Fermentative profile, chemical composition, *in vitro* gas production, and ruminal degradation kinetics of sugarcane silages associated with increasing levels of butterfly pea hay

Perfil fermentativo, composición química, producción de gas y cinética de la degradabilidad ruminal in vitro de ensilajes de caña de azúcar asociados a niveles incrementales de heno de guisante mariposa

Perfil fermentativo, composição química, produção de gases in vitro e cinética da degradação ruminal de silagens de cana-de-açúcar associadas a níveis incrementais de feno de cunhã

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Abstract

Background: The ensiling process of sugarcane promotes yeast proliferation during fermentation, requiring the use of additives. *Clitorea ternatea* can be used as a natural additive in sugarcane silages to reduce dry matter losses and modifying the fermentation profile of the silage. **Objective:** To evaluate the fermentative profile, chemical composition, *in vitro* gas production and ruminal degradation kinetics of sugarcane silages associated with different levels of butterfly pea hay. **Methods:** Increasing levels of butterfly pea hay (0, 10, 20, and 30% on dry matter basis) were added to sugarcane silages. A completely randomized design was adopted, with four treatments and four repetitions, totaling 16 experimental silos that were opened after 60 days of ensiling. **Results:** Positive changes were observed in terms of fermentative losses, fermentative profile, chemical composition, *in vitro* gas production, and ruminal degradation kinetics with the addition of butterfly pea hay to sugarcane silage ($p<0.05$). **Conclusion:** The inclusion of up to 20% butterfly pea hay in sugarcane silage reduces fermentation losses and improves silage quality, such as increase in protein and energy content and reduction of the fibrous fractions of the silage, making silage an excellent ingredient to be included in ruminant diets.

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Keywords: *acetic acid; additives; chemical composition; diet; dry matter; ensilage; fermentative profile; forage; forage conservation; gas production; ruminants.*

Resumen

Antecedentes: El proceso de ensilaje de caña de azúcar promueve la proliferación de levaduras durante la fermentación, por lo que se requiere usar aditivos. La *Clitorea ternatea* se puede utilizar como aditivo natural en ensilajes de caña de azúcar para reducir la pérdida de materia seca y modificar el perfil de fermentación del ensilaje. **Objetivo:** Evaluar el perfil fermentativo, la composición química, la producción de gas *in vitro* y la cinética de degradación ruminal de ensilajes de caña de azúcar asociados con varios niveles de heno de guisante mariposa. **Métodos:** Se agregaron niveles incrementales de heno de guisante mariposa (0, 10, 20 y 30% con base a materia seca) a los ensilajes de caña de azúcar. Se adoptó un diseño completamente al azar, con cuatro tratamientos y cuatro repeticiones, totalizando 16 silos experimentales que se abrieron después de 60 días de ensilado. **Resultados:** Se observaron cambios positivos en las pérdidas fermentativas, perfil fermentativo, composición química, producción de gas *in vitro* y cinética de degradación ruminal con la adición de heno de guisante mariposa al ensilaje de caña de azúcar ($p<0,05$). **Conclusión:** La inclusión de hasta 20% de heno de guisante mariposa en el ensilaje de caña de azúcar reduce las pérdidas por fermentación y mejora la calidad del ensilaje, aumentando el contenido proteico y energético y reduciendo la fracción fibrosa del ensilaje de caña de azúcar, haciendo del ensilaje un excelente ingrediente a incluir en la dieta de rumiantes.

Palabras clave: *ácido acético; aditivos; composición química; conservación de forrajes; dieta; ensilado; forraje; materia seca; perfil fermentativo; producción de gas; rumiantes.*

Resumo

Antecedentes: A ensilagem da cana-de-açúcar promove a proliferação da levedura durante a fermentação, sendo necessário o uso de aditivos. *Clitorea ternatea* pode ser utilizado como aditivo natural em silagens de cana-de-açúcar atuando na redução da perda de matéria seca e modificando o perfil fermentativo da silagem. **Objetivo:** Avaliar o perfil fermentativo, composição química, produção de gases *in vitro* e cinética da degradação ruminal de silagens de cana-de-açúcar associadas a diferentes níveis de feno de cunhã. **Métodos:** Níveis incrementais de feno de cunhã (0, 10, 20 e 30% na matéria seca) foram adicionados às silagens de cana-de-açúcar. Adotou-se o delineamento inteiramente casualizado, com quatro tratamentos e quatro repetições, totalizando 16 silos experimentais que foram abertos após 60 dias de ensilagem. **Resultados:** Foram observadas alterações positivas nas perdas fermentativas, perfil fermentativo, composição química, produção de gases *in vitro* e cinética da degradação ruminal com a adição de feno de cunhã em silagens de cana-de-açúcar ($p<0,05$). **Conclusão:** A utilização de até 20% de cunhã em silagens de cana-de-açúcar reduz as perdas fermentativas e melhora a qualidade da silagem, com aumento do teor de proteína e energia e redução da fração fibrosa das silagens de cana-de-açúcar, tornando as silagens um excelente ingrediente a ser incluído nas dietas para ruminantes.

Palavras-chave: *ácido acético; aditivos; composição química; conservação de forragem; ensilagem; dieta; forragem; matéria seca; perfil fermentativo; produção de gás; ruminantes.*

Introduction

Due to seasonality of forage plants during the dry season, tropical forages do not provide sufficient nutrients for maintain productive response of small ruminants in arid and semiarid regions. Therefore, alternatives to meet the demand for roughage during this period, such as silage production, are necessary. Thus, storage of surplus forage produced during the rainy season for use in the dry season is a viable strategy (Amorim *et al.*, 2020).

Sugarcane (*Saccharum officinarum* L.) is widely used in tropical regions due to its high production of dry matter (DM; 25–40 t/ha) (Del Valle *et al.*, 2019), climate adaptability, and resistance to pests and diseases. Its high content of water-soluble carbohydrates (396 g/Kg DM) and buffering power (25.81%) allow the pH of the silage made with this forage plant to drop to values close to 3.5 due to generation of organic acids such as lactic acid. In addition, the high content of soluble sugars can result in proliferation of yeasts, which generate ethanol, and high loss of gases and effluents, resulting in low DM recovery (Reis *et al.*, 2022).

To reduce DM losses during the ensiling process, chemical and microbial additives are traditionally used in sugarcane ensiling (Rabelo *et al.*, 2019). Besides additives, mixed silages are capable to modify the fermentation profile and reduce DM losses. Compared to other additives, mix silages can result in even greater increase in chemical composition and degradability (Del Valle *et al.*, 2020). The effects depend on the forages chosen for the association with sugarcane in the ensiling process, as observed in the report by Carvalho *et al.* (2018), with the addition of *Manihot pseudoglaziovii*; Silva *et al.* (2014), with addition of *Atriplex numularia*; and Queiroz *et al.* (2015) with addition of *Typha domingensis*.

Butterfly pea (*Clitorea ternatea* Linn) is an excellent option as an absorbent additive for sugarcane ensilage. Butterfly pea belongs to the Fabacea tribe and originates from tropical Asia. It can be found in India, China, Africa, Central America, and Brazil. Its common names are telang flower, samsamping, dậu biếc, kordofan pea, guisante mariposa and cunhã. It is a perennial plant that can grow from 1 to 1,800 m.a.s.l., with rainfall between 650–1,250 mm, and temperatures above 27 °C (Pratiwi, 2022; Surya *et al.*, 2022). Butterfly pea is a legume with root nodules that play a role in fixing nitrogen in the soil, acting as a natural fertilizer in agricultural land (Suarna and Wijaya 2021). Its flowers can be white, lilac, light blue and dark blue, with corolla in normal and multilayered variations. The seeds are brown or green, olive green, 4-7 mm long and 3-4 mm wide (Pratiwi, 2022).

Due to its high dry matter content (286 to 291 g/Kg in fresh matter) (Araújo *et al.*, 2022) and crude protein (254.8 g/Kg DM) (Jusoh and Nur Hafifah, 2018), butterfly pea is widely used in diets for ruminants (Araújo *et al.*, 2022). In addition, it contains phenolic compounds, terpenes and alkaloids with antioxidant and bactericidal potential (Jaafar *et al.*, 2020; Hariad *et al.*, 2023) that can modify silage fermentation and improve the quality of the ensiled mass. Thus, butterfly pea hay can contribute to improve the nutritional and fermentative quality of sugarcane silages.

The association of butterfly pea with elephant grass provides a good fermentative profile of the silage, with reduced levels of butyric acid, ammoniacal nitrogen, total losses, and increased DM recovery, thus improving the nutritional value of silage (Costa *et al.*, 2022); however, it can reduce the *in vitro* digestibility of dry matter (Lemos *et al.*, 2021). Butterfly pea hay has been also associated with cactus pear meal in diets for goats. A mix of 67% butterfly pea hay and 33% cactus pear meal increased digestibility and weight gain of animals (Araújo *et al.*, 2022), increased carcass yield, and improved the fatty acid profile of goat meat (Pereira *et al.*, 2020) when compared to feeding a diet of 70% elephant grass. However, to the best of our knowledge, there is a scarcity of studies on the effects of including butterfly pea hay in sugarcane silage. Thus, our hypothesis is that butterfly pea hay promotes protein increase and improves fermentation kinetics in sugarcane silage.

Therefore, the aim of this study was to evaluate the fermentative profile, chemical composition, *in vitro* gas production, and ruminal degradation kinetics of sugarcane silage associated with different levels of butterfly pea hay.

Materials and Methods

Ethical considerations

This study was approved and certified by the Ethics Committee on Animal Use of the Federal University of Vale do São Francisco Univasf (protocol 0010/18042018), Brazil.

Experimental site

The experiment was conducted at the Federal University of Vale do São Francisco (Univasf), in Petrolina-PE, Brazil (9º 19 '28" South latitude, 40º 33' 34" West longitude, with an altitude of 393 m.a.s.l.). The climate is hot semi-arid with rainy season (BSh), with 376 mm average annual precipitation. Maximum and minimum temperatures during the experimental period were 33.83 and 24.56°C, respectively, with relative humidity between 50.50 and 73.56%.

Experimental design and elaboration of silages

Levels of butterfly pea hay inclusion (0, 10, 20 and 30% on dry matter basis) were evaluated in sugarcane silage, in a completely randomized experimental design, with four treatments and four repetitions, totaling 16 experimental silos.

The whole sugarcane plant used was cultivar VAT90212, harvested manually seven months after the last cut (regrowth). Butterfly pea (leaf, branches, petioles, and pods) was harvested manually at 90 days after planting (DAP), when the plants were at the flowering stage, cut at 10 cm from the ground. Butterfly pea hay elaboration was conducted in the field, by natural sun drying. After harvesting, before processing, butterfly pea (leaf, branches, petioles and pods) was spread over a plastic canvas to reduce losses, and remained under dehydration for 48 h, with the material being turned over after 24 h to uniform drying. During the night, to avoid losses, the material was covered with canvas. When the hay point (87.5% of dry matter) was obtained, the material was collected and stored in a dry place.

Sugarcane and butterfly pea hay were crushed in a stationary forage chopper (Nogueira PN PLUS 2000, São Paulo, Brazil) to an average particle size of approximately 2.0 cm. The material was manually mixed and ensiled in experimental silos (25 L capacity) equipped with a Bunsen valve to allow for fermentation gas escape. A total of 2 Kg of sand were deposited at the bottom of the silos and protected with a cotton fabric to prevent the ensiled material from meeting sand and allowing the effluent to drain.

Table 1. Chemical composition of sugarcane and butterfly pea hay.

Variable (g/Kg dry matter)	Sugarcane	Butterfly pea**	Butterfly pea hay
Dry matter*	259	829	875
Mineral matter	44	88.9	73
Ether extract	27	33.7	27
Crude protein	16	198	150
Neutral detergent fiber	622	448	470
Acid detergent fiber	360	254	319
Total carbohydrates	913	-	751
Non-fiber carbohydrates	291	-	281
Total digestible nutrients	574	-	603

*in g/Kg natural matter; **Araújo *et al.* (2022).

The material was compacted aiming to reach a minimum density of 600 Kg/m³ of natural matter. Samples of the non-ensiled material (original material) were collected for further laboratory analysis (Table 1). After being sealed, the silos were kept for 60 days in a covered shed.

Determination of density and fermentative losses of silages

The silos were weighed empty, after ensiling and weighed again at 60 days of ensiling, during opening. The density of ensiled mass was determined by the equation:

$$D\ (Kg/m^3) = m/V \quad \text{(Equation 1)}$$

where: D=density; m=weight of the ensiled material; V=volume of ensiled material.

Total dry matter losses (TDML) were obtained by adding the production of gases and effluents (Amorim *et al.*, 2020). Dry matter recovery (DMR), gas losses (GL), and effluent losses (EL) in silages were determined according to the equations proposed by Amorim *et al.* (2020):

$$DMR=((FMo*DMo) / (FMc*DMc))*100 \quad \text{(Equation 2)}$$

where: FMo=forage mass at the opening; DMo=dry matter content at the opening; FMc=forage weight at closing; DMc=dry matter content of forage at closing.

$$GL=((WSc - WSo)/FMs*FDMc)*100 \quad \text{(Equation 3)}$$

where: WSc=weight of the silo at closing, WSo=weight of the silo at opening, FMs=forage mass in silage; FDMc=forage dry matter content at silo closure (Amorim *et al.*, 2020).

$$EL=((WSSo - WSS)/GMSF)*100 \quad \text{(Equation 4)}$$

where: WSSo=weight of the set (silo + sand + screen) at the opening; WSS=weight of the set (silo + sand + screen) in the silage; GMSF=green mass of silage forage.

Fermentation profile of silages

Sample pH was measured with a portable digital pH meter (Marconi® MA-552, Piracicaba, São Paulo, Brazil) immediately after opening the silos and collecting the material. Organic acids concentration: acetic (AA), propionic (PA), and lactic (LA), was measured according to the methodology of Kung Jr and Ranjit (2001). In 2 mL of filtrate, 1 mL of metaphosphoric acid 20% v/v was added, and this sample was centrifuged. Analyzes of organic acids were performed by high performance liquid chromatography (HPLC).

Chemical composition of silages

Silage samples were collected during the opening of silos, with the top layer (10 cm) of each silo being discarded. Samples were pre-dried in a forced-ventilation oven at 55 °C for 72-h. Then, they were individually processed in a knife mill (Wiley, Marconi, MA 580, Piracicaba, Brazil) at 3 mm mesh sieve to determine gas production and *in vitro* degradability, and at 1 mm mesh sieve to determine dry matter (DM; method 967.03), mineral matter (MM; method 942.05), crude protein (CP; method 981.10), ether extract (EE; method 920.29), and acid detergent fiber (ADF; method 973.18) (AOAC 2016). Neutral detergent fiber (NDF) was determined according to Van Soest *et al.* (1991). Klason lignin (KL) content was determined according to Theander and Westerlund (1986), and total digestible nutrients (TDN) was obtained with the equation proposed by Harlan *et al.* (1991):

$$TDN=82.75 – (0.704*ADF) \quad \text{(Equation 5)}$$

Total carbohydrates (TC) were measured according to Sniffen *et al.* (1992):

$$TC\ (g/Kg\ DM)=1000 – (CP + EE + MM) \quad \text{(Equation 6)}$$

Non-fiber carbohydrates (NFC) were measured according to Hall (2003):

$$NFC\ (g/Kg\ DM)=1000 – (\%\ CP +\%\ EE +\%\ MM + NDF) \quad \text{(Equation 7)}$$

In vitro gas production

Gas production and rumen degradation were carried out according to the methodology proposed by Menezes *et al.* (2015). The inoculum was obtained from rumen fluid jointly and homogenized from two ruminally fistulated cattle which were fed a diet with 70% sugarcane and 30% concentrate based on cottonseed meal and ground corn and offered water *ad libitum*. Ruminal inoculum was collected through the cannula and stored in an anaerobic environment in a thermal bottle. The solid part was collected from the rumen through the cannula and manually pressed to separate the solid from the liquid part. Ruminal inoculum was filtered through four layers of gauze, constantly injecting CO_2 to maintain the anaerobic environment, and kept in a water bath at 39 °C.

One gram of sample was added to glass vials (160 mL), to which 90 mL nutrient medium (buffer solution, pH indicator solution, macro, and micro mineral solution, 1 molar sodium hydroxide solution, and reducing solution) was added. Subsequently, 10 mL of ruminal fluid was added to each flask, which was kept under a CO_2 atmosphere. Then they were sealed with rubber stoppers and aluminum seals. The same procedure was applied to the blanks (flask containing inoculum and medium, without samples). Four flasks were used as a blank. The pressure (P; in psi) originated by the gases accumulated in the upper part of the vials was measured with a portable pressure transducer (GE Druck Series DPI 705) connected at its end to a needle (0.6 mm). Pressure readings were taken more frequently during the initial fermentation period and subsequently reduced (2, 4, 6, 8, 10, 12, 14, 17, 20, 24, 28, 34, 48, 72, and 96 h of incubation).

Pressure data were converted to gas volume (1 psi=4.859 mL gas). From each pressure reading, the total produced by the bottles without substrate (blank) was subtracted from each sample. Cumulative gas production data were analyzed by the Gompertz two-compartment model (Schofield *et al.*, 1994):

$$V=Vf1 / (1 + \exp (2 - 4*m1*(T - L))) + Vf2 / (1 + \exp (2 - 4*m2*(T - L))) \quad \text{(Equation 8)}$$

where: V=total gas volume; Vf1=maximum volume of gas production from non-fibrous carbohydrates; Vf2=maximum volume of gas production from fibrous carbohydrates; m1=degradation rate (%/h) of the fraction of non-fibrous carbohydrates; m2=rate of degradation (h) of the fibrous carbohydrate fraction; T=incubation time (h); L=time of colonization (h).

The Gompertz two-compartment model was chosen assuming that gas production rate is proportional to microbial activity, but proportionality decreases with incubation time, which can be attributed to the loss of efficiency of fermentation rate in time (Cunha *et al.*, 2022).

In vitro ruminal degradation kinetics

In vitro DM degradability was estimated by inserting nylon bags (20 mg cm-2 weight and 50 microns of porosity) containing 600 mg sample in flasks with 60 mL buffer solution (combination of solutions A + B with pH 6.8) and 15 mL ruminal inoculum. Samples were incubated for 0, 2, 6, 12, 24, 48, 96 and 120 h. After *in vitro* fermentation, bags were washed, and oven dried at 105 ºC for 4-h and weighed. Samples at time 0 were just washed with distilled water at 39 °C for 5 minutes and then dried and weighed (Tilley and Terry 1963).

To determine potential degradability (PD), effective degradability (ED), and the non-degradable fraction C, the Ørskov and McDonald (1979) models were used:

$$PD=A + B (1 -\exp-ct) \quad \text{(Equation 9)}$$

where: PD=is potential degradability; A=is the water-soluble fraction; B=is the water-insoluble fraction, but potentially degradable; c=is the degradation rate of fraction B and t=is incubation time (h).

$$ED=a + (b*c) / (c + kp) \quad \text{(Equation 10)}$$

where: kp=is the rate of passage (a 5%/h pass rate was admitted).

The undegradable fraction (C) was calculated with the following equation:

$$C=100 - (A + B) \text{ (Equation 11)}$$

Gas production rate obtained by the semi-automated gas production technique (m1+m2) was used to estimate the rate of passage used in the degradability test.

Statistical analysis

Data were subjected to the normality test and analysis of variance. When significant, the parameters of the regression equations were determined by GLM and REG procedures, respectively, with 5% significance. The results were analyzed with the SAS program, version 9.4 (2013) software (Statistical Analysis System; SAS Institute Inc., Cary, NC, USA; 2013).

Results

Increased levels of butterfly pea hay in sugarcane silage provided a linear reduction in density ($p<0.001$) and EL ($p<0.001$) silages (Table 2). Quadratic effect was observed for GL ($p<0.001$), TDML ($p<0.001$), and DMR ($p<0.001$), with the increase in the levels of butterfly pea hay in the sugarcane silage (Table 2). The pH ($p<0.001$) and PA ($p<0.001$) of the sugarcane silages increased linearly according to increased inclusion of butterfly pea hay (Table 2).

A quadratic effect was observed for AA ($p<0.001$) and TFA ($p<0.001$) content in silages of sugarcane associated with increasing levels of butterfly pea hay (Table 2). There was no effect of the inclusion of butterfly pea hay in sugarcane silage on LA ($p>0.05$) (Table 2).

In relation to chemical composition, the DM ($p<0.001$), MM ($p<0.001$), CP ($p<0.001$), KL ($p<0.001$) and TDN ($p<0.001$) content increased linearly as the proportions of butterfly pea hay increased in the composition of sugarcane silages (Table 3). The opposite effect was observed NDF ($p<0.001$) and ADF ($p<0.001$), whose content decreased linearly according to increased levels of butterfly pea hay in the composition of the sugarcane silages (Table 3).

Table 2. Density, losses, and fermentative profile of sugarcane silages with increasing levels of butterfly pea hay.

Variable	Butterfly pea hay levels (%DM)				SEM	P-value	
	0	10	20	30		L	Q
Density (Kg/m^3)	566.35	434.90	405.75	398.55	17.57	<0.0011	<0.511
Gas losses (g/Kg DM)	89.0	131.8	103.0	74.0	0.56	<0.341	<0.0012
Effluent losses (Kg/ton NM)	25.54	6.07	3.63	2.45	2.43	<0.0013	<0.3201
Total dry matter losses (g/Kg DM)	112.8	137.3	106.5	76.5	0.57	<0.201	<0.0014
Dry matter recovery (g/Kg DM)	887.1	862.8	893.7	923.5	0.57	<0.425	<0.0015
pH	3.27	3.64	3.74	3.86	0.06	<0.0016	<0.451
Lactic acid (g/Kg DM)	4.54	4.57	4.62	4.73	0.05	0.257	0.729
Acetic acid (g/Kg DM)	5.11	10.30	11.60	10.15	6.69	<0.471	<0.0017
Propionic acid (g/Kg DM)	0.47	0.64	1.08	1.40	0.10	<0.0018	0.175
Total fatty acids (g/Kg DM)	5.58	10.94	12.68	11.55	6.75	<0.121	<0.0019

SEM: Standard error of the mean; DM: Dry matter; NM: Natural matter; L: Linear effect; Q: Quadratic effect. Significant at the 5% probability level. Equations: [1]$\hat{y}$=531.27-5.325x, R2=0.77; 2$\hat{y}$=9.256+0.465x-0.018x², R2=0.83; $\hat{y}$3=20.181-0.717x, R2=0.72; $\hat{y}$4=11.555+0.269x-0.014x², R2=0.88; $\hat{y}$5=88.423-0.265x+0.013x², R2=0.88; $\hat{y}$6=3.345+0.019x, R2=0.89; $\hat{y}$7=51.675+6.611x-0.166x2, R2=0.91; $\hat{y}$8=0.415+0.033x; R2=0.92; $\hat{y}$9=52.127+6.634x-0.165x2, R2=0.91.

Table 3. Chemical composition of sugarcane silages associated with increasing levels of butterfly pea hay.

Variable (g/Kg dry matter)	Butterfly pea hay levels (%DM)				SEM	P-value	
	0	10	20	30		L	Q
Dry matter*	205.7	282.8	347.6	413.0	1.99	<0.0011	0.026
Mineral matter	52.8	61.6	62.6	63.9	0.12	<0.0012	0.003
Crude protein	18.2	47.2	67.2	82.2	0.62	<0.0013	<0.001
Neutral detergent fiber	740.6	679.8	626.9	599.7	1.40	<0.0014	0.0003
Acid detergent fiber	461.3	431.6	425.7	386.3	0.72	<0.0015	0.316
Klason lignin	74.2	81.8	85.4	88.0	0.14	<0.0016	0.061
Total digestible nutrients	497.7	524.1	530.3	553.5	0.52	<0.0017	0.526
Acetic acid (g/Kg DM)	5.11	10.30	11.60	10.15	6.69	<0.471	<0.0017
Propionic acid (g/Kg DM)	0.47	0.64	1.08	1.40	0.10	<0.0018	0.175
Total fatty acids (g/Kg DM)	5.58	10.94	12.68	11.55	6.75	<0.121	<0.0019

*g/Kg natural matter; SEM: Standard error of the mean; L: Linear effect; Q: Quadratic effect. Significant at the 5% probability level. Equations: ŷ1=20.927+0.686x, R2=0.99; ŷ2=5.509+0.034x, R2=0.67; ŷ3=2.199+0.212x, R2=0.97; ŷ4=73.306-0.476x, R2=0.96; ŷ5=46.086-0.231x, R2=0.86; ŷ6=7.561+0.045x, R2=0.82; ŷ7=50.038+0.174x, R2=0.91.

For the gas production kinetics and ruminal degradability *in vitro*, the increase in the levels of butterfly pea hay in the composition of the sugarcane silages increased the Vf1 ($p<0.001$), A ($p<0.001$), Kd ($p<0.001$), and IVDMD ($p<0.001$), reduced L ($p<0.001$) and B ($p<0.001$) (Table 4) and promoted a quadratic effect for Vf2 ($p<0.001$), V ($p<0.001$), C ($p<0.001$), PD ($p<0.001$), ED ($p<0.001$), and pH ($p=0.002$) (Table 4).

Higher cumulative rates of *in vitro* gas production were observed in the initial incubation times. In 48 h of incubation, the average values of cumulative gas production were close between the control treatment and the sugarcane silages containing 10 and 20% butterfly pea hay. Sugarcane silages containing 10, 20 and 30% butterfly pea hay stabilized the cumulative gas production at 72 and 96 h of incubation, while the control treatment (0% butterfly pea hay) continued to increase gas production (Figure 1).

Discussion

The association of butterfly pea hay with sugarcane in mixed silages increased the dry matter content (Table 1). This increase difficulted the process of compaction, reducing silage density. A similar result was reported by Reis *et al.* (2022) who, by including *Moringa oleifera* hay in sugarcane silage, increased DM content (from 253.1 to 441.1 g/Kg NM) and reduced silage density (from 503.9 to 408.3 Kg/m^3) with increasing levels of *Moringa oleifera*. The increased DM content -reduced moisture- allowed for proper fermentation of the ensiled material, which possibly reduced the development of yeasts (which consume water-soluble carbohydrates and produce ethanol, CO_2, water, and ATP, generating losses) (Auerbach *et al.*, 2020; Kim *et al.*, 2021), thus improving DMR at increasing levels of butterfly pea. In addition, butterfly pea hay acted as a hygroscopic barrier absorbing the effluent generated by sugarcane fermentation, preventing it from leaching, thus reducing effluent losses.

Gas losses derive from carbohydrate and protein fermentation, which results in the production of CO_2, N_2O and N-NH_3, representing most of the TDML. The fermentative profile of ensiled material influences gas losses (Zanine *et al.*, 2020). The highest GL were obtained with 12.94% butterfly pea hay inclusion, representing a loss of 122.6 g/Kg DM. The lowest GL was obtained with 30% inclusion of butterfly pea hay in the silage.

Table 4. *In vitro* gas production and ruminal degradation kinetics of sugarcane silages with increasing levels of butterfly pea hay inclusion.

Variable	Butterfly pea hay levels (%DM)				SEM	P-value	
	0	10	20	30		L	Q
Vf1 (mL/g DM)	56.32	60.47	60.31	64.67	3.34	<0.0011	0.892
Vf2 (mL/g DM)	37.99	20.90	19.55	21.45	7.91	<0.371	<0.0012
V (ml/g DM)	94.31	81.37	79.86	86.12	5.92	<0.561	<0.0013
L (h)	17.55	15.69	13.45	10.47	2.81	<0.0014	0.157
A (g/Kg DM)	152.0	191.8	194.9	192.2	1.87	<0.0015	<0.521
B (g/Kg DM)	457.0	469.5	367.9	355.5	5.32	<0.0016	0.183
C (g/Kg DM)	391.0	338.8	437.2	452.3	4.64	<0.001	<0.0017
Kd (%/h)	0.048	0.070	0.075	0.088	0.02	<0.0018	0.199
PD (g/Kg DM)	609.0	661.3	562.8	547.7	4.64	<0.421	<0.0019
ED (g/Kg DM)	375.8	462.5	414.3	418.7	3.33	0.278	<0.00110
pH	6.64	6.96	6.92	6.92	0.04	0.129	0.00211
IVDMD (g/Kg)	358.3	364.4	381.0	452.7	0.88	<0.00112	<0.291

Vf1: Maximum volume of gas production from non-fibrous carbohydrates; Vf2: Maximum volume of gas production of fibrous carbohydrates; V: Total gas volume; L: Time of colonization (h); A: Water-soluble fraction; B: Water-insoluble fraction, but potentially degradable; C: Non-degradable fraction; PD: Potential degradability; ED: Effective degradability; IVDMD: *In vitro* dry matter degradability; DM: dry matter; SEM: Standard error of the mean; L: Linear effect; Q: Quadratic effect; Significant at the 5% probability level. Equations: $\hat{y}1=56.708+0.249x$, $R^2=0.74$; $\hat{y}2=37.3647-1.934x+0.048x2$, $R^2=0.93$; $\hat{y}3=94.122-1.701x+0.048x2$, $R^2=0.96$; $\hat{y}4=17.812-0.235x$, $R^2=0.93$; $\hat{y}5=16.419+0.127x$, $R^2=0.58$; $\hat{y}6=47.335-0.406x$, $R^2=0.79$; $\hat{y}7=37.929-0.223x+0.017x2$, $R^2=0.63$; $\hat{y}8=0.052+0.0013x$, $R^2=0.78$; $\hat{y}9=62.071+0.223x-0.017x2$, $R^2=0.63$; $\hat{y}10=38.518+0.697x-0.021x2$, $R^2=0.48$; $\hat{y}11=6.663+0.032x-0.00081x2$, $R^2=0.67$; $\hat{y}12=34.413+0.299x$, $R^2=0.76$.

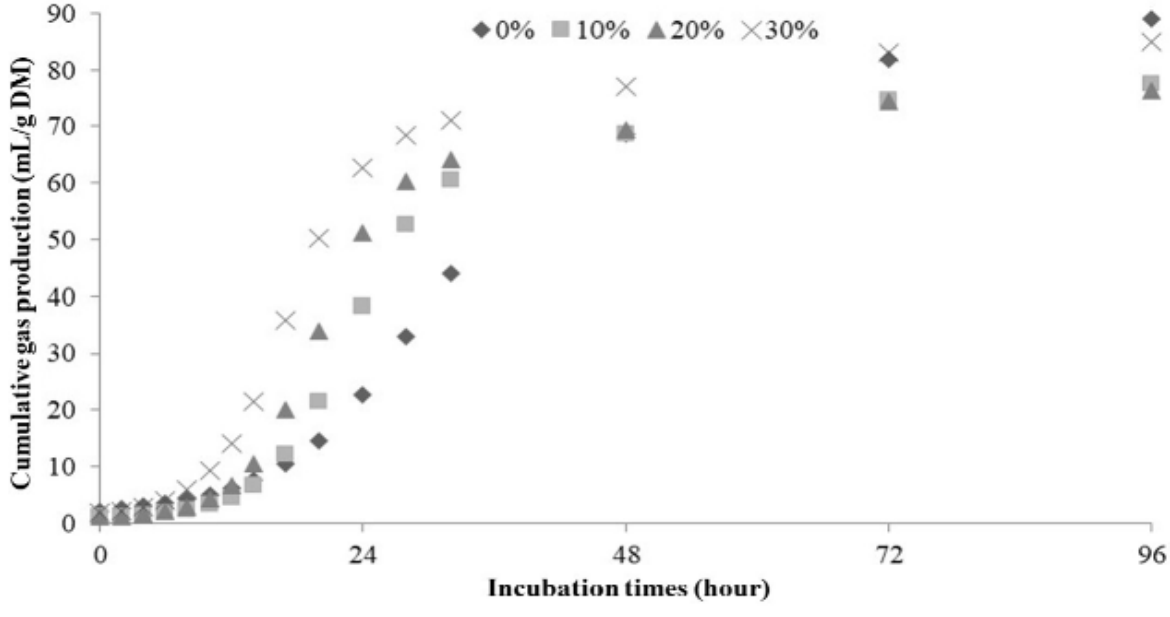

Figure 1. Cumulative gas production (mL/g DM) at different incubation times of sugarcane silages associated with butterfly pea hay levels (0, 10, 20 and 30% on dry matter basis).

The efficiency in reducing GL with the greatest inclusion of butterfly pea hay may be related to greater production of acetic acid, which, according to Ávila and Carvalho (2020), is negatively correlated with CO_2 production inside the silo, which may indicate low occurrence of gas-producing microorganisms such as clostridia and possible absence of secondary fermentations (Costa *et al.*, 2022). Corroborating our findings, when Costa *et al.* (2022) included butterfly pea in elephant grass silage also observed reduced GL compared to exclusive elephant grass silage.

Addition of butterfly pea hay increased the pH of sugarcane silage. However, the pH value considered adequate for properly fermented silages (3.8 to 4.2) (Pereira *et al.*, 2019) was only observed for sugarcane silages containing 30% butterfly pea hay. This increase could possibly be related to reduce levels of soluble carbohydrates in sugarcane silage with increasing butterfly pea hay proportions. In addition, by increasing pH and osmotic pressure of the environment (Figueiredo *et al.*, 2022), a greater inclusion of butterfly pea hay in the sugarcane silages changed the environment -previously favorable to the development of yeasts- becoming inappropriate, thus reducing GL. As butterfly pea has antioxidant and bactericidal substances (Jaafar *et al.*, 2020) such as anthocyanins, anthocyanidin, and flavanol (Multisona *et al.*, 2023), these compounds may have inhibited growth of undesirable microorganisms during fermentation, resulting in decreased ethanol production and fermentative losses.

Contrarily, Carvalho *et al.* (2014) observed a pH reduction (3.7 – 3.4) in sugarcane silages at increasing levels of *Manihot pseudoglaziovii*. They emphasized that pH is not a good indicator of quality for sugarcane silage when considered in isolation, since the main concern when ensiling sugarcane is the occurrence of yeasts which develop even at low pH, and ethanol itself can act as microbial inhibitor; therefore, control of losses and ethanol production should be the focus in sugarcane silage.

Among the fatty acids evaluated, butyric acid concentration was below the detection limit. Acetic acid is a potential inhibitor of undesirable fungi and yeasts in silage. High levels of AA in sugarcane silage containing butterfly pea hay may result from enterobacteria and secondary fermentations in the silos. Acetic acid increased with inclusion of up to 20% butterfly pea hay; however, this concentration decreases as the fermentation process progresses. Studying mixed sugarcane silage with forage peanut, Costa *et al.* (2022) observed that AA increased up to 50% forage peanut inclusion in the sugarcane silages, then declined. They emphasized that high AA content (above 2%) is desirable for silages rich in water-soluble carbohydrates, as they decrease yeast activity, reducing gas losses and improving aerobic stability after opening the silos. Thus, addition of up to 20% butterfly pea hay in sugarcane silage could be an alternative to reduce yeast activity during fermentation in sugarcane silage since microorganisms represent one of the main problems when ensiling this crop. However, with mean values between 10.15 and 11.60 g/Kg DM, AA concentrations in all silages containing butterfly pea hay are considered acceptable, according to Santos *et al.* (2020).

Propionic acid is associated with conversion of lactic acid to acetic acid and 1,2-propanediol, which in turn is converted to PA and 1-propanol by naturally occurring microorganisms in silages (Gonzalez-García *et al.*, 2017). Increased butterfly pea hay levels in sugarcane silage increased the PA content in silages from 0.47 (control) to 1.40 g/Kg DM, staying below 5 g/Kg DM, without affecting silage quality (Borreani *et al.*, 2018). As butterfly pea has high buffering capacity (57.25 e.mg/100 g DM) (Lemos *et al.*, 2021) heterofermentative species possibly increased -corroborated by PA increase- which have antifungal properties and inhibit yeast growth and, consequently, decrease ethanol production (Costa *et al.*, 2022). However, as ethanol was not quantified in this study, additional research is needed.

The increase in DM, MM, CP, and TDN contents in silage is directly related to the

nutritional characteristics of butterfly pea hay in relation to sugarcane (Table 1). This result was already expected and corroborates previous research by Carvalho *et al.* (2018), Costa et al. (2022) and Reis *et al.* (2022) by including legumes in the composition of sugarcane silage.

The DM value of sugarcane silage associated with 20% butterfly pea hay is within the limit of 30 to 35% established by McDonald *et al.* (1991) for good forage fermentation. Dry matter values below 30% make the silage susceptible to the action of undesirable microorganisms, promoting effluent losses. Percentages above 35%, make compaction difficult, causing undesirable phenomena due to air entering the silo, which was verified for control, 10, and 30% butterfly pea hay inclusion.

Mixed silages intercropping legumes and grass forage plants aims to increase CP content of the silage. Since sugarcane has low CP content (16 g/Kg DM) (Table 1), protein content of the silage was favored by butterfly pea hay, which has 150 g/Kg DM (Table 1). However, only with 30% butterfly pea hay inclusion in the sugarcane silage it was possible to obtain the necessary CP content (70 g/Kg DM) (Pereira *et al.*, 2019) for adequate ruminal fermentation.

Reduction in NDF and ADF content in the silages was promoted by adding butterfly pea hay due to reduction in the sugarcane: butterfly pea hay ratio; the concentration of digestible components probably increased. Carvalho *et al.* (2018) also observed a reduction of NDF and ADF contents of sugarcane silages by including *Manihot pseudoglaziovii*. The highest concentration of NDF and ADF was found in the silage not added with butterfly pea hay (Control) (Table 3), which would be related to sugarcane composition (Table 1).

Lignin behaved opposite to other cell wall components. Lignin content is an important parameter to be considered because it is the main limiting factor during degradation of the fibrous fraction of silages (Pereira *et al.*, 2019). Even with the reduction of NDF, with increased inclusion of butterfly pea hay, the result is a linear increase in lignin that influenced the quality of potentially degradable fraction (fraction B) and non-degradable fraction (fraction C) (Table 4). This reduction is explained by the lower concentration of structural carbohydrates in the legume cell wall compared to the grass, contributing to improve silage digestibility (Silva *et al.*, 2018). Agreeing with our results, Hawu *et al.* (2022) also observed that legume hay addition to corn silage reduced fiber concentration, probably due to hemicellulose hydrolysis into monosaccharides, which provides extra carbohydrates for generating lactic acid during fermentation. Our results are like the maximum NDF limit recommended by Van Soest (1994) for ruminant diets, which is 60% NDF.

Butterfly pea hay increased cumulative gas production from non-fibrous carbohydrates in sugarcane silage, as well as fraction A concentration. Regarding gas production from the fibrous fraction, inclusion of up to 20% butterfly pea hay reduced gas production as well as fraction B concentration; however, gas production increased at 30% butterfly pea hay inclusion due to increased degradation of this fraction, which was reflected in the total volume of gas produced. This is possibly associated with the fibrous carbohydrate content, which is related to the rate of degradation and the time of colonization by ruminal microorganisms (Ribeiro *et al.*, 2019).

The latency phase is the time between the beginning of incubation and the microbial action on the substrate. Latency depends on the presence of readily fermentable substrates and chemical characteristics of the sample, which can favor microbial fermentation (Hamill *et al.*, 2020). Addition of butterfly pea hay to sugarcane silage reduced the latency period, which is positive, since microorganisms will adhere to the particles more quickly, promoting rapid fiber degradation (Yansari *et al.*, 2017). According to Xue *et al.* (2020), the energy used by microorganisms during the first hours of incubation comes from fermentation

of non-fibrous carbohydrates, which are readily available for degradation, resulting in shorter fermentation time. After reducing non-fibrous carbohydrates, fermentation of fibrous carbohydrates continues, since they are fermented more slowly.

Although IVDMD increased linearly due to increased soluble fraction (A), potential and effective degradability were influenced by increase in fraction C and remained below levels considered adequate for satisfactory degradability (Magalhães *et al.*, 2019).

In conclusion, inclusion of butterfly pea hay in sugarcane silage improves fermentation, loss reduction, and nutritional value. Inclusion of up to 20% butterfly pea hay in sugarcane silage reduces fermentation losses, improving silage quality: specifically, by increasing protein and energy content and reducing the fibrous fractions of sugarcane silage.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

Conceptualization: MAAQ, DRM; data acquisition and design of methodology: EJNR, BASA, APRS, TSSN; data analysis: EJNR, MAAQ; writing original draft: EJNR, MAAQ, GCG; writing-review and editing: MAAQ, GCG.

Use of artificial intelligence (AI)

No AI or AI-assisted technologies were used during the preparation of this work.

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