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In vitro degradation and total gas production of byproducts generated in the biodiesel production chain

Raissa Kiara Oliveira de Morais[1], Aderbal Marcos de Azevedo Silva[2], Leilson Rocha Bezerra[3*], Heloisa Carneiro[4], Milenna Nunes Moreira[1] and Fabiola Franklin de Medeiros[1]

*[1]Programa de Pós-graduação em Zootecnia, Universidade Federal de Campina Grande, Patos, Paraíba, Brazil. [2]Unidade Acadêmica de Medicina Veterinária, Universidade Federal de Campina Grande, Patos, Paraíba, Brazil. [3]Universidade Federal do Piauí, Rod. Bom Jesus Viana, km 3, 64900-000, Bom Jesus, Piauí, Brazil. [4]Empresa Brasileira de Pesquisa Agropecuária, Juiz de Fora, Minas Gerais, Brazil. *Author for correspondence. E-mail: leilson@ufpi.edu.br*

ABSTRACT. This study aimed to evaluate the *in vitro* degradation and total gas production of different oil seed press cakes from a biodiesel production chain gas through the use of a semi-automatic technique of gas production *in vitro*. The treatments consisted of substituting elephant grass in increasing levels, 0%, 30, 50 and 70%, with the byproducts of *Gossyypium hirsutum*, *Ricinus communis*, *Moringa oleifeira*, *Jatropha curcas* and *Helianthus annus*. The oil seed press cakes of *Moringa oleifeira* had the highest rate of *in vitro* degradation of dry matter compared with other foods but did not result in a higher final volume of gases production. *Gossyypium hirsutum*, *Pinhão manso curcas* and *Ricinus communis* showed a higher *in vitro* degradability of similar dry matter. The highest total gas production was obtained by the oil seed press cakes of *Helianthus annus*. The oil seed press cakes of *Moringa oleifeira* can replace elephant grass up to 70% and therefore reduce both greenhouse gas emissions and energy loss for the animal.

Keywords: degradability, *Gossypium hirsutum*, greenhouse gases, *Ricinus communis*, ruminal fermentation.

Degradação *in vitro* e produção total de gás de subprodutos gerados na cadeia produtiva do biodiesel

RESUMO. Objetivou-se avaliar a degradação *in vitro* e produção total de gás de bolos diferentes prensas de sementes de óleo de um gás cadeia produtiva do biodiesel através do uso de uma técnica semi-automática de produção de gás *in vitro*. Os tratamentos consistiram na substituição do capim elefante por níveis crescentes, 0, 30, 50 and 70%, dos coprodutos da prensagem de *Gossyypium hirsutum*, *Ricinus communis*, *Moringa oleifeira*, *Jatropha curcas* and *Helianthus annus*. As tortas oriundas da prensagem de sementes de óleo de *Moringa oleifeira* apresentaram a maior taxa de degradação *in vitro* da matéria seca quando comparado com as outras tortas, porém este comportamento não resultou em um volume final maior de produção de gases. Os copodutos do *Gossyypium hirsutum*, Pinhão-manso e *Ricinus communis* apresentaram maior degradabilidade *in vitro* da matéria seca e não diferiram entre si. A maior produção total de gases foi obtida pelos bolos de prensagem de sementes oleaginosas de Helianthus annus. A torta oriundas da prensagem de sementes de óleo de *Moringa oleifeira* pode substituir capim-elefante até 70% e, portanto, reduzir as emissões de gases de efeito estufa e perda de energia para o animal.

Palavras-chave: degradabilidade, *Gossypium hirsutum*, gases de efeito estufa, *Ricinus communis*, fermentação ruminal.

Introduction

Research involving the production of greenhouse gases has globally increased because of alterations in the ozone layer and its influence on human health (Giger-Reverdin et al., 2002). Brazil has the world's second largest cattle herd and has signed the Kyoto Protocol; therefore, the country monitors the amount of greenhouse gases produced by the national herd. The introduction of non-conventional foods with characteristics that favor an amelioration in greenhouse gases levels and consequently reduce environmental impacts are now prerequisites for funded projects that benefit the production system for these animals and that contribute to the development of the various production chains (Moreira et al., 2014).

In addition to environmental aspects is the economic aspect in which Brazil, a large amount of agricultural byproducts are produced, and agribusiness products with potential use in animal feeding, especially those from the biodiesel chain (pies and sharps), can be used as sources of nutrients for animals for immediately degradation in the rumen (Muller & Aubert, 2014).

Moreover, when the mandatory blending of biodiesel to diesel was only 3%, Brazil had a potential production of pies and/or sharps in the order of 14,746 kg^{-1} ha^{-1} $year^{-1}$. This production can vary depending on the culture, can demonstrate different performance levels and can generate a larger or smaller volume of waste (Abdalla et al., 2008).

The existence and use of such discharges should be considered an important step in the process of oil extraction for biodiesel production because environmental laws have become increasingly strict in relation to the processing units of plant materials with respect to the fate of these residues (Manika et al., 2013). The production of such waste also represents a loss of biomass and nutrients and is associated with the wasteful use of materials, especially materials that are generated alongside the agribusiness chain that have no apparent economic value. These materials have lately been considered for feeding ruminant animals (Tahseen et al., 2014).

The byproducts generated by the diversity of fatty materials used in the biodiesel chain, such as cotton, castor, moringa, pinhão manso and sunflower, may represent a desirable good quality protein concentrate for ruminants. However, its use in the diet depends on the use of byproduct technologies to maximize livestock productivity and add value to the biodiesel production process (Oliveira et al., 2013).

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. In this context, we aimed to evaluate the use of several byproducts of the biodiesel production chain through *in vitro* ruminal degradability testing using the gas production technique.

Material and methods

The experiment was conducted in April 2012 at the Experimental Field José Henrique Bruschi, Coronel Pacheco, owned by Embrapa Dairy Cattle, located in Minas Gerais State, Brazil.

The substrates used for *in vitro* incubations were elephant grass (*P. purpureum*), with a cutoff age of 50 days, as a treatment control and the byproducts of the biodiesel industry. These byproducts were: castor (Family Euphorbiaceae; *R.s communis*), cotton (Family Malvaceae; *G. hirsutum* L.), moringa (Family Moringaceae; *M. oleifera* Lam.), pinhão manso (Pinhão manso curcas) and sunflower (Family Asteraceae; *H. annuus*).

Samples of approximately 300 g 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.

The substrates consisted of material and byproducts which were pre-dried in a forced greenhouse at 55°C for 48 hours ventilation. After milling in a Wiley mill (1.0 mm screen perforations at 105°C), we determined the dry matter (DM) content and crude protein (CP), according to the Kjeldahl method according to the general procedures described by AOAC (2005); neutral detergent fiber (NDF), and acid detergent fiber (ADF) according to the method of (Van Soest et al., 1991), acid detergent lignin (ADL) and ether extract (EE) were determined using an ANKOM®XT10 system. The nonfibrous carbohydrates (NFC) were obtained according to Mertens (2002) and total carbohydrates (TC) were calculated using the equation 100 - (%CP + %EE + %ash) described by (Sniffen et al., 1992).

Diets were formulated by replacing elephant grass (control) with byproducts in the following proportions: 100/0, 70/30, 50/50 and 30/70 (elephant grass / byproduct). After being dried at 55°C for 48h, the ingredients of the diet were milled to 1 mm. Next, we weighed 0.5 g of dry matter into a sample bag (ANKOM® (F57) with 6 replicates/treatment and then sealed and placed the bags into glass serum vials (amber color, 50 mL), which were previously washed with distilled water, dried the samples in an oven and identified them.

Three rumen fistulated lactating Holstein-Friesian dairy cows were fed a diet consisting of 60% forage and 40% concentrate pellets (22% CP and 12.6% NDF in DM) on a DM basis for the *in sacco* study. An inoculum for the *in vitro* incubation was obtained from three rumen fistulated cows grazing beard grass and that were fed 4 kg of concentrate (22.0% CP and 12.6% NDF in DM) each day as a supplement. Rumen fluid was collected 2h before morning milking from 4 distinct sites in the rumen, filtered through four layers of cheesecloth, combined in equal portions from each animal and transported in a pre-warmed Thermos® flask to the laboratory. The inoculum was prepared by mixing rumen fluid and a mineral buffer with 0.5 mL of a cysteine sulfide solution (Vitti et al., 1999) at a ratio of 1:3. The inoculum (30 mL) was then transferred into pre-loaded, pre-warmed (39°C) vials under stream of O_2-free nitrogen (N) gas. The vials were sealed and placed on an orbital shaker rack set at 120 oscillations per minute in an incubator set at 39°C.

The incubation of 126 white bottles, with six containing only rumen fluid and culture as a buffering control, was used to determine the production of gas from the rumen contents for the later correction of net gas production. The remaining bottles corresponded to 6 replicates per treatment.

The profiles of gas production *in vitro* in each vial were measured 48 hours after incubation using a graduated water displacement apparatus to measure in milliliters. After the incubation period, the bags of waste (ANKOM®) were removed and placed on ice to stop the fermentation and were then washed with water and dried in an oven at 55°C for 48 hours. The *in vitro* dry matter degradability (IVDMD) was obtained by calculating the difference in weight between the dry matter of the sample before and after incubation.

The experimental design used a randomised 5 x 4 factorial arrangement (byproduct levels x replacement). When the variable was independent of the main factors of the byproducts, we applied the mean test, and replacement levels determined the most representative regression model. Those variables where the effect of the main factors was dependent, the regression model that best represented the data were applied to the byproducts according to the level of substitution of sugar cane. Additionally, the effect of the byproducts at each level of substitution was subjected to the mean test. When choosing the regression models that best represented the behaviour of the data, we considered significant at a p -value of ≤ 0.05 using the tukey's test (SAS®, 2003).

Results

The chemical composition of byproducts and elephant grass (control), shown in Table 1, indicates that the elephant grass had protein levels of 126.1 g kg^{-1}; fibrous constituent (NDF, ADF and lignin) values of 555.0, 351.1 and 144.4 g kg^{-1}, respectively; and a rate of 591.4 g kg^{-1} *in vitro* digestibility of dry matter.

Among the byproducts, the pinhão manso and sunflower showed the lowest crude protein values (356.9 and 329.4 g kg^{-1}, respectively), but the byproducts of moringa and cotton had the highest values in this analysis (577. 6 and 549.9 g kg^{-1}, respectively). Note that the highest levels of ether extract were found in the byproducts of sunflower and pinhão manso (162.0 and 110.6 g kg^{-1}, respectively), and the smallest levels were found in the byproducts of cotton and castor bean (40, 3 and 43.8 g kg^{-1}, respectively).

The lowest values of NDF were found in the byproduct of moringa and cotton (202.7 and 303.6 g kg^{-1}). For the byproduct sunflower, a value of 439.7 g kg^{-1} was determined, and for the byproduct of castor, a value of 423.3 g kg^{-1} was determined.

The highest levels of ADF byproducts were found in sunflower (384.0 g kg^{-1}) and castor oil (383.4 g kg^{-1}), which also showed the highest percentage rates of lignin (120.4 and 154.4 g kg^{-1}, respectively).

The highest values found for IVDMD were 595.6 and 791.3 g kg^{-1} of the byproducts of cotton and moringa, respectively, and the lowest rates determined were those of the byproducts of castor oil (497.5 g kg^{-1}) and sunflower (463.1 g kg^{-1}).

Analyzing IVDMD (Table 2), there was a dependent interaction between the effect of byproducts and replacement levels of elephant grass.

Table 1. Chemical composition (g kg^{-1}) of elephant grass (control), cotton, moringa, pinhão manso, sunflower and castor.

Substrates	DM	CP	NDF	ADF	ADL	EE	ASH	NFC	TC	IVDMD
Elephant grass	882.3	126.1	555.0	351.1	144.4	14.2	25.4	279.3	834.3	591.4
Cotton	929.1	549.9	303.6	207.7	32.1	40.3	68.3	37.9	341.5	595.6
Castor	912.6	420.2	423.3	383.4	154.4	43.8	42.3	70.3	493.6	497.1
Moringa	901.2	577.6	202.7	80.5	10.3	84.8	49.8	85.1	287.8	791.3
Pinhão manso	920.7	356.9	391.4	334.5	43.4	110.6	79.5	61.6	453.0	571.3
Sunflower	914.5	329.4	439.7	384.0	120.4	162.0	41.3	27.6	467.3	463.1

Abbreviations: DM, dry matter; CP, crude protein; NDF and ADF, neutral and acid detergent fiber; ADL, acid detergent lignin; EE, ether extract; NFC, ASH, ashes; non fiber carbohydrate, TC, total carbohydrates; IVDMD, *in vitro* dry matter degradability.

Table 2. Mean values of *In vitro* Dry Matter Degradability (IVDMD) (%) and regression equations representing the replacement levels of elephant grass within the byproducts after 48 hours of incubation.

Byproducts	Levels of substitution %				Equation	R^2	P-value
	0	30	50	70			
Cotton	52.93	55.34^{A}	53.22^{A}	54.44^{A}	Y = 53.50	0.01	0.51
Castor	52.93	46.49^{B}	42.48^{B}	35.00^{C}	Y = 53.55-0.24x	0.89	< 0001
Moringa	52.93	52.06^{AB}	52.93^{A}	59.80^{A}	Y =51.51+0.08x	0.50	0.004
Pinhão manso	52.93	48.48^{B}	43.64^{B}	41.59^{B}	Y= 52.99-0.16x	0.84	<0001
Sunflower	52.93	53.53^{AB}	51.05^{A}	47.54^{B}	Y= 53.93-0.07x	0.30	0.007

*Different letters in the same column differ significantly by the Tukey test ($p < 0.05$). R^2, regression coefficient and p-value, probability.

Examining the byproducts within each level of substitution, we noted that at the levels of 30 and 50%, the byproduct cotton presented an *in vitro* degradability of dry matter of 55.33 and 53.22%, respectively, exceeding the byproducts of castor and pinhão manso ($p < 0.05$) but not differing from the byproducts of moringa and sunflower ($p > 0.05$). However, at the level of 70% substitution, the byproducts of moringa and cotton had higher levels of DMS (59.80 and 54.44%, respectively) than the levels of other byproducts analyzed ($p < 0.05$).

Considering the replacement levels of elephant grass with byproducts alone, we observed that the byproducts of pinhão manso and castor showed a negative correlation, i.e., as the byproducts increased the percentage unit reduction occurred in the degradation of this food (0.16 and 0.24%, respectively). The byproduct of moringa showed a linear correlation; therefore, with increasing levels there was an increase of 0.10% percentage replacement units. The behavior of the byproduct of sunflower showed a decrease in production by 0.07% percentage replacement units.

The effect of byproducts and replacement levels of elephant grass were dependent (Table 3). We noticed that for all levels of substitution, the byproduct of sunflower showed superior total production when compared to the gas analysis of other byproducts.

However, the byproduct of pinhão manso differed statistically from the other byproducts levels at 50 and 70% by producing lower total gas levels (44.01 and 43.03 g mL^{-1}, respectively).

Analyzing the replacement levels of elephant grass by byproducts within each byproduct, it is observed that the byproduct of sunflower had the highest final volume of gas production (123.96 mL g^{-1} DM incubated). Averages for the final production of the byproducts of cotton, castor beans and moringa had the highest rates at the 30% level and the lowest rates at 70% substitution.

Regression analysis of the total gas production showed that the byproducts that had higher and lower gas production (sunflower and pinhão manso, respectively) were represented by a linear model, as were other byproducts (from castor beans and moringa), except for the byproduct of cotton.

Discussion

A major downfall of their use in the diet is the presence of high levels of lignin in some byproducts, such as sunflower and castor beans, which most likely negatively influence the degradation of fiber and non-fiber (Mizubuti et al., 2011). The protein content of these byproducts cited in literature is on average 35%, ranging from 14-60% (Abdalla et al., 2008). In this study, the byproducts that had the lowest values of CP were the castor bean and sunflower, which remained within the varying levels cited by the author above.

The highest levels of EE achieved by the byproducts were sunflower (162 g kg^{-1}) and pinhão manso (120.4 g kg^{-1}), which can be considered beneficial because the inclusion of oil in the diet can assist in the mitigation of total gases, as cited in the literature (Farias et al., 2012). Nevertheless, care is required when adding oleaginous content to the diet of ruminants because levels above 7% may interfere with the intake of fiber and have the same deleterious effect for ruminal microorganisms (Azevedo et al., 2013; Tamminga & Doreau, 1991).

Another constituent of the diet affects the digestibility and the total gas production is the fibrous content. The presence of lignin, for example, tends to increase the indigestible portion, thereby reducing the digestible portion (Zeoula et al., 2003). The fiber content levels (NDF and ADL) presented in this study were higher than those values reported by Goes et al. (2010), except for the byproducts of moringa and cotton. This variation can be explained by the lack of standardization in relation to crop management and processing of seeds.

Table 3. Means values of the total gas production (mL g^{-1}); the regression equations of replacement levels of elephant grass within the byproduct values.

Byproducts	Levels of substitution%				Equation	R^2	p-value
	0	30	50	70			
Cotton	72.17	73.98^B	70.10^B	65.78^B	Y = 73.97	0.09	0.14
Castor	72.17	59.43^C	53.08^C	45.00^C	Y =71.80-0.38x	0.93	< 0001
Moringa	72.16	57.80^C	54.58^C	46.78^C	Y = 70.96-0.35x	0.88	< 0001
Pinhão manso	72.16	52.00^C	44.01^D	43.03^D	Y = 68.89-0.42x	0.83	< 0001
Sunflower	72.16	119.90^A	121.83^A	123.96^A	Y = 84.27+0.69x	0.78	0.0004

Different letters in the same column differ significantly by tukey test ($p < 0.05$). R^2, regression coefficient and p-value, probability.

In this study, regarding the byproduct pinhão manso and byproduct of castor, the decrease in IVDMD, in conjunction with the increase of substitution, can be explained by two hypotheses: the first involves high lignin content because foods that have less lignin promote better access to micro-organisms (Goes et al., 2010) and the second involves the presence of anti-nutritional compounds. Such byproducts interfere with the degradation process. In the case of castor, the toxic substance is ricin, and in the case of a pinhão manso, toxicity is mainly due to the presence of saponins (Rakshit et al., 2008). The higher degradability exhibited by the byproduct of moringa was the result of a lower concentration of fiber and lignin and a higher carbohydrate concentration when compared to other byproducts.

In the process of gas production after replacement levels of 0, 30, 50 and 70% of the forage, the byproduct of sunflower showed the highest final volume of total gas production at all levels ($p < 0.05$).

The byproduct of sunflower, despite having the highest final volume of gases, did not obtain the highest rate of *in vitro* degradation of dry matter. This opposed the results of Mizubuti et al. (2011), which confirmed that the greater the degradation of food during ruminal fermentation, the better the quality because there is an improved balance between energy and nitrogen compounds available to the rumen microorganisms. According to the literature, the volume of gas produced in the same incubation period is reflected in the production of volatile fatty acids, which serve as an energy source for the animal (Getachew et al., 2005). In the case of the sunflower byproduct, it is most likely that the microorganisms colonized fibrous food particles and did not contact enough lignified structures, which produced more acetate, free hydrogen and carbon dioxide, thereby increasing the production of gas.

The byproduct of pinhão manso, however, had the lowest total gas production. This decrease in production is related to the lower degradability, corroborating the results obtained by Makkar et al. (1999). According to Neiva Júnior et al. (2010), the addition of pinhão manso cakes to elephant grass silage resulted in a marked reduction in the *in vitro* digestibility of dry matter for silage with pinhão manso. Therefore, the byproduct of pinhão manso, despite its high protein content, should be detoxified and included in the diet of animals.

The behavior of the byproduct of moringa, which had the highest degradability at the level of 70% substitution and lower gas production compared to the sunflower, indicates that moringa may contain antibacterial properties that allow binding to ruminal microorganisms. At low levels, these proteins may be protected from degradation in the rumen, but high levels of these proteins may inhibit the process of rumen fermentation (Beauchemin & McGinn, 2005).

The gas production is a reflection of the effectiveness and extent of degradability of food because forages that have a high ruminal degradability of dry matter tend to have high gas production (Njidda & Nasiru, 2010). On the production side, the moringa byproduct stands out among the rest. It showed indications for use in dairy diets because the high rate of degradability by the rumen promotes increased availability of energy, which likely results in higher milk production.

The results obtained with the byproducts of pinhão manso, moringa and castor allow us to infer that these foods have the same behavior as rumen fermentation. This was in agreement with Abdalla et al. (2008), who, in a survey conducted with these byproducts, showed that over 50% of the replacement material lowered the total production of gas.

Conclusion

The byproduct of Moringa at 70% level of replacing elephant grass increases the *in vitro* dry matter degradability (IVDMD) while the byproduct of Pinhão manso at 70% level has the highest potential in the total reduction of gases. Further more research are necessary for checking if the gases were reduced are beneficial in promoting the reduction of the greenhouse effect and the byproducts influence the ruminal metabolism.

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