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The Impact of Diet on Expression of Genes Involved in Innate Immunity in Goat Blood

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Abstract

Sericea Lespedeza (SL), is a high-quality, low input forage that suppresses gastro-intestinal parasites in goats. The effect of dietary SL on the expression of genes involved in innate immunity in goats has not been established. The objective of this study was to evaluate the impact of a diet containing SL on the expression of genes involved in innate immunity in goat blood. Blood was collected by jugular venipuncture from goats fed a diet of 75% SL (n = 9) and a control group (n = 7), fed a SL free diet. Blood was used to evaluate expression of (CD-14, TLR-2, TLR-4, IL-10, IL-8, IL-2, INF-γ, and TNF-α). Serum was extracted and used for evaluation of the secretion of pro-inflammatory cytokines (TNF-α, IFNr, granulocyte colony stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GMCSF), IL-1α, IL-8, IP-10 and RANTES) using a commercial ELISA kit. The level of gene expression of CD-14, TLR-2, TLR-4, IL-10, IL-8, IL-2, INF-γ, and TNF-α was higher in treated animals compared to control. The *Sericea Lespedeza* diet affected the secretion of pro-inflammatory cytokines by increasing the serum levels of TNF-α, IFNr, GCSF, GMCSF, IL-1α, IP-10 ($P < 0.0002$), and by decreasing ($P < 0.0001$) IL-8 and RANTES in blood from goats fed SL. This suggests that dietary tannins modulate gene expression and may affect the goat's innate immune response in blood. Further research is needed to understand and harness the effect of dietary condensed tannins to modulate innate immunity in goats.

1. Introduction

Internal parasites pose the greatest challenge to goat production (Sahlu et al., 2009) in humid areas largely due to anthelmintic resistance (Kaplan et al., 2007). Gastrointestinal parasites are typically controlled with anthelmintic drugs, but due to reduced efficacy of this strategy, alternative approaches are being evaluated (Mortensen et al., 2003; Worku et al., 2009). One approach the use of Condensed tannin containing plants, has been studied as a promising alternative remedy (Paolini et al., 2003a; Burke et al., 2011). Condensed tannins (Proanthocyanidins) are a type of secondary compound found in some plants and are classified based on their chemical structure (Hagerman & Butler, 1991). These particular tannins are found in abundance in the leaves of plants from grasses and legumes (Mueller-Harvey, 2006). Consuming plants high in tannins has been found to be effective in reducing internal parasites in herbivorous animals (Min et al., 2005). Condensed tannin (CT) containing forages such as sericea lespedeza [SL, *Lespedeza cuneata* (Dum-Cours., G. Don)] have been effective as a natural dewormer in goats (Terril et al., 2009). Sericea Lespedeza is a drought resistant legume, widely planted throughout the southern USA as a grazing, hay, soil restoration and conservation crop, and is rich in CT (Lange et al., 2006; Shaik et al., 2006). Sericea lespedesza (SL) hay drastically reduced fecal egg count (67%-98%) and worm numbers (67.2%). Feeding sheep with SL was proven as a practical means to reduce pasture contamination by *H. contortus* (Lange et al., 2006). Its impact on growth rate has been investigated in both sheep and goats (Lange et al., 2006; Moore et al., 2008).

Plants containing tannins are able to modulate the immune response by acting on immune cells such as T cells in man and cows (Holderness et al., 2008). This results in changes in the expression of innate immunity markers and cytokine secretion (Graff et al., 2009) resulting in either pro- or anti-inflammatory activity (Holderness et al.,

2008). The innate immune system is considered to be the first line of defense, providing immediate protection against infection (Akira et al., 2006). The innate immune system is divided into three functions: barrier function, phagocytosis, and inflammation. These functions are mediated by a network of innate immune cells. Interfaces between the host and the environment, such as the skin, mucosa, and intestine, highlight the interconnectedness of these three functions (Basset et al., 2003; Butler, 2004; Gribar et al., 2008). Innate and adaptive immune systems are regulated by a complex network of chemical signals, including enzymes, immunoglobulins and cytokines (Provenza & Villalba, 2010). Throughout these barriers are cells containing receptors to detect pathogens and induce immune programs to destroy them, either by altering the environment or recruiting other cell mediators to do so. These barriers also regulate inflammatory responses since overt inflammation destroys the integrity of this barrier and exposes the host to the external environment (Basset et al., 2003; Butler, 2004).

Cells of the innate immune system sense the external environment by Pattern Recognition Receptors recognizing pathogen associated molecular patterns (PAMPs) to rapidly control disease and tissue damage (Basset et al., 2003). Toll-like receptor (TLR) signaling by commensal bacteria plays an essential role in maintaining immunity (Imler & Hoffmann, 2001). Of particular interest to early innate responses is the γδ T cell. Early in infection, many immune responses are regulated by the γδ T cell's ability to respond to PAMPs, and thus eliciting appropriate responses (Holderness et al., 2008).

Several types of chemicals that are able to modulate immune response have been identified in mammals, including proteins such as interferon gamma (IFN-γ), granulocyte colony-stimulating factor (GCSF) and granulocyte-macrophage colony-stimulating factor (GMCSF) (Schepetkin & Quinn, 2006). Besides these signaling compounds, some carbohydrates isolated and purified from microorganisms have immune stimulating properties such as lipopolysaccharide (LPS). Scientific research revealed that some plants compounds could be a promising alternative source of anthelmintic for the treatment of gastrointestinal trichostrongylids in small ruminants (Hördegen et al., 2003). Polyphenol and polysaccharide fractions from plant-derived nutritional supplements are known to activate γδ T cell responses (Hollderness et al., 2011). The present study focuses on evaluation of innate immune markers through comparing gene expression profiles of a control group and SL treated group. The objective of the current study was to evaluate the effect of a diet containing SL on the expression of markers of innate immunity in goat blood in response to dietary SL.

2. Materials and Methods

2.1 Animals

Animals used in the study were reared on the North Carolina Agricultural and Technical State University farm. Initially, thirty Boer Spanish cross goats (n = 30) were used in the study, males (n = 15) and females (n = 15). Goats were assigned to three levels of SL treatment with alfalfa pellets of 0%, 50% and 75% for a period of four weeks. The animals were naturally infected with nematodes through pasture grazing (Burke et al., 2011).

2.2 Blood Sampling

Blood samples were collected by jugular venipuncture at the end of the study. For serum extraction and isolation of total RNA. For serum extraction blood was collected in non-treated tubes and used to evaluate immune cytokines and serum protein levels. For RNA isolation 2.5 ml of blood was collected into PAXgene Blood RNA tubes (PreAnalytiX Gmb, Feldbachstrasse, 8634 Hombrechtikon, Switzerland).

2.3 Evaluation of Total Serum Protein Concentration

Total serum protein concentration was evaluated using the Pierce bicinchonninic (BCA) assay kit (Fisher Scientific, Rockford, IL USA). Samples of extracted serum from treated and control goats were separately pooled for evaluation and the concentration of total protein was read using a spectrophotometer (BioTek) at 562 nm. A standard curve was obtained by plotting the average blank corrected 562 nm measurement for each BCA standards against its concentration in μg/ml.

2.4 Quantitative Profiling of Pro-Inflammatory Cytokines in Serum

The concentration of 8 pro-inflammatory cytokines; Tumor necrosis factor (TNFα), interferon production regulator (IFNr), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GMCSF), interleukin-1α (IL-1α), interleukin-8 (IL-8), IFN-inducible protein-10 (IP-10), and Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) in serum was evaluated using the Human inflammation ELISA Strip (Signosis, Inc.). Standards and samples were pipetted (100 μl) into pre-coated wells, and then incubated for 1 hour at room temperature with gentle shaking. Wells were aspirated, and washed by adding 200 μl of 1X assay wash buffer then repeated three times. Diluted biotin-labeled antibody (100 μl) was added to each well and incubated for one hour at room temperature with gentle shaking. The aspiration/wash

process was repeated, and 100 µl of substrate was added to each well and incubated for 30 minutes. Stop solution (50 µl) was added to each well and the OD at 450 nm was determined using a microplate reader (BioTek). The observed readings were used to produce a standard curve to obtain average concentrations of the samples in Pico-gram per microliter (pg/ml).

2.5 Isolation of RNA from Goat Blood

The ZR Whole-Blood RNA Miniprep kit, (ZYMO Research corp.) was used to isolate total RNA from white blood cells in goat blood collected in PAXgene tubes (PreAnalytiX Gmb, Feldbachstrasse, 8634 Hombrechtikon, Switzerland). Tubes were kept at -20 °C before transfer to 4 °C for 24 hours, and then left at room temperature for 2 hours. Tubes were centrifuged (Eppendorf, centrifuge 5810 R) at 4000 × g for 10 minutes. The extraction of total RNA was followed as suggested by the manufacturer (PreAnalytiX Gmb).

2.6 Gene Expression Profiling of Innate Immunity Markers

Isolated total RNA was used to synthesize complementary DNA (cDNA) using RETROscript Kit (Ambion, Austin, TX) following the manufacturer's instructions. The cDNA concentration was evaluated using a Nanodrop spectrophotometer.

The prepared cDNA was used as a template for quantitative real time PCR using SsoAdvanced SYBR Green Supermix kit (BioRad Laboratories, CA) following the manufacturer's instructions to evaluate gene expression of immune markers. Primers (Table 1) for the immune markers; Cluster of Differentiation antigen 14 (CD-14), Toll-Like Receptor-2 (TLR-2), Toll Like Receptor-4 (TLR-4), Interlukin-10 (IL-10), Interlukin-8 (IL-8), Interlukin-2 (IL-2), Interferon-γ (INF-γ), and Tumor Necrosis Factor-α (TNF-α) were used in the quantitative real time PCR. All primers used were purchased from MWG Inc (High Point, NC). Reaction was set by adding SsoAdvanced SYBR Green Supermix (10 µl), forward and reverse primers (250 nM), cDNA (100 ng), and DNase/RNase free water to 20 µl. To determine the relative expression of the target genes in the test samples and calibrator samples using reference gene (GAPDH) for normalization, the expression levels of both the target and the reference genes were determined using RT-qPCR. Real time PCR results were then analyzed using the Livak's method (Schmittgen & Livak, 2001) (also known as the 2 -ΔΔCq method) to compare gene expression profile between treatment and control animals. The determined cycle threshold (Ct) values were used to calculate fold change in expression of select genes involved in immunity.

Table 1. List of primers and primer sequences used for the study

Gene	Primer sequence	Primer size (bp)	Reference
GAPDH	Forward:5' CTGGAGAAACCTGCCAAGT 3'	200	Menzies and Ingham (2006)
	Reverse:5'GCCAAATTCATTGTCGTACCA3'		
TLR2	Forward:5'ACGCCTTTGTGTCCTAC 3′	192	Menzies and Ingham (2006)
	Reverse: 5' CCGAAAGCACAAAGATGGTT 3'		
TLR4	Forward:5'-AACCACCTCTCCACCTTGATACTG-3'	410	Worku and Morris (2009)
	Reverse: 5'-CCAGAAAGACCTTGAATACAGG-3'		
CD14	For: 5' -GACGACGATTTCCGTTGTGT-3'	600	Worku and Morris (2009)
	Rev: 5' -TGCTAGCGCTAGATATTGGA-3'		
TNF-α	Forward:5'-CTGCACTTCGGGGTAATCGG-3'	549	Worku and Morris (2009)
	Reverse:5'-CAGGGCGATGATCCCAAAGTA-3'		
IL-10	Forward:5'- TGCTGGATGACTTTAAGGGTTACC -3'	186	Konnai et al. (2003)
	Reverse:5'- TCATTTCCGACAAGGCTTGG -3'		
IL-8	Forward:5'- GGAAAAGTGGGTGCAGAAG -3'	443	Worku and Morris (2009)
	Reverse:5'- GGTGGTTTTTTCTTTTTCATGG-3'		
IL-2	Forward: 5' CCTCAA CTCCTG CCACAA TGTA-3'	376	Coussens et al. (2004)
	Reverse: 5' GTTTGC AACGAG TGCAAG AGTTA-3'		
IFNγ	Forward:5'TGGAGG ACTTCA AAAAGC TGATT 3'	261	Coussens et al. (2004)
	Reverse:5'TTTATG GCTTTG CGCTGG AT 3'		

2.7 Statistical Analysis

Concentrations of pro-inflammatory cytokines, total serum protein, as well as concentration and purity of DNA were determined using a standard curve. Means were considered significant at the 5% level of probability. One-way Analysis of variance was performed using SAS software (SAS Institute, Cary, NC) to evaluate differences between treatment and control group for ELISA of pro-inflammatory cytokines.

3. Results

3.1 Total Serum Protein Concentration

A slight decrease in total serum protein in the treatment group (147.23 µg/ml) compared to the control group (152.80 µg/ml) was observed (Figure 1). The decrease was however not significant (P > 0.05).

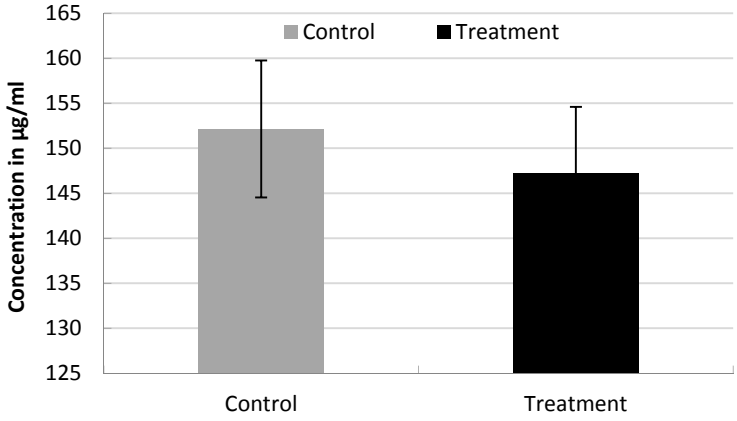

Figure 1. Mean concentration of total serum protein cytokines measured in sericea lespedeza treated (Treatment) and control groups

3.2 Pro-Inflammatory Cytokine Concentration in Serum

All cytokines on the array were detected in serum of both SL and control animals. Cytokines TNF-α, IFNr, G-CSF, GM-CSF, IL-1a and IP-10 showed a highly significant increase in animals fed SL (P < 0.0002), whereas IL-8 and RANTES showed a highly significant decrease (P < 0.0001) (Figure 2).

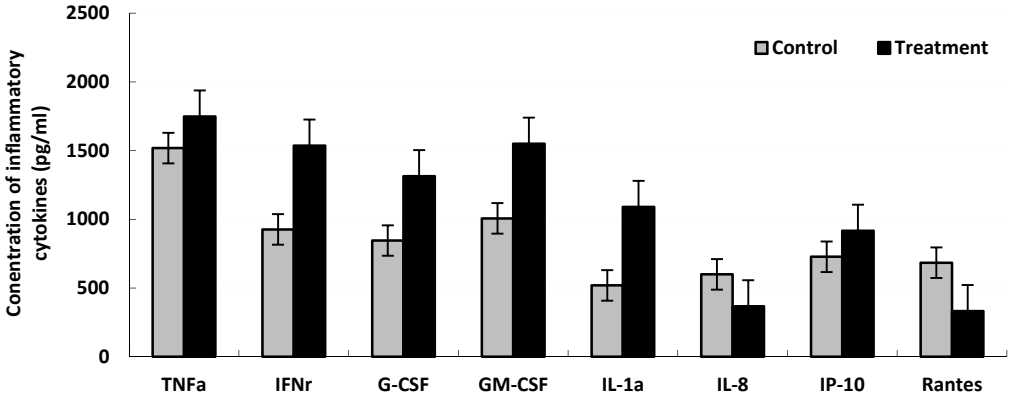

Figure 2. Mean concentration of inflammatory cytokines measured in goats fed sericea lespedeza (Treatment) and control groups

3.3 Concentration of Synthesized cDNA

The average concentration of synthesized cDNA from the reverse transcription of collected RNA for control and SL receiving groups were 1587.11 ng/µl and 1631.14 ng/µl respectively (Figure 3). Differences in observed means of cDNA concentration were not significant (P < 0.8148).

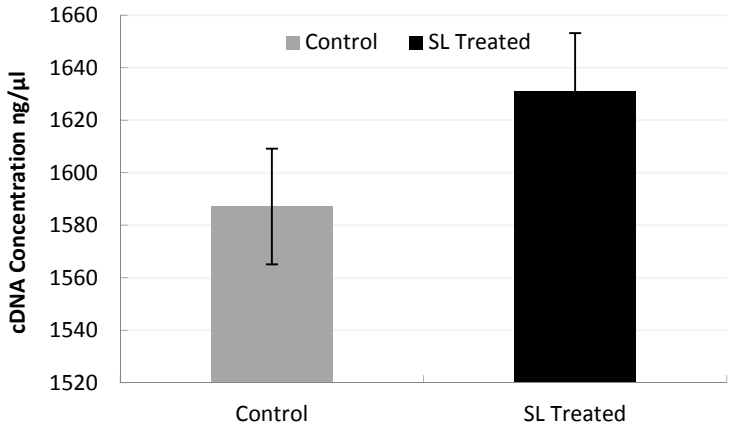

Figure 3. Isolated cDNA concentration in sericea lespedeza (SL) receiving goats and control group

3.4 Gene Expression Profiles of Innate Immunity Markers

The aim behind performance of gene expression studies was to explore possible systemic effect of dietary SL on select genes involved in innate immunity. Gene expression results indicated a fold increase in gene expression in all examined genes, thus supporting the hypothesis that there is an effect of SL treatment on innate immunity in goat blood (Table 2).

Table 2. Fold change in gene expression of innate immune genes in goat blood showing the effect of sericea lespedeza diet

Gene name	Fold change[a]	Gene function [b]
TLR2 (Toll-like receptor 2)	22	A member of the Toll-like receptor family and relevant for pathogen recognition and activation of innate immunity. Associated with host response to Gram positive bacteria and yeast
TLR4 (Toll-like receptor 4)	7	A member of the Toll-like receptor family and relevant for pathogen recognition and activation of innate immunity. Mostly associated with Gram negative bacteria (LPS) induced signal transduction
IL-2 (Interleukin 2)	16	A secreted cytokine important for T and B lymphocytes proliferation
IL-8 (Interleukin 8)	10	A member of the CXC chemokine family, functions as a chemoattractant and is one of the key mediators of inflammation response
IL-10 (Interleukin 10)	0.1	A cytokine with a pleotropic function in immunoregulation and inflammation.
TNF-α (Tumor necrosis factor-α)	8	A proinflammtory cytokine secreted by macrophage and function in regulation of cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation.
IFNγ (Interferon, gamma)	23	A member of the type II interferon family and function as an activator of macrophages. The protein encoded by this gene is a soluble cytokine having antiviral, immunoregulatory and antitumor properties.
CD14(CD14 Molecule)	10	A surface antigen expressed on monocytes and macrophages and it mediate innate immune response to bacteria lipopolysaccharide.

Note. [a] Fold changes presented are significant $P < 0.05$; [b] Gene function information was obtained from GeneCards (Human gene database) http://www.genecards.org

4. Discussion

Our study is an attempt to shed light on the effect of SL, a tannin-rich legume, on the innate immune response in blood through monitoring the activation of markers of innate immunity. Two general traits of tannins relevant to grazing ruminants are the prevention of bloat (Lees, 1992) and the suppression of internal parasites (Hoste et al.,

2006). Studies in sheep and cattle showed that natural dietary components may directly prime innate immune cells residing in the intestinal wall, independent of antigens, and provides further evidence for the use of CT to control gastrointestinal parasites in grazing (Schreurs et al., 2010). The possible systemic effect of a SL diet on important genes important to innate immunity was evaluated in goats. Serum total protein is an important immunological and health indicator. Total serum protein can be affected by the type of nutrition that the animal receives. Feeding leaves from tannin containing plants resulted in an increase in total serum protein (Oni et al., 2012). No serious effects were reported on the hematology or serum biochemistry of lambs in response to SL feeding (Acharya et al., 2015). However, our results showed a slight decrease in total serum protein concentration in goats. This may be due to differences in the sources of tannins or duration of the study.

In goats infected with *H. contortus* larvae SL feeding did not affect mucosal inflammatory cellular responses (Joshi et al., 2011). Studies have shown that plant polyphenols such as tannin are effective at increasing the potency of γδ T cell and cell responsiveness (Graff et al., 2009; Holderness et al., 2007). In goats, our results show that SL treatment increased the expression of cytokines and may be associated with stimulation of γδ T cell responsiveness. The results obtained from Enzyme-linked immunosorbent assays of cytokines showed increases in TNF-α, IFNr, G-CSF, GM-CSF, IL-1a and IP-10 in the treatment group in comparison to the control group, the levels of IL-8 and Rantes decreased after treatment. Increased production of cytokines activates macrophages, stimulating these cells to increase microbicidal activity, up-regulating the level of class II Major Histocompatibility complex (MHC), and secretion of other cytokines such as IL-12, which induces TH cells to differentiate into the TH1 subset (Mosser & Edwards, 2008). In goats, the DRB1*1101 gene of the MHC II class has been reported as a potential genetic marker for *Haemonchus contortus* (Corley & Salvage, 2015). Interferon gamma (IFN-γ) secretion by T helper 1 (TH1) cells also induces antibody-class switching to immunoglobulin G (IgG) classes (Trinchieri, 1997) that support phagocytosis and fixation of complement. Interferon-gamma (IFN-gamma)-inducible protein-10 (IP-10), a member of the C-X-C sub-family of chemokines, is known to be produced by monocytes, lymphocytes, keratinocytes and endothelial cells in response to IFN-gamma (Cassatella et al., 1997). Interleukin 8 (IL-8) has suppressive effects on the macrophage and serves to further diminish the biologic consequences of TH1 activation (Kindt, 2007). Studies in sheep have shown that the Th1 immune response is the protective immune response to *H. contortus*, while the Th2 immune response is an adaptive immune response for the host (Zhong et al., 2014). Our results suggest a significant alteration in cytokine profiles in support of our hypothesis that using SL in the goat diet also has a significant effect on host innate immunity.

Plant tannins induce cytokine secretion and cell division (Holderness et al., 2007). Plant polyphenols are capable of modulating TLR-mediated signals (Provenza & Villalba, 2010). An i*n vitro* assay with tannic acid inhibited viability of *H. contortus* L3 larvae and modulated the immune response of sheep WBC stimulated by *H. contortus* crude larval antigen by inhibiting Th1 cytokines and increasing Th2 cytokine expression (Zhong et al., 2004). Genes encoding the pattern recognition receptors Toll-like receptors (TLR2, 4 and 9) associated with the early inflammatory response arr reported to be more abundantly expressed in lambs that are resistant to *H. contortus* and *Trichostrongylus colubriformis* infections (Reverter et al., 2008).

In the present study, increased expression of TLR2 and TLR4 was observed following SL treatment. The TLR2 gene expression profile exhibited a high fold change of 22, indicating that SL treated group expressed TLR2 at a 22 times higher level compare to the control. Tannic acid (Zhong et al., 2014) and polyphenols (Schroecksnadel et al., 2007) suppress the Th1-type immune response in human blood phenolic compounds decrease IFN-γ production (Zhong et al., 2014). In goat blood, interferon-gamma (INF-γ) was expressed the highest with a fold change of 24 in the treatment group compared to the control group. The two genes TLR-2 and INF-γ showed high fold change compare to the other innate immunity markers. These findings suggest stimulation of γδ T cells and NK cells as a result of SL treatment in goats.

Comparison of fold change level for TLR-4 and CD14 showed increase in the expression of those genes in levels of 7 and 10 respectively (see Table 2). These genes encode PRR relevant in recognizing PAMPS by immune cells and activation of the innate immune response. Moreover, our results revealed an increase in expression of IL-2 by 16 folds in the treatment group compared to the control group. Interleukin -2 (IL-2) is important in activation of T cells and B cells. Tumor necrosis factor alpha (TNF-α) also works to increase innate immunity through a synergistic effect with INF-γ and interferes with the initiation of chronic inflammation. The expression of TNF-α was increased by 9 folds in blood from SL treated compared to control goats. These findings indicate an effect of SL through increased expression of markers of innate immunity. The expression of interleukin 10 (IL-10) was the lowest. This cytokine is important in regulation of inflammatory response and known for its inhibitory effect on macrophage activation. Our results revealed that IL-8 expression at the trasncrpit level

increased by almost 10 folds in treatment groups compared to the control group. Interleukin 8 (IL-8) is known to be associated with boosting responsiveness of γδ T cells, and this action can result from tannin treatment according to previous studies (Holderness et al., 2007). Cytokines associated with TLR activation include TNFα, IL-6, and IL-1β, as well as chemokines, such as IL-8 (Cerenius et al., 2010) similar observation was obtained in our study.

5. Conclusion

This study explored the possible effects of dietary CT on markers of innate immunity in goat blood. Increased expression of all examined genes and differential effects on secretion of cytokines were found. This supports the hypothesis that a diet containing SL has an effect on the expression of genes involved in innate immunity in goat blood. The observed effect on TLR2 in goat blood may contribute to inhibition of parasites and warrants further study. Such studies will help increase our understanding and harnessing of the effect of dietary condensed tannins to modulate innate immunity in goats.

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