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Effect of organic acids or monensin-sodium addition on fatty acid production of short chain and methane through the ruminal fermentation “in vitro”

Efeito da adição de ácidos orgânicos ou monensina sódica na produção de ácidos graxos de cadeia curta e metano através da fermentação ruminal “in vitro”

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Abstract

Food additives may increase food and animal production efficiency, as well as reduce the production of enteric methane (CH_4). With the aim to evaluate the influence of organic additives in two concentrations (250 and 500 ppm) in the production of CH4 (mL), short chain fatty acids (SCFA), ammonia nitrogen (N-NH3) and pH, an experiment “in vitro” gas was conducted, having two control groups, one as a negative control without the presence of additive and another as a positive control, with the addition of monensin-sodium (30ppm). The experiment was arranged in a completely randomized design with three replications. The treatment with monensin-sodium increased the propionate production ($p<0.05$) and decreased ($p<0.05$) CH_4, acetate, butyrate, valerate, isobutyrate, isovalerate production, the acetate/propionate ratio and total SCFA production compared to treatment without additive, the $N\text{-}NH_3$ concentration being unchanged. Among CTX 250 and 500 organic acids treatments, only 500 CTX showed a trend ($p<0.10$) to decrease in $N\text{-}NH_3$ concentrations, with no significant changes ($p>0.05$) in the remaining parameters related to treatment without additives. In this sense, monensin-sodium shows characteristics of modulation of rumen environment.

Key words: Food additives, ionophores, ammonia nitrogen, pH

Resumo

Aditivos alimentares podem aumentar a eficiência dos alimentos e a produção animal, bem como reduzir a produção de metano (CH_4) entérico. Com objetivo de avaliar a influência de aditivos orgânicos

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em duas concentrações (250 e 500ppm) na produção de CH_4 (mL), ácidos graxos de cadeia curta (AGCC), nitrogênio amoniacal ($N\text{-}NH_3$) e no pH, foi conduzido um experimento "in vitro" gás, tendo dois controles, um negativo sem a presença de aditivo e outro positivo, com à adição de monensina sódica (30ppm). O experimento foi organizado em um delineamento inteiramente casualizado, com três repetições. O tratamento com monensina sódica aumentou ($p<0.05$) a produção de propionato e diminuiu ($p<0.05$) a produção de CH_4, acetato, butirato, valerato, isobutirato, isovalerato, a relação de acetato/propionato e a produção total AGCC em relação ao tratamento sem aditivo, não alterando a concentração de $N\text{-}NH_3$. Dos tratamentos com ácidos orgânicos CTX 250 e 500, somente o CTX 500 mostrou tendência ($p<0.10$) a diminuição nas concentrações de $N\text{-}NH_3$, sem alterações significativas ($p>0.05$) nos demais parâmetros avaliados em relação ao tratamento sem aditivos. Neste sentido a monensina sódica apresenta características de modulação do ambiente ruminal.
Palavras-chave: Aditivos alimentares, ionóforos, nitrogênio amoniacal, pH

Introduction

Scientific progress on animals requirements and food nutritive values made technical knowledge and necessary information available to researchers for accurate balancing diets. Therefore, the discovery of substances that control animal metabolism, increasing the efficiency of feed utilization, has led to a new class of substances known as food additives. Additives are substances or mixtures of substances added to feed in order to increase animal production, and also enhancing the products quality of animal origin. Among additives: ionophore and non-ionophore antibiotics, yeast, probiotics, prebiotics, essential oils, plant extracts and organic acids are currently used in ruminants' diets (BERCHIELLI et al., 2011).

Ionophores were initially used as coccidiostats in poultry, becoming also used as growth promoters for cattle since 1975. In the rumen they inhibit gram-positive bacteria (acetate producers), but not the growth of gram-negative propionate producers, causing decrease in acetate: propionate ratio, promoting a reduction in methane production, increasing consequently the energy efficiency of ruminants (RUSSELL; STROBEL, 1989).

The monensin is certainly the most studied and used however in recent years, its use has undergone several constraints, mainly from the European Union, which in 2006 banned from its market, any animal product that has been produced with promoting growth antibiotic usage (EUROPEAN UNION, 2003). This legislation also abolished animal products importation from countries that make use of this ingredient.

The WHO (World Health Organization) also considers antibiotics use in animal production an increasing risk to the human health, due to the microorganism's possible resistance to antibiotics (FAO et al., 2004). Given these constraints, there has been a growing interest in the additives research that offers similar results as ionophores, however with no human health risks. As a result, organic products have been studied in order to manipulate rumen fermentation, increasing animal feed efficiency, thereby reducing the limitations imposed by some consumers markets, regarding milk and beef consumption, produced with growth-promoters of synthetic origin.

With the aim of evaluating the use of a product based on organic acids, resulting from the activation of ascorbic acid with natural organic acids (citric and lactic) in a matrix glycerin against a monensin-sodium additive in the production of short chain fatty acids and methane was developed the present work using the production technique of "in vitro" gas.

Material and Methods

The study was conducted from December 2011 to May 2012, at Food Science and Nutrition of Ruminants Laboratory (LABRUMEN), of Animal

Science Department, Federal University of Santa Maria, in Santa Maria, RS, Brazil, located in the physiographic region known as Central Depression, with the coordinates 29º and 43' South Latitude and 53º and 42' West Longitude.

Two types of additives were tested, totaling four treatments: CTL (control, no additive), MON (ionophore monensin-sodium), CTX250 (250ppm organic additive) and CTX500 (500ppm organic additive). For MON treatment Rumensin (Elanco®) commercial product was used at the dosage recommended by the manufacturer, 2g/animal/day. Minimal dosage of monensin-sodium in the product is 100mg *per* gram of Rumensin (Elanco®). As for CTX treatments a commercial product based on organic acids was used, resulting on the ascorbic acid activation with natural organic acids (citric and lactic) in a glycerin matrix at 250 and 500ppm concentrations. The 250ppm concentration being the one recommended by the manufacturer.

For calculation basis a bovine with an average live weight of 400kg was taken as standard and a 60L rumen volume (15% of body weight), then 1,67mg Rumensin® were added to the MON treatment, 1,69mg and 3,38mg of organic product for CTX250 and 500 treatments respectively, in each bottle containing 50mL of the incubation solution.

The gas production in vitro technique (THEODOROU et al., 1994) was adapted to the semi-automatic system (MAURICIO et al., 1999), using a pressure transducer (800 Date Pressure Press, Piracicaba, SP - Brazil), to estimate digestibility, the SCFA production, N-NH_3 and CH_4.

As food elements, Cynodon *spp* hay cv. tifton 85 (bulky) and corn (concentrate) were used, pre-dried under forced air in an oven (55-60ºC/72h), ground into a 1mm Willey sieve. These foods were used as fermentation substrate, obeying the bulky/concentrate proportion of 55:45 ratio. Treatments with additives were manually added to the food mixture, at concentrations described above. Substrate samples weighing 0.5g, were placed into fermentation bottles of 100mL volume capacity, in which was added 40 mL of culture medium (buffer), as described by Theodorou et al. (1994). The bottles were sealed with rubber stoppers (14mm) and sealed with aluminum bands. To prevent the occurrence of any type of fermentation, bottles were stored in a refrigerator at 4°C for the period (8-12h) prior to inoculation.

The bottles containing the substrate and culture medium were removed from the refrigerator and placed in a water bath at 39°C five hours before the inoculation. The rumen fluid was obtained from fistulated cattle and fed with the same experimental diet based on forage and concentrate; the material was collected and stored, according to the gas production in vitro technique (MAURICIO et al., 1999). The rumen fluid was filtered through two layers of cotton gauze under continuous CO_2 injection and kept in a water bath at 39°C. Volumes of 10mL of filtrated rumen fluid were injected into the bottles of the above respective treatments. Flasks containing rumen liquid and culture medium (buffer) were used as white, to discount the gas production coming from the ruminal fluid. After the rumen fluid inoculation, bottles were sealed and placed in a water bath at 39°C and constant stirring for 48 hours.

The pressure caused by gases accumulated at the bottles top, was measured via a pressure transducer connected at its end to a needle (0.6mm). Pressure readings were taken at times 2, 4, 6, 8, 10, 12, 18, 24, 30, 36 and 48 hours after the incubation. From the needle insertion into the rubber stopper, the pressure produced within the bottles was read on the digital player pressure transducer.

For the pressure readings interpretation (psi = pressure per square inch) was used in the following equation $V = 4,9515p + 0.656$. Where V= gas volume (mL); p= measured pressure (psi), this equation allows the conversion of the gas measurements, made in psi through the pressure transducer, to

millimeters. The total gas production was taken as the sum of each reading partial production.

Relating to SCFA production analysis, 1mL samples of the bottles fermentation liquid were collected with 1mL syringe and needle at incubation times 0, 6, 12, 24 and 48 hours, these samples stored in Eppendorf tubes, frozen and sent in isothermal boxes to the LANA-UNESP Laboratory, Jaboticabal, SP, Brazil. In the laboratory, for the SCFA concentrations determination, samples were centrifuged at 30,000rpm for 30 minutes at 10°C, after the 0.5mL supernatant, 0.1mL of formic acid was added for the gas chromatography analysis (SHIMADZU, GC-2014 model), equipped with a flame ionization detector (FID), using a HP-INNOwax 19091N-213 capillary column (30m, 0.32mm, 0.5µm). The injector temperature was 200°C and the detector 250°C. The injection was in "split" mode, with 1:30 ratio, using nitrogen as a carrier gas. The external calibration curve was made with chromatographic patterns (Chem Service, West Chester, PA, USA) for acetic acid (99.5%; CAS 64-19-97), propionic acid (99%; CAS 09-04-79), isobutyric acid (99%; CAS 79-31-2), butyric acid (98.7%; CAS 107-92-6), isovaleric (99%; CAS 503-74-2) and valeric acid (99%; CAS 109-52-4).

For $N\text{-}NH_3$ determination, 100µL of the fermentation liquid sample was collected at the same time of SCFA collections and, diluted and analyzed by the hypochlorite phenol method (WEATHERBURN, 1967). After 48 hours of incubation, the bottles were opened and pH was measured with a digital potentiometer (Digimed DM21, São Paulo, SP - Brazil).

The CH_4 determination was performed in an independent test measurement, with pressure and gas collection higher intervals, following the same methodology above. The scan times were 6, 12, 24 and 48 hours, where the pressure reading was done at each time, with the use of a three-way stopcock system coupled, a 10mL sample of produced gas was collected, through a syringe that was stored in glass bottles with rubber cover and double silicone layer, which were sent for analysis in "extainer" type vacuum tubes and sent to the Environmental Biogeochemistry Laboratory, Department of Soils Science at UFRGS, Porto Alegre, RS, Brazil. The CH_4 concentration in air samples was analyzed through gas chromatography (GC-2014 mod. "Greenhouse") in a FID detector, operating at 250°C from one mL auto-sampler and using N_2 as carrier gas (MOTERLE et al., 2013).

To calculate the methane amount produced, the equation " $CH_4 = (GP + HS) \times Conc$" was used; where CH_4 is the volume (ml) of methane, GP the volume (mL) of produced gas at the end of each incubation period, HS the volume (mL) of the bottle "headspace" and Conc is the methane concentration in the gas of sample analyzed (TAVENDALE et al., 2005).

The experimental design for "in vitro" production gases was completely randomized with 4 treatments and 3 replications (bottles) and repeated measures through time. Also for SCFA, $N\text{-}NH_3$ and CH_4 quantification, the experimental design was completely randomized with three replications (bottles), in a 4 x 4 factorial design and the following factors: 4 treatments (CTL, MON, CTX250 and CTX 500) and 4 incubation times (6, 12, 24, 48h.).

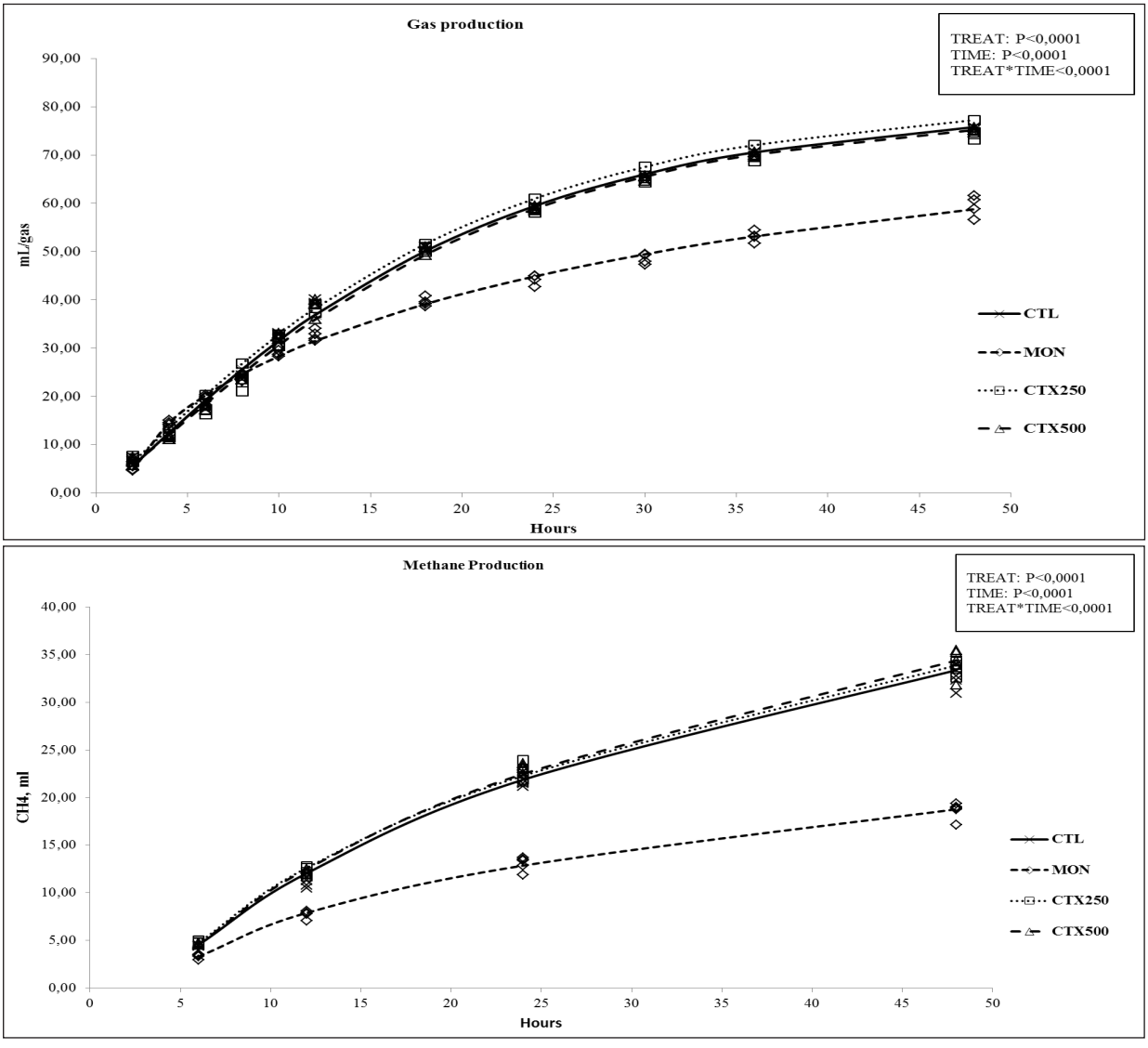
The SCFA, $N\text{-}NH_3$ and CH_4 gas production data were subjected to variance analysis and F test using the PROC MIXED procedure. When the F test was significant, the treatment averages were compared using the Tukey's test, with significance level at $p \leq 0.05$ and trends at $p > 0.06$ and < 0.10, using the SAS (Statistical Analysis System, version 9.2).

Results and Discussion

The monensin-sodium decreased ($p < 0.05$) the gas production compared to control, which was not observed in the CTX 250 and 500 organic acids treatments. This decrease is due mainly to

its action in methanogenic bacteria, providing a lower methane production and consequently lower total gas production, as can be seen in figure 1. On average, the gas production reduction was 15% compared to control, while the methane decrease was more significant, around 60%. This gas production reduction was also observed by Araujo et al. (2011), that testing monensin-sodium in gas "in vitro" system technique found an average decrease of 20%, with variations between 15 and 25%. "In vitro" experiments indicate that monensin reduces the methane production, which can reduce emissions by 25% (VAN NEVEL; DEMAYER, 1995), reaching reductions in CH_4 total production of 48, 52 and 58%, as were observed when using in "in vitro" gas technique with 2.5; 5.0 and 12.5mg/L of monensin, respectively (RUSSELL; STROBEL, 1989).

Figure 1. Gas production accumulated values (mL) and methane (mL) observed for different treatments: control, without the use of additives (CTL), monensin-sodium (MON) and organic additive 250 and 500 ppm (CTX 250 e CTX500) respectively, within 48 hours of "in vitro" fermentation gas. TREAT= effect of treatment; TIME= effect of incubation time; TREAT*TIME= interaction between treatment and incubation time.



Most studies done with organic acids, such as nutritional additives, concentrate in the malic acid and/or fumaric acid use, intermediates of the citric acid cycle (KHAMPA; WANAPAT, 2007) than when used "in vitro", the same effect of monensin on the gas and methane production reduction are observed (CARRO; RANILLA, 2003; MOHAMMED et al., 2004). Other studies (CALLAWAY; MARTIN, 1996; GOMEZ et al, 2005), found similar results, where there was no gas and methane production reduction through the use of organic acids "in vitro".

There are two hypotheses for the gas and CH_4 production decline, caused by monensin-sodium. Firstly, monensin reduces the "in vitro" organic matter degradation, actually sustained by a lower (p<0.05) degradation rate as observed in table 1, for monensin treatment compared to control. This organic matter degradation reduction is a typical limitation of "in vitro" experiments of short duration, for monensin inhibits Gram-positive bacteria related to fermentation, which includes some *Ruminococcus* sp. cellulolytic. Under "in vivo" conditions, no deleterious effects on fiber degradation are observed, once cellulolytic bacteria tolerant to monensin, under these conditions, are able to replace the sensitive ones (RUSSELL; STROBEL, 1989). The second explanation for the decreased gas and CH_4 production is that monensin increases the propionate production (RUSSELL; STROBEL, 1989). According to the stoichiometry of the gas production, the propionate formation is associated to decreases of CO_2 and CH_4 production (WOLIN, 1960).

Table 1. Parameter estimations of gas production, degradation rate, methane (CH_4) production, short chain fatty acids production (SCFA), ammonia nitrogen ($N\text{-}NH_3$) and pH observed by "in vitro" gas fermentation under the effect of Monensin-sodium addition (Rumensin ®) and additive based on organic acids.

Item[1]	Treatments[2]				Significance of P	CV (%)
	CTL	MON	CTX250	CTX500		
Gas, mL (48h)	75,20[a]	59,60[b]	76,25[a]	74,06[a]	0,0001	2,04
Degradation Tax. %/h	3,47[a]	2,26[b]	3,50[a]	3,52[a]	0,0001	4,00
CH_4, mL (48h)	32,61[a]	18,61[b]	33,52[a]	34,15[a]	0,0001	3,59
SCFA, mM (48h)						
Total	93,77[a]	81,87[b]	92,26[a]	93,82[a]	0,0001	1,78
Acetate	60,66[a]	47,93[b]	59,89[a]	60,95[a]	0,0001	1,60
(%)	(64,69)	(58,54)	(64,91)	(64,96)		
Propionate	18,45[b]	24,04[a]	18,17[b]	18,43[b]	0,0001	2,98
(%)	(19,67)	(29,36)	(19,69)	(19,64)		
Isobutirate	1,13[a]	0,79[b]	1,13[a]	1,12[a]	0,0001	4,27
(%)	(1,20)	(0,96)	(1,22)	(1,19)		
Butirate	10,57[a]	6,88[b]	10,10[a]	10,36[a]	0,0001	3,86
(%)	(11,27)	(8,40)	(10,94)	(11,04)		
Isovalerate	1,86[a]	1,33[b]	1,87[a]	1,84[a]	0,0001	4,49
(%)	(1,98)	(1,62)	(2,02)	(1,96)		
Valerate	1,08[a]	0,88[b]	1,08[a]	1,09[a]	0,0017	4,66
(%)	(1,15)	(1,07)	(1,17)	(1,16)		
C_2:C_3	3,28[a]	1,99[b]	3,29[a]	3,30[a]	0,0001	2,01
$N\text{-}NH_3$, mg dL^{-1} (48h)	28,34[a]	27,79[ab]	26,66[ab]	25,99[b]	0,0619	3,52
pH	6,22	6,26	6,20	6,21	0,4494	0,66

1SCFA = Short chain fatty acids; C2:C3 = acetate/propionate ratio.
2CTL = Control; MON = Monensin at 30 ppm; CTX = Organic additive to at 250 and 500 ppm.
Different letters in the same row indicate a significant difference ($P \leq 0.05$) according to the Tukey test.

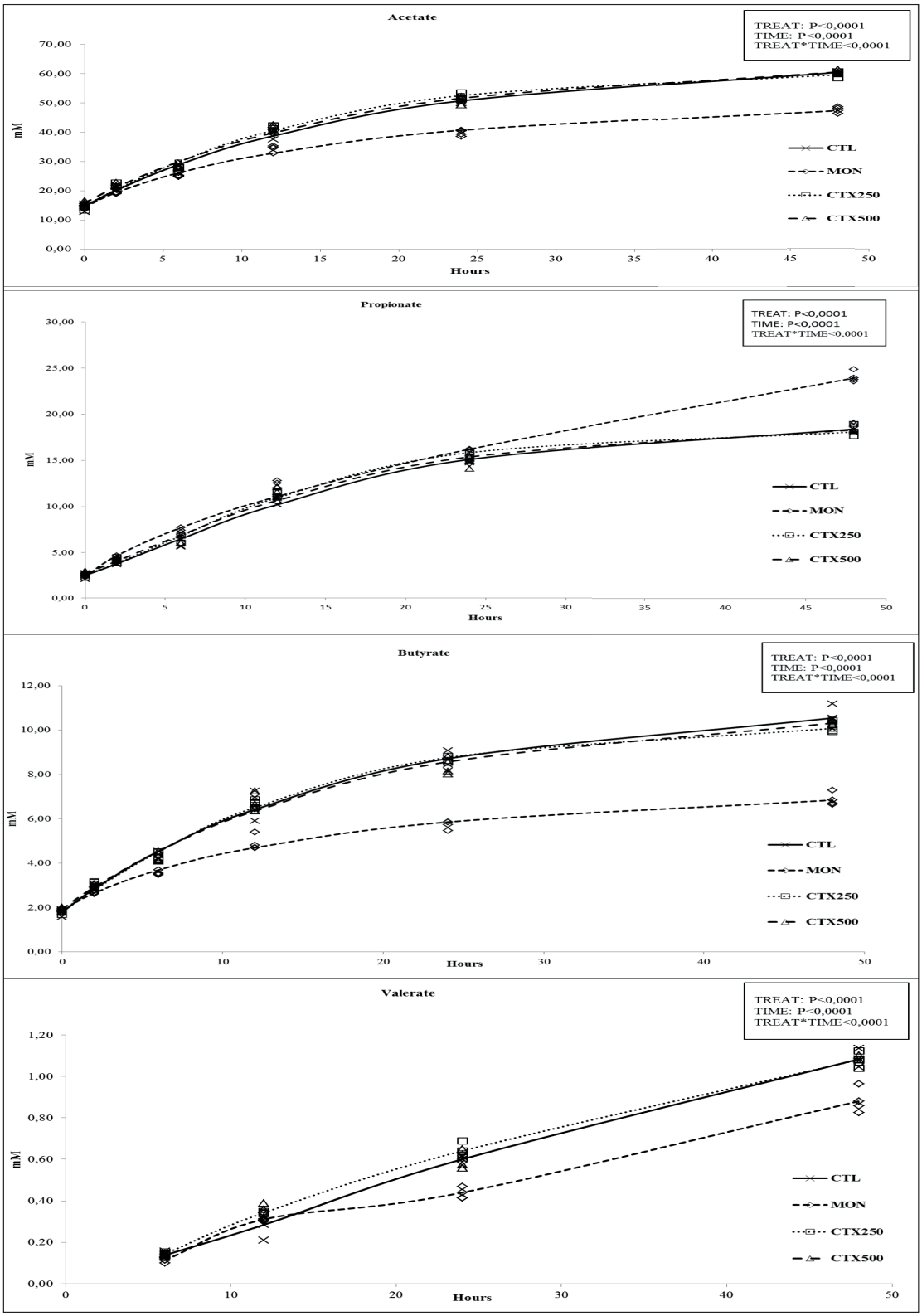
The concentrations of total SCFA and each SCFA (acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) were similar (p<0.05) among organic acids treatments and control, within 48 hours of gas "in vitro" incubation. Carro and Ranilla (2003), observed an increase in SCFA total concentration, and also a propionate and butyrate production increase, with no change in the acetate production, when the malic acid is added to the "in vitro" culture system. However, Callaway and Martin (1996), using different levels of malic and fumaric organic acids, observed no changes in SCFA total concentration, and similar results were observed in treatments with the organic acids mixture in the present work.

While in the monensin-sodium treatment there was a change in SCFA molar ratio (Figure 2). In SCFA total production there was a decrease of around 12% compared to control. These results differ from those reported by Castillejos et al. (2008) and Makkar (2004), who observed the same SCFA total production compared to control. The acetate concentration in the monensin-sodium group was approximately 21% lower compared to control (Table 1). This occurs because gram-positive bacteria are sensitive to monensin, for they are large acetate producers (RUSSELL, 2002). Due to the acetate lower production, there is consequently a reduction in CO_2 and H_2 production, a fact which partially explains the lower CH_4 production due to the monensin use (CALLAWAY et al., 2003).

The propionate concentration increased (p<0.05) due to monensin inclusion of approximately 21% over the control. It is known that stimulating of propionate production causes reduction in the CH_4 production. The propionate producing metabolic pathways compete with methanogenesis for H_2 (RUSSELL, 2002). Several microorganisms that produce propionate (Ex: *Selenomonas ruminantium* and *Megasphaera elsdenii*) are not affected by monensin supply (CALLAWAY et al., 2003). The butyrate and valerate concentrations were lower (p<0.05) when monensin was used as additive. The butyrate production is usually reduced by monensin, once ionophores inhibit *Butyrivibrio fibrisolvens* gram-positive bacterium, which is considered the largest butyrate producer (RUSSELL, 2002).

As for iso-acids, monensin reduced (p<0.05) isobutyrate and isovalerate concentration around 30% (Table 1). The iso-acids concentrations reductions indicate a lower deamination, as they are originated from the catabolism of amino acids of branched chain, such as valine and leucine. The lowest dietary deamination in rumen is compensated by its increased use in the small intestine (RUSSELL, 2002). This leads to a lower conversion of proteins and amino acids into ammonia, resulting in a decrease of ammonia loss in urine by urea, providing a nitrogen better use of diet. Busquet et al. (2006) also observed a lower "in vitro" iso-acids concentration with 12.5mg/L of monensin. The C2:C3 ratio decreased by monensin addition (p<0.05), but did not changed by the organic acids treatment, once acetate and propionate production values were similar to control. Despite a lower C2:C3 ratio provided by monensin, we have to point out that SCFA total production was reduced with its use.

Figure 2. The concentration of short chain fatty acids; acetate, propionate, butyrate and valerate observed for different treatments: control, without the use of additives (CTL), monensin-sodium (MON) and organic additive 250 and 500ppm (CTX250 e CTX500) respectively, within 48 hours of fermentation "in vitro" gas accumulated data over time. TREAT= effect of treatment; TIME= effect of incubation time; TREAT*TIME= interaction between treatment and incubation time



When analyzing the ammonia nitrogen production, we realize that there was a decrease tendency ($p<0.10$) of $N\text{-}NH_3$ concentration to treatment with organic acids in 500ppm concentration over control, while the monensin-sodium treatment and 250ppm organic acids did not differ ($p>0.05$) compared to control at 48 hours of incubation. This possibly indicates that organic acids of the present work control the proteolytic and deamination bacteria, providing reduced production of ammonia nitrogen. However, no change in the iso-acids production, which are products of amino acids and proteins degradation, was observed. Therefore, we should treat with caution the $N\text{-}NH_3$ reduction, requiring further studies to confirm this inhibitory action of proteolysis and deamination afforded by organic acids.

There were no significant differences ($p>0.05$) in pH values among the different "in vitro" treatments. The monensin-sodium "in vivo" may cause pH elevation, mainly by inhibiting lactate-producing bacteria, ex: *Streptococcus bovis* (RUSSELL; STROBEL, 1989). While organic acids (malate, fumarate and aspartate) in rich lactate medium, stimulate the *Selenomonas ruminantium* bacteria development. This bacterium has advantages over other microorganisms, surviving to rumen, by being able to ferment different soluble carbohydrates and using lactate, therefore delaying pH decrease and metabolic problems appearance in the animal (CALLAWAY; MARTIN, 1996). However, the effects inexistence on pH "in vitro" is quite common, once it is controlled by the buffering agents of the technique culture medium. If they exist, the effect is commonly related to the fermentation inhibition and SCFA lower production (BUSQUET et al., 2006).

Conclusions

The analyzed organic acids did not achieve results similar to monensin-sodium, not changing the "in vitro" gas there is no change in gas production, methane and the concentration of fatty acids total short chain, as well as the molar ratios of acetate, propionate, butyrate, valerate, isobutyrate, isovalerate and pH. Organic acids tends to decrease the ammonia nitrogen concentration.

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