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In Vitro Screening of the Foliage of Eucalyptus Species Harvested in Different Seasons for Modulating Rumen Fermentation and Methane Production

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Abstract: The aim of this study was to assess the effects of five eucalyptus species (*Eucalyptus camaldulensis*, *E. leucoxylon*, *E. astringens*, *E. sideroxylon*, and *E. lehmannii*), harvested in spring, autumn, or winter from two Tunisian arboretums, on in vitro rumen fermentation and methanogenesis. Batch cultures were performed to determine rumen fermentation kinetics and end-product formation after 24 h of incubation. The foliage of the species *E. sideroxylon* and *E. lehmannii* showed the greatest digestibility coefficients, whereas *E. leucoxylon* was the least digestible. Acetate-to-propionate ratio was reduced when *E. sideroxylon* and *E. lehmannii* were incubated, and these species were also the most efficient at reducing methane emission. Foliage harvested in winter showed greater digestibility and an increase in the acetate-to-propionate ratio than in other seasons, without an increase in methane emission. Foliage from *E. sideroxylon* and *E. lehmannii* showed a potential to decrease enteric methane production without depressing effects on ruminal fermentation. In vivo studies would be necessary to conclusively validate these effects as the first step towards proposing the inclusion of eucalyptus leaves in ruminant diets.

Keywords: browse tree; rumen; in vitro fermentation; methane; ruminant feedstuff

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1. Introduction

Greenhouse gas (GHG) emissions have been being linked to the livestock sector. It is estimated that cattle may be responsible for up to one-third of the anthropogenic CH_4, and enteric fermentation may represent up to 39% of the global GHG emissions from the livestock sector [1]. According to Deuri et al. [2], methane has a warming potential of 21 CO_2-eq over a 100-year time horizon. Furthermore, enteric CH_4 reduces energy efficiency representing a loss of 2 to 12% of the gross energy intake in ruminants [3]. Therefore, reducing enteric methane yields by ruminants may contribute to improving nutrient utilization and to mitigating the risks of global warming.

Agrosilvopastoral systems that integrate livestock, crops, grasslands, and forestry have been considered as an alternative for improving the sustainability of animal farming [4,5] These systems combine the beneficial effects of trees on animal comfort, forage quality, and the preservation of natural resources [6], in addition to providing foliage as a feedstuff from which animals can obtain nutrients and secondary compounds with functional activities, such as phenolics and tannins.

Eucalyptus is a genus of tall evergreen trees with many species native to many parts of the world [7–9] Eucalyptus is a noble wood and has a rapid growth, which has increased its cultivation in several integrated livestock and forestry areas. There is not much information about the potential feeding value of eucalyptus foliage when included in ruminant diets, and the few available reports seem to indicate that, nutritionally, it would be a poor roughage resource [7] However, eucalyptus leaves, twigs, and fruits contain phytochemicals with biological properties, such as anti-inflammatory, antiseptic, bacteriostatic, fungistatic, archaeacidal, and antiprotozoal effects [10–12] The antimethanogenic activity of eucalyptus has received considerable attention as a promising nutritional alternative for reducing GHG emissions from ruminants.

While numerous chemical additives and antibiotics have been proven to mitigate methane production, consumer demands for products from sustainable and environmentally friendly systems raise concerns about their use. The use of antibiotics as feed additives is not authorized in the EU, and the ban could be extended to many other countries to prevent the emergence and spread of antimicrobial resistance. Plant material, extracts, or essential oils rich in phytochemicals are perceived as safer and more environmentally friendly products [1,13] and therefore have high levels of acceptance, raising fewer animal- and food-safety concerns [11].

Some studies have shown a potential for eucalyptus leaves or compounds to modify ruminal fermentation. However, there is scarce information on the interspecies differences or on the seasonal variation of such an activity. Therefore, our study aimed to assess the differences among five eucalyptus species harvested in spring, autumn, and winter from silvopastoral lands in Tunisia on in vitro rumen fermentation kinetics, end-products and methanogenesis.

2. Materials and Methods

2.1. Foliage Collection

Eucalyptus trees (average age: 52 years) were sampled in two areas located in the region of Nabeul (North East Tunisia) with a subhumid bioclimate: Korbous (36°50′ N 10°23′ E, 180 m altitude) and Djebel Sidi Abderrahman (36°40′ N 10°40′ E, 255 m altitude). In each area, there was an arboretum with a diversity of eucalyptus trees, and specimens were identified at the species level at the Institut National de Recherche en Génie Rural, Eaux et Forêts (INRGREF). Then, foliage was collected from the trees of five eucalyptus species, namely *Eucalyptus camaldulensis*, *E. leucoxylon*, *E. astringens*, *E. sideroxylon*, and *E. lehmannii*. In each arboretum, five experimental plots were established (one for each species), and the position of each sampled tree in the canopy was recorded, so that the same trees could be sampled in winter, spring, and autumn. Therefore, the study was designed according to a layout with five eucalyptus species harvested in three seasons. The sampling procedure has been described in detail by Horst et al. [7] Briefly, ten trees were selected within each plot (based on health status and size), and a branch (ca. 3–4 m high and 1 m long) was cut from each tree, handpicking ca. 100 g of fresh matter sample of mature foliage. Samples were immediately taken to the laboratory and then air-dried under shade for 15 days. Dried leaves were ground and stored in sealed plastic bags.

2.2. Chemical Composition

Standard methods were used to determine chemical composition. The methods for chemical composition have been described comprehensively, and the observed results have been reported by Horst et al. [7] Average values for each species and season are summarized in Table 1 only as supporting data, and a more exhaustive description of these data can be found in Horst et al. [7]

Table 1. Chemical composition of foliage from eucalyptus species harvested in different seasons Horst et al. [7]

	Ash	EE	CP	NDF	ADF	Lignin	NSC
				Dry Matter Basis (%)			
***Eucalyptus* species**							
E. camaldulensis	7.35	5.81	8.62	35.6	25.3	11.7	42.6
E. leucoxylon	5.35	5.12	7.51	37.5	24.6	10.9	44.5
E. astringens	5.69	6.53	7.02	31.3	22.3	12.9	49.5
E. sideroxylon	5.22	4.57	6.65	29.5	20.8	10.3	54.0
E. lehmannii	5.05	9.06	5.40	25.2	18.3	9.2	55.3
Season							
Autumn	5.90	6.43	6.63	29.7	21.0	10.1	51.4
Winter	5.81	6.36	7.42	34.9	24.2	12.9	45.5
Spring	5.48	5.86	7.07	31.0	21.5	9.9	50.6

EE: ether extract (crude fat); CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; NSC: nonstructural carbohydrates.

2.3. Fermentation Kinetics

Three adult sheep were used for ruminal fluid collection. The sheep were cared for and handled following the protocols of European Directive 2010/63/EU on the protection of animals used for scientific purposes, and experimental procedures were approved by the University of León (Spain) Institutional Animal Care and Use Committee.

The in vitro gas-production technique was performed as described by Theodorou et al. [14] Samples (500 mg) were incubated in 120 mL serum vials (two vials per sample) containing 40 mL of culture medium (buffer and minerals) and 10 mL of rumen fluid. The procedures for preparing the incubation medium have been described in detail by Horst et al. [7] Vials containing only diluted rumen fluid (no sample incubated) were used as blanks to correct for background gas production. Once filled, the vials were sealed so as to be airtight, shaken, and then incubated at 39 °C. The volume of fermentation gas released to the headspace in each vial was determined at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48, 60, 72, 96, and 144 h after inoculation. Pressure rising in the headspace was gauged by using a pressure transducer (DeltaOhm, Caselle di Selvazzano, Italy). Then, the volume of cumulative fermentation gas produced at each incubation time was calculated from the pressure values as proposed by López et al. [15] To evaluate the fermentation kinetics, the exponential model derived by France et al. [16] was fitted to the gas-production profiles:

$G = A\left[1 - e^{-c(t-L)}\right]$, where G is the cumulative gas production (mL g^{-1} DM incubated) at time t (h), A is the asymptotic gas production (mL g^{-1} DM incubated), c is the fractional fermentation rate (per h), and L is the lag time (h).

After 144 h of incubation, vial contents were filtered using glass crucibles with sintered filter plates under vacuum. The incubation residue after 144 h was oven-dried at 55 °C for 48 h to determine apparent DM disappearance (D144). Then, the recovered residue was weighed into filter bags (ANKOM Technology, Macedon NY, USA), and neutral detergent fiber was determined in an ANKOM fiber analyzer (ANKOM Technology). The residual NDF after 144 h of incubation was used to calculate the potential DM degradability of each substrate (PD). Other fermentation kinetics parameters, such as average fermentation rate (AFR), partitioning factor (PF), and extent of degradation in the rumen (ED), were calculated as described by García-Rodríguez et al. [17]

2.4. In Vitro Incubations for 24 h in Batch Cultures, and Fermentation End-Product Analytical Determinations

In another trial, samples of eucalyptus foliage were incubated in serum bottles following the same steps as described above. In this case, the vials remained in the incubator for 24 h (with no sequential recordings of gas production), and then the pressure in the vial was measured with the transducer and the volume of gas accumulated in the headspace

after 24 h was quantitatively collected in a 100 mL syringe. The gas collected was sampled for further methane (CH_4) analysis by transferring a 10 mL sample to a Vacutainer® tube (BD Diagnostics, Franklin Lakes, NJ, USA). Fermentation was stopped by placing the bottle in a cooling bath. Then, then the bottles were opened, and a sample of the liquid contents (5 mL) was collected from each bottle. This sample was acidified, centrifuged, and frozen at −20 °C for volatile fatty acid (VFA) analysis. The residue recovered after 24 h of incubation was dried, weighed, and then washed out with neutral detergent to determine the in vitro DM digestibility and the amount of DM digested after 24 h of incubation. The concentration of CH_4 in the fermentation gas, and the concentrations of VFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric) were determined by gas chromatography following the procedures described by García-González et al. [18] and Horst et al. [19]

2.5. Statistical Analysis

Data were subjected to analysis of variance using a split-plot model where arboretum (sampling location) was the blocking factor, and the two fixed effects were eucalyptus species as the whole-plot factor and season as the subplot factor (within each whole plot). The random effects were species × block (used to test species effects) and the residual error (to test seasonal effects and the interaction). The Tukey test was used for the multiple comparison of means. The PROC GLM procedure of SAS (v. 9.2; SAS Institute Inc., Cary, NC, USA) was used for the analysis of variance, whereas pairwise correlation coefficients were derived using the PROC CORR procedure.

3. Results

Interactions between eucalyptus species (ES) × season (S) were not significant ($p > 0.05$) for any of the variables considered. Therefore, only the main effects of species and season are reported. The asymptotic gas production (*A*) did not differ among seasons and was greatest for the foliage of the species *E. camaldulensis*, but there was no difference with the species *E. leucoxylon* and *E. sideroxylon* (Table 2). The fractional rate of fermentation (*c*) was not different among species or seasons. The average fermentation rate (in mL h^{-1}) was greatest for foliage harvested in autumn, while foliage harvested in winter and spring were no different from each other. The gas partitioning factor (PF) was greatest for the foliage of *E. astringens*, *E. sideroxylon,* and *E. lehmannii*, with no differences ($p > 0.05$) among seasons. The foliage of the species *E. sideroxylon* and *E. lehmannii* also showed the greatest coefficients for D144, PD, and ED, but did not differ from the species *E. camaldulensis* for ED. Likewise, the greatest coefficients for D144 and PD were observed in foliage harvested in winter.

As with longer incubations, DM digestibility (after incubation in ruminal fluid for 24 h) was greatest for *E. lehmannii,* followed by *E. sideroxylon* (Table 3). However, no significant effects of species or seasons on VFA production were observed. There were no differences among seasons in methane production. Methane production in any of the units (expressed as a percentage of the total gas of fermentation produced, or in mmol per g DM incubated, per g of DM digested, or per mol VFA produced) was greatest when the foliage of the species *E. leucoxylon* and *E. camaldulensis* were fermented in vitro, and it significantly decreased when the species incubated were either *E. lehmannii* or *E. sideroxylon*, with no significant differences between these two species and *E. astringens*.

Table 2. In vitro gas-production kinetics of foliage from eucalyptus species harvested in different seasons.

	A	*c*	AFR	PF	D144	PD	ED
	(mL g^{-1} DM)	(h^{-1})	(mL h^{-1})		% DM	% DM	% DM
***Eucalyptus* species (ES)**							
E. camaldulensis	125.20 ^{a}	0.0414	3.69	5.48 ^{b}	52.67 ^{b}	67.93 ^{b}	37.29 ^{ab}
E. leucoxylon	117.40 ^{ab}	0.0374	3.14	5.27 ^{b}	46.51 ^{b}	60.76 ^{c}	32.05 ^{b}
E. astringens	84.90 ^{c}	0.0768	4.34	8.13 ^{a}	51.72 ^{b}	68.29 ^{b}	41.59 ^{a}
E. sideroxylon	100.92 ^{abc}	0.0458	3.19	7.68 ^{a}	64.22 ^{a}	73.63 ^{a}	42.11 ^{a}
E. lehmannii	91.75 ^{bc}	0.0450	2.87	8.42 ^{a}	60.63 ^{a}	74.61 ^{a}	42.20 ^{a}
S.E.M.	1.773	0.001	0.484	0.484	2.961	0.648	0.830
p-value	0.002	0.279	0.082	<0.001	<0.001	<0.001	<0.001
Season (S)							
Autumn	103.43	0.0637	4.16 ^{A}	7.01	52.82 ^{B}	67.68 ^{B}	39.93
Winter	106.09	0.0425	3.11 ^{B}	7.17	59.06 ^{A}	71.56 ^{A}	39.60
Spring	102.57	0.0417	3.06 ^{B}	6.81	53.57 ^{B}	67.89 ^{B}	37.61
S.E.M.	2.289	0.001	0.625	0.625	4.936	1.476	1.071
p-value	0.087	0.260	0.003	0.793	0.005	0.015	0.297
ES × S (*p*-value)	0.587	0.488	0.467	0.221	0.590	0.345	0.332

$^{a–c}$ Within a column, mean values not sharing a common superscript represent significant ($p < 0.05$) differences among species. ^{A,B} Within a column, mean values not sharing a common superscript represent significant ($p < 0.05$) differences among seasons. DM = dry matter; *A*: asymptotic gas production; c: fractional rate of fermentation; PF: partitioning factor (mg DM digested mL^{-1} gas); D144: apparent in vitro DM disappearance after 144 h of incubation; PD: potential DM degradability after 144 h of incubation; ED: extent of DM degradation in the rumen (effective degradability); S.E.M.: standard error of the mean.

Table 3. Methane and total volatile fatty acid (VFA) production when the foliage of eucalyptus species harvested in different seasons was incubated in vitro in buffered rumen fluid for 24 h.

				Methane		Total VFA
	DM Digestibility %	µmol g^{-1} DM Incubated	µmol g^{-1} DM Digested	% GP	mmol mol^{-1} VFA	mmol g^{-1} DM Incubated
***Eucalyptus* species (ES)**						
E. camaldulensis	59.4 ^{b}	238.4 ^{a}	402 ^{a}	10.44 ^{a}	210.9 ^{a}	1.19
E. leucoxylon	52.0 ^{c}	205.4 ^{a}	395 ^{a}	10.54 ^{a}	194.0 ^{a}	1.10
E. astringens	57.4 ^{bc}	122.6 ^{b}	214 ^{b}	7.54 ^{b}	136.4 ^{ab}	0.99
E. sideroxylon	62.7 ^{b}	84.3 ^{b}	138 ^{b}	5.41 ^{b}	73.6 ^{b}	1.12
E. lehmannii	68.6 ^{a}	81.5 ^{b}	120 ^{b}	5.47 ^{b}	82.9 ^{b}	1.11
S.E.M.	1.32	12.82	22.2	0.489	20.19	0.116
p-value	<0.001	<0.001	<0.001	<0.001	0.001	0.801
Season (S)						
Autumn	61.0	137.3	239	7.15	143.3	1.01
Winter	59.0	154.4	267	8.37	136.2	1.15
Spring	60.1	147.7	256	8.12	139.2	1.14
S.E.M.	1.02	9.93	17.2	0.379	15.64	0.090
p-value	0.381	0.488	0.518	0.088	0.955	0.533
ES × S (*p*-value)	0.365	0.612	0.572	0.351	0.319	0.446

$^{a–c}$ Within a column, mean values not sharing a common superscript represent significant ($p < 0.05$) differences among species. DM: dry matter; GP: gas production; S.E.M.: standard error of the mean.

The incubation of foliage harvested in autumn showed a lower acetate-to-propionate ratio than that collected in winter, with intermediate values for that picked in spring (Table 4). No seasonal effects on VFA molar proportions were observed. The acetate molar proportion was greatest for *E. leucoxylon* and lowest for *E. sideroxylon*, whereas the molar proportion of propionate showed the opposite pattern, greatest values for *E. sideroxylon* and lowest for *E. leucoxylon*. Butyrate and valerate did not differ among the eucalyptus species and seasons, while the yield of iso-acids was greatest for *E. camaldulensis*, which differed only from the species *E. sideroxylon*, for which the production of iso-acids was not detected.

Table 4. Molar proportions of volatile fatty acids (mmol per mol of total VFA) when the foliage of eucalyptus species harvested in different seasons was incubated in vitro in buffered rumen fluid for 24 h.

	Acetate	Propionate	Butyrate	Valerate	Iso-Acids	C2-to-C3
***Eucalyptus* species (ES)**						
E. camaldulensis	752 ^{ab}	198 ^{bc}	40.2	3.27	6.09 ^{a}	3.73 ^{ab}
E. leucoxylon	778 ^{a}	169 ^{c}	50.2	1.77	0.76 ^{ab}	3.93 ^{a}
E. astringens	718 ^{abc}	246 ^{ab}	35.2	0.35	0.59 ^{ab}	3.45 ^{bc}
E. sideroxylon	660 ^{c}	289 ^{a}	50.2	0.55	0.00 ^{b}	3.16 ^{c}
E. lehmannii	705 ^{bc}	239 ^{ab}	53.7	0.06	2.11 ^{ab}	3.38 ^{c}
S.E.M.	14.3	14.4	5.24	0.876	1.291	0.077
p-value	0.001	0.001	0.144	0.127	0.034	<0.001
Season (S)						
Autumn	709	248	40.1	1.38	1.63	3.40 ^{B}
Winter	739	208	49.5	1.51	2.19	3.69 ^{A}
Spring	721	228	48.0	0.71	1.91	3.51 ^{AB}
S.E.M.	11.1	11.2	4.06	0.678	1.000	0.059
p-value	0.214	0.087	0.288	0.685	0.932	0.011
ES × S (*p*-value)	0.945	0.997	0.288	0.681	0.944	0.849

$^{a–c}$ Within a column, mean values not sharing a common superscript represent significant ($p < 0.05$) differences among species. ^{A,B} Within a column, mean values not sharing a common superscript represent significant ($p < 0.05$) differences among seasons. C2-to-C3: acetate-to-propionate ratio. S.E.M.: standard error of the mean.

4. Discussion

Several studies have focused efforts on evaluating ruminal fermentation (using the in vitro gas-production technique) of eucalyptus foliage and to trace correlations with digestibility [7,20,21] Some results have indicated a negative correlation between fiber content in eucalyptus foliage and digestibility or gas production [22] In our study, the digestibility of eucalyptus foliage was affected by the NDF content, with a negative correlation between ED and NDF content (−0.62). Horst et al. [7] have suggested that chemical attributes other than fiber may have an effect on the digestibility of eucalyptus foliage as there are variations among species in the biological activity of tannins or other secondary compounds that can affect their digestive utilization in ruminants. The results reported by Horst et al. [7] have shown that foliage from eucalyptus trees would be a feedstuff of rather limited nutritional value for ruminants. Results presented by Thao et al. [23] and Wang et al. [24] showed that, at low levels of inclusion, eucalyptus foliage does not affect intake or digestibility. The secondary compounds of eucalyptus foliage limit the use of this material as a ruminant feed due to the astringent taste and low level of palatability. In the case of tannins, their effects can be positive or negative depending on the concentration in the foliage [25] Levels below 4% can promote the tannin-protein complex, reducing methane production and protein degradation [24] Levels greater than 6% have been shown to negatively affect growth and milk production [26]

Some of these secondary compounds contained in the tree leaves may exhibit functional properties, in particular antimicrobial activity that may cause changes in the ruminal fermentation pattern. These changes may occur due to the action of these compounds against the cell membranes of the rumen microbes [27], especially against cellulolytic bacteria [28] At a suitable concentration, these effects may be beneficial, for instance if methanogenesis is reduced without inhibiting microbial fermentation to a substantial extent. A number of studies have shown a significant level of in vitro antimethanogenic activity of eucalyptus leaves, extracts, and essential oils. The most studied species showing this activity have been *E. camaldulensis* [23,29] and *E. globulus* [21,30–34] It is worth mentioning that some of these studies have shown that *E. camaldulensis* can be effective in decreasing methane production by rumen fermentation, whereas our study shows that other species (i.e., *E. lehmannii* or *E. sideroxylon*) could have a more noticeable effect on this fermentation gas. Wang et al. [24] observed significant dose-dependent effects of a eucalyptus essential

oil on methane production in vitro, but these effects could not be reproduced with sheep in vivo. As highlighted by Bueno et al. [35] through in vitro assays, the biological activity of secondary compounds varies among eucalyptus species [36,37] However, there are no studies in the literature examining the differences among species in their effects on rumen fermentation, or on the impact of other sources of variation (e.g., season) that can affect the concentration of plant secondary compounds in the leaves and thus their biological activity. Our data, obtained from in vitro fermentation studies, suggest that these differences exist not only among species, but also that there may be some variations among seasons since the fermentation rate was higher in autumn, even though there were no chemical differences in eucalyptus foliage among seasons. Fermentation efficiency (assessed in terms of PF values [17]) was similar to that reported by Chouchen et al. [29] in diets with the inclusion of essential oil of eucalyptus at concentrations of 1.6 to 2.4 mL L^{-1} of ruminal liquid (5.26 and 7.46 mg DM digested mL^{-1} gas) in in vitro studies. In our study, there were significant differences among eucalyptus species in fermentation PF values. Based on our evidence, it is plausible to assume that there are substantial interspecies variations in the structural form, concentration, and mechanism of action of the secondary compounds contained in eucalyptus leaves. Other studies have reported altered rumen fermentation and decreased gas production due to reduced archaea, protozoa, and cellulolytic bacteria [30] although Cobellis et al. [31] did not observe any effect of *E. globulus* on total bacteria or archaea.

Mukharji and Srivastava [38] described a reduction in gas production, methane, and total VFA concentration, and an increase in propionate molar proportion in response to the addition of eucalyptus oil. These changes in VFA concentrations deserve further investigation as, in our study, the concentrations of acetate and propionate and the acetate-to-propionate ratio were different among eucalyptus species and seasons. The reduction in enteric methane production can occur either by inhibiting H_2 production or by a change in H_2 allocation [39] Any activity reducing the numbers of archaea or depressing their methanogenic activity would constrain the use of metabolic H_2 to reduce CO_2 to CH_4. The accumulation of H_2 would favor the formation of propionate, shifting the fermentation pattern as reflected in reduced acetate-to-propionate ratios [24] Some other studies have shown that, when eucalyptus foliage is incubated in rumen fluid, the acetate-to-propionate ratio is reduced [24,40,41], improving production efficiency [42]

Changes in deamination are another important modification reported as a result of the inclusion of eucalyptus in in vitro ruminal fermentation assays [23,29,31,32], which may partially explain the variations observed in the concentration of iso-acids in our study. Condensed tannins, present at considerable levels in eucalyptus foliage [7], build complexes with nitrogen compounds [43] via hydrogen bonds [44], leading to a reduction in protein degradation.

5. Conclusions

Although the use of eucalyptus foliage as a feedstuff for ruminants is questionable, our results showed that material from some eucalyptus species could be included in ruminant diets with the purpose of modulating fermentation processes in the rumen. *Eucalyptus sideroxylon* and *E. lehmannii* foliage seem to be the most digestible and with some potential to reduce methane emission and, therefore, are the most suitable eucalyptus species to be used for this purpose. During the winter, the foliage showed greater digestibility and an increase in the acetate-to-propionate ratio but lacked an effect on methane emission. The antimethanogenic activity of eucalyptus foliage should be validated in vivo, and dose–response studies will be required to establish the best level of inclusion of eucalyptus leaves in ruminant diets to improve feed efficiency and favorably affect the environmental impact of animal husbandry.

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