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Effects of Isomix® and virginiamycin on *in vitro* ruminal fermentation

Efeitos do Isomix® e virginiamicina na fermentação ruminal *in vitro*

Sara Adriana Salinas Laura*[1], Rogério de Paula Lana[2], Márcia das Neves Saores[1], Paulo Sérgio Dornelas Silva[2], Ruth Ccalta Hancco[1]

1 Universidade Federal de Lavras (UFLA), Lavras, Minas Gerais, Brazil
2 Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil

*corresponding author: sarasalinas0697@hotmail.com

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Abstract: Additives improve cattle feed efficiency by altering microbial populations. This study evaluated the effects of varying doses and the interaction between two additives on *in vitro* dry matter digestibility (IVDMD), neutral detergent fiber digestibility (IVNDFD), and ruminal fermentation parameters using rumen fluid from fistulated cows. Diets with a 70:30 forage-to-concentrate ratio were incubated for 48 h using the Tilley and Terry method, with *Brachiaria decumbens* as the forage and a concentrate composed of corn meal, soybean meal, and urea. The additives tested were Isomix® (from 0.0 to 4.8 % of diet dry matter) and virginiamycin (from 0.0 to 0.80 %). The significant effects ($P<0.05$) of virginiamycin included increased pH, NH_3, and soluble protein (SP) and decreased IVDMD, IVNDFD, microbial protein (MP), and volatile fatty acids (VFA). Isomix had no significant effects on these variables, but increased isobutyrate concentration ($P < 0.05$). Additive interactions affected acetate, propionate, butyrate, isovalerate, and the acetate:propionate ratio ($P<0.05$). While neither additive improved digestibility, Isomix enhanced propionate production and reduced the acetate:propionate ratio, potentially improving energy balance and performance. Further research is recommended, particularly on diets with low protein and urea supplementation in tropical grazing systems.

Key-words: Branched-chain volatile fatty acids; *in vitro* digestibility; tropical pastures.

Resumo: O uso de aditivos melhora a eficiência alimentar de bovinos ao alterar as populações microbianas. Este estudo avaliou os efeitos de diferentes doses e da interação de dois aditivos sobre a digestibilidade *in vitro* da matéria seca (DIVMS), a digestibilidade da fibra em detergente neutro (DIVFDN) e os parâmetros de fermentação ruminal, utilizando líquido ruminal de vacas fistuladas. Dietas com relação 70:30 de forragem para concentrado foram incubadas por 48 h pelo método de Tilley & Terry, utilizando *Brachiaria decumbens* como forragem e um concentrado de milho moído, farelo de soja e ureia. Os aditivos testados foram Isomix® (0,0 a 4,8 % da matéria seca da dieta) e virginiamicina (0,0 a 0,80 %). Efeitos significativos ($P<0,05$) da virginiamicina incluíram aumento do pH, NH_3 e proteína solúvel (PS), com redução da DIVMS, DIVFDN, proteína microbiana (PM) e ácidos graxos voláteis (AGV). O Isomix não apresentou efeitos significativos sobre essas variáveis, mas aumentou ($P<0,05$) a concentração de isobutirato. As interações entre os aditivos afetaram o acetato, propionato, butirato, isovalirato e a relação acetato:propionato ($P<0,05$). Embora nenhum dos aditivos tenha melhorado a digestibilidade, o Isomix aumentou o propionato e reduziu a relação acetato:propionato, potencialmente melhorando o balanço energético e o desempenho. Recomenda-se mais pesquisas, especialmente em dietas com baixo teor de proteína e suplementação de ureia em sistemas de pastejo tropical.

Palavras-chave: Ácidos graxos voláteis de cadeia ramificada; digestibilidade *in vitro*; pastagens tropicais.

1. Introduction

Brazil has the largest herd of beef cattle in the world, raised predominantly on pasture systems using tropical forages. The high proportions of structural carbohydrates found in fodder limit voluntary intake and digestion in ruminants by reducing fermentation rates [1]. The search for new technology to enhance animal performance has been considered as an alternative to reduce costs in ruminant nutrition [2].

Ruminal fermentation provides several advantages in terms of digestive and metabolic processes. Ruminal fermentation products such as volatile organic acids and microbial proteins are the main sources of energy and nutrients for ruminants. Controlling certain metabolic processes in the rumen to enhance animal performance has been an attractive concept for rumen nutritionists and microbiologists [3]. Thus, the use of food additives can provide benefits, such as weight gain, improvement in feed conversion, decreased risk of acidosis and improvement in immune response, which results in gains for the production system [4,5].

Virginiamycin is a non-ionophore antibiotic that inhibits the synthesis and growth of gram-positive bacterial cells [6]. Branched chain volatile fatty acids (BCVFA, i.e., isobutyric, isovaleric, valeric and 2-methylbutyric) are considered essential for the growth of cellulolytic microorganisms, which are necessary to degrade the fibrous components of diets [7]. Fibrolytic bacteria may benefit the most from the addition of BCVFA in the diet, while other microbes may benefit from increased fiber degradation [8].

Previous research has shown variable effects of BCVFA supplementation on ruminal activity; however, there is little information on their supplementation under our conditions. Likewise, knowledge of the effects of virginiamycin in high-forage diets are also lacking [18, 21, 22].

The aim of this study was to analyze the effects of different levels of virginiamycin and Isomix (product containing BCVFA) on ruminal microorganisms using the *in vitro* technique, by evaluating the digestibility of dry matter and neutral detergent fiber in the diet, as well as possible modifications in selected ruminal fermentation parameters.

2. Material and methods

The experiment was conducted at the Animal Nutrition Laboratory and Rumen Microbiology Laboratory of the Department of Animal Science and at the Laboratory of Anaerobic Microbiology of the Department of Agricultural Microbiology of the Federal University of Viçosa (UFV), Viçosa, MG, Brazil. The experimental procedures were approved by the Ethics Committee on the Use of Production Animals of the university (CEUAP-UFV; Protocol 032/2020).

Two Holstein females with an average live weight of 550 kg fistulated in the rumen were subjected to the same conditions, adapted to the same diet for 15 days before rumen fluid collection, and offered water *ad libitum*. The diet consisted of corn silage as forage and a concentrate containing corn meal and soybean meal, supplied twice a day, after morning and afternoon milking. The cows were kept in individual stalls with a cement floor equipped with a water dispenser and a feeder for forage and concentrate.

In the experiment, the effects of the inclusion of different levels of virginiamycin and a commercial product (Isomix®) on ruminal fermentation *in vitro* in a pasture-based diet of *Brachiaria decumbens* and forage:concentrate ratio of 70:30 were evaluated. The fermentation parameters evaluated were pH; total volatile fatty acids (VFA); acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids (in mM and in percentage); acetic:propionic ratio; ammoniacal nitrogen (in mM or mmol/L and mg of $N\text{-}NH_3$/100 mL); and protein and microbial protein (mg/L).

The forage was harvested at the end of the rainy season, by manual grazing simulation, in a paddock of Boa Vista farm, district of Cachoeira of Santa Cruz, Viçosa-MG, belonging to the Department of Animal Science of UFV. The feed consisted of *Brachiaria decumbens* grass, corn meal, soybean meal and urea in the ratio: 70.0; 19,3; 9.7 and 1.0 % in the air-dried sample, respectively. A total of 75 g of feed were prepared and then homogenized and processed in a Willey mill, with a 1 mm porosity sieve to also perform bromatological analyses. The composition obtained by laboratory analyses were: crude protein, 16.7 %; neutral detergent fiber, 66.7 %; acid detergent fiber, 32.4 %; lignin, 5.21 %; ether extract, 2.29 %; and ash, 6.03 %.

The treatments were administered in a 6 × 6 factorial design, consisting of six levels of Isomix (0.0, 1.5, 3.0, 6.0, 12, and 24 mg of Isomix or at concentrations of 0.0, 0.3, 0.6, 1.2, 2.4 and 4.8 %) and six virginiamycin levels (0.0, 0.25, 0.5, 1, 2, and 4 mg of virginiamycin 10 % or at concentrations of 0.0, 0.05, 0.1, 0.2, 0.4 and 0.8 %) in 500 mg of dry matter ration (or 550 mg of air-dried matter), plus two additional controls, with three repetitions, totaling 114 experimental units. The highest levels chosen for Isomix and virginiamycin *in vitro* were well above the commercially recommended levels in vivo; however, the lower levels were close to the recommended levels. This approach was adopted to verify the range of possible effects under *in vitro* conditions.

In the preparation of the six treatments containing Isomix, 0.873 g of Isomix and 9.13 g of air-dried diet were added to a bottle. This mixture contained 24 mg of Isomix for every 275 mg of air-dried sample (named 24 mg; to be used in the second paragraph ahead). Dry dilutions were then made by taking 5 g from this bottle and adding it to 5 g of feed (12 mg), resulting in concentrations of 6.0, 3.0, and 1.5 mg of Isomix. The control bottle (0 mg) contained pure feed.

In the preparation of the six treatments containing virginiamycin, 0.145 g of virginiamycin (10 %) and 9.86 g of the air-dried diet were added. This mixture contained 4 mg of virginiamycin for every 275 mg of air-dried sample (named 4 mg; used in the next paragraph). Dry dilutions were then made by taking 5 g from this bottle and adding it to 5 g of feed (2 mg), resulting in concentrations of 1.0, 0.5 and 0.25 mg of virginiamycin. The control bottle (0 mg) contained pure feed.

For *in vitro* fermentation, 0.275 g of sample with or without Isomix was mixed with 0.275 g of sample with or without virginiamycin, depending on the different combinations of treatments (6 × 6), totaling 0.550 g of air-dried sample. We added 10 mL of rumen fluid and 40 mL of McDougall [8] buffer in a 100 mL penicillin bottle (1:4 inoculum ratio and buffer solution). The experiment followed the standard method for *in vitro* digestibility studies described by Tilley and Terry [9].

The day before incubation, McDougall's [8] buffer solution was prepared in an air-conditioned room at 39 °C for temperature stabilization. Before each repetition, the ruminal digest (liquid and solids) was removed from different points of the liquid–solid interface of the ruminal environment, and packed

in thermal containers suitable for the maintaining temperature at 39 °C, using preheated thermal bottles. Subsequently, the digest was transferred to a blender in the air-conditioned room at 39 °C and homogenized for 30 seconds. The homogenized material was filtered through three layers of gauze in a 2-L Erlenmeyer [10].

The free space of the vials was immediately saturated with CO_2, being closed with rubber caps and aluminum seals. The vials were kept under agitation on an orbital agitator table (40 rpm) in an air-conditioned room (39 ºC). The gases from fermentation were removed every three hours, with the aid of needles. After 48 hours of incubation, the vials were removed from the air-conditioned room and submitted to pH measurement with digital pot, and the contents were transferred to filter crucibles, with the aid of distilled water (temperature higher than 90 ºC). Then, the crucibles were dried (105 ºC/24 hours) and weighed, obtaining the residue apparently not digested from dry matter.

For the evaluation of *in vitro* digestibility of neutral detergent fiber (IVDNDF), crucibles containing incubation residue were introduced inside autoclavable universal collectors (120 mL), adding 80 mL of neutral detergent solution, 16 produced according to Mertens [11] with omission of sodium sulphite, and 250 µL of thermostable α-amylase (Termamyl 2X). The collectors with crucibles packed inside were closed with their lids and autoclaved (105 ºC/1 h) according to the method described by Detmann *et al.* [12]; INCT-CA F-002/1). After removing from the autoclave, the crucibles were again washed with hot distilled water and, at the end, with 30 mL of acetone, being dried (105 °C/24 h) and weighed to obtain NDF residue.

After pH measurement, the liquid was collected from the vials in Eppendorf tubes in triplicate per sample. Organic acids were analyzed using high-efficiency liquid chromatography (HPLC). Ruminal fluid samples (1.5 mL) were collected and centrifuged (10,000 × g, 10 min) to remove cells, and the cell-free supernatant was processed as described by Siegfried *et al.* [13]. The samples were separated using a Phenomenex Rezex ROA column, 300 × 7.8 mm, maintained at 45 ºC, using Dionex Ultimate 3000 Dual chromatograph coupled to a Shodex RI-101 refraction index (RI) detector, maintained at 45 ºC. The mobile phase was sulfuric acid (H2SO4; 5.0 mM), with a flow of 0.7 mL/min. Calibration curves were constructed using propionic acid (60 mM), acetic acid (60 mM), butyric acid (20 mM), valeric acid (20 mM), isovaleric acid (5 mM), and isobutyric acid (10 mM) as the external patterns. External patterns were analyzed at the concentrations described and at dilutions of 1:2, 1:4, 1:16, and 1:32. Crotonic acid (12.5 mM) was used as an internal standard for the samples and calibration curve. The concentration of the organic acids was normalized to the response factor and expressed as a fraction of the total fatty acids produced (mol/100 mol).

Ammonia concentration in the ruminal fluid samples was determined using the colorimetric method described by Chaney and Marbach [14]. Absorbance was measured at 630 nm using a Spectronic 20D spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) and ammonium chloride (NH_4Cl) was used as the standard.

The soluble protein concentration in the cell-free supernatant was determined after centrifuging the samples (Eppendorf 5417C, Hamburg, Germany) for 10 min (10,600 × g). Protein was quantified according to the methodology described by Bradford [15], using bovine serum albumin (BSA) as a standard.

The microbial protein concentration was determined after centrifuging 1 mL of the sample for 10 min (10,600 × g) in a microtube centrifuge (Eppendorf 5417C, Hamburg, Germany). The supernatant was discarded and the pellet digested with NaOH (0.2 N) for 5 min at 100 °C. The microbial protein concentration was quantified by the Bradford colorimetric method [15], using BSA as the standard.

Statistical analyses was performed using Statistical Analysis System [16] software and a generalized linear model was generated, adopting a probability level of 0.05 for the type I error. The treatments were in a 6 × 6 factorial scheme, composed of combinations of Isomix and virginiamycin concentrations, plus two additional controls, and three replicates, totaling 114 experimental units, and the effects of the treatments and their interactions were evaluated. The difference between the means of treatments was analyzed using the LS-means Tukey test, adopting a significance level of 5 %.

3. Results and discussion

Virginiamycin influenced pH, IVDMD, and IVDNDF (Table 1). The Isomix and its interaction with virginiamycin had no effect on these variables (P>0.05).

Table 1. P-values of some analyzed variables.

Variation source	pH	IVDDM (%)	IVDNDF (%)
% V	0.0040	<0.0001	<0.0001
% I	0.6919	0.8488	0.5967
%V*%I	0.9994	0.9983	0.9911

IVDDM - *in vitro* digestibility of dry matter; IVDNDF - *in vitro* digestibility of neutral detergent fiber; V - virginiamycin; I - Isomix.

In a study by Roman [17], with the inclusion of BCVFA, no effect was observed on pH, as was the case in the present study. The final pH values of incubations between different forages and the addition of BCVFA ranged from 6.64 to 6.86, which was within the range for the optimal digestion of fibers in the rumen [18].

The lack of an effect of Isomix, which contains BCVFA, on the digestibility of dry matter and neutral detergent fiber contrasts with previous studies in which effects on digestibility were found. For example, Liu *et al.* [19] reported an increase in ruminal concentration of VFA and total digestibility of nutrients. Zhang *et al.* [20] evaluated the inclusion of branched chain amino acids (BCAA) in a wheat diet at concentrations of 0, 2, 4, 7, and 10 mmol/L and observed an increasing linear effect in BCVFA with the supplementation of BCAA, and that the lowest concentration of 2 mmol/L of BCAA allowed for better efficiency in ruminal fermentation and digestibility of dry matter and fiber. Suryapratama and Suhartati [21] suggested that the supplemented amount of 0.05 mM is not sufficient to influence the growth of ruminal bacteria.

Other *in vitro* studies have shown that BCVFA increases ruminal fermentation and fiber digestion by stimulating cellulolytic microorganisms, since BCVFA supplementation improves the activity of carboxymethylcellulase, xylanase, and b-glucosidase [22a].

Variations in the effects of supplementation may be related to the dietary composition used in the studies because the results show that the effectiveness of grass-based diets is greater than that of legume diets owing to the higher crude protein content of legumes [22b]. Previous studies have shown that the response of ruminal fermentation and fiber degradation is limited by supplementation with BCVFA (at a dose of 2 mM) in a diet with high crude protein content and a rapid rate of degradation of neutral detergent fiber [23] or with a low content of fermentable carbohydrates [24].

In the present study, the pH of the medium increased with the use of virginiamycin (Table 2), according to Maciel *et al.* [25], who reported that virginiamycin potentially increases the ruminal pH of animals receiving different diets with varying forage:concentrate ratios. This can be explained by an increase in the population of bacteria that use lactic acid, and a reduction in lactic acid-producing bacteria [26].

The addition of virginiamycin increased IVDDM and IVDNDF levels (Table 2). These results are similar to those of Maciel *et al.* [25], who evaluated the digestibility of Marandu grass with 33.5 mg/100 kg body weight of virginiamycin in Nellore cows in April and May, which may be related to the lower crude protein content of the pasture, reduced degradation of the protein, and the supply of nutrients to cellulolytic bacteria. Furthermore, Thorniley *et al.* [27] evaluated different concentrations of virginiamycin in ewes fed wheat straw and found that at concentrations of 80 and 160 mg virginiamycin/day (the highest in the study), a decrease in digestibility was observed, which was associated with decreased dry matter intake.

The behavior of IVDNDF was similar to that reported by Oliveira *et al.* [28], who evaluated virginiamycin supplementation (150 mg/kg DM) in the diet of grazing dairy cows and observed a decrease in fiber digestibility, which may be related to ammonia reduction with the use of virginiamycin or the toxic effect of virginiamycin on cellulolytic ruminal microbiota. On the other hand, in a study by Silva *et al.* [29], supplementation of 22.5 mg/kg DM concentrate for crossbred cows fed a diet based on sugarcane tended to increase the digestibility of DM and NDF, showing a better ruminal environment and better use of fiber.

Table 2. Values (means ± standard error)1 of pH and *in vitro* digestibility of dry matter and neutral detergent fiber as a function of virginiamycin levels.

Virginiamycin (%)	pH	IVDDM (%)	IVDNDF (%)
0.0	6.87 ± 0.02 B	65.58 ± 1.05 A	66.98 ± 1.10 A
0.05	6.85 ± 0.02 B	63.42 ± 1.11 AB	64.32 ± 1.15 A
0.1	6.87 ± 0.02 B	59.77 ± 1.11 B	59.51 ± 1.15 B
0.2	6.90 ± 0.02 AB	53.96 ± 1.11 C	52.57 ± 1.15 C
0.4	6.89 ± 0.02 AB	50.85 ± 1.11 CD	47.18 ± 1.15 D
0.8	6.95 ± 0.02 A	47.14 ± 1.11 D	39.40 ± 1.15 E

[1]P values in Table 1; IVDDM, *in vitro* digestibility of dry matter; IVDNDF, *in vitro* digestibility of neutral detergent fiber; A,B,C,D,Emeans with the same letters in same column do not differ by Tukey's test at 5 % probability.

IVDNDF and IVDDM showed a strong positive correlation (0.89) ($P<0.05$). Another important variable that has been analyzed is the concentration of ammoniacal nitrogen in the rumen, as this may compromise the activity of rumen microorganisms, especially those that degrade fibrous carbohydrates. Ruminal microbiota selector additives promote ammoniacal nitrogen levels suitable for microbial growth [30]. The concentration of N-NH_3 in the rumen can influence fiber degradation, and values above 4 mg/dL can maximize fiber degradation [31, 32]. In our results, the values of N-NH_3 (mg/dL = mM*1,4) were lower than the adequate level to improve fiber degradation for the four lowest levels of virginiamycin (Tables 3 and 4). Ferreira *et al.* [33] found no differences between treatments, with 3.961, 3.876, and 4.147 mg in the control, and 108 and 216 mg virginiamycin/animal/day, respectively. Similar behavior was found in a study by Salinas-Chavira [34], where virginiamycin supplementation did not affect the amount of N-NH_3

or the net microbial efficiency (21g microbial N per kg of fermented OM), indicating that the effects of virginiamycin on ruminal parameters were very small. Virginiamycin increased pH, which was associated with increased concentrations of soluble protein and ammonia. In contrast, Moreira *et al.* [35] reported that the application of virginiamycin reduced ammonia concentration, attributing this effect to its ability to inhibit proteolysis and amino acid deamination, which may reduce ammonia accumulation in the rumen, enhancing dietary protein retention in the host.

Costa [36] and Neto *et al.* [37] reported results similar to ours (Tables 3 and 4), in which the presence of virginiamycin increased the availability of N-NH_3, suggesting that virginiamycin had limited effects on the growth control of amino acid-fermenting bacteria and protein deamination (*Clostridium aminophilum* and *Clostridium sticklandii*, which are gram-positive bacteria). On the other hand, we also observed that the crude protein content present in forage and in the supplements offered to the animal influenced the concentration of N-NH_3 in the rumen [28]. Protein is degraded at a faster rate because of the larger readily soluble fractions, which are rapidly degraded in the ruminal environment, thereby increasing the concentration of NH_3 [33]. Another reason for this behavior is that virginiamycin can increase the population of protozoa. In a study by Costa *et al.* [38], an increase in *Entodinium* was observed to result in 70–75 % of bacterial lysis.

Table 3. P-values of selected variables related to fermentation.

Fonte de variação	pH	DIVMS (%)	DIVFDN (%)
% V	0.0040	<0.0001	<0.0001
% I	0.6919	0.8488	0.5967
%V*%I	0.9994	0.9983	0.9911

SV - Source of variation; V - virginiamycin; I - Isomix; SP - soluble protein; MP - microbial protein; CPD - crude protein degradability; VFA - volatile fatty acids; A:P - acetate:propionate. 1Mean ± SE = 288 ± 64.3 mg/L; 2Mean ± SE = 0.46 ± 0.17 %.

Table 4. Means ± standard error1 of significant variables, without interaction, for virginiamycin.

% V	NH_3 (mM)	SP (mg/L)	CPD (%)	Total VFA (mM)
0.0	2.24±0.74 C	166.64±43.43 B	34.47±1.82 B	82.23±16.19 BDE
0.05	2.61±0.74 BC	189.57±43.43 BD	38.25±1.82 A	82.12±16.19 BDE
0.1	2.34±0.73 BC	200.20±43.43 ABC	37.72±1.82 A	72.63±16.19 ACE
0.2	2.77±0.73 BC	198.62±43.43 ABC	40.14±1.82 A	68.06±16.19 AC
0.4	3.01±0.74 AB	233.70±43.43 AC	42.43±1.82 A	62.29±16.19 AC
0.8	3.45±0.74 A	213.33±43.43 ACD	44.46±1.82 A	61.98±16.19 A

[1]P values in Table 3; V - virginiamycin; SP - soluble protein; CPD - crude protein degradability; VFA - volatile fatty acids; A,B,C,D,Emeans with same letters in same column do not differ by Tukey test at 5 % probability..

Microbial protein can supply up to 100 % of the amino acids required by the animal, depending on the quality and/or quantity of non-degradable protein in the rumen [39], and its limitation may affect animal performance. In our study, this was not affected by the addition of virginiamycin (Table 3), although the soluble protein was increasing with the increase in virginiamycin concentration. Similarly, in the study by Moreira *et al.* [35], the addition of virginiamycin did not affect the concentration of microbial protein. However, a decrease in specific deamination activity was observed with the addition of 10 µmol. L^{-1}, and

ammonia concentration was inhibited with addition of 20 µmol. L^{-1}. The study by Souza *et al.* [40] suggests that the absence of soluble carbohydrates, such as pasture sugars or insoluble carbohydrates such as starch, seems to have been limiting microbial synthesis.

Virginiamycin caused a decrease in total VFA (Tables 3 and 4), while Coe *et al.* [41] and Costa *et al.* [38] found no effect on total VFA concentration, intake and digestibility. The authors attributed the moderate influence of virginiamycin to ruminal fermentation due to a low presence of *Lactobacillus* sp and *Streptococcus bovis* compared to the control treatment. Other factors, such as the relationship between rumen concentrations of VFA and the flow of these from the rumen need to be considered when interpreting the proportions of VFA [42].

BCVFA and $N\text{-}NH_3$ are obtained by diet protein degradation and these are also used for bacterial growth and microbial protein synthesis [43], although in our results (Table 3) no effect of BCVFA supplementation via Isomix on the concentrations of $N\text{-}NH_3$, microbial protein, soluble protein and total VFA was found. Other studies also maintain that high forage diets show no effects on $N\text{-}NH_3$ concentration [44]. On the other hand, studies have shown decreased ruminal protease activity with BCVFA supplementation [45, 46, 47]. Liu *et al.* [19] showed an increase in microbial protein synthesis, which is related to the result of the decrease in ruminal $N\text{-}NH_3$ while increasing BCVFA supplementation.

In the present study, the total VFA had no effect of the incorporation of BCVFA, unlike the result obtained by Zhang *et al.* [20], by supplementing BCAA separated, having found, in the three cases, increase in the concentration of total VFA. They also obtained an increase in the final pH, while in our case, the pH did not suffer the effect of adding BCVFA via Isomix. In the study by Wang *et al.* [22], the increase in VFA is justified by the increase of cellulolytic bacteria and enzymatic activity, in which BCVFA supplementation produces an increase in dry matter intake, which offers more substrate of microbial fermentation and thus increases the concentration of VFA. They found a decreasing linear behavior of propionate, associated with an increase in the molar proportion of acetate and in the proportion of acetate propionate. The authors suggested, therefore, that ruminal fermentation was affected, with a higher production of acetic acid, similar to the results of other studies [48,19]. On the other hand, the concentration of valerate did not vary in this study, similar to the result obtained by Wang *et al.* [22]. In the case of isobutyrate, there was growth as the concentration of BCVFA was increased (Table 5), agreeing with the results of Liu *et al.* [48], which was justified by exogenous supplementation of BCVFA, in this case of the product Isomix.

Table 5. Means ± standard error1 of significant variables, without interaction, for Isomix.

Item	% Isomix					
	0.0	0.3	0.6	1.2	2.4	4.8
Isobutyrate %	0.35±0.48C	0.42±0.48C	0.52±0.48C	0.64±0.48C	0.99±0.48B	1.40±0.48A

[1]P values in Table 3; I - isomix; a-c means with same letters do not differ by Tukey test at 5 % probability.

In this study, interactions were found between the additives and acetate, propionate, butyrate, isovalerate, and acetate:propionate ratio (Table 6). The in vivo recommended dosage in g of Isomix/g of DM of the diet reproduced the best *in vitro* results, as can be seen in the VFA, with an increase in propionate and a reduction in the acetate:propionate ratio. Levels above and below the in vivo recommendations of 28 g Isomix/kg DM were used *in vitro*. It may be that in Isomix, the addition of ionophores such as monensin reduces the acetate:propionate ratio [49]. The results obtained by Liu *et al.*

[24] showed that an increase in total VFA suggests that ruminal fermentation and nutrient use improved with BCVFA supplementation, and acetate increased as the digestibility of NDF and ADF increased. Propionate production was not affected, indicating that BCVFA supplementation did not affect the growth of propionate-producing bacteria. In addition, previous studies have shown that amylolytic bacteria and amylase levels were not altered [45, 46, 47], but the total VFA and acetate:propionate ratio increased with BCVFA supplementation.

Table 6. Test of means of significant variables, with interaction, for virginiamycin and Isomix.

Isomix	Virginiamycin					
	0.0	0.05	0.1	0.2	0.4	0.8
	Acetate					
0.0	61.95 Aab	55.91 Abc	54.48 ABc	63.05 Aa	62.41 Aab	52.01 Ac
0.3	66.60 ABabc	56.08 Aabc	53.21 ABbc	59.27 Aab	62.33 Aa	50.02 Abc
0.6	62.40 Aa	53.14 Ab	58.06 ABab	62.21 Aa	60.84 Aa	41.84 Cc
1.2	43.95 Cd	49.91 Acd	55.32 ABbc	62.64 Aa	61.25 Aab	48.49 ABCcd
2.4	53.86 Bb	52.12 Abc	52.51 Bbc	64.01 Aa	61.49 Aa	45.74 ABCc
4.8	54.8 Bb	54.52 Ab	59.74 Aab	62.79 Aa	60.03 Aab	44.89 BCc
	Propionate					
0.0	27.30 Bbc	34.66 Aab	34.82 ABab	25.83 Ac	26.66 Abc	37.36 Ba
0.3	34.38 Babc	32.21 Abc	36.91 ABab	30.11 Abc	28.34 Ac	41.45 Ba
0.6	27.82 Bb	35.16 Ab	31.54 ABb	27.52 Ab	27.40 Ab	54.00 Aa
1.2	47.02 Aa	39.06 Aab	33.34 ABbc	26.91 Ac	28.76 Ac	37.96 Bb
2.4	33.91 Bab	36.33 Aa	38.45 Aa	25.29 Ac	26.84 Abc	40.25 Ba
4.8	32.46 Bb	31.72 Ab	28.30 Bb	25.39 Ab	24.58 Ab	43.01 Ba
	Butyrate					
0.0	8.89 Aab	7.63 Ab	8.67 Aab	10.20 Aa	10.09 Aab	8.02 Aab
0.3	8.53 Aa	8.59 Aa	8.29 Aa	9.29 Aa	8.85 Aa	7.90 Aa
0.6	8.40 Aa	8.21 Aa	9.38 Aa	9.65 Aa	10.25 Aa	3.73 Ab
1.2	6.78 Ab	7.56 Aab	8.30 Aab	9.53 Aa	8.72 Aab	8.07 Aab
2.4	8.21 Aa	7.54 Aa	9.43 Aa	8.64 Aa	9.52 Aa	7.38 Aa
4.8	8.23 Aa	8.04 Aa	8.23 Aa	8.14 Aa	9.52 Aa	7.32 Ba
	Isovalerate					
0.0	0.71 Ba	0.81 Ba	1.41 Aa	0.15 Ba	0.31 Ba	2.28 BCa
0.3	0.36 Ba	1.90 ABa	1.14 Aa	0.35 ABa	0.12 Ba	0.07 Da
0.6	0.59 Ba	2.06 ABa	0.50 Aa	0.29 ABa	0.33 Ba	0.30 CDa
1.2	1.78 ABb	2.07 ABb	2.38 Ab	0.34 ABb	0.50 Bb	4.66 Aa
2.4	2.43 ABb	2.71 ABb	1.51 Ab	0.75 ABb	0.74 Bb	5.60 Aa
4.8	3.90 Aa	3.84 Aa	1.63 Ab	2.43 Aab	4.02 Aa	3.99 ABa
	Acetate: Propionate					
0.0	2.27 Aa	1.62 Ab	1.58 ABb	2.45 Aa	2.35 Aa	1.43 Ab
0.3	1.69 ABab	1.75 Aab	1.44 Bb	2.06 Aa	2.20 Aa	1.23 ABb
0.6	2.25 Aa	1.51 Ab	1.85 Aab	2.27 Aa	2.23 Aa	0.79 Bc
1.2	0.94 Cc	1.28 Abc	1.66 ABb	2.34 Aa	2.14 Aa	1.30 ABbc
2.4	1.60 Bbc	1.44 Ac	1.40 Bc	2.55 Aa	2.29 Aa	1.14 ABc
4.8	1.71 ABb	1.72 Ab	2.13 Aab	2.48 Aa	2.47 Aa	1.05 ABc

I-isomix; V-virginiamycin; A,B,C,D, a,b,c,dmeans with thr same letters in the same column (uppercase) or same row (lowercase), within each variable, do not differ from each other by the Tukey test at 5 % probability; 1,2,3,4,5Standard error for acetate = 3,698, propionate = 4,635, butyrate = 1,083, isovalerate = 0,889, and A:P=0,289.

In a study by Costa [36], virginiamycin increased the proportion of propionate and decreased those of acetate and butyrate, coinciding with the results of Wolin [50], indicating that it is the most efficient route of carbohydrate fermentation in the rumen. In the same study by Costa [36], it was shown that in 33.3 % of the studies, there was an increase in propionate and in 66.7 % of the studies, there was no change or reduction in propionate with the use of virginiamycin. According to Neto *et al.* [37], virginiamycin induces an increase in butyrate-producing bacteria, such as Butyrivibrio, which may explain the improvement in the efficiency of animals, since butyrate is the main source of energy for epithelial cells, and an increase in the production of this component can increase the absorption of nutrients. Moreira *et al.* [35] reported an increase in pH and a decrease in the VFA and acetate:propionate ratio, which may have occurred because of the ability to inhibit the production of organic acids associated with the use of amino acids and energy sources, such as isobutyric, valeric, and isovaleric acids. They also reported an increase in propionate levels for higher concentrations of virginiamycin, similar to our findings (Table 6).

4. Conclusion

Isomix and virginiamycin did not improve digestibility. However, Isomix enhanced propionate production and reduced the acetate:propionate ratio, potentially improving energy balance and performance. However, further research is recommended, especially on diets with low protein and urea supplementation levels in tropical grazing systems.

Conflicts of interest statement

The authors declared no conflict of interest.

Data availability statement

The data will be provided upon request.

Author contributions

Conceptualization: S. A. Salinas and R. Hancco. Data curation: R. Lana. Funding acquisition: R. Lana. Supervision: R. Lana. Investigation: S. A. Salinas and P. S. Dornelas. Visualization: S. A. Salinas and M. Soares. Writing (original draft): S. A. Salinas. Writing (proofreading and editing): R. Lana.

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