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# Effects of macauba cake on profile of rumen protozoa of lambs

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**ABSTRACT** - The present study was conducted to determine the effects of the inclusion of macauba cake, from biodiesel processing, on profile of rumen protozoa of Santa Ines lambs. Twenty-four lambs were randomized in block design supplemented with macauba cake at 0, 100, 200, and 300 g/kg of dry weight of the diet. Concentrations of small, medium, and large protozoa had quadratic relationships with inclusion of macauba cake, with maximum protozoa occurring at 100 g/kg. High genus diversity occurred in rumen fluid of lambs that did not feed macauba cake, comprising 13 protozoa genera. However, only the genera *Isotricha*, *Charonina*, *Entodinium*, *Diplodinium*, *Eodinium*, *Diploplastron*, and *Polyplastron* were detected in lambs fed 300 g/kg macauba cake, indicating that these protozoa were resistant to the effects of the cake. Addition of macauba cake levels greater than 100 g/kg show antiprotozoal effect in the rumen.

Key Words: *Acrocomia aculeata*, byproduct, protozoa diversity, sheep

## Introduction

Macauba (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.) is an oleaginous palm tree native to dry hillsides and open forests from Central America to Southern Brazil. It is highly productive and adapted to semiarid ecosystems. The oil of macauba may be used to produce biodiesel, cosmetics, and foods (Coimbra and Jorge, 2012; Souza et al., 2016). During the process of oil extraction by hydraulic pressing, large quantities of biomass residue are generated, representing 500 g/kg of the processed fruit. This residual biomass, macauba cake, contains high levels of fiber and lipids and it can be indicated as an alternative ruminant feed (Rufino et al., 2011; Azevedo et al., 2013).

Feeds rich in lipids contribute to energy supply of high-producing animals, optimizing the use of the digestible energy (Hess et al., 2008). However, high lipid level in the ruminant diets (>70 g/kg of dry matter) can inhibit rumen fermentation (Palmquist and Jenkins, 1980) and, consequently, impair performance of finishing animals. According to Byers and Schelling (1989), this inhibition could be due to the reduction of the microbial adhesion in vegetal fiber or, possibly, a direct toxic effect of unsaturated fatty acids on rumen microbes via alteration in the lipid composition and physicochemical properties of the microbial cellular membranes (Jenkins, 1993). The Gram-positive bacteria (Nagaraja et al., 1997) and ciliate protozoa (Doreau and Ferlay, 1995) are the most sensitive microorganisms to toxic effect of unsaturated fatty acids.

Ciliate protozoa suppression may reduce methane production and increase the efficiency of energy utilization and the microbial protein levels for ruminant, improving animal production (Doreau and Ferlay, 1995; Eugene et al., 2004; Martin et al., 2010), but it also negatively affects fiber digestion, which is the main function of the rumen (Mosoni et al., 2010).

Macauba cake might alter the rumen profile of protozoa populations due to the high levels of lipid and fiber contents. In goats fed macauba cake inclusion at 100 and 150 g/kg of dry matter (Rufino et al., 2011), the concentration of medium protozoa populations increased significantly. However, the effects of macauba cake inclusion in the rumen microbial ecosystem of sheep, as well as their relation on animal performance, are not known. This research evaluated the effects of macauba cake inclusion on rumen protozoa populations of confined lambs.

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## Material and Methods

All animal experimental procedures were performed according to the Institutional Committee on animal use Ethics under case no. 46/2009.

Twenty-four Santa Ines lambs (average age 5±0.1 months (mean±SD) and weighing 23.9±0.6 kg) were randomly assigned to the four treatment diets in a randomized block design. Blocks were formed according to their initial live weight and lambs were located in individual cages of 1.10 × 1.10 m equipped with waterers and feed bunks.

The experimental period lasted 70 days, including 10 days for adaptation to installation and diets. At the beginning of the adaptation period, the lambs were individually identified, treaded with anthelmintic (Aldazol 10 CO® laboratory Vallée SA, Montes Claros, Minas Gerais, Brazil), and immunized for clostridiosis (POLI-R® laboratory Vallée SA, Montes Claros, Minas Gerais, Brazil).

Four experimental diets were formulated according to National Research Council (NRC, 2007) for a weight gain of 200 g per day; levels of macauba cake in the diet were: 0, 100, 200, and 300 g/kg of dry matter (DM) (Table 1). Forage and concentrate were mixed and fed twice daily at 07.00 and 16.00 h. The feeding rate was adjusted daily to yield refusal rates of ~200 g/kg of total feed delivered. Refusals were removed before the morning feeding and weighed daily to determine intake. Animals were weighted at the start of the experiment and then weekly before morning feeding.

Lambs were subjected to visual observation at the beginning, middle, and end of the experiment with trained observers positioned strategically so as not to disturb the lambs. Observations occurred every 5 min during 24 h, divided into three periods of 8 h to determine the time spent on eating, ruminating, and idling, according to Johnson and Combs (1991). For nocturnal observations, the environment was maintained with artificial lighting.

Samples of diets and refusals were daily collected, identified, and stored in a freezer at –20 °C for later laboratory analysis. All samples were pre-dried in a ventilated oven at 55 °C, ground to pass through 1-mm sieve screen, using a laboratory mill, and stored in airtight plastic containers until analysis.

Samples were analyzed according to the Association of Official Analytical Chemists (AOAC, 1998) for DM (method 934.01), ash (method 942.05), crude protein (CP; method 954.01), and ether extract (EE; method 920.39). Neutral detergent fiber (aNDF; Van Soest et al., 1991) and acid detergent fiber (ADF) were analized (AOAC, 1998, method 973.18) using ANKOM200 Fiber Analyzer unit (ANKOM Technology Corporation, Fairport, New York, USA). Sodium sulphite and alpha amylase were used in the determination of aNDF. Non-fiber carbohydrates were calculated as: NFC = 100 – (% aNDF + %EE + %CP + %Ash), according to NRC (2001). The DM, organic matter (OM), CP, EE, NFC, and aNDF intakes were

Table 1 - Ingredients and chemical composition (g/kg of dry matter) of the diets and macauba cake

| Item | Level of dietary macauba cake (g/kg) | | | | Macauba cake[1] |
|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | |
| Ingredient | | | | | |
| Sorghum silage | 300 | 300 | 300 | 300 | - |
| Soybean meal (450 g CP/kg) | 149.8 | 153.0 | 156.1 | 159.3 | - |
| Corn grain | 486.5 | 384.0 | 281.4 | 178.9 | - |
| Cottonseed seed | 50 | 50 | 50 | 50 | - |
| Macauba cake | 0 | 100 | 200 | 300 | - |
| Dicalcium phosphate | 3.3 | 3.3 | 3.4 | 3.4 | - |
| Limestone | 7.4 | 6.7 | 6.1 | 5.4 | - |
| Sodium chloride | 2.8 | 2.8 | 2.8 | 2.8 | - |
| Vitamin-mineral mix[2] | 0.2 | 0.2 | 0.2 | 0.2 | - |
| Chemical composition | | | | | |
| Dry matter | 729.2 | 739.4 | 743.0 | 748.0 | 949.6 |
| Crude protein | 150.1 | 158.8 | 158.1 | 157.4 | 81.8 |
| Ether extract | 39.0 | 49.5 | 62.0 | 67.9 | 149.5 |
| Ash | 42.8 | 46.8 | 51.9 | 58.6 | 34.5 |
| Acid detergent fiber | 167.5 | 203.3 | 249.6 | 272.3 | 493.9 |
| Neutral detergent fiber | 320.9 | 363.5 | 408.8 | 431.7 | 620.3 |
| Non-fibrous carbohydrates | 447.2 | 381.4 | 319.2 | 284.4 | 113.9 |

CP - crude protein.
[1] Fatty acid composition of the macauba cake (per kg fatty acids): 15.3 g $C_{12:0}$; 14.7 g $C_{14:0}$; 178.0 g $C_{16:0}$; 40.6 g $C_{16:1}$; 18.0 g $C_{18:0}$; 550.3 g $C_{18:1}$; 150.0 g $C_{18:2}$; 17.6 g $C_{18:3}$; 15.5 g other acids; 1.0/3.4 saturated/unsaturated.
[2] Provided (per kg of dry matter): 140 g Ca; 70 g P; 80 g Na; 30 g Mg; 15 mg Se; 500,000 IU vitamin A; 120,000 IU vitamin D3; 2,500 mg vitamin E.

calculated by the difference of the daily weight offered and refused by lambs. Fatty acid composition of the macauba cake was determined according to Machmu Èller and Kreuzer (1999), subsequent to chloroform-methanol extraction (2:1, v/v) as methyl esters by an HP 5890A gas chromatograph (Hewlett-Packard, Avondale, Pennsylvania, USA), equipped with a Supelcowax 10-column (fused silica capillary column, 30 m ×0.32 mm).

At the end of the experiment, the animals were fasted for 14 h. Immediately after slaughter, rumen fluid was obtained from each lamb by a 10-cm incision in the ventral rumen sac. Approximately 15 mL was collected with sterile inverted pipettes coupled to a rubber pipettor and allocated in test tubes and immediately transported in isothermal boxes at 4 °C to the laboratory. Tubes were homogenized in a vortex for 1 min and an aliquot of 1000 µL was transferred to tubes containing 9 mL of 10% formalin (10 mL/100 mL distilled water) for preservation of protozoa. Subsequently, serial dilutions in saline solution were conducted in Sedgewick Rafter counting chambers (S52 glass, Pyser-SGI, Edenbridge, Kent, UK) for determination of numbers of small (up to 40 × 60 µm), medium (up to 100 × 150 µm), and large (larger than 100 × 150 µm) protozoa per mL of rumen fluid by light microscopy at 10x magnification (Dirksen, 1993). Two subsamples of 1 mL were evaluated, counting all 50 columns of the Sedgewick Rafter chambers and the average represented individual count for each animal.

For protozoa identification, subsamples were placed on slides with cover slips with a drop of Lugol iodine solution. Identifications were performed in optical microscope at 40x objective to characterize a minimum of 200 protozoa per lamb (Rufino et al., 2011). In this study, 9,345 protozoa were evaluated and genera classified in accordance with the morphologic characteristics described by Dehority (1993).

Results were analyzed according to the GLM procedure of SAS (Statistical Analysis System, version 9.1) in a randomized block design to test effects of macauba cake inclusion.

Protozoa counts were analysed after logarithmic transformation (log10) and regression analysis was performed to determine the relationship of macauba cake level of the diets and feed intake, ruminating time, concentrations of small, medium, large, and total protozoa. For analysis of the population profiles, the relative values (counts/100) of the genus identified were subjected to regression analysis.

In the regression study, the model was: $\gamma ij = \beta 0 + \beta 1xi + \beta 2xi2 + \beta 3xi3 + \alpha j + \varepsilon ij$, in which $\gamma ij$ = dependent variables; $\beta$ = regression coefficients; xi = macauba cake levels; $\alpha j$ = deviations from the regression; and $\varepsilon ij$ = random residual error.

## Results

There was no difference among treatments for weight gain and intake of DM, OM, and CP (Table 2). There was an increase in the intake of EE and aNDF ($P<0.01$) and in time spent ruminating ($P = 0.04$) and a decrease in intake of NFC ($P<0.01$) with inclusion of macauba cake.

The concentration of ciliate protozoa ranged from 1.35 to 4.24 × $10^6$/mL of rumen fluid (Table 3). Concentrations of small, medium, and large protozoa, as well as the total, were maximal at 100 g/kg level in the diet. Above 100 g/kg, the protozoa concentrations decreased substantially. Intake of 200 and 300 g/kg reduced the ruminal protozoa population by 26% and 50% (in relation to diet without macauba cake), respectively.

The genera of the subclasses Holotricha (*Buetschilia* and *Isotricha*) and Entodiniomorpha (*Charonina*, *Entodinium*, *Diplodinium*, *Eodinium*, *Eremoplastron*, *Eudiplodinium*, *Diploplastron*, *Polyplastron, Ostracodinium*, *Metadinium*, and *Enoploplastron*) were identified in sheep in the diet without macauba cake, with Entodiniomorphs representing more than 95% of the fauna. With inclusion of macauba cake, there was a reduction in the diversity of fauna ($P = 0.05$) (Table 4), with only seven protozoa genera in the rumen contents of lambs fed 300 g/kg (*Isotricha*, *Charonina*, *Entodinium*, *Diplodinium*, *Eodinium*, *Diploplastron*, and *Polyplastron)*.

*Entodinium* spp. was predominant in controls, with 53 counts/100 of the identified ciliate protozoa and in the 100 g/kg macauba cake group with 43 counts/100. *Charonina* spp. was the most frequent in diets of 200 g/kg and 300 g/kg macauba cake at 42 and 50 counts/100 of ciliates, respectively. Increases in the level of macauba cake resulted in increase ($P<0.01$) in the proportion of *Charonina* spp. and *Diplodinium* spp. and decreases ($P<0.01$) in the proportion of *Entodinium* spp., indicating this genus as the most sensitive to antiprotozoal activity of the macauba cake.

## Discussion

The macauba cake inclusion did not alter the intake of DM, OM, and CP (Table 2), even when considering the increase of aNDF (320.9 to 620.3 g/kg DM) provided by macauba cake inclusion. Possibly, the small particle size of the fiber constitution of the macauba cake favored the normal traffic of fibers on digestive tract and did not

promote repletion of the pre-stomachs. Burke et al. (2011) and Nunes et al. (2011) reported that lambs fed different levels of palm kernel expeller, an aNDF-rich feed, showed no significant differences between diets for total DM intake and DM intake adjusted for metabolic weight.

Van Wyngaard et al. (2015) reported that, recently, high maize prices have forced nutritionists to partly replace maize with byproduct feeds. Macauba cake is a fiber-fat source, which can be used to replace maize in diets. The effects of substitution of starch by fiber-fat source depend on the fiber content and oil present in the byproduct. According to Jesus et al. (2012), high levels of digestible fiber increase the population of the rumen protozoa, while the presence of lipids and other compounds inhibits the growth of these microorganisms.

Although levels above 100 g/kg of macauba cake induced higher aNDF intake and longer rumination time, there was substantial decline in the total number of protozoa (Table 3), possibly due to increase in intake of EE from macauba cake. The latter could have caused toxic effect on ciliate protozoa due to its high levels of unsaturated fatty acids, considerable levels of lauric ($C_{12:0}$) and myristic ($C_{14:0}$) acids (Table 1). Both *in vitro* and *in vivo* studies have demonstrated protozoa sensitive to linolenic ($C_{18:3}$), linoleic ($C_{18:2}$), and oleic ($C_{18:1}$) unsaturated fatty acids, in this order (Machmüller et al., 1998; Hristov et al., 2004), and to lauric and myristic saturated fatty acids (Dohme et al., 1999; Faciola et al., 2013), with greater antiprotozoal effects observed with medium chain saturated lipids (Machmüller et al., 1998).

There is consensus that lipids can promote antiprotozoal effects. However, the mechanisms by which it occurs are still unclear. Jenkins (1993) proposed that antimicrobial effects of lipids in the rumen may be similar to cytotoxic effects of fatty acids on biological membrane functions, such as oxidative phosphorylation. Other mechanisms for lipid toxicity that have been proposed were: perturbation of ether-lipid metabolism; inhibition of a specific-acyl-CoA-acyltransferase, an enzyme involved in lipid-remodelling (Lux et al., 2000); and inhibition of methylation of phosphatidylcholine, which would block neo synthesis (Lira et al., 2001). Even though these studies were in flagellated protozoa, which have different membrane structure than rumen ciliates, these mechanisms may shed light into possible lipid toxicity mechanisms in rumen ciliates (Faciola et al., 2013).

In the present study, low concentration of *Isotricha* spp. was observed (Table 4), suggesting an escape of this or other Holotricha from rumen to the reticle during the obligatory fasting period that preceded the animal slaughter. These protozoa translocations from the rumen to the reticle during fasting and the subsequent migration from the reticle to the rumen after animal feed have been reported in cattle

Table 2 - Live weight gain, ruminating time, and feed intake of sheep fed different levels of macauba cake inclusion in the diet

| Item | Level of dietary macauba cake (g/kg) | | | | SEM | P-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | | Linear | Quadratic |
| Initial live weight (kg) | 23.6 | 24.2 | 23.7 | 24.1 | 0.6 | 0.83 | 0.94 |
| Final live weight (kg) | 35.9 | 36.2 | 35.9 | 35.4 | 0.9 | 0.84 | 0.84 |
| Live weight gain (g/d) | 205 | 200 | 203 | 188 | 10 | 0.57 | 0.80 |
| Ruminating time (min/d) | 450 | 512 | 592 | 561 | 24 | 0.04 | 0.31 |
| Feed intake (g/d) | | | | | | | |
| Dry matter | 1094 | 1093 | 1133 | 1179 | 48.1 | 0.32 | 0.72 |
| Organic matter | 1056 | 1048 | 1079 | 1114 | 45.6 | 0.47 | 0.73 |
| Crude protein | 157 | 176 | 179 | 185 | 15.3 | 0.10 | 0.57 |
| Ether extract | 42 | 56 | 72 | 80 | 4.1 | <0.01 | 0.46 |
| Non-fibrous carbohydrates | 543 | 450 | 381 | 359 | 19.4 | <0.01 | 0.19 |
| Neutral detergent fiber | 314 | 366 | 447 | 489 | 18.3 | <0.01 | 0.83 |

SEM - standard error of the mean.

Table 3 - Concentrations of ciliate protozoa ($\times 10^5$/mL of rumen fluid) of sheep fed different levels of macauba cake inclusion in the diet

| Item | Level of dietary macauba cake (g/kg) | | | | SEM | P-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | | Linear | Quadratic |
| Protozoa[1] | | | | | | | |
| Small | 12.7 | 14.9 | 10.0 | 5.93 | 0.70 | <0.01 | <0.01 |
| Medium | 14.1 | 27.0 | 9.77 | 7.46 | 1.59 | <0.01 | <0.01 |
| Large | 0.14 | 0.47 | 0.09 | 0.06 | 0.04 | <0.01 | <0.01 |
| Total | 26.9 | 42.4 | 19.9 | 13.5 | 2.26 | <0.01 | <0.01 |

[1] Small (up to 40 × 60 μm), medium (up to 100 × 150 μm), and large (larger than 100 × 150 μm).
SEM - standard error of the mean.

Table 4 - Genus profile (counts/100) of rumen protozoa in sheep fed different levels of macauba cake in the diet

| Item | Level of dietary macauba cake (g/kg) | | | | SEM | P-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | | Linear | Quadratic |
| Protozoa genera | | | Counts/100 | | | | |
| *Isotricha* | 1.3 | 1.1 | 1.5 | 0.8 | 0.31 | 0.66 | 0.23 |
| *Charonina* | 32.6 | 37.6 | 42.0 | 50.4 | 1.62 | <0.01 | 0.37 |
| *Entodinium* | 53.0 | 42.5 | 36.3 | 30.1 | 2.02 | <0.01 | 0.22 |
| *Diplodinium* | 1.8 | 2.5 | 2.9 | 5.3 | 0.30 | <0.01 | 0.06 |
| *Eodinium* | 8.6 | 13.7 | 15.2 | 11.3 | 0.83 | 0.07 | 0.06 |
| *Diploplastron* | 1.1 | 1.2 | 1.1 | 1.2 | 0.12 | 0.97 | 0.48 |
| *Polyplastron* | 0.8 | 0.9 | 0.8 | 0.9 | 0.11 | 0.97 | 0.78 |
| Other genera | 0.8 | 0.5 | 0.2 | 0.0 | 0.10 | 0.05 | 0.26 |
| Total identified[1] | 2406 | 3576 | 1923 | 1440 | - | - | - |

[1] Total number of protozoa identified by treatment group.
SEM - standard error of the mean.

(D'Agosto and Guedes, 2000; Martinele et al., 2007). Similar to our previous observations in goat and cow (Rufino et al., 2011; Dos Santos et al., 2015), *Entodinium* was the main genus, constituting more than 40% of the total protozoa in the rumen of the lambs fed or not 100 g/kg macauba cake. However, in sheep fed 200 or 300 g/kg, *Entodinium* spp. did not predominate among rumen ciliates, being supplanted by *Charonina* spp. (Table 4). This suggests that *Entodinium* spp. is more sensitive to toxic effects of macauba cake lipids in the rumen. According to Williams (1986), *Entodinium* spp., which consumes fiber, normally readily uses available carbohydrates. Therefore, the decrease in NFC intake with higher macauba cake inclusion could have also contributed to the reduction of this genus. This reduction could have positive effects, since methanogens were found living in the cytoplasm of *Entodinium* spp., using $H_2$ evolved by the host ciliate to form methane (Finlay et al., 1994). Increases in the macauba cake level also resulted in increases ($P<0.01$) in the proportion of *Charonina* spp. and *Diplodinium* spp., suggesting these genera as the most tolerant to antiprotozoal effects of macauba cake lipids in the rumen. This may also be associated with increased aNDF intake since entodiniomorphs can adhere to fibers, engulf particulate matter, and may produce cellulases and xylanases (Williams and Coleman, 1985).

The antiprotozoal activity of macauba cake could have positive effect on efficiency of dietary energy use because protozoa may be responsible for up to 37% of methanogenesis (Finlay et al., 1994) and, thus, reduction in ciliates may result in lower energy loss from the rumen.

## Conclusions

Macauba cake offered up to 300 g/kg does not alter feed intake of Santa Ines lambs; however, it alters ruminal protozoa concentrations. The maximum protozoa population occurs at 100 g/kg and, above this level, it promotes antiprotozoal effect with reduction of the genus diversity.

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