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Research article

THE IMPACT OF A SERICEA LESPEDEZA DIET ON *BIFIDOBACTERIA* IN GOAT RUMEN

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ABSTRACT: Gastrointestinal nematodes in goats have developed resistance to chemical anthelmintics resulting in the need for alternative control strategies. One approach to this problem is the incorporation of condensed tannin- rich (CT) forages that suppress gastrointestinal parasites in goats. In this study, the effect of supplementing goat diets with sericea lespedeza (SL), a high-quality CT-rich forage, on rumen microorganisms was evaluated in goats. A diverse collection of microorganisms is found in the goat rumen. *Bifidobacteria* are important organisms involved in gastrointestinal tract immunity, however, their presence and modulation in the goat rumen has not been fully investigated. The objective of this study was to evaluate the impact of a diet containing SL on rumen microorganisms, especially *Bifidobacteria*, in goats. Isolated microbial DNA from rumen samples of goats receiving a diet with SL (n=5) and goats receiving an SL free diet (n=6) were used for detection of *Bifidobacteria*. The isolated microbial DNA was amplified using 16S rDNA universal bacterial primers. Amplified DNA from goats receiving SL in the diet and control groups by Polymerase chain reaction were used for denaturing gradient gel electrophoresis (PCR-DGGE) analysis of total microbial profile. Genus-specific primers of 16S rDNA for *Bifidobacterium* were used to amplify the specific DNA sequence. The results of the DGGE analysis showed similar band patterns in the control group compared to the groups receiving the SL diet. A 580-bp *Bifidobacterium*-specific band was observed in samples from goats fed the SL free diet ($P<0.002$). *Bifidobacterium* DNA was not detected in goats fed a diet containing SL. This suggests that dietary tannins may affect the goat's rumen microbial profile. Further, studies are needed to determine the significance of changes in *Bifidobacteria* on goat health.
Keywords: *Bifidobacteria*, Gastrointestinal nematodes, anthelmintics, goat rumen.

INTRODUCTION

Gastrointestinal nematodes represent a major hazard for goat health and production in hot humid areas such as the southeastern United States [1]. Internal parasites pose the greatest challenge to goat production, and the extensive use of anthelmintic to control nematode infections in the past has resulted in increased levels of anthelmintic resistance [2]. Alternative control strategies including enhancement of immunity via nutrition, vaccination, pasture

management, and genetic resistance are being explored [1]. Consuming plants high in tannins such as sericea lespedeza (SL), *Lespedeza cuneata*, has been found to be effective in reducing internal parasites in herbivorous animals [2-4]. Sericea lespedeza contains a CT amount of 46-152 g/kg of dry matter [3,5]. The use of SL, when grazed or fed as hay and pellets has been shown to reduce fecal egg count and reduce worm burdens [2]. Tannins in SL can cause fluctuations in rumen microbes, which have been found to play a significant role in animal health [6,7].

The innate immune system is composed of physical, chemical and microbiological barriers that include normal rumen microorganisms. A diverse collection of microorganisms has been shown to exist in the goat rumen [8]. Tannins are generally regarded as inhibitory to the growth of microorganisms, but the mechanisms involved are poorly understood. One of the suggested explanations for the inhibitory effect of tannins is their ability to complex with bacteria through reactivity of the polyphenolic group with the cell wall of bacteria and secreted extracellular enzymes. Either one of these interactions is likely to inhibit the transport of nutrients into the cell and retard the growth of the organism [9]. The effects of tannins on rumen microorganisms such as *Fibrobacter succinogenes* based on a cell-associated and extracellular endoglucanase activity have been reported. Under *in vitro* conditions, CT (100–400 ug/ml) from *Lotus corniculatus* had an inhibitory effect on *F. succinogenes* [10,11]. The growth of proteolytic bacteria (*Butyrivibrio fibrisolvens, Ruminobacter amylophilus* and *S. bovis*) was reduced by condensed tannins, but a strain of *Prevotella ruminicola* was tolerant to CT (<600 ug/ml) from *Onobrychis viciifolia*) [10,12].

Studies to determine the effect of SL on foodborne pathogens in meat goats have shown, that high CT in the diet influenced rumen volatile fatty acid composition, but did not reduce the bacterial loads in gastrointestinal tracts. *Bifidobacterium* is a genus of gram-positive, non-motile, often branched anaerobic bacteria inhabiting the gastrointestinal tract and vagina [13]. *Bifidobacteria* belong to the dominant gut microbiota in humans and animals. *Bifidobacteria* aid in digestion, are associated with a lower incidence of allergies [14] and are used as probiotics [15]. Furthermore, the impact of polyphenols on human gut microbiota has been reviewed by Ref. [16,17], have shown that exposure to tannins inhibits the growth of *Bifidobacterium animalis ssp. lactis*. There is interest in the use of *Bifidobacteria* as a probiotic in goat feed supplements. Commercial probiotics use and its effects on growth performance, diet digestibility, and the fecal bacterial population has been evaluated in goats [18]. *Bifidobacteria* were found to be a subdominant group of fecal microflora in 1-day old goats, reaching the counts of 6.41 log CFU g-1 and milk [19]. Using PCR techniques, which are more rapid and sensitive than culture-based method [20], *Bifidobacteria* has been detected in goat feces.

Bifidobacteria is an important organism in the immunity of the gastrointestinal tract, and the impact of diet on its presence in the goat rumen has not been fully studied. Such studies are needed in goats in light of the use of SL as a supplement beneficial to goat health through nematode control. The objective of the current study was thus to explore the effect of a diet containing SL on rumen microbial populations, especially *Bifidobacteria* in goats

MATERIALS AND METHODS

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, both males (n=15) and females (n=15). The goats were divided into three groups, and each group received an SL dosage at different concentration levels for a period of four weeks. Goats were assigned to one of three levels of SL treatment with alfalfa pellets of 0%, 50%, and 75%. The animals were naturally infected with nematodes through pasture grazing [2]. At the end of the study, rumen samples were collected to explore the effect of the treatment on rumen microorganisms, particularly *Bifidobacterium*.

Rumen Microbes

Rumen content was collected at slaughter from 16 goats, control (n=7), diets containing 50% SL (n=4), and 75% SL (n=5) under sterile conditions using clean sterile glass jars and stored at -20°C. Frozen rumen samples were thawed at room temperature and aliquots of 10 ml rumen content were transferred to clean 30 ml tubes using clean new graduated pipettes for each sample. Samples were homogenized using a bio-homogenizer and centrifuged at 6500 × g using Eppendorf 5810 rotor for 30 mins. The pellets were collected and used for further analysis.

Extraction of Microbial DNA

Total microbial DNA was isolated from rumen contents using the QIAmp DNA stool kit (QIAGEN Sciences, Maryland, USA) following the manufacturer's protocol. After thawing samples at room temperature, 200 ml of rumen sample were obtained. Stool lysis buffer (ASL Buffer), (QIAGEN Sciences, Maryland, USA) was added to the sample (1.4 ml), then vortexed until homogenized. Samples were placed in a 95°C water bath for 5 mins, vortexed for 15 seconds and centrifuged for 1 min. The supernatant was transferred into a new tube, and one inhibitor adsorption tablet (inhibitEX tablet, QIAGEN Sciences, Maryland, USA) was added to each tube, vortexed and incubated for 1 min at room temperature. Samples were centrifuged for 3 mins in 14,000 × g, supernatants were collected in a new 1.5 ml, and the pellets were discarded. Proteinase K (15 µl) was added to a new 1.5 ml tube then 200 µl of the supernatants were added to the 1.5 ml tube containing proteinase K. After that, 200 µl of the lysis buffer (AL buffer, QIAGEN Sciences, Maryland, USA) were added to each tube, vortexed for 15 secs, and then transferred to 95°C water bath for 10 minutes. Ethanol 100% was added to the samples (200 µl), transferred to spin columns and centrifuged for 1 minute at full speed. The spin columns were then transferred to new 2 ml tubes and 500 µl of wash buffer 1 (AW1 buffer, QIAGEN Sciences, Maryland, USA) was added to the columns and centrifuged for 1 min at full speed. The washing step was repeated again using washing buffer 2 (AW2 buffer, QIAGEN Sciences, Maryland, USA) and centrifuged for 3 mins at full speed. The columns were then removed to a new 2 ml tube and 200 µl of DNA elution buffer (AE) buffer were added to each and then centrifuged for 1 min at full speed to collect isolated DNA.

Determination of DNA Concentration and Purity

The concentration and purity for the isolated DNA were determined using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). The measurement was performed by taking one microliter of isolated DNA and evaluated at Optical Density (OD) of 260 nm/230 nm for purity and 260 nm/280 nm.

Amplification of Microbial DNA from rumen samples using PCR

Isolated total DNA was used in PCR, and 125 ng DNA was used as a template. The following primers were used for PCR amplification across the 16S rDNA variable (V3) region: forward, 5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3'; reverse, 5'-ATTACCGCGGCTGCTGG-3' [21,22]. Polymerase chain reaction was conducted using Super Taq Plus Polymerase kit (Ambion, Austin, TX). The PCR condition used was: one cycle (94°C for 5 min), 25 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) and one cycle (72°C for 7 min), [21]. Aliquots (5 µl) of the amplified PCR products were examined by electrophoresis on 2% agarose gel (w/v), and stained with 1 µg/ml Ethidium Bromide.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis of Microbial DNA

Amplified DNA samples were re-run using Denaturing Gradient Gel Electrophoresis (DGGE) procedure to observe the presence of any difference in the amplicon band migration patterns. Isolated DNA from rumen samples of SL-fed goats (n=5), and control animals, 0% SL (n=6), were used as representative samples for DGGE analysis. Denaturing gradient gel electrophoresis in parallel gradient gel ranging from 40% to 60% (10% acrylamide) was run at 130 V, 60°C for 5 h, on the DCode Universal Mutation Detection system (16-cm system; Bio-Rad, Hemel

Hempstead, UK). As recommended by the manufacturer (Bio-Rad) gels were stained with Ethidium Bromide solution, and visualized using a gel transilluminator (Gel Doc 2000, Bio-Rad).

Detection of *Bifidobacterium*

Bifidobacterium genus-specific primers shown in Table 1 were used to investigate the presence of *Bifidobacterium* in the rumen contents of goats (n=16). Those primers were designed on the basis of 16S rDNA sequences [23]. Treatment groups used included 50% SL fed goats (n=4), 75% SL fed goats (n=5) and control group (n=7) goats fed 0% SL diet. Controls used in this study included a DNA isolated from *Bifidobacterium longum* (ATCC 1507) as positive control, and isolated microbial DNA without primers served as negative control.

Table 1: *Bifidobacterium* genus specific primers set. Bif164-PCR (Forward) and Bif662-PCR (Reverse)

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

The PCR reaction included 250 ng of the isolated DNA as a template, 5 µl of 10x PCR buffer, 2.5 µl deoxynucleotide triphosphates (dNTPs), 0.5 µl each primer and 1 µl Taq polymerase in 25 µl reactions. All procedures were performed on ice. The PCR conditions consisted of one cycle of 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 4 minutes followed by t 1 cycle of 72°C for 6 minutes using (MWG-BIOTECH primus 96) thermocycler. Tubes containing PCR products were then stored at 4°C until used. Electrophoresis was conducted to visualize the amplified sequence of 16S rDNA to determine the presence of *Bifidobacterium* species in rumen samples. Agarose gel 2% (w/v) was prepared by adding 1 g of agarose (LE, analytical grade agarose, Promega Corporation, Madison, WI USA) to 50 ml 1x Tris-acetate-EDTA (TAE). Amplified PCR products were electrophoresed along with a 100 bp molecular ruler (Bio-Rad Laboratories, Inc.) to determine the size of amplified products [24]. Electrophoresis was run at 150 V for 20 mins, gels were stained with Ethidium Bromide and viewed using a gel transilluminator (BioRad).

The Statistical Analysis Software System (SAS Institute, Cary, NC) frequency procedure was used to analyze gels for *Bifidobacteria* detection.

RESULTS AND DISCUSSION

Microbial DNA was isolated and successfully amplified using 16S rDNA primers. The results of the DGGE analysis showed similar banding patterns in the control group compared to the SL-fed animals. However, individual band detection indicates possible differences in tolerance to tannin-rich diets. A 580-bp *Bifidobacterium*-specific band was observed in samples from control goats, 0% SL diet ($P<0.002$). *Bifidobacterium* DNA was not detected in goats fed a diet containing SL.

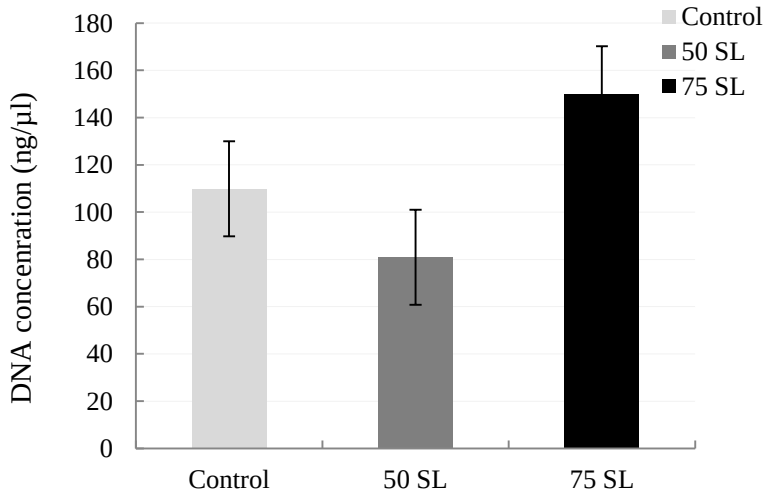
The rumen harbors various types of bacteria which are active in degradation of lignocellulosic components of the feed [6]. The microbial ecology of the rumen can be modulated by environmental factors such as the type of

nutrition. Adaptation of ruminal metabolism to counter anti-nutritive effects of forage tannins may involve the microbial degradation of these compounds [10]. Condensed tannins have been shown to be a suppressive substance for rumen bacterial growth and/or alter their metabolism [23]. In this study, we focused on the effects of SL diet with high CT content on rumen microorganisms.

Concentration and Purity of Isolated DNA

The concentration and purity of isolated DNA were reported from three groups of animals treated with 0%, 50% and 75% SL in the diet (Figure 1). Means for the concentration of isolated DNA were calculated for the three groups and showed no significant difference (P=0.5215). The concentration of isolated DNA was the highest in the treatment group of SL 75% and the control group with a mean concentration of 150.11 ng/µl and 109.89 ng/µl respectively. The lowest mean concentration was obtained from the treatment group that had received 50% SL (80.88 ng/µl). The mean purity was 1.83, 1.92, and 1.86 for DNA isolated from 0%, 50%, and 75% SL diet respectively, indicating adequately pure DNA for downstream PCR application.

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



Comparison of Rumen Microbes DNA

Amplification of 16S rDNA gene was performed using PCR, and after running the gel electrophoresis for the amplified products, a specific band of size 200 bp was observed, indicating primer specificity for the target sequence of the microbial 16S rDNA gene (Figure 2). This step is important to ensure that all samples used in the experiment contain the amplified sequence necessary for the establishment of legitimate comparison between treatment and control group in the denaturing gel electrophoresis.

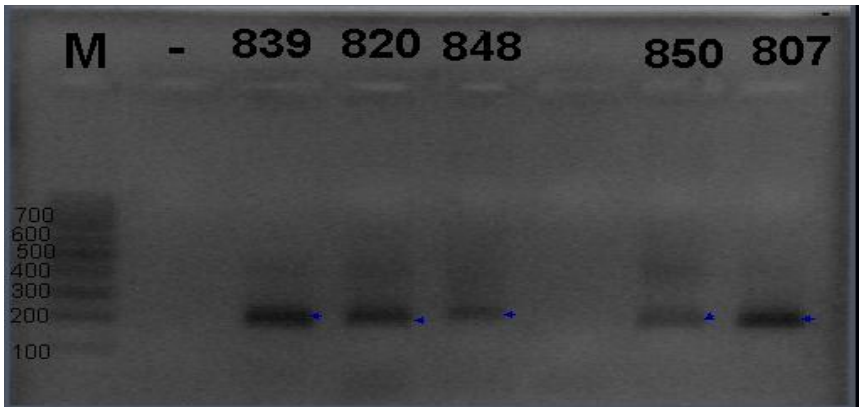


Figure 2: Representative samples of amplified sequence of microbial 16S rDNA gene. M: Molecular marker (100 bp marker); -: No primer negative control. Negative controls showed no bands, whereas selected amplified samples (839, 820, 848, 850 and 807) showed the expected 200 bp band.

The amplified sequences were then used in a denaturing gradient gel to detect changes in microbial populations. The result from DGGE experiments showed an overall similarity in patterns in both the control group and treatment groups. Several specific bands observed in the control group were absent in SL 75% and SL 50% groups (see Figure 3). Control and SL treatment groups showed conserved bands indicated in Figure 3, as 1, 2 and 3. Bands 1, 2 and 3 may represent tannin tolerant microorganisms since these bands continue to be present under SL treatment conditions in lanes for SL 50% and SL 75%. Bands indicated as (4, 5 and 6) in control group lanes (0% SL), were not observed in SL 75% and SL 50% treatment groups. These bands may represent tannin intolerant microorganisms since their specific bands were consistently not detected on the gel. Animals treated with 0% SL maintained similar band patterns indicated by numbers (1, 2, 3, 4, 5 and 6), whereas specific bands 4, 5, and 6 were absent in 75% and 50% SL treated animals (Figure 3).

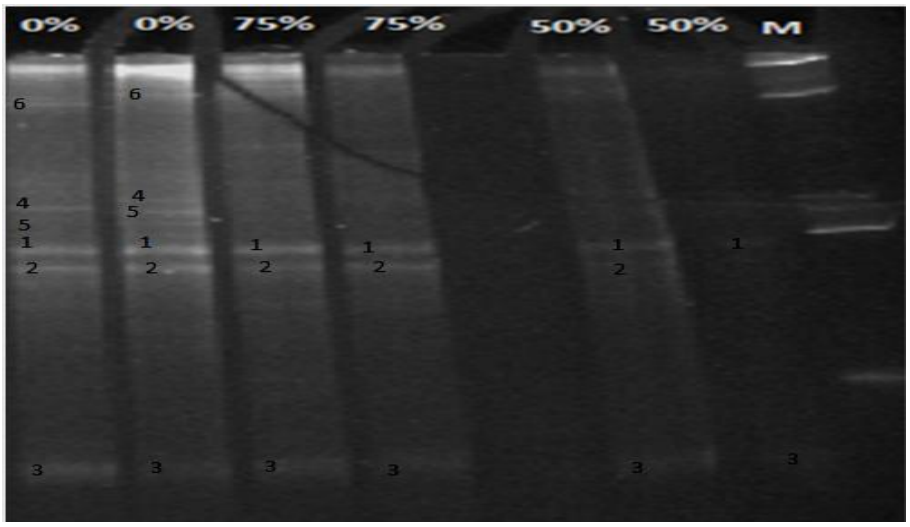


Figure 3: Denaturing gradient gel electrophoresis of amplified rumen microbial DNA. 75%- Samples from goats fed 75% Sericea lespedeza (SL), 50%- Samples from goats fed 50% SL, 0%- Samples from goats fed SL- free diet and M: molecular marker.

Denaturing gradient gel electrophoresis results suggest that the occurrence of total rumen microbial changes after SL treatment. The role of rumen microbes extends from its significance in the fermentation of plant nutrients and

immunity to environmental effects through rumen methanogen which has been identified as the single largest source of anthropogenic methane [6,24]. Thus, rumen microbial population shifts will not only affect host nutrition and immunity but would reflect on the environment [24,25]. In our study, the results obtained through DGGE analysis suggest that the SL diet influenced the rumen microbial population. Similar studies using this approach (DGGE) have been conducted to investigate rumen methanogenic *Archaea* microbes in goats to help reduce methane emissions [26].

The influence of SL treatment on the rumen microbial population our study results was expected, since SL is considered to be a high tannin containing plant [27]. Several studies have also indicated, highly significant effects of plant tannins on rumen microbes by suppressing and/or alteration of the microbe metabolism [23]. Studies done by Ref. [17], have shown that exposure to tannins from pomegranate inhibits *Bifidobacterium animalis ssp. lactis* growth. Tannins have been shown to prevent or, at least, interfere with the attachment of rumen microorganisms to plant cell walls, and it is well known that such attachment is essential for degradation of plant cells to occur [28]. Tannins might have a direct effect on ruminal microorganisms by altering the permeability of their membranes, yet this effect varies based on the rumen microorganisms' tolerance to tannins [28].

Detection of *Bifidobacterium*

Following the procedure mentioned above, amplified sequences of 16S rDNA gene were obtained from control animals (820, 824, 846, 850, 848, 839 and 810), and *Bifidobacteria*-specific bands of ≈ 523 bp were observable in the controls, except for one goat (810) which failed to show any specific band (see Figures 4 a, b, and d). In contrast, animals fed 50% SL diet (816, 836, 803 and 807), and 75% SL diet (828, 852, 814, 809 and 827), exhibited no observable *Bifidobacteria*-specific bands (see Figure 4 a, b, c and d). The effect of SL treatment groups on *Bifidobacteria* detection in rumen fluid was found to be significant ($p<0.002$), using a frequency procedure and Chi-square test. Six animals (820, 824, 846, 850, 848, 839) out of the seven control animals of 0% SL, showed *Bifidobacteria* genus specific band of 523 bp, but bands were not detected in either of SL treatment groups. This approach may aid in the further exploration of the molecular diversity of rumen *Bifidobacteria* and its distribution through the gastrointestinal tract of goats.

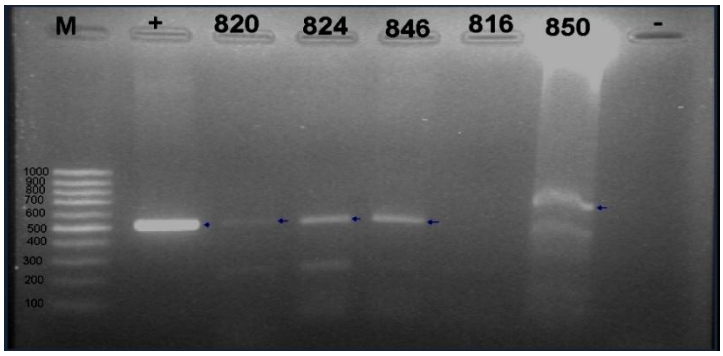


Figure 4a: Amplified 16S rDNA sequence of *Bifidobacteria*, M: marker, +: *Bifidobacterium longum* (ATCC 1507) positive control, 820: control animal (0% SL), 824: control animal (0% SL), 846: control animal (0% SL), 816: treatment group (50% SL), 850: control animal (0% SL), -: No primer negative control.

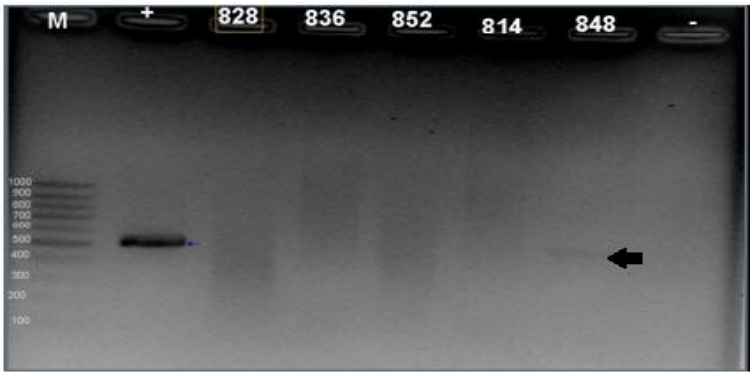


Figure 4b: Amplified 16S rDNA sequence of *Bifidobacteria*, M: marker, +: *Bifidobacterium longum* (ATCC 1507) positive control, 828: treatment group (75% SL), 836: treatment group (50% SL), 852: treatment group (75% SL), 814: treatment group (75% SL), 848: control animal (0% SL), -: No primer negative control.

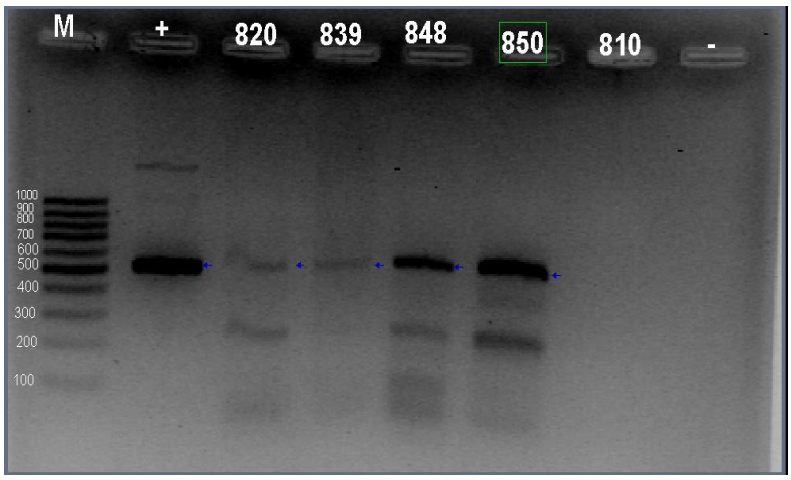


Figure 4c: Amplified 16S rDNA sequence of *Bifidobacteria*, M: marker, +: *Bifidobacterium longum* (ATCC 1507) positive control, 820: control animal (0% SL), 839: control animal (0% SL), 848: control animal (0% SL), 850: control animal (0% SL), 810: control animal (0% SL), -: No primer negative control.

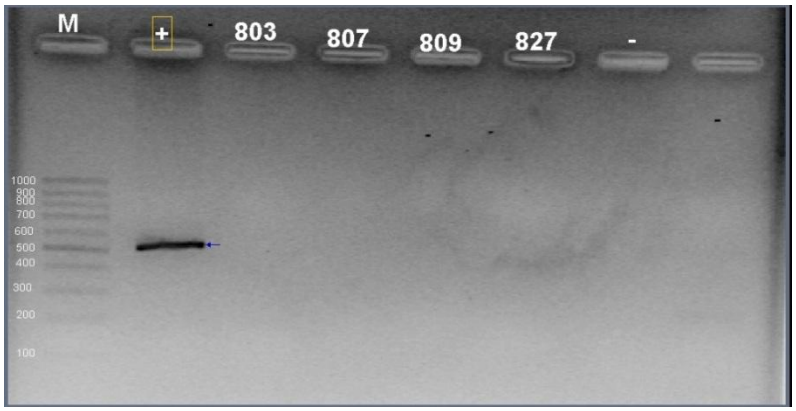


Figure 4d: Amplified 16S rDNA sequence of *Bifidobacteria*, M: molecular marker, +: *Bifidobacterium longum* (ATCC 1507) positive control, 803: treatment group (50% SL), 807: treatment group (50% SL), 809: treatment group (75% SL), 827: treatment group (75% SL), -: No primer negative control.

Interestingly, control animals showed consistent specific bands of *Bifidobacteria* in comparison to animals receiving an SL diet, indicating a highly significant change in the *Bifidobacteria* population ($p<0.002$). Detection of *Bifidobacteria* in the gastrointestinal tract of ruminants has been investigated [29] and its beneficial effect on innate immunity has also been studied [15].

This study explored the possible effects of an SL diet on goat rumen microbial populations and *Bifidobacteria.* A change in band patterns in response to SL supplementation indicates a change in rumen microbial composition [30]. Furthermore, specific changes were observed in *Bifidobacteria* after consumption of an SL supplemented diet. These changes may have a negative effect on nutrition and the immunity in goats and need further study. The molecular approach used to investigate *Bifidobacteria* proved successful in detection of microbial profile changes in goat rumen [31].

CONCLUSION

Results obtained the present study suggests that dietary tannins may affect the rumen microbial profile in goats. This approach may be useful in future studies to determine the significance of *Bifidobacteria* in this process.

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