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In vitro evaluation of novel crude extracts produced by actinobacteria for modulation of ruminal fermentation

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ABSTRACT - We evaluated the inclusion of two crude extracts produced by *Streptomyces* genus on *in vitro* dry matter and organic matter digestibility (IVDMD; IVOMD), cumulative gas production, volatile fatty acids (VFA), and methane (CH_4) and ammoniacal nitrogen (NH_3-N) concentration. The experimental design was randomized blocks with three blocks and four treatments: AMC (1.2 mg/25 mL ruminal inoculum), Caat (1.2 mg/25 mL ruminal inoculum), negative control (no inclusion of extracts), and positive control (sodium monensin, 1.7 mg/25 mL ruminal inoculum). Ruminal fluid samples were collected from three multiparous Holstein dairy cows fitted with ruminal cannula and incubated in a 24-h fermentation assay. There was no effect of crude extract inclusion in comparison with negative control on cumulative gas production in 24 h. However, cumulative gas production was lower when Caat extract was included in comparison with AMC inclusion. The Caat inclusion increased propionic acid concentration and reduced the concentration of butyric acid and acetate:propionate (A:P) ratio in relation to negative control. The CH_4 concentration was lower with Caat inclusion in relation to AMC, and the ratio of CH_4 concentration to digestible dry matter was lower in the negative control compared with all additives. Caat inclusion decreased the NH_3-N concentration, and IVDMD was not altered compared with negative control. Additionally, the inclusion of Caat crude extract increased propionic acid concentration and reduced butyric acid concentration and A:P ratio, without reducing the IVDMD and IVOMD. Caat extract modulates rumen fermentation, increasing available energy and decreasing gas production without causing changes in dry matter and organic matter digestibility.

Keywords: actinobacteria, extracts, *in vitro* fermentation, monensin

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Introduction

Ionophores are a class of feed additives used in ruminant nutrition to increase the efficacy of ruminal fermentation. Gram-positive bacteria are the most affected by the action of ionophores, since they do not have the peptidoglycan layer in the cell wall and produce less ATP per mole of fermented glucose (Bergen and Bates, 1984) than Gram-negative bacteria. Ionophores alter the passage of cations through the cell membrane and, thus, the concentration gradient is unbalanced. In an attempt to balance the environment, bacteria die from energy depletion and disappear from the rumen environment (Ipharraguerre and Clark, 2003). The reduction of methane (CH_4) concentration, total gas production, protein degradation and deamination, and increased concentration of propionic acid are the main

effects of ionophore inclusion in ruminant diets (Narvaez et al., 2013). As a consequence, the effects of ionophores in rumen fermentation usually increase the fermentation efficiency as well as decrease the risks of digestive disorders in feedlot animals. There are about 120 ionophores described, but sodium monensin is the most studied and widely used as a food additive (Nagaraja et al., 1997).

Although the effects of monensin on modulation of ruminal fermentation have been well studied, there is a constant demand for the development of alternatives to increase ruminal fermentation efficiency. This demand can be explained by possible negative interference with food digestibility, which can vary according to diet, forage:concentrate ratio, or quality of ingredients used. Because of this problem, some compounds have been studied as alternatives to ionophores, such as essential oils and natural extracts (Tomkins et al., 2015; Vendramini et al., 2016). Bioactive compounds produced by soil microorganisms have been described to have anti-inflammatory, antibiotic, and ionophore functions (Oliveira et al., 2010; Priya et al., 2015). Recently, our research group reported the production of two crude extracts, AMC (Crevelin et al., 2014) and Caat, produced *in vitro* from the actinobacteria fermentation isolated from mangrove and caatinga biomes, respectively.

A previous companion paper (Alves et al., 2018) evaluated the dose-response of AMC and Caat crude extracts, and an optimum dose response for the crude extracts was observed at 1.2 mg/25 mL ruminal inoculum 160 mL (glass bottle) with a reduction of gas production during 72 h of *in vitro* fermentation. In addition, the inclusion of Caat also increased ruminal propionic acid concentration and reduced ruminal butyric acid concentration.

Thus, we hypothesized that AMC and Caat crude extracts have similar modulation of *in vitro* ruminal fermentation compared with a positive control (monensin). The objective of this study was to evaluate the effect of AMC and Caat on *in vitro* dry matter (IVDMD) and organic matter digestibility (IVOMD), cumulative gas, CH_4 production, volatile fatty acids (VFA), and ammoniacal nitrogen (NH_3-N) concentrations.

Material and Methods

Research on animals was conducted according to the guidelines of the institutional committee on animal use (2038140514). The study was conducted in Pirassununga, SP, Brazil (latitude: 21°59'46" S, 47°25'33" W).

The isolates of actinobacteria were cultivated in Petri dishes using potato-dextrose-agar medium (PDA) for 10 days, at 27 °C. Crude extracts were obtained by simple liquid culture filtration and purified by direct bioautography tests, according to methodology described by Smedsgaard (1997). At the end of the purification process, all extracts obtained were subjected to liquid chromatography-mass spectrometry (LC-MS), so that the spectra were compared to an existing database (Chemical Abstract - CAS) and the Dictionary of Natural Products (DNP), allowing the identification and characterization of the compounds present in the extracts, according to methodology described by Crevelin et al. (2014).

After comparison with the database, the crude extracts were classified as macrotetrolids, according to Jani et al. (2008) classification. It was possible to identify molecular structures that were similar with ionophore antibiotics, two of which, AMC and Caat 2-63, were selected for the present study. The AMC extract was produced by actinobacteria of mangrove (red mangrove rhizosphere) biome, while the Caat extract was produced by the Brazilian caatinga biome (Brazilian caatinga rhizosphere). Our research group (Crevelin et al., 2013) recently characterized the AMC crude extract, the composition of which indicated the presence of macrotetrolids, with bafilomycin as the main compound. The identification of main secondary metabolites present in actinobacteria Caat 2-63 (actinomycin D) was based on the dereplication data employed in high-resolution mass spectrometry (HRMS) and collision-induced dissociation (CID) experiments, as described by Alves et al. (2018).

Three multiparous Holstein cows in mid-lactation (150±50 days; mean±SD), weighing approximately 542±32.2 kg, were used as donors of rumen inoculum. Donor cows were housed in individual stalls equipped with individual intake control and water supply *ad libitum.* Cows fed two daily meals

composed of corn silage as forage, soybean meal, urea, ground corn, and mineral supplement, in a forage:concentrate ratio of 60:40, formulated based on the nutritional requirements of lactating cows producing 30 kg of milk/day, 3.5% fat, and 3.1% protein, according to NRC (2001). The composition of the substrate (diet) used in the *in vitro* fermentation assay was determined by the chemical analysis previously reported by Alves et al. (2018).

The experimental design was randomized blocks (with each cow considered a block) with three replications and four treatments: negative control (no additive inclusion), monensin (positive control, at the dose of 1.7 mg of Bovensin® 200 – Phibro, Brazil), AMC, and Caat (both extracts at 1.2 mg dose). Twenty-four glass bottles (160 mL) were used for each study block: 12 bottles for the four treatments and 12 blank bottles (each glass bottle was considered as the experimental unit). All treatments and blank bottles were made in triplicate, totaling 72 bottles (3 replicates × 24 bottles per block). Blanks were included to adjust the variation among each gas production reading and were used for each inoculum to measure the fraction of total gas production due to substrate. These values were subtracted from the total of cumulative gas production in each treatment (negative control, positive control, AMC, and Caat). Out of 12 bottles, three were used as blanks composed only of the ruminal inoculum without the substrate addition, and the other blank bottles (n = 9) were composed only of ruminal inoculum plus added treatments (positive control, AMC, and Caat), to correct the isolated effects of the treatments on animal feeding.

The inoculum was collected via ruminal cannula (approximately 6 L/donor) and was composed of the mixture of liquid and solid phases of the ruminal content of donors. The collection was performed by electric vacuum pump and a vacuum flask, and the liquid phase was rapidly conditioned in thermal bottles, while the solid phase was packed in plastic bags, both previously sprinkled with CO_2, until sent to the laboratory. Both liquid and solid phases were mixed for 10 s in a blender previously sprayed with CO_2, so the microorganisms adhered to the solid fraction were recovered. Afterwards, the ruminal inoculum was filtered on a nylon filter and kept in an oven at 39 °C.

Sodium monensin (1.7 mg of Bovensin – 5mM) was weighed into the bottles for the positive control. For each crude extract treatment, 1.2 mg of crude extracts previously diluted in 10 mg/mL of dimethylsulfoxide (DMSO) was weighed. All bottles, except for the blank ones, received the substrate (0.5 g), 50 mL of buffer solution, prepared according to Lowe et al. (1985), and 25 mL of ruminal inoculum. Blank bottles received only the ruminal inoculum and the buffer solution. All bottles were sealed with rubber stoppers, shaken, and incubated in forced-circulation air oven at 39 °C (zero fermentation time) for 24 h.

The *in vitro* gas production readings were performed at 4, 8, 12, 16, 20, and 24 h after inoculation (zero fermentation time) by a DATTALOGGER pressure transducer (Press Data 800), according to methodology of Mauricio et al. (1999). After each reading, the gas produced inside the bottles was released and a second reading was taken to ensure that the pressure read-out was zero. Then, all bottles were shaken and returned to the oven until the next reading.

After 24 h of incubation, all bottles were immersed in ice for the interruption of fermentation process. All bottles were opened and aliquots of the supernatant, as well as the total mixture, were collected and stored at −20 °C until further analysis.

To perform VFA concentration analysis of the ruminal fluid, the aliquots were thawed at room temperature and centrifuged at 14,500 × *g* for 10 min. The supernatant was transferred to another flask with 200 µL of 98-100% formic acid with 100 µL of internal standard (100 µM 2-ethyl butyric acid, Chemservice, West Chester, PA, USA). The prepared samples were subjected to gas chromatography (GC-2014, Shimadzu, Japan) using a capillary column (Stabilwax®, Restek Corp., Bellefonte, PA, USA) at 145 °C and a splitless injector and dual FID detector at 250 °C, according to methodology proposed by Erwin et al. (1961) and adapted by Getachew et al. (2004) with external standard according to the manufacturer's specifications (Chemservice, West Chester, PA, USA). GCSolution® software (Shimadzu, Japan) was used for calculations.

The solid sediment present in the *in vitro* digestibility bottles after 24 h of incubation was obtained by filtering the contents of the bottles in glass crucibles with porous plate and known empty weight. After filtration, the crucibles with the solid pellet were dried at 105 °C for 24 h, and the IVDMD was calculated as original dry sample weight minus dry residue weight divided by the original sample weight. This value was then multiplied by 100. Afterwards, the solid residues were ashed in a muffle furnace (600 °C) for the estimation of IVOMD. The IVDMD and IVOMD coefficients were determined according to Menke et al. (1979).

The ruminal NH_3-N concentration was determined by the colorimetric method of salicylic acid (Kulasek, 1972), adapted by Foldager (1977). From each bottle, 2 mL of the contents were collected and placed in glass tubes with rubber stoppers, adding 1 mL of 1N sulfuric acid solution, and stored at −20 °C until analysis. Samples were thawed at room temperature, added with 10% sodium tungstate, and then centrifuged at 2,000 *g* for 15 min. Then, 20 µL of the supernatant were added in a glass tube, with 1 mL of the salicylic acid buffer and 1 mL of the sodium hypochlorite oxidizing solution. The tubes were homogenized and incubated in a water bath at 37 °C for 20 min until they became greenish. After cooling at room temperature, the absorbance reading was performed in a spectrophotometer (Bioclin 100® Semi-Automatic, Photometry 0~3.5 Abs, Belo Horizonte, Brazil).

The CH_4 concentration in the ruminal liquid was measured by gas chromatography (GC-2014, Shimadzu, Japan) according to recommendations of Abdalla et al. (2008). For analysis, 2.5 mL of the gas produced in each fermentation bottle were collected during each gas production reading with the aid of disposable syringes, totaling 15 mL of gas at the end of the incubation period. The gas was stored in glass tubes with rubber stoppers. From the total samples collected during the incubation, about 1 mL was used for CH_4 quantification, and the last reading and collection of CH_4 was performed 24 h after the start of sample incubation. For gas chromatography methodology, we used helium as the entrainment gas, with an injector at 100 °C and a ShinCarbon Micropacked/60 °C column with flame ionization detector (FID) at 120 °C. The calibration was carried out with 200 µL of standard and the curve established in GCSolution software (Shimadzu, Japan). The concentration of CH_4 in each fermentation bottle was expressed in mL of gas produced and in mL/digestible dry matter.

Statistical Analysis System® software (version 9.3) was used to analyze the results. Residual normality and homogeneity of variances was verified by PROC UNIVARIATE, and data were analyzed as a completely randomized block design. The Satterthwaite method (DDFM = SATTERTH) was used to calculate the degrees of freedom, and the *in vitro* ruminal fermentation variables were evaluated by the MIXED procedure. The variables VFA, IVDMD, IVOMD, CH_4, and NH_3-N were analyzed in relation to the main effects of the treatments (negative control, positive control, AMC, and Caat), according to the following model:

$$Y_i = \mu + T_i + B_j + e_{ij},$$

in which Y_i = dependent variable, μ = overall mean, T_i = fixed effect of treatment i, B_j = random effect of cow, and e_{ij} = random error associated with each observation. Means were obtained and adjusted by LSMEANS statement, and the differences of means were performed by DIFF statement. Comparisons were performed using orthogonal contrasts: C1 (negative control vs others); C2 (positive control vs crude extracts), and C3 (Caat vs AMC). For all statistical analysis, the level of significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

Results

An effect of treatments was observed on the accumulated gas production after 24 h (Table 1). Considering the contrast C1, no difference was observed among the inclusion of additives (monensin, AMC, and Caat) and negative control, but when the comparison was made between the crude extracts (C3), Caat inclusion reduced gas production compared with AMC inclusion (reduction of 8.82%, SEM = 3.88) (Table 1; Figure 1). The positive control inclusion reduced gas production after 24 h compared with

the negative control. However, no difference in gas production means was observed between negative control and Caat crude extract.

According to contrast C2, only the monensin inclusion reduced the IVDMD by approximately 30% (monensin = 46.34), compared with the inclusion of both extracts (AMC = 60.75; Caat = 61.61; SEM = 0.02). No effect was observed on IVOMD after inclusion of any additive (crude extracts or positive control) in comparison with negative control (Table 1).

When the crude extracts AMC and Caat were compared to each other (C3), Caat inclusion reduced the acetic acid concentration (55.22%), compared to AMC inclusion (55.98%) (SEM = 0.21; Table 2). Caat inclusion increased by 17 and 26% the proportions of propionic acid in relation to negative control (C1) and AMC extract (C3), respectively. The A:P ratio was reduced with Caat inclusion by approximately 17% compared with negative control (2.71 vs 2.3 mM; SEM = 0.08).

Caat inclusion decreased butyrate concentration by approximately 34% compared with AMC extract (C3; SEM = 0.54). The inclusion of any of the additives had no effect on the isovaleric acid concentration in comparison with the negative control (C1), expressed in both mM and percentage. Finally, a decrease of valeric acid concentration was observed with the inclusion of monensin and crude extracts compared with the negative control (C1; SEM = 0.04) (Table 2).

Table 1 - Cumulative gas production in 24 h (mL/g), IVDMD, and IVOMD of crude extracts produced by actinobacteria (AMC and Caat) on *in vitro* fermentation with ruminal dairy cow inoculum

Variable	Treatment[1]				Mean	SEM	Effect[2]			
	CO	MON	AMC	Caat			Treatment	C1	C2	C3
Cumulative, 24 h	143.90b	122.13d	156.70a	142.89bc	141.40	3.88	0.0005	0.29	<.0001	0.006
IVDMD (%)	62.09a	46.34b	60.75a	61.61a	57.69	0.02	0.04	0.19	0.01	0.86
IVOMD (%)	70.50	79.60	81.72	80.28	78.02	0.03	0.50	0.24	0.87	0.88

IVDMD - *in vitro* dry matter digestibility; IVOMD - *in vitro* organic matter digestibility; SEM - standard error mean.
[1] CO - negative control, no additive inclusion; MON - monensin (5 µM), positive control; AMC and Caat - crude extracts (at 1.2 mg).
[2] C1: orthogonal contrast control vs others; C2: orthogonal contrast monensin vs AMC + Caat; C3: orthogonal contrast AMC vs Caat.
Means followed by different letters are significantly different at P<0.05.

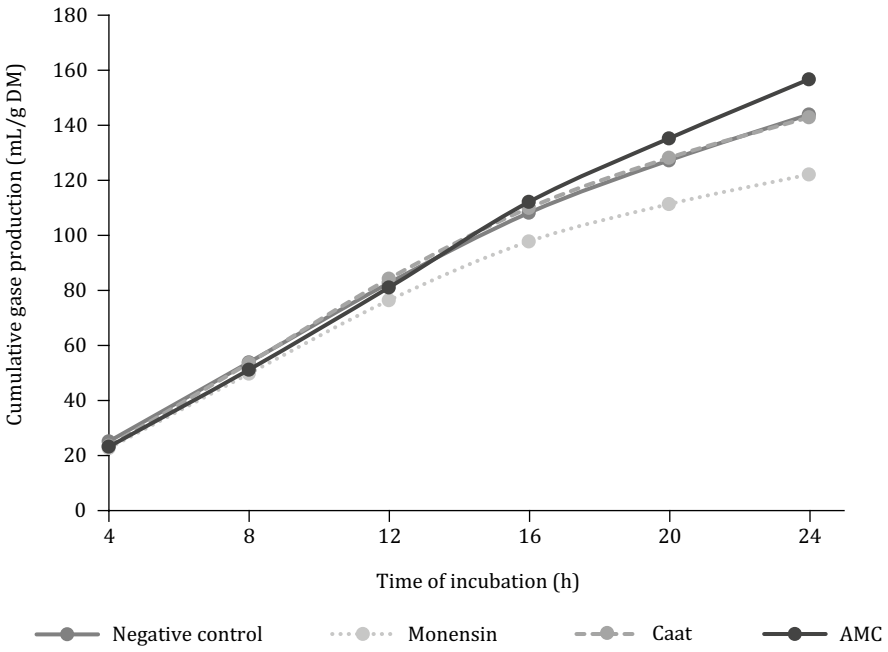

Figure 1 - Profiles of the *in vitro* fermentation kinetics of two crude extracts, derived from actinobacteria, and sodium monensin on total diet of dairy cows.

There was no difference between the negative control and the other additives (C1) or between the crude extracts (C3) in relation to the ruminal NH_3-N concentration (Table 3). Only the monensin inclusion decreased the ruminal concentration of NH_3-N after 24 h of *in vitro* ruminal fermentation assay (24.46 mg/dL) in relation to crude extracts (AMC = 29.76 mg/dL; Caat = 26.07 mg/dL; SEM = 0.65) (C2).

There was a trend towards reduction of CH_4 concentration when additives were included in comparison with the negative control (13.87 mL; SEM = 0.53). The CH_4 concentration was higher (AMC = 9.47 mL; Caat = 9.02 mL) with inclusion of both crude extracts compared with the monensin inclusion (8.31 mL) (C2); however, when the crude extracts were compared to each other (C3), Caat was more efficient than AMC in reducing CH_4 concentration (Table 3).

The CH_4 concentration/digestible DM was decreased with the inclusion of additives in comparison with the negative control (C1). However, there was no difference between the monensin inclusion vs crude extracts (C2), or between crude extracts (C3), on CH_4 concentration/digestible DM.

Discussion

The results of the present study indicate that the Caat crude extract obtained from actinobacteria fermentation was capable of modulating the *in vitro* ruminal fermentation because it decreased the CH_4 concentration, and was also able to modulate VFA concentration by increasing propionic acid and decreasing butyric acid and A:P ratio without changing IVDMD or IVOMD. While several food

Table 2 - Effect of the inclusion of monensin and crude extracts produced by actinobacteria (AMC and Caat) on the *in vitro* production of volatile fatty acids with ruminal dairy cow inoculum

VFA	Treatment[1]				Mean	SEM	Effect[1]			
	CO	MON	AMC	Caat			Treatment	C1	C2	C3
			mM							
A:P ratio	2.71b	1.86d	2.95a	2.3c	2.45	0.08	<.0001	0.0002	<.0001	<.0001
Total SCFA	126.69	117.84	129.31	123.76	124.40	2.28	0.242	0.519	0.090	0.341
			%							
Acetic	54.9c	53.19d	55.98a	55.22bc	54.82	0.21	<.0001	0.64	<.0001	0.01
Propionic	20.5cd	28.88a	19.13d	24.15b	23.16	0.75	<.0001	<.0001	<.0001	<.0001
Isobutyric	1.81	1.62	1.77	1.82	1.76	0.10	0.89	0.76	0.49	0.88
Butyric	18.43a	12.73c	18.84a	13.97b	16	0.54	<.0001	<.0001	<.0001	<.0001
Isovaleric	2.72bc	2.3d	2.72c	3.48a	2.80	0.11	<.0001	0.56	0.0005	0.003
Valeric	1.6a	1.26c	1.53a	1.33bc	1.43	0.04	<.0001	0.02	0.13	0.12

VFA - volatile fatty acids; SCFA - short-chain fatty acids.
[1] CO - negative control, no additive inclusion; MON - monensin (5 µM), positive control; AMC and Caat - crude extracts (at 1.2 mg).
[2] C1: orthogonal contrast control vs others; C2: orthogonal contrast monensin vs AMC + Caat; C3: orthogonal contrast AMC vs Caat.
Means followed by different letters are significantly different at P<0.05.

Table 3 - Effect of the inclusion of monensin and crude extracts produced by actinobacteria (AMC and Caat) on the ammoniacal nitrogen (NH_3-N) and methane (CH_4) concentration of *in vitro* fermentation with ruminal dairy cow inoculum

Variable	Treatment[1]				Mean	SEM	Effect[2]			
	CO	MON	AMC	Caat			Treatment	C1	C2	C3
NH_3-N (mg/dL)	29.20a	24.46b	29.76a	26.07b	27.37	0.65	<.0001	0.18	0.0009	0.72
Methane (mL)	13.87a	8.31b	9.47b	9.02b	10.17	0.53	<.0001	0.07	0.0006	0.0001
mL/g digestible DM	48.92a	41.92ab	35.21bc	32.42c	39.62	1.83	0.0026	0.01	0.97	0.0038

[1] CO - negative control, no additive inclusion; MON - monensin (5 µM), positive control; AMC and Caat - crude extracts (at 1.2 mg).
[2] C1: orthogonal contrast control vs others; C2: orthogonal contrast monensin vs AMC + Caat; C3: orthogonal contrast AMC vs Caat.
Means followed by different letters are significantly different at P<0.05.

additives–including ionophores, oil extracts, and essential oils–have been commercially available for the past decade, to our best knowledge, this is the first study on the use of crude extracts derived from actinobacteria in the modulation of *in vitro* ruminal fermentation. This study was the first to report the CH_4 and NH_3-N concentration, after the *in vitro* dose-response effects reported previously by our group (Alves et al., 2018), although previous studies have used essential oils or chitosan as alternatives to ionophores (Benchaar et al., 2006; Khorrami et al., 2015; Tomkins et al., 2015; Vendramini et al., 2016).

In this study, Caat inclusion resulted in lower cumulative gas production compared with AMC, although among all treatments, the positive control showed the lowest gas production, probably associated with reduction in DM digestibility. Salem et al. (2014) evaluated the plant extract of *Salix babylonica* on the *in vitro* ruminal fermentation and, differently from the results of our study, they observed an increase in gas production over the incubation time. However, the plant extract used by Salem et al. (2014) was mainly composed of phenols and alkaloids, which may have made digestion difficult for ruminal microorganisms. In our study, the inclusion of both crude extracts did not change the IVDMD or IVOMD.

However, the reduction of total gases after the inclusion of extracts may vary according the *in vitro* assay characteristics, such as substrate quality, anaerobic conditions, buffer quality, as well as the included dose (Alves et al., 2018; Bueno et al., 2005; Rymer et al., 1999). In the present study, we evaluated two crude extracts diluted in DMSO, and probably this dilution may have affected the magnitude of the crude extract mode of action in comparison with a pure extract. One suggestion would be to use higher doses than those reported by Alves et al. (2018) for diluted compounds, if it is not possible to use them in the most concentrated or pure form.

The IVDMD reduction was observed only in the positive control with the monensin inclusion, while the inclusion of crude extracts did not affect the digestibility of the substrate included in the fermentation bottle. Contrary to what was reported by our team, an *in vivo* study conducted by Salem et al. (2011) evaluated the effects of secondary compounds from *Salix babylonica* (SB) and *Leucaena leucocephala* (LL) on feed intake, nutrient digestibility, and growth of crossbred male lambs. They observed that the extracts improved the digestible dry matter by 5 and 3% with SB and LL, respectively, compared with control. However, this difference in the effects of DM digestibility can be explained by the substrate limitation found in *in vitro* assays, whereas in *in vivo* assays, this does not happen, since the diet is adjusted for DM intake. If in these studies, the crude extracts studied here were used, there could probably be effects of increasing diet digestibility.

In the present study, the ruminal VFA concentration was modulated by Caat inclusion, as there was an increase of propionic acid concentration and a reduction of butyric acid and A:P ratio concentrations. Using an *in vitro* methodology, Jiménez-Peralta et al. (2011) evaluated different doses of *Leucaena leucocephala* and *Salix babylonica* extracts on the production of VFA and observed higher total production of VFA for the extracts in relation to the control, although production of individual acids were not reported. Similar to the present study, Ponce et al. (2012) observed an increase in propionic acid concentration and a decrease of A:P ratio when comparing six different compositions of ionophores (lasalocid sodium commercial premix, lasalocid sodium mycelium cake, laidlomycin sodium salt, laidlomycin propionate commercial premix, monensin sodium salt, and monensin sodium commercial premix) to negative control on *in vitro* fermentation variables.

Although the mode of action of crude extracts is not defined, some selection of ruminal bacteria is suggested, similar to what happens after the inclusion of monensin, since effects on the reduction of gas production, CH_4, and modulation of VFA production were observed. Actinomycin, the main compound of Caat extract, binds specifically to bacterial DNA and inhibits RNA (transcription) synthesis (Reich et al., 1962), and, to some extent, the Gram-negative lipopolysaccharide cell wall can act as a physical barrier against this action.

In the present study, Caat inclusion reduced the A:P ratio around 15% compared with negative control, a similar reduction to that found by Kholif et al. (2018) when evaluating doses of *Moringa oleifera* leaf

extract on feed intake, nutrient digestion, and ruminal and blood serum measurements in goats. On the other hand, Takiya et al. (2017) evaluated the increasing dietary doses of an *Aspergillus oryzae* extract with alpha-amylase activity on ruminal fermentation of lactating dairy cows. They observed that treatments did not affect acetate, propionate, and total production of VFA. However, this difference on VFA modulation found between the study of Takiya et al. (2017) and the present study can be explained by some individual traits of each diet or substrate, such as particle size and neutral detergent fiber digestibility, which can affect the digestion rate by bacteria. In this case, Takiya et al. (2017) suggested that the small effects on ruminal fermentation were due to alpha-amylase activity in rumen. However, the reduction of A:P ratio corroborate the positive effects of secondary metabolites of natural extracts, potentially favoring propionate-producers that increase the rumen energy metabolism.

Additionally, we observed in this study that CH_4 concentration was reduced by approximately 5% with the Caat inclusion compared with AMC inclusion. Similarly, Kholif et al. (2018) described a 7% decrease in CH_4 (mmol/L) in ruminal fluid of goats fed *Moringa oleifera* leaf extract. Moreover, a reduction of 19% in CH_4 (g/DM) production was reported by Cattani et al. (2016), when evaluating the inclusion of pure oils extracts (limonene, allyl sulfide, cinnamaldehyde, and eugenol) on *in vitro* ruminal fermentation. These latter authors may have observed that short-term incubations, such as those performed in the present experiment (24 h), may well evidence the effects of plant extracts on rumen fermentation, such as the positive control. Both studies confirm that the CH_4 reduction from ruminal fermentation by secondary metabolites derived from extracts can be achieved by direct inhibition or by reduction of the processes involved in methanogenesis (Bodas et al., 2012).

However, the present study evaluated the CH_4 concentrations in digestible DM basis, and no effect of positive control was observed in comparison with crude extracts. Therefore, the effect of CH_4 concentration reduction in mL was associated with the IVDMD reduction caused by the monensin inclusion. In comparison with monensin, the extracts were even more efficient, evidencing that the lower CH_4 concentration observed with the monensin inclusion was due to its decrease of IVDMD, which was not observed with the inclusion of both crude extracts. This result indicated that CH_4 reduction found with the monensin inclusion (mL) was related to their IVDMD reduction, as previously described. This fact indicates the need for an *in vivo* study by inclusion of crude extracts in dairy cow diet, as the use of these compounds could have an advantage over the productive performance, since it would not reduce fiber digestibility.

In this study, no effect of the inclusion of crude extracts was observed in the NH_3-N concentration, which may be associated with the reduction of ruminal protein deamination (Ghorbani et al., 2008). However, Van Soest (1994) stated that ionophores have a larger effect on the reduction of rumen degradation of dietary protein in concentrate-based diets than in hay- and silage-based diets. Thus, considering that, in this study, we used substrates based on corn silage, this may have contributed to the absence of observed effect on rumen deamination after the inclusion of crude extracts.

In the present study, both crude extracts used were characterized as a mixture of active compounds with more than one active metabolite present. Therefore, the observed effects in the analyzed variables were higher after the positive control inclusion, although it was possible to observe effects on some variables after extract inclusion, mainly Caat. Caat extract increased propionate concentration and decreased A:P ratio and butyrate concentration, as well as ammonia nitrogen and CH_4 reduction, while AMC extract was effective in decreasing CH_4 production, both compared with negative control.

Our study evaluated crude extract inclusion without quantification of the active metabolites present in each one. Even so, gas production and ruminal fermentation results in 24 h demonstrate that Caat crude extract is a promising additive for ruminant diets, whether used alone or in addition to other groups of additives. Because it is of natural origin, it is assumed that there is less risk of inducing cross-resistance with other metabolites. The decrease in CH_4 production and the increase of propionate concentration emphasize the importance of the use of Caat, since it maximizes ruminal fermentation without affecting DM digestibility. However, further studies are needed to evaluate these effects using an *in vivo* ruminal fermentation model.

Conclusions

The inclusion of Caat crude extract modulates the volatile fatty acid production on *in vitro* ruminal fermentation compared with the negative control. In addition, Caat crude extract reduces CH_4 per kg of digested dry matter without altering the digestibility of dry and organic matter.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.G. Alves, L.A.B. Moraes and M.V. Santos. Data curation: B.G. Alves. Formal analysis: B.G. Alves, C.M.M.R. Martins and A.P.F. Peti. Investigation: B.G. Alves. Methodology: B.G. Alves and A.P.F. Peti. Project administration: B.G. Alves, C.M.M.R. Martins, L.A.B. Moraes and M.V. Santos. Software: C.M.M.R. Martins. Supervision: M.V. Santos. Writing-original draft: B.G. Alves. Writing-review & editing: B.G. Alves, C.M.M.R. Martins, A.P.F. Peti, L.A.B. Moraes and M.V. Santos.

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Rerefences

Abdalla, A. L.; Silva Filho, J. C.; Godoi, A. R.; Carmo, C. A. and Eduardo J. L. P. 2008. Utilização de subprodutos da indústria de biodiesel na alimentação de ruminantes. Revista Brasileira de Zootecnia 37:260-268. https://doi.org/10.1590/S1516-35982008001300030

Alves, B. G.; Martins, C. M. M. R.; Arcari, M. A.; Peti, A. P. F.; Moraes, L. A. B. and Santos, M. V. 2018. Dose-response effect of crude extracts produced by actinobacteria on *in vitro* rumen fermentation. Brazilian Journal of Veterinary Research and Animal Science 55:e141243. https://doi.org/10.11606/issn.1678-4456.bjvras.2018.141243

Benchaar, C.; Petit, H. V.; Berthiaume, R.; Whyte, T. D. and Chouinard, P. Y. 2006. Effects of addition of essential oils and monensin premix on digestion, ruminal fermentation, milk production, and milk composition in dairy cows. Journal of Dairy Science 89:4352-4364. https://doi.org/10.3168/jds.S0022-0302(06)72482-1

Bergen, W. G. and Bates, D. B. 1984. Ionophores: Their effect on production efficiency and mode of action. Journal of Animal Science 58:1465-1483. https://doi.org/10.2527/jas1984.5861465x

Bodas, R.; Prieto, N.; García-González, R.; Andrés, S.; Giráldez, F. J. and López, S. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animal Feed Science and Technology 176:78-93. https://doi.org/10.1016/j.anifeedsci.2012.07.010

Bueno, I. C. S.; Cabral Filho, S. L. S.; Gobbo, S. P.; Louvandini, H.; Vitti, D. M. S. S. and Abdalla, A. L. 2005. Influence of inoculum source in a gas production method. Animal Feed Science and Technology 123-124:95-105. https://doi.org/10.1016/j.anifeedsci.2005.05.003

Cattani, M.; Maccarana, L.; Rossi, G.; Tagliapietra, F.; Schiavon, S. and Bailoni, L. 2016. Dose-response and inclusion effects of pure natural extracts and synthetic compounds on *in vitro* methane production. Animal Feed Science and Technology 218:100-109. https://doi.org/10.1016/j.anifeedsci.2016.05.014

Crevelin, E. J.; Canova, S. P.; Melo, I. S.; Zucchi, T. D.; Silva, R. E. and Moraes, L. A. B. 2013. Isolation and characterization of phytotoxic compounds produced by *Streptomyces* sp. AMC 23 from Red Mangrove (*Rhizophora mangle*). Applied Biochemistry and Biotechnology 171:1602-1616. https://doi.org/10.1007/s12010-013-0418-5

Crevelin, E. J.; Crotti, A. E. M.; Zucchi, T. D.; Melo, I. S. and Moraes, L. A. B. 2014. Dereplication of *Streptomyces* sp. AMC 23 polyether ionophore antibiotics by accurate-mass electrospray tandem mass spectrometry. Journal of Mass Spectrometry 49:1117-1126. https://doi.org/10.1002/jms.3432

Erwin, E. S.; Marco, G. J. and Emery, E. M. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. Journal of Dairy Science 44:1768-1771. https://doi.org/10.3168/jds.S0022-0302(61)89956-6

Foldager, J. 1977. Protein requirement and non protein nitrogen for high producing cow in early lactation. Ph.D. Thesis. Michigan State University, East Lansing, MI.

Getachew, G.; Robinson, P. H.; DePeters, E. J. and Taylor, S. J. 2004. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. Animal Feed Science and Technology 111:57-71. https://doi.org/10.1016/S0377-8401(03)00217-7

Ghorbani, B.; Ghoorchi, T.; Amanlou, H. and Zerehdaran, S. 2008. Effects of monensin and increasing crude protein in early lactation on performance of dairy cows. Pakistan Journal of Biological Sciences 11:1669-1675.

Ipharraguerre, I. R. and Clark, J. H. 2003. Usefulness of ionophores for lactating dairy cows: a review. Animal Feed Science and Technology 106:39-57. https://doi.org/10.1016/S0377-8401(03)00065-8

Jani, P.; Emmert, J. and Wohlgemuth, R. 2008. Process analysis of macrotetrolide biosynthesis during fermentation by means of direct infusion LC-MS. Biotechnology Journal 3:202-208. https://doi.org/10.1002/biot.200700174

Jiménez-Peralta, F. S.; Salem, A. Z. M.; Mejia-Hernández, P.; González-Ronquillo, M.; Albarrán-Portillo, B.; Rojo-Rubio, R. and Tinoco-Jaramillo, J. L. 2011. Influence of individual and mixed extracts of two tree species on *in vitro* gas production kinetics of a high concentrate diet fed to growing lambs. Livestock Science 136:192-200. https://doi.org/10.1016/J.LIVSCI.2010.09.008

Kholif, A. E.; Gouda, G. A.; Anele, U. Y. and Galyean, M. L. 2018. Extract of *Moringa oleifera* leaves improves feed utilization of lactating Nubian goats. Small Ruminant Research 158:69-75. https://doi.org/10.1016/j.smallrumres.2017.10.014

Khorrami, B.; Vakili, A. R.; Mesgaran, M. D. and Klevenhusen, F. 2015. Thyme and cinnamon essential oils: potential alternatives for monensin as a rumen modifier in beef production systems. Animal Feed Science and Technology 200:8-16. https://doi.org/10.1016/j.anifeedsci.2014.11.009

Kulasek, G. A. 1972. A micromethod for determination of urea in plasma, whole blood and blood cells using urease and phenol reagent. Polskie Archiwum Weterynaryjne 15:801-810.

Lowe, S. E.; Theodorou, M. K.; Trinci, A. P. J. and Hespell, R. B. 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. Journal of General Microbiology 131:2225-2229.

Mauricio, R. M.; Mould, F. L.; Dhanoa, M. S.; Owen, E.; Channa, K. S. and Theodorou, M. K. 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. Animal Feed Science and Technology 79:321-330. https://doi.org/10.1016/S0377-8401(99)00033-4

Menke, K. H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D. and Schneider, W. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. The Journal of Agricultural Science 93:217-222. https://doi.org/10.1017/S0021859600086305

Nagaraja, T. G.; Newbold, C. J.; Van Nevel, C. J. and Demeyer, D. I. 1997. Manipulation of ruminal fermentation. p.523-632. In: The rumen microbial ecosystem. Hobson, P. N. and Stewart, C. S., eds. Springer, Dordrecht. https://doi.org/10.1007/978-94-009-1453-7_13

Narvaez, N.; Wang, Y. and McAllister, T. 2013. Effects of extracts of *Humulus lupulus* (hops) and *Yucca schidigera* applied alone or in combination with monensin on rumen fermentation and microbial populations *in vitro*. Journal of the Science of Food and Agriculture 93:2517-2522. https://doi.org/10.1002/jsfa.6068

NRC - National Research Council. 2001. Nutrient requirements of dairy cattle. Seventh Revised Edition. The National Academies, Washington, DC.

Ponce, C. H.; Smith, D. R.; Branine, M. E.; Hubbert, M. E. and Galyean, M. L. 2012. Effects of type of ionophore and carrier on *in vitro* ruminal dry matter disappearance, gas production, and fermentation end products of a concentrate substrate. Animal Feed Science and Technology 171:223-229. https://doi.org/10.1016/j.anifeedsci.2011.11.003

Oliveira, T. L.; Lima, E. O.; Souza, I. A. and Cornejo, L. C. Z. 2010. Atividade antifúngica e cinética de morte microbiana de extratos obtidos de *Streptomyces* spp. isolados de solos paraibanos. Revista Brasileira de Farmacognosia 20:60-64. https://doi.org/10.1590/S0102-695X2010000100013

Priya, M.; Anandaraj, B. and Ganesan, T. 2015. Antimicrobial activity of estuarine actinobacteria against selected human pathogens. International Journal of Current Microbiology and Applied Sciences 4:120-125.

Reich, E.; Franklin, R. M.; Shatkin, A. J. and Tatum, E. L. 1962. Action of actinomycin D on animal cells and viruses. Proceedings of the National Academy of Sciences of the United States of America 48:1238-1245. https://doi.org/10.1073/pnas.48.7.1238

Rymer, C.; Huntington, J. A. and Givens, D. I. 1999. Effects of inoculum preparation method and concentration, method of inoculation and pre-soaking the substrate on the gas production profile of high temperature dried grass. Animal Feed Science and Technology 78:199-213. https://doi.org/10.1016/S0377-8401(99)00006-1

Salem, A. Z. M.; Kholif, A. E.; Elghandour, M. M. Y.; Buendía, G.; Mariezcurrena, M. D.; Hernandez, S. R. and Camacho, L. M. 2014. Influence of oral administration of *Salix babylonica* extract on milk production and composition in dairy cows. Italian Journal of Animal Science 13:2978. https://doi.org/10.4081/ijas.2014.2978

Salem, A. Z. M.; Olivares, M.; Lopez, S.; Gonzalez-Ronquillo, M.; Rojo, R.; Camacho, L. M.; Cerrillo, S. M. A. and Mejia, H. P. 2011. Effect of natural extracts of *Salix babylonica* and *Leucaena leucocephala* on nutrient digestibility and growth performance of lambs. Animal Feed Science and Technology 170:27-34. https://doi.org/10.1016/j.anifeedsci.2011.08.002

Smedsgaard, J. 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. Journal of Chromatography A 760:264-270.

Takiya, C. S.; Calomeni, G. D.; Silva, T. H.; Vendramini, T. H. A.; Silva, G. G.; Consentini, C. E. C.; Bertoni, J. C.; Zilio, E. M. C. and Rennó, F. P. 2017. Increasing dietary doses of an *Aspergillus oryzae* extract with alpha-amylase activity on nutrient digestibility and ruminal fermentation of lactating dairy cows. Animal Feed Science and Technology 228:159-167. https://doi.org/10.1016/j.anifeedsci.2017.04.017

Tomkins, N. W.; Denman, S. E.; Pilajun, R.; Wanapat, M.; McSweeney, C. S. and Elliott, R. 2015. Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. Animal Feed Science and Technology 200:25-34. https://doi.org/10.1016/j.anifeedsci.2014.11.013

Van Soest, P. J. 1994. Nutritional ecology of the ruminants. 2nd ed. Cornell University, Ithaca. 476p.

Vendramini, T. H. A.; Takiya, C. S.; Silva, T. H.; Zanferari, F.; Rentas, M. F.; Bertoni, J. C.; Consentini, C. E. C.; Gardinal, R.; Acedo, T. S. and Rennó, F. P. 2016. Effects of a blend of essential oils, chitosan or monensin on nutrient intake and digestibility of lactating dairy cows. Animal Feed Science and Technology 214:12-21. https://doi.org/10.1016/j.anifeedsci.2016.01.015