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IN VITRO FERMENTATION AND GAS PRODUCTION OF OILSEED PRESS CAKE

FERMENTACIÓN Y PRODUCCIÓN DE GAS *IN VITRO* DE TORTAS DE SEMILLAS OLEAGINOSAS

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ABSTRACT

The utilization of byproducts from biodiesel production is an alternative for animal feed that allows for an increased nutritional value of the diet at a low cost and reduce green house gases (GHG). The objective of this study was to evaluate the production of volatile fatty acids (VFAs) and gases of biodiesel production byproducts in replacement of elephant grass. We used elephant grass (*Pennisetum purpureum*) and the following byproducts: soybean meal [SBC, Family Fabaceae (alt. Leguminosae; *Glycine max*)], canola cake (CC, Family Brassicaceae; *Brassica napus* L.), sunflower cake (SC, Family Asteraceae; *Helianthus annuus*), and fodder radish cake (FRC, Family Brassicaceae; *Raphanus sativus* L.) using an *in vitro* semiautomatic gas production system and four different levels (0, 30, 50 and 70%) of elephant grass hay (EGH) replacement. The inoculum for the *in vitro* incubation was obtained from three Holstein cows with rumen fistulas. Gas production was measured 3, 6, 12, 24 and 48 hours after incubation. The experimental design was completely randomized in a 4×4 factorial arrangement with the factors being the byproduct type and levels of inclusion in diet. Ruminal incubation using a 0.3-g sample in buffered culture medium was performed in order to determine the yield of methane (CH_4), carbon dioxide (CO_2) and ammoniacal nitrogen (N-NH_3), as well as pH and concentration of volatile fatty acids (VFAs). The production of gas increased ($p < 0.05$) with increasing levels of diet substitution. The addition of different levels of byproducts influenced ($p < 0.05$) the production of CH_4, CO_2 and NH_3 of VFAs, and the molar concentration of acetic, propionic and butyric acids. The acetate:propionate ratio and pH were affected ($p < 0.05$) by the inclusion of studied byproducts. The byproducts of soybean generated greater gas production and VFAs, while the lowest yield was observed with the byproduct fodder radish in the highest level tested (70%).

Key words: animal nutrition, byproducts, methane, ruminal fermentation

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RESUMEN

La utilización de los subproductos de la producción de biodiesel es una alternativa para la alimentación animal, por cuanto permite un mayor valor nutricional de la dieta a un bajo costo y reducir los gases de efecto invernadero (GEI). El objetivo de este estudio fue evaluar la producción de ácidos grasos volátiles (AGV) y la producción de gas de los subproductos de la producción de biodiesel en sustitución del pasto elefante. Se utilizó pasto elefante (*Pennisetum purpureum)* y los siguientes subproductos: harina de soja [SBM, Familia Fabaceae (alt. Leguminosae; *Glycine max)*], canola (Familia Brassicaceae; *Brassica napus* L), girasol (Familia Asteraceae; *Helianthus annuus*) y nabo forrageiro (fodder radish) (Familia Brassicaceae; *Raphanus sativus* L) utilizando un sistema de producción de gas in vitro semiautomático y cuatro niveles (0, 30, 50 y 70%) de reemplazo de pasto elefante. El inóculo para la incubación in vitro se obtuvo de tres vacas Holandés con fístulas en el rumen. La producción de gas se midió 3, 6, 12, 24 y 48 horas después de la incubación. El diseño experimental fue completamente al azar en un arreglo factorial 4 × 4; los factores fueron el tipo de subproducto y niveles de inclusión en la dieta. Para determinar el rendimiento de CH_4, CO_2 y NH_3 y para determinar concentración de los ácidos grasos volátiles (AGV) y pH ruminal. Se realizó la incubación ruminal usando una muestra de 0,3 g en medio de cultivo tamponado. La producción de gas aumentó ($p < 0,05$) con el aumento de los niveles de sustitución de la dieta. La adición de diferentes niveles de subproductos influenciado ($p < 0,05$) la producción de CH_4, CO_2 y NH_3 de AGVs, y la concentración molar de los ácidos acético, propiónico y butírico. La relación acetato:propionato y pH fueron afectados ($p < 0,05$) mediante la inclusión de los subproductos estudiados. Los subproductos de soja generaron una mayor producción de gas y AGVs, mientras que el rendimiento más bajo se observó con nabo forrageiro en el nivel más alto que se usó (70%).

Palabras clave: nutrición animal, fermentación ruminal, metano, subproductos

INTRODUCTION

The consumer market has undergone major changes, requiring animal production systems that offer both direct benefits, such as palatable food, adequate nutritional value and food safety, and indirect benefits, such as promoting animal welfare and environmental sustainability (Main et al., 2014). Brazil is a country in which the main food of ruminants is pasture, which makes Brazil a major worldwide producer of methane gas, contributing to the greenhouse effect. The study of byproducts from biodiesel production as an alternative for animal feed allows for an increased nutritional value of the diet at a low cost (Moreira et al., 2014).

The cattle industry is a major producer of GHG due to the emitted carbon dioxide (CO_2) and methane (CH_4). This process is associated with the large number of ruminants in the world, especially cattle, which produce large amounts of CH_4 during food digestion (Goel and Makkar, 2012; Hristov et al., 2013).

The changes made to ruminant feed in order to mitigate methane also alter the rumen microorganisms (fungi, protozoa and bacteria) that are responsible for the fermentation and synthesis of nutrients. Additionally, the amount of volatile fatty acids (VFAs) produced or synthesized by the ruminal microbiota also changes. VFAs are the main source of energy for ruminants, supplying over 85% of the energy required by the animal according to Van Soest et al. (1991). The rumen produces acetic, propionic and butyric acid through microbial fermentation of carbohydrates or, in certain cases, protein (Oliveira et al., 2013).

In this scenario, byproducts from the biodiesel production chain in Brazil have been studied as potential ingredients for ruminant diets. Studies and techniques that characterize the ruminal metabolism of these byproducts, such as *in vitro* gas production, are needed to identify potential ingredients that could be used efficiently in the diet of ruminants to replace conventional ingredients (Mizubuti et al., 2011; Olivares-Palma et al., 2013). Therefore, this study aimed at evaluating the production of volatile fatty acids (VFAs), CH_4, CO_2, and amoniacal nitrogen ($N\text{-}NH_3$) and the ruminal pH of diets including byproducts resulting from the industrial production of biodiesel chain as replacement of elephant grass in cattle feed.

MATERIALS AND METHODS

Feeds and substrates

This research was conducted at the Experimental Station of Coronel Pacheco, Minas Gerais State (21°35′ S, 43°15′ W; 435 m above sea level), which is owned by Embrapa Gado de Leite - CNPGL in Minas Girais, Brazil.

Four different oilseed press cakes of plants commonly used for biodiesel production were tested in the *in vitro* study. Cakes were soybean cake [SBM, Family Fabaceae (alt. Leguminosae; *Glycine max*)], canola cake (Family Brassicaceae; *Brassica napus* L.), sunflower cake (Family Asteraceae; *Helianthus annuus*), and fodder radish cake (Family Brassicaceae; *Raphanus sativus* L.), while elephant (*Pennisetum purpureum*) fresh cutted was used as a control.

The oilseed press cakes were obtained from Embrapa Dairy Cattle in Juiz de Fora, Minas Gerais State, Brazil. The cakes were obtained by double pressing of whole grains mechanical press-type "expeller" stainless steel, with capacity for extraction 150 kg h^{-1}. It was obtained with average temperatures of cake output between 90 and 110°C. Seeds and cakes were homogenized in an analytical laboratory (IKA model A11 basic) and stored at -18°C until analysis completion.

Elephant grass (*Pennisetum purpureum*) was harvested at 60 d of re-growth at the EMBRAPA Dairy Cattle Research Center, Coronel Pacheco, MG, Brazil.

In vitro incubations

The substrates that were used for the *in vitro* incubations were elephant grass (*Pennisetum purpureum*) supplemented with oilseed press cake at ratios of 100/0, 70/30, 50/50 and 30/70% (elephant grass/byproduct) for each treatment. The feed ingredients were dried at 55°C for 24 h and then ground to pass through a 1-mm screen. Each *in vitro* incubation was conducted according to Meale et al. (2012). The entire incubation procedure was repeated twice (*i.e.,* two incubation runs × three replicates per treatment, resulting in a total of six replicate vials per treatment).

The inoculum for the *in vitro* incubation was obtained from three ruminally fistulated cows grazing elephant grass (*Pennisetum purpureum*) that was supplemented with 2 kg of concentrate (22 g kg^{-1} crude protein (CP) and 12.6 g kg^{-1} neutral detergent fiber (NDF) in dry matter (DM). The rumen fluid was collected 2 h before the morning milking from 4 distinct sites in the rumen, filtered through 4 layers of cheesecloth, combined in equal portions from each animal and immediately transported in a pre-warmed flask (Thermos®) to the laboratory. The inoculum was prepared by mixing the rumen fluid and a mineral buffer with 0.5 mL of cysteine sulfide solution (Vitti et al., 1999) in a ratio of 1:5. The inoculum (30 mL) was then transferred into pre-loaded, pre-warmed (39°C) vials under a stream of O_2-free N gas. The vials were sealed and placed on an orbital shaker rack (Crystal SilentShaker®, SYC-2102A, SP, Brazil) at 120 oscillations per min in an incubator (Bibby Scientific Limited, Beacon Road, Stone, Staffordshire, ST15 OSA, UK) at 39°C.

Net gas production of each vial was measured at 6, 12, 24 and 48 h of incubation using a water displacement apparatus (Fedorak and Hrudey, 1983). At 6 h and 12 h prior to the gas measurement, the headspace gas was sampled from each vial using a 20-mL syringe and immediately transferred to a 5.9-mL evacuated tubes Exetainer® (Labco, Ltd., High Wycombe, Buckinghamshire, UK), which was then analyzed for the CH_4 concentration by gas chromatography (Hewlett Packard HP, Model 32 Embrapa, São Carlos, SP, Brazil). Methane was expressed as mg of CH_4 g DM^{-1} that disappeared and the total net gas production as ml g^{-1} of incubated DM. After the gas was sampled for CH_4 and the total gas production was measured at 48 h of incubation, the fermentation vials were opened, and the pH of the culture was measured using a pH meter (Orion Model 260A, Fisher Scientific, Toronto, Ontario Canada). Nylon bags made from polyester filter cloth (Model F57, Ankom Technology, USA) with the residues were then removed from the bottles, rinsed thoroughly with distilled water and dried at 55°C for 48 h to a constant weight to estimate the *in vitro* dry matter degradability (IVDMD).

A subsample (1.6 mL) of the culture media from each vial was transferred to 2-ml microcentrifuge tubes and centrifuged at 14,000×*g* for 10 min at 4°C (Spectrafuge 16M, National Labnet Co., Edison, New Jersey, USA) to precipitate particulate matter and protein. The supernatant was transferred into 2-mL micro-centrifuge tubes and analyzed for ammonia-N. In addition, a subsample (1.5 mL) of each vial was collected, acidified with 300 μl of metaphosphoric acid (0.25; w/v), and centrifuged as previously described for ammonia-N analysis. The supernatant was frozen at -20°C until analyzed for the VFA concentration. The 0-h samples were also analyzed for ammonia-N and VFA to calculate the net ammonia-N and net total VFA production (Holtshausen et al., 2009).

Chemical analysis

The substrates and the byproducts were predried in a forced air oven at 60°C for 48 h and then ground in a Willey mill that was equipped with a sieve of 1.0 mm to determine dry matter (DM) content at 105°C according to the general procedures described by Silva and Queiroz (2002). Crude protein (CP) content was determined by the Kjeldahl method and samples were also analyzed for ether extract (EE) (AOC, 1995). Neutral fiber (NDF) and acid detergent fiber (ADF) were determined by the method described by Van Soest et al. (1991). The forage and byproducts were bagged XT4 (Ankon)® and subjected to extraction by the official high temperature method AOCS (2009) using an XT10 extractor (Ankon)®. The IVDMD was assessed following the methodology of Tilley and Terry (1963).The total carbohydrates (TC) were obtained through the equation 100 - (% CP +% EE +% ash) and non-fiber carbohydrates (NFC) were calculated using the expression NFC = [100 – (%CP + %EE + %MM + %NDF)], as described by Sniffen et al. (1992).

Statistical analyses

The experimental design that was used to evaluate the cumulative gas production and degradability of dry matter was completely randomized in a 4 × 4 factorial arrangement (byproducts and substitution levels). The total gas production and degradability of the DM were subjected to an analysis of variance (PROC ANOVA) where, to measure the interaction effects, we applied Tukey's test ($p < 0.05$) between the byproducts within each level of substitution and the levels substitution within each byproduct. The increasing levels were interpreted through regression models using PROC REG of SAS (2003).

RESULTS

The soybean presented the highest CP, NFC and in vitro digestibility of dry matter, lower NDF and ADF (Table 1).

In this study we observed an interaction effect between the types of byproducts and the levels of the replacement values on methane production *in vitro* with a time interval of 48 hours (Table 2).

When assessing byproducts in replacement levels at 30% by elephant grass, sunflower cake and canola cake presented higher methane production and were similar in gas production ($p < 0.05$), outperforming the elephant grass and soybean cake, which produced less CH_4. The levels of 50% and 70% substitution exhibited the same behavior; the byproduct fodder radish had the lowest methane production ($p < 0.05$), differing from the values observed with the other byproducts, which presented higher yields and did not differ ($p > 0.05$).

The replacement of elephant grass by soybean caused a linear increase for each percentage replacement unit. There was an increase of 0.23 mL g^{-1} in the CH_4 production by ruminal microorganisms.

The methane production of canola cake, fodder radish cake and sunflower cake presented ($p < 0.05$) a quadratic relationship with 62.5% of the minimum point (31.25 mL g^{-1}) to canola cake, 66.5% of the minimum point (20.00 mL g^{-1}) to fodder radish cake, and sunflower cake that had 6.5% as a minimum point (3.38 mL g^{-1}) of CH_4 production.

The interaction types and levels of byproducts replacement affected the cumulative production of CO_2. When analyzing the different levels of substitution of elephant grass, it was observed that at the levels of 30 and 50%, canola and sunflower cake produced more CO_2 than fodder radish and soybean cake ($p < 0.05$). At the level of 70% replacement, soybean, canola and sunflower cake did not significantly differed ($p > 0.05$) and produced more CO_2 ($p < 0.05$) that fodder radish, being the byproduct that produced the lowest quantity of this gas.

The replacement of elephant grass with soybean cake caused a linear increase ($p < 0.05$); for every 1% replacement level, there was an increase of 1.1 mL g^{-1} in the production of enteric CO_2.

For canola, fodder radish cake and sunflower that replaced elephant grass, there was a quadratic effect in relation to CO_2 production, being

Table 1. Chemical composition (g kg^{-1}) and in vitro DM digestibility of elephant grass and byproducts from biodiesel production.

Tabla 1. Composición química (g kg^{-1}) y digestibilidad in vitro de MS de pasto elefante y los subproductos de la producción de biodiesel.

Ingredients	DM	CP	NDF	ADF	ADL	EE	ASH	TC	NFC	IVDMD
Elephant grass	882.3	126.1	555.0	351.1	144.4	14.2	25.4	83.43	279.3	591.4
					Byproducts					
Soybean cake	869.9	528.4	184.5	106.7	15.9	18.3	66.0	38.73	202.8	808.9
Canola cake	922.1	375.1	410.3	378.3	120.5	24.3	57.7	54.29	132.6	689.0
Fodder radish cake	935.6	393.7	380.5	154.2	69.6	28.4	50.6	27.16	146.8	644.8
Sunflower cake	901.1	342.6	390.1	243.6	34.3	32.1	54.9	57.04	180.3	582.3

Abbreviations: DM: Dry matter; CP: Crude protein; NDF and ADF: Neutral and Acid Detergent Fiber, respectively; ADL: Acid detergent lignin; EE: Ether Extract; NFC: Non-fiber Carbohydrates; ASH: Ashes; TC: Total carbohydrates; IVDMD: In vitro dry matter degradability.

Table 2. Mean values, probability (P-value) and regression equation-dependent effects of CH_4, CO_2 and $N\text{-}NH_3$ (mL g^{-1}) of byproducts from biodiesel production at different levels of substitution of elephant grass after 48 hours of incubation *in vitro*.

Tabla 2. Valores medios, probabilidad (P-valor) y de regresión de efectos de ecuaciones que dependen de CH_4, CO_2 y $N\text{-}NH_3$ (mL g^{-1}) de los subproductos de la producción de biodiesel en diferentes niveles de sustitución de pasto elefante después de 48 horas de incubación in vitro.

Byproduct	Levels of substitution				Regression equations	R^2	P-value
	0%	30%	50%	70%			
Methane production (CH_4)							
Soybean cake	0.865	8.043 B	11.863 A	17.865 A	$\hat{Y} = 0.74+0.23x$	0.82	0.0001
Canola cake	0.865	13.158 A	15.248 A	16.538 A	$\hat{Y} = 1.01+0.50x -0.004x^2$	0.96	0.0001
Fodder radish cake	0.865	5.523 B	7.328 B	7.407 B	$\hat{Y} = 0.83+0.20x-0.001x^2$	0.88	0.0004
Sunflower cake	0.865	13.553 A	13.952 A	14.368 A	$\hat{Y} = 1.10 +0.52x-0.004x^2$	0.94	0.0001
Carbon dioxide (CO_2)							
Soybean cake	10.47	36.68 B	56.49 B	89.79 A	$\hat{Y} = 6.90+1.1x$	0.76	0.0001
Canola cake	10.47	91.41 A	100.78 A	93.32 A	$\hat{Y} = 11.15+3.63x -0.03x^2$	0.97	0.0001
Fodder radish cake	10.47	34.87 B	36.75 B	32.88 B	$\hat{Y} = 10.67+1.12x-0.01x^2$	0.90	0.0001
Sunflower cake	10.47	81.23 A	82.75 A	77.13 A	$\hat{Y} = 11.54 +3.10x-0.03x^2$	0.97	0.0001
Ammoniacal nitrogen ($N\text{-}NH_3$)							
Soybean cake	21.46	41.767 A	54.83 A	58.80 A	$\hat{Y} = 21.14+0.87x-0.004x^2$	0.95	0.030
Canola cake	21.46	36.633 A	44.10 BC	56.46 A	$\hat{Y} = 21.34+0.48x$	0.97	0.001
Fodder radish cake	21.46	35.467 A	41.06 C	47.13 B	$\hat{Y} = 22.62+0.36x$	0.97	0.001
Sunflower cake	21.46	39.200 A	50.63 AB	59.73 A	$\hat{Y} = 11.81-0.27+0.003x^2$	0.98	0.001

*Means in the same column with different letters differ statistically by Tukey's test at a 5% probability.

60.5% of the maximum point (219.62 mL g^{-1}) for the CO_2 production of canola, 56% of the maximum point (62.75 mL g^{-1}) of fodder radish cake, and 62.75% of the maximum point (160.18 mL g^{-1}) of sunflower cake.

For ammonia production, there was an interaction effect in replacing the elephant grass with the byproducts of biodiesel. When analyzing the different replacement levels, it was observed that levels of 30% soybean cake produced more $N\text{-}NH_3$ that sunflower, canola and fodder radish cake cakes ($p < 0.05$). Levels of 50% soybean cake and sunflower cake produced the highest yields of $N\text{-}NH_3$ and at 70% level of sunflower, soybean and canola produced the highest yields of $N\text{-}NH_3$ ($p < 0.05$).

For the production of $N\text{-}NH_3$, canola presented a linear model: as the percentage unit increased, the production of $N\text{-}NH_3$ increased by 0.48 mL g^{-1}.

The production of NH_3 of fodder radish cake was affected ($p < 0.05$) by replacing the elephant grass, exhibiting a linear increase as there was an increase of 0.36 mL $g^{-1}$for each percentage unit in replacement level.

The soybean and sunflower cake were affected ($p < 0.05$) by substitution, with a quadratic relationship to the production of $N\text{-}NH_3$ levels by elephant grass replacement, with the 10.87 level promoting the minimum point (9.45 mL g^{-1}) for the production of $N\text{-}NH_3$ for soybean. The sunflower cake at 45% level was equivalent to the minimal point (12.15 mL g^{-1}) of $N - NH_3$ production.

An effect ($p < 0.05$) of the levels of substitution of the elephant grass by byproducts was observed on the concentration of VFAs within 48 hours (Table 3). Soybean and fodder radish cake yielded the highest concentration of acetic acid with 30% and 50% in replacement to elephant grass. The lowest yields were obtained with sunflower and canola cake. The sunflower cake yielded a lower production but did not differ statistically from the of canola cake ($p > 0.05$).

The level of 70% replacement by soybean and fodder radish cake exhibited a higher concentration of acetic acid, and the lowest value was found for canola ($p < 0.05$).

Substitution with soybean cake showed a linear increase in acetic acid concentration ($p < 0.05$); as the percentage of soybean cake increased, there was an increase of 0.24 mL g^{-1} in the production of acetic acid via the rumen fermentation process. No effect ($p > 0.05$) was observed due to substitution levels of canola. The fodder radish cake exhibited an increasing linear response that

Table 3. Means values, probability (P-value) and regression equation-dependent effects of the production of volatile fatty acids (VFAs) of biodiesel production byproducts at different levels of substitution of elephant grass.

Tabla 3. Medios valores, probabilidad (P-valor) y regresión efectos de ecuaciones que dependen de la producción de ácidos grasos volátiles (AGVs) de subproductos de la producción de biodiesel en los diferentes niveles de sustitución de pasto elefante.

Byproduct	Levels of substitution				Regression equations	R^2	P-value
	0%	30%	50%	70%			
			Production of acetate (μmol mL^{-1})				
Soybean cake	26.517 ^{A}	36.097 ^{A}	43.555 ^{A}	42.136 ^{A}	$\hat{Y}$ = 27.98+0.24x	0.61	0.002
Canola cake	26.517 ^{A}	20.821 ^{B}	23.510 ^{B}	22.540 ^{C}	$\hat{Y}$ = 25.08	-	-
Fodder radish cake	26.517 ^{A}	32.792 ^{A}	41.822 ^{A}	34.455 ^{AB}	$\hat{Y}$ = 28.25+0.15x	0.40	0.090
Sunflower cake	37.729 ^{A}	16.692 ^{B}	17.344 ^{B}	26.291 ^{BC}	$\hat{Y}$ = 26.61-0.59+0.008x^2	0.90	0.010
			Production of propionate (μmol mL^{-1})				
Soybean cake	11.764 ^{A}	14.282 ^{A}	18.202 ^{A}	19.122 ^{A}	$\hat{Y}$ = 11.62+-0.11x	0.76	0.0002
Canola cake	11.764 ^{A}	9.348 ^{AB}	10.935 ^{B}	10.839 ^{B}	$\hat{Y}$ = 11.03	-	-
Fodder radish cake	11.764 ^{A}	13.587 ^{A}	17.852 ^{A}	15.107 ^{AB}	$\hat{Y}$ = 12.17+0.06x	0.42	0.020
Sunflower cake	11.764 ^{A}	7.329 ^{B}	7.603 ^{B}	11.723 ^{B}	$\hat{Y}$ = 11.81-0.27+0.003x^2	0.92	0.001
			Production of butyrate (μmol mL^{-1})				
Soybean cake	3.343 ^{A}	5.651 ^{A}	7.131 ^{A}	6.937 ^{A}	$\hat{Y}$ = 3.28+0.11x+0.0008x^2	0.85	0.040
Canola cake	3.343 ^{A}	3.052 ^{B}	3.531 ^{B}	3.334 ^{C}	$\hat{Y}$ = 3.25	-	-
Fodder radish cake	3.343 ^{A}	4.804 ^{A}	6.025 ^{A}	5.299 ^{B}	$\hat{Y}$ = 3.26+0.08x-0.0007x^2	0.79	0.009
Sunflower cake	3.343 ^{A}	2.510 ^{B}	2.825 ^{B}	3.557 ^{C}	$\hat{Y}$ = 3.33-0.04x+0.0007x^2	0.69	0.001

*Means in the same column with different letters differ statistically by Tukey's test at the 5% probability.

was estimated at 0.15 mL g^{-1} for each percentage unit in the concentration of acetic acid.

The sunflower cake described a quadratic model for the concentration of acetic acid, estimated at 36.87% for the minimum replacement level that promoted by the elephant grass (21.75 mL g^{-1}) the concentration of acetic acid.

The concentration of propionic acid obtained was also significant ($p < 0.05$) by replacing the elephant grass within 48 hours (Table 3). When analyzing the byproducts substitution levels, the 30% level of substitution of elephant grass for soybean, oilseed and fodder radish cake, presented more propionic acid concentration ($p < 0.05$), with fodder radish cake producing amounts that were similar to those of canola and soybean ($p > 0.05$). The sunflower cake exhibited a lower production that did not differ statistically from canola cake ($p > 0.05$).

The 50% level of the substitution of elephant grass for soybean cake also showed a higher concentration of this acid ($p < 0.05$) that did not differ from that of fodder radish cake ($p > 0.05$). In addition, a lower production was observed for sunflower, being equivalent to canola. The level of 70% substitution for soybean again showed a higher concentration of propionic acid similar to that of fodder radish cake; lower concentration was observed for canola and sunflower ($p < 0.05$).

The replacement of elephant grass by soybeans linearly increased the concentration of propionic acid ($p < 0.05$), with an increase of 0.11 mL g^{-1} for each percentage unit in the production of this acid. The canola cake did not affect ($p > 0.05$) the levels of substitution of elephant grass. The fodder radish cake influenced linearly concentration of propionic acid, increasing 0.06 mL g^{-1} of this acid for every 1% replacement level. In turn, sunflower cake showed a quadratic behavior, with the level of 45% of the minimum point promoting (12.15 mL g^{-1}) the concentration of this acid.

When analyzing the concentration of butyric acid, an interaction effect ($p < 0.05$) was observed between the levels of substitution of elephant grass for the byproducts for 48 hours (Table 3). At levels of 30% and 50%, canola and sunflower cake produced more butyric acid ($p < 0.05$) and reached similar values ($p > 0.05$). The fodder radish cake and soybean exhibited a lower concentration of VFA ($p < 0.05$). At 50% replacement level, the byproducts canola and sunflower also presented higher yields of this acid. The lowest yields were observed for fodder radish and soybean cake. At 70% replacement level, soybean exhibited a higher concentration of butyric acid compared to the other byproducts ($p < 0.05$).

For the concentration of butyric acid, the soybean cake presented a linear increase of 1.1 mL g^{-1}. The canola cake, fodder radish cake and sunflower cake described a quadratic model for the concentration of butyric acid when their levels increased, with the 60.5% level promoting the maximum point (219.62 mL g^{-1}) of the concentration of butyric acid by canola cake, the 56% level promoting the maximum point (62.75 mL g^{-1}) of fodder radish cake and 62.75% level promoting the maximum point (160.18 mL g^{-1}) of sunflower.

DISCUSSION

During ruminal fermentation, when there is a decrease in the NDF and an increase in the EE, there is no decrease in the CH_4 production. According to the stoichiometry of gas production, the quantity of fiber in the diet increases the production of acetate, which increases methane production. Therefore, less fiber is expected to produce less CH_4 due to the inclusion of byproducts. Furthermore, increased lipids in the diet aid in the removal of H_2^+ from the rumen via biohydrogenation to transform unsaturated fatty acids into saturated fatty acids, sequestering two molecules of H_2^+ for every molecule of formed saturated fatty acid; when free, the H_2^+ is used by methanogenic bacteria to produce CH_4 (Kozloski, 2009).

The increased levels of byproducts from zero to 70% increase the production of CH_4. It was hypothesized that a reduction occurred in the production of acetate / propionate and, consequently, CH_4. This did not occur in relation to the replacement by elephant grass but may be related to the carbohydrate ratio since the elephant grass has a larger amount in relation to the byproducts. This effect may also be related to the incubation time, as the low methane production in early times occurred due to the fact that this period includes the lag phase, i.e., no methanogenesis until there are saturated spots available for microbial attachment to synthesize these structures and enzymes (Franco et al., 2011). Lee et al. (2011) conducted incubations of up to 72 hours for various types of diets and suggested that the slowly digestible fraction of the diet (i.e., structural fibers) is associated with the increased production of CH_4 and reported that methanogenesis is inhibited after 24 hours of incubation, averaging 81% of CH_4 production.

The industrialization of biodiesel can affect the production of CH_4. The canola cake produced the most enteric methane because this food contains a higher amount of fiber compared with the other byproducts. The fodder radish cake exhibited a lower amount of CH_4 because the fodder radish cake is rich in polyunsaturated fatty acids, promoting an increase in the ruminal process. However, these byproducts increased the CH4 production compared to the elephant grass (Abdalla et al., 2012; Oliveira et al., 2013). Regarding the production of CH_4, the byproduct of fodder radish cake decreased the CO_2 in the rumen, playing an important role, as CO_2 functions in the continuous removal of H_2, which is the result of the fermentation of organic matter, promoting the reduction of methanogenesis (Manatbay et al., 2014).

The 10.87% level of soybean cake described a minimum point of N-NH_3 of 9.45 ml g^{-1}. This concentration was greater than 5 mg 100 mL^{-1}, which is considered ideal for maximum microbial growth (Van Soest et al., 1991), indicating that nitrogen availability is not a limiting factor for the development of microorganisms that are responsible for the degradation of structural carbohydrates.

Acetate is the main VFA that is produced in the rumen by ruminal bacteria and may even reach 75% of the total VFAs when the diet is composed of forage (Li et al., 2014), which is responsible for higher energy and CH_4 production. This was observed in this study with a higher concentration of acetic acid in the VFA. A higher production of the acids in soybeans was induced by less sunflower byproduct (Faciola and Broderick, 2014). However, it was observed during fermentation that replacing the elephant grass with these byproducts resulted in an increase in the production of acetate and no significant increase in the production of CH_4, as has been described in other studies (Martínez-Fernández et al., 2014).

An increase in propionic acid at different levels was observed, with soybean byproducts producing the highest concentration (mmol mL^{-1}) and sunflower byproducts producing the lowest concentration at 30% substitution. This behavior can be explained by the low amount of fiber and a high concentration of water-soluble carbohydrates in soybean byproducts.

Only 0.87 mol of VFA production was indirect, whereas the production of acetate produces 2 moles of CO_2 per mole of glucose by direct production. A total of 0.87 mol of VFA production was indirect (Kozloski, 2009), which can be verified with the increased production of CO_2 and acetate.

Consequently, the production of propionic acid was lower for the sunflower byproducts due to a high concentration of carbohydrates. This can be correlated with the decrease in the production of CO_2 and CH_4 due to the stoichiometry of rumen fermentation, in which the production of propionic acid energy losses do not occur in the form of CO_2 or CH_4 (Mizubuti et al., 2014).

The largest production of butyric acid was obtained with the soybeans cake at the level of 50%, while the lowest production was obtained with sunflower cake on the 30% level. The largest production of butyric acid was found in the soybean cake and is directly related to the increased gas production that is associated with the structural components of fermentation byproducts (Romero-Huelva and Molina-Alcaide, 2013). Butyric acid can be the source of acetate, propionate and other components necessary for the production of microbial protein that is synthesized in the rumen, the majority of which is an absorbable source of amino acids in the small intestine of ruminants (Vyas et al., 2014).

The ruminal pH alters ruminal fermentation depending on the type of diet and the amount of time after food ingestion. The inclusion of high proportions of non-fibrous carbohydrates in the diet, which have high degradation rate, generally results in a decrease in the pH and fiber digestibility level of the rumen (Kozloski, 2009).

The ruminal pH is also directly related to the end products of fermentation and also with the growth rate of rumen microorganisms. The pH range for microbial activity in the rumen is typically 6.7 ± 0.5 (Van Soest et al., 1991). According to Orskov (1988), when the ruminal pH is below 6.2, there is a reduction in fiber digestion due to the sensitivity of fibrolytic bacteria, and the optimum fiber digestion occurred at pH values between 6.7 and 7.1.

In this study, the average pH was 6.5. Therefore, reducing the digestibility of the nutritional components of byproducts does not occur due to the pH of the rumen but to other factors. The mechanisms that are associated with the effects of rumen pH are not well elucidated but include effects on the adhesion process the fiber fraction and mainly on bacterial metabolism (Kozloski, 2009).

CONCLUSIONS

The replacement of elephant grass with byproducts from biodiesel production chain altered the pattern of fermentation *in vitro* because of the greater substitution of the production of greenhouse gases emissions (CH_4, CO_2 and NH_3) as well as increased production of VFAs; pH remained constant. Soybean and fodder radish cakes present greater efficiency in generating feed energy when replacing the elephant grass because they showed the highest concentrations of VFAs propionate and butyrate, which have higher energy density compared to acetate. The byproduct fodder radish cake showed higher gas mitigation capacity *in vitro*.

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