 10-fold diluted sera.

Sera can be diluted with 1X

Diluent buffer. When serum-containing conditioned media is required, be sure to use serum as a control.

Dilute 50 times of biotin labeled antibody mixture with 1X Diluent buffer.

Dilute 200 times of streptavidin-HRP with 1X Diluent buffer.

Assay procedure

1. Cut the sealing film over the plate and remove it from the desired number of well strips. Make sure the rest of wells are well sealed.

2. Add 100 µl of Standard, control, or sample per well and incubate for 1 hour at room temperature with gentle shaking.

3. Aspirate each well and wash by adding 200µl of 1X Assay wash buffer. Repeat the process three times for a total of three washes. Complete removal of liquid at each wash. After the last wash, remove any remaining liquid by inverting the plate against clean paper towels.

4. Add 100µl of diluted biotin-labeled antibody mixture to each well and incubate for 1 hour at room temperature with gentle shaking.

5. Repeat the aspiration/wash as in step 3.

6. Add 100 µl of diluted streptavidin-HRP conjugate to each well and incubate for 45 min at room temperature with gentle shaking.

7. Repeat the aspiration/wash as in step 3.

8. Add 100µl substrate to each well and incubate for 10- 30 minutes.

9. Add 50µl of Stop solution to each well. The color in the wells should change from blue to yellow.

10. Determine the optical density of each well with a microplate reader at 450 nm within 30 minutes.

Appendix D

Source: Bio-Rad, DCode Universal Detection System Handbook

Tsai and Frasch Silver Stain

Materials and Equipment needed:

- Orbital Shaker
- Staining tray
- Ethanol Acetic Acid
- Periodic acid
- Silver nitrate NaOH
- Citric acid
- Formaldehyde
- DH2O

1. Use Powder-free gloves throughout the silver staining procedure.

2. Transfer gels from gel cassettes into staining trays filled with fixative (1).

3. Place on a shaker overnight to fix the samples. Perform all steps at room temperature.

4. After the overnight fixing step, discard the fixative and add the oxidizer solution (2). Make fresh daily. Place the gel on the shaker for 5 minutes.

5. Wash the gel in DH2O for 15 minutes. Repeat this step twice.

6. Incubate the gel 30 minutes in silver stain solution (3). Prepare fresh and follow with three 10 minute washes.

7. Develop the gel in developer solution.

Solutions

1) Fixative

Ethanol	400 ml
Acetic acid	50 ml
DH2O	550 ml

2) Oxidizer (Prepare fresh daily)

Solution (1)	100 ml
Periodic acid	0.7 g

3) Silver Stain (Prepare fresh daily)

DH 2 O	115 ml
NaOH	(0.4 g/ 100ml DH 2O) 28.0 ml
*AgNO3	(1 g/5ml) 5.0 ml
*Conc NH4OH	2.0 ml

*Add last and simultaneously

4) Developer

Citric acid	50 mg
37% Formaldehyde	0.5 ml
DH2O	1 liter

5) Stop Solution (10% Acetic acid)

Acidic acid	100 ml
DH2O	900 ml

Appendix E

Source: Qiagen Human Toll-like Receptor pathway handbook

Symbol	Description
BTK	Bruton agammaglobulinemia tyrosine kinase
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCL2	Chemokine (C-C motif) ligand 2
CD14	CD14 molecule
CD80	CD80 molecule
CD86	CD86 molecule
CHUK	Conserved helix-loop-helix ubiquitous kinase
CLEC4E	C-type lectin domain family 4, member E
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
CSF3	Colony stimulating factor 3 (granulocyte)
CXCL10	Chemokine (C-X-C motif) ligand 10
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2
ELK1	ELK1, member of ETS oncogene family
FADD	Fas (TNFRSF6)-associated via death domain
FOS	FBJ murine osteosarcoma viral oncogene homolog
HMGB1	High mobility group box 1
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSPA1A	Heat shock 70kDa protein 1A
HSPD1	Heat shock 60kDa protein 1 (chaperonin)
IFNA1	Interferon, alpha 1
IFNB1	Interferon, beta 1, fibroblast
IFNG	Interferon, gamma
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL10	Interleukin 10
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
IL1A	Interleukin 1, alpha
IL1B	Interleukin 1, beta
IL2	Interleukin 2
IL6	Interleukin 6 (interferon, beta 2)
IL8	Interleukin 8
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRF1	Interferon regulatory factor 1
IRF3	Interferon regulatory factor 3
JUN	Jun proto-oncogene
LTA	Lymphotoxin alpha (TNF superfamily, member 1)
CD180	CD180 molecule

LY86	Lymphocyte antigen 86
LY96	Lymphocyte antigen 96
MAP2K3	Mitogen-activated protein kinase kinase 3
MAP2K4	Mitogen-activated protein kinase kinase 4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
TAB1	TGF-beta activated kinase 1/MAP3K7 binding protein 1
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
MAPK8	Mitogen-activated protein kinase 8
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3
MYD88	Myeloid differentiation primary response gene (88)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1
NFRKB	Nuclear factor related to kappaB binding protein
NR2C2	Nuclear receptor subfamily 2, group C, member 2
PELI1	Pellino homolog 1 (Drosophila)
PPARA	Peroxisome proliferator-activated receptor alpha
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
RIPK2	Receptor-interacting serine-threonine kinase 2
SARM1	Sterile alpha and TIR motif containing 1
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain
ECSIT	ECSIT homolog (Drosophila)
TBK1	TANK-binding kinase 1
TICAM2	Toll-like receptor adaptor molecule 2
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR1	Toll-like receptor 1
TLR10	Toll-like receptor 10
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5

TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TOLLIP	Toll interacting protein
TRAF6	TNF receptor-associated factor 6
TICAM1	Toll-like receptor adaptor molecule 1
UBE2N	Ubiquitin-conjugating enzyme E2N
UBE2V1	Ubiquitin-conjugating enzyme E2 variant 1
B2M	Beta-2-microglobulin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPL13A	Ribosomal protein L13a
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	Actin, beta
HGDC	Human Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control
PPC	Positive PCR Control
PPC	Positive PCR Control

Appendix F

The scatter plot compares the normalized expression of every gene on the array between two groups by plotting them against one another to quickly visualize large gene expression changes. The central line indicates unchanged gene expression.

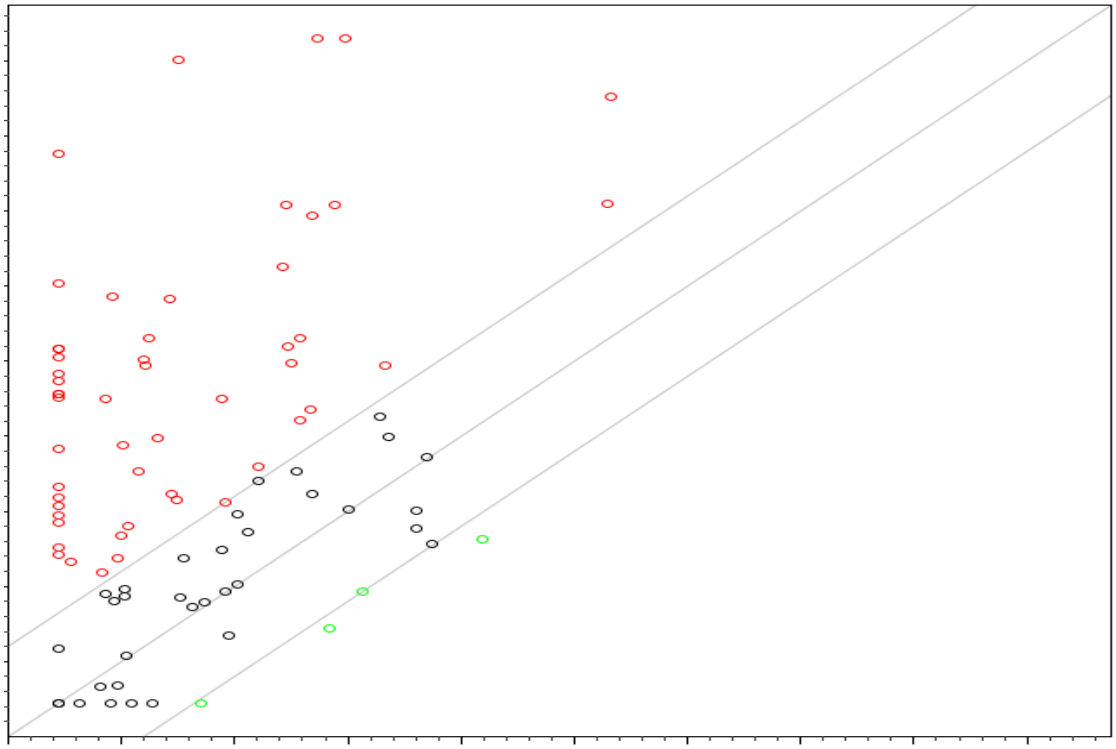

Figure 18. Scatter plot showing number of up regulated genes (red), down regulated genes (green) and unchanged genes (black) for hot treatment week 0.

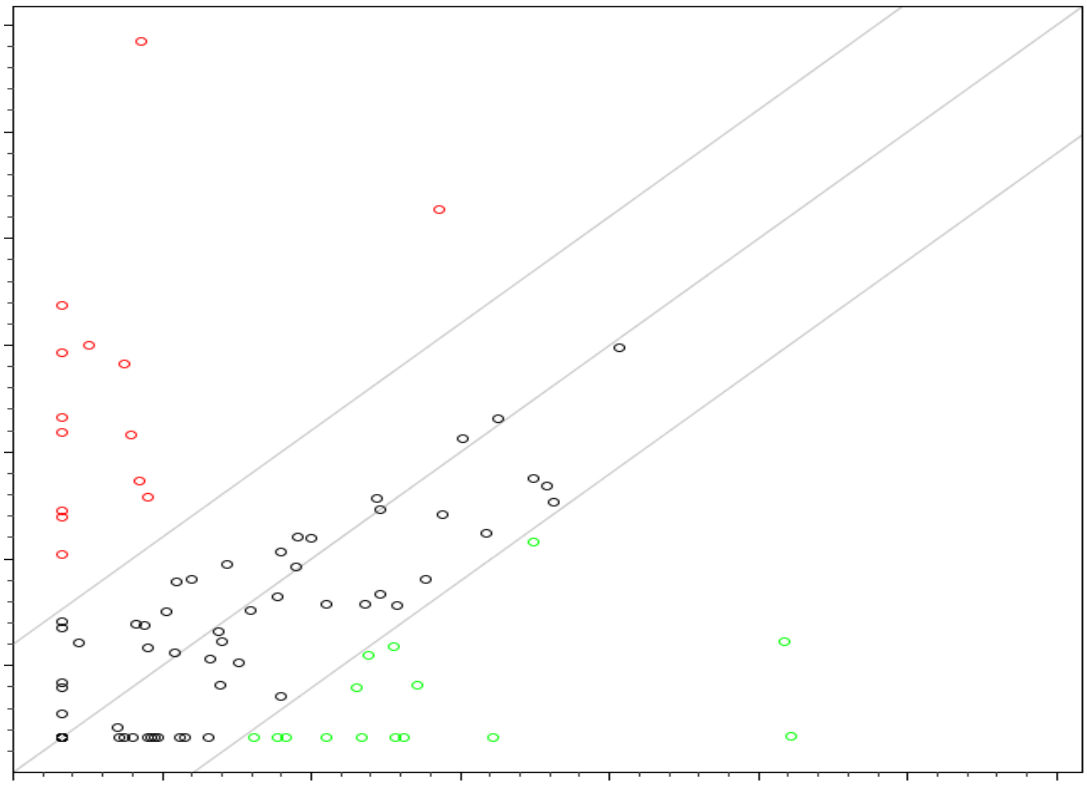

Figure 19. Scatter plot showing number of up regulated genes (red), down regulated genes (green) and unchanged genes (black) for cold treatment week 0.

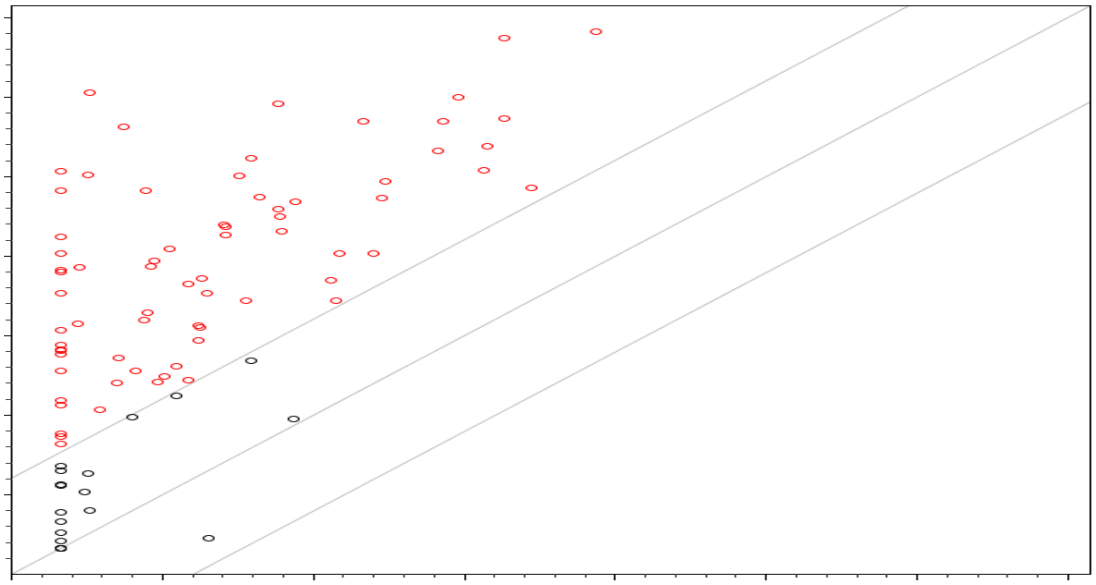

Figure 20. Scatter plot showing number of up regulated genes (red), down regulated genes (green) and unchanged genes (black) for hot treatment week 8.

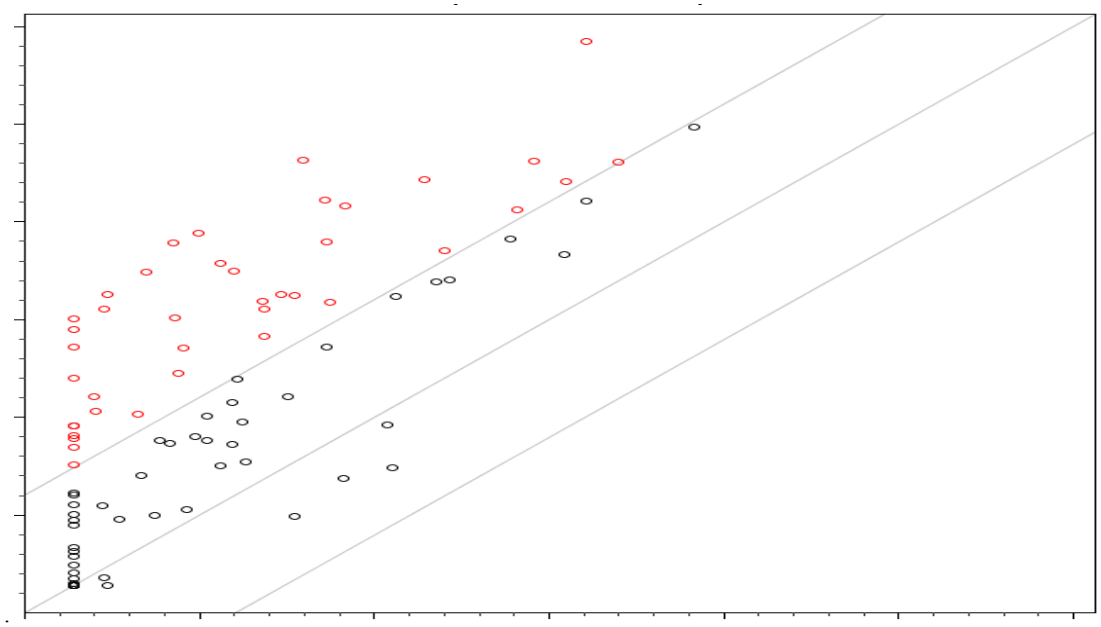

Figure 16. Scatter plot showing number of up regulated genes (red), down regulated genes (green) and unchanged genes (black) for cold treatment week 8.

Appendix G

Table 3.

Summary of Effect of Treatments

	Hot	Cold
FAMACHA	-	-
Body Weight	-	-
Body Condition	-	-
Fecal Egg Count	-	-
PCV	-	-
White Blood Cell Differential	-	-
RNA Concentration	-	-
Plasma Protein Concentration	+	+
Cytokines	+	+
Gene Regulation		
Up Regulation	75	40
Down Regulation	8	44
Unchanged	0	0

⌖-no effect, + effect

Appendix H

Source: Cayman ELISA Handbook

Addition of EIA Buffer to wells

One Hundred (100) µl of EIA Buffer was added to NSB wells. Fifty (50) µl of EIA Buffer was also added to the B0 wells

Addition of Prostaglandin E2 EIA Standards to wells

Fifty (50) µl from tube #8 was added to both of the lowest standard wells (S8). Fifty (50) µl from tube #7 was also added to each of the next two standard wells (S7). The procedure was continued until all the standards were aliquoted. The same pipette tip should was used to aliquot all the standards

Addition of Samples to wells

Fifty (50) µl of sample was added per well. Each sample should be assayed was assayed in triplicates

Addition of Prostaglandin E2 AChE Tracer

Fifty (50) µl of AChE Tracer was added to each well except the TA and the Blk wells.

Addition of Prostaglandin E2 Monoclonal Antibody

Fifty (50) µl was added to each well except the TA, the NSB, and the Blk wells

Incubation of plate

The plate was covered with a plastic film and incubated over night at 4°C.

Development of the Plate

Wells were emptied and rinsed five times with Wash Buffer. Two hundred (200) µl of Ellman's Reagent was added to each well. Five (5) µl of tracer was added to the TA well. The

plate was covered with plastic film and placed on an orbital shaker equipped with a large, flat cover for 90 minutes to develop in the dark.

Reading the Plate

The bottom of the plate was wiped with a clean tissue to remove fingerprints, dirt, etc. The plate cover was removed. The plate was inserted into a micro plate reader (BioTek) read at wavelengths 405, 410, 415, 417 and 420 nm.

Standard Curve

SAS was used to plot %B/B0 for standards S1-S8 versus PGE2 concentration using a logit transformation.