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ORIGINAL ARTICLE

Fermentation Parameters and In Vitro Degradability of Diets Containing *Cratylia argentea* Hay as a Substitute for Tifton-85 Hay

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ABSTRACT

Cratylia argentea has potential for use in ruminant feed; however, the presence of condensed tannins (CT) can interfere with rumen fermentation dynamics. This study aimed to evaluate the fermentation parameters and in vitro degradability of diets containing *C. argentea* hay as a substitute for Tifton-85 hay. The experiment was conducted in a randomised block design with a 3×4 factorial arrangement, using incubation times of 24, 48, and 96 h, and varying the inclusion of *C. argentea* hay at 0%, 20%, 40%, or 100% concentration, with three replicates per treatment. The production of CH_4 [mL/true organic matter (TOM)], gas [mL/dry matter (DM)], CH_4 mL/g DM, the in vitro DM digestibility (IVDMD), organic matter (IVOMD), TOM (TOMD), the partition factor (PF), which quantifies the fraction of total organic matter available for microbial fermentation, pH, the ammonia nitrogen concentrations (NH_3-N), short-chain fatty acids (SCFA), isoacids and the acetate to propionate ratio were evaluated. Substitution levels did not significantly influence ($p > 0.05$) CH_4 production, gas, PF, pH, NH_3-N, SCFA, or isoacids. CH_4 mL/g DM showed quadratic behaviour ($p < 0.05$), whereas digestibility coefficients showed a linear reduction at 96 h of incubation, probably due to fibre and CT concentrations in legume hay. However, these coefficients at the 40% inclusion level were similar to the treatment without legume hay at 96 h of incubation. Therefore, the fermentation and in vitro degradability parameters suggest that *C. argentea* hay can replace Tifton-85 hay by up to 40% in ruminant feed with the potential to reduce enteric CH_4 production.

1 | Introduction

Agricultural production has been operating with increasingly tight margins and with strong pressure to mitigate its impact on the environment (Yue et al. 2022; IPCC 2023). This scenario requires the sector to be more efficient in the application and use of resources to meet global demands without neglecting the activity's profitability. One of the reasons for the intensification of production systems is the need to optimise resource use through the implementation of new technologies.

In the livestock sector, the search for alternative feed sources for ruminant production is constant. Recent studies have explored the potential of forage legumes as a source of nitrogen-rich roughage and secondary compounds (Berça et al. 2023; Ugbogu et al. 2019) for feeding these animals. Among these

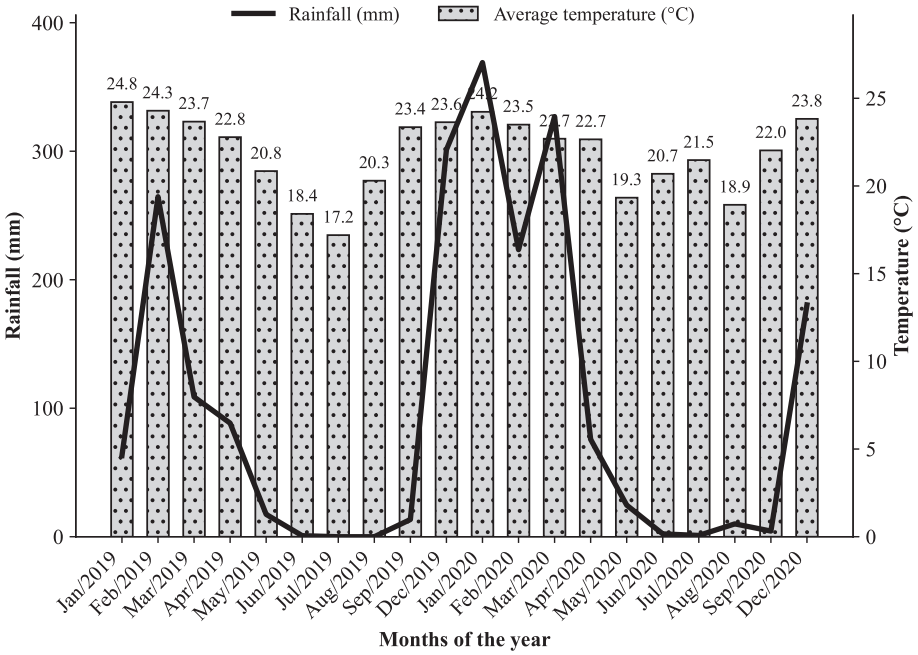

FIGURE 1 | The rainfall regime and maximum and minimum temperatures during the harvesting of *C. argentea* for hay production between 2019 and 2020.

compounds, condensed tannin (CT) stands out as a biomolecule from the intermediate metabolism of plants. These compounds have the capacity to reduce the CH_4 enteric production (Battelli et al. 2024; Jayanegara et al. 2012; Ugbogu et al. 2019), one of the main greenhouse gases. The shrubby legume *Cratylia argentea* has good tolerance to acidic soils with high aluminium saturation and low fertility (Mass 1995), a vigorous root system, and good productivity throughout the year, making it an alternative for cultivation in regions with water deficit (Lascano 1995; Luz et al. 2015).

Recent studies have evaluated the growth curve (Climaco et al. 2023), chemical composition (Abreu et al. 2023), and use of *C. argentea* in ruminant feeding (Teixeira et al. 2023), but studies evaluating its fermentation profile, microbial dynamics, and potential to reduce CH_4 production are scarce in the literature. The in vitro gas production technique has been used for these evaluations with the advantage of allowing the rate and extent of substrate digestion to be measured continuously, generating useful results for comparing feeds and their processing methods, pre-selecting materials to be evaluated in vivo, and providing information applicable to the practice of ruminant nutritionists (Owens and Basalan 2016).

Blümmel et al. (1997) suggested that the technique should be associated with a degradability parameter, as it is an indicator of the amount of substrate fermented, whereas the gas produced indicates how much of this material was used for the synthesis of short-chain fatty acids (SCFA) and gas. A high amount of degraded substrate in the first few hours of incubation indicates a higher dry matter intake (DMI), and higher values for the partition factor can be interpreted as more microbial protein (MP) being digested in the small intestine. Considering that the high crude protein (CP) concentration of legume hay can reduce the protein cost of the diet, this study aimed to evaluate the fermentation parameters and in vitro degradability of diets containing *C. argentea* hay as a substitute for *Cynodon* spp. cv. Tifton-85 hay.

2 | Materials and Methods

2.1 | Experimental Planning and Sample Preparation

The experiment was conducted at the University of São Paulo, Piracicaba–São Paulo (22°42′28.4″ S 47°38′43.2″ W). The samples used for this trial consisted of *C. argentea* hay harvested at the farm of the Brazilian Agricultural Research Corporation (EMBRAPA) Corn and Sorghum in Sete Lagoas/MG (19°29′15″ S 44°10′35″ W) and at the Santa Rita experimental farm belonging to the Minas Gerais Agricultural Research Corporation (EPAMIG) in Prudente de Morais/MG (19°27′09″ S 44°09′25″ W), contiguous areas where *C. argentea* has been cultivated since December 2009. The units are located in the Cwa climate, according to Köppen, a savannah climate with dry winters and humid summers with rain. Figure 1 shows the rainfall and the maximum and minimum temperatures during the period when the material was collected and prepared for the production of *C. argentea* hay.

To produce *C. argentea* hay, the plants were pruned to even out the forage canopy in January 2019. The plants were cut at 25 cm from the ground every 90 days for a year (April 2019 to April 2020), including the entire plant. Temperatures during hay production ranged from 17.7°C to 29.2°C (Figure 1). The accumulated rainfall was 885 mm, and the relative humidity was 66.6%. The harvested material was processed in a forage machine, spread out in a 10 cm layer for drying in the sun, and turned frequently until it reached a dry matter (DM)

TABLE 1 | *C. argentea* substitution levels and chemical composition of the experimental diets.

Chemical composition	Substitution levels (%)				Hay	
	0	20	40	100	*C. argentea*	Tifton-85
Dry matter (g/kg)	894	885	893	886	911	903
Organic matter (g/kg DM)	918	921	919	919	917	933
Mineral matter (g/kg DM)	82.1	79.0	81.2	81.4	82.7	66.6
Crude protein (g/kg DM)	216	194	194	194	202	119
NDF (g/kg DM)	497	510	453	453	715	725

Abbreviations: DM, dry matter; NDF, neutral detergent fibre.

TABLE 2 | Phenolic compounds of *C. argentea* in the edible, woody, and whole plant materials.

Phenolic compounds	Plant parts		
	Inedible material*	Woody*	Entire plant
Total phenols (g/kg DM)*	15.9	7.05	10.5
Total tannins (g/kg DM)*	8.67	3.83	5.49
Condensed tannin (g/kg DM)**	1.04	1.83	1.07

Abbreviation: DM, dry matter.
*Grams tannic acid/kg DM.
**Equivalent grams leucocyanidin/kg DM.

content of 85%. The interval of exposure to the sun until the material reached the hay stage varied between 48 and 72 h. During the night or on rainy days, the material was covered with a plastic sheet to prevent moisture build-up. The hay produced was stored in raffia bags and stored in drums to avoid contamination until the experimental diets were prepared in November 2020.

The Tifton-85 and *C. argentea* hays were ground to a particle size of 5 mm, homogenised, and then mixed with the other ingredients to make up the experimental rations, according to the substitution levels and chemical composition described in Table 1. The diets were formulated to meet the nutritional requirements of finishing lambs, with 16% CP and 75% total digestible nutrients (NDT), considering DMI of 4% of live weight and an average daily gain of 200 g (NRC 2007).

The samples of *C. argentea* collected from the experimental areas were separated into edible material (leaves and stems less than 5 mm in diameter) and inedible material (stems more than 5 mm in diameter), dried at room temperature, and submitted to analysis to determine the phenolic compounds (Table 2). The contents of total phenols (TP) and total tannins (TT) were analyzed using the butanol-HCl method (Folin and Ciocalteu 1927) and the CT butanol-HCl method (Makkar 2003a), with the *C. argentea* samples being ground and sieved through a mesh with a diameter of 0.25 mm. The method involves the oxidative conversion of proanthocyanidins to anthocyanidins in a heated acidic medium, which generates a reddish-coloured compound. The absorbance of this compound was measured at 550 nm using a spectrophotometer, and the results were expressed as leucoanthocyanidin equivalents.

2.2 | In Vitro Gas Production

For the in vitro gas production test, rumen fluid collected from three adult male Santa Ines sheep with rumen fistulae and an average body weight of 65.0 ± 2.30 kg was used as inoculum. The animals were fed rations containing Tifton 85—Cynodon spp. hay ad libitum (891 g DM/kg; 876 g NDF/kg DM; 492 g ADF/kg DM; 78.3 g CP/kg DM; 62.1 g MM/kg DM, where NDF is neutral detergent fibre, ADF is acid detergent fibre, and MM is mineral matter), water, and mineral salt. Before the daily feed intake, liquid and solid fractions of the rumen contents of the three animals were collected individually in thermal containers, using a silicone tube adapted to a 60 mL syringe (Becton-Dickson Indústria Cirúrgica, Curitiba, Brazil) and crucible forceps, respectively. Adopting a solid/liquid ratio of 50:50 (based on volume) (Bueno et al. 2005), three inoculums were prepared, using three different combinations of each animal's rumen content.

The experimental design used was a randomised block design (inoculum) in a 3 × 4 factorial arrangement, with three incubation periods: 24, 48, and 96 h, four levels of *C. argentea* hay at 0, 20, 40, or 100% and three replicates per treatment. Incubation was carried out according to the methodologies described by Theodorou et al. (1994) and Mauricio et al. (1999), adopting the adaptations proposed by Bueno et al. (2005) and Longo et al. (2006). One gram of each total diet was weighed in Ankom bags (Ankom F57, Macedon, NY, USA) and transferred to 160 mL bottles along with 50 mL of incubation medium (Menke's buffer medium) (Menke and Steingass 1988) and 25 mL of inoculum. Three bottles of each treatment were incubated, each containing a different inoculum. Once incubation was complete, the bottles were sealed with rubber caps and placed in a forced-air ventilation oven at 39°C.

The periods of 24, 48, and 96 h after incubation were used to measure the internal pressure of the bottles, using a pressure sensor (PressData 800, LANA/CENAUSP, Piracicaba/SP) and a datalogger. Net gas production (mL/g MS) was estimated using the equation: $V = P \times 6.1432 + 0.0451$; ($n = 328$, $R^2 = 0.994$) defined in the laboratory for the sensor and

TABLE 3 | Estimates of gas production parameters of diets with inclusion levels of *C. argentea* hay (0, 20, 40, and 100%) as a substitute for Tifton-85 hay in the three incubation periods (24, 48, and 96 h).

	Hay inclusion (%)					*p*				
Parameter estimates	**0**	**20**	**40**	**100**	**SEM**	***I***	***T***	***I*×*T***	***L***	***Q***
CH_4 production (mL/g TOM)										
CH_4	44.4	43.9	50.1	40.3	3.61	0.0740	<0.0001	0.8797	—	—
Gas production (mL/g DM)										
CH_4	27.4	27.5	30.4	23.2	2.64	0.0416	<0.0001	0.7010	0.0508	0.0485
Gas	126	133	132	123	5.21	0.0993	<0.0001	0.0711	—	—

Abbreviations: DM, dry matter; *I*, inclusion level of *C. argentea* hay as a substitute for Tifton 85 hay; *I*×*T*, interaction; *L*, linear effect; OM, organic matter; *p*, probability values; *Q*, squared effect; SEM, standard error of the mean; *T*, incubation period; TOM, true organic matter.

datalogger used, where: *V* = gas volume (mL) and *P* = measured pressure (Psi).

After measuring the pressure, a gas sample (2 mL) was collected from each bottle and transferred to vacuum test tubes with a capacity of 10 mL (BD Vacutainer, Curitiba, Brazil). Three *pools* of these samples were formed for the 24-, 48- and 96-h incubation periods, to quantify the net production of CH_4 (mL/g MS) in vitro using gas chromatography, as described by Lima et al. (2018), using equipment (Shimadzu 2014, Chiyodaku, Tokyo, Japan) with a flame ionisation detector and an HP-molesieve capillary column (GC 30 m × 0.53 mm × 25 μm). After reading the pressure and collecting the gas, the bottles were depressurized and their contents were homogenised by shaking before returning them to the oven.

2.3 | In Vitro Degradability Test

Degradability was assessed at 24, 48, and 96 h of incubation. After pressure measurement and gas collection, fermentation was stopped for the group of bottles for the corresponding period by opening the bottles and placing the F57 bags in ice water. The bags were then washed with a neutral detergent solution for 1 h at 90°C, followed by five washes with distilled water at 90°C and dried in an oven at 105°C for 24 h. They were weighed again and reduced to ash in a muffle furnace at 550°C for 5 h to determine the true organic matter digestibility (TOMD), calculating the difference between the incubated true organic matter (TOM), which is the organic matter incubated, and the remaining undegraded TOM, according to Goering and Van Soest (1975).

After removing the F57 bags, an aliquot from each bottle was collected in 20 mL glass vials and stored at −20°C for the determination of SCFA, using the methodology described by Lima et al. (2018), ammonia nitrogen concentration (NH_3-N), using micro-Kjeldahl steam distillation with a 5% sodium tetraborate solution according to Preston (1995), and pH, using a digital pH-meter (model TEC-2, Tecnal, Piracicaba/São Paulo–Brazil). The partition factor (PF) was used to estimate microbial efficiency, which was estimated by Blümmel et al. (1997), calculating the ratio between TOMD and total gas production (TG) in 24 h of incubation.

2.4 | Statistical Analysis

The data was tested for the probability distribution model and homogeneity of variances using the Shapiro–Wilk and Bartlett tests, respectively. Variables that did not meet the two criteria of normal probability distribution and homogeneity of error variance were transformed using the logarithmic function. Variables which, even when transformed, violated normality and homogeneity of variance were assessed using non-parametric statistics, using the Kruskal-Wallis test followed by Dunn's post-test. Analysis of variance (ANOVA) was carried out to test the effects of the individual factors (level of inclusion of *C. argentea* and incubation periods) and the interaction between the factors in the randomised block experimental design. Linear and quadratic regression models were used, assuming an error rate of $\alpha = 0.05$, to measure the effects of replacing *C. argentea* hay with Tifton-85 hay on gas production kinetics and nutrient degradability. To compare the means of the variables in the three incubation periods, orthogonal contrasts were adjusted, respecting the significance of the ANOVA of the interaction or the main effect (isolated). Orthogonal contrasts were also used to compare the inclusion levels with the treatment that did not include *C. argentea* hay. Pearson's correlation test was used to assess the relationship between independent variables. Statistical analyses were carried out using R Core Team (2024).

3 | Results

3.1 | In Vitro Gas Production

The in vitro gas production data showed a normal probability distribution and homogeneity of variances. There was no significant interaction between *C. argentea* hay inclusion levels and incubation periods for the gas production parameter estimates (Table 3). As for the main effects, only CH_4 production (mL/g DM) had a significant effect of legume inclusion levels, showing quadratic behaviour (Figure 2), with significant predictive power ($R^2 = 88.8\%$). CH_4 production increased with the inclusion of *C. argentea* hay, reaching maximum production at an inclusion level of 39.1% and then decreasing.

In the main effect of incubation periods, CH_4 and gas production increased ($p < 0.05$) as incubation time progressed (Table 4).

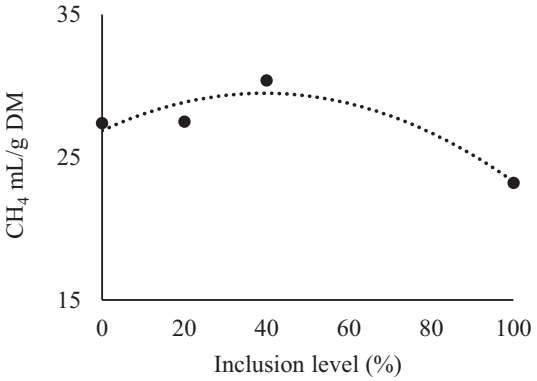

FIGURE 2 | CH_4 production mL/g DM incubated as a function of *C. argentea* hay inclusion level. Equation: $Y = -0.0017x^2 + 0.1328x + 26.863$. $R^2 = 0.8881$.

TABLE 4 | Orthogonal contrasts of parameter estimates for in vitro gas production during incubation periods of 24, 48, and 96 h, regardless of the levels of inclusion of *C. argentea* hay (0%, 20%, 40%, and 100%) as a substitute for Tifton-85 hay.

Contrasts	CH_4 mL/g TOM*	CH_4 mL/g DM*	Gas mL/g DM*
		Média	
24 h	24.9	13.0	93.1
48 h	38.6	21.9	129
96 h	70.3	46.4	134
		p	
24 h × 48 h	0.0002	0.0002	< 0.0001
24 h × 96 h	< 0.0001	< 0.0001	< 0.0001
48 h × 96 h	< 0.0001	< 0.0001	< 0.0001
SEM	3.61	2.64	5.21

Abbreviations: DM, dry matter; SEM, standard error of mean; TOM, true organic matter.
*Orthogonal contrasts by Fisher's test at 5%.

3.2 | In Vitro Degradation and Fermentation Parameters

The NH_3-N and SCFA data were transformed using the logarithmic function. The acetate: propionate and isoacid variables did not fit the transformation functions and were therefore analysed using non-parametric methods. The other variables had a normal probability distribution and homogeneity of variances. There was a significant interaction between inclusion levels of *C. argentea* hay and incubation periods for the estimates of degradation parameters (Table 5). In relation to fermentation parameters, all variables were influenced by inclusion levels ($p < 0.05$), with the exception of the acetate: propionate ratio.

The estimates of degradability parameters showed no significant effect between the incubation periods (24 and 48 h) and the levels of inclusion of *C. argentea* hay, when the interaction was split (Table 6). However, they reduced linearly (Figure 3) as a function of legume inclusion levels at 96 h of incubation, showing significant predictive power IVDMD, $R^2 = 94.8\%$; TOMD, $R^2 = 95.2\%$ and IVOMD, $R^2 = 92.9\%$, where IVDMD is in vitro dry matter digestibility and IVOMD is in vitro organic matter digestibility. However, only the maximum inclusion level differed from the treatment without legume hay inclusion ($p < 0.05$).

For the main effect of incubation periods, only the PF and pH variables showed no difference at (24 and 48 h) and (24 and 96 h), respectively (Table 7). For the other variables, the parameter estimates increased as the incubation time progressed ($p < 0.05$).

A positive and statistically significant correlation was found between the degradability variables and the gas production parameters (Table 8). Specifically, IVDMD and IVOMD showed a strong and significant relationship with methane production per g of true organic matter (CH_{4mov}, $r = 0.890$ and $r = 0.890$), methane production per g of dry matter (CH_4, $r = 0.931$ and $r = 0.929$), and total gas production (Gas_{total}, $r = 0.902$ and $r = 0.919$). All of these correlations were highly significant ($p < 0.01$).

Among the gas production parameters, only total gas production showed a strong and significant relationship with ammonia nitrogen (NH_3-N) concentration ($r = 0.883$, $p < 0.05$). The other gas production parameters (CH_{4mov} and CH_4) had a moderate but still significant relationship with both NH_3-N and SCFA concentrations. The IVDMD also had a strong and significant correlation with NH_3-N ($r = 0.746$) and a moderate and significant correlation with SCFA ($r = 0.531$).

4 | Discussion

4.1 | In Vitro Gas Production

The in vitro gas production technique provides indicators of rumen kinetics and is used to assess fermentation potential and feed degradability before in vivo studies.

Gas production is the result of the fermentation of feed substrates by rumen microorganisms. It is influenced by factors such as DMI, passage and degradation rates, the chemical and physical composition of the feed, the roughage: Concentrate ratio, the pH of the rumen fluid, and the predominant microbial population. The CH_4 produced during this process occurs as an indispensable mechanism for the survival of rumen microorganisms through the consumption of surplus H^+ ions (Janssen 2010) and the regeneration of reducing equivalents (Nicotinamide adenine dinucleotide). (Janssen and Kirs 2008; Morgavi et al. 2010; Thauer et al. 2010). Although beneficial to the rumen microbiota, excess CH_4 compromises the energy efficiency of diets, as it is eliminated by eructation without being used by the host.

High CH_4 production occurs when ruminants are fed diets with a high inclusion of forage and low nutritional value, due to the higher proportion of the end products of fermentation, acetate and butyrate, when compared to diets with lower and higher inclusion of forage of good nutritional value and concentrates, respectively (Agle et al. 2010; Hills et al. 2015; Jiao et al. 2014). Diets such as these increase the proportion of propionate (Getachew et al. 1998), which does not result in the production of CH_4, and

TABLE 5 | Estimates of degradation parameters and in vitro fermentation of diets with inclusion levels of *C. argentea* hay (0, 20, 40, and 100%) as a substitute for Tifton-85 hay in the three incubation periods (24, 48, and 96 h).

	Hay level (%)					*p*				
Parameter estimates	**0**	**20**	**40**	**100**	**SEM**	***I***	***T***	***I*** × ***T***	***L***	***Q***
Degradation parameters										
IVDMD (g/kg DM)	832	835	812	753	12.7	0.0092	< 0.0001	0.0281	—	—
TOMD (mg/kg DM)	0.678	0.679	0.661	0.611	0.011	0.0097	< 0.0001	0.0426	—	—
IVOMD (g/kg OM)	825	832	805	750	13.1	0.0100	< 0.0001	0.0316	—	—
Fermentation parameters										
PF (mL gas/kg TOM)*	8.12	8.36	7.85	8.53	0.813	0.8409	< 0.0001	0.9153	—	—
pH	6.56	6.57	6.58	6.61	0.022	0.2929	< 0.0001	0.2049	—	—
NH_3-N (mg/dL)	3.82	3.73	3.92	3.75	0.176	0.6382	< 0.0001	0.2001	—	—
SCFA (mol/d)	115	115	122	110	3.07	0.0845	< 0.0001	0.4445	—	—
Acetate: Propionate	1.92	1.90	1.93	1.99	NA	0.9158	0.8304	—	—	—
Isoacids (mmol/L)	2.50	2.70	2.30	3.30	NA	0.9978	< 0.0001	NA	NA	NA

Abbreviations: DM, dry matter; *I*, inclusion level of *C. argentea* hay as a substitute for Tifton 85 hay; *I* × *T*, interaction; IVDDM, in vitro dry matter degradability; IVOMD, in vitro organic matter degradability; *L*, linear effect; NA, not applicable; NH_3-N, ammonia nitrogen; OM, organic matter; *p*, probability values; *Q*, squared effect; SCFA, short-chain fatty acids; SEM, standard error of mean; *T*, incubation period; TOM, true organic matter; TOMD, in vitro true organic matter degradability.
*Partition factor that is presented at time 24 h, according to Blümmel et al. (1997).

TABLE 6 | Estimates of in vitro degradability parameters of diets with inclusion levels of *C. argentea* hay (0%, 20%, 40%, and 100%) as a substitute for Tifton-85 hay per incubation period (24, 48, and 96 h).

	Hay level (%)					*p*	
Incubation (h)	**0**	**20**	**40**	**100**	**SEM**	***L***	***Q***
In vitro dry matter degradability (g/kg DM)							
24	639	647	649	659		Ns	NS
48	688	715	701	659		Ns	Ns
96	832	835	812	753*	12.7	0.0001	0.3804
In vitro true organic matter degradability (mg/kg DM)							
24	0.512	0.520	0.517	0.526		Ns	Ns
48	0.564	0.581	0.574	0.538		Ns	Ns
96	0.678	0.679	0.661	0.611*	0.011	0.0002	0.4331
In vitro organic matter degradability (g/kg OM)							
24	623	638	630	646		Ns	Ns
48	687	712	700	661		Ns	Ns
96	825	832	805	750*	13.1	0.0002	0.3757

Abbreviations: DM, dry matter; *L*, linear effect; Ns, not significant; OM, organic matter; *p*, probability values; *Q*, squared effect; SEM, standard error of mean; TOM, true organic matter.
*On the same line, it differs significantly from the 0% inclusion level by orthogonal contrasts using the Dunnett test ($p < 0.05$).

make it possible to make better use of the energy available to the host. For this reason, ruminant nutritionists have been improving forage production and harvesting techniques with a goal of improving their nutritional value, as well as maximising the inclusion of concentrates in diets to meet the animals' energy requirements and increase the efficiency of their use. Similarly, synthetic additives as well as secondary compounds (Min et al. 2020) produced by plants have been included in diets to optimize rumen fermentation, reducing energy losses and maximizing the synthesis of fermentation products MP and SCFA.

Legumes are widely known for their higher concentration of nitrogen than tropical forage grasses (Durmic et al. 2017). In contrast, they have higher concentrations of secondary compounds

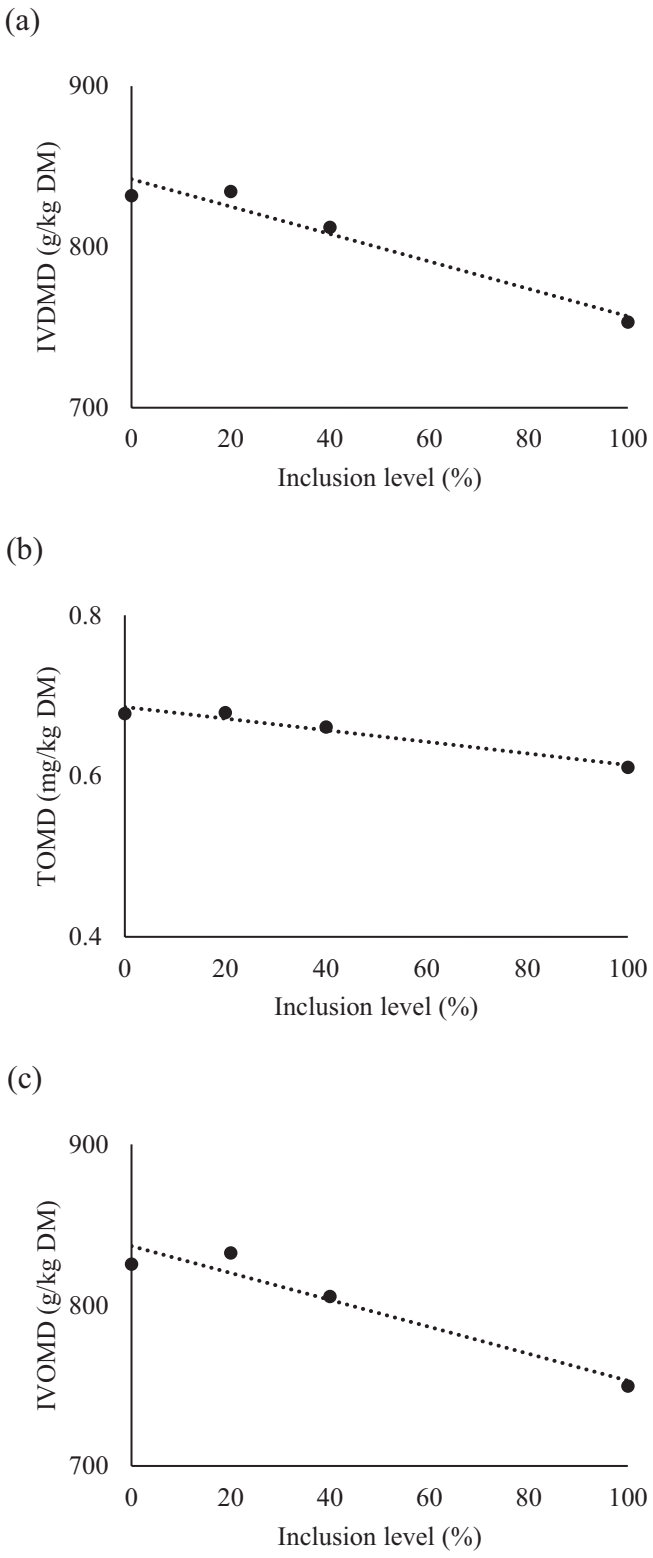

FIGURE 3 | In vitro dry matter digestibility (IVDMD) (a), True organic matter digestibility (TOMD) (b), and in vitro organic matter digestibility (IVOMD) (c), at 96 h of incubation as a function of the levels of inclusion of *C. argentea* hay. Equations: (a) $y=-0.8525x+842.12$. $R^2=0.9481$; (b) $y=-0.0007x+0.6859$. $R^2=0.9515$ 0.9515; (c) $y=-0.8372x+836.74$. $R^2=0.929$.

such as CT. These, in turn, have an affinity for carbohydrates and proteins, leading to the formation of insoluble complexes (Hagerman et al. 1992; Makkar 2003b; Naumann et al. 2017) with lower fermentation potential and rumen degradability. This mechanism has been explored (Ku-Vera et al. 2020) as a strategy to reduce enteric CH_4 production, considering its potential to reduce the energy use of diets and its contribution as a greenhouse gas (GHG) (Tamminga 1995).

High concentrations of CT (60–120 g/kg DM) have been shown to reduce the palatability and rumen degradability of diets, resulting in low animal productivity (Frutos et al. 2002). However, these effects seem to depend more on the type of tannin (Mueller-Harvey 2006) and its efficiency (Naumann et al. 2017) in forming insoluble complexes than on its quantity. According to Mueller-Harvey (2006), concentrations of CT lower than 50 g/kg DM promote beneficial effects such as an increase in undegraded protein in the rumen. Makkar (2003b) and Paengkoum et al. (2021) cited the effect of CT in reducing microbial attack on food particles, decreasing the rate of in vitro gas production. Getachew et al. (1998) concluded that the reduction in in vitro gas production was caused by the presence of CT. In this sense, we hypothesise that both the presence of CT (Table 2) and the composition of the fibrous fraction of *C. argentea* explain the quadratic behaviour observed for the accumulated production of CH_4 mL/g DM. Increasing the inclusion levels of *C. argentea* hay possibly provided a favourable rumen environment for the growth and multiplication of cellulolytic microorganisms, resulting in greater release of H^+ ions, which were used to reduce CO_2 to CH_4. However, the levels of fibre and the amount of CT, which increased proportionally with the inclusion levels of legume hay, may have reduced the fermentation rate due to the accumulation of slow-degrading material (fibre) and the formation of insoluble compounds (CT plus carbohydrates and proteins), reducing CH_4 production from inclusion level 39.1% onwards. Meza-Bone et al. (2022) suggested that the main causes of the variation observed in *C. argentea*'s accumulated in vitro CH_4 production (96 h of incubation) were the presence of CT and the concentrations of fibrous carbohydrates. These authors observed values of 79.5, 110, and 141 mL CH_4/0.400 g DM for 30, 45, 60, and 75 days, respectively, after regrowth in the rainy season. According to Carmona et al. (2005), the physical–chemical composition of forage influences enteric CH_4 production. This, in turn, is reduced in the presence of high concentrations of fibrous carbohydrates (cellulose and hemicelluloses) (Vanegas et al. 2017), which accumulate as the plant reaches physiological maturity (Andino et al. 2019; Ortiz-Tirado et al. 2019).

Cumulative gas production mL/g DM and CH_4 production intensity mL/g TOM ranged from 40.3 to 50.1 and 123 to 133, respectively, with no interference from *C. argentea* hay inclusion levels. In addition to CH_4, CO_2, and N_2 gases are also produced during rumen fermentation (Castañeda-Rodríguez et al. 2023). Both the amount of gas and the intensity of CH_4 production depend on the type and chemical composition of the fermented substrates, as well as their potential degradability and rumen degradation rate. As already mentioned, diets with a high inclusion of forage favour the growth and multiplication of cellulolytic microorganisms whose main

TABLE 7 | Orthogonal contrasts of estimates of in vitro fermentation parameters during incubation periods of 24, 48, and 96 h, regardless of the levels of inclusion of *C. argentea* hay (0%, 20%, 40%, and 100%) as a substitute for Tifton-85 hay.

Contrasts	PF*	pH*	NH_3-N mg/dL*	SCFA (mol/d)*	Isoacids (mmol/L)**
			Mean		
24 h	5.61	6.53	2.68	103	1.85
48 h	4.40	6.66	3.78	113	2.50
96 h	14.6	6.55	4.92	131	3.92
			p		
24 h × 48 h	0.0976	< 0.0001	< 0.0001	0.0077	0.0148
24 h × 96 h	< 0.0001	0.4840	< 0.0001	< 0.0001	< 0.0001
48 h × 96 h	< 0.0001	0.0001	< 0.0001	< 0.0001	0.0177
SEM	0.813	0.022	0.002	3.07	NA

Abbreviations: DM, dry matter; NA, not applicable; NH_3-N, ammonia nitrogen; *p*, probability values; PF, partition factor; SCFA, short-chain fatty acids; SEM, standard error of mean.
*Orthogonal contrasts by Fisher's test at 5%.
**Contrasts by Dunn's test at 5%.

TABLE 8 | Estimates of pearson's correlation coefficients between variables.

	CH_{4mov}	CH_4	Gas_{total}	IVDMD	IVOMD	NH_3-N	SCFA	pH
CH_{4mov}	—	—	—	0.890*	0.890*	0.685*	0.485*	—
CH_4	—	—	—	0.931**	0.929**	0.699*	0.498*	—
Gas_{total}	—	—	—	0.902**	0.919**	0.883*	0.665*	—
IVDMD	—	—	—	—	—	0.746*	0.531*	—

Note: **, * significant at 1% and 5% probability of error, by *t*-test, respectively. —, no correlation.
Abbreviations: CH_4, methane production in mL/g of dry matter; CH_{4mov}, methane production in mL/g of true organic matter; Gas_{total}, gas production in mL/g of dry matter; IVDMD, in vitro dry matter degradability; IVOMD, in vitro organic matter degradability; NH_3-N, ammonia nitrogen; SCFA, short chain fatty acids.

metabolic route is the production of CH_4. We hypothesize that the composition of the diets in this study favoured the growth of this microbial population, but the presence of CT possibly contributed to controlling the intensity of CH_4 production. Increased levels of legume hay inclusion were accompanied by the inclusion of concentrates, which may have contributed positively to maintaining rumen fermentation (gas production), including reducing the intensity of CH_4 production. Fagundes et al. (2020) compared the in vitro gas production of *C. argentea* with that of Tifton-85 hay. The gas production values mL/g DM for *C. argentea* were similar to those found in the present study (Table 3), being approximately 60, 120, and 140 at 24, 48, and 96 h of incubation, respectively. However, *C. argentea* showed 82.5% ($p < 0.05$) lower cumulative gas production compared to Tifton-85 hay (254 vs. 139 mL/g DM). The authors suggested that the high concentration of potentially fermentable carbohydrates (743 g NDF/kg DM) and the absence of CT in Tifton-85 explain the difference found. The lignin concentration (133 g/kg DM) of Tifton-85 probably also contributed, considering that it was numerically 23.3% lower than that of *C. argentea* (164 g/kg DM), which may also have occurred in the present study.

The increases observed in the production of CH_4 mL/g TOM and DM and gas mL/g DM over the incubation periods were probably due to the greater availability of degraded substrates, which, once fermented, generate volatile fatty acids (VFAs), CO_2, CH_4, H_2, and MP as end products. The high and strong positive correlation found between intensity (CH_{4mov}), production (CH_4 and Gas_{total}), and IVDMD and IVOMD supports our hypothesis (Table 8). However, the similar correlation behaviour found for Gas_{total} production and NH_3-N concentration ($r = 0.883$) suggests that, in addition to the potential degradability of the feed, the availability of N and energy sources is a determinant of the fermentation process, considering that they are used as nutrients for microbial growth and multiplication, resulting in gas production.

4.2 | In Vitro Degradation and Fermentation Parameters

The degradability coefficient corresponds to the fraction of feed that disappears during the rumen fermentation process, and is considered a determining factor in the supply of energy and nutrients essential for the maintenance and productive performance of animals. It can be expressed in relation to DM, OM, and degraded TOM. Apparent IVOMD is usually expressed as g of substrate per kg of OM that has apparently disappeared from the rumen. However, the apparent disappearance of substrates differs from the amount that has actually been digested, considering that around 24%–40% of

the weight of the digesta is incorporated into microbial cells (Owens and Goestsch 1988).

The potential degradability of feed in the rumen environment is a function of the DMI, the physical and chemical composition of the feed, and the predominant microbial population. Under in vivo conditions, higher DMI and concentrate feeds tend to reduce the degradability of substrates by stimulating an increase in the ruminal passage rate of digesta (Kp) (Pina et al. 2010). Conversely, diets with a high roughage: concentrate ratio can potentially increase feed degradability, depending on their physical and chemical composition. In general, roughage feeds have a lower specific density than concentrates (Bargo et al. 2003), which increases their rumen retention time (lower Kp) and favours their degradability. Processing these feeds (chopping) increases the contact surface for microbial adhesion (Ehle et al. 1982), but by itself does not guarantee good rumen degradation, being highly dependent on chemical composition.

Lignin, a polyphenol with a structural function in plant cell walls, is a polymer that has no nutritional value because it is not digested by animal or microbial enzymes. The greater its degree of association with hemicelluloses (xylose, arabinose, and mannose), the lower the degradability of the fibrous fractions (hemicelluloses and cellulose) (Hartley and Harris 1986; Jung and Fahey 1983; Lawoko et al. 2005). It can be found in high concentrations in leguminous plants, especially in their stems (Bhandari et al. 2023). In this study, the stems of *C. argentea* were part of the composition of the hay, which may explain the linear reduction in IVDMD, IVOMD, and TOMD as inclusion levels increased at 96 h of incubation. The presence of lignin probably increased the amount of fractions with a slow degradation rate, as described by the Cornell Net Carbohydrate and Protein System (CNCPS) (Fox et al. 2004). This system proposes fractioning the diet's carbohydrates into *A* (sugars and organic acids) with a fast degradation rate, B_1 (starch and pectic substances) with a medium degradation rate, B_2 (available fibre) with a slow degradation rate, and *C* (unavailable fraction with zero degradation rate). The CP, in turn, is fractionated into *A* (readily available and instantly degraded in the rumen), B_1 (kp 120%–400%/h), B_2 (kp 3%–16%/h), B_3 (kp 0.06%–0.55%/h), and *C* (non-degradable CP). Yungán et al. (2022) and Fagundes et al. (2020) observed IVDMD (248 and 553 g/kg DM) and IVOMD (340 and 554 g/kg DM) coefficients for *C. argentea* that were numerically lower than those found in this study. On the other hand, Teixeira et al. (2023) observed degradability coefficients similar to those found in this study when they evaluated the inclusion levels (0%, 20%, 40%, and 100%) of *C. argentea* hay as a substitute for Tifton-85 hay in sheep diets. These authors found an average IVDMD of 688 g/kg DM, but IVOMD, degradability of NDF, and CP were lower ($p < 0.05$) at the highest level of legume inclusion (100%) when compared to the lowest (20%). Corroborating our hypothesis, the authors suggested that this behaviour seems to be related to the high lignin content of legumes (Hess et al. 2004), but cited the haymaking process. During the making of *C. argentea* hay, the stems and leaves were not separated, which possibly contributed to increasing its lignin content and reducing its ruminal degradability. In addition, according to Van Soest et al. (1991) and Dzowela et al. (1995), drying the plant in the sun to make hay may induce the Maillard reaction between the plant's carbohydrates and proteins and reduce rumen degradability.

Maximising MP synthesis and flow to the small intestine has been one of the nutritional strategies adopted in ruminant nutrition. MP has a high biological value, little variation in its composition, and makes up a large part of metabolizable protein. The efficiency of MP synthesis has been used as one of the indicators of feed quality for ruminants. It can be expressed as g of microbial cells per amount of energy, degradable OM, and fermentable carbohydrates in the rumen (Firkins 2021) or even by adopting the PF, which represents the amount of OM available for microbial fermentation, resulting in gas production and microbial growth (Caregnato et al. 2019). High PF values suggest that a large amount of OM is converted into MP and SCFA and, consequently, less is lost in the form of gas (Blümmel et al. 1997). Clark et al. (1992) cited the fermentable OM intake in the rumen (FOMI) as the main factor affecting the efficiency of microbial synthesis. In this sense, under in vivo conditions, higher FOMI tends to increase the efficiency of microbial synthesis. On the other hand, Russell et al. (1992) suggested that MP synthesis efficiency is mainly affected by the availability and synchronisation of energy and nitrogen compounds in the rumen. The treatments tested in this study seem to have met these conditions, as PF and NH_3-N concentrations remained unchanged, even at the highest levels of legume hay inclusion. It is possible that the inclusion of concentrate in the diets also contributed positively as a source of fermentable OM, compensating for the slow degradation of the legume's fibrous fractions. In addition to the inclusion of concentrate, the incubation time explains the higher PF value observed at 96 h. During this period, there was more available degraded OM, which was probably used for MP synthesis more efficiently.

Energy is the main limiting factor in the ability of fermenting microorganisms to use substrates, but the quantity and availability of N sources determine the efficiency of this process (Oba and Allen 2003). In addition to the evolutionary advantage of ruminants in taking advantage of fibrous plant fractions, thanks to the microbiota present in the fermentation chambers (Lapierre et al. 2006), these animals can convert non-protein N sources (NPN) into protein of high biological value (MP). Cellulolytic microorganisms perform this conversion more efficiently than amylolytic ones because they use ammonia as their N source (Russell et al. 1992). The concentration of NH_3-N is a parameter used to infer the efficiency of the utilisation of nitrogenous fractions and the availability of energy in the diet by the rumen microbiota. Synchrony between the two is desired in order to maximise MP synthesis and prevent excess NH_3-N from being eliminated with energy expenditure. Clark et al. (1992) and Satter and Slyter (1974) suggested between 2 and 5 mg/dL of NH_3-N as suitable values for maximising the passage of MP to the small intestine. The values found for NH_3-N in the present study, as a function of the inclusion levels of *C. argentea* hay (average 3.80 mg/dL), are within the range proposed by the authors, indicating that legume hay seems to be a good source of energy and protein for rumen microorganisms when associated with concentrate feed. The different NH_3-N concentrations observed during the incubation periods were probably due to the greater amount of degraded substrate accumulated over time,

corroborating the high and strong positive correlation ($r = 0.746$) between NH_3-N and IVOMD found.

SCFA, the products of rumen microbial fermentation and considered to be microbial excretion waste, are the main source of energy for ruminants. During fermentation, acetate, propionate, and butyrate are synthesised to a greater extent, coming mainly from the fermentation of dietary carbohydrates (Nozière et al. 2010). The predominance of microbial populations determines the composition of SCFA, and is highly dependent on the level of DMI and dietary composition, especially the nature and rate of carbohydrate degradation. Under physiological conditions, the decreasing order of SCFA production is acetate, propionate, and butyrate. However, diets rich in soluble (non-fibrous) carbohydrates increase the proportion of propionate, whereas those rich in fibrous carbohydrates increase the proportion of acetate (Wang et al. 2020). This second scenario would have been the most expected for the present study due to the increase in the inclusion levels of the legume, which is rich in fibrous carbohydrates; however, the production of SCFA and isoacids and the acetate: propionate ratio did not change. These results corroborate those found for PF, NH_3-N, and gas production, which were also not influenced by the inclusion levels of *C. argentea* hay, indicating that legume hay did not hurt the fermentation profile when substituted for Tifton-85 hay under the conditions of this study. As with PF, the greater availability of degraded substrates stimulating microbial growth and multiplication may explain the increasing values of SCFA and isoacids observed as the incubation period progressed.

A high proportion of concentrated feed in the diet tends to reduce the pH of rumen fluid due to the high rate of production of VFA and the lower buffering potential. This is an unfavourable condition for the activity of the cellulolytic microbiota, which has its cell membrane potential altered, requiring energy expenditure to maintain intracellular pH (Strobel and Russell 1986). On the other hand, it may favour the activity of amylolytic microorganisms and reduce the engulfment of bacteria by protozoa, contributing positively to microbial efficiency. The pH values found in this study were within the expected range, given that the diets consisted mainly of bulk feed (Van Soest 1994). This, in turn, plays a role in capturing H^+ ions from the medium, preventing sudden drops in pH. As the production of SCFA was not influenced by the levels of legume inclusion, the hypothesis of a drop in pH caused by the accumulation of SCFA in an in vitro fermentation system is ruled out. The significant increase in pH at 48 h of incubation does not seem to be of great importance, considering that it remained within the expected range for diets rich in roughage (6.2–7.0) (Owens and Goestsch 1988).

5 | Conclusion

C. argentea showed good in vitro fermentation parameters. Declines in degradability coefficients may occur as the fermentation period progresses, as substitution levels increase, but the presence of secondary compounds in the plant seems to contribute positively to reducing enteric CH_4 production. The results indicate that *C. argentea* hay can replace Tifton-85 hay in ruminant diets by up to 45%. However, in vivo studies are needed to corroborate the results.

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Ethics Statement

The procedures involving the use of animals in this study were approved by the Animal Use Ethics Committee of the "Luiz de Queiroz" School of Agriculture—CEUA/USP (Protocol 008-2018).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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