Effect of a regulator product of the fermentation with coconut oil on the methanogenic population and other microbial of the sheep rumen Pelibuey

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To reduce ruminal methanogenic population, a research with six male sheep from Pelibuey breeds was carried out. The animals were kept in metabolism cages, located in a polyethylene tunnel, and they had a cannula in the dorsal sac of the rumen. A product that modulates the ruminal fermentation was used, with two inclusion levels of coconut oil: A) control, without coconut oil and B) 15% of coconut oil. The remainder of the diet consisted on star grass (*Cynodon nlemfuensis*). A Latin square design with six repetitions was applied. The samples were made before the food intake (hour 0), at 2, 4 and 12 hours after the ingestion of the regulator. The regulator with coconut oil reduced the population of methanogenic bacteria and its counts were 43.44 and 15.94 x10^9 cfu. mL^{-1} for treatments without and with coconut oil, respectively. The cellulolitic bacteria population was 29 and 18 x 10^6 cfu. mL^{-1} for treatments A and B, respectively. The coconut oil did not modify the population of proteolytic and viable total bacteria, cellulolitic fungi and total protozoa, although the Entodiniomorphos were reduced to a half of its initial population. The concentration of SCFA and NH_3 was not modified as a result of the inclusion of coconut oil in the regulator. It is concluded that the inclusion of a regulator with 15% of coconut oil reduces the population of methanogens and Entodiniomorphos protozoa. The decrease effect on the population of cellulolitic bacteria allows the recommendation of its cautious use in fibrous diets for optimizing the fiber degradation.

Key words: *male sheep, coconut oil, rumen, methanogens, cellulolitic bacteria, fungi, protozoa*

The methane is generated, mainly, by the fermentative process of the food that enters the rumen (Beauchemin *et al.* 2008). It is the result of the carbohydrate fermentation in the rumen and is considered an inefficient process that causes loss (2-12%) of the gross energy that ruminants consume. It also causes damages to the environment (Johnson and Johnson 1995, Johnson *et al.* 2000 and Soliva *et al.* 2003).

This gas is one of the major contributors to the greenhouse effect, and it is responsible for the 18-21% of this phenomenon (Moss *et al.* 2000). For its reduction, different means have been used (Anderson *et al.*2003 and Greathead 2003). One of the most used is the application of chemical additives, like methane halogenated analogous, ionophore antibiotics, unsaturated fatty acids, fumaric acid and others compounds (Santoso *et al.*2004).

Fatty acids make toxic effect in the methanogenic bacteria. The methanogenesis inhibitors more effective include fatty acids like linoleic and cisoleic acid and some saturated fatty acids, like estearic. It is also recommended the use of miristic and lauric acid mixtures. Among the oils, those that are used are linseed, coconut, canola, radish, sunflower and fish oils based on n-3-eicosapentaenoic (EPA) and n-3-docosahexaenoic (DHA) acids (Demeyer and van Nevel 1979, Gil 2004, Soliva *et al.* 2004, Beauchemin and McGim 2006 and Beauchemin *et al.*2008).

It has been informed that the coconut oil is a product able to waive the methanogenesis, due to its high content in fatty acids of long chain. Its content in saturated fatty acids is approximately 90%.In fatty acids, its average composition is miristic (17%), palmitic (9%), estearic (2.5%), oleic (7%) and linoleic (1.8%) (Blas *et al.* 2003).

The objective of this research was to reduce the population of ruminal in sheep of Pelibuey breed by means of a fermentation regulator with coconut oil.

Materials and Methods

Experimental treatments. Two treatments were evaluated, according to a Latin square design in factorial arrangement (two treatments x four sampling times). The treatments were designed according to the level of coconut oil in the regulator: 1) star grass (*Cynodon nlemfuensis*) + regulator without coconut oil, control and 2) star grass+ regulator, with15% of coconut oil. The level of coconut oil represents 7% of the fat concentration of the total diet. The regulator composition, with or without coconut oil, is shown in table 1.

The chemical composition of the star grass (% of DM) was 94.20; 7.26; 74.57; 10.11; 0.42 and 0.18 for the residual DM; CP, NDF, ash, calcium and phosphorous, respectively (AOAC 1995).

Experimental procedure. The experiment was conducted under *in vivo* conditions. Six sheep from Pelibuey breed were used, with average live weight of 25 kg ± 3.5, with a cannula in the dorsal sack of the rumen. They were located in metabolism cages, which were inside polyethylene tunnel that contributed to capture the volume of methane gas that was the result of fermentation. The animals were distributed in treatments at a rate of three x treatment and received the properly weigh foods, in the morning and at the end of the afternoon.

Table 1.Composition of the modulative produts of the ruminal fermentation with or without coconut oil

Ingredients	%	
	0 % coconut oil	15 % coconut oil
Soy bean meal	49.0	52.8
Maize meal	46.3	27.4
Coconut oil	0.0	15.0
Dicalcic carbonate	0.4	0.5
Calcium phosphate	0.4	0.3
Zeolite	2.0	2.0
Sodium chloride	1.0	1.0
Mineral premixing	1.0	1.0
Composition		
ME, MJ. kg^{-1} DM	12.54	15.00
CP, %	28.2	28.2
NDF,%	30.10	21.20
Ca,%	1.62	0.92
P, %	0.86	0.75

The experimental periods were of 21 days.14 days were dedicated to the adaptation to the diet, and seven to the collection of data. The samples of ruminal liquid were took before food intake (hour 0), at 2, 4 and 12 hours after the food supply.

Culture techniques and microorganisms count. A culture technique of Hungate (1950) in roll tubes and in strict anaerobiosis conditions was used. The sowing of viable total bacteria, and cellulolytic was made in the cultivation medium of Caldwell and Bryant (1966), modified by Elias (1971) and Galindo (1988).For the determination of fungal population the Joblin (1981) medium of cultivation was used. The methanogenic bacteria were counted by the same method; but a mixture of hydrogen and carbon dioxide (60:40) in the gaseous phase was used. The medium of cultivation was described by Anderson and Horn (1987).

The protozoa were perserved in formol at 10%, in a dissolution 1: 1(v/v).The preserved samples were keep in refrigerator at 4ºC.They were count later on by means of the optical microscope in Neubauer chamber.For it , the protozoa were tinted in a solution of gentian violet at 0.01%in glacial acetic acid at 1%.

The ammoniac concentration was determined by Conway (1957) and totals SCFA concentration by Cottyn and Boucque (1968) technique.

Statistical treatment of microorganism counts. The statistical analysis of the experimental results was carried out according to the experimental design that was applied. The interaction between treatments (level of coconut oil in the regulator) and the fermentation times (sample hours) were to identify. The Duncan (1955) test for $P < 0.05$ was used in necessary cases.

The microorganism counts were transformed according Log N, to guarantee the normality conditions in the growth curve. The formula (K+N).10x was applied for the analysis, where:

K is the constant that represents the dilution logarithm where the microorganism was inoculated.

N is the logarithm of the colonies counting, determined as cfu.mL^{-1}, tfu.mL^{-1}, or cells.mL^{-1}

10 is the base of logarithms

X is the inoculation dilution

Results

There was no significant interaction among the regulator with coconut oil and the sample times for the methanogens, total viable bacteria, cellulolytic, proteolyticals, fungus and protozoan, neither in the AGCC and NH_3 concentration.

The coconut oil in the regulator reduces the population of ruminal methanogenic. When the sheep received the regulator with coconut oil, the population of methanogenic bacteria was reduced at 1/3 of the initial population, regarding to the animals that do not consumed the coconut oil in the regulator (table 2).The cellulolytic bacteria also reduced their population with coconut oil inclusion.

The effect of coconut oil in some members of the ruminal microbial population. There was not effect of the inclusion of coconut oil in the regulator in the total number of viable bacteria neither in groups of proteolytic bacteria, cellulolitic fungus and total protozoa in the sheep rumen. However the Entodiniomorphos reduced their population.

There was not significant effect in the concentration of ammoniac (NH_3) and total fatty acids of short chain in the rumen (SCFAt) (table 3)

There was significant interaction between treatments or inclusion levels of coconut oil in the regulator, and

Table 2. Effect of coconut oil of the regulator on the population of total viable bacteria, proteolytic, fungi and protozoa in the sheep rumen

Microorganisms	Without coconut oil	With coconut oil	SE ±
Methanogenic Archaea, 10^9 cfu. mL^{-1}	3.56 (43.44)	1.78 (15.94)	0.16**
Cellulolitic bacteria, 10^6 cfu. mL^{-1}	3.32 (29.00)	2.55 (18.00)	0.23*
Total viable bacteria, 10^{11} cfu. mL^{-1}	3.61 (42.97)	3.36 (36.66)	0.15
Proteolytic bacteria, 10^6 cfu. mL^{-1}	3.20 (25.98)	3.22 (27.44)	0.10
Cellulolitic fungi, 10^5 tfu. mL^{-1}	3.26 (40.93)	3.24 (32.17)	0.28
Total protozoa, 10^5 cell. mL^{-1}	2.71 (22.83)	2.37 (14.52)	0.18
Enthodiniomorphos, 10^5 cell. mL^{-1}	2.84 (29.25)	2.13 (13.40)	0.16*

cfu. Colony forming units; tfu talum forming units.
data Transformed according log X
Original means between parenthesis,*P < 0.05; **P < 0.01

Table 3. Effect of coconut oil in the regulator in ammoniac and SCFA concentration in the sheep rumen

Indicator	Without coconut oil	Coconut oil	SE ±
SCFA, mMol. L^{-1}	116.46	109.70	0.26
NH_3, meq. L^{-1}	10.15	7.75	0.72

Table 4. Effect of the coconut oil in the modulator and the time after feeding on the sheep ruminal ph

Treatments	Time after fermentation, h				SE ±
	0	2	4	12	
Modulator without coconut oil	6.85^{c}	6.55^{bc}	6.33^{b}	6.49^{bc}	0.95 **
Modulator with coconut oil	6.84^{c}	6.21^{ab}	5.90^{a}	5.81^{a}	

^{abc}Values with different letters difer at P<0.05 (Duncan 1955) **P < 0.01

Table. 5 Effect of the time after fed in protozoa population and ruminal ammoniac concentration in sheep.

Measure	0	2	4	12	SE ±
Protozoan, 10^5 cel. mL^{-1}	2.65 (24.33)	2.40 (13.33)	2.40 (14.50)	2.74 (23.27)	0.26
NH_3, meq. L^{-1}	11.86^{b}	9.11^{b}	7.98^{ab}	6.86^{a}	0.95*

^{abc}Values with different letters difer at P<0.05 (Duncan 1955) **P < 0.01
( ) Original value. *P<0.05

in after the supply of foods on the pH ruminal liquid (table4).The higher pH was found before feed, but when the animals received coconut oil decrease at 4 and 12 hours after being fed.

The time after the feeding did not produce significant changes in the population of ruminal protozoa, but the ammonia concentration decreased with time. Table 5 shows that at 4 and 12 h the ammonia concentration in the sheep rumen is lower.

Discussion

When 15% of coconut oil was included on the modulatorof the ruminal fermentation of sheep, the ruminal methanogens population was reduced to 1/3, approximately, or the initial one.

Beauchemin *et al.* (2008) informed that there wasmarked differences in the response to the supplementation with lipid sources, with regard to the decrease of the methane production in the rumen. These differences depend on the diet base and level. Reductions of 63% in the methane production occurred when 7% of coconut oil was used (Machmuller and Kreuser 1999).

The results of this study coincide with those informed by Machmuller and Kreuser (1999), Machmuller *et al.* (2001) and Machmuller *et al.* (2003). These authors verified that the supplementation with medium-sized chain fatty acids or vegetable oils, like oils of coconut, sunflower (McGinn *et al.* 2004), fish (Fievez *et al.*2003) or olive (Ungerfeld *et al.* 2005) reduces the methane emissions by means of the competition of the methanogenic micoorganisms for the reducers equivfalents or for the toxic direct effect on ruminal microorganisms. Due to the reduction of some protozoa species in the rumen it is obvious the indirect effect that mantains the symbiotic relations with methanogenic microorganisms.

It is important to highlight the decrease effect of the coconut oil in the population of cellulolytic bacteria of the rumen. In studies carried out with sheep Delgado *et al.* (2010) informed that this same level of coconut oil reduced in 66% and 69% the DM and OM consumption, respectively. These are enough to use with caution the coconut oil to reduce the microbial populations responsible of producing methane in diets with high fiber content. The level of coconut oil used was of 15% in the regulator or 7% of the total diet. Galindo *et al.* (2009) reports, with the use of 3.5% of the same source, that there were no decreasing effects in the population of ruminal cellulolitic organisms. This aspect suggests the importance of considering the inclusion levels for these purposes, if it is wish to optimize the degradation of the fiber in the ruminant animals.

It is evident that coconut oil produce modifications in the fermentative dynamic of the rumen, aspect that is evident in the pH decrease. There are different opinions about the effect that different lipid sources have on the fermentation extension, rumen capacity to metabolize them and consequently, the balance among fermentation capacity, metabolization and absorption through the rumen wall.

When evaluating the glycerol effect as lipids source in the rumen fermentative capacity, Farkasova *et al.* (2008) found decreases in the rumen pH at four and ten hours after the supply of the diet that contained the source. These results coincide with reports giving by Galvini (2008), when finding low values of postprandial pH, attribute to the lowest degradability of the DM when high levels of lipids are used in the diet.

Kijora *et al.* (1997) informed that the ruminal pH is affected by the inclusion of lipids on the diet. These authors provided a source of lipids at 10% of DM intake, and reported a pH fall that was not explained by the increase of SCFA production. When giving 8% of DM from the concentrate to high concentrate diets, destined to Holstein bulls, Mach *et al.* (2008) informed lower ruminal pH. These results can be influenced by a tendency to the highest intake of concentrated in this treatment group. Similarly, Wang *et al.* (2009) informed lineal decrease of pH when increase the doses (100, 200, and 300 g/d) of a lipid source in steers.

It is known that the lipids that get to the rumen, as consequence of the diet ingestion, are fermented by microorganisms and processes of fatty acids hydrogenization are produced, as well as the hydrolysis of glycerides and phospholipids, and the fermentation of glycerol coming from glycerides and phospholipids (Stuart 2009). As it was previously stated, the coconut oil contained saturated fatty acids, mainly myristic, palmitic, and estearic (Blas *et al.* 2003), during its fermentation in rumen experiment, in lower grade, the hydrogenization action of the unsaturated fatty acids, of two or three double links. These reasons lead to search biochemical mechanisms that explain the reasons for which this source of fatty acids make effect in methanogenic population, and in the fermentative ruminal process in general.

Ciappezoni (2011) showed that free fatty acids in the rumen, when are in excess (over 8% of the diet that the animals intake), lead to mixed to foods particles and microbes, what prevents the fermentation of structural carbohydrates.

It is concluded that the inclusion of 15% of coconut oil in a regulator of ruminal fermentation, reduces the population of methanogens and protozoa Entodiniomorphos. The decreasing effect on the population of cellulolitic bacteria allows the recommendation of its use with caution in fibrous diets, if you want to optimize the fiber degradation.

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