Journal of Agricultural Science; Vol. 8, No. 9; 2016
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

An Extract of Sericea Lespedeza Modulates Production of Inflammatory Markers in Pathogen Associated Molecular Pattern (PAMP) Activated Ruminant Blood

Emmanuel K. Asiamah[1], Sarah Adjei-Fremah[1], Bertha Osei[1], Kingsley Ekwemalor[1] & Mulumebet Worku[2]

[1] Department of Energy and Environmental Systems, North Carolina A&T State University, Greensboro, NC, USA

[2] Department of Animal Sciences, North Carolina A&T State University, Greensboro, NC, USA

Correspondence: Mulumebet Worku, Department of Animal Sciences, North Carolina Agricultural and Technical State University, 1601 E Market St, Greensboro, NC 27411, USA. Tel: 1-336-285-4816 E-mail: worku@ncat.edu

Received: June 15, 2016 Accepted: July 20, 2016 Online Published: August 15, 2016

doi:10.5539/jas.v8n9p1 URL: http://dx.doi.org/10.5539/jas.v8n9p1

Abstract

Programs based on antibiotics are failing to control diseases due to increase in resistance of pathogens to antibiotics. Food safety, animal welfare and public health concerns have fueled interest in the use of plant-based alternatives. This study was conducted to evaluate the effect of a plant (Sericea Lespedeza, SL), and pathogen associated molecular patterns (PAMPs) (Lipopolysaccharide (LPS) and peptidoglycan (PGN)) on gene activation in ruminant blood. A water extract of SL, was used as a source of plant-derived tannins. Blood was collected from Holstein-Friesian cows (N = 4), Spanish × Boer goats (N = 4), St Croix sheep (N = 4) and incubated with 100 ng/mL of SL in the presence or absence of LPS or PGN. Samples maintained in Phosphate-buffered saline (PBS) served as negative control. The total protein concentration, WNT5a, and prostaglandin E2 in plasma were determined. Total RNA was isolated, reverse transcribed and Real time-PCR was performed using gene specific primers for TLR2, TLR4, WNT5a, and FZD. TLR2 and FZD were up-regulated in response to PAMPs. WNT5a and TLR4 genes were undetected in PAMP treated blood. SL regulated protein and prostaglandin concentration in all species. SL reduced PGE2 in sheep and cow blood. WNT5a was only secreted in LPS treated cow blood. Transcription and translation of genes involved in innate and adaptive immunity and the WNT signaling pathway in ruminant blood were responsive to diverse PAMPS, and can be modulated by SL. This suggests that dietary tannins may promote the health of ruminants. Further studies are needed to determine the significance of these changes in immune gene expression on ruminant health.

Keywords: Sericea Lespedeza, PAMPs, prostaglandin E2, transcription, WNT

1. Introduction

Elimination of pathogens depends on how efficiently the immune system of the host coordinates the delivery of blood components (plasma and leukocytes) to the site of infection or injury (Boermans et al., 2009). Improper orchestration of the immune response to microbial infections may lead to sepsis (Werners et al., 2005). Sepsis is a condition caused by an overwhelming immune response (Wiersinga et al., 2014). The sequencing of the cattle, sheep, and goat genomes have led to increased understanding of the genetics underlying immune response mechanism. Innate immunity especially plays a critical role in host defense against infection since it is the first line of defense. The innate immune response is triggered by recognition through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (Barton et al., 2009).

Toll-like receptors are PRRs that recognize highly conserved structural motifs known as pathogen-associated microbial pattern (PAMPs). The TLR family comprises a total of 13 genes. Ten TLR genes have been identified in human, pig, mouse, cattle, sheep and goat (Chang et al., 2006, 2009; Werling & Coffey, 2007; McGuire et al., 2006; Raja et al., 2011). Bacterial peptidoglycan (PGN) and lipopolysaccharide (LPS) designated PAMPs, are recognized by TLRs (Adib-Conquy & Cavaillon, 2007). Specifically, LPS is a ligand for TLR-4 and PGN is a ligand for TLR-2. The linkage between PAMPs, TLR, activation of the prostaglandin pathway, and the promotion of wingless (Wnt) signaling in inflammatory response has been studied (Blumenthal et al., 2006;

Hiroko et al., 2009). The Wnt signaling pathway is a conserved pathway associated with cellular function and biological processes (Clevers & Nusse, 2012). Plants rich in phenolic compounds have been studied to modulate expression of Wnt signaling pathway genes (Tarapore et al., 2012; Adjei-Fremah et al., 2016). Understanding the innate immune mechanism and inflammatory response mediated through TLRs and Wnt may provide more understanding about disease resistance in cattle, sheep, and goats, as well as aid in drug design and animal selection through breeding programs.

Control programs based on antibiotics are failing to control diseases. This is due to the increase in resistance of pathogens to antibiotics. Food safety, animal welfare, and public health concerns have fueled the interest in plant-based based alternatives. *In vitro* studies have shown that forages containing condensed tannins (CT) have anthelmintic and antibacterial effects and potentially could be used to control gastrointestinal nematodes (GIN) and bacterial infections (Molan et al., 2000; Min et al., 2008). Scientific literature indicates that plant derived compounds like tannins and other polyphenols can trigger the immune response through the TLR pathway (Holderness, 2012). Sericea lespedeza (SL), also known as *Lespedeza cuneate* is one of the high tannin containing legumes that are being studied extensively for possible health boosting benefits. The benefits of SL as a feed component for control of gastrointestinal nematodes in small ruminants has been reported (Min et al., 2008). A recent study has also shown that cowpea, which is also a plant rich in polyphenols activate the wnt pathway in cows (Adjei-Fremah et al., 2016).

This study was conducted to evaluate the effect of treatment with water extract of SL and known pathogen associated molecular patterns (LPS and PGN) on gene activation in ruminant (cow, sheep, and goat) blood.

2. Materials and Methods

2.1 Animals

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

2.2 Blood Sampling

Whole blood (10 mls) was collected aseptically from the jugular vein of the animals into vacutainer tubes containing 1ml of the anti-coagulant Acid Citrate Dextrose.The tubes were gently mixed and placed on ice immediately after collection. The samples were transported to the laboratory.

2.3 Total White Blood Cell Count and Cell Viability

Cell Viability was assessed using the Trypan blue dye exclusion method and cells were counted using the TC20 automatic cell counter (Bio-rad). Samples of whole blood were diluted 1:100 in Phosphate Buffered Saline (PBS). Cell counting was done in duplicate and an average was taken. Cell viability was expressed as a percentage of [(total viable and non- viable cells/total cells)]. White blood cell differential counts were conducted on whole blood treated with each of the ten treatments and PBS control using Wright Staining procedure. A thin smear of blood was made on a glass slide and left to dry at room temperature overnight. The air-dried slide was dipped in Wright's stain for 10 seconds. Excess stain was washed off the stained slide with deionized water. The slide was then air dried before reading under a light microscope (Carolina Biologicals). Smears were read under oil immersion for cell counts. The different cells were counted up to 100 for numerical representation of various cells present in the blood sample.

2.4 Preparation of Bacterial PAMPS

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

2.5 Preparation of Sericea Lespedeza (SL) Extract

Fifty grams (50 g) of powdered SL leaves kindly provided by Dr Niki Whitley (FVSU), was mixed with 500 ml of PBS and stirred for 30 minutes. Afterwards, the entire content in the volumetric flask was then filtered through a filter paper. The filtrate was again passed through a 0.2-micron filter to obtain a fine filtrate free of particles and microbes. Bicinchoninic acid assay (BCA assay) was done on the filtrate to determine the total protein concentration (Thermo Scientific Inc., Waltham, MA).

2.6 Treatment of Blood

One mL of blood from each animal was incubated with 1ml of the 100 ng/ of LPS or PGN and water extract of Sericea Lespedeza (SL) either individually or in pairwise combinations to assess the expression of select genes and other inflammation indicators. Samples were incubated at 37 ^{o}C, with 85% humidity and 5% CO_2 for 30 minutes as described by Adjei-Fremah et al. (2015). Samples incubated with PBS served as negative control. All the reagents used were prepared with diethyl pyrocarbonate (DEPC)-treated endotoxin free water. At the end of the incubation period, cells were spun down at 1700 × g at 4 ^{o}C for 5 minutes. Supernatants were collected and stored at -80 ^{o}C to measure total protein concentration and prostaglandin levels. Trizol was added to cell pellets and stored for RNA isolation.

2.7 Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated using Trizol according to the manufacturer's instruction (Sigma-Aldrich St. Louis, MO). The appropriate precautions were used to avoid RNase contamination throughout the entire procedure. The RNA concentration (ng/µl) and purity (260/280) were assessed using a Nanodrop Spectrophotometer ND 1000 (Thermo Scientific Inc., Waltham, MA). Total RNA was pipetted into an RNA 6000 Nano LabChip® (Agilent Technoliogies, DE) and RNA integrity was determined using Agilent® Bioanalyzer following manufacturer's protocol. Complimentary DNA (cDNA) synthesis was performed with 500 ng/µl RNA (purity 260/280 = 1.8, RIN = 7). Retroscript kits (Bio-rad Laboratories, CA) were used to synthesize cDNA for real-time Polymerase chain reaction.

2.8 Gene Expression Profiling of Markers for Innate Immunity

Primers specific for TLR2, TLR4, WNT5A, and Frizzled were purchased from MWG, Biotech Huntsville AL (Table 1). GAPDH was used as an internal control and for normalization.

Table 1. Sequences of primers used for study

Gene	Primer	Sequence 5' > 3'	Primer size(bp)	Reference
WNT5a	Forward	*CGCTTCAGCTCCGGTTCACT*	384	Person (2010)
	Reverse	*CGACGCTGGAGTTCCAGCTT*		
Frizzled	Forward	*CTAGCGCCGCTCTTCGTGTACCTG*	403	Rhee(2002)
	Reverse	*CAGCGTCTTGCCCGACCAGATCCA*		
TLR2	Forward	*ACGCCTTTGTGTCCTAC*	192	Menzies and Ingham(2006)
	Reverse	*CCGAAAGCACAAAGATGGTT*		
TLR4	Forward	*AACCACCTCTCCACCTTGATACTG*	208	Menzies and Ingham(2006)
	Reverse	*CCAGAAAGACCTTGAATACAGG*		
GAPDH	*Forward*	*GGAAGCTCACTGGCATGGC*	200	Worku and Morris(2009)
	Reverse	*TAGACGGCAGGTCAGGTCCA*		

2.9 Real Time PCR

The CFX connect machine (Bio-rad Laboratories, CA) was used for the real time PCR. The intercalating dye, SYBR green was used to detect amplification. The machine was programmed as follows: 95 ^{o}C for 15 seconds for denaturing, 60 ^{o}C for 30 seconds for primer annealing, and 72 ^{o}C for elongation. Real-time PCR data was analyzed using the Livak's method. Housekeeping gene GAPDH and samples treated with PBS were used to determine the ΔΔCt. Where ΔCt = (Target genes$_{treat}$ − GAPDH$_{treat}$) − ΔCt (Target genes $_{PBS}$ − GAPDH$_{PBS}$). Fold change = $2^{(-\Delta\Delta Ct)}$ (Livak & Schmittgen, 2001). Amplified samples were run on a 0.8% agarose gel and visualized after staining with Ethidium bromide to determine amplicon size (Figure 2).

2.10 Evaluation of Total Plasma Protein Concentration

Total protein concentrations in plasma harvested from control (PBS) and treated whole blood was measured using the Bicinchoninic acid assay (BCA) following the manufacturer's instructions (Thermo Scientific™ Pierce, Waltham, MA).

2.11 Evaluation of Prostaglandin (PGE2) Concentration in Plasma

Prostaglandin (PGE2) concentration in plasma from control (PBS) and treated whole blood was evaluated using a commercial Enzyme-linked immunosorbent assay kit (ELISA) (Cayman Chemicals, MI) following the manufacturer's instructions.

2.12 Determination of WNT5a Secretion

Levels of WNT5a were determined in plasma aliquots (100 µl) by using commercially available ELISA kits (Cusabio Biotech, Waltham, MA) for human WNT5a, according to the manufacturer's instructions.

2.13 Data Analysis

Means of white blood cell differential cell counts and total cell count of all groups were compared with one-way Analysis of variance (ANOVA) using SAS 9.2 (SAS Institute, Cary, NC) statistical analysis software. Results were considered statistically significant at ($P \leq 0.05$). Concentrations of total plasma protein, WNT5a and PGE2 ELISAs were determined using standard curves. Data for RNA concentration and purity are represented as means. Real-time PCR data was analyzed using the Livak's method. Housekeeping gene GAPDH and samples treated with PBS were used to determine the ΔΔCt., as described above.

3. Results

3.1 Species-Specific Variation of Total Cell Count and Viability in Whole Blood

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

Table 2. Percentage cell viability after whole blood incubation with various treatments

Treatments	Cow	Sheep	Goat
LPS	93%	70%	60%
SL	87%	68%	70%
PGN	75%	71%	66%
PGN/SL	90%	74%	79%
LPS/SL	91%	63%	94%
LPS/PGN	83%	73%	70%
PBS	97%	80%	66%

Note. LPS = Lipopolysaccharide, PGN = Peptidoglycan, SL = Sericea, LPS/PGN = Lipopolysaccharide + Peptidoglycan, LPS/SL = Lipopolysaccharide + Sericea, PGN/SL = Peptidoglycan + Sericea PBS = Phosphate Buffered Saline.

3.2 Species-Specific Variations in WBC Populations

Sheep: Average lymphocyte was 70% in all samples. Basophils and Eosinophils were less than 1%. The neutrophil percentage was 31%. There was no change in WBC after incubation ($p > 0.05$).

Cow: The percentage of lymphocytes was lower in treated samples compared to the control ($p < 0.0001$). Average lymphocyte percentage in all treatments was 70% and 90% in control samples. Monocytes in PBS ranged from 0-2% whereas monocytes in treatments ranged from (1-10%) in almost all the samples. Lymphocytes percentage in samples incubated with LPN, SL and PGN/SL were significantly greater than the control ($p < 0.0001$). All others were not. Neutrophil concentration increased from 7-56% in all samples compared to the control ($p < 0.001$). There was no treatment effect on Basophil and Eosinophil percentage (0.84 and 0.87 respectively).

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

3.3 Concentrations of Total RNA

The concentration and purity of total RNA extracted from whole blood was measured to evaluate the treatment effect on RNA transcription. Concentration and purity levels were not affected by treatment. There was no species variation observed as well.

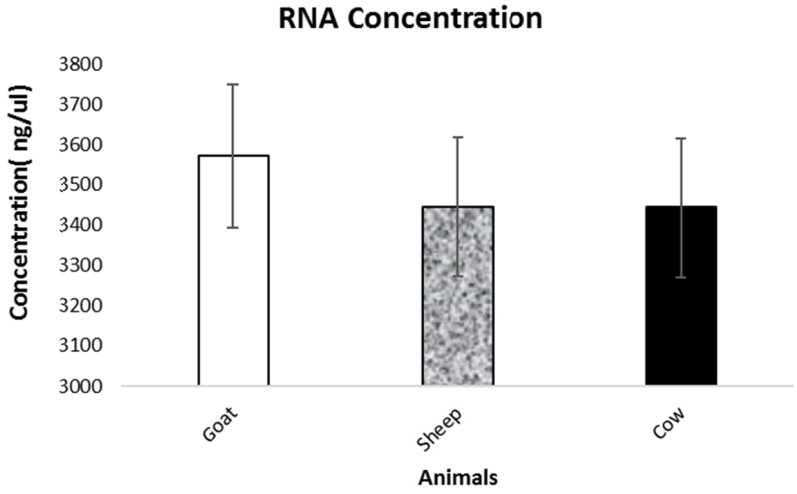

Figure 1. Average RNA concentration of treated and control samples in all 3 species

3.4 Detection of WNT5a, FZD, TLR2 and TLR4 in Blood

Genes encoding TLR2 and FZD were expressed in both PAMP and PBS treated blood. Fold change in gene expression for TLR 2 and FZD genes are shown in Tables 3 and 4 below. Genes encoding WNT5a and TLR4 however were not detected in treatment groups but were observed in control samples (PBS). Therefore, fold changes could not be calculated. Figure 2 shows RT-PCR amplicons for WNT5A, TLR2, TLR4 and, FZD detected in PBS samples in cow, sheep and goat blood (sizes are indicated in Table 1). Thus, even though the genes were present in all 3 species, the treatment did not increase their levels. *In vitro* stimulation of blood with 100 ng/ml of LPS, PGN and SL for 30 minutes does not lead to upregulation of these genes.

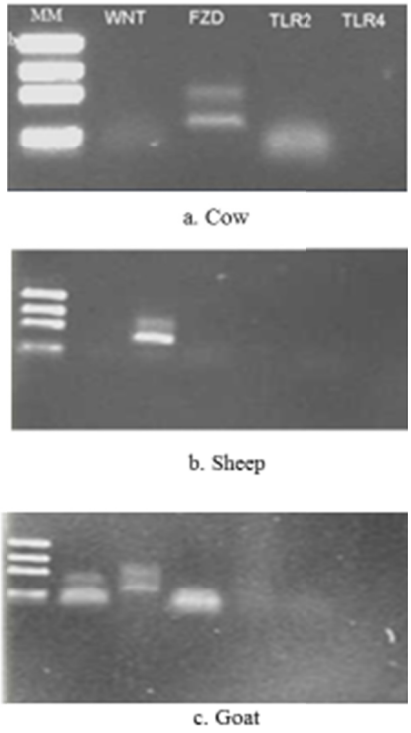

Figure 2. Visualization of amplicons of WNT5a, FZD, TLR2 and, TLR4 in cow(a), sheep(b) and goat (c) blood *following* real time PCR

3.5 Fold Change in TLR2 Expression

Variable increases in expression of TLR2 were observed. Cow samples exposed to individual treatments (LPS, PGN, or SL) had lower transcript levels (0.3, 0.5, and 0.6 folds respectively) compared to samples that were incubated with mixtures of treatments. Exposure to SL in combination with PGN was also associated with increased transcript levels (1.5 fold). Similarly, a high fold change (≥ 2) was observed in sheep samples exposed to PGN (2.4 fold). The highest fold change increase was observed in samples exposed to LPS and SL together. However, samples exposed to individual treatments (LPS, or SL) had lower transcription levels (0.1, and 0.1 folds respectively) compared to samples that were incubated with mixtures of treatments. In Goat blood, the highest fold changes in the sample were observed in samples exposed to PGN/SL, LPS/SL and SL only. It was observed from this study that goats responded more to PAMPS combined with SL.

Table 3. Increased expression calculated as fold changes in TLR2 expression for each species

Treatments	Cow	Sheep	Goat
LPS	0.6	0.1	0.0
SL	0.5	0.1	1.7
PGN	0.4	2.4*	1.3
PGN/SL	1.5	0.6	4.1*
LPS/SL	0.5	1.8	4.8*
LPS/PGN	0.4	2.7*	0.0

Note. * = Fold changes ≥ 2 is considered significant.

3.6 Fold Change in Frizzled Receptor

Variable increases in Frizzled receptor expression were observed. In Cow blood the highest fold change in Frizzled Receptor was observed when PGN and SL were used together (13.5). That was the only significant fold change (≥ 2). All others were low (<1). In goat blood however, significant fold changes were observed

when PGN was combined with SL (4), LPS/PGN (2) and LPS/SL (2). Sheep, samples stimulated with LPS and SL showed the highest fold change (2.7). Followed by SL alone (2.4). In all 3 animals, it was observed that samples co-incubated with water extract of SL and PAMPS showed increase in fold change (Table 4).

Table 4. Fold changes in Frizzled receptor expression for each species

Treatments	Cow	Goat	Sheep
LPS	0.2	0.0	0.0
PGN	0.0	1.0	0.1
SL	1.0	1.0	2.4
PGN/SL	13.5*	4.0*	0.6
LPS/PGN	0.1	2.0*	1.8
LPS/SL	0.7	2.0*	2.7*

Note. * = Fold changes ≥ 2 is considered significant.

3.7 Evaluation of Total Plasma Protein Concentration

Stimulation with PAMPs had an impact on plasma protein concentration. Protein levels were high in plasma of samples exposed to PGN and SL ($p < 0.05$). In cow and goat blood, protein concentration following activation by PAMPs and SL was higher than in PBS. However, stimuli did not have that much effect on protein concentration in sheep blood (Figure 3).

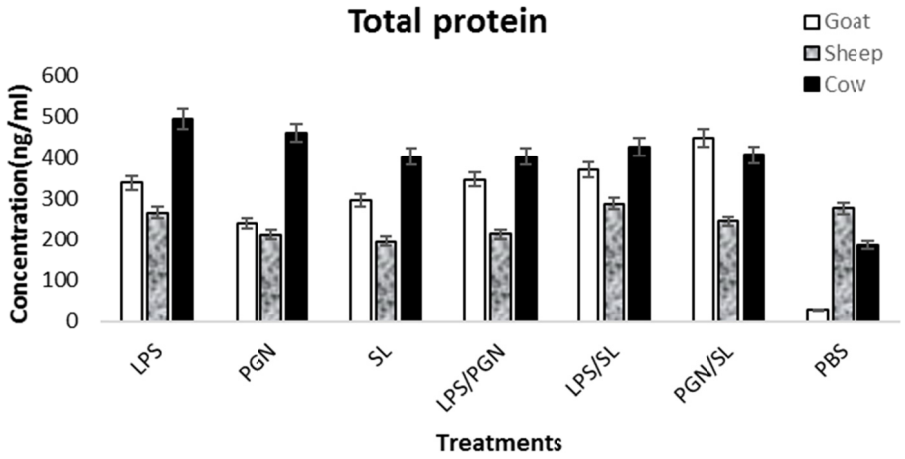

Figure 3. Total protein concentration in cow, sheep and goat plasma

Total protein concentration in all cow blood samples incubated with the various treatments was significantly different from the control. Samples incubated with LPS alone had the highest protein level (495 µg/ml). All others were not significantly different from each other ($p < 0.05$). The average concentration of the rest of the samples was 428 µg/ml. In goat blood, total protein concentration in all samples was significantly higher than that of the control (PBS) ($p < 0.05$). The highest concentration was recorded in samples incubated with PGN/SL. However, there was no significant difference between the 3 samples ($p < 0.05$). The average concentration of the three was 443.3 µg/ml. In sheep blood, total protein concentration in samples incubated with LPS, LPS/SL, PGN/SL, were not significantly different from blood incubated with PBS (258.7 µg/ml and 276 µg/ml respectively). Inflammation causes extra protein to be released from the site of inflammation and circulates in the bloodstream. These studies show species-specific variation in the secretion of proteins in plasma is response to PAMPs.

3.8 Detection of Prostaglandin Levels (PGE2) in Plasma

Overall, goats had the lowest PGE2 levels among the 3 ruminants (Figure 4). In goat blood, LPS had the highest mean of 110 pg/ml, followed by PGN (86 pg/ml). However, when blood was incubated with LPS/SL, prostaglandin concentration dropped to 67.6. Similarly, when goat blood was incubated with PGN/SL, prostaglandin levels dropped by 62 pg/ml. All treatments were significantly different from the control (PBS) which has 24.7 pg/ml of prostaglandin. Sheep samples incubated with PGN alone, LPS alone and LPS/PGN had the most prostaglandin concentration levels of 565 pg/ml, 349 pg/ml, 313 pg/ml and 251 pg/ml respectively. Prostaglandin levels also decreased significantly when LPS and PGN were both paired with SL. That is, LPS/SL (22.9 pg/ml) and PGN/SL (71 pg/ml) respectively ($p < 0.001$). In cow blood, the highest prostaglandin level was found in samples incubated with LPS/PGN and PGN (313 and 254 respectively). However, prostaglandin levels dropped significantly when LPS and PGN were paired with SL. That is, 123 pg/ml and 94 pg/ml ($p < 0.001$). This was similar to what happened in sheep blood. From the results, treatment, and species interaction effect was significant ($p < 0.0001$). As observed from the plasma protein secretion previously, PGE2 secretion was also species and PAMP dependent.

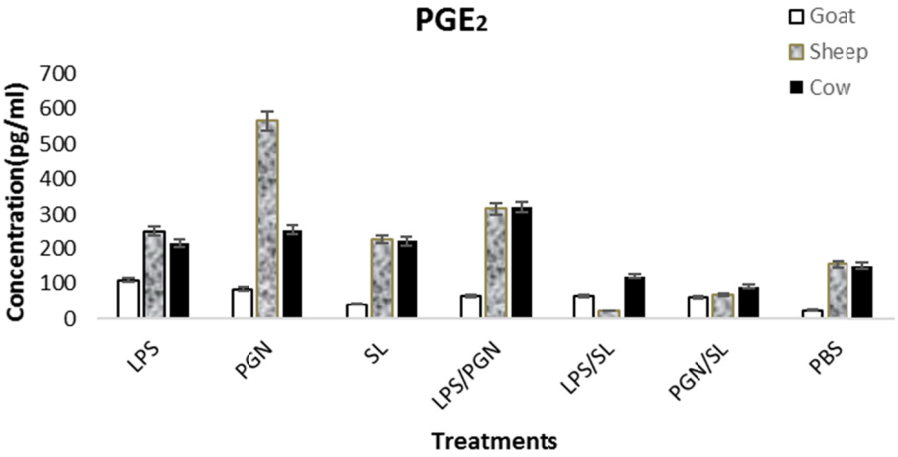

Figure 4. Prostaglandin (PGE2) levels in cow sheep and goat blood plasma

3.9 Detection of WNT5a in Plasma

Secretion of WNT5a was only observed in the plasma of LPS treated cow blood (Figure 5). No WNT5a was detected in sheep and goat blood.

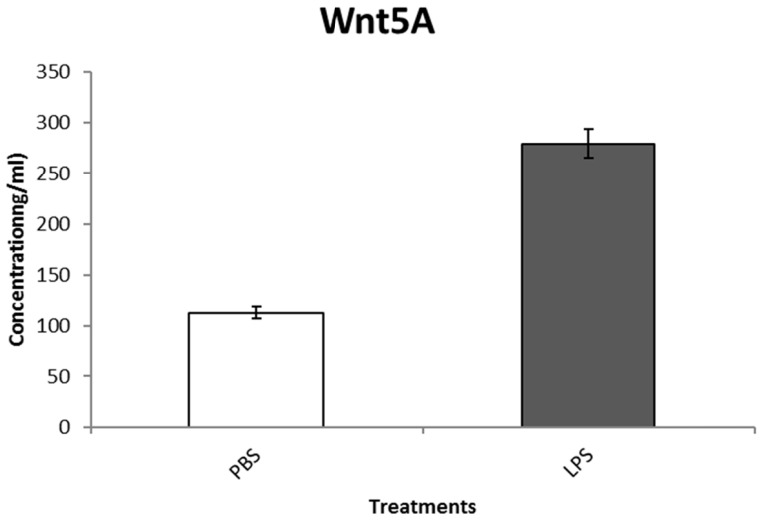

Figure 5. WNT5a detection in plasma of PBS and LPS treated cow blood

4. Discussion

In this study, we demonstrate that Sericea Lespedeza, a tannin-rich legume is involved in cell activation and modulates the response to bacterial cell wall components (PAMPS) in ruminant blood. Under the conditions of this study, SL was effective in activating genes involved in the Wnt signaling as well as TLR pathways. Total protein and prostaglandin levels were also modulated in the presence of SL. The results show that there is variation in how various species respond to SL and different bacteria cell wall stimuli.

Cell viability may be dependent on the type of stimuli and animal species. Cow had the highest viable cells after treatment. Also, results from white blood cell differential count showed that there is variation in how the three different species respond to SL and different bacteria cell wall stimuli. The principal function of leukocytes is to defend the animal against invading microorganisms by phagocytosis or the release of cytokines into the blood stream, thus contributing to cellular inflammatory responses (Luster, 2005). The variation in change of the number of leukocytes in different species is an indication of differences in general inflammatory response to infection between species. Lymphocytes and Neutrophil populations were observed to be higher than the rest of the leukocytes in all three ruminants. There was no significant change in their population compared to the normal leukocytes levels in ruminants. Both control and treated samples were within the normal white blood population range for all the animals. Hence treatment did not have an effect on WBC population.

Variation in fold changes in WNT and TLR expression was also observed in all species. Furthermore, differences in gene expression were observed when bacterial cell wall components were in concomitant exposure with Sericea Lespedeza. Scientific literature indicates that plant derived compounds like tannins and other polyphenols can modulate immune response through a number of pathways. These include the anti-inflammatory polyphenol and the oligomeric procyanidins (Holderness, 2012). Polyphenols produced by plants exhibit antifungal and anti-inflammatory properties (Leiro et al., 2004). Those studies also indicated the existence of plant polyphenols capable of modulating TLR-mediated signals. The data from the RT-PCR experiment showed a fold change in gene expression of TLR 2 in SL treated samples, supporting the suggested effect of tannins, in this case, that of Sericea Lespedeza on innate immunity. Our results corroborate with previous study findings by Worku et al., (2016), that TLR2 was observed to be increased in the blood of SL fed goats.

Furthermore, SL was observed to regulate protein secretion and prostaglandin concentration in all species. The concentrations of PAMPS used in this study were selected based on the minimum concentrations that stimulated significant TNF production from ruminant whole blood. Although WNT5a was detected in LPS treated cow samples, it was not detected in all other samples. LPS, PGN, and SL failed to induce WNT5a production under these conditions in goat and sheep as well. Previous studies have suggested that Wnt5a signaling is essential for the general inflammatory response of human macrophages (Blumenthal, 2006). Wnt5a has also been shown to be induced by mycobacterial cell wall components like lipopolysaccharide and lipopolypeptides in humans. It is clear from this study that Wnt5a secretion in ruminants may be species and PAMP dependent. The FZD receptor is a coreceptor involved in Wnt signaling. While TLRs promote inflammation, Wnt signaling pathway ensures order by homeostasis. Secondary metabolites derived from natural products including bioactive compounds such as tannins and phenols have been reported to inhibit inflammation (Olszanecki et al., 2002). The observed upregualtion of the frizzled receptor in the SL samples may be important in aiding the Wnt signaling pathway to keep inflammation levels in check. Hence SL may possess some anti-inflammatory properties. The expression of Frizzled receptor after stimulation with SL in ruminant blood is a novel discovery that needs to be explored.

With overuse, most synthetic anthelmintic and antibiotics are becoming increasingly ineffective due to parasite and pathogen resistance worldwide. There is also a growing concern over chemical residues in animal products and on pastures, and a worldwide increased demand for organic agricultural products for which use of synthetic chemicals is minimal (Hördegen et al., 2003). These factors all contribute to an increasing effort to find alternative and novel approaches to parasite and pathogen control. Condensed tannin containing plants have gained interest as a promising alternative to antibiotics and anthelmintics (Min, 2008). Several studies have been conducted to ascertain their effectiveness *in vivo* (Athanasiadou et al., 2001; Min & Hart, 2003; Paolini et al., 2003a; Paolini et al., 2003b; Paolini et al., 2003c; Shaik et al., 2004) and *in vitro* (Athanasiadou et al., 2001; Molan et al., 2002; Bahuaud et al., 2006).

Ruminants such as cattle, sheep and goats rely on plants for nutrtion. Our results suggest that SL, a tannin rich legume, affects gene transcription and translation in ruminant blood. In summary, the effect of SL on the expression of innate immune markers in response to microbial products may offer an avenue for the exploitation of plant-derived tannins to regulate inflammatory response and enhance the cow, goat and sheep innate response. In addition, this study demonstrated that a TLR2, TLR4 and/or WNT -dependent process may serve as the

molecular basis for the immumomodualtory properties of SL. This study expands the concept that certain plant-based drugs may serve as agonists for TLR and WNT, whose consequent activation may mediate beneficial drug effects.

5. Conclusion

Transcription and translation of genes involved in innate and adaptive immunity and the WNT signaling pathway in cow, sheep, and goat blood is responsive to diverse PAMPS and can be modulated by SL. Further studies are needed to determine the significance of SL and other tannin containing legumes on changes in immune gene expression on ruminant health and production.

References

Adib-Conquy, M., & Cavaillon, J. M. (2007). Stress molecules in sepsis and systemic inflammatory response syndrome. *FEBS Letters, 581*(19), 3723-3733. http://dx.doi.org/10.1016/j.febslet.2007.03.074

Adjei-Fremah, S., Asiamah, E. K., Ekwemalor, K., Jackai, L., Schimmel, K., & Worku, M. (2016). Modulation of Bovine Wnt Signaling Pathway Genes by Cowpea Phenolic Extract. *Journal of Agricultural Science, 8*(3), 21.

Athanasiadou, S., Kyriazakis, I., Jackson, F., & Coop, R. L. (2000). Consequences of long-term feeding with condensed tannins on sheep parasitised with Trichostrongyluscolubriformis. *International Journal for Parasitology, 30*(9), 1025-1033. http://dx.doi.org/10.1016/S0020-7519(00)00083-7

Bahuaud, D., De Montellano, C. M. O., Chauveau, S., Prevot, F., Torres-Acosta, F., Fouraste, I., & Hoste, H. (2006). Effects of four tanniferous plant extracts on the in vitro exsheathment of third-stage larvae of parasitic nematodes. *Parasitology, 132*(04), 545-554. http://dx.doi.org/10.1017/S0031182005009509

Barton, G. M., & Kagan, J. C. (2009). A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nature Reviews Immunology, 9*(8), 535-542. http://dx.doi.org/10.1038/nri2587

Boermans, H. J., Korosteleva, S. N., & Smith, T. K. (2009). Effects of feed naturally contaminated with Fusarium mycotoxins on metabolism and immunity of dairy cows. *Journal of Dairy Science, 92*(4), 1585-1593. http://dx.doi.org/10.3168/jds.2008-1267

Chang, Z. L. (2010). Important aspects of Toll-like receptors, ligands, and their signaling pathways. *Inflammation Research, 59*(10), 791-808. http://dx.doi.org/10.1007/s00011-010-0208-2

Holderness, J. S. (2012). Induction of innate immune responses by plant-derived procyanidins and polysaccharides.

Hördegen, P., Hertzberg, H., Heilmann, J., Langhans, W., & Maurer, V. (2003). The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. *Veterinary Parasitology, 117*(1), 51-60. http://dx.doi.org/10.1016/j.vetpar.2003.07.027

Leiro, J., Arranz, J. A., Parama, A., Alvarez, M. F., & Sanmartin, M. L. (2004). *In vitro* effects of the polyphenols resveratrol, mangiferin and (-)-epigallocatechin-3-gallate on the scuticociliate fish pathogen Philasteridesdicentrarchi. *Diseases of Aquatic Organisms, 59*(2), 171-174. http://dx.doi.org/10.3354/dao059171

Livak, K. J., & Thomas, D. S. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25*(4), 402-408. http://dx.doi.org/10.1006/meth.2001.1262

Luster, A. D., Alon, R., & von Andrian, U. H. (2005). Immune cell migration in inflammation: present and future therapeutic targets. *Nature Immunology, 6*(12), 1182-1190. http://dx.doi.org/10.1038/ni1275

McGuire, K., Jones, M., Werling, D., Williams, J. L., Glass, E. J., & Jann, O. (2006). Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors. *Animal Genetics, 37*(1), 47-50. http://dx.doi.org/10.1111/j.1365-2052.2005.01364

Menzies, M., & Ingham, A. (2006). Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. *Veterinary Immunology and Immunopathology, 109*(1), 23-30. http://dx.doi.org/10.1016/j.vetimm.2005.06.014

Min, B. R., & Hart, S. P. (2003). Tannins for suppression of internal parasites. *Journal of Animal Science, 81*(14, Suppl. 2), E102-E109. http://dx.doi.org/2003.8114_suppl_2E102

Min, B. R., Hart, S. P., Miller, D., Tomita, G. M., Loetz, E., & Sahlu, T. (2005). The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. *Veterinary Parasitology, 130*(1), 105-113. http://dx.doi.org/10.1016/j.vetpar.2005.03.011

Min, B. R., Pinchak, W. E., Merkel, R., Walker, S., Tomita, G., & Anderson, R. C. (2008). Comparative antimicrobial activity of tannin extracts from perennial plants on mastitis pathogens. *Scientific Research and Essay, 3*(2), 066-073.

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

Olszanecki, R., Gebska, A., Kozlovski, V. I., & Gryglewski, R. J. (2002). Key words: Flavonoids, nitric oxide synthase, macrophages. *Journal of Physiology and Pharmacology, 53*(4), 571-584.

Paolini, V., Bergeaud, J. P., Grisez, C., Prevot, F., Dorchies, P., & Hoste, H. (2003). Effects of condensed tannins on goats experimentally infected with Haemonchuscontortus. *Veterinary Parasitology, 113*(3), 253-261. http://dx.doi.org/10.1016/S0304-4017(03)00064-5

Person, A. D., Beiraghi, S., Sieben, C. M., Hermanson, S., Neumann, A. N., Robu, M. E., ... Mazzeu, J. F. (2010). WNT5A mutations in patients with autosomal dominant Robinow syndrome. *Developmental Dynamics, 239*(1), 327-337. http://dx.doi.org/10.1002/dvdy.22156

Raja, A., Vignesh, A. R., Mary, B. A., Tirumurugaan, K. G., Raj, G. D., Kataria, R., ... Kumanan, K. (2011). Sequence analysis of Toll-like receptor genes 1-10 of goat (*Capra hircus*). *Veterinary Immunology and Immunopathology, 140*(3), 252-258. http://dx.doi.org/10.1016/j.vetimm.2011.01.007

Rhee, C. S., Sen, M., Lu, D., Wu, C., Leoni, L., Rubin, J., ... Carson, D. A. (2002). Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. *Oncogene, 21*(43), 6598-6605.

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

Tarapore, R. S., Siddiqui, I. A., & Hasan Mukhtar, H. (2012). Modulation of Wnt/β-catenin signaling pathway by bioactive food components. *Carcinogenesis, 33*(3), 483-491. http://dx.doi.org/10.1093/carcin/bgr305

Werling, D., & Coffey, T. J. (2007). Pattern recognition receptors in companion and farm animals–The key to unlocking the door to animal disease. *The Veterinary Journal, 174*(2), 240-251. http://dx.doi.org/10.1016/j.tvjl.2006.10.010

Werners, A., Bull, S., & Fink-Gremmels, J. (2005). Endotoxaemia: A review with implications for the horse. *Equine Veterinary Journal, 37*, 371-383. http://dx.doi.org/10.2746/0425164054529418

Wiersinga, W. J., Leopold, S. J., Cranendonk, D. R., & van der Poll, T. (2014). Host innate immune responses to sepsis. *Virulence, 5*(1), 36-44. http://dx.doi.org/10.4161/viru.25436

Worku, M., & Morris, A. (2009). Binding of different forms of lipopolysaccharide and gene expression in bovine blood neutrophils. *Journal of Dairy Science, 92*(7), 3185-3193. http://dx.doi.org/10.3168/jds.2008-1905