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In vitro degradation and gas production of *brachiaria* grass with levels of biodiesel byproducts

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ABSTRACT. This study aimed to evaluate the *in vitro* degradability and gas production in diets containing byproducts from the national biodiesel industry (castor bean, canola, forage radish and black sunflower) replacing *Brachiaria* grass in four levels (0, 30, 50 and 70%). The inoculum for *in vitro* incubation was obtained from three fistulated Holstein cows. The experimental design was 4 x 4 factorial completely randomized experimental design consisting of four byproducts and four levels. All byproducts studied had a significant effect ($p < 0.05$) on *in vitro* digestibility. The castor bean byproducts promoted the lowest cumulative gas production at the end of 48 hours incubation. Regarding digestibility, the byproducts of canola and radish at 70% level did not affect the degradability of dry matter.

Keywords: digestibility, ruminal fermentation, protein.

Degradação e produção de gases *in vitro* de *capim brachiaria* com níveis de coprodutos do biodiesel

RESUMO. Objetivou-se avaliar a degradabilidade e produção de gás *in vitro* em dietas com coprodutos produzidos pela indústria nacional do biodiesel (mamona, canola, nabo forrageiro e girassol preto) em substituição ao *capim brachiaria* em quatro níveis (0, 30, 50 e 70%). O inóculo para incubação *in vitro* foi obtido de três vacas da raça Holandesa com fistulas ruminais. O delineamento experimental foi inteiramente casualizado, em um arranjo fatorial 4 x 4 , com os fatores representados pelos quatro coprodutos e os quatro níveis. Todos os coprodutos estudados apresentaram significativo efeito sobre a digestibilidade *in vitro* ($p < 0,05$). O coproduto da mamona promoveu a menor produção cumulativa de gases no final de 48h de incubação. Com relação à digestibilidade, os coprodutos da canola e do nabo forrageiro em nível de 70% não prejudicou a degradabilidade da MS.

Palavras-chave: digestibilidade, fermentação ruminal, proteína.

Introduction

In Brazil, a large number of byproducts from agroindustry have potential for use in animal feed; planning and proper allocation allows for the establishment of new partnerships and solutions between industry and livestock (Geron, Mexia, Garcia, Silva, & Zeoula, 2012). Many cultivated oleaginous crops are being studied in Brazil to be used in biodiesel industrialization process, such as sunflower (*Helianthus annuus L.*), castor bean (*Ricinus communis L.*), forage radish (*Raphanus sativus L.*), canola (*Brassica napus* L.), among others.

Studies and techniques that characterize these byproducts, such as the *in vitro* degradability technique by gas production, enable the identification of byproducts that are efficient in replacing conventional foods. These techniques are less costly and facilitate control of experimental conditions being efficient, reproducible, and highly correlated with *in vivo* results (Getachew, Blummel, Makkar, & Becker, 1998). Gas production is directly proportional to the microbial fermentation of the food and, as it can be measured at frequent intervals, allows to evaluate the way the microbial attack occurs in the degradation of the food in the rumen.

The goal of this study was to evaluate the effects on *in vitro* degradability, by total gas production, of different levels of biodiesel chain byproducts replacing brachiaria grass in the diet for cattle.

Material and methods

The experiment was carried out at the Experimental Field of Coronel Pacheco, owned by Embrapa Dairy Cattle - National Research Center,

located in Zona da Mata of the state of Minas Gerais.

The substrates used for *in vitro* incubation were *Brachiaria* grass (*Brachiaria brizantha* at 28 days of cutting) as control and byproducts resulting from the biodiesel industrialization: castor bean (*Ricinus communis L.*), canola (*Brassica napus L.*), black sunflower (*Helianthus annus L*), and forage radish (*Raphanus sativus L.*).

The substrates composed of the byproducts and the forage were pre-dried in a forced ventilation oven at 55°C for 48 hours, then ground in a Wiley mill with 1.0 mm sieves to determine dry matter (DM) (Method 967.03 - Association of Official Analytical Chemists [AOAC], 1990). Ash was determined by the method 942.05 (AOAC), crude protein (CP) by the method 981.10 (AOAC) and ether extract (EE) by the method 920.29 (AOAC, 1990). To determine the NDF and the FDA, we used the methodology of Van Soest, Robertson, and Lewis (1991) was used, due to modifications proposed in the Ankon device manual (Ankon Technology Corporation Macedon, New York, USA)

The DDM was evaluated following the methodology proposed by Tilley and Terry (1963). Total fiber carbohydrates were determined by the methodology described by Detmann and Valadares Filho (2010).

Treatments were formulated, replacing the *brachiaria* grass (control) with the byproducts in the following proportions 100/0, 70/30, 50/50 and 30/70 (forage/by-product). The ingredients composing the treatments were dried at 55°C for 48 hours and then ground to 1 mm. We then weighed 0.5 g dry matter (DM) of the sample, placed into a bag of ANKOM® (F57) with 6 replicates per treatment, sealed and placed into 50 mL pre-washed serum-amber glass bottles with distilled water, dried in an oven and identified.

Rumen fluid was collected from three fistulated Holstein cows with average weight of 600 kg and transferred to thermal bottles previously heated at 39°C and taken immediately to the laboratory. In the laboratory, the ruminal contents were homogenized and filtered through two layers of cotton cloth, kept in a water bath at 39°C under CO_2 saturation until adding the other solutions (buffer, macro- and micro-minerals,resazurin solution and buffer B) for the culture medium.

It was used rumen fluid and buffer solution (Vitti et al., 1999) at a ratio of 5:1. The inoculum (30 mL) was then transferred to incubation flasks, then sealed and placed on a orbital shaker rackset at 120 oscillations per minute in an incubator at 39°C.

Flasks (n = 102) were incubated, 6 of which contained only the rumen liquid and the buffer culture medium as control (blank), used to determine the production of gas from the rumen content for later correction of the net gas production, the other flasks corresponded to six repetitions of each treatment.

The cumulative *in vitro* gas production profiles of each flask were measured at 3, 6, 12, 24 and 48 hours after incubation using a water displacement apparatus graduated in mL.

After 48 incubation hours, ANKOM® bags with the residues were removed and placed on ice to stop fermentation, then washed with abundant water and oven dried at 55°C for 48 hours. The apparent dry matter degradability was obtained by the difference in weight between the dry matter of the sample before and after the incubation.

The statistical design used to evaluate cumulative gas production and dry matter degradability was a 4x4 factorial completely randomized design (four byproducts and four levels of replacement).

The total gas production and the DM degradability were subjected to an analysis of variance (Proc Anova) of SAS, and Tukey's test ($p < 0.05$) was applied to the effects of the main factors between the byproducts and of these within each level of replacement and of replacement levels within each byproduct. The results of the increasing levels were analyzed statistically through the regression models by PROC REG (Statistical Analysis System [SAS], 2003).

Results and discussion

The chemical composition and the results are presented in Table 1, where the high protein content was observed for the coproducts, mainly for castor bean and forage radish (420.2 and 393.7 g kg^{-1}, respectively). The latter still has a large amount of residual ether extract from the process of cold extraction (pressing). Regarding the NDF content, castor bean and canola (523.3 and 410.3 g kg^{-1}) showed the highest levels. Silva et al. (2013) report that high NDF and EE content decrease the degradability of diets, leading to rumen defaunation, due to the deleterious action of unsaturated fatty acids.

Table 1. Chemical composition (g kg^{-1}) of *Brachiaria* grass and the four substrates resulting from biodiesel industrialization, on a dry matter basis.

Ingredient	DM (g kg^{-1})	CP (g kg^{-1})	NDF (g kg^{-1})	ADF (g kg^{-1})	LIG (g kg^{-1})	EE (g kg^{-1})	ASH (g kg^{-1})	NFC (g kg^{-1})	DDM (g kg^{-1})
Brachiaria grass	870.8	122.2	556.2	277.7	34.7	32.2	86.1	203.3	652
Byproducts									
Forage radish	935.6	393.7	217.1	137.1	69.6	284.1	50.6	54.5	644.8
Castor bean	912.6	420.2	423.3	383.4	154.4	43.8	42.3	70.4	497.1
Black sunflower	901.1	342.6	390.1	243.6	34.3	32.1	54.9	180.3	582.3
Canola	922.1	375.1	410.3	378.3	120.5	24.3	57.7	132.6	689

DM: dry matter; CP: crude protein; ND: neutral detergent fiber; ADF: acid detergent fiber; LIG: lignin; EE: ether extract; CZ: Mineral matter; NFC: non-fiber carbohydrates, DDM: in vitro dry matter digestibility.

Considering the levels of replacement of *brachiaria* grass with biodiesel byproducts (Table 2), it was found that the linear regression model that best explained the degradability of the canola byproduct with a 0.22% increase in the digestibility of DM for the addition of a percent unit of the byproduct (Table 2). The same behavior was observed for the forage radish and black sunflower byproducts, which showed an increase in digestibility of 17 and 11%, respectively. For the castor bean byproduct with increasing participation per percent unit, degradability was reduced by 0.24%.

Table 2. Mean values and regression equations for *in vitro* dry matter digestibility (DDM %) of byproducts according of replacement levels of brachiaria grass.

Byproduct	Replacement levels				Regression equation	R^2	P-value
	0%	30%	50%	70%			
Forage radish	52.76	61.40^{A}	62.20^{A}	65.36^{AB}	$\hat{Y}$ = 53.947+0.172x	0.81	< 0001
Castor bean	52.76	46.10^{B}	41.16^{C}	35.50^{C}	$\hat{Y}$ = 53.081-0.245x	0.95	< 0001
Black sunflower	52.76	57.36^{A}	58.06^{B}	60.76^{B}	$\hat{Y}$ = 53.151+0.109x	0.86	< 0001
Canola	52.76	60.06^{A}	63.33^{A}	68.36^{A}	$\hat{Y}$ = 52.941+0.218x	0.95	< 0001

*Different letters in the same column indicate significant differences by Tukey's test at 5% probability, R^2 = regression coefficient and P = probability.

Canola, forage radish and black sunflower showed superior digestibility to castor bean. This performance was possibly due to the lower lignification of DM and lower fiber concentration, reducing the rumenfilling effect. As for the castor byproduct, it presented the lowest rate of degradability, which may be associated to the high content of fiber and lignin in its composition. The presence of lignin tends to increase the indigestible fraction, thus reducing the potentially digestible fraction and causing a great rumenfilling effect, limiting dry matter intake (Mizubuti et al., 2011; Medeiros et al., 2015). In addition, castor oil has antinutritional compounds. Ricin is a protein located only in the castor bean endosperm, which promotes cell death by inhibiting protein synthesis, leading to death or inhibition of rumen microorganisms, thus reducing food degradation (Oliveira et al., 2010).

The effect of the main factors was dependent on the cumulative gas production within 48 hours of incubation (Table 3). As for the replacement levels of brachiaria grass within each byproduct, it was observed that the by-product of castor bean had a decreasing linear behavior ($p < 0.001$), that is, the increase of a percentage unit of that byproduct reduced by 0.74 mL g^{-1} in total gas production.

In relation to the production of gases of canola, forage radish and black sunflower, there was a quadratic behavior. The byproduct of castor bean promoted the lowest cumulative gas production at the end of 48 incubation hours, because the high protein concentration of this byproduct acts in the formation of bicarbonate from CO^2, favoring the reduction of gas production (Cieslak, Szumacher-Strabel, Stochmal, & Oleszek, 2013).

Moreover, the antinutritional factors of ricin are able to bind to the ribosomes, stopping intracellular protein synthesis and causing cell death (Oliveira, Antoniassi, Freitas, & Muller, 2013), making it highly detrimental to rumen microorganisms and explaining the reduction in gas production.

The highest cumulative gas production occurred with the byproduct of canola, followed to a lesser extent by the black sunflower and the forage radish. Lana (2007) emphasizes that the volume of gases produced from an incubated food reflects on the production of short chain fatty acids, which are the main source of energy for ruminants.

Table 3. Mean content, P-probability and regression equations of the effects dependent on total gas production (mL g^{-1}) of biodiesel production byproducts at different levels of replacement of *Brachiaria* grass after 48 hours of in vitro incubation in culture medium.

By-product	Replacement levels				Regression equation	R^2	P
	0%	30%	50%	70%			
Canola	121.63	181.17^{A}	188.40^{A}	193.40^{A}	$\hat{Y} = 122.587+2.492x-0.021x^2$	0.96	< 0001
Forage radish	121.63	123.47^{C}	120.03^{C}	109.47^{C}	$\hat{Y} = 121.508+0.272x- 0.006\ x^2$	0.81	< 0002
Black sunflower	121.63	147.70^{B}	163.70^{B}	148.97^{B}	$\hat{Y} = 120.548+1.534x\ 0.015x^2$	0.86	< 0001
Castor bean	121.63	94.83^{D}	82.37^{D}	69.73^{D}	$\hat{Y} = 119.850-0.738x$	0.98	< 0001

*Different letters in the same column indicate significant differences by Tukey's test at 5% probability, R^2 = regression coefficient and P = probability.

The byproduct of canola proved to be able to provide nutrients for *in vitro* rumen microorganisms in relation to the others, and this can be explained by the high NDF content, thus producing acetate, CO_2 and hydrogen in the fermentation process, which contributes to a higher gas production. The preferential metabolic pathway for degradation of fibers used by cellulolytic bacteria generates a large part of the ruminal methane through the interspecific hydrogen transfer. It is observed that the reduction reactions of CO_2 are coupled to the supply of electrons by H_2. Biochemically, methanogenic organisms, when using H_2 present in the medium, are fundamental for the regeneration of cofactors, such as NAD + and NADP+. Therefore, they are fundamental for the maintenance of the whole set of fermentative processes in the rumen (Arcuri, Lopes, & Carneiro, 2006). The reaction occurs by the elimination of hydrogen from the combination of H_2 and CO_2, according to the following equation: $CO_2 + 4H_2 = CH_4 + 2H_2O$ (Morgavi, Martin, Jouany, & Ranilla, 2012).

Conclusion

The most expressive digestibility was achieved with the use of canola, but its use produced greater amount of gases. On the other hand, the use of forage radish byproduct promoted high digestibility and gas production significantly lower than canola.

At 70%, the byproducts of canola and forage radish increased digestibility, allowing a reduction in the emission of polluting and better use of energy by the animals.

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