RAPHAELA CENCI VIDAL

ASSOCIATION OF VIRGINIAMYCIN AND MULTIPLE SUPPLEMENT FOR CATTLE FED A HIGH-QUALITY TROPICAL FORAGE

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Edenio Detmann

Co-adviser: Marcio de Souza Duarte

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Assent:

Raphaela Cenci Vidal
Author

Edenio Detmann
Adviser

I dedicate my master's degree to my grandmother Florestina Vidal, who during my experiment was called by her mother to rest in peace. Thank you for all your love and for being part of my childhood and my evolution as a human being and woman. You will always be an example of brave women. I'm sorry. I didn’t say goodbye to you.

You will be forever in my pray and heart.

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ABSTRACT

VIDAL, Raphaela Cenci, M.Sc., Universidade Federal de Viçosa, March, 2021. **Association of virginiamycin and multiple supplement for cattle fed a high-quality tropical forage.** Adviser: Edenio Detmann. Co-adviser: Márcio de Souza Duarte

The aim of this study was to evaluate the effect of adding virginiamycin to either mineral mixture or multiple supplement on intake, digestion, ruminal fermentation profile, rumen microbial production, blood metabolites, and liver metabolism of zebu heifers fed high-quality tropical forage. Eight heifers were used and assigned to a 4 × 4 Latin Square design. The treatments were: mineral mixture (MM), mineral mixture with virginiamycin (MM + V), multiple supplement (MSUP), and multiple supplement with virginiamycin (MSUP + V). The mineral mixture was provided daily at 120 g/ animal for the treatments MM and MM + V. The multiple supplement was formulated to provide 300 g of crude protein (CP)/ kg as fed and contained mineral mixture, corn grain, and urea: ammonium sulfate, and was daily provided at 200 g/animal. The amount of supplemental virginiamycin was based on a maximum theoretical response on animal performance (50 mg/100 kg body weight) and daily mixed to the supplements types. The treatments were compared according to a 2 × 2 factorial arrangement (mineral mixture and multiple supplement, with or without virginiamycin). All analyzes were performed using the MIXED procedure of SAS 9.4. Significant effects were declared at $P<0.10$. There was no interaction between virginiamycin and supplement type ($P\geq0.58$) or effects of virginiamycin ($P\geq0.66$) on voluntary intake. The multiple supplement decreased voluntary dry matter intake ($P<0.07$). The multiple supplement decreased digested organic matter (DOM) intake ($P<0.10$), but increased dietary CP:DOM ratio ($P<0.01$). There were no effects of virginiamycin ($P\geq0.44$) or interaction between virginiamycin and supplement type ($P\geq0.61$) on total digestibility or dietary DOM content. The multiple supplementation increased ($P<0.08$) the CP digestibility. The ruminal ammonia N concentration was only affected by supplement type ($P<0.04$). The molar proportions of individual volatile fatty acids were affected by the provision of multiple supplement ($P<0.07$), except for butyrate ($P>0.76$). There was an interaction between virginiamycin and supplement type on urine N excretion ($P<0.08$). The fecal-N excretion was decreased by multiple supplementation ($P<0.06$). Provision of virginiamycin decreased the blood hormone, IgF-1 ($P<0.07$), and the multiple supplementation increased blood urea-N ($P<0.01$). The gene expression for propionyl-CoA carboxylase was affected by virginiamycin (P<0.01). An interaction between supplement type and virginiamycin was observed on the pyruvate carboxylase (P<0.01), and for citrate synthase gene expression (P<0.02). However, using a low-intake multiple supplement with high CP content for cattle fed high-quality forage causes a substitutive effect on forage intake, keeps the nitrogen retention unchanged. The virginiamycin supplementation seems to cause some post-prandial influences, which may vary according to the type of supplement. Therefore, this statement deserves further studies in order to obtain a clearer understanding.

Keywords: Virginiamycin. Supplementation. N balance.

RESUMO

VIDAL, Raphaela Cenci, M.Sc., Universidade Federal de Viçosa, março de 2021. **Associação de virginiamicina e suplemento múltiplo para bovinos alimentados com forragem tropical de alta qualidade**. Orientador: Edenio Detmann. Coorientador: Márcio de Souza Duarte.

O objetivo deste estudo foi avaliar o efeito da adição de virginiamicina à mistura mineral ou suplemento múltiplo na ingestão, digestão, perfil de fermentação ruminal, produção microbiana no rúmen, metabólitos sanguíneos e metabolismo hepático em novilhas zebuínas alimentadas com forragem tropical de alta qualidade. Oito novilhas foram utilizadas e atribuídas a um delineamento de Quadrado Latino 4 × 4. Os tratamentos utilizados foram: mistura mineral (MM), mistura mineral com virginiamicina (MM + V), suplemento múltiplo (MSUP) e suplemento múltiplo com virginiamicina (MSUP + V). A mistura mineral foi fornecida diariamente a 120 g/ animal para os tratamentos MM e MM + V. O suplemento múltiplo foi planejado para apresentar 300 g de proteína bruta (PB) / kg em matéria natural e era composto por mistura mineral, grão de milho e ureia: sulfato de amônio, e foi fornecido diariamente na dose de 200 g/ animal. A quantidade de virginiamicina foi baseada no objetivo de uma resposta máxima no desempenho animal (50 mg/ 100 kg de peso corporal) e misturada diariamente aos tipos de suplementos. Os tratamentos foram comparados segundo arranjo fatorial 2 × 2 (mistura mineral e suplemento múltiplo, com ou sem virginiamicina). Todas as análises foram realizadas usando o procedimento MIXED do SAS 9.4. Efeitos significativos foram declarados em $P<0,10$. Não houve interação entre a virginiamicina e o tipo de suplemento ($P\geq0,58$) ou efeitos da virginiamicina ($P\geq0,66$) na ingestão voluntária de matéria seca. O suplemento múltiplo diminuiu o consumo voluntário de matéria seca ($P<0,07$). A ingestão de matéria orgânica digerida (MOD) foi suprimida pelo suplemento múltiplo ($P<0,10$), porém a ingestão deste suplemento aumentou a proporção de PB: MOD ($P<0,01$). Não houve efeitos da virginiamicina ($P\geq0,44$) ou interação entre a virginiamicina e o tipo de suplemento ($P\geq0,61$) na digestibilidade total ou nos componentes de MOD da dieta. A suplementação múltipla aumentou ($P<0,08$) a digestibilidade da PB. A concentração de NAR foi afetada apenas pelo tipo de suplemento ($P<0,04$). As proporções molares de AGV individuais foram afetadas pelo fornecimento de suplemento múltiplo ($P<0,07$). Houve interação entre a virginiamicina e o tipo de suplemento na excreção de N na urina ($P<0,08$). A excreção fecal de N foi reduzida pela suplementação múltipla (P<0,06). O fornecimento de virginiamicina diminuiu o hormônio, IgF-1 no sangue (P<0,07), e a suplementação múltipla aumentou o N ureico sanguíneo (P<0,01). A expressão gênica da propionil-CoA carboxilase foi afetada pela virginiamicina (P<0,01). Interação entre o tipo de suplemento e virginiamicina foi observada na expressão gênica da piruvato carboxilase (P<0,01) e da citrato sintase (P<0,02). Portanto, o uso de um suplemento múltiplo de baixo consumo com alto teor de PB para bovinos alimentados com forragem de alta qualidade causa um efeito substitutivo no consumo de forragem, mantendo inalterada a retenção de nitrogênio. A suplementação de virginiamicina parece causar algumas influências pós-prandiais, que podem variar de acordo com o tipo de suplemento. No entanto, essa afirmação merece mais estudos para obter uma compreensão mais clara.

Palavras-chave: Virginiamicina. Suplementação. Balanço de N.

SUMMARY

1. INTRODUCTION

In tropical regions, most beef cattle production takes place under grazing conditions. However, tropical pastures cannot be considered balanced diets for an optimized production (Detmann et al., 2014). This particular aspect may result in a lowered beef production caused by low weight gain and advance slaughter age. In order to improve production, technologies must be adopted, such as adequate pasture management, supplementation, and, or using feed additives.

The application of nutritional technologies to grazing animal systems must be supported by understanding and exploring the interaction between forage and supplements aiming at optimizing the economic and productive variables of the system (Detmann et al., 2008). In the tropics, during the rainy season, we have available forage that undergoes intense growth and it has better quality than the dry-season forage. However, despite this better quality improve the animals performance, the utilization of basal nutritional resources is not thought to be optimized (Detmann et al., 2014). In this context, there is a growth potential of approximately 200g/animal/d that is unused and can be achieved by using supplemental resources (Paulino et al., 2008). So, for animals grazing on medium- to high-quality forages, the supplementa must be based on the supply of rumen-degradable N to increase the ruminal ammonia nitrogen and it can be optimized by the association of supplements and additives that modified the rumen fermentation (Lemos et al., 2016).

Additives are substances without nutritional value, which are intentionally added to a diet aiming at improving either its nutritional characteristics or feeding efficiency. In terms of ruminant feeding, some additives can be used to change ruminal fermentation profile by altering the molar proportion of volatile fatty acids (VFA), mitigating methane production, and increasing the efficiency of microbial protein synthesis (Lemos et al., 2016). The main fermentation modifiers used in ruminant feeding are: ionophore antibiotics (e.g., monensin, lasalocid, salinomycin), non-ionophore antibiotics (e.g., virginiamycin, tylosin), essential oils, and probiotics.

In Brazil, monensin and virginiamycin are the most used additives for beef cattle production, including their utilization in supplements for grazing cattle. Both of them have antimicrobial action mainly on gram-positive bacteria, whose species are responsible for decreasing rumen pH and, consequently, causing metabolic disorders (Nagaraja et al., 1987).

By controlling gram-positive bacteria growth, we may change the equilibrium among ruminal microbial populations and benefit the proliferation of gram-negative bacteria.

Produced by *Streptomyces virginae*, virginiamycin is a cyclic depsipeptides antibiotic that has a strong synergistic bactericidal activity against a wide range of gram-positive bacteria. Its structural formula comprises two factors: factor M (responsible for 80% of the molecular weight) and factor S. The factor M is known for its action on *Streptococus spp*. and *Staphlycoccus spp*. However, its action is enhanced by the presence of factor S, which, alone, is not effective against most microorganisms. The factors M and S pass through cell membrane of the gram-positive bacteria and reach the cytoplasm, where they exert their effect. Both factors irreversibly link to the 50S subunit of bacterial ribosomes, preventing the formation of the peptide bounds. Thus, they act at tRNA level, avoiding the transcription of new proteins. Consequently, there would be either a decreased growth (bacteristatic effect) or death (bactericidal effect) of the bacterial cells (Cocito et al., 1974).

In the tropics, the supply of virginiamycin to Nellore cattle was able to increase the ruminal degradation of dry matter (DM), neutral detergent fiber (NDF), and starch compared to the use of monensin and the combination of monensin and virginiamycin. Simultaneously, virginiamycin caused a decrease in the rumen protozoa population, which may imply in a decreased predation activity (Squizatti et al., 2019a; 2019b). Moreover, studies on the inclusion of virginiamycin in grazing cattle diets indicated either an improvement in animal performance (Alves Neto et al., 2018; Costa et al., 2018) or a maintenance of performance but with improvement in feed efficiency (Maciel et al., 2019).

Since tropical forages cannot be considered as equilibrate diets when fed exclusively for cattle, multiple supplementation constitutes the main nutritional tool to improve diet quality and nutrient utilization (Detmann et al., 2014) In this sense, the additives for modulating ruminal fermentation should be associated with either multiple supplements or mineral mixtures in order to improve the utilization of supplementary nutrients for animals under grazing system during rainy season in tropical regions.

However, the literature is still scarce of studies that evaluate any possible metabolic alteration in animals fed mineral mixture or multiple supplement associated or not with virginiamycin. Such evidence could add to the current knowledge about the mechanisms of action of virginiamycin in animal production and efficiency.

Thus, considering the potential effects caused by virginiamycin supplementation, we hypothesize that its effects on rumen may affect the animal metabolism. In addition, this effect can be altered when an additional supply of nutrients is provided to the animal as a mineral mixture or a multiple supplement.

Therefore, the objective of this experiment was to evaluate the effect of adding virginiamycin to either mineral mixture or multiple supplement on intake, digestion, ruminal fermentation profile, microbial production in the rumen, blood metabolites, and liver metabolism in zebu heifers fed a high-quality tropical forage.

2. MATERIAL AND METHODS

The experiment was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Minas Gerais, Brazil. All surgical and animal care procedures were approved by the Institutional Animal Care and Use Committee of the Universidade Federal de Viçosa (CEUAP, protocol 57/2020).

2.1. Animals and management

Eight 3-years old Brahman heifers, averaging 520±24.6 kg of body weight (BW), were used. The animals were housed in individual stalls (2 × 5 m) equipped with concrete floors, individual feeders, and water drinkers, which assured unrestricted access to feed and water.

The animals were previously adapted to the experimental facilities, management, and feeding for 20 d before beginning of the experiment. The basal diet consisted of a high-quality Tifton 85 hay (*Cynodom sp*.) chopped at 10-cm particle size and fed twice daily at 0600 and 1800 h. To ensure ad libitum forage intake, the amount of hay was monitored daily in order to provide, at least, 300 g orts/kg of offered forage.

2.2. Experimental design and treatments

The experiment was performed according to a complete and duplicate 4 × 4 Latin square design, balanced for residual effects (Cochran and Cox, 1957). Animals were allocated to the different squares according to their BW. Each experimental period lasted 25 d, with 14 d for adaptation to the treatments (Machado et al., 2016) and 11 d for sample collections. Heifers were weighed at the beginning and the end of each experimental period in order to calculate the average BW and relative intake.

The four treatments were: mineral mixture (MM), mineral mixture with virginiamycin (MM+V), multiple supplement (MSUP), and multiple supplement with virginiamycin (MSUP+V). The mineral mixture contained 90 g phosphorus/kg as fed (Table 1) and was provided daily at 120 g/animal for the treatments MM and MM+V. This specific daily amount of mineral mixture was provided so that the animals could achieve an *ad libitum* intake. The multiple supplement was planned to present 300 g of crude protein (CP)/kg as fed and contained mineral mixture (600 g/kg as fed), corn grain (300 g/kg as fed), and urea: ammonium sulfate (9:1, 100 g/kg as fed). The multiple supplement was daily provided at 200 g/animal so the amount of offered mineral mixture was the same for all treatments. The amount of supplemental virginiamycin (VMAX 10®, Phibro Animal Health Corporation, Teaneck, New Jersey, USA) was based on the findings by Alves Neto et al. (2018) aiming at a maximum theoretical response on animal performance (50 mg/100 kg BW or 250 mg/d assuming an average BW of 500 kg). The virginiamycin was daily mixed to the mineral mixture or multiple supplement just before offering to the animals. The supplements (mineral and multiple) were offered at 0600 h in a separate feeder and their intake was monitored daily.

2.3. Sample collections

Voluntary intake and digestibility

Voluntary forage intake was quantified from d 15 to d 21 of each period. The calculations considered the amount of forage offered from d 15 to d 20, and the orts obtained from d 16 to d 21. Representative samples of hay and orts were collected daily, stored in plastic bags, and blended manually at the end of each period in order to obtain pooled samples per animal. Samples of the supplements were collected after each batching in the ration factory of the Universidade Federal de Viçosa. All samples were ground in a knife mill to pass through a 1-mm screen sieve.

Total fecal output was measured at d 16, d 18, and d 20 of each period. The feces were collected immediately after spontaneous defecation and stored in 35-L polyethylene containers. After 24 hours, the collected feces were weighted and manually homogenized. Then, a representative sample (50 g/kg) was collected and oven-dried (55ºC). The samples were ground as previously described and pooled samples proportional to each collection day were produced per animal.

Urine output

The urine was fully collected from d 21 to d 23 of each period using a 2-way Foley probe (n° 26, Rush Amber, Kamuting, Malaysia) which has a 30-mL balloon. A polyethylene tube was connected to the free end of the probe for conducting the urine to clean containers (20-L). To avoid N losses, the containers were kept into Styrofoam boxes with ice (Van Niekerk et al., 1963). At the end of each 24-h period, the urine was weighed and thoroughly mixed. Two aliquots (10 mL/L) were taken and filtered through four layers of cheesecloth. The first one was immediately analyzed for the total N concentration (method N-001/1;

Detmann et al., 2012). The second aliquot had its pH corrected down to 3.0 with the addition of concentrated sulfuric acid (H_2SO_4) and frozen (-20°C) for later analyzes.

Blood sampling

On d 24 of each period, blood samples were collected at 0600, 1200, 1800, and 2400 h using needles and 4-mL vacuum tubes (BD vacutainer, Bencton Dickson Franklin Lakes, New Jersey, USA) with sodium fluoride/EDTA (for glucose analyzes) and with clot activator (for the other analyzes). The samples were then centrifuged (1.200 × *g*, 15 min at 4ºC) for plasma separation. The blood serum was kept refrigerated (4ºC). At the end of the collections, a pooled sample was prepared per animal and frozen (-20°C) for later analyzes.

Rumen liquid sampling

On d 24 of each period, sampling of rumen liquid was performed at 1200 h using an oral probe. The liquid was filtrated through a triple layer of cheesecloth and two 40-mL aliquots were taken. The first was fixed with 1 mL of a H_2SO_4 solution (500 mL/L) and frozen (-20ºC) for ruminal ammonia N (RAN) analyzes. The second aliquot was fixed with 0.4 mL of H_2SO_4 solution (500 mL/L) and kept at -20°C for subsequent VFA analyzes. This procedure was performed with only four animals from one of the Latin squares.

Liver biopsy

On the last day of each period (d 25), hepatic biopsy was performed in the animals from one Latin square (opposite to the one used for rumen liquid sampling). The sampling procedure took place in the intercostal space between the 11th and 12th ribs on the right side of the animals. A 5 × 5-cm area was trichotomized, cleaned with ethanol (700 mL/L), and anesthetized with lidocaine (20 g/L). After this procedure, a 1.0-cm incision was made with a scalpel, and through it the tru-cut needle was inserted to drain the liver sample (Herdt, 2013). The liver samples were washed with sterile saline solution (NaCl, 9 g/L) and immediately frozen in liquid nitrogen. After that, the samples were kept in an ultra-freezer (-80°C) for subsequent analyzes of gene expression.

2.4. Laboratory analyzes

All feed, ort, and fecal samples were analyzed for dry matter (DM; dried overnight at 105ºC; method G003/1), ash (complete combustion in a muffle furnace at 550°C; method M-

001/1), and N (Kjeldahl procedure; method N-001/1) contents according to the standard analytical procedures of the Instituto Nacional de Ciência e Tecnologia de Ciência Animal (INCT-CA; Detmann et al., 2012). The neutral detergent fiber (NDF) contents were evaluated using a heat-stable α-amylase and omitting sodium sulfite according to Mertens (2002). The NDF contents were expressed exclusive of residual ash and protein (NDFap).

The urinary concentrations of urea (colorimetric kinetic test, Bioclin® K056), creatinine (enzymatic-colorimetric method, Bioclin® K067), and uric acid (enzymatic-colorimetric method, Bioclin® K139) were quantified with an automated chemistry analyzer (BS200E, Mindray, China). A colorimetric method was used to measure the concentration of urinary allantoin (Chen and Gomes, 1992). The microbial production in the rumen was estimated using the equations proposed by Barbosa et al. (2011).

Blood plasma samples were analyzed for glucose (enzymatic glucose oxidase-peroxidase method, Bioclin® K082), urea (enzymatic-colorimetric method, Bioclin® K056), total protein (colorimetric kinetic test, Bioclin® K031), albumin (bromecresol green method, Bioclin® K040), aspartate transaminase (AST, U.V. kinetic – IFCC, Bioclin® K048), alanine transaminase (ALT, U.V. kinetic – IFCC, Bioclin® K049), and gamma-glutamyl transferase (GGT, SZASZ IFCC method, Bioclin® K080). The blood concentration of insulin-like growth factor (IgF-1) was analyzed by chemiluminescence in a commercial laboratory (ViçosaLab, Viçosa, Minas Gerais, Brazil). The blood globulins were estimated as the difference between blood total protein and albumin.

The concentration of RAN was quantified using the colorimetric technique described by Detmann et al. (2012, method N-006/1). For VFA analyzes, rumen fluid samples were centrifuged (12,000 × g, 10 min, 4ºC) and supernatants were treated as described by Siegfried et al. (1984). Ruminal VFA were analyzed by HPLC (Shimadzu HPLC class VP series, model SPD 10A; Shimadzu Corporation, Kyoto, Japan) using a reverse-phase column (mobile phase 0.15 Morpho-phosphoric acid) and UV detector at a wavelength of 210 nm.

The frozen samples of liver were powdered in liquid N and total RNA was extracted from 0.07 g of tissue using Trizol® (Invitrogen TM, Thermo Fischer Scientific®, Oregon USA) following the manufacturer’s recommentadions. Subsequently, washing and RNA isolation were performed using RNase free silica membrane columns (PureLink TM RNA Mini Kit – Invitrogen TM, Fischer Scientific®, Oregon USA). After extraction, the RNA was quantified by a NanoVue spectrophotometer (GE Healthcare Life Sciences Inc.) and its integrity was verified using an agarose gel (1%). The RNA samples were then reverse transcribed into cDNA using the GoScriptTM Reverse Transcription System Kit (Promega Corporation, Madison, Wisconsin, USA). The primers (Table 2) for amplification of target and endogenous genes were designed using PrimerQuest software (www.idtdna.com/Scitools/Applications/PrimerQuest) with sequences obtained using GenBank (www.ncbi.nlm.nih.gov). Real-time quantitative PCR was performed in thermal cycler ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the detection method SYBR Green (Applied Biosystems – Foster City, CA, USA) with GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA) using the following cycle parameters. The mRNA expression was determined by $2^{-\Delta Ct}$ method as described by Livak and Schmittgen (2001).

2.5. Statistical analyzes

The experiment was carried out according to a duplicated 4 × 4 Latin Square design. The response variables measured in both squares were analyzed according to the model:

$$Y_{ijkl} = \mu + Q_i + T_i + A_{(i)k} + P_{(i)l} + QT_{ij} + \varepsilon_{ijkl} \qquad (1),$$

where Y_{ijkl} is the response measured on the animal k and period l within square i, and submitted to the treatment j; μ is the general constant; Q_i is the effect of the square i (random); T_j is the effect of treatment j (fixed); $A_{(i)k}$ is the effect of the animal k nested to the square i (random); $P_{(i)l}$ is the effect of experimental period l nested to the square i (random); QT_{ij} is the interaction effect between square i and treatment j (random); and ε_{ijkl} is the random error, which is supposed to be NID (μ, σ2).

We performed a first analyzis of variance in order to evaluate the interaction between Latin squares and treatments. We anticipate that we did not find any significant interaction ($P>0.10$). Then, the interaction was removed from the model as it did not interfere on casualization and its removal increases the residual degrees of freedom. For the response variables measured in only one square, the model (1) was re-parameterized by excluding Latin square effect and its interaction with treatments.

The treatments were compared according to a 2 × 2 factorial arrangement (mineral mixture and multiple supplement, with or without virginiamycin). All analyzes were performed using the MIXED procedure of SAS 9.4. Significant effects were declared at $P<0.10$.

3. RESULTS

Intake and digestibility

On average, the mineral mixture intake was 86 and 91 g/d without and with virginiamycin inclusion, respectively. Moreover, the multiple supplement intake averaged 173 and 168 g/d without and with virginiamycin inclusion, respectively (Table 3). We planned that the animals should consume 250 mg/d of virginiamycin. However, as the supplement intake was lower than amount offered, the estimated virginiamycin intake was 186 and 207 mg/d for mineral mixture and multiple supplement, respectively.

There was no interaction between virginiamycin and supplement type ($P \geq 0.58$) nor effects of virginiamycin ($P \geq 0.66$) on voluntary intake characteristics (Table 3). However, using the multiple supplement decreased voluntary DM intake in both kg/d ($P < 0.07$) and g/kg BW ($P < 0.05$). This pattern was a direct reflex of a decrease in forage intake ($P < 0.05$), despite of supplement intake has been slightly higher when the multiple supplement was provided to the animals. Due to the decreased forage intake, the multiple supplementation did not affect the CP intake ($P = 0.60$). Thus, from those effects, providing multiple supplement decreased digested organic matter (DOM) intake ($P < 0.10$), but increased dietary CP:DOM ratio ($P < 0.01$).

There were no effects of virginiamycin ($P \geq 0.44$) or interaction between virginiamycin and supplement type ($P \geq 0.61$) on total digestibility or dietary DOM content (Table 4). The multiple supplementation increased ($P < 0.08$) the CP digestibility, but did not affect the other digestion characteristics ($P \geq 0.34$). The average dietary DOM across treatments ($P \geq 0.44$) was 562 g/kg DM.

Ruminal fermentation profile

There were no treatment effects ($P \geq 0.46$) on rumen pH and VFA concentrations (Table 5). The RAN concentration was only affect by supplement type ($P<0.04$). On average, when compared to mineral mixture, multiple supplement increased RAN from 6.16 to 8.16 mg/dL. The molar proportions of individual VFA were only affected by providing multiple supplement, which increased the proportion of acetate ($P<0.07$) and acetate-to-propionate ratio ($P<0.06$), but decreased the molar proportion of propionate ($P<0.06$). No effects of treatments were verified on molar proportion of butyrate ($P \geq 0.46$).

Nitrogen balance and microbial production

The N intake followed the same pattern described for CP intake (Table 6). However, there was an interaction between virginiamycin and supplement type on urine N excretion ($P<0.08$). The slicing of this effect revealed that virginiamycin did not affect urine N when animals received mineral mixture ($P>0.64$), but increased urinary N excretion when multiple supplementation was used ($P<0.05$). On the other hand, the fecal-N excretion was decreased on average by multiple supplementation ($P<0.06$). Despite of these patterns, there were no treatment effects on N retention ($P \geq 0.48$) or efficiency of N utilization ($P \geq 0.27$). The same was verified for microbial N production and efficiency ($P \geq 0.24$), whose average values were 99 g N/d, 0.75 g N/g N intake, and 137 g microbial CP/kg DOM.

Blood characteristics and liver function

Providing virginiamycin decreased the blood IgF-1 ($P<0.07$), whereas multiple supplementation increased blood urea-N ($P<0.01$). The other blood characteristics and liver function indicators shown at Table 7 were not altered by any effect ($P \geq 0.11$).

Liver biopsy

Hepatic gene expressions for phosphoenolpyruvate carboxykinase ($P \geq 0.23$) and carbamoyl phosphate synthetase ($P \geq 0.77$) were not affected by treatments (Table 8). The gene expression for propionyl-CoA carboxylase was not affected by either multiple supplementation ($P>0.23$) or interaction between multiple supplementation and virginiamycin ($P>0.67$). However, it was increased by virginiamycin ($P<0.01$). Interaction between supplement type and virginiamycin was observed ($P<0.01$). The slicing of this effect indicated that virginiamycin increased the gene expression when mineral mixture was provided

(P<0.01), but decreased it when animals were fed multiple supplement (P<0.04). The interaction effect was also observed for citrate synthase gene expression (P<0.02). The slicing of this effect showed the same pattern observed for pyruvate carboxylase.

4. DISCUSSION

Generally, virginiamycin did not cause any effect on voluntary intake and digestibility. This pattern agrees with several experiments carried out in the tropics (Monção, 2017; Alves Neto et al., 2018; Costa et al., 2018). On the other hand, some authors stated that virginiamycin could decrease the voluntary intake of supplements (Maciel et al., 2019), which was not verified in our study, as the multiple supplement intake was approximately the same with or without virginiamycin inclusion. According to Monção (2017), the voluntary intake should not be affected by the virginiamycin because the diet quality would not be altered by its inclusion.

However, the forage intake was depressed by the multiple supplementation with an average substitutive effect of 10 g of forage per g of supplement (Table 3). Such a negative effect reflected a lack of compensation on total DM intake, which was also decreased when compared to the animals fed mineral mixture. Overall, the control of voluntary intake in ruminants cannot be attributed to a single factor as it has a multifactorial influence (Forbes, 2003). In the tropics, the protein-to-energy ratio (CP:DOM) in the total diet has been pointed out as valuable indicator of the animal comfort/discomfort towards the voluntary intake behavior. This ratio allows integrating the supplemental effects on both rumen and body metabolism (Poppi and McLennan, 1995; Detmann et al., 2014). In this aspect, the intake of tropical forages would be maximized when CP:DOM of the diet is adjusted close to 210 g/kg through supplementation (Reis et al., 2016). In our experiment, the average CP:DOM with mineral mixture supplementation was 176 g/kg and was increased by the multiple supplement, on average, up to 187 g/kg. Despite of being lower than the optimal ratio (Reis et el., 2016), the multiple supplementation did not change CP:DOM towards a more comfortable ratio, and a decrease in voluntary intake should not have occurred. An excessive ruminal ammonia caused by protein supplements also can impair forage intake (Reis et al., 2020) due to its possible neurotoxicity (Kertz et al., 1982) or impairment in liver metabolism (Allen et al., 2009). However, the increase in RAN caused by the multiple supplement was only marginal and any constraint to intake caused by ammonia is unlikely. It is known that substitutive effect caused by supplementation increases as the forage quality increases (Minson, 1990). It seems to be the case we observed, even though a better scientific explanation for the causes of substitution could not be obtained from our results.

The only effect detected on digestibility was the increased CP digestion when the multiple supplement was provided to the animals. Considering the substitution of forage by supplements, part of the protein from forage (mainly fiber-bounded protein) was replaced by urea, which increased, on average, the urinary N excretion. Therefore, a lower amount of N from forage escaped to the hindgut (indicated by the lowered fecal N) and, then, the apparent digestion of CP increased.

Despite of its expected action on gram-positive bacteria (Cocito et al., 1974), we did not observe any apparent impact of virginiamycin on rumen fermentation profile or microbial production. According to Salinas-Chavira et al. (2009), virginiamycin tends to improve feed efficiency in cattle, but its effects on ruminal fermentation and digestion are small and cannot be responsible for improvements in performance. Several researches also have demonstrated either absence of impacts or only marginal effects of virginiamycin on rumen fermentation characteristics (Nagaraja et al., 1987; Moção, 2017; Alves Neto et al., 2018; Costa et al., 2018). Particularly, Costa et al. (2018) found an increment in microbial N production in the rumen with virginiamycin supplementation, which was probably caused by a decreased predation activity. However, we did not find a similar pattern in our study.

Nevertheless, ruminal fermentation characteristics were affected by multiple supplementation. The RAN concentration was increased by the multiple supplement, on average, from 6.2 to 8.2 mg/dL, making the rumen ammonia availability more adequate for action of fibrolytic microorganisms (Detmann et al., 2009). Despite of virginiamycin has no effect on total VFA concentration, multiple supplementation increased the molar proportion of acetate and decreased molar proportion of propionate. The positive effects of supplemental protein on molar proportion of acetate were also described by Figueiras et al. (2016).

Nonetheless, multiple supplementation did not affect the microbial N production in the rumen. However, this pattern should not be seen as a complete absence of effects of supplemental N on rumen microorganisms. The protein supplementation is able to increase the contribution of fibrolytic species in the total bacterial population in the rumen, even though a concomitant improvement in fiber digestion was not observed (Silva-Marques et al., 2019). The change in the fermentation profile from propionate to acetate seems to indicate an increased growth of fibrolytic bacteria, which was caused by supplemental N. However, microbial growth in the rumen and microbial flow could not necessarily be related to each other (Reis et al., 2020) and an increased growth of particular species cannot reflect an increased microbial flow to intestines (Belanche et al., 2012; Fanchone et al., 2013). On the other hand, an increased fiber degradation in the rumen could not be detected by total digestion measurements, as it is not able to account for the influence of hindgut fermentation. An increased escape of potentially degradable NDF from rumen increases the amount of potentially degradable fiber in the hindgut (Oliveira et al., 2020). Therefore, we may affirm that multiple supplement did affect bacterial growth in the rumen. However, due to integration with other nutritional events, it was not able to improve total microbial flow to intestines and the amount of fiber digested in the gastrointestinal tract.

At first glance, the treatments here evaluated would have no potential action on animal performance, as no effect was detected on body N accretion. However, that result must be evaluated in an integrated way by considering simultaneously other evidences obtained in our work. First, the multiple supplementation decreased voluntary intake, but did not affect N accretion. It constitutes a clear evidence that low-intake protein supplements can improve feed efficiency of cattle fed high-quality forages. Our basic reasoning relies on the fact that total energy intake decreased (which is here represented by DOM intake), but efficiency of N utilization remained unchanged.

Second, virginiamycin supplementation decreased blood IgF-1, but also did not affect N accretion. The IgF-1 is an endocrine regulator of muscle growth and it is also an important link between growth hormone and the metabolic processes of growth (Drewnoski et al., 2014; Barclay et al., 2019). Blood IgF-1 has been positively correlated with N accretion in cattle fed tropical forages (Reis et al., 2020; Rufino et al., 2020; Franco et al., 2021). In this sense, the decreased IgF-1 caused by virginiamycin should have implied a decreased N accretion, which did not happen. There are two connected implications here. First, the virginiamycin improved metabolic efficiency of the animals, which corroborate the results obtained by Salinas-Chavira et al. (2009). Second, virginiamycin could change the metabolic regulation in the animals and prioritizes other ways to keep anabolism unaltered. These effects could not be caused directly by supplemental virginiamycin. Rather, they could be attributed to any change in digestion process that signals those possible changes in animal metabolism.

We may support our hypothesis from the results obtained for gene expression of key enzymes for hepatic energy metabolism. Virginiamycin increased gene expression for propionyl-CoA-carboxylase, which is a key enzyme to insert propionate into the Krebs cycle (Nelson and Cox, 2014) for either oxidation or gluconeogenesis. This is particularly intriguig, as virginiamycin had no effect on VFA concentration and molar proportion. However, we measured only concentration of VFA, which represents a static picture of rumen fermentation and not necessarily is associated with VFA production. The amount of VFA absorbed is influenced by substrate type, fermentation rate and routes, absorption through rumen wall, and outflow to abomasum. Thus, this dynamic process cannot be accurately described by a single and statical measurement of concentrations in rumen fluid. Therefore, the pattern of propionyl-CoA-carboxylase caused by virginiamycin seems to indicate a significant impact on rumen fermentation and, consequently, an alteration of metabolite profile for intermediate metabolism and synthesis processes in the animal body. This could have, at least partially, some influence on the improvement of the animal efficiency caused by virginiamycin supplementation.

On the other hand, the virginiamycin effects on pyruvate carboxylase, which interacted with the type of supplement, seems to indicate another post-prandial influence of this additive. Despite of being non-significant ($P>0.23$), the gene expression of phospoenolpyruvate caboxykinase followed the same interaction pattern (Table 8). Both enzymes are key for the gluconeogenesis (Nelson and Cox, 2014) and their gene-expression pattern may indicate some indirect influence of virginamycin (perhaps from ruminal fermentation as well) on glucose production in ruminant. Interestingly, the same influence was observed on gene expression for citrate synthase, which indicates some influence in the hepatic oxidation of acetate. However, the actual meaning of the interaction between virginiamycin and supplement type could not be understood from our results and further studies are suggested in order to obtain a clearer understanding of this post-prandial virginiamycin influence.

5. CONCLUSIONS

Using a low-intake multiple supplement with a high CP content for cattle fed high-quality forage causes a substitutive effect on forage intake, but keeps nitrogen accretion unchanged. That pattern indicates an improvement in feed efficiency. On the other hand, virginiamycin supplementation seems to cause some post-prandial influences, which may vary according to the type of supplement. Those influences apparently improve animal efficiency. However, this statement deserves further studies in order to obtain a clearer understanding.

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7. RESULTS TABLES

Table 1. Chemical composition of forage and supplements

Item[a]	Forage	MM[b]	MSUP[c]
DM, g/kg as fed	898±3.6	979	932
OM, g/kg DM	951±0.9	31.2	394
CP, g/kg DM	100±3.7	-	305
NDFap, g/kg DM	670±11.1	-	32.8

[a] DM, dry matter; OM, organic matter; CP, crude protein; NDFap, neutral detergent fiber assayed with a heat-stable alpha amylase and expressed exclusive of residual ash and protein.

[b] Mineral mixture: Ca (183,0 g/kg) ;Co (79,0 g/kg); Cu (1.328,0 mg/kg); S (22,0 g/kg); Fe (1.726,0 mg/kg); Fl (734,0 mg/kg); P (90,0 g/kg); I (79,0 mg/kg); Ma (17,50 g/kg); Mg (2.646,0 mg/kg); Se (22,0 mg/kg); Na (80,70 g/kg); Zn (4.427,0 mg/kg).

[c] Multiple supplement [mineral mixture, 600 g/kg as fed; corn grain, 300 g/kg as fed; urea: ammonium sulfate (9:1), 100 g/kg as fed].

Table 2. List of primers for mRNA relative abundance analyzes by RT-qPCR

Gene	Gene abbreviation	NCBI access code	Primer
Pyruvate-carboxylase	*PC*	NM_177946.4	F: CGTCTTTGCCCACTTCAA R: CTCAAACTCCTCTGCAATC
Citrate synthase	*CS*	NM_001044721.1	F: GGGAATCTGGCTCTTCTTTC R: AGGGTGGACAGAGTAGAAC
Propionyl- CoA- carboxylase	*PCCA*	NM_001083509.1	F: CTCGTGGAACTGGAATGAAG R: GCCGAACGCCTGAATAAA
Phosphoenolpyruvate carboxykinase	*PCK2*	NM_001205594.1	F: CTGGAAAGTGGAGTGTGTG R: GCCATTCTCAGGGTTGATG
Carbamoyl- phosphate synthetase	*CPS1*	NM_001192258.1	F: CAGTGACAGGTTGGAAAGAG R: GAACACCCATGGCATCAA
18 S ribosomal RNA	*18S*	NM_001033614	F: CCTGCGGCTTAATTTGACTC R: AACTAAGAACGGCCATGCAC

Table 3. Effects of feeding multiple supplement and, or virginiamycin on voluntary intake of Brahman heifers fed a high-quality tropical forage

	Treatments[a]					P-value[c]		
Item[b]	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V × S
	kg/d							
DM	8.61	8.44	7.81	7.88	0.559	0.87	0.066	0.73
Forage	8.53	8.35	7.65	7.72	0.558	0.87	0.046	0.72
Supplement	0.086	0.091	0.173	0.168	-	-	-	-
SE (g/g)[d]	-	-	-10.1	-9.9	-	-	-	-
V (mg/d)[e]	-	186	-	207	-	-	-	-
OM	8.11	7.94	7.34	7.40	0.528	0.87	0.067	0.72
CP	0.867	0.834	0.829	0.833	0.070	0.68	0.60	0.61
NDFap	5.68	5.53	5.09	5.16	0.408	0.85	0.057	0.64
DOM	4.95	4.72	4.40	4.43	0.359	0.69	0.094	0.58
DNDFap	3.35	3.18	2.95	2.96	0.298	0.66	0.085	0.60

CP:DOM	176	176	188	186	6.0	0.82	0.002	0.77
	g/kg BW							
DM	16.7	16.4	15.0	15.2	1.02	0.99	0.044	0.69
Forage	16.5	16.2	14.6	14.9	1.02	0.99	0.031	0.69
OM	15.7	15.5	14.0	14.3	0.967	0.98	0.042	0.69
NDFap	11.0	10.7	9.70	9.92	0.730	0.95	0.044	0.62

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] DM, dry matter; SE, substitutive effect on forage intake compared to MM treatment; OM, organic matter; CP, crude protein; NDFap, neutral detergent fiber assayed with a heat-stable alpha amylase and expressed exclusive of residual ash and protein; DOM, digested OM; DNDFap, digested NDFap; CP:DOM, dietary ratio of CP to DOM (g/kg).

[c] V, S, and V × S, effects of virginamycin, supplement type, and their interaction, respectively.

[d] Substitutive effect (SE = [Forage intake – Forage intake(MM)]/[Supplement intake – Supplement intake(MM)].

[e] Estimated intake of virginiamycin.

Table 4. Effects of feeding multiple supplement and, or virginiamycin on total digestibility and total dietary content of digested organic matter in Brahman heifers fed a high-quality tropical forage

	Treatments[a]					P-value[c]		
Item[b]	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V × S
	g/g							
OM	0.606	0.597	0.597	0.591	0.011	0.44	0.46	0.85
CP	0.621	0.623	0.646	0.637	0.024	0.71	0.077	0.61
NDFap	0.581	0.578	0.575	0.561	0.018	0.48	0.34	0.65
DOM	571	561	562	555	11.1	0.44	0.46	0.86

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] OM, organic matter; CP, crude protein; NDFap, neutral detergent fiber assayed with a heat-stable alpha amylase and expressed exclusive of residual ash and protein; DOM, digested OM.

[c] V, S, and V × S, effects of virginiamycin, supplement type, and their interaction, respectively.

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Table 5. Effects of feeding multiple supplement and, or virginiamycin on ruminal fermentation characteristics of Brahman heifers fed a high-quality tropical forage

	Treatments[a]					P-value[c]		
Item[b]	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V×S
pH	7.18	7.36	7.11	7.22	0.201	0.46	0.58	0.87
RAN (mg/dL)	6.61	5.70	7.94	8.39	0.932	0.76	0.036	0.40
VFA (mM/dL)	26.7	25.6	27.7	25.8	3.12	0.56	0.82	0.86
VFA, mol/100 mol								
Acetate	66.9	66.8	68.1	69.6	1.13	0.47	0.065	0.41
Butyrate	11.3	10.5	10.8	10.6	0.76	0.46	0.76	0.62
Propionate	21.9	22.7	21.1	19.8	0.76	0.76	0.052	0.20
A:P	3.08	2.96	3.25	3.53	0.159	0.61	0.057	0.25

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] RAN, ruminal ammoniacal N; VFA, volatile fatty acids; A:P, acetate to propionate ratio.

[c] V, S, and V × S, effects of virginiamycin, supplement type, and their interaction, respectively.

Table 6. Effects of feeding multiple supplement and, or virginiamycin on N utilization characteristics of heifers fed a high-quality tropical forage

	Treatments[a]					P-value[b]		
Item	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V×S
N intake (g/d)	139	133	133	133	11.3	0.68	0.60	0.61
N excretion								
Fecal N (g/d)	51.4	49.9	46.2	47.8	2.97	0.98	0.059	0.40
Urine N (g/d)	59.1	57.9	60.4	66.1	3.22	0.23	0.020	0.079
N retention (g/d)	28.4	25.7	26.1	22.0	8.92	0.48	0.54	0.88
N efficiency (g/g)	0.179	0.187	0.175	0.132	0.055	0.49	0.27	0.35
Microbial production								
g N/d	102	102	88	105	9.49	0.33	0.52	0.35
g N/g N intake	0.741	0.783	0.692	0.794	0.08	0.24	0.75	0.62
g CP/kg DOM	132	137	129	148	14.5	0.29	0.73	0.53

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] V, S, and V × S, effects of virginiamycin, supplement type, and their interaction, respectively.

Table 7. Effects of feeding multiple supplement and, or virginiamycin on blood characteristics of heifers fed a high-quality tropical forage

	Treatments[a]					P-value[c]		
Item[b]	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V×S
Glucose (mg/dL)	59.9	58.5	59.0	59.2	1.06	0.63	0.99	0.49
IgF-1 (mg/dL)	196	183	205	182	27.6	0.065	0.66	0.59
Urea N (mg/dL)	13.7	13.9	15.6	14.8	0.75	0.61	0.008	0.32
AST (U/L)	71.2	86.4	75.4	71.9	9.56	0.42	0.48	0.20
ALT (U/L)	23.9	24.2	24.7	23.0	1.09	0.25	0.78	0.11
GGT (U/L)	16.5	14.3	16.9	15.	1.67	0.14	0.49	0.67
Total protein (g/dL)	7.11	7.11	7.17	7.16	0.138	0.91	0.38	0.92
Albumin (g/L)	3.03	3.02	3.07	3.01	0.055	0.23	0.81	0.32
Globulin (g/L)	4.07	4.07	4.10	4.15	0.136	0.65	0.36	0.71

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyltransferase.

[c] V, S, and V × S, effects of virginiamycin, supplement type, and their interaction, respectively.

Table 8. Effects of feeding multiple supplement and, or virginiamycin on the hepatic gene expression of some key enzymes of energy metabolism and urea cycle of heifers fed a high-quality tropical forage

	Treatments[a]					P-value[b]		
Item	MM	MM+V	MSUP	MSUP+V	SEM	V	S	V×S
Pyruvate - carboxylase (×10^{-3})	5.69	9.31	2.97	0.27	1.069	0.50	0.002	0.004
Citrate synthase (×10^{-2})	1.71	3.14	2.46	0.67	0.666	0.72	0.12	0.019
Propionyl - CoA-carboxylase (×10^{-2})	0.72	1.70	0.58	1.42	0.383	0.001	0.23	0.67
Phosphoenolpyruvate carboxykinase (×10^{-3})	2.71	5.21	4.85	0.26	0.297	0.71	0.61	0.23
Carbamoyl phosphate synthase (×10^{-2})	2.79	3.01	2.58	3.26	1.343	0.77	0.98	0.88

[a] MM, mineral mixture; MSUP, multiple supplement, V, virginiamycin.

[b] V, S, and V × S, effects of virginiamycin, supplement type, and their interaction, respectively.