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Short Communication

Capacity of ensilage of *Jatropha curcas* L. cake to degrade forbol esters

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ABSTRACT - The objective of this study was to evaluate the capacity of the ensilage of *Jatropha curcas* L. expeller cake to reduce the phorbol esters and its effect on fermentative losses, by adding soluble carbohydrates or microbial inoculants. The design was completely randomized with four replications in a 3 × 2 factorial arrangement, with three sources of soluble carbohydrates (SC, control, 50 g sucrose/kg or 50 g crude glycerin/kg as fed) and two doses of microbial inoculants (MI, 0 or 5×10^5 ufc *Lactobacillus plantarum* + 3.33×10^5 ufc *Propionibacterium* per g as fed). Twenty-four mini-silos (982 cm^3) of polyvinyl chloride were created and opened after 60 days of fermentation at room temperature. The pre-hydrated *Jatropha curcas* L. cake (282 g of water/kg) contained 0.424 mg of phorbol esters/g of dry matter. Ensiling reduced the phorbol esters in 47.4%, on average, regardless of the SC or MI. There was no interaction effect between SC and MI on effluent, gases or total dry matter losses. However, both losses were increased when SC were added, and it was higher with glycerin that than sucrose. The addition of MI reduced all fermentation losses. The process of ensiling, although partially to reduce the phorbol esters of pre-hydrated *Jatropha curcas* L. cake, is not indicated as a biodestoxification procedure.

Key Words: additives, biodestoxification, biodiesel, co-products, glycerin

Introduction

Jatropha curcas L. is a perennial plant, native to Central and South America, present in various soil and climatic conditions, whose seeds have high oil content (350 g/kg) (Oliveira et al., 2010.) Due to these characteristics, researchers have elected it as one of the potential sources of vegetable oil for biodiesel production.

The products resulting from the oilseed extraction of *Jatropha curcas* L. have chemical composition that is suitable for use in ruminant diets as protein sources. However, the presence of toxic compounds limits the use in animal feed, which reduces the competitiveness of *Jatropha curcas* L. over other oilseeds (Oliveira et al., 2010).

The phorbol esters represent the main toxic component of seeds, oil and products of oil extraction (Makkar et al., 2009). The biological effects of these compounds include inflammatory and carcinogenic ones and are correlated with activation of protein kinase C, which leads to a variety of cellular responses by phosphorylation (Azzi et al., 1992).

Together, chemical (alkaline) and physical (autoclaving) procedures effectively reduce the phorbol esters of jatropha curcas meal, and they were patented (Kumar et al., 2010). The biodestoxification shows as a promising alternative. Lipolytics fungi cultivated in *Jatropha curcas* L. seed meal were able to reduce up to 97% of the phorbol esters (Barros et al., 2011). The phorbol esters were biodegraded in soil between 9-19 days depending on the temperature and environmental humidity (Devappa et al., 2009). Since at the ensiling process there is intense biochemical modification of organic compounds by microbial action, it was hypothesized that ensilage can reduce levels of phorbol esters in the *Jatropha curcas* L. seed meal and that addition of soluble carbohydrate sources or microbial additives may boost this effect. However, so far, no evidence of this effect in national and international literature has been found.

Therefore, this research investigated if the ensiling process is able to reduce the level of phorbol esters in the *Jatropha curcas* L. cake, and if addition of soluble

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carbohydrate (sucrose or glycerol) sources or microbial inoculant (*Latobacillus pantarum* and *Propionibacterium*) influences these effects.

Material and Methods

The experiment was conducted in the laboratory of Animal Nutrition and Forage of Universidade Federal do Mato Grosso (UFMT), Campus Sinop, in the period from November 30, 2010 to January 31, 2011. The design was completely randomized in a 3 × 2 factorial arrangement, with three sources of soluble carbohydrates (control, 5% sucrose or 5% crude glycerin, natural matter basis) and two doses of microbial inoculant (with and without), in four replications.

The *Jatropha curcas* L. cake was obtained after *expeller* extraction, supplied by Bioauto (Nova Mutum, Mato Grosso). The crude glycerin (800 g of glycerol/kg and 150 mg methanol/kg, as feed) was obtained after the transesterification of soybean oil by Frialto (Lucas do Rio Verde, Mato Grosso).

Microbial inoculants recommended for high moisture grain silages (KERA-SIL Grão Úmido®) were used at a dose of 5×10^5 cfu of *Lactobacillus plantarum* (homofermentative bacteria> 85% of the acid produced in the form of lactic acid) + 3.33×10^5 cfu of *Propionibacterium* (propionic acid producing bacteria, inhibitory of fungi and yeasts growth) per gram of the ensiled material, as the minimum recommended for Muck (1989).

The *Jatropha curcas* L. cake (10% moisture) was hydrated with distilled water immediately before ensiling to achieve 30% moisture. Twenty-four 24 mini-silos of polyvinyl chloride (PVC, 50 cm of high and 5 cm of diameter, totaling 982 cm^3) were used, with caps provided of silicone valve type Bunsen to allow escape of gases derived from the fermentation process and quantify losses. Bags of nonwoven textiles containing 100 grams of dry sand (ar force for 24 hours at 55 °C) were added to the bottom of the mini-silo for uptake of the fermentation effluents. A total of 871 g/mini-silo of *Jatropha curcas* L. cake hydrated were added, comprising an average density of 887 kg mass ensiled/m^3.

Before ensiling, a sample of *Jatropha curcas* L. cake hydrated was collected, the pH was determined immediately (Silva & Queiroz, 2002) and stored in a freezer (-20 ºC) for subsequent determination of dry matter (DM, method 934.01 In; AOAC, 1990), ash (method 942.05 in; AOAC, 1990), crude protein (CP, method No 954.01, AOAC, 1990), ether extract (EE, method No 920.39, AOAC, 1990), neutral detergent fiber (NDF, Mertens, 2002) and phorbol esters (Makkar et al., 2007).

The effluent losses, gases losses and total dry matter losses were quantitatively determined based gravimetric difference. Sets of silo + cover + sand (SCSpre) were weighed prior to ensiling. Immediately after sealing, sets of silo + cover + sand + *Jatropha curcas* L. cake (SCSJpre) were weighed. After sealing, the silos were stored at room temperature and opened after 60 days. Before the opening of the silos, sets of silo + cover + sand + *Jatropha curcas* L. cake silage (SCSJpos) and sets of silo + cover + sand (SCSpos) were weighed.

Losses were estimated according to the following equations:

EL (g/kg of DM) = [(SCSpos - SCSpre) / (SCSJpre - SCSpre) × dry matter content of the material pre-ensiled (g/kg)] × 1.000.000;

GL (g/kg of DM) = [(SCSJpos - total weight of SCSJpre) / (SCSJpre - SCSpre) × dry matter content of the material pre-ensiled (g/kg)]×1.000.000;

TDML (g/kg of DM) = EL + GL.

in which: EL = effluent losses; GL = gases losses; TDML = total dry matter losses.

After weighing, three samples were taken from the post-ensiled material: one for immediate determination of pH (Silva & Queiroz, 2002), the other stored in a freezer (-20 ºC) for subsequent analysis of phorbol esters, and another one immediately pre oven-dried (55 °C) for 3 days, ground in a knife mill with a sieve of 1 mm and stored in sealed plastic bottles with a cover for further determination of the dry matter content.

The phorbol esters were determined as described by Makkar et al. (2007). The crude extracts were obtained by stirring the sample *Jatropha curcas* L. cake solution of dichloromethane and five sequential filtrations. A volume of 20 µL of extract was analyzed by high performance liquid chromatography in reverse phase (C18 4.6 × 250 mm). The results were expressed as equivalent phorbol-12-myristate 13-acetate (Sigma, USA).

The data were subjected to analysis of variance, considering soluble carbohydrate sources, microbial inoculants and the interaction between these factors. In occurrence of interaction soluble carbohydrates *versus* microbial inoculants, the effects were unfolded. Tukey test was adopted for multiple comparison of means with 0.05 as the probability level for type I error.

Results and Discussion

The pre-ensiled hydrated *Jatropha curcas* L. cake showed DM content of 717.8 g/kg, similar to adequate for typical moisture corn grain silage (Jobim et al., 1997). The

Jatropha curcas L. cake used may be classified as protein concentrates feed (> 200 g CP/kg dry matter) (Table 1). The CP content was lower than those found in oilseeds meal, mainly due to the full presence of seed hull in *Jatropha curcas* L. cake, which increases NDF and lignin fractions and reduces the CP content (Oliveira et al., 2010).

The mean value of phorbol esters (0.425 mg/g DM) found was lower than that classified as toxic (1.6 to 3.5 mg/g DM for *Jatropha curcas* cake or meal), but above the levels *Jatropha curcas* L. cake of edible seeds meal found (0.11 mg/g DM) in Mexico (Makkar et al., 1998; Aregheore et al., 2003, Martinez-Herrera et al., 2006). Therefore, the *Jatropha curcas* L. cake used in this study can still be considered potentially toxic if ingested by animals (Oliveira et al., 2010), requiring detoxification procedure.

Four peaks of phorbol esters were observed, between 28 and 31 minutes of chromatography of crude extract of *Jatropha curcas* L. cake pre-ensilage; the firsts two were more pronounced (Figure 1). Makkar et al. (2007) described the methodology of analysis (adopted in this study) considering a typical chromatogram containing four peaks of phorbol esters from 27 to 29 minutes.

The silage *Jatropha curcas* L. cake presented phorbol esters level mean of 0.223 mg/g of DM (Table 2): an efficiency of 47.4% reduction in toxic compounds by ensilage. This effect indicates a probable stearic enzymatic activity by the microbiota, but it is still necessary to investigate the

Table 1 - Means of pH, dry matter (DM), chemical composition and phorbol esters in pre-ensiled hydrated *Jatropha curcas* L. cake

Item	Value
pH	6.37
Dry matter (g/kg)	717.8
Ash (g/kg DM)	53.0
Crude protein (g/kg DM)	213.0
Ether extract (g/kg DM)	173.1
Neutral detergent fiber (g/kg DM)	519.7
Neutral detergent soluble carbohydrates (g/kg DM)	41.2
Phorbol ester[1] (mg/g DM)	0.424

[1] Expressed in equivalent phorbol-12-myristate 13-acetate (Sigma).

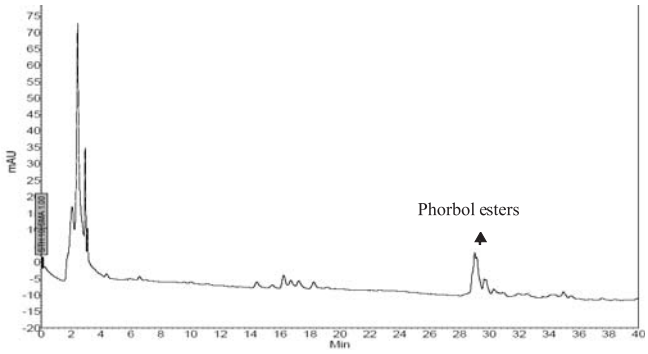

Figure 1 - Typical chromatogram obtained from crude extract of *Jatropha curcas* L. cake pre-ensiled with four peaks of phorbol esters from 28 to 31 minutes of chromatography.

populations responsible. It should be noted; however, that phorbol esters residue at the ensiled *Jatropha curcas* L. cake still cannot be considered safe for animal feed.

Barros et al. (2011) observed that the cultivation of lipolytic fungi (*Bjerkandera adusta and Phebia rufa*) in *Jatropha curcas* L. meal (0.80 mg phorbol esters/g of DM) decreased level phorbol esters in 91 and 97%. Thus, the presence of fungi during ensiling can be considered responsible potential for degradation, even partial, of phorbol esters. However, they are considered undesirable populations for ensiling, because they reduce the nutritional value of material and may negatively affect animal and human health, especially by the production of mycotoxins (Pereira et al., 2002).

The additions of soluble carbohydrate sources or microbial inoculates (homofermentative and propionic acid producers) did not affect (P>0.05) the phorbol esters in *Jatropha curcas* L. cake silage (Table 2). Thus, it can be concluded that lactic acid bacteria does not reduce phorbol esters at the ensiling of *Jatropha curcas* L. cake, even combining lactic acid bacteria population with substrate for growth increase (soluble carbohydrates).

There was no effect (P>0.05) of interaction between soluble carbohydrates and microbial inoculates on effluent

Table 2 - Effect of soluble carbohydrates sources (CS) and microbial inoculates (MI) on the phorbol esters (PE) level of hydrated *Jatropha curcas* L. cake silage (282 g water/kg)

Item	Effects					Effect (P-value)			CV (%)
	CS			MI[2]		SC	MI	SC × MI	
	0	Suc	Gli	Without	With				
PE (mg/g DM)[1]	0.225	0.212	0.232	0.244	0.203	0.99	0.99	0.99	40.3

Suc = sucrose (50 g/kg as fed); Gli = 50 g of crude glycerin (800 g glycerol/kg)/kg as fed. SC × MI = interaction between SC and MI; CV = coefficient of variation.
[1] Expressed in equivalent phorbol-12-myristate 13-acetate (Sigma).
[2] MI - KERA-SIL Grão Úmido®, dose of 5 × 10^5 cfu of *Lactobacillus plantarum* + 3.33 × 10^5 cfu of *Propionibacterium* per gram of ensiled material).

losses, gases losses or total dry matter losses during the ensilage of *Jatropha curcas* L. cake (Table 3). However, both losses were increased (P<0.05) with addition of soluble carbohydrate source; higher (P<0.05) for glycerin than than sucrose (Table 3).

Effluents from ensilage mainly comprise nitrogen compounds, carbohydrates, organic acids and soluble minerals (Pereira & Bernadino, 2004). Thus, probably, soluble carbohydrate fractions of sucrose and glycerin added are not fermented and used for microbial growth, which consequently increases the effluent fraction in the silo. The higher (P<0.05) effluent losses with glycerin addition also indicates that this substrate was used less efficiently than sucrose for microbial growth. The increase (P<0.05) of gases losses by the addiction of soluble carbohydrates is probably due to the increase in total fermentation brought about by substrate addition for microbial growth in the silo.

On the other hand, the addition of microbial inoculates based on lactic acid and propionic bacteria reduced (P<0.05) all losses (effluents, gases and total dry matter losses). With microbial addiction of inoculates, the utilization of soluble organic compounds in the silo was expanded, fixing in the microbial cell inoculated and reducing their availability in the medium with consequent reduction of effluent losses and gases losses.

There was interaction effect (P<0.05) between carbohydrate sources and microbial inoculates. Thus, the interaction was unfolded (Table 4). The addition of microbial inoculates decreased (P<0.05) the pH of silage without addition of soluble carbohydrates and with addition of sucrose, but did not affect (P>0.05) the pH when crude glycerin was added. This behavior confirms the earlier argument that addition of microbial inoculates increased fermentation and consequently the production of organic acids during ensilage. However, this did not occur when crude glycerin from biodiesel (80 g glycerol/kg) was added, indicating some inhibitory effect of crude glycerin on the microbiota added.

Table 4 - Deployment of interaction between soluble carbohydrates sources (SC) and microbial inoculates (MI) on pH of hydrated *Jatropha curcas* L. cake silage (282 g water/kg)

MI[1]	Carbohydrates source (g/kg as fed)		
	0	50 g sucrose	50 g glycerin
Without	5.45aA	5.04aC	5.31aB
With	5.38bA	4.80bC	5.31aB

[1] IM - KERA-SIL Grão Úmido®, dose of 5 × 10^5 cfu *lactobacillus plantarum* + 3.33 × 10^5 cfu *Propionibacterium* per gram of ensiled material).
Means followed by same uppercase letters are not different (P>0.05) in the same column. Means followed by same lowercase letters are not different (P>0.05) in the same row.

Sucrose was more effective in reducing (P<0.05) the pH of the ensiled *Jatropha curcas* L. cake than glycerin, regardless of addition of microbial inoculates. This indicated that sucrose is more effective in stimulating fermentation and acid production than glycerin and also corroborates the results and arguments presented on the most negative effect of glycerin on fermentation losses.

Although the addition of fermentation stimulators (soluble carbohydrates) increased losses during ensiling of *Jatropha curcas* L. cake, it can be noted that they can be considered low (<50 g/kg DM), compared with reference maximum total losses in ensilage of 70 g/kg suggested by McDonald (1981). Furthermore, the negative effects of glycerin on fermentations losses cannot be considered conclusive at the definition of real impact on animal performance, requiring therefore, investigation on animal performance and metabolism.

Table 3 - Effect of soluble carbohydrates sources (SC) and microbial inoculates (MI) on fermentation losses and pH of hydrated *Jatropha curcas* L. cake silage (282 g water/kg)

Item[1]	Effects					Effect (P-value)			CV (%)
	SC			MI[2]		SC	MI	SC × MI	
	0	Suc	Gli	Without	With				
EL (g/kg DM)	11.7c	13.7b	19.0a	18.3	11.3	0.013	0.001	0.440	30.6
GL (g/kg DM)	16.5c	18.4b	23.5a	22.4	16.5	0.045	0.015	0.999	27.3
TDML (g/kg DM)	28.9c	32.1b	42.5a	41.2	27.8	<0.001	<0.001	0.999	18.5
pH	5.42	4.92	5.31	5.27	5.17	<0.001	<0.001	<0.001	0.27

Suc = 50 g of sucrose/kg as fed, Gli = 50 g of crude glycerin (800 g glycerol/kg)/kg as fed; SC × MI = interaction between SC and MI; CV = coefficient of variation.
Means followed by different letters are different (P<0.05).
[1] EL = effluent losses; GL = gases losses; TDML = total dry matter losses.
[2] MI - KERA-SIL Grão Úmido®, dose of 5 × 10^5 cfu *Lactobacillus plantarum* + 3.33 × 10^5 cfu *Propionibacterium* per gram of ensiled material).

Conclusions

The ensiling process, in spite of partially reducing (47%) the levels of phorbol esters of hydrated *Jatropha curcas* L. cake (282 g of water/kg), is not indicated as biodestoxification procedure, regardless of the addition of soluble carbohydrate sources or lactic acid homofermentative and propionic bacteria.

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