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Greenhouse gases, short-chain fatty acids and ruminal pH *in vitro* of biodiesel byproducts to replace corn silage

Gases de efeito estufa, ácidos graxos de cadeia curta e pH ruminal in vitro de coprodutos do biodiesel em substituição à silagem de milho

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SUMMARY

The aim of the study was evaluate the production potential for methane (CH_4) and carbon dioxide (CO_2), short-chain fatty acids, ammonia nitrogen (N-NH_3) and pH by semi-automated technique *in vitro* from biodiesel byproducts cottonseed cake (*Gossypium hirsutum*), castor bean (*Ricinus communis*), moringa cake (*Moringa oleifera*), jatropha cake (*Jatropha curcas*) and sunflower cake (*Helianthus annuus*) substituting corn silage in increasing levels, 0, 30, 50 and 70%. The experimental design used was completely randomized in a 5 x 4 factorial arrangement (byproducts and substitution levels). The inoculum for the in vitro incubations was obtained from three Holstein cows with rumen fistulas. In the experiment, the conditions were verified for the differences in potential gas production among the ingredients. The byproduct of cotton was the ingredient with the greatest potential to produce acetate, butyrate, CO_2 and CH_4. The byproduct of moringa had the lowest potential for the production of acetate, butyrate, CO_2 and CH_4 from *in vitro* degraded dry matter and a greater potential for the production of propionate. Among the byproducts studied, moringa was distinguished for promoting mitigation of CH_4 and obtaining levels of pH and N-NH_3 satisfactory for maximum rumen fermentation; thus, it is recommended the byproduct of moringa to replace corn silage because reduces environmental impact without impairing *in vitro* rumen fermentation.

Keywords: acetate, CO_2, CH_4, propionate, *Ricinus communis*

RESUMO

O objetivo do estudo foi avaliar o potencial de produção de metano (CH_4), dióxido de carbono (CO_2), ácidos graxos de cadeia curta, nitrogênio amoniacal (N-NH_3) e pH pela técnica semi-automática técnica *in vitro* a partir de coprodutos do biodiesel como torta de algodão (*Gossypium hirsutum*), torta de mamona (*Ricinus communis*), torta de moringa (*Moringa oleifera*), torta de pinhão manso (*Jatropha curcas*) e torta girassol (*Helianthus annuus*) em substituição a silagem de milho em níveis crescentes, 0, 30, 50 e 70%. O delineamento foi inteiramente casualizado, em um arranjo fatoria 5 x 4 (coprodutos and níveis de substituição). O inóculo para as incubações *in vitro* foi obtido a partir de três vacas da raça Holandesa com fístulas ruminais. No experimento, as condições foram verificadas para observer as diferenças na potencial produção de gás entre os ingredientes.

O coproduto de algodão foi o ingrediente com o maior potencial para produção de acetato, butirato, CO_2 e CH_4. O coproduto de Moringa teve o menor potencial para a produção de acetato, butirato, CO_2 e CH_4 e digestibilidade *in vitro* da matéria seca e um maior potencial para a produção de propionato. Entre os coprodutos estudados, a moringa destacou-se pela promoção da mitigação de CH_4 e obtenção de níveis de pH e N-NH_3 satisfatórias para a fermentação máxima no rúmen. Assim, recomenda-se utilizar o coproduto de moringa para substituir a silagem de milho, pois reduz o impacto ambiental sem prejudicar a fermentação ruminal *in vitro*.

Palavras-chave: acetato, CO_2, CH_4, propionato, *Ricinus communis*

INTRODUCTION

Ruminant feeds are based on tropical grasses because of the ability of ruminants to ingest and digest feed rich in fiber. Thus, to be highly efficient, animal production needs to maximize the use of the energy supplied in the forage, but observations of biological indices suggest that production is below its potential. In addition, ruminants throughout the world are permanent sources of discussion and continue to be cited as the main producers of greenhouse gas (GHG) emissions because of their enteric fermentation digestive process, which produces primarily methane (CH_4) and carbon dioxide (CO_2); the accumulation of these gases is considered to be the main cause of global warming (HULSHOF et al., 2012).

Byproducts originating from the biodiesel production chain have been studied as potential ingredients for ruminant diets to reduce GHG production. Studies aimed at characterizing the ruminal metabolism of cakes are needed to identify potential and use efficiently these foods in the diet of ruminants to replace conventional ingredients (SILVA et al., 2013; GONZAGA NETO et al., 2014).

This research is important because Brazil has the largest commercial cattle herd in the world and is currently the target of criticism due to deforestation to expand pastures and low production indices recorded in cattle farming systems that have degraded pastures or are below their production potential. As a result, these farming systems generate greater amounts of GHG per pound of meat and milk produced (GOEL & MAKKAR, 2012; HRISTOV et al., 2013).

The byproducts derived from the production of biodiesels can be used as alternatives in ruminant diets, which could contribute to the growth of agricultural productivity and reduction the emission of greenhouse gases is used in the diet of cattle. Furthermore, these byproducts have great potential, with considerable concentrations of proteins and lipids that characterize them as energy or protein-rich feeds; For this, studies and techniques that characterize the ruminal metabolism of these byproducts, such as *in vitro* technical gas production, are necessary to identify potential equipment that could be used to replace conventional ingredients without harming the health of the animal byproducts and production of greenhouse gases (COTTLE et al., 2011; MOREIRA et al., 2014; MEDEIROS et al., 2015; MORAIS et al., 2015; SILVA et al., 2015a; SILVA et al., 2015b).

Thus, we determined the optimum application levels of biodiesel byproducts for the ruminant diet, searching for lower production of CH_4 and CO_2 and higher yields of enteric short-chain fatty acids (VFAs).

MATERIAL AND METHODS

This research was conducted at the Experimental Station of Coronel Pacheco, MG (owned by Embrapa Gado de Leite – CNPGL), located in the Mata of Minas Gerais State, Brazil. In this experiment, the following feeds from industrial biodiesel byproducts were used: cottonseed cake (*Gossypium hirsutum*), castor bean (*Ricinus communis*), moringa cake (*Moringa oleifera*), jatropha cake (*Jatropha curcas*) and sunflower cake (*Helianthus annuus*), with corn silage as a control from the experimental field of Embrapa in Coronel Pacheco in Minas Gerais state.

Samples of approximately 300g of each byproduct resulting from the processing or extraction of vegetable oil were collected and sent to the Laboratory of Food Analysis, Embrapa Dairy Cattle, Juiz de Fora city, Minas Gerais State, for chemical analysis and analysis of gases.

At the start of the analyses, the samples were thawed, pre-dried using forced ventilation at 55°C for 72h, ground in a Wiley mill equipped with a 5 mm sieve, packaged in plastic bags, and sent for laboratory analyses at Embrapa Dairy Cattle, Juiz de Fora, MG. In the laboratory, samples of each cake were mixed and homogenized to form a single sample. From this sample, a portion (100g) was used for chemical analyses, and the remainder of the material was used to formulate the test diets. Each diet was formulated with different levels of the byproducts in the diet, with ratios of 100/0, 70/30, 50/50 and 30/70% (corn silage/byproducts).

The substrates consisting of forage materials and the byproduct were pre-dried in forced air ovens at 60°C for 48h and then ground in a Willey mill equipped with a 1.0 mm stored in air tight plastic containers, and sealed properly until the laboratory analysis of the levels of the dry matter (DM) (Method 967.03 - AOAC, 1990), ash (Method 942.05 -AOAC, 1990), crude protein (CP) (Method 981.10 - AOAC, 1990), and ether extract (EE) (Method 920.29 - AOAC, 1990). To determine the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents, the methodology of Van Soest et al. (1991) was used with the modifications that were proposed in the Ankon device manual (Ankon Technology Corporation, Macedon, New York, US). Acid detergent lignin (ADL) was determined according to method 973.18 (AOAC, 2002), in which the ADF residue was treated with 72% sulfuric acid. The total carbohydrates (TC) were obtained through the equation 100 - (% CP +% EE +% ash) described by Sniffen et al. (1992).

The assay for *in vitro* dry matter digestibility (IVDMD) was performed according to the methodology of Tilley & Terry (1963). Three fistulated steers were used for this in vitro test. The ruminal contents were collected in the morning, transferred to preheated thermo-flasks (39ºC) and transported immediately to the laboratory. In the laboratory, the rumen fluid was filtered using a fine sieve. Mineral buffer solution was later added to the rumen fluid in a water bath maintained at 39ºC with continuous CO_2 injection. The samples were held in an incubator, in a state of rotation, for 72h. Peptides were added to the incubators within 48 h to act as intermediate compounds for the action of microorganisms.

The substrate used for in vitro incubations was glycerin, which was used to replace the byproduct fodder in ratios of 100/0, 70/30, 50/50 and 30/70 % (corn silage/byproducts). The feed ingredients were dried at 55ºC for 24 h and then ground to pass through a 1 mm screen. Each in vitro incubation was conducted according to the methods reported by Meale et al. (2012). The entire incubation procedure was repeated twice (i.e., two incubations x three replicates per treatment, for a total of six replicate vials per treatment).

The inoculum for the in vitro incubations was obtained from three fistulated cows grazing on beard grass supplemented with 2 kg of concentrate (22 g kg^{-1} CP and 12.6g kg^{-1} NDF in 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 Thermos® flask to the laboratory. Inocula were prepared by mixing rumen fluid and a mineral buffer with 0.5 mL of cysteine sulfide solution (VITTI et al., 1999) in a ratio of 1:5. Inoculum (30 mL) was then transferred into pre-loaded, pre-warmed (39ºC) vials under a stream of oxygen (O_2) free nitrogen (N_2) gas. The vials were sealed and placed on an orbital shaker rack at 120 oscillations per min in an incubator at 39ºC.

The net gas production of each vial was measured after 6, 12, 24 and 48 h of incubation with a water displacement apparatus (FEDORAK & HRUDEY, 1983). At 6 h and 12 h before the gas measurement, the headspace gas was sampled from each vial with a 20 mL syringe and immediately transferred into a 5.9 mL evacuated Exetainer® (Labco Ltd., High Wycombe, Buckinghamshire, UK), which was analyzed to determine the CH_4 concentration using gas chromatography. Methane is expressed as mg CH_4 g^{-1} of DM disappeared, and total net gas production is expressed 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 cultures was measured using a pH meter (Orion Model 260A, Fisher Scientific, Toronto, ON, Canada). The ANKOM® bags with the residues were then removed from the bottles, rinsed thoroughly with distilled water and the residues dried at 55ºC for 48h to a constant weight to estimate IVDMD.

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

The experimental design used was completely randomized in a 5 x 4 factorial arrangement (byproducts and substitution levels). The model consisted of dietary treatment, and periods were random effects Nutrient digestibility, gas production Ruminal pH, ammonia, and VFAs were analyzed as a 5 × 4 Latin square with repeated measures using the MIXED procedure of SAS® version. 9.3. (SAS, 2008). Effects were considered significant at a P-value of ≤ 0.05, 0.01 and 0.001. The results of increasing levels were interpreted through regression models using Proc Reg of SAS. The total gas production and degradability of DM were subjected to analysis of variance (PROC ANOVA). Interaction effects were assessed using Tukey's test between the byproducts within each level of substitution and levels substitution within each byproduct.

RESULTS AND DISCUSSION

All the cakes had high crude protein and ether extracts, particularly the byproduct moringa cake because of a higher rate of digestibility and a lower amount of NDF (Table 1).

The production of acetate, propionate and butyrate from the byproducts that replaced the corn silage showed dependent effects (Table 2). Analyzing the byproducts within the levels of substitution of the corn silage, the 30% substitution with the moringa cake, castor bean and jatropha cake were similar, obtaining the highest average productions of acetate. Cotton and sunflower cakes had lower production of acetate ($P<0.05$).

At the 50% and 70% levels of substitution, the highest production of acetate was obtained with the byproduct of cottonseed cake and was significantly different ($P<0.05$), while the lowest acetate production was with the byproducts of moringa, castor and jatropha cakes, which were similar to each other statistically ($P>0.05$). The production of acetate from the byproduct of moringa with 70% replacement of the corn silage was lower than that obtained at all levels of byproducts and treatments ($P<0.05$).

Examining the levels of substitution of corn silage with biodiesel byproducts, it appeared that the regression model that best explained acetate concentration from the byproducts of cotton ($P = 0.0006$), moringa ($P = 0.0002$) and sunflower ($P = 0.0014$) was quadratic. For cottonseed cake, the maximum output point that represented acetate was 20.62% (30.68 mmol mL^{-1}). For the moringa cake byproduct, 2.38% was the point of maximum concentration of acetate (37.93 mmol mL^{-1}). Additionally, 29.47% was the point of the maximum concentration of acetate from sunflower cake (47.96 mmol mL^{-1}). The castor bean presented linearly (*P* = 0.0007) decrease and for every 1% of the castor replacing corn silage the acetate concentration decreased 0.09 mmol mL^{-1}. Similarly, 1% of jatropha cake (*P* = 0.0014) replacing corn silage reduced acetate concentration 0.10 mmol mL^{-1}.

Table 1. Chemical composition (g kg^{-1}) of corn silage and biodiesel byproducts

Ingredients	DM	CP	NDF	ADF	ADL	EE	ASH	IVDMD	TC
Corn silage	842.7	62.5	469.3	314.5	29.7	22.2	51.7	607.0	863.6
Cottonseed cake	929.1	549.9	303.6	207.7	32.1	40.3	68.3	595.6	341.5
Moringa cake	901.2	577.6	202.7	80.5	10.3	84.8	49.8	791.3	287.8
Jatropha cake	920.7	356.9	391.4	334.5	43.4	110.6	79.5	571.3	453.0
Sunflower cake	901.1	342.6	390.1	243.6	34.3	32.1	54.9	582.3	570.4
Castor bean	912.6	420.2	423.3	383.4	154.4	43.8	42.3	497.1	493.6

DM = dry matter; CP = crude protein; NDF and ADF = neutral and acid detergent fiber; ADL = acid detergent lignin; EE = ether extract; ASH = ashes; IVDMD = *in vitro* Dry Matter Degradability; TC = total carbohydrates.

Table 2. Mean values, regression, coefficient of determination and equations probability (*P*-value) illustrating the effects on concentration of short-chain fatty acids with the substitution of silage corn with industrial biodiesel byproducts

Byproducts	Levels of substitution				Regression Equations	R^2	P
	0%	30%	50%	70%			
Production of Acetate (µmol mL^{-1})							
Cottonseed cake	37.729^A	34.458^B	41.500^A	47.900^A	$\hat{Y}$ =37.4384-0.2186x+0.0053x^2	0.80	0.0006***
Moringa cake	37.729^A	37.087^A	32.167^C	29.418^C	$\hat{Y}$ =37.9397-0.0081x-0.0017x^2	0.85	0.0002***
Jatropha cake	37.729^A	36.401^A	32.634^C	30.507^C	$\hat{Y}$ =38.3611-0.1078x	0.65	0.0014**
Sunflower cake	37.729^A	34.854^B	35.837^B	41.377^B	$\hat{Y}$ =37.8106-0.2299x+0.0039x^2	0.76	0.0014**
Castor bean	37.729^A	35.803^{AB}	35.360^{BC}	30.534^C	$\hat{Y}$ =38.34690-0.09308x	0.69	0.0007***
Production of Propionate (µmol mL^{-1})							
Cottonseed cake	19.636^A	18.297^B	16.709^B	15.173^B	$\hat{Y}$ =19.8595-0.0641x	0.76	0.0002***
Moringa cake	19.636^A	22.386^A	20.342^A	18.300^A	$\hat{Y}$ =19.7511+0.1418x-0.0024x^2	0.71	0.0036**
Jatropha cake	19.636^A	18.624^B	15.470^{BC}	13.646^{BC}	$\hat{Y}$ =20.19723-0.08941x	0.79	0.0001***
Sunflower cake	19.636^A	18.815^B	18.628^{BA}	19.208^A	$\hat{Y}$ =19.0719	-	0.1670^{ns}
Castor bean	19.636^A	15.996^C	14.617^C	11.455^C	$\hat{Y}$ =19.65040-0.11265x	0.96	<.0001***
Production of Butyrate (µmol mL^{-1})							
Cottonseed cake	12.414^A	7.928^{AB}	9.822^A	10.305^A	$\hat{Y}$ =12.2197-0.1841x+0.0023x^2	0.54	0.0282*
Moringa cake	12.414^A	6.850^B	4.449^B	4.267^C	$\hat{Y}$ =12.4573-0.2494x+0.0018x^2	0.94	<.0001***
Jatropha cake	12.414^A	11.270^A	10.387^A	8.666^B	$\hat{Y}$= 10.2315 + 0.0357x – 0.0008x^2	0.43	0.0023**
Sunflower cake	12.414^A	8.394^{AB}	7.531^{AB}	7.082^{AB}	$\hat{Y}$ =12.3763-0.1666x+0.0013x^2	0.83	0.0004***
Castor bean	12.414^A	10.599^A	8.674^A	6.712^{BC}	$\hat{Y}$ =12.66258-0.08166x	0.85	<.0001***

$^{A-C}$Different letters within same column differ significantly (P < 0.05).
In same line differ significantly *P < 0.05, ** P < 0.01, *** P < 0.001 and ^{ns} to no significance.

In this study, the cottonseed bean showed greater potential for the concentration of acetate. This can be justified by comparing with the other cakes, which had higher fiber content, but had antinutritional toxic compounds such as ricin and trypsin inhibitors that decreased the digestibility and hence the fermentation and production of gases. The cottonseed cake also had high levels of EE, which in the diet aid in the removal of H_2^+ free in the rumen by a biohydrogenation process, the transformation of unsaturated fatty acids into saturated fatty acids. This process secretes two molecules of H_2+ for each molecule of saturated fatty acid formed, and this H_2+ when available is used for the production of CH_4 by methanogenic bacteria. The more that H_2+ is removed from the medium, the greater the proportion of NADH that is converted to H_2^+ and the greater the yield of acetate (KOZLOSKI, 2009).

For the production of propionate, the byproducts with the levels of substitution of the corn silage at 30%, 50% and 70% were analyzed, and generally the moringa cake had the highest average concentration of propionate, and castor bean had the lowest (P<0.05). Regardless of the level of substitution, the byproducts of castor presented concentration less propionate.

Analyzing the levels of substitution of corn silage by the biodiesel byproducts, the regression model that best explained the concentration of propionate from moringa cake was quadratic (P = 0.0036). The point of minimum concentration (21.84 mmol mL^{-1}) of propionate was observed as 30.82%. The propionate concentration of the cottonseed cake (P = 0.0002), castor bean (P<0001) and jatropha cake (P = 0.0001) decreased linearly; the concentration of propionate was reduced by 0.06 mmol mL^{-1}, 0.11 mmol mL^{-1} and 0.089 mmol mL^{-1} for every 1% replacement of corn silage with the cottonseed cake, castor bean and jatropha cake, respectively. The byproduct of sunflower showed no significant regression for propionate concentration.

In the concentration of propionate, only the levels of 30 and 50% replacement with the moringa cake had a higher production of propionate compared with the control group; propionate concentration decreased with the other byproducts. The substitution of fibrous carbohydrates for non-fibrous carbohydrates, obtained by increasing the roughage concentrate ratio or the use of better nutritional quality of forage, favored the concentration of propionate instead of acetate (PATRA & Yu, 2012). The current study found similar results when it replaced corn silage rich in fiber with the moringa cake that was low in fiber and high in protein.

Reviewing the concentration of butyrate from the byproducts for the 30%, 50% and 70% levels of substitution of the corn silage, generally the moringa cake differed (P<0.05) from the others with lower average concentration of butyrate. At the 70% level of substitution, the cottonseed and sunflower cake showed the highest yields of butyrate and were similar to each other. This can be associated with the high protein content these byproducts, which resulted in the formation of bicarbonate of ammonium, from CO_2 and ammonia, thereby reducing the contribution of CO_2 to the total gas production (HRISTOV et al., 2013).

Comparing the levels of substitution of the corn silage by biodiesel byproducts on butyrate concentration, the regression that best explained the concentration of butyrate of the cottonseed cake (P = 0.0282), moringa cake (P<0001), jatropha cake(P = 0.0023) and sunflower cake (P = 0.0004) was quadratic.

For the cottonseed cake, 40% was identified as the point of minimum concentration of butyrate (1.11 mmol mL^{-1}). For the moringa cake, 69.28% was observed as the point of maximum concentration of butyrate at 3.82 mmol mL^{-1}, and 64 % was the maximum yield point of 7.03 mmol mL^{-1} for butyrate for the sunflower cake. The castor bean (P<0001) had a linear decreasing relationship with the level of substitution, and for every 1% of corn silage replaced with butyrate concentration was reduced in 0.08 mmol mL^{-1} (Table 2).

For production of CH_4 and CO_2, the substitution of corn silage with the biodiesel byproducts had a dependent effect (Table 3).

Table 3. Mean values, regression, coefficient of determination and equations probability (*P*-value) illustrating the effects on production of CH_4 (mL g^{-1}), CO_2 (mL g^{-1}), N-NH_3 and pH with the substitution of corn silage with industrial biodiesel byproducts

Byproducts	Levels of substitution				Regression Equations	R^2	P
	0%	30%	50%	70%			
Methane production (CH_4; mL g^{-1})							
Cottonseed cake	187.663^A	125.317^B	142.803^A	170.020^A	$\hat{Y} = 186.2901-3.0596x+0.0409x^2$	0.68	0.0059**
Moringa cake	187.663^A	117.490^B	70.223^B	97.410^{BC}	$\hat{Y} =190.4031-3.9011x+0.0357x^2$	0.90	<.0001***
Jatropha cake	187.663^A	127.557^B	99.653^B	95.067^C	$\hat{Y} =178.77542-1.36774x$	0.89	<.0001***
Sunflower cake	187.663^A	147.643^{AB}	132.090^{AB}	133.160^B	$\hat{Y} =187.9460-1.8305x+0.0149x^2$	0.96	<.0001***
Castor bean	187.663^A	163.927^A	94.490^B	66.060^C	$\hat{Y} =197.26981-1.84626x$	0.90	<.0001***
Carbon Dioxide (CO_2; mL g^{-1})							
Cottonseed cake	42.317^A	27.450^B	38.410^A	49.657^A	$\hat{Y} =41.7159-0.7760x+0.0129x^2$	0.60	0.0157*
Moringa cake	42.317^A	25.250^B	10.587^C	12.940^C	$\hat{Y} =43.0358-0.8841x+0.0062x^2$	0.92	<.0001***
Jatropha cake	42.317^A	28.213^B	22.743^{BC}	23.680^{BC}	$\hat{Y} =42.4360-0.6567x+0.0055x^2$	0.87	<.0001***
Sunflower cake	42.317^A	32.377^{AB}	30.967^{AB}	34.800^B	$\hat{Y} =42.3558-0.5104x+0.0057x^2$	0.85	0.0002***
Castor bean	42.317^A	41.403^A	24.150^B	18.760^{BC}	$\hat{Y} =45.4774-0.3685x$	0.80	<.0001***
Ammonia Nitrogen (N-NH_3; mL g^{-1})							
Cottonseed cake	6.533^A	7.800^D	21.533^B	26.367^C	$\hat{Y} =5.8301+0.0602x+0.0036x^2$	0.90	<.0001***
Moringa cake	6.533^A	23.100^A	24.167^{BA}	31.73^B	$\hat{Y} =8.55389+0.34212x$	0.91	<.0001***
Jatropha cake	6.533^A	12.667^C	15.767^C	23.367^C	$\hat{Y} =5.94081+0.23047x$	0.95	<.0001***
Sunflower cake	6.533^A	16.333^B	25.900^A	40.833^A	$\hat{Y} =4.40935 +0.47975x$	0.95	<.0001***
Castor bean	6.533^A	17.267^B	19.133^{BC}	25.900^C	$\hat{Y} =7.30530+0.26408x$	0.94	<.0001***
pH							
Cottonseed cake	5.600^A	5.866^{AB}	5.866^B	5.933^B	$\hat{Y} =5.6076+0.0098x-0.0001x^2$	0.86	0.0001***
Moringa cake	5.600^A	5.900^A	6.066^A	6.133^{BA}	$\hat{Y} =5.63178+0.00782x$	0.93	<.0001***
Jatropha cake	5.600^A	5.833^{AB}	6.000^A	6.133^{BA}	$\hat{Y} =5.60312+0.00769x$	0.93	<.0001***
Sunflower cake	5.600^A	5.733^B	5.966^{AB}	6.033^B	$\hat{Y} =5.58567 +0.00660x$	0.90	<.0001***
Castor bean	5.600^A	5.833^{AB}	6.000^A	6.200^A	$\hat{Y} =5.58941+0.00850x$	0.98	<.0001***

$^{A-C}$Different letters within same column differ significantly ($P < 0.05$)
In same line differ significantly *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ^{ns} to no significance.

Reviewing the byproducts at the 30% level of replacement, the castor bean had the highest average CH_4 production ($P<0.05$), which was similar to the sunflower cake ($P>0.05$) and different of the moringa, jatropha and cottonseed cakes, which had similar behavior ($P<0.05$). At the 50% level of replacement, the byproduct of cottonseed cake had the highest average methane production ($P<0.05$), which was similar to sunflower cake and different from the others cakes ($P>0.05$). The moringa cake had the lowest production and was similar to the jatropha cake. At the level of 70% replacement, the cottonseed cake again had the highest production of methane and differed significantly from the other byproducts ($P<0.05$). The moringa cake was the best at mitigation of CH_4 at all levels of substitution; the 50% replacement level promoted greater mitigation of methane than the 100% corn silage.

The total gas production and the proportion of CH_4 and CO_2 from rumen fermentation can vary depending on the diet used. The potential to produce CH_4 and CO_2 chemical composition of the diets, wherein feeds rapidly fermentable tended to produce minor amounts of CH_4 g^{-1} dry mass in comparison with fermented ingredients with higher fiber (MORGADO et al., 2013).

Reviewing the substitution levels for each byproduct, it appeared that the regression model that best explained the CH_4 production of the cottonseed cake ($P = 0.0059$), moringa cake ($P<0001$) and sunflower cake ($P<0001$) was quadratic. For moringa cake, the point of maximum CH_4 production was 71.30% (11.53 ml gDM^{-1}), but because this result exceeds the preferred level, it was determined using a linear model. The point of maximum production of CH_4 (30 ml gDM^{-1}) was 30.1% for the cottonseed cake, 44.77% for the sunflower cake (30.93 ml gDM^{-1}). The model of the castor bean ($P<0001$) and jatropha ($P<0001$) best explained that the decrease was linear, i.e., approximately 1% of byproduct diminished the production of CH_4 by 0.37%.

Reviewing the byproducts for the production of CO_2 at the levels of substitution of the silage, at the level of 30% (Table 3), the castor bean had the highest average CO_2 output and was similar to the sunflower cake but different from the moringa, jatropha and cottonseed cakes ($P<0.05$). At the 50% level of replacement, the cottonseed and sunflower cakes had the highest average CO_2 production and were different from the other byproducts ($P<0.05$). At the 70% level of replacement, the cottonseed cake had the highest average CO_2 production and differed significantly ($P<0.05$) from the castor, jatropha and moringa cakes obtained, which had the lowest average CO_2 output. Moringa cake, jatropha cake, sunflower cake and castor bean had lower production of CO_2 compared with the control fodder (100% corn silage) (Table 3). This can be explained by the difference in the fiber content of these byproducts, with the byproduct of moringa having better nutritional quality because of a lower fiber content and higher digestibility (Table 1), which is directly related to the production of acetate, as the formation of pyruvate is degraded to CO_2 (KOZLOSKI, 2009; MANATBAY et al., 2014).

Examining the substitution levels within each byproduct, it appeared that the regression model that best explained the CO_2 production of the cottonseed ($P = 0.0157$), moringa ($P<0001$), jatropha ($P<0001$) and sunflower cakes ($P = 0.0002$) was quadratic, with 54.64% of the minimum yield point (83.82 ml gDM^{-1}) for the moringa cake, 37.40% of the minimum point of production (100.47 ml gDM^{-1}) for the cottonseed cake, and 61.43% of the point of minimum CO_2 production (131.73 ml gDM^{-1}) for the sunflower cake. For the castor bean, the model best explained that the decrease was linear, i.e., every 1% of the byproduct reduced CO_2 production by 1.84% (castor bean).

The production of ruminal N-NH_3 from the biodiesel byproducts replacement of corn silage showed a dependent effect. Comparing the byproducts that replaced corn silage at increasing levels, at the level of 30% replacement, the moringa cake had the highest average yield of N-NH_3 and was significantly different (P<0.05) compared to the other byproducts and cottonseed cake had the lowest levels of N-NH_3. At the level of 50% replacement, the sunflower and moringa cakes had the highest average production of N-NH_3, and the byproduct of jatropha had the least. At the 70% level, the sunflower and jatropha cakes had the highest and lowest values of N-NH_3, respectively. For N-NH_3 concentration, the substitution of corn silage with biodiesel byproducts increased NH_3 in all fodder types and levels of replacement, and the values obtained were above the optimal level of 10 mg dL^{-1} (VAN SOEST et al., 1991). This increase can be explained by the large amount of protein found in these byproducts (Table 1).

Observing levels of substitution for each byproduct, the cottonseed cake (P<0001) byproduct was described with a quadratic model with 8.36% at the lowest point of N-NH_3 production (6.33 mg dL^{-1}); other cakes showed a linear model (P<0001) with each 1% increase in level of substitution of corn silage by the byproduct resulting in an increase of 0.26 N-NH_3 for the castor bean, 0.34 for the moringa cake, 0.48 for the sunflower cake and 0.23 for the jatropha cake.

The pH was dependent on the effects of the main factors (Table 3). Reviewing the byproducts within the levels of substitution of corn silage by biodiesel byproducts, at the level of 30%, the moringa cake had the highest measurement and was similar to the pH of the cotton and castor cake and pH of the meek. The pH was different for jatropha cake, and sunflower cake had the lowest average. At the level of 50% replacement, the castor bean, jatropha cake, moringa cake and sunflower cake showed similar behavior with higher mean pH (P>0.05), differing from the cottonseed cake, which had the lowest average pH (P<0.05). When the level observed was 70% replacement, the castor bean, similar to the byproducts of moringa and jatropha cakes, had higher mean pH compared with the sunflower and cottonseed cakes, which were similar in pH measurement.

The ruminal pH below 6.2 reduced the activity of cellulolytic bacteria and the digestion of straw (KANG et al., 2014). These results can be explained because the moringa cake increased the production of propionate, removing H_2 + for the formation of short-chain fatty acids. The castor bean had a high fiber content, which explained the greater increase pH.

Studying the substitution levels for each byproduct pH, the cottonseed cake (P = 0.0001) presented quadratic behavior with 49% of the maximum measurement point (5.85); the other byproducts showed a linear model (P<0001), with every 1% increase in byproduct substitution the increase in pH was 0.0085 with the castor bean, 0.0078 with the moringa cake, 0.0066 with the sunflower cake, and 0.0076 with the jatropha cake.

Among the byproducts studied, moringa cake at levels of 30, 50 and 70%, substituting corn silage was distinguished for promoting mitigation of methane and carbon dioxide and obtaining levels of pH and $N\text{-}NH_3$ satisfactory and VAFs concentration, for maximum rumen fermentation; thus, it is recommended to use the moringa cake to replace corn silage because adding this byproduct to the diet provides for a lower environmental impact.
Other byproducts (cotton cake, jatropha cake, sunflower cake and castor bean) also are likely to be used as a protein supplement in the diet of cattle, and it is advisable to use up to 30% substitution with byproducts so as not to harm the process of ruminal fermentation.

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