CHANGES IN BACTERIAL DIVERSITY IN THE RUMEN OF CATTLE FED RIPE ACACIA [*Samanea saman* (Jacq.) Merr.] PODS

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ABSTRACT

To determine the effect of Acacia pods meal (APM) on the rumen bacterial diversity, three fistulated cattle were fed at 3% of BW with Napier, Napier-rice bran-copra meal mix (RBC) and Napier-APM following a 3 x 3 Latin square design. Isolated 16S rDNA from the rumen were sequenced and analyzed *in silico*. Growth performance of cattle was also evaluated using 18 heifers divided into three groups and fed with 70% Napier grass and 30% of the following treatments as concentrate portion: 30% RBC, 15% RBC:15% APM and 30% APM. Secondary metabolites extracted from APM were qualitatively assayed by paper chromatography (PC) and thin-layer chromatography (TLC). Tannin content was determined spectrophotometrically. Bacterial diversity analysis showed that feeding APM resulted in the proliferation of tannin-resistant bacterium *S. ruminantium* and loss of cellulolytic bacteria *A. ruminis.* Feeding APM in growing heifers reduced average daily gain and feed conversion ratio but not average daily feed intake. Hot water extract contained hydrolyzable tannins as determined by PC. TLC of *n*-hexane extract did not show secondary compounds. Quantitative analysis showed 16.42% tannins in APM. The study showed that reduction in growth performance of heifers is related to tannins in APM that could have direct bioactivity against important bacteria in the rumen.

Key words: Acacia pods, bacterial diversity, growing heifers, secondary compounds, tannins

INTRODUCTION

The decreasing land resources for pasture production in the Philippines resulted in researches for alternative feedstuffs for ruminant. Tree legumes, for example, can be utilized as ruminant feed especially during summer months when traditional feed resources are scarce. Aside from leaves, pods can be utilized as protein and carbohydrate sources for growth and reproduction. In addition to the nutrients they provide, various studies reported growth performance-regulating effects in ruminant animals by modifying rumen metabolism. The presence of secondary compounds in these plants may cause regulative effect to rumen function (Ukoha *et al*., 2011 and Hosmani *et al*., 2005). At the right dietary levels, changes in

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bacterial diversity may favor growth of economically-important cellulolytic microbial groups that results in better utilization of dietary cellulosic materials.

Samanea saman (Jacq.) Merr. or locally known as Acacia or Rain-tree is not only known as a shade tree but has leaves that are good sources of dry matter especially during the summer months. Acacia bears copious pods every December and ripe sweet pods fall from March to May of each year. Traditionally, fallen Acacia pods are fed during summer months and are claimed to fatten cows even when traditional dry matter sources (*i.e.* grasses) are scarce (Sevilla, *personal communication*). Too much intake of Acacia pods leads to temporary gait problems similar to alcohol toxicity in cattle (JALIRI Farm, 2011, *personal communication*). These observations suggest two things; Acacia pods may possibly serve as good source of essential nutrients or may contain compounds that alter gastro-intestinal function.

This study aimed to determine the effects of feeding Acacia pods meal (APM) on bacterial diversity in the rumen of mature cattle and growth performance of growing heifers. It also intended to explain the reason behind the rumen-regulative property of APM by qualitatively and quantitatively identifying the secondary products that may have caused the observed bacterial diversity changes in mature cattle and reduction in growth performance of heifers.

MATERIALS AND METHODS

Collection and preparation of Acacia pod meal

Fallen ripe Acacia pods were collected from different areas of Laguna, Philippines. Fresh and damaged-free pods were stored at room temperature or were manually chopped (1.0-2.0 cm in length) prior to feeding to the experimental animals. Chopping was carried out in a manner that makes sure that seeds were also crushed or chopped. In this study, the chopped whole Acacia pod was called Acacia pod meal (APM). APM for chemical analyses were oven-dried at 105ºC for 8 h prior to grinding using 2 mm screen. Ground samples were stored at 4ºC until use.

Sample preparation and chemical analyses

Ground whole pods, pulp and seeds were subjected to moisture content (MC), crude protein (CP), crude fiber (CF), ether extract (EE), crude ash (CA), calcium (Ca) and phosphorus (P) analyses using standard methods (AOAC, 1995). Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined gravimetrically following the procedure of Van Soest (1963). Cellulose content was computed from the ADF and ADL values.

16s rDNA bacterial diversity analysis

Three fistulated cattle housed in individual elevated metabolism stalls were fed at 3% of their BW (DM basis) with three treatments which were replicated over time following 3 x 3 Latin Square Design. Dietary treatments include; 1) all Napier grass (AN); 2) Napier grass + rice bran-copra meal mix (NR); and 3) Napier grass +

Acacia pod meal (NA). The nutrient composition of the feedstuffs is shown in Table 1. Napier and concentrate feed were given at 70:30 ratio. For each round, animals were fed at 8:00 h and 14:00 h for 10 d and were given free access to clean water. On the 11th day, samples of rumen fluid were obtained for DNA extraction. After rumen fluid collection, animals were rested for 7 d by allowing them to graze freely in the pasture area planted with Napier and Guinea grasses. After the rest period, the animals were brought to the metabolism stalls and were given the next dietary treatment rotation. Body weights of the animals were recorded before and after each round.

Table 1. Nutrient composition of feedstuffs included in the experimental diets of growing cattle.

Nutrient	Napier grass[1]	Rice bran-copra Concentrate mix	Acacia pod meal
Dry matter, % DM	30.60	88.40	88.70
Crude protein, % DMB	7.97	15.45	15.37
Total digestible nutrient, % DMB	65.00	68.86	67.51[2]
Neutral detergent fiber, % DMB	61.07	43.60	42.86
Cellulose, % DMB	24.00	8.62	9.77
Hemicellulose, %DMB	29.80	27.72	10.53
Lignin, %DMB	5.87	0.60	4.50

[1]Feed composition table for the Philippines, PCAARRD.
[2]Hosamani *et al*. (2005).

DNA was extracted from rumen fluid samples using DNA easy Plant DNA Minikit (Quiagen). Extracted DNA quality and quantity were verified spectrophotometrically (NANOdrop).

Extracted DNA was first amplified by PCR using universal primers to fish-out the whole 16S rDNA. PCR mixture (20 µl) contains 2 µl 10x buffer, 0.6 µl $MgCl_2$, 0.16 µl dNTP, 0.5 µl forward primer, 0.5 µl reverse primer, 0.06 µl Taq polymerase and 3 µl of DNA (template). It was run with initial denaturation step of 94ºC for 2 min followed by 30 cycles: 94ºC for 1 min, 45ºC for 30 sec and 72ºC for 90 sec, and final elongation of 72ºC for 10 min.

The product was then subjected to second PCR amplification using 926r and 341f-GC primers to fish-out the V3 region of 16S rDNA. Second PCR mixture (20 µl) contained the same components that were used in the first amplification, except for primers; 0.3 µl of 926r and 341f-GC of each were used. It was run with initial denaturation step of 94ºC for 1 min followed by 28 cycles: 94ºC for 1 min, 53ºC for 1 min and 72ºC for 1 min, and final elongation of 72ºC for 30 min. PCR products were used in the denaturing gradient gel electrophoresis (DGGE) using the procedures of Muvzer (1998) and Ercolini (2004). Bands were viewed using gel photodoc (Bio-rad). Distinct bands were excised and subjected to PCR amplification. PCR

products were sent to Macrogen, Inc., Seoul, South Korea for nucleotide sequencing. *In silico* analysis involved the comparison of quality sequences to known sequences in the nucleotide databases for 16S rDNA. Sequences with >90% homology to the queried sequences were identified.

Feeding trial in growing heifers

Eighteen growing cattle with BW range of 223±20 kg were divided into three treatment groups with 6 animals per replicate per treatment. The animals were fed with diets based on feeding rate corresponding to 3.5% of the animal's body weight (BW) dry matter (DM) basis. Dietary treatments contained concentrate supplementation provided at 70:30 roughage to concentrate ratio with varying APM inclusion. The treatment diets include: 1) 30% Rice bran-copra meal mix (RBC); 2) 15% RBC-15% Acacia pod meal combination (RCA); and 3) 30% Acacia pod meal (APM). RBC concentrate was formulated to contain crude protein and total digestible nutrient level very close to APM.

Daily feed consumption was monitored by recording the amount of feed offered and feed refused. Thirty and 60 d average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) based on dry matter (DM) were compared among all treatments. All data obtained were subjected to analysis of variance following a completely randomized design. Total tannic acid intake (TAI) and cellulose intake (CI) were also correlated with growth performance and FCR.

Determination of secondary products in APM and other feedstuffs

To determine the water soluble secondary products present in the feeds, paper chromatography of hot water extract was performed following the procedure of Ody (1993). Five hundred mg of dried and ground (1 mm mesh) Napier grass, whole Acacia pods, Acacia pod pulp, Acacia pod seeds and concentrates were placed in 15 ml test tube containing 10 ml of hot water and incubated in hot-water bath (80ºC) for 10 min. The extract was centrifuged at 3000 x *g* for 10 min at room temperature. The clear supernatant was kept at 4ºC until use.

Supernatants were spotted on the chromatographic paper (Whatman) one inch from the bottom with spots not exceeding 2 mm in diameter. Compounds from the sample were resolved using 1:4:5 (v/v/v) acetic acid: *n*-butanol: distilled water as mobile phase. The process was stopped by removing the paper from the chamber and was allowed to dry and develop using 25% ammonia solution and iodine vapors. The spots were located and marked with pencil. Retardation factors were calculated using the formula below:

$$\text{Retardation factor (Rf)} = \frac{\text{distance travelled by the sample spot}}{\text{distance travelled by solvent front}}$$

Five hundred mg of dried and ground (1 mm mesh) Napier grass, whole Acacia pods, pulp seeds and concentrate were individually wrapped in filter paper and placed in separate Soxhlet apparatus with *n*-hexane as solvent. Samples were refluxed for 8 h at 40ºC. After *n*-hexane extraction, the solvent fraction was removed from the mixture by rotary evaporator with water bath set at 60ºC and rotation at 70

rpm. TLC procedure was based on the procedures of Ukoha (2011).

Tannin acid content of APM, Napier and concentrate mix was estimated using tannic acid equivalent (TAE) following the spectrophotometric procedure by Burns (1963).

RESULTS AND DISCUSSION

Changes in bacterial diversity analysis

Predominant bacterial groups as represented by their 16S rDNA V3 (variable region 3) regions in the rumen of cattle in the *in situ* study include 5 distinct bands in DGGE gel shown in Figure 1. Band B (lane 1), D (lane 2) and F (lane 4) were identified as *Selenomonas ruminantium, Acetitomaculum ruminis* and *Olsenella*

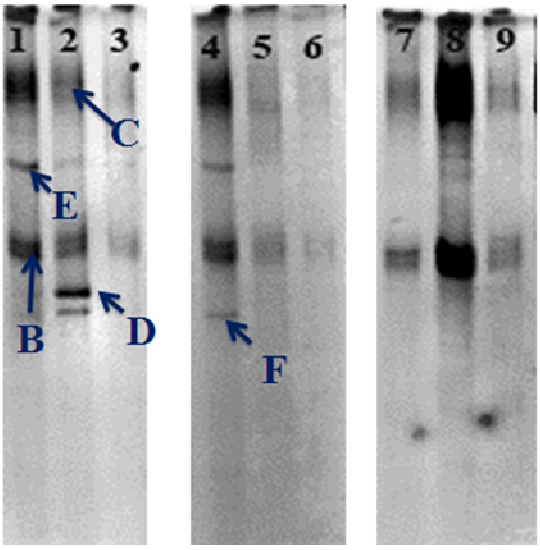

Figure 1. 16S rDNA DGGE profile of bacterial groups from cattle fed with different dietary treatments as resolved by denaturing gradient gel electrophoresis (DGGE): Lanes 1, 6 and 8 are Napier grass fed animals; Lanes 2, 4 and 9 are Napier-concentrate feed fed animals; Lanes 3, 5 and 7 are Napier-Acacia pod meal fed animals.

profuse, respectively. Accession numbers and corresponding percentage similarity to the queried sequences are shown in Table 2. Two other distinct bands (band C and E) were observed and sequenced which have lower homology with the known sequences in NCBI database. In this study, *S. ruminantium* proliferated in animals fed with all Napier (AN) and Napier + rice bran copra meal mix (NR) fed diets and tend to be less adaptable when the NA diet was supplemented with APM. This is indicated by lighter banding patterns in 2 out of 3 animals fed NA diet. *S.*

Table 2. PCR-DGGE bands, with accession number and percentage similarity to known sequences submitted to Nucleotide BLAST-NCBI database.

PCR-DGGE Band ID	Accession No.	Description	Sequence Identity (%)
B	NR. 044734.1	*Selenomonas ruminantium*	96
D	NR. 044713.1	*Acetitomaculum ruminis*	100
F	NR 036821.1	*Olsenella profuse*	100

ruminantium is not only cellulolytic but also a saccharolytic bacterium with starch as principal substrate (Van Soest, 1992). This observation was not expected since Acacia pod contains 10% total sugars (Hosamani *et al*., 2005) as manifested by its sweet tasting characteristics. Also, Kamra (2005) reported that *S. ruminantium* is a tannin-resistant bacterium so that its survival in diets containing tannin-rich ingredient should not be affected.

A. ruminis (band D) was found only in one cattle fed with Napier-concentrate (NC) treatment but was not consistently present in the other two replicate animals. *O. profuse* was only observed in 2 out 3 animals fed with NR diet and absent in AN and NA treatment diet. *O. profuse* is a bacterium commonly found in human mouth and would logically thrive in substrate with high soluble carbohydrates similar to those found in rice bran and copra meal present in the NR treatment.

Effect on growth performance of heifers

Average daily gain (ADG) and FCR were affected as the inclusion of APM was increased in both 30 ($P<0.05$) and 60 d ($P<0.01$) feeding periods. ADG was highest in treatment diet containing rice bran-copra (RBC) mix in both 30 and 60 d periods with ADG values of 1.03 and 0.67 kg, respectively. Feed conversion ratio computations showed consistent and highly significant reduction with RBC, RCA and APM groups, exhibiting 13.41, 21.48 and 30.53 kg of diet (DM basis) per kg BW, respectively. Cellulolytic activities in the rumen by microorganisms were likely affected by compounds present in Acacia since calculated cellulose intake as % of BW was almost similar (data not shown). Differences in voluntary intake were not significant although numerically, increasing APM in the ration resulted in higher feed intake. This validates the earlier stated possibility that secondary compounds maybe present in APM causing the reduction in growth performance as dietary intake increases (Van Soest, 1992).

Determination of secondary product in Acacia pods

Water soluble extract from Napier, concentrate mix and Acacia pods fractions including whole pod, seed and pulp, showed characteristic bands similar to hydrolysable tannins using standard tannins (J. T. Baker). Paper chromatographic profile of each sample is shown in Figure 2. Computed retardation factor (Rf) values (data not shown) indicate that hydrolyzable tannins are predominant in whole pod (Rf = 0.50 – 0.89), pulp (Rf = 0.69-0.93) and Napier (Rf = 0.49-0.93) as described

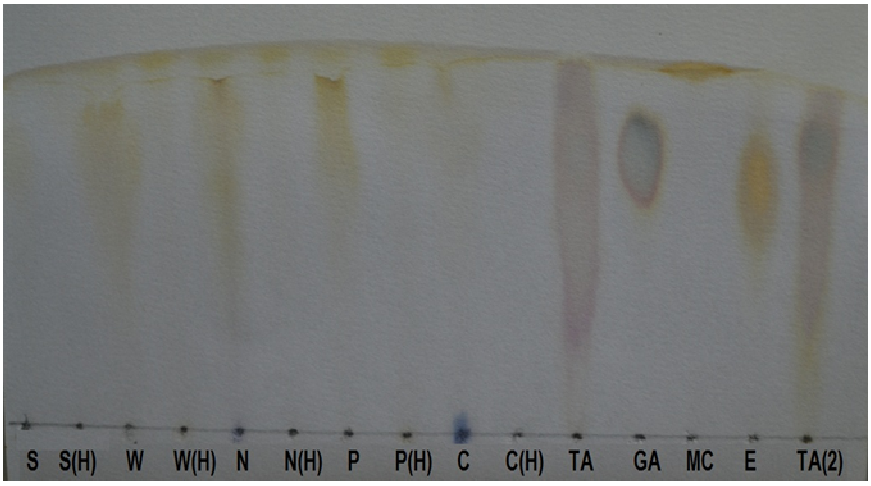

Figure 2. Paper chromatographic profile of hot water (80ºC) soluble extract of Acacia pod fractions, Napier grass, rice bran-copra meal concentrate mix and some analytical standards. S-seed, S(H)-hydrolyzed, W-whole pod, W(H)-hydrolyzed whole pod, P-pulp, P(H)-hydrolyzed pulp, N-Napier, N(H)-hydrolyzed Napier, C-Rice bran-copra meal mix, C(H)-hydrolyzed concentrate, GA-gallic acid, MC-methyl cathecol, PA-Phytic acid, E-epicathechin, TA and TA2-tannic acid (hydrolysable, J.T. Baker).

by long stretch of band similar to the banding pattern of the standard hydrolysable tannins whose Rf values range from 0.23 to 0.97. An attempt to further characterize the specific tannins in the samples was done by hydrolysing the samples with acid-butanol (Porter *et al*., 1986) to yield monomeric units like gallic acid and/or ellagic acid for hydrolyzable tannin and cathechin and/or epicathechin for condensed tannins. PC of hydrolyzed samples did not yield both monomeric products as shown in Figure 2.

Secondary products determination of *n*-hexane extracts from same samples did not show distinct bands in TLC (banding profile not shown). This suggest that condensed tannins which are less soluble in water but more soluble in organic solvent like hexane were below detectable amount using qualitative profiling by TLC.

Tannic acid quantitative determination by spectrophotometry yielded hydrolysable tannic acid content expressed as tannic acid equivalent (TAE). Although the procedure by Burns (1956) intends to analyze total phenolic compounds in the sample, utilizing an analytical grade of blended hydrolyzable tannins suggests that estimation can be expressed as equivalent relative to the standard used. Analysis by TAE resulted in tannin contents of 6.82, 1.22, 16.42, 7.36 and 22.77% for Napier, concentrate mix, whole pod, Acacia seed and Acacia pulp fractions, respectively. In contrast to the tannin level reported by Hosamani *et al*. (2005), TAE of whole Acacia pods in this study is 5.6 x greater than their

reported value of 2.95%. The assay was done several times yielding consistent amounts of tannins in all of the fractions.

Correlation of growth performance to the level of Acacia pod in the diet

Average daily gain and FCR significantly deteriorated at increasing level of APM even at 30 d after feeding. This result is contrary to the report of Hosamani *et al*. (2005) in a 21-d feeding trial in growing heifers which concluded that Acacia pod is comparable with a regular concentrate feed. The report of Morais (2012) showed comparable negative effects on ADG and FCR of Acacia pod meal fed growing goats. Calculation of dietary tannin intake based on the assayed tannin contents of feed components and ADFI showed significant differences in the average daily tannin intake (ADTI) among all treatments (Table 3).

Table 3. Average daily tannin intake (ADTI), average daily gain (ADG) and feed conversion ratio (FCR) at 30-d and 60-d period.

Treat-ment	ADTI, kg (Ave±SD)		ADG, kg (Ave±SD)		Feed:Gain (Ave±SD)	
	30 d**	60 d**	30 d*	60 d**	30 d**	60 d**
RBC	0.39±0.01^{a}	0.45±0.01^{a}	1.03±0.13^{a}	0.67±0.07^{a}	7.52±1.05^{a}	13.41±1.6^{a}
RCA	0.60±0.03^{b}	0.65±0.03^{b}	0.75±0.23^{ab}	0.39±0.11^{ab}	11.21±2.8^{ab}	21.48±2.9^{ab}
APM	0.90±0.04^{c}	0.87±0.03^{c}	0.67±0.20^{b}	0.25±0.12^{b}	12.16±3.3^{b}	30.53±21.8^{b}
CV, %	4.42	3.48	23.69	22.78	19.44	32.07

*- significant (P<0.05).
**- significant (P<0.01).
Means within column with the same letter subscripts are not different.

Tannins may reduce intake of forage legumes by decreasing palatability or by negatively affecting digestion. Low total intake and low growth rates were also observed in animals eating *A. sieberiana* pods. The negative effect of tannins on growth rate was caused by a combination of reduced intake and low true digestibility of protein (Reed, 1995). In this study, there were no marked reduction of the animal's voluntary intake and that astringency attributed to tannins was very much tolerated by growing heifers. Poor digestibility of nutrients, especially protein, carbohydrates and minerals resulted in poor feed conversion. In addition, tannin from APM could have possibly prevented the growth of cellulolytic microorganisms and may have deactivated fibrolytic enzymes that they secrete.

CONCLUSION

Feeding Acacia pod meal resulted in the proliferation of tannin-resistant bacterium *S. ruminantium* and loss of cellulolytic bacteria *A. ruminis.* Increasing dietary inclusion of Acacia pod meal at 30% of the DM requirement of growing heifers resulted in the significant reduction of average daily gain and feed conversion ratio but not average daily feed intake. Hot water extract predominantly contain hydrolysable tannins as determined by PC. TLC of *n*-hexane extract did not yield detectable amounts of secondary compounds. Tannic acid analysis showed that whole Acacia pod contains 16.42% tannins. The study showed that reduction in growth performance of heifers can be directly related to high dietary tannins that could possibly have direct bioactivity against economically important microorganism in the rumen.

It is recommended that feeding Acacia pod meal should be limited to 15% of the DM requirement of the animal to prevent significant reduction in growth performance. Further study should be done focusing on possible processes that will help eliminate the tannin-rich fraction (pulp) in APM. Also, feeding trial with APM inclusion levels below 15% of the animal's BW will help identify the optimal utilization of APM.

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