Rumen Defaunation: Determining the Level and Frequency of *Leucaena leucocephala* Linn. Forage

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***Abstract*—A rumen defaunation trial was conducted using *Leucaena leucocephala* forage as defaunating agent. Defaunation was performed by supplementing fresh *L. leucocephala* forage at two levels and frequencies as follows: T1 = 0.75% of BW, DM basis + 1 follow-up administration in a month), T2 = 0.75% BW + 2 follow-up administration in a month, T3 = 1.25% BW + 1 follow-up administration in a month, T4 = 1.25% BW + 2 follow-up administration in a month and T5 = control treatment, without defaunating agents added. The experiment was set up in a Randomized Complete Block Design (RCBD) having age and sex of the animals as basis for blocking. Rumen fluid was collected in each animal through stomach tubing before and after the experiment for bacterial and protozoal counting. Results revealed that *L. leucocephala* was significantly (P<0.001) effective in reducing protozoal numbers and increasing the bacterial population in all levels and frequencies as compared to the control. However, T2 and T4 appeared to be the best treatments in reducing protozoal numbers with a corresponding greater increase in bacterial counts. Therefore, *L. leucocephala* can effectively manipulate the rumen environment by reducing the protozoa without negative effects on bacteria. At any level of feeding, its use as defaunating agent must have two (2) follow-ups administration in a month.**

***Index Terms*—bacterial population, defaunating agent, *L. leucocephala*, levels and frequency, protozoal population, rumen defaunation**

I. Introduction

Removing the protozoa (defaunation) in the rumen improves productivity of animals fed with low quality diets [1] by increasing the amount of microbial proteins that flow into the abomasum and small intestines. Though protozoa contribute fiber digestion thus increasing the availability of energetic substrates for the animal [2], it has a negative impact since it preys on bacteria to supply its needs for amino acids [3] especially if the ruminant animal is fed low-nitrogen diet.

Ruminant animal depends on the ability of rumen fermentation to yield nutrients such as the Short-Chain Fatty Acids (SCFA) and microbial biomass to meet animal requirements [4]. Microorganisms, particularly bacteria, therefore, play a great role in ruminant feeding, and one way to improve the protein to energy ratio of the absorbed nutrients is through manipulation of rumen microbial population. However, no satisfactory techniques are currently available for defaunating animals under field condition. Several existing defaunation techniques or methods include rumen emptying and washing [5], [6] use of chemicals that are toxic to protozoa, isolating young animals within few hours of birth before protozoa become established [6], and changing the diet into pure milk or even prolonged starvation, and copper sulphate administration [1].

Such methods appeared to be impractical, that [3] recommended the use of leaf materials voluntarily eaten by the animals as an alternative approach to lessen the negative effects of defaunation. This can be done by supplementation of forages rich in saponin and tannins [7] which are chemicals produced by plants that limit growth of rumen microorganisms [8]. Some tree fodder contain tannins and saponins which both metabolites show defaunating properties [9]. However, these forages have also detrimental effects on rumen fermentation and rumen microorganisms, such that it decreases the number of rumen bacteria [10].

Leucaena leucocephala is well-known forage in tropical countries for ruminants. This plant species can play a dual role for ruminants given low-quality diets by being a source of supplemental nitrogen and a potential defaunating agent [11]. Extracts of *Leucaena leucocephala* together with *Centrosema pubescens*, *Gliricidia sepium* and *Desmodium heterophylum* were effective defaunating agents however, giving them as a component in normal diets warrants further testing. Hence, this study was conducted to determine the defaunating capacity of *Leucaena leucocephala* given at varying levels and frequencies of feeding.

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II. Materials and Methods

Extracts of *L. leucocephala* forage was found to be the best among the forages tested in defaunating rumen protozoa [12]. However, in the light of its practical use as defaunating agent, the forage was given directly as feed supplement to the animals.

A. Preparation and Feeding of Test Animals

The experimental animals were placed in individual metabolism cages and dewormed before the start of the study. Disinfection of the cages was done prior to the conduct of the study.

The basal diet of Napier grass (*Pennisetum purpureum*) was given at 3% of the body weight, DM basis, in fresh form.

The goats in each group were fed twice a day: one half in the morning, the other half in the afternoon feeding. The animals were given clean drinking ad libitum water free choice over the experimental period.

B. Treatment Design

This experiment used *L. leucocephala* which was found to have a greatest defaunating property [12]; it was administered at two levels and frequencies of defaunation Instead of giving 150-300ml plant extract [13], [14], the following levels and frequencies of administration were adopted (Table I).

TABLE I. Level and Frquencies of Administration of *L. Leucocephala* Forages as Defaunating Agent

Treatments	Factor A (Level - L)	Factor B (Frequency - F)
T1 - L1F1	0.75% BW	F1 - three consecutive days + 1 follow-up administration in a month
T2 - L1F2	0.75% BW	F2 - three consecutive days + 2 follow-up administration in a month
T3 - L2F1	1.25% BW	F1 - three consecutive days + 1 follow-up administration in a month
T4 - L2F2	1.25% BW	F2 - three consecutive days + 2 follow up administration in a month
T5 - Control	No *L. leucocephala* forage	

The levels of feeding were based on the experiments of [15] to achieve a "supplementary effect" where voluntary intake of basal diet increased by the level of supplement feeding rather than a "substitution effect" where addition of a certain level of supplement causes reduction in the voluntary intake of the basal diet.

C. Experimental Design

Each combination of level and frequency of feeding represents one (1) treatment. In the control group (T5), the experimental animals were not receiving any defaunating agent. These 5 treatments were replicated 4 times and a total of 20 goats were used. This study was conducted using a Randomized Complete Block Design (RCBD) with sex - age combination as basis for blocking.

D. Rumen Defaunation of Animals

1) Day 1-6 (Adjustment period)

In two periods, a basal diet of Napier grass was given to all experimental animals constituting about 70% of the total daily allowance. This allowed the animals to get conditioned to the experimental area as well as to the diet. During this period, the ruminal fluid of the animals was examined every three days to monitor changes in the numbers of protozoa [5].

2) Day 7-10 (Defaunation procedure)

The following day after the adjustment period, the feed offered to the animals was reduced into half of their requirement. On the second day, the defaunation procedure started with consideration on the frequency and level of administration of the defaunating forage as presented in Table II. The defaunating forage was given as fresh supplement one (1) hour before the morning feeding of the basal diet while the animals are hungry.

TABLE II. Procedure and Schedule of Administrating the Defaunating Agent (*L. Leucocephala* Forage)

Days of administration	Activities	Frequency of administration	
		F1	F2
Day 1	*The feed given was reduced into half of their requirement at the same time, rumen fluid was collected and served as initial reading	/	/
Day 2-4 (defaunation period)	*The feeds was reduced into half of their ration ***First dose:** the defaunating agent was given fresh in three (3) consecutive days.	/	/
Day 5	*Full fed onward, rumen fluid collection and analysis	/	/
Day 15	*Rumen fluid collection and analysis **Second dose:** administration of the defaunating agent *The animals were given *ad libitum* feed until the end of the experiment	/	/
Day 25	***Third dose:** administration of the defaunating agent	x	/
Day 30	*Rumen fluid was collected and was analyze in the laboratory (final reading)	/	/

E. Rumen Fluid Collection and Measurement

A rumen fluid volume of about 8-10ml was collected through stomach tubing procedure [16] two hours before the morning feeding on the first day of the defaunating agent was administered. The second collection was done on the 5th day; the last administration of the defaunating agent. The third collection was done on the 15th day.

Another collection was done before the morning meal on the last day of the experiment. Rumen contents were collected via a stomach tube and were strained through 4 layers of cheesecloth to yield rumen fluid [17].

The fluid was analyzed for its pH, protozoal and bacterial count. Measurement of the rumen pH was done immediately after collection of the fluid using a digital pH meter. Ref. [18] reported that the optimum pH for ruminal fermentation is 6.0-6.4.

F. Protozoal Counting Procedure

The collected rumen fluid was immediately placed in a test tube and was serially diluted into 1:10 dillutions similar to the procedure used by [19]. Loguls solution

was added into the fluid before protozoal counting to fix the protozoa for this purpose [20].

The protozoa were counted using a microscope and a hand tally counter. Protozoal counts were expressed as cell counts (cc/ml) = Number of protozoal cells x 1000X objective x dilution rate [19].

During protozoal counting, three (3) measurements were scanned from each treatment replicate, and the mean of these three (3) determinations were calculated.

G. Bacterial Counting Procedure

Collected rumen digesta was also used in bacterial counting. This was brought immediately to the Microbiology Laboratory for isolation and counting. The rumen fluid was serially diluted into 1:10 to 1:10,000,000 dilutions and a representative 1ml drop were poured into a petri plate with a prepared medium for growth.

After incubating for 24-48 hours the bacterial colonies were counted using a colony counter. The counted colonies were then expressed as colony forming units/ml fluids as follows:

Colony forming unit (cfu/ml) = Number of bacterial colonies x dilution rate.

H. Laboratory Analyses

Basal diet and forage defaunating agent were analyzed for its DM content using a convection oven set at 100 ° Celcius for about 24 hours [21]. The DM content was used in the calculation of the feed requirement of the experimental animals.

I. Data Gathered

1) Ruminal pH
2) Protozoal count (cell count/ml) and morphological identification
3) Bacterial count (cfu/ml) and morphological identification
4) DMI (Dry Matter Intake)
5) Animal body weight gain

J. Analysis of Data

Data were analyzed using two – way analysis of variance (ANOVA). Comparison of treatment means were done by Honestly Significant Difference (HSD) test using the Statistical Package for Social Sciences (SPSS) ver. 17.0 software.

III. Results and Discussion

A. Changes in Protozoal Population

Percent reduction in protozoal and bacterial numbers showed significant ($p<0.05$) differences among treatment means (Table III). All animals that received *L. leucocephala* supplement as defaunating agent showed higher percent reduction ($P<0.01$) in protozoal count compared to the control treatment. *L. leucocephala* given at 1.25% of the BW appeared to have a greater effect on reducing protozoal population compared to 0.75% level as measured on day 5 and 15.

On the 30th day, significant reduction on protozoa was observed at 99% confidence level of significance. Measurement showed that L2F2 got the highest percent reduction followed by L1F2, indicating the effectiveness of frequency 2 (three consecutive days + 2 follow up administration in a month) as compared to frequency 1 (three consecutive days + 1 follow up administration in a month). Defaunating the animal twice a month showed higher reduction in protozoal numbers and was able to maintain reduced protozoal population.

TABLE III. Actual Count and Percent Change in Protozoal Population as Affected by Level and Frequency of Feeding *L. leucocephala* Forage

Treatments	Protozoal Count (1×10^4)				Day 5	Day 15	Day 30
	Initial count	Day 5	Day 15	Day 30	% reduction in Protozo[a]	% reduction in Protozo[a]	% reduction in Protozo[a]
T1 (L1F1)	9.08	5.4	5.3	6.0	40.39[b]	41.82[ab]	34.04[b]
T2 (L1F2)	6.00	3.2	3.8	2.9	47.74[b]	35.73[ab]	51.49[ab]
T3 (L2F1)	21.17	2.6	10.1	12.3	87.80[a]	51.60[a]	40.70[b]
T4 (L2F2)	10.33	3.6	4.2	3.8	65.22[ab]	58.54[a]	62.28[a]
T5 (Control)	18.92	19.4	15.8	20.3	-2.36[c]	12.14[b]	-6.29[c]
P –value					*0.000***	*0.016***	*0.000***

Means of the same superscript within a column are not significantly different each other
** - Highly significant, * - Significant, ns - Not significant

This result could be attributed to the secondary metabolites present in forages affecting rumen microorganisms. The defaunating effects of tree fodders have been demonstrated in vitro and in vivo, and it has been attributed to both Condensed Tannin (CT) and saponins [4]. This coincides with the result where the effectiveness of *Leucaena leucocepala* in eliminating protozoa can be attributed to the saponin content of the forage which appeared to be positive in the froth test analysis. Saponin is high in molecular weight glycosides, consisting of a sugar unit(s) linked to a triterpene or a steroid aglycone and widely distributed in higher plants. The sensitivity of ciliate protozoa towards saponins may be attributed to the sterols present in protozoa; it is different in bacterial membranes. Hence, the sterol-binding ability of saponins most likely results to the damage of protozoal cell membranes [22].

Some literature that suspected tannin was responsible in defaunating the protozoal population. Condensed tannins reduce the total protozoal population in the rumen.

Tannins modified the Entodiniomorphos, like a Holotrichia. This demonstrates once again that the plant secondary compounds act as defaunating agents [23].

Ref. [24] lend support to the presented result in Table III where supplementation of *Leucaena leucocephala* as defaunating agent is effective in reducing protozoal count to about 4×10^5. The same is true with the data reported by [4] where *L. leucocephala* trees which were included in the grazing system together with the mixture of natural pastures (inclusion level 30 or 100% of the area) reduced the ruminal protozoa in cows. *Leucaena leucocephala* has tannin compounds and possesses anti-protozoal effect in such a way that the protozoal cell membranes are fluid and semi-permeable. The capability of protozoal membrane to bind to sterol or its capability to alter cell membrane permeability is being reduced by tannin hence, disintegrating the protozoal cell membranes. Therefore, the function of tannin is similar to saponins which are lipid compounds that alter cell membrane structures of protozoa which have a potency to destroy protozoal growth and change the pattern of fermentation in the rumen system [24].

Therefore, utilizing tree leaves as defaunating agent is more beneficial than using the existing chemical agent (ex. Sodium lauryl sulfate, copper sulfate, Nonyl phenol ethoxylate and Dioctyl sodium sulfosuccinate). This is supported by [25] who said that the use of tree leaves or plant extracts reduces the protozoal numbers in the rumen and improves fiber digestion. Besides it supplying additional nutrients such as protein from legumes, it minimizes the stress in regular drenching that may limit the feed intake [3].

B. Changes in Bacterial Population

Table IV shows the actual count and percent change in bacterial population. It was observed that bacterial population was significantly ($p<0.01$) increased in response to the different levels and frequencies of administration of the defaunating agent. Bacterial counts in day 15 appeared that T1, T2, T3 and T4 were not significantly ($p>0.01$) indicating the similar effect of the defaunating agents on bacterial population at this period. Notably, these four treatments were significantly higher to the control treatment. In day 30, the pattern of effects was already different to that of day 15 where, T2, T3 and T4 got the highest percent increase in bacteria compared to other treatments. Frequency 2 is therefore effective in increasing the bacterial population whether using Level 1 (0.75%) and Level 2 (1.25%). If Level 2 is to be used, frequency 1 (F1) could also be followed since they were statistically comparable to each other.

The positive effect on bacterial count *of L. leucocephala* supplementation as defaunating agent is supported by [24] who also used *L. leucocephala* and found out an increase in bacterial population to about 5×10^{10}. In addition, the increase in bacterial population by *L. leucocephala* supplementation is due to the decrease in protozoal numbers since protozoa is responsible in engulfing the bacteria [26]. Besides, *L. leucocephala* is a protein source that encourages increase in bacterial numbers due to the increase in VFAs produced during the fermentation of leaves in the rumen.

It appears definitely that there will be more microbial and dietary protein available to the ruminant when protozoa are absent from the rumen. It follows therefore that in the absence of protozoa in the rumen, there is an increase in the total protein available relative to the VFA produced and absorbed from the rumen [3].

TABLE IV. ACTUAL COUNT AND PERCENT CHANGE IN BACTERIAL POPULATION AS AFFECTED BY LEVEL AND FREQUENCY OF FEEDING *L. LEUCOCEPHALA* FORAGE

Treatments	Bacterial Count (1×10^7)			Day 15	Day 30
	Initial count	Day 15	Day 30	% increase in bacteria	% increase in bacteria
T1 (L1F1)	3.83	9.08	6.38	60.37^{a}	41.8^{b}
T2 (L1F2)	3.92	10.00	17.75	60.47^{a}	78.5^{a}
T3 (L2F1)	10.67	21.75	24.13	50.69^{a}	55.7^{ab}
T4 (L2F2)	8.92	16.92	22.63	46.96^{a}	60.9^{ab}
T5 (Control)	9.42	10.33	10.88	8.29^{b}	3.7^{c}
P –value				*0.000***	*0.000***

C. Changes in Rumen pH

Rumen is a continuous anaerobic culture system in which the pH and temperature are maintained between 5 to 7 and 39 to 40 ℃, respectively [1]. Table V shows the rumen pH of goats collected in the morning before feeding. Only at day 5 of both pH reading and percent reduction showed significant difference among treatments at 95% level of significance while the rest were not significantly different ($P>0.05$) among treatment means. It should different from each other be noted, however, that the pH values in day 5, 15, and 30 were reduced compared to the initial reading although still is high since it was collected before the morning feeding.

TABLE V. RUMEN PH OF GOAT AS INFLUENCED BY THE LEVELS AND FREQUENCIES OF ADMINISTRATION OF *L. LEUCOCEPHALA* FORAGE

Treatments	Initial pH	Actual Rumen pH		
	Day 1	Day 5	Day 15	Day 30
T1 (L1F1)	7.45	7.30^{a}	6.98	6.95
T2 (L1F2)	7.43	7.23^{ab}	7.00	7.08
T3 (L2F1)	7.28	7.15^{ab}	6.83	6.90
T4 (L2F2)	7.25	7.20^{ab}	6.95	7.03
T5 (Control)	7.53	6.90^{b}	7.00	6.88
P –value	0.357^{ns}	0.05*	0.747^{ns}	0.016*

Means of the same letter-superscripts within a column are not significantly different

** - Highly significant, * - Significant, ns - Not significant

This is supported by [1] and [16] that the ruminal pH is high before the morning feeding because of intensive rumination and limited feed intake at night. Protozoa are generally more sensitive to dietary changes than the bacterial population and there appears to be greater host animal to animal variation in the protozoal population than with bacterial populations [1].

D. Goat Performance in Response to Defaunation

The weight gain of goats after being defaunated with *L. leucocephala* forage appeared to be significantly affected by the level and frequency of the defaunating agents (Table VI). Weight gain was higher on T4 however, it is not significantly different from T1, T2, T3 and T4 but it

is different from the control treatment in terms of dry matter intake.

TABLE VI. ANIMAL WEIGHT GAIN AND DRY MATTER INTAKE OF GOAT'S DEFAUNATED WITH VARYING LEVELS AND FREQUENCY OF *L. LEUCOCEPHALA* FORAGE

Treatments	Weight gain (kg)	DMI as % BW
T1 (L1F1)	4.60^{ab}	2.87^{b}
T2 (L1F2)	4.69^{ab}	3.63^{ab}
T3 (L2F1)	3.85^{ab}	2.99^{b}
T4 (L2F2)	7.28^{a}	4.19^{a}
T5 (Control)	0.78^{b}	2.87^{b}
p - value	*0.044**	0.016*

Means of the same superscript within a column are not significantly different each other *- Significant

Ref. [11] reported that the supplementation of L. foliage at 27% in a 63% basal diet increased ($P<0.01$) the DM intake (%BW) and OM (kg) in 19%, compared with the control treatment. However [13] added that it did not affect the DM and NDF apparent digestibility.

Ref. [3] also reported the effectiveness of *Leucaena leucocephala* in defaunating protozoa of either *in vitro* or *in vivo*. *Lotus pendunculatus, Acacia dealbata, Centrosema pubescens Desmodium intortum*, Fern leaf, *Vigna parteri*, and *Desmodium uncinatum* showed anti-protozoal activity.

Several literatures reported the symbiotic relationship of rumen protozoa and methanogens. It is proven that defaunation results in a decrease in CH_4 gas production [27].

Ref. [11] presented a study about the effectiveness of *Leucaena leucocephala* in reducing methane gas production, that inclusion of 27% of *L. leucocephala* in a basal diet of *P. purpureum* reduced methane gas production by 15.6% in L/kg consumed DM without affecting the apparent digestibility of nutrients in sheep. Therefore, this study will give a preliminary idea that the reduction in protozoal population will reduce methanogenic bacteria, hence, reducing the production of methane gas.

IV. CONCLUSION AND RECOMMENDATION

The use of *L. leucocephala* forage can effectively defaunate the rumen, significantly decreasing protozoal population. The frequency of administration of L. leucocephala is an important consideration when using it as a defaunating agent, such that indicating the effectiveness of frequency 2 (T2 and T4) in maintaining lower protozoal count. As regards to increasing bacterial population, providing a high level of forage with one follow-up treatment (T3) and the lower level of forage with two follow-up treatments (T2 and T4) are effective.

It is, therefore, recommended to use *L. leucocephala* supplement as defaunating agent with two follow-up treatments using at least 0.75% BW level on DM basis.

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