UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

PERFORMANCE, CARCASS TRAITS AND RUMEN FERMENTATION FROM FEEDLOT NELLORE CATTLE FED CRUDE GLYCERIN AND VIRGINIAMYCIN

Pablo de Souza Castagnino

Zootecnista

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Orientador: Profa. Dra. Telma Teresinha Berchielli

Coorientador: Dr. Giovani Fiorentini

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TÍTULO DA TESE: PERFORMANCE, CARCASS TRAITS AND RUMEN FERMENTATION FROM FEEDLOT NELLORE CATTLE FED CRUDE GLYCERIN AND VIRGINIAMYCIN

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DADOS CURRICULARES DO AUTOR

PABLO DE SOUZA CASTAGNINO, filho de Angelo Carlo Bragança Castagnino e Círia de Souza Castagnino, nasceu em 02 de Janeiro de 1988 em Roca Sales - RS. Em fevereiro de 2012, graduou-se no curso de Zootecnia pela Universidade Federal de Santa Maria. Em março de 2012, ingressou no mestrado em Zootecnia pela Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Câmpus de Jaboticabal (FCAV–UNESP/Jaboticabal) sob orientação da Profa. Dra. Telma Teresinha Berchielli. De Outubro a Dezembro de 2013 realizou estágio no Rowett Institute of Nutrition and Health, Aberdeen, Scotland sob orientação do Dr. Robert John Wallace. Em março de 2014 ingressou no curso de Doutorado em Zootecnia na Faculdade de Ciências Agrárias e Veterinária da Universidade Estadual Paulista “Júlio de Mesquita Filho”, campus de Jaboticabal, sob orientação da Profa. Dra. Telma Teresinha Berchielli. De novembro de 2016 a Agosto de 2017 realizou Doutorado – Sanduíche no “Department of Animal Sciences/The Ohio State University, Columbus, USA”, sob orientação do Prof. Dr. Jeffrey L. Firkins.

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo nº 021119/11 do trabalho de pesquisa intitulado **"Balanço de gases de efeito estufa e estratégias de mitigação em pastos de Brachiaria submetidos a diferentes manejos"**, sob a responsabilidade da Profª. Drª. Telma Teresinha Berchielli está de acordo com os Princípios Éticos na Experimentação Animal, adotado pelo Colégio Brasileiro de Experimentação (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 07 de Outubro de 2011.

Jaboticabal, 11 de Outubro de 2011.

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DESEMPENHO, CARACTERISTICAS DE CARCAÇA E FERMENTAÇÃO RUMINAL DE BOVINOS NELORE ALIMENTADOS COM GLICERINA BRUTA E VIRGINIAMICINA

RESUMO – O glicerol é um substrato utilizado por bactérias que metabolizam o lactato ruminal e a virginiamicina é um antibiótico não ionóforo que inibe o crescimento de bactérias gram-positivas produtoras de lactato. Foram realizados dois experimentos para avaliar os efeitos da glicerina bruta (GB) e da virginiamicina (VM) na ingestão, digestibilidade, fermentação ruminal, população microbiana, desempenho, características de carcaça e perfil de ácidos graxos da carne de bovinos Nelore. Os tratamentos experimentais foram organizados em um arranjo fatorial 2 × 2: dietas sem virginiamicina (VM-) ou 25 mg de virginiamicina/kg de matéria seca (VM+) combinadas com dietas sem glicerina bruta (GB-) ou 100 g de glicerina bruta/kg de matéria seca (GB+). O bagaço de cana-de-açúcar foi usado como forragem na proporção de 20% na matéria seca (MS) da dieta e a GB substituiu o milho na formulação da dieta. No primeiro experimento, foram utilizados oito bovinos Nelore fistulados no rúmen (Peso corporal = 600 ± 34 kg, 26 ± 3 meses) em um quadrado latino 4×4 replicado (período= 21 dias) para se avaliar a digestibilidade dos nutrientes, fermentação ruminal e população microbiana. A ingestão de MS teve uma tendência a aumentar em dietas com GB (P = 0,07). As digestibilidades aparentes totais dos nutrientes foram semelhantes entre as dietas (P ≥ 0,10). As dietas com GB ou VM apresentaram valores de pH similares (média = 6,15; P ≥ 0,10). A proporção de propionato aumentou 27,5% nas dietas com GB+, independentemente da inclusão VM (P = 0,01). No segundo experimento, foram utilizados quarenta e oito bovinos com peso corporal inicial (408,4 ± 22,2 kg, 21 ± 2 meses) em um delineamento de blocos completos ao acaso para avaliação das características de carcaça e do desempenho animal. A ingestão de MS, o peso da carcaça fria e o rendimento de carcaça fria foi superior nas dietas com GB (P < 0,05). VM teve uma tendência para aumentar ganho médio diário e a eficiência alimentar (P < 0,1). A concentração total de ácidos graxos insaturados (AGI) no músculo longissimus aumentou em 6,08% nas dietas dietas com GB (P < 0,05), porém a relação n-6/n-3 permaneceu constante entre todos os tratamentos (P > 0,10). A GB teve efeitos positivos sobre os produtos de fermentação e pode substituir a virginiamicina com aumento na abundância de *Megasphaera elsdenii* e da deposição de AGI na carne. No entanto, a administração simultânea de VM e GB não interfere positivamente nos produtos de fermentação ruminal, desempenho e características de carcaça de bovinos Nelore em confinamento.
Palavras-chave: glicerina bruta, microbiologia, qualidade da carne, virginiamicina

PERFORMANCE, CARCASS TRAITS AND RUMEN FERMENTATION FROM FEEDLOT NELLORE CATTLE FED CRUDE GLYCERIN AND VIRGINIAMYCIN

ABSTRACT – Glycerol is a substrate used for bacteria that metabolize ruminal lactate and virginiamicyn is a non-ionophore antibiotic that inhibits the growth of gram-positive lactate-producing bacteria. Two experiments were conducted to evaluate the effects of crude glycerin (CG) combined with virginiamycin (VM) on intake, digestibility, ruminal fermentation, microbial population, performance, carcass traits and fatty acid profile of meat from feedlot Nellore cattle. Treatments were arranged in 2 × 2 factorial design: diets without virginiamycin (VM-) or virginiamycin at 25 mg/kg DM (VM+) combined with diets without crude glycerin (CG-) or CG (80% glycerol) at 100 g/kg DM (CG+). The sugar cane bagasse was used as the exclusive roughage in the proportion of 20% in the dry matter (DM) of diet and crude glycerin replaced corn in the diet formulation. In the first experiment, eight rumen fistulated bulls (BW= 600 ± 34 kg; 26 ± 3 months) were used in a replicated 4 × 4 Latin square (21-d periods) to evaluate the digestibility, ruminal fermentation and microbial population. The intake of DM had a tendency to be greater in CG+ than CG- diets (P = 0.07). Apparent total tract digestibilities of nutrients were similar among diets (P ≥ 0.10). Diets with CG or VM had similar values of pH (mean=6.15; P ≥ 0.10). The proportion of propionate increased 27.5% in CG+ diets, regardless of VM inclusion (P = 0.01). In the second experiment, forty-eight bulls with initial BW (408.4 ± 22.2 kg; 21 ± 2 months) were used in a randomized complete block design for carcass traits and animal performance evaluation. The intake of DM, cold carcass weight and cold carcass dressing was greater in crude glycerin diets (P < 0.05). VM had a slightly tendency to increase ADG and feed efficiency (P < 0.1). Total unsaturated fatty acids (UFA) concentration in the longissimus muscle increased 6.08% in diets CG+ diets (P < 0.05), however n-6/n-3 ratio remained constant among all treatments (P > 0.10). Glycerin had a positive effects on fermentation products and could replace virginiamycin with increment of *Megasphaera elsdenii* abundance and UFA deposition on meat. However, simultaneous administration of VM and CG does not interfere positively on rumen fermentation products, performance and carcass traits of feedlot Nellore cattle.
Keywords: crude glycerin, meat quality, microbiology, virginiamycin

CHAPTER 1 – GENERAL CONSIDERATIONS

Beef production from Brazilian feedlots has increased in recent years with market demands on efficiency and consumer satisfaction. According to Anualpec (2015) between 2006 and 2014 there was an increase of more than 100%, in feedlot finished cattle in Brazil with an approximate contribution of 10% of total cattle slaughtered. In addition to system efficiency, as ingredient costs are currently high, there is concern about the risk of metabolic disorders (e.g. acidosis) caused by a high intake of carbohydrates with faster rate of degradation. In a survey of Brazilian nutritionists, Millen et al. (2009) showed that acidosis represents the second largest health problems in feedlots.

High-concentrate diets increase the accumulation of VFA and potentially lactate in the rumen fluid by altering composition and metabolic activity of rumen microbiota, leading to an increment of lactate accumulation and metabolic disorders (e.g. ruminal acidosis) (NAGARAJA; TITGEMEYER, 2007; FERNANDO et al., 2010; ZEBELI et al., 2015).

The majority of bacteria stimulated by feeding high concentrate diets were *Proteobacteria*, *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* populations followed by *Rumminococcus* spp reduction during high grain adaptation (FERNANDO et al., 2010; ZEBELI; METZLER-ZEBELI, 2012). In addition to bacteria fermentation ciliate protozoa can influence the rate and site of starch degradation by engulfment of starch granules (NAGARAJA et al., 1992). Strains of lactate utilizer and producers bacteria can metabolize starch in different pathways (Figure 1).

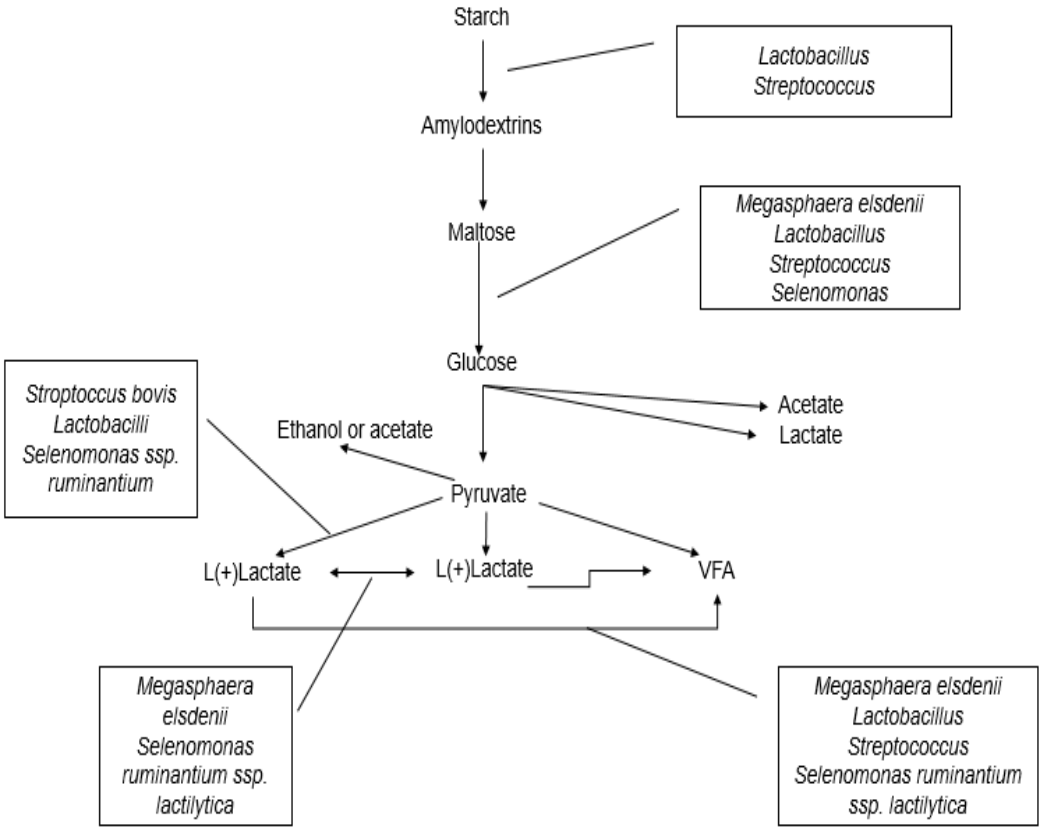

Figure 1- Starch fermentation by different strains of bacteria that produce and utilize lactate. Adapted from Nagaraja and Titgemeyer (2007).

Megasphaera elsdenii belongs to the phylum *Firmicutes* and metabolize lactate to propionate by the acrylate pathway (MAROUNEK; FLIEGROVA; BARTOS, 1989). During glucose fermentation, butyric and caproic acids are produced from an acetic acid intermediate (HINO; KURODA, 1993). Studies with *Megasphaera elsdenii* administration have demonstrated different results on acidosis manifestation both in beef and dairy cattle (KLIEVE et al., 2003; HAGG et al., 2010; MEISSNER et al., 2010; AIKMAN et al., 2011; MUYA et al., 2015). Some general effects of *Megasphaera elsdenii* supplementation include acetate:propionate ratio reduction and lower daily pH fluctuation.

The rumen influx of high fermentable carbohydrate (e.g. starch) may exceed the production of VFA during fermentation without the same removal synchronism by the

epithelium or saliva neutralization, and therefore cause a reduction of pH below 6. This in turn, will lead to a development of lactate producing species such as *Streptococcus bovis* and *Lactobacilus* spp (AL JASSIM; GORDON; ROWE, 2003). *Streptococcus bovis* is a gram-positive bacteria homofermentative that grows extremely rapidly and becomes predominant in the rumen with lactate accumulation (HERNANDEZ et al., 2007). Some other microbes' species involved in lactate production in the rumen include *Bifidumbacterium* spp. and *Lactobacilus* spp. (STEWART; FLINT; BRYANT, 1997).

The decrease of pH changes the proportions of ruminal microbial populations. Cellulolytic bacteria can not develop in low pH environments due to the accumulation of VFA anions in the cell compared to bacteria resistant to acidification of the medium, and an increase in the pH gradient can cause anionic toxicity (RUSSEL; WILSON, 1996). In an experiment with high and low concentrate diets Fernando et al. (2010) found an average of 398 taxonomic units (OTU's) in cattle fed high-forage diets and approximately 315 OTU's in high-concentrate diets. However, they only share 24 OTU´s in similarities.

The main accumulation of lactic acid or VFA may cause two forms of acidosis; (1) an acute ruminal lactic disorder presenting ruminal pH less than 5.0 or (2) a subacute ruminal acidosis that is characterized by a range of 5.2-5.5 for at least 3 hours per day (METTE DANSCHER et al., 2015). The subacute ruminal acidosis is associated with the production of immunogenic factors in the rumen environment when the balance between host and rumen bacteria is disrupted. For example, the increase of Gram-negative bacteria and their subsequent lysis is accompanied with dramatic rises in the concentration of lipopolysaccharide, a cell-wall component known as endotoxins that can be translocated through disrupted epithelial junctions of the rumen epithelium and activate a cascade of inflammation events expressed through general signs like fever, lowered feed intake, changes in metabolism, lipolysis and stress (ZEBELI; METZLER-ZEBELI, 2012)

Problems linked with acidosis often follow an abrupt change in the diet such as from a fiber-rich roughage to one of a starch-rich concentrate diet (lacking physically effective fiber). Its severity is a function of the extent of decline in rumen pH and the duration of exposure to low pH (HERNANDEZ et al., 2007).

The contribution of starch present in cereal grains to the acidogenic power depends on size, shape and embedment within the protein matrix (GONZÁLEZ et al. 2017). Furthermore, processing methods such as reduction of particle size, thermal treatment (e.g. steam flaking) and shear forces are known to increase the rate of cleavage of starch to glucose (OWENS et al., 1998; GONZÁLEZ et al., 2017). The increase of starch degradation in the small intestine by feeding processed grains or using diets rich in corn versus barley and wheat or grains can relieve the generation of protons in the rumen environment and increase glucose pools, which may prevent excessive lipolysis and keep concentration of non-esterified fatty acids (NEFA) and beta-hydroxy-butyrate (BHBA) at rather normal levels (ZEBELI et al., 2015).

Several strategies have been used to modulate ruminal fermentation to increase performance and mitigate rumen acidosis risks in high concentrate diets of beef cattle: (1) feeding management (BEVANS et al., 2005); (2) use of buffers (CRAWFORD et al., 2008) and antibiotics such as virginiamycin (VM) or ionopheres (COE et al., 1999); and (3) administration of direct fed microbes such as *Megasphera elsdenii* or *Sacchararomyces cerevisiae* (MEISSNER et al., 2010). Furthermore, to reduce costs associated with cereal grains, the utilization of co-products from bio-diesel plants such as crude glycerin can be an alternative to replace part of corn in the diet formulation.

The use of antibiotics such as VM in sub-therapeutic doses has been used in the feeding of several species of animals as a growth promoter. The VM is a non-ionophore antibiotic of the streptogramins class, produced by *Streptomyces virginiae* that inhibits the growth of gram-positive lactate-producing bacteria. The VM structure has two major factors, M and S (GOTTSCHALL; WANG; KINGSTON, 1988) that function synergistically impairing bacteria protein synthesis by blocking peptide chain elongation (COCITO, 1979).

In the gastrointestinal tract of pigs VM acts decreasing inhibition of gram-positive bacterial growth, degradation of protein and ammonia production and therefore enhance nutrient availability for the animal. The majority of VM effects on ruminal environment are similar to those of monensin, such as increasing propionate content at the expense of acetate and methane (HEDDE; SHOR; QUACH, 1983; NAGARAJA et al., 1997) and protecting ruminal protein degradation (IVES et al., 2002). Virginiamycin has demonstrated to be very efficient in the inhibition of lactic-

acid producing microorganisms (e.g., *Lactobacillus and Streptococcus spp.*) without interfering in the growth of lactic-acid consuming micro-organisms like *Megasphaera elsdenii* (ARAÚJO, 2016). The incidence of liver abscess decreased with VM administration by inhibiting *Fusobacterium necrophorum* and *Actinomyces pyogenes* (NAGARAJA; CHENGAPPA, 1998).

Rogers et al. (1995) have shown in a series of dose–response trials (19 mg to 27 mg VM/kg of the dry matter [DM]) with steers and heifers that VM enhanced average daily gain (4.6%) and gain to feed ratio (3.6%) and reduced the incidence of liver abscess (38%). Coe et al. (1999) evaluated different doses of VM (17.5 or 25mg/kg of DM) in diets with 85% of concentrate on the fermentative parameters as VFA, lactate and ammonia. The concentration of 25 mg/kg of DM was efficient in reducing ammonia (-1.40%), increasing propionate (+22.05%) and reducing butyrate (-15.89%), but in this study the lactate concentration was not altered.

Salinas-Chavira et al. (2009) did not show any effects on carcass characteristics (hot carcass weight, dressing percentage, fat thickness, quality grade and retail yield) of Holstein feedlot steers fed with control (no antibiotic) compared with VM (16 or 22.5 mg/kg of the DM). However VM inclusion tended to increase longissimus muscle area which was likely on the final indirect effect of virginiamycin on final body weight. In a second trial with cannulated Holstein steers VM administration increased molar proportion of acetate and tended decreased propionate molar proportion.

According to VAN BOECKEL et al. (2015) the top countries consuming antimicrobials, such as VM, in livestock production are China, the U.S, India, Brazil and Germany. By 2030, the authors project that antimicrobial consumption will rise by 50% in Brazil, Russia, India, China and South Africa due to consumer demand for livestock products and a shift to large-scale farms where antimicrobials are used routinely. The use of medically antimicrobials in food animals for production purposes is an important global issue due to the possible transfer of resistance genes and bacteria among animals and animal products and the environment (MCEWEN; FEDORKA-CRAY, 2002). On January 2006 the EU banned the use of antibiotics, including VM in animal feed. In 2017, the U.S Food and Drug Administration (FDA) published a Veterinary Feed Directive (VFD) regulation for medicated feeds, which restricts the use of antimicrobial drugs in feeds without veterinary prescription.

Another strategy to prevent or attenuate excessive lactate production and increase animal performance would be to increase the number of lactate-utilizing bacteria (e.g., *Megasphaera elsdenii* and *Selenomonas ruminantium*) in the diet. According to Owens et al. (1988) the maintenance of *Megasphaera elsdenii* or *Selenomonas ruminantium* (lactate utilizers) for a long-term phase in the rumen can be limited by substrate availability and competition from other microbial species (i.e. lower turnover time compared with *Streptococcus bovis*). Furthermore, many of the candidate microbes are obligate anaerobes, limiting cell yield and complicating their culture in commercial fermentation facilities (MCALLISTER et al., 2011). One alternative would be the use of a substrate, such as glycerol that makes *Megasphaera elsdenii* or *Selenomonas ruminantium* grows in the rumen environment (STEWART; FLINT; BRYANT, 1997).

Crude glycerin (65-85% pure glycerol), a byproduct from the biodiesel industry, could be used to modulate ruminal fermentation and lactate metabolism. The growth of the biofuels industry has increased availability of crude glycerin and has directed its use to livestock production (DONKIN, 2008; MACH et al., 2009; LAGE et al., 2014). Glycerol can be fermented by ruminal microrganisms, be absorbed in the epithelium or flow to the duodenum (REMOND; SOUDAY; JOUNAY, 1993). Ruminants can ferment glycerol to propionate in the rumen and utilize its molecules at gluconeogenesis via hepatic metabolism.

Glycerol and starch from corn grains present similar attributes as glucose suppliers (BAJRAMAJ et al., 2017). Ruminal degradation of glycerol and corn in the rumen increase the proportions of propionate and butyrate at the expense of acetate. The small intestine digestion of starch in grain corn (30-55%) may release glucose to be partially absorbed into the bloodstream (RÉMOND et al., 2004). Glycerol can be absorbed at the half percentage in the rumen wall and enhance glucose circulation via gluconeogenesis in the liver (REMOND; SOUDAY; JOUNAY, 1993).

Rumen bacteria oxidizes glycerol to dihydroxyacetone by glycerol dehydrogenase and then phosphorylatizes to dihydroxyacetone phosphate by dihydroxyacetone kinase. Triose phosphate isomerase in the glycolysis directs the dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. Each mole of glycerol converted to pyruvate generates two moles of NADH, which must then go through the

propionic acid synthesis pathway in order to completely convert the NADH back to NAD^+ and maintain the redox balance (ZHANG; YANG, 2009).

In vitro trial with pure culture of cellulolytic microbes and glycerol addition at 0.1, 0.5, 1, 2 and 5%, inhibited the growth of *Ruminococcus flavefasciens* and *Fibrobacter succinogenes* and impaired growth and cellulolytic activity of fungus *Neocallimastix frontalis* when included at 5% using cellobiose as the sole energy source (ROGER et al., 1992). ABO EL-NOR et al. (2010) evaluated diets consisting of 600 g/kg alfalfa hay and 400 g/kg concentrate (DM basis) with glycerol replacing corn at 0, 36, 72 and 108 g/kg dry matter in a continuous fermenter. They found a decreased of NDF digestibility and population of *Butirivibrio fibrisolvens*, as well as *Selenomonas ruminantium* and *Clostridium protoclasticum* by increasing concentrations of glycerol. However no effects were observed on pH, NH_3-N and dry matter digestibility.

Crude glycerin has demonstrated variable results when administered in livestock diets. Dry matter intake has been shown to decrease in diets containing more than 40 g glycerol /kg of DM of a steam-flaked corn finishing diet of crossbred heifers (PARSONS et al., 2009). Inclusion of crude glycerin up to 150 g/kg of DM in diets of finishing lambs increased DMI and feedlot performance in the first 14 days (GUNN et al., 2010). In feedlot diets of beef cattle, glycerin levels up to 100 g/kg of DM have also been used to replace cereals grains with positive results on performance of beef cattle (MACH et al., 2009; PARSONS et al., 2009; LAGE et al., 2014).

Carcass and meat traits can be altered in the meat of animals fed with crude glycerin by increasing marbling deposition as a function of glycogenic precursors absorption (SCHOONMAKER et al., 2003) or by increasing unsaturated fatty acid content on meat (CARVALHO et al., 2014; EIRAS et al., 2014; FAVARO et al., 2016) possibly due to the ruminal lipolysis inhibition (KRUEGER et al., 2010; EDWARDS et al., 2012).

Improvements of beef cattle performance through manipulation of rumen fermentation may be an alternative to increase feedlots' profitability and to reduce acidosis risks. The VM is a non-ionophore antibiotic that inhibits the growth of gram-positive lactate-producing bacteria (e.g., *Streptococcus bovis* and *Lactobacillus spp*). Crude glycerin (CG) is a substrate that stimulates the growth by bacteria that metabolize lactate, such as *Megasphaera elsdenii* and *Selenomonas ruminantium*.

We hypothesize that CG could replace VM without impairing ruminal fermentation, carcass traits and fatty acid profile of meat from feedlot Nellore cattle or that combining VM and CG would increase the positive effects on ruminal fermentative parameters and animal performance .

The objective of this study was to evaluate the effect of VM and CG on rumen fermentation parameters, performance, carcass traits and fatty acid profile of meat from feedlot Nellore cattle. In order to evaluate these parameters we quantified the following variables: intake and digestibility of nutrients, average daily gain, rumen VFA, pH, NH_3-N, ruminal microbial population, meat and fat color and fatty acid composition of longissimus muscle.

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CHAPTER 2

O artigo a seguir está redigido conforme normas de publicação do *Animal Feed Science and Technology*, exceto o posicionamento das tabelas.

Changes in ruminal fermentation and microbial population of feedlot Nellore cattle fed crude glycerin and virginiamycin

Abstract

The objective of this experiment was to evaluate the effects of crude glycerin (CG) and virginiamycin (VM) diets on ruminal fermentation and microbial population of feedlot Nellore cattle. Eight rumen fistulated bulls (BW= 600 ± 34 kg; 26 ± 3 months) were used in a replicated 4 × 4 Latin square (21-d periods) with 2 × 2 factorial arrangement of treatments: diets without virginiamycin (VM-) or virginiamycin at 25 mg/kg DM (VM+) combined with diets without crude glycerin (CG-) or CG (80% glycerol) at 100 g/kg DM (CG+). The sugar cane bagasse was used as the exclusive roughage in the proportion of 200 g/kg in dry matter of diet and crude glycerin replaced corn in the diet formulation. Data were analyzed in a replicated 4 × 4 Latin square with a 2 × 2 factorial using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). There were no CG×VM interactions for any variable measured ($P \geq 0.10$). The intake of DM had a tendency to be greater in CG+ than CG- diets ($P = 0.07$). Apparent total tract digestibilities of nutrients were similar among diets ($P \geq 0.10$). Diets with CG or VM had similar values of pH (mean=6.15; $P \geq 0.10$). Data showed that CG or VM did not affect the concentration of total VFA (116.92 mM; $P \geq 0.10$). The proportion of propionate increased 27.5% in CG+ diets, regardless of VM inclusion ($P = 0.01$). Acetate:propionate ratio was lower in CG+ compared to CG- diets (3.58 vs. 2.12; $P \geq 0.10$). Valerate and butyrate proportion was greater in CG+ than CG- diets ($P < 0.05$). The inclusion of VM or CG did not alter the relative abundance of fibrolityc bacteria (P≥ 0.10). Total protozoa counts ($P = 0.05$) and *Metadinium* spp. ($P = 0.058$) had a tendency to decrease in VM+ than VM- diets ($P \leq 0.10$). The CG+ diets had a tendency to increase the microbial N flow ($P = 0.08$), however, the efficiency of Microbial N synthesis based on intake of digestible organic matter was similar among treatments ($P \geq 0.10$). Crude glycerin had positive effects on rumen fermentation products and can replace virginiamycin with increment of *Megasphaera elsdenii* abundance. However, combining virginiamycin and glycerin does not affect positively rumen fermentation and growth of bacteria that metabolize lactate.

Keywords: beef cattle**;** crude glycerin; fermentation; finishing diets; virginiamycin

Abbreviations: aNDFom, NDF without sodium sulfite, with alpha amylase and corrected for residual ash; CG, crude glycerin; CP, crude protein; DM, dry matter; DMI, dry matter intake; EE, ether extract; BW, body weight; OM, organic matter; VFA, volatile fatty acids; VM, virginiamycin.

1. Introduction

Feedlots diets of beef cattle are generally rich in grains with high starch content. The great concentration of starch present in finishing diets increment the accumulation of VFA and potentially lactate that can alter metabolic activity of rumen microbiota, leading the increment of lactate accumulation and metabolic disorders (Nagaraja and Titgemeyer, 2007; Fernando et al., 2010; Zebeli et al., 2015). Several strategies have been used to manipulate the rumen microbial environment to increase beef cattle performance and to reduce acidosis risks: feeding management (Bevans et al., 2005); use of buffers (Crawford et al., 2008) and antibiotics such as virginiamycin (VM) or ionopheres (Coe et al., 1999); administration of direct fed microbes such as *Megasphera elsdenii* or *Sacchararomyces cerevisiae (*Meissner et al., 2010) .

The antibiotics, such as VM, have been used at subtherapeutic levels in the feeding of several species of animals as a growth promoter. The VM inhibits the growth of gram-positive lactate-producing bacteria by impairing protein synthesis (Cocito, 1979) . Rogers et al. (1995) have shown an increase in the average daily gain (4.6%) and gain to feed ratio (3.6%) of steers and heifers receiving VM at 25 mg/kg DM in a series of dose–response trials (19 mg to 27 mg VM/kg DM). However, in recent years there has been a growing concern about hazards of antibiotic utilization in animal feed. On January 2006, the EU banned the use of antibiotics, including virginiamycin in animal feed. In 2017, the U.S Food and Drug Administration (FDA) published Veterinary Feed Directive (VFD) regulations for medicated feeds, which restrict the use of antimicrobial drugs in feeds without veterinary prescription.

Thus, another strategy to prevent or attenuate excessive lactate production could be increasing the availability of substrate, such as crude glycerol, to lactate-utilizing bacteria (e.g, *Megasphaera elsdenii*). Glycerol promotes the growth of *Megasphaera elsdenii* and *Selenomonas ruminantium* in the rumen environment (Stewart et al., 1997). Glycerol and starch from corn grains present similar attributes

as glucose suppliers by increasing the proportions of propionate and butyrate at the expense of acetate (Remond et al., 1993;Wang et al., 2009; Bajramaj et al., 2017). The small intestine digestion of starch in grain corn (30-55%) may release glucose to be partially absorbed into the bloodstream (Remond et al., 2004). Glycerol can be absorbed at half percentage in the rumen wall and enhance glucose circulation via gluconeogenesis in the liver (Remond et al., 1993). Crude glycerin can replace cereal grains with levels up to 100g/kg DM of feedlot diets of beef cattle without detrimental effects on performance (Parsons et al., 2009; Mach et al., 2009; Lage et al., 2014) and it potentially enhance unsaturated fatty acid deposition on meat (Carvalho et al., 2014; Eiras et al., 2014; Favaro et al., 2016)

We hypothesizes that CG (substrate promoter of lactate-utilizer bacteria) could replace VM (inhibitor of gram-positive lactate-producing bacteria) without impairing rumen fermentation or that combining VM and CG would increment the positive effects on fermentation and promote the growth of bacteria that metabolize lactate. The objective of this study was to evaluate the effect of combining VM (0 or 25 mg/kg DM) and CG (0 or 100g/kg DM) on fermentation and microbial population of feedlot Nellore cattle. To solve this issue we evaluated the intake and digestibility of dry matter and nutrients, pH, ammonia content, volatile fatty acids, efficiency of microbial protein synthesis and evaluation of the ruminal microbiota.

2. Material and methods

2.1. Animals and feed management

This study was conducted in accordance with the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal) and it was approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA; protocol number 021119/11).

Eight rumen cannulated bulls (BW= 600 ± 34 kg 26 ± 3 months) were used in a replicated 4 × 4 Latin square design with 2 × 2 factorial arrangement of treatments: diets without virginiamycin (VM-) or virginiamycin at 25 mg/kg DM (VM+) combined

with diets without crude glycerin (CG-) or crude glycerin (80% glycerol) 100 g/kg DM (CG+). Crude glycerin was acquired from a soybean oil based biodiesel production company ADM, Rondonópolis, Brazil (80.3% of glycerol; 1.59% of ether extract; 5.03% of ash and 12.0% of water). Animals were submitted to 21 days of adaptation to experimental installations and diets. The experiment was conducted for four periods and each lasted for 21 days. Each period contained 14 days for diet adaptation and 7 days of data and sample collection. Animals were housed in individual stalls and fed twice daily at 07:00 and 16:00 hours for 10% refusals. The ration comprising sugarcane bagasse and concentrates in a 20:80 ratio on a DM basis (Table 1) were weighed individually and manually mixed in the troughs of each animal.

Table 1. Chemical composition of the treatments

	Treatments[a]			
Proportion of ingredients, g/kg DM	CG-		CG+	
	VM-	VM+	VM-	VM+
Sugarcane bagasse	200	200	200	200
Ground corn	625	625	528	528
Soybean meal	136	136	131	131
Crude glycerin	0.00	0.00	100	100
Urea	9.00	9.00	11.0	11.0
Mineral premix[b]	30.0	30.0	30.0	30.0
Virginiamycin (mg/kg of DM)	0.00	25.0	0.00	25.0
Nutrients				
DM, g/kg	852	852	828	828
Crude protein, g/kg DM	157	157	156	156
aNDFom[c], g/kg DM	308	308	296	296
EE, g/kg DM	25.0	25.0	24.0	24.0
Ash, g/kg DM	74.0	74.0	76.0	76.0
Starch, g/kg DM	372	372	322	322
NFC[d], g/kg DM	436	436	447	447

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Composition (mg/kg of DM) = Calcium: 8000; Phosphoros:3300; sodium: 2700; magnesium: 1400; sulphur: 6300; zinc: 82; cupper: 21; maganase: 52; cobalt: 1.3; iodine: 1.1: selenium: 0.35.
[c]NDF assayed with a heat stable amylase and expressed exclusive of residual ash
[d]Non fiber carbohydrates =1000 - (CP+ EE + Ash + NDF)

2.2. Sampling and chemical analysis

Samples of diets (forage and concentrate) and orts from each animal were collected in 5-d sampling period (from days 15 to 19), then composited every period to determine dry matter intake (DMI). Dry matter and nutrients intake were calculated as the difference between amounts offered and refused based on chemical analysis of the composited sample within animals on each period. From days 15 to 19, feces were collected immediately after each spontaneous defecation, stored in 20-L plastic bags, and at the end of each 24-hours collection period the bags were changed and the feces were weighed, manually blended, and aliquots of 10% of the daily feces excretion were collected. Each fecal sample obtained per day, animal, and period was pre dried in a forced-air oven at 60 °C for 72 to 96 hours. Then, 10 g of each of the pre-dried samples from each day were used to compose the final sample. Apparent total tract digestibility of nutrients was calculated as follows: [Nutrient intake (kg/d)-fecal residue (kg/d)]/nutrient intake (kg/d) × 1000.

The composite samples of each material (concentrate, sugarcane bagasse, orts and feces were used to determine DM (method 934.01; AOAC, 1990), ether extract (AOAC, 1990), N was determined by combustion (Leco Instruments Inc., method 976.06) and multiplied by 6.25 to obtain CP, ash (method 924.05; AOAC, 1990) and neutral detergent fiber (NDF) analyses were performed without sodium sulfite and with alpha amylase, and the data were corrected for residual ash (Mertens, 2002).

At day 21, spot urine samples (Chizzotti et al., 2008) were taken at 16:00 h to determine the purines derivatives excretion and estimate the microbial protein synthesis (Barbosa et al., 2011). After collected, urine was placed in a plastic pot and stored at 4°C. Immediately after sampling, subsamples (10 mL) of urine was diluted in 40 mL of a 0.036 N H_2SO_4 solution before storage at − 20°C. Allantoin in urine was analyzed colorimetrically, following the technique of Fujihara et al. (1987) as described by Chen and Gomes (1992). Colorimetric method was used to determine uric acid concentrations in urine (Labtest Diagnostic S.A., Lagoa Santa, Brazil).

Ruminal samples were taken immediately before feeding and at 3, 6, 12, 18 h post feeding on day 20 and day 21 of the sampling week to determine the pH value (measured after the contents were filtered), and the concentrations of NH_3-N (mg/dL) and VFA (mM). Two 20-mL aliquots were stored at -10ºC and were later used to

determine the NH_3-N concentration as describing by Fenner (1965), adapted for use in Kjeldahl distillation. The VFA concentrations were measured using a gas chromatograph (GC2014, Shimadzu Corporation, Kyoto, Japan) with an HP-INNOWax capillary column (30 m × 0.32 mm; 0.50 µm film thickness; Agilent Technologies, CO, USA) at an initial temperature of 80ºC which was increased by 20ºC/min until a final temperature of 240°C was reached.

2.3. Microbiology

Ruminal samples used for the quantification of bacteria and protozoa were collected before feeding on day 21 of the sampling week. Ciliate protozoa were identified using a Sedgewick-Rafter chamber as described by Dehority (1984), with the modifications suggested by D'Agosto and Carneiro (1999). Total DNA was extracted using a FastDNA® Spin Kit for Soil (MP Biomedicals) according to the manufacturer´s instructions.

The relative abundances of archaea (Denman et al., 2007), *Megasphaera elsdenii* (Ouwerkerk et al., 2002), *Streptococcus bovis, Selenomonas ruminantium and Lactobacillus* sp. (Khafipour et al., 2009), and three cellulolytic bacterial species, namely, *F. succinogenes* (Denman and McSweeney, 2006), *R. albus* (Mosoni et al., 2007), and *R. flavefaciens* (Denman and McSweeney, 2006), were determined by qPCR using methods of primers standardization of Castagnino et al. (2015).

Data were normalized using the 16S rRNA gene amplified by the total bacterial primer as a ‘housekeeping gene’(DENMAN; MCSWEENEY, 2006) and qPCR was performed using an ABI Prism 7500 and SYBR Green (Applied Biosystems®) technology.

2.4. Statistical analysis

Data of feed intake, digestibility, and ruminal parameters were evaluated in a replicated 4 × 4 Latin square design, in a factorial arrangement 2 × 2, glycerol and virginiamycin using the MIXED procedure of SAS (version 9.2). Statistical model included the fixed effect of virginiamycin (1 degree of freedom, DF), crude glycerin

(1DF) and all interactions. Random effects were period (3DF), bulls (7DF), square (1DF) and residual error. When the interaction between the main effects (i.e., VM and CG) was not significant, data are reported showing the main effect.

Ammonia, VFA concentrations and pH were analyzed in a replicated 4 × 4 Latin square design with repeated measurements in time using the MIXED procedure of SAS (version 9.2). Statistical model included the fixed effect of virginiamycin (1 degree of freedom, DF), crude glycerin (1DF), time (4DF) and all interactions. Random effects were period (3DF), bulls (7DF), square (1DF) and residual error. The covariance structures was selected by the lowest corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC). Data were subjected to analysis of variance using the PROC MIXED procedure of SAS (2004). When significant, the means between treatments were compared using Tukey´s minimum significant difference (i.e., option PDIFF adjust=Tukey of the command LSMEANS). A probability of $P<0.05$ was accepted as being statistically significant, but tendencies with $P \leq 0.10$ were noted and discussed.

3. Results

There are not interactions for intake and total tract digestibility ($P \geq 0.10$; Table 2). The intake of DM and MO was greater for animal fed with CG+ than CG-, independent of VM inclusion ($P < 0.05$). The total tract digestibility, % DM, OM, CP, starch, NFC remained relatively constant in all diets ($P \geq 0.10$).

Table 2. Effect of crude glycerin (CG) and virginiamycin (VM) on intake and total tract apparent digestibility of Nellore bulls fed with finishing diets

	Treatments[a]							
	CG-		CG+			*P-value*		
	VM-	VM+	VM-	VM+	SEM[b]	CG	VM	CG ×VM
Intake, kg								
DM	12.8	12.6	13.6	13.4	0.52	0.07	0.59	0.98
OM	11.5	11.2	12.0	12.3	0.31	0.01	0.98	0.13
CP	2.16	2.06	2.16	2.57	0.01	0.24	0.33	0.58
NDF	3.50	3.40	3.40	3.81	0.15	0.07	0.51	0.15
Starch	4.22	4.81	4.10	4.30	0.36	0.23	0.15	0.45
NFC	5.60	5.81	5.43	5.74	0.13	0.44	0.74	0.20
Total tract apparent digestibility, g/kg								
DM	732	733	685	730	37.1	0.43	0.39	0.44
CP	817	753	768	820	36.9	0.81	0.79	0.20
aNDFom[c]	535	533	510	570	36.0	0.84	0.32	0.29
Starch	919	892	891	883	18.2	0.22	0.12	0.77
NFC[d]	907	909	871	884	23.5	0.66	0.12	0.76

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Standard error of mean
[c]NDF assayed with a heat stable amylase and expressed exclusive of residual ash
[d]Non fibrous carbohydrates=1000 - (CP + EE + Ash + NDF)

The VFA, mM concentration and pH was similar among treatments ($P \geq 0.10$; Table 3). The Acetate and A:P ratio decreased in CG+ than CG- diets ($P < 0.05$). The propionate, butyrate and valerate proportion was greater in CG+ than CG- diets ($P < 0.05$). The proportion of propionate increased 27.5% in CG+ diets, regardless of VM inclusion. The NH_3-N (mg/dL) concentration remained constant among treatments ($P \geq 0.10$).

Table 3. Effect of crude glycerin (CG) and virginiamycin (VM) on VFA, pH and NH_3-N of Nellore bulls fed with finishing diets

	Treatments[a]									
	CG-		CG+			*P*-value				
	VM-	VM+	VM-	VM+	SEM[b]	CG	VM	CG *VM	T[c]	CG×VM×T
Total VFA, *m*M	130	117	123	124	15.5	0.98	0.67	0.60	0.01	0.70
Individual, mol/100 mol										
Acetate	61.3	64.2	50.6	53.0	2.43	0.01	0.21	0.91	0.01	0.91
Propionate	20.0	17.7	25.7	26.3	2.31	0.01	0.68	0.49	0.01	0.88
Isobutyrate	0.95	1.11	0.99	0.98	0.18	0.56	0.35	0.27	0.01	0.18
Butyrate	12.7	12.0	16.7	14.1	1.32	0.03	0.25	0.48	0.05	0.36
Isovalerate	3.64	3.89	3.41	3.66	0.35	0.58	0.56	0.99	0.01	0.41
Valerate	1.47	1.23	2.55	1.98	0.16	0.01	0.01	0.25	0.06	0.69
Ratio A:P	3.34	3.82	2.13	2.12	0.36	0.01	0.42	0.40	0.01	0.47
pH	6.15	6.27	6.07	6.09	0.13	0.18	0.47	0.63	0.01	0.52
NH_3-N, mg/dL	28.1	27.5	23.7	26.7	4.89	0.46	0.72	0.60	0.01	0.14

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Standard error of mean.
[c]Time

There is no interaction between CG and VM for the microbial population ($P \geq 0.10$; Table 4). The relative abundance *Megasphaera elsdenii* doubled in CG+ compared with CG- diets ($P < 0.05$). The inclusion of CG decreased *Streptococcus bovis* ($P < 0.02$). The inclusion of VM or CG did not alter the relative abundance of fibrolityc bacteria ($P \geq 0.10$).

Table 4. Effect of crude glycerin (CG) and virginiamycin (VM) on relative abundance (n x 10^{-3}) of rumen bacteria of Nellore bulls fed with finishing diets

	Treatments[a]							
	CG-		CG+			*P*-value		
	VM-	VM+	VM-	VM+	SEM[b]	CG	VM	CG × VM
Lactobacilus spp.	4.3	4.12	0.01	0.17	0.21	0.79	0.59	0.64
Streptococcus bovis	0.58	0.56	0.42	0.16	0.19	0.02	0.22	0.48
Megasphaera elsdenii	0.14	0.05	0.17	0.20	0.10	0.03	0.73	0.48
Selenomonas ruminantium	30.3	51.1	17.6	14.0	2.4	0.15	0.61	0.46
Ruminococcus albus	0.58	1.86	1.52	1.42	0.5	0.62	0.17	0.24
Fibrobacter succinogenes	0.07	0.07	0.089	0.093	0.02	0.14	0.30	0.39

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Standard error of mean.

There is no interaction for protozoa counts ($P \geq 0.10$; Table 5). The species of *Entodinium, Polyplastron, Isotricha* and *Eudiplodinium* were unaffected by all diets ($P \geq 0.10$).Total protozoa counts ($P = 0.05$) and *Metadinium* spp. ($P = 0.058$) had a tendency to decrease in VM+ than VM- diets.

Table 5. Effect of virginiamycin (VM) combined with crude glycerin (CG) on rumen protozoa counts of Nellore bulls fed with finishing diets

	Treatments[a]							
	CG-		CG+			P-value		
	VM-	VM+	VM-	VM+	SEM[b]	CG	VM	CG ×VM
Protozoa ,n x 10^4/ ml								
Entodinium	4.58	4.41	4.88	3.51	1.06	0.71	0.37	0.48
Isotricha	2.81	1.24	1.78	2.26	0.69	0.99	0.42	0.49
Metadinium spp.	2.39	1.18	2.37	0.66	0.73	0.74	0.058	0.72
Polyplastron	1.64	1.24	2.43	2.05	0.92	0.11	0.22	0.49
Eudiplodinium	1.23	1.82	2.15	1.14	0.81	0.79	0.70	0.22
Total protozoa	12.6	10.4	14.9	9.73	2.63	0.64	0.05	0.39

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Standard error of mean.

Animals that received CG+ diets had a greater purine derivatives excretion ($P < 0.05$; Table 6). The microbial N flow estimated and the absorbed purine derivatives had a tendency to be higher in CG+ than CG- diets (P=0.08).

Table 6. Effect of virginiamycin (VM) combined with crude glycerin (CG) on purine derivatives, urea excretion and microbial N flow and efficiency of microbial synthesis of Nellore bulls fed with finishing diets

	Treatments[a]							
Item	CG-		CG+		SEM[b]	P-value		
	VM-	VM+	VM-	VM+		CG	VM	CG×VM
Allantoin, mmol/da	116	117	166	155	24.52	0.05	0.79	0.77
Uric Acid,mmol/da	11	10.4	9.76	13.5	1.49	0.38	0.18	0.09
PD[c], mmol/day	114	112	157	147	21.12	0.04	0.71	0.77
AP[d], mmol/day	89.3	101	143	132	25.63	0.08	0.99	0.57
Urea excretion/day	227	211	213	207	42.32	0.74	0.78	0.79
NMIC[e], g/day	49.1	55.6	81	72.5	14.38	0.08	0.94	0.57
NMIC/kg DOMI[f]	17.2	13.4	13.4	19.3	4.70	0.85	0.39	0.27

[a]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[b]Standard error of mean.
[c]Total urinary excretion of purine derivatives (allantoin + uric acid).
[d]Absorbed purine derivatives.
[e]Microbial N flow to the small intestine.
[f]Efficiency of microbial synthesis based on intake of digestible organic matter

4. Discussion

The studies with CG inclusion in feedlot diets at 10 % DM did not show interferences in the DMI (Mach et al., 2009; Lage et al., 2014). In our experiment, the increase in the intake of DM and OM in CG+ diets may be occurred due to the physical characteristics of glycerol, such as viscosity, which could to maintain feed particles aggregated (i.e., sugarcane bagasse particles) and to reduce ruminal fill. Furthermore, glycerol can be metabolized within 2 hours in adapted animals (Kijora et al., 1998) by the following routes: fermentation, epithelium absorption or flow to the duodenum (Remond et al., 1993).

In our experiment total VFA content remained relatively constant in all diets. The lack of effect in CG+ diets is in accord with experiments with bulls receiving diets

containing up to 12% crude glycerol (Mach et al., 2009) or diets of dairy cows with glycerol replacing corn starch (DeFrain et al., 2004). Coe et al. (1999) did not show effects of VM on total VFA content of Holstein steers fed during adaptation to a high concentrate diet. The authors attributed the moderating influence of VM on ruminal fermentation by a lower *Lactobacillus* sp. and *S. bovis counts* compared with control treatments. Other factors, such as, the relationship between rumen concentrations of VFA and flux of these from the rumen needs to be considered when interpreting rumen VFA proportions (Clayton et al., 1999).

The inclusion of VM normally alters the fermentation by increasing propionate relative to other VFA (Nagaraja et al., 1995;Coe et al., 1999; Ives et al., 2002). However, in our experiment VM+ diets only decreased the valerate proportion. On the other hand, the CG+ diets increased propionate and butyrate proportion at expense of acetate in the rumen. Glycerol is a substrate that is more reduced than the glucose and to maintain the oxidation–reduction balance, the metabolism is directed to formation of more reduced end products, such as propionate (Castagnino et al., 2015). The increase in molar proportion of propionate and acetate: propionate ratio reduction in CG+ diets are in agreement with the finds of others (Defrain et al., 2004; Trabue et al., 2007; Krueger et al., 2010; Lage et al., 2017).

Glycerol can be metabolized by *Megasphaera elsdenii* and *Selenomonas ruminantium* (Stewart et al., 1997). The higher concentration of butyrate in CG+ diets possibly occurred due to an increase in the *Megasphaera elsdenii* abundance as an active lactate utilizer. The oxidation of lactate to pyruvate generates two hydrogen atoms, and formation of butyrate from acetate may act as an electron sink providing some protection against acidosis due to H_2 use (Coe et al., 1999). In our experiment, another factor that facilitated the increase in the *Megasphaera elsdenii* abundance in CG+ diets was the pH value higher than 6.0. According to Mackie and Gilchrist (1979) a higher ruminal pH would allow a more active population of lactate-utilizing bacteria to ferment lactate to VFA.

The pH values seem to be in accordance with nutrients digestibilities and total VFA content. The average value of pH (6.15) for all diets in our experiment may occurred due to the adaption of the animals to concentrate over a number of weeks with the proportion of forage in the diet decreasing over that period. According to Klieve

et al. (2003) adaptation process would allow time for the resident populations of lactic acid-utilizing and other starch-fermenting bacteria to keep up with the growth of *S. bovis* and prevent acidosis. Although VM inclusion has been attributed functions of stabilize the ruminal pH (Godfrey et al., 1995), the lack of effect on pH values in our experiment can be attributed to a fast rate of lactate metabolism in the rumen and the lower retention time with increase in the absorption of VFA.

Diets with CG and VM did not affect fibrolytic bacteria abundance, which can explain the constant value of NDF digestible among diets. Hales et al. (2013) reported no effect of glycerin at 10% of diet DM on steers DM digestibility. Others have reported a decrease in NDF digestibility at 5% of pure glycerol in DM of dairy cows diets (Donkin et al., 2009). In vitro trial with pure culture of cellulolytic microbes and glycerol addition at 0.1, 0.5, 1, 2 and 5%, inhibited the growth of *Ruminococcus flavefasciens* and *Fibrobacter succinogenes* and impaired growth and cellulolytic activity of fungus *Neocallimastix frontalis* when included at 5% using cellobiose as the sole energy source (Roger et al., 1992).

The N metabolism seems not be affected by VM+ diets as the NH_3-N concentration and apparent digestibility of crude protein remained constant compared with VM- diets. According to *in vitro* studies of Van Nevel et al. (1984) VM decreased deamination and casein degradation. Ives et al. (2002) evaluating finishing diets for steers fed with or without corn gluten feed combined with VM or monensin plus tylosin showed a higher content of α-amino nitrogen in the rumen for VM diets, which could potentiate an increase in metabolizable protein supply to cattle. However an additional *in vitro* trial testing the same diets did not corroborate the hypothesis of a protein-sparing effect. Therefore, more research is needed to clarify the effect of VM on ruminal proteolytic bacteria and intestinal digestion of protein.

Although *Megasphaera elsdenii* has potential to degrade amino acids in the rumen and to produce branched chain volatile fatty acids (Stewart et al., 1997), its greater abundance in CG+ than CG- diets did not increase the release of NH_3-N in the rumen. *Megasphaera elsdenii* is deficient in peptidase activity (Rychlik et al., 2002) which can help explain the lack of differences on NH_3-N content and branch chain volatile fatty acid proportions in diets with crude glycerin. Defrain et al. (2004) reported

no effect on ammonia concentration when crude glycerol replaced corn starch in diets of dairy cows.

Ruminal selenomonads are classified into 2 subspecies, *ruminantium* and *lactilytica*, strains that utilize lactate and glycerol are placed in the subspecies *lactilytica*, and all other strains are grouped under the subspecies *ruminantium* (Ricke et al., 1996). *Selenomonas ruminantium* was not different across the all treatments in our experiment, which can be explained by substrate specificity as demonstrated by previous authors or inefficacy of VM to inhibit Gram-negative bacteria.

Protozoa contributes to microbial N reaching the duodenum and it releases in the rumen significant amounts of H_2 that is used by methanogens for methane production. In our experiment total protozoa counts decreased in diets with VM. Protozoa counts have generally decreased (Murray et al., 1992; Nagaraja et al., 1995) or not been affected by VM addition (Coe et al., 1999; Ives et al., 2002) in previous studies. The decrease of protozoa counts should allow a reduction of NH_3-N concentration due to a decrease of protozoal proteolytic and deaminative enzymes (Hristov and Jouany, 2005). Notwithstanding, in our experiment the reduction of protozoa counts did not compensate for a decrease in NH_3-N concentration.

According to Hackmann and Firkins (2015) the production of microbial protein is inefficient because microbes can direct ATP toward maintenance functions, synthesis of reserve carbohydrate, or energy spilling (futile cycles that dissipate heat) instead of use it for growth. Although the microbial N flow had a tendency to increase in diets with CG, the efficiency of microbial N synthesis based on intake of digestible organic matter remained constant. Glycerol is a readily fermentable feedstuff that increase energy availability for microbial syntheses in the rumen, in this case the increased of microbial N flow probably occurred due to greater intake of OM in diets with crude glycerin. However, the efficiency of N utilization possible was not altered as the NH_3-N and urea concentration remained constant among the treatments.

5. Conclusions

Crude glycerin had positive effects on rumen fermentation products and can replace virginiamycin with increment of *Megasphaera elsdenii* abundance. However, combining virginiamycin and glycerin does not affect positively rumen fermentation and the growth of bacteria that metabolize lactate.

Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 3

O artigo a seguir está redigido conforme normas de publicação do *Meat Science*, exceto o posicionamento das tabelas.

Fatty acid profile and carcass traits of feedlot Nellore cattle fed crude glycerin and virginiamycin

Abstract: Forty-eight bulls with an initial body weight (BW) of 408.4 ± 22.2 kg (age = 21 ± 2 months) were used in a randomized complete block design arranged in a 2 × 2 factorial design. The treatments were diets without virginiamycin (VM-) or with virginiamycin, at 25 mg/kg of dry matter (DM, VM+), and diets without crude glycerin (CG-) or with crude glycerin, at 100 g/kg DM (CG+). The cold carcass weight and cold carcass dressing ($P \leq 0.05$) was greater in bulls fed crude glycerin diets. Total unsaturated fatty acids (UFA) were 6.08% higher, and cooking weight loss was 10% lower in bulls fed CG+ diets, compared to bulls fed CG- diets ($P = 0.0081$). Crude glycerin at 100 g/kg DM could be a suitable replacement for VM, as it led to a slight increase in UFA deposition in meat. However, simultaneous administration of VM and CG did not positively affect performance and carcass traits of feedlot Nellore cattle.
Keywords: beef cattle, fatty acid profile, feedlot, glycerin, meat quality, virginiamycin

1. Introduction

Brazilian beef production from feedlots has increased in recent years, and market demands regarding efficiency and meat quality have also increased. Consumers are increasingly concerned and critical about the health characteristics of meat that can arise from intensive production practices, including those with a natural origin (fat content), or those associated with external compounds added to animals diets, such as antibiotics (T. McAllister & Cameron, 2016). On the other hand, modifying meat composition by feeding animals with byproducts or substrates that modulate fermentation may be a method to improve meat quality, and to reduce environmental impacts (Monteschio et al., 2017).

Feedlot diets are generally rich in concentrates. The faster rate of starch degradation from these diets increases the risk of metabolic disorders (e.g. acidosis). Several strategies have been used to prevent acidosis and increase beef cattle performance, including adaptation to grain diets (Bevans, Beauchemin, Schwartzkopf-Genswein, McKinnon, & McAllister, 2005), use of ruminal buffers (Crawford et al.,

2008), use of antibiotics such as ionophores or virginiamycin (Coe et al., 1999), and administration of microbes such as *Megasphaera elsdenii* or *Sacchararomyces cerevisiae* (Meissner et al., 2010*)*.

Antibiotics such as virgiamycin (VM) have been used in the feeding of livestock animals as a growth promoter. The VM inhibits the growth of gram-positive lactate-producing bacteria by disrupting protein synthesis (Cocito, 1979). Rogers et al. (1995) demonstrated, in a series of dose–response trials (19 mg to 27 mg VM/kg DM) with steers and heifers, that VM enhanced the average daily gain (4.6%), and gain to feed ratio (3.6%). However, studies describing the effect of VM on meat traits are still limited in beef cattle (Lemos et al., 2016; Salinas-Chavira et al., 2009), and, to our knowledge, no previous study has described fatty acid profiles of meat from feedlot Nellore cattle supplemented with VM.

The utilization of microbes that are directly fed to livestock, such as *Megasphaera elsdenii,* could be an alternative for antibiotics such as VM, by reducing ruminal accumulation of lactate (Counotte, Prins, Janssen, & Debie, 1981). However, many of the candidate microbes are obligate anaerobes, limiting cell yield, and complicating their culture in commercial fermentation facilities (T. A. McAllister et al., 2011). Thus, the use of glycerin, a highly available substrate from the biodiesel industry, that promotes the growth of *Megasphaera elsdenii* and *Selenomonas ruminantium* (Stewart, Flint, & Bryant, 1997) in the rumen, could be used to increase the proportion of lactate utilizing bacteria, and improve animal performance.

Crude glycerin (CG) has been demonstrated to have varying results when administered in livestock diets. In feedlot diets of beef cattle, glycerin levels up to 100 g/kg DM have been used to replace cereal grains, with positive effects on performance of beef cattle (Lage et al., 2014; Mach, Bach, & Devant, 2009; Parsons, Shelor, & Drouillard, 2009). Glycerin has also been demonstrated to enhance unsaturated fatty acid concentration of meat (Carvalho et al., 2014; Eiras et al., 2014; Favaro et al., 2016), possibly due to ruminal lipolysis inhibition (Edwards et al., 2012; Krueger et al., 2010).

We hypothesized that CG could replace VM without causing detrimental effects on the performance of feedlot Nellore cattle and fatty acid profiles of meat, and that the combination of VM and CG could increase animal performance and improve carcass

traits of feedlot Nellore cattle. The objective of this study was to evaluate the effect of VM (0 or 25 mg/kg of DM) combined with CG (0 or 100 g/kg of DM) on the performance, and carcass and meat quality of feedlot Nellore Cattle.

2. Material and methods

2.1. Animals and feed management

This study was conducted in accordance with the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal), and it was approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA; protocol number 021119/11). The experiment was designed as a completely randomized block and forty-eight Nellore bulls (21 months of age) with initial body weights (BW) of 408.4 ± 22.2 kg were individually fed feedlot diets without virginiamycin (VM-) or with virginiamycin, at 25 mg/kg of dry matter (DM, VM+/ V-Max, Phibro Animal Health, Ridgefield Park, NJ), and diets without crude glycerin (CG-) or with crude glycerin at 100 g/kg DM (CG+).

Crude glycerin was acquired from a soybean oil based biodiesel production company ADM, Rondonópolis, Brazil (80.3% glycerin; 1.59% ether extract; 5.03% ash, and 12.0% water). Cattle were first given 21 days of adaptation to experimental procedures and diets, and the experimental period to evaluate cattle performance and meat traits lasted 81 days.

Cattle were fed two times daily, at 07:00 and 16:00, and feed refusals were recorded daily for each pen. Amounts of feed offered to animals were calculated according to previous dry matter intake (DMI) and adjustments were made when needed, so that refused feed did not exceed 10% of daily intake. Orts daily weights and samplings were performed for the diet quantities provided for each animal. The ingredient proportions and chemical compositions of the experimental diets are presented in Table 1.

Table 1. Chemical composition of the treatments

Item		Treatments[1]			
		CG-		CG+	
		VM-	VM+	VM-	VM+
Ingredients g/kg DM					
Sugarcane bagasse		200	200	200	200
Ground corn		625	625	528	528
Soybean meal		136	136	131	131
Crude glycerin		0.00	0.00	100	100
Urea		9.00	9.00	11.0	11.0
Mineral premix[2]		30.0	30.0	30.0	30.0
Virginiamycin (mg/kg of DM)		0.00	25.0	0.00	25.0
Chemical composition, g/kg DM		852	852	828	828
Crude protein		157	157	156	156
Neutral detergent fiber		308	308	296	296
Ether Extract		25.0	25.0	25.0	25.0
Ash		74.0	74.0	76.0	76.0
Starch		372	372	322	322
Non fiber carbohydrates		436	436	447	447
Fatty acid (g/100 g of total FA)[3]					
Caprylic	8:0	0.03	0.03	0.02	0.02
Capric	10:0	0.02	0.02	0.02	0.02
Myristic	14:0	0.05	0.05	0.04	0.04
Palmitic	16:0	17.1	17.08	13.2	13.2
Margaric	17:0	0.17	0.17	0.11	0.11
Stearic	18:0	3.46	3.46	2.80	2.80
Palmitoleic	16:1 *cis*-9	0.15	0.15	0.12	0.12
Oleic	18:1 *cis*-9	39.6	39.6	37.0	37.0
Linoleic	18:2 n-*6*	35.0	35.0	41.0	41.0
α-Linolenic	18:3 n-3	1.14	1.14	1.51	1.51
SFA		20.96	20.96	16.3	16.3
UFA		75.74	75.74	79.6	79.6
MUFA		39.75	39.75	37.2	37.2
PUFA		36.14	36.14	42.5	42.5

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]Composition (mg/kg of DM)= Calcium:8000; Phosphoros:3300; sodium: 2700; magnesium: 1400; sulphur: 6300; zinc: 82; cupper: 21; maganase: 52; cobalt: 1.3; iodine: 1.1: selenium: 0.35.
[3]SFA = saturated fatty acids; UFA=unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

2.2. Sampling and chemical analysis

Forage and concentrate samples and orts from each animal were first composited weekly, and then composited every month. Feed samples were frozen at -18 °C for later analysis of DM and ether extract (AOAC, 1990). Nitrogen (N) content

was determined by combustion (Leco Instruments Inc), and multiplied by 6.25 to obtain crude protein (CP) content. Neutral detergent fiber (NDF) analyses were performed without sodium sulfite and with alpha amylase, and the data were corrected for residual ash (Mertens, 2002) .

2.3. Slaughter, carcass data, and sample collection

At the beginning of the trial, all the cattle were weighed after a 16-h solid fast before the first feeding in the morning. The average daily gain (ADG) was obtained by weighing the cattle at the beginning and the end of the experiment, always after a 16-h solid fast. After 81 days of feeding, all the cattle (497.3 ± 41.9 kg) were transported to a slaughterhouse (Minerva Foods, Barretos, Sao Paulo, Brazil) 90 km from the feedlot. Transport to the slaughterhouse took approximately 1.5 h. On arrival at the slaughterhouse, cattle were kept in resting pens and were humanely slaughtered under Brazilian federal inspection. To compensate for potential differences in final BW due to variation in digestive tract fill and other factors, such as mud or manure on the animal, carcass adjusted final BW was calculated by dividing hot carcass weight for each animal by the average dressing percentage across all treatments within each study. After slaughter, carcasses were weighed and then refrigerated at 4 °C for approximately 24 h. After the postmortem chill period, the cold carcass weight (CCW), ultimate carcass pH (pHu), 12^{th} rib fat thickness (RFT), and 12^{th} rib longissimus muscle area (LMA) were measured on the left side of each carcass.

The LMA were traced on transparencies and measured later with a planimeter, and RFT measurements were taken at 3/4 of the length, ventrally over the longissimus muscle (Greiner, Rouse, Wilson, Cundiff, & Wheeler, 2003). Cold carcass dressing percent (CCD) was calculated using CCW divided by final shrunk body weight (SBW) and then multiplying the result by 100. A boneless longissimus section (10 cm thick) was removed from the posterior end of the whole rib.

Longissimus muscle samples were individually vacuum-packaged and held at -20 °C for two days. Thereafter, each frozen longissimus sample was standardized from the posterior end into one 2.54-cm-thick steak sample (AMSA, 1995) for Warner-Bratzler shear force measurement, and other analyses, as described later. All steaks were vacuum-packed (99% vacuum, with a 200 Selovac Sealer machine, Selovac, São Paulo, SP, Brazil), in polyamide/polyethylene pouches of 120 μm and 1 $cm^3/m^2/24$ h

0_2 permeability, 3 cm^3/m^2/24 h CO_2 permeability measured at 5 °C and 75% relative humidity; water vapor transmission rate (WVTR) was 3 g/m^2/24 h at 38 °C and 100% RH. The vacuum value 20 (50 Pa) was used to pack the steaks, and steaks were stored at -20 °C for 10 days until analysis.

For proximate analysis, the epimysium was removed from the samples prior to lyophilization for 36 h. The cooking weight loss (CKL) was determined using thawed samples, as the difference between the weight of a steak before and after cooking in an oven pre-heated to 175 °C.

2.4. Meat and subcutaneous fat color

The determination of meat and fat color was performed as described by Cañeque et al. (2004), using a Minolta colorimeter (Model CR 400, Minolta Camera Co. Ltd., Osaka, Japan), and lightness (L*), redness (a*), and yellowness (b*) were evaluated. The color aspects were assessed by the CIE L*a*b* color system, using 0°/45°. Thirty minutes prior to the assessment, samples were removed from vacuum packages, and surface samples were exposed to air for oxygenation of myoglobin. The same procedure was used for the fat color measurement. After this step, the color was measured at three different points, and average values were calculated. The colorimeter was calibrated before analyzing the samples against white and black standards.

2.5. Warner–Bratzler shear-force measurement and cooking weight loss

The Warner–Bratzler shear force (WBSF) steaks were thawed at 4 °C for 24 h and oven-broiled in an electric oven (Layr, Luxo Inox) preheated to 150 °C. Internal steak temperatures were monitored by 20-gauge copper-constantan thermocouples (Omega Engineering, Stamford, CT) placed in the approximate geometric center of each steak, and attached to a digital monitor. When the internal steak temperature reached 35 °C, the steak was turned over and allowed to reach an internal temperature of 70 °C, before removal from the oven. Cooked WBSF steaks were cooled for 24 hours at 4 °C (AMSA, 1995). Six round cores (1.27 cm diameter) were removed from each steak parallel to the long axis of the muscle fibers (Wheeler & Koohmaraie, 1999). Each core was sheared once through the center, perpendicular to the fiber direction, by a Warner-Bratzler shear machine (G-R Manufacturing Company, Manhattan, KS -

USA). Cooking weight loss was evaluated for the steaks that were also used for WBSF measurement. Total cooking weight loss was calculated as the difference between the weight of the steaks before and after oven-broiling.

2.6. Fatty acid profile and fat content

For determination of the fatty acid composition of the fresh meat, samples of the transversal section were collected from the longissimus muscle, lyophilized, and frozen for lipid extraction and methylation. The fatty material was extracted using a mixture of chloroform–methanol, as reported by Bligh and Dyer (1959), and the fatty acid methyl esters (FAME) were obtained using the ISO 5509 method (1978). After phase separation, the upper layer was discarded, and 10 mL was transferred to glass beakers that had been previously tarred. After evaporation, beakers were re-weighed, and fat content was calculated based on the difference in weights. Qualitative and quantitative measurements of fatty acid content were performed by gas chromatography using a chromatograph (Model GC-14B, Shimadzu, Kyoto, Japan, with a Communication Bus Module - CBM 102) with a flame ionization detector (FID), and fused silica capillary column (Omegawax 250), which was 30 m in length and 0.25 mm in diameter, and had a film thickness of 0.25 μm (Supelco SP-24136). Helium was used as a carrier gas at a flow of 1 mL/min. A 1 μL aliquot of the sample was injected into a “split” at a division ratio of 1/100 and a temperature of 250 °C. The temperature of the oven was programmed to remain at 100 °C for 2 min, and then increase to 220 °C at 4 °C/min for 25 min, while the detector was set at 280 °C. Identification and quantification of the methyl esters of the fatty acids was achieved by comparison with the retention times and concentrations of methyl esters of standard fatty acids.

The indices of enzyme activities of Δ^9-desaturase for C18 fatty acids and C16 fatty acids and elongase were estimated according to Malau-Aduli et al. (1997):

Δ^9-desaturase 16 = 100[(C16:1cis9)/(C16:1cis9 + C16:0)]

Δ^9-desaturase 18 = 100[(C18:1cis9)/(C18:1cis9 + C18:0)]

Elongase= 100[(C18:0 + C18:1cis9)/(C16:0 + C16:1cis9 + C18:0 + C18:1cis9)]

The atherogenicity index and saturation index (SI) was estimated according to Ulbricht and Southgate (1991):

Atherogenicity = [C12:0 + 4(C14:0) + C16:0]/ΣUFA

SI = (C14:0 + C16:0 + C18:0) /ΣMUFA + PUFA.

2.7. Statistical analysis

The data on animal performance and carcass variables were analyzed as a completely randomized block design, arranged in a 2 × 2 factorial design, including dietary virginiamycin and crude glycerin. The block was represented by two different feedlots, and the Nellore bulls were housed in individual pens (bull = experimental unit). Statistical models included virginiamycin (1 degree of freedom, DF), crude glycerin (1 DF), and all interactions as fixed effects. Block (1 DF) and residual error were included as random effects. Data were analyzed using the PROC MIXED procedure in SAS statistical software. When significant, the means between treatments were compared using the Fisher's least significant difference test (i.e., the option 'pdiff command LSMEANS'). A probability of $P < 0.05$ was considered to be statistically significant, but tendencies with $P \leq 0.10$ were noted and discussed.

3. Results

There were no significant interactions between performance and carcass traits. Thus, the main effects of VM or CG on intake, average daily gain, and feed efficiency are reported independently (Table 2). The DMI, as a percentage of BW was greater in cattle fed CG+ (2.06%) diets, than in cattle fed CG- diets (1.95%), regardless of VM addition (Table 2; $P = 0.0351$). The ADG was 1.31 kg/day for cattle fed CG+ diets, and it was 1.13 kg/day for cattle fed CG- diets ($P = 0.0452$). The ADG was 1.30 kg/day for cattle fed VM+ diets, and 1.14 kg/day for cattle fed VM- diets ($P = 0.0832$). The NDF intake was statistically similar for all diets ($P = 0.1843$). Cattle fed diets with VM+ tended to have higher feed efficiency ($P = 0.0954$).

Table 2. Effect of virginiamycin (VM) and crude glycerin (CG) on intake, average daily gain (ADG) and feed efficiency (FE) of feedlot Nellore cattle

	Treatments[1]							
Item	CG-		CG+			*P-value*		
	VM-	VM+	VM-	VM+	SEM[2]	CG	VM	CG×VM
Intake, kg								
Dry matter	9.00	10.1	10.2	10.4	0.41	0.0421	0.6112	0.4124
Organic matter	8.26	9.21	9.33	9.51	0.37	0.0443	0.2418	0.3884
Crude protein	1.29	1.39	1.48	1.52	0.07	0.0112	0.6635	0.3277
Neutral detergent fiber	2.46	2.73	2.69	2.80	0.13	0.1843	0.5843	0.1366
DM, % of BW	1.91	1.98	2.09	2.02	0.06	0.0351	0.9823	0.1525
ADG	1.04	1.22	1.23	1.38	0.08	0.0452	0.0832	0.7643
FE	0.11	0.12	0.11	0.13	0.01	0.2651	0.0954	0.3965

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]Standard error of mean

The final BW, RFT, LMA, and WBSF were similar among the treatments ($P \geq 0.1$; Table 3). Cattle fed CG+ diets had lower CKL and higher HCW, CCW, and CCD when compared with cattle fed CG- diets ($P < 0.1$). The fat content did not change in cattle fed diets with CG+ or VM+ ($P \geq 0.1$).

Table 3. Effect of virginiamycin (VM) and crude glycerin (CG) on carcass traits of feedlot Nellore cattle

	Treatments[1]							
Item[2]	CG-		CG+			*P-value*		
	VM-	VM+	VM-	VM+	SEM[3]	CG	VM	GB×VM
No. of bulls	12	12	12	12	-	-	-	-
Initial BW, kg	410	419	414	412	14.6	0.8712	0.6745	0.4643
Final BW, kg	495	514	515	525	18.3	0.1634	0.2022	0.7363
HCW	282	287	298	304	9.05	0.0078	0.3232	0.8643
CCW, Kg	277	282	290	297	8.86	0.0328	0.3836	0.9073
CCD, %	56.9	55.7	58.2	58.3	0.91	0.0077	0.3557	0.4254
RTF, mm	4.1	5.58	4.66	4.79	0.54	0.8354	0.1582	0.2263
LMA, cm^2	69.9	71.2	74.0	74.9	3.05	0.1153	0.6522	0.9273
LMA/100kg	25.2	25.3	25.6	25.2	0.70	0.8328	0.8645	0.7187
WBSF, kgf	4.85	4.32	4.69	4.78	0.40	0.6876	0.5564	0.3945
CKL, %	31.5	29.5	27.2	27.7	0.99	0.0081	0.4372	0.2148
Fat	2.29	2.34	2.46	2.59	0.23	0.4334	0.7354	0.8954

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]HCW= hot carcass weight; CCW = cold carcass weight; CCD = cold carcass dressing; RFT = rib fat thickness; LMA = longissimus muscle área; LMA/100kg = longissimus muscle area in relation to cold carcass weight; WBSF = Warner-Bratzler shear force; CKL = cooking loss;
[3]Standard error of mean

The fat content did not change in cattle fed diets with CG+ or VM+ ($P \geq 0.1$). Meat color, subcutaneous fat color, and pH did not change in cattle fed CG+ diets ($P \geq 0.1$; Table 4). However, the b* value of fat color tended to be lower in cattle fed VM+ diets ($P = 0.0901$).

Table 4. Effect of virginiamycin (VM) and crude glycerin (CG) on meat pH and subcutaneous fat color (L*, a* and b*)[2] of feedlot Nellore cattle

	Treatments[1]							
Item[2]	CG-		CG+			*P-value*		
	VM-	VM+	VM-	VM+	SEM[3]	CG	VM	GB × VM
No. of bulls	12	12	12	12	-	-	-	-
pH	5.52	5.52	5.56	5.53	0.03	0.1867	0.4765	0.4432
Meat colour								
L*	36.8	37	35.8	36.1	1.07	0.4233	0.8156	0.9822
a*	17.4	17.0	17.5	17.0	0.74	0.9465	0.4322	0.8776
b*	8.79	8.87	8.72	8.23	0.52	0.4934	0.6845	0.5765
Fat colour								
L*	64.2	62.7	62.2	63.1	1.20	0.5033	0.8235	0.3445
a*	6.12	6.10	6.52	7.06	0.73	0.3455	0.7733	0.6733
b*	14.1	12.2	13.2	13.0	0.65	0.9567	0.0901	0.1934

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]L* = luminosity; (0 = black and 100 = white); a* = index from green (-) to red (+); b* = index from blue (-) to yellow (+).
[3]Standard error mean

The saturated and monounsaturated fatty acid profiles of longissimus muscles are reported in Table 5. There was an interaction effect between CG and VM for myristoleic acid ($P = 0.0815$). Myristoleic acid concentration had a tendency to be greater in longissimus muscles from cattle fed diets with VM (VM+) but without CG (CG-) than in longissimus muscles from cattle fed diets with CG+ combined with VM+ ($P = 0.0614$). The inclusion of dietary CG or VM did not affect the capric, lauric, myristic, and palmitic acid content of meat ($P \geq 0.1$). Heptadecanoic, margaric, and stearic acid concentration were greater in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets, regardless of VM inclusion ($P < 0.05$).

Table 5- Effect of virginiamycin (VM) and crude glycerin (CG) on saturated and monounsaturated fatty acids (mg/100g of meat) from longissimus muscle of feedlot Nellore cattle

Fatty acid[2] (mg/100 g of meat)		Treatments[1]				SEM[3]	*P-value*		
		CG-		CG+					
		VM-	VM+	VM-	VM+		CG	VM	CG ×VM
No. of bulls		12	12	12	12				
SFA									
Capric	10:0	1.54	1.24	1.55	1.41	0.26	0.7322	0.1945	0.6964
Lauric	12:0	1.83	1.62	1.78	1.64	0.17	0.9031	0.1444	0.9344
Myristic	14:0	82.4	76.0	76.6	76.7	6.37	0.9416	0.1854	0.9858
Palmitic	16:0	688	679	642	667	20.1	0.1734	0.9568	0.4768
Margaric	17:0	71.2	65.7	76.7	73.5	1.72	0.0077	0.2345	0.1076
Stearic	18:0	426	459	389	366	21.9	0.0082	0.8887	0.1254
MUFA									
Myristoleic	14:1 *cis*-9	17.2	15.4	17.3	20.4	1.87	0.2223	0.8535	0.0815
Palmitoleic	16:1 *cis*-9	77.9	73.7	77.1	85.9	4.35	0.1645	0.4764	0.4753
Heptadecenoic	17:1	18.7	18.1	24.8	31.4	1.37	0.0254	0.2355	0.1177
Oleic	18:1 *cis*-9	1057	1000	1084	1112	24.6	0.0210	0.8534	0.1898
Eicosenoic	20:1 *cis*-9	4.17	3.82	4.52	4.50	0.54	0.6133	0.4943	0.1423

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids.
[3]Standard error of mean

The polyunsaturated fatty acid profiles are shown in Table 6. There was a tendency for docosahexaenoic acid (DHA) to be greater in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets (3.35 *vs.* 2.38 mg/100 g of meat; P = 0.0865). UFA (P = 0.0403) and monounsaturated fatty acids (MUFA) contents (P = 0.0391) were greater in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets. The saturated fatty acid (SFA) content was 7.38% lower in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets (P = 0.0396). The UFA/SFA (P = 0.0808) and MUFA/SFA ratio (P = 0.0320) was lower in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets. However, the PUFA/SFA ratio was not affected by CG or VM ($P \geq 0.1$). The n-6/n-3 ratio was similar in meat from cattle fed any of the diets ($P \geq 0.1$). The Δ^9-desaturase enzyme activity index was greater for C18 fatty acids than for C16 fatty acids. Meat from cattle fed CG+ diets had a greater index of Δ^9-desaturase for C16 (P = 0.0725) and C18 (P = 0.0101) than meat from cattle fed diets without glycerin (CG-). The index of elongase and atherogenicity enzyme activity was not altered by VM or CG inclusion ($P \geq 0.1$). The saturation index was lower in meat from cattle fed CG+ diets than in meat from cattle fed CG- diets (P = 0.0294).

Table 6. Effect of virginiamycin (VM) and crude glycerin (CG) on polyunsaturated fatty acids (mg/100g of meat), Δ9-desaturase and elongase enzyme activity indixes, and atherogenicity index from longissimus muscle of feedlot Nellore cattle

	Treatments[1]							
Fatty acid (mg/100 g of meat)[2]	CG-		CG+			*P-value*		
	VM-	VM+	VM-	VM+	SEM[7]	CG	VM	VM×CG
No. of bulls	12	12	12	12				
PUFA								
Linoleic (18:2 n-6)	131.3	141	162	127	14.8	0.5876	0.3021	0.1722
α-Linolenic (18:3 n-3)	13.8	13.2	14.0	11.5	1.29	0.4644	0.1844	0.5348
CLA (18:2 *cis*-9 *trans*-11)	12.1	10.5	11.1	11.9	0.93	0.8955	0.3613	0.1212
Eicosatrienoic (20:3 n-6)	7.42	8.72	11.0	7.67	1.44	0.5967	0.1334	0.1388
Arachidonic (20:4 n-6)	34.8	39.4	48.0	38.4	6.38	0.3248	0.5861	0.3835
EPA (20:5 n-3)	10.2	10.9	12.8	10.0	1.92	0.3134	0.9827	0.3022
DTA (22:4 n-6)	3.90	4.69	5.45	5.21	0.73	0.2788	0.8343	0.1734
DPA (22:5 n-3)	18.7	19.7	24.8	19.6	3.13	0.4334	0.5712	0.2755
DHA (22:6 n-3)	1.80	2.97	3.71	3.0	0.53	0.0865	0.8167	0.1465
Total SFA	1225	1242	1140	1145	42.1	0.0396	0.9514	0.7314
Total MUFA	1108	1179	1141	1180	30.6	0.0391	0.4555	0.2611
Total PUFA	204	221	256	203	25.5	0.5055	0.3842	0.2486
Total UFA	1312	1299	1397	1383	38.7	0.0403	0.9277	0.9454
UFA/SFA	1.08	1.02	1.13	1.16	0.05	0.0808	0.4845	0.7835
n-6/n-3	4.08	4.10	4.00	3.98	0.19	0.9848	0.5855	0.9223
Δ^9-desaturase 16[3]	10.4	9.80	10.6	11.5	0.59	0.0725	0.7066	0.3168
Δ^9-desaturase 18[4]	70.9	69.2	72.8	75.3	1.37	0.0101	0.7843	0.1334
Elongase[5]	65.7	66.5	66.8	66.3	0.73	0.5824	0.8113	0.3531
Atherogenicity[6]	0.80	0.77	0.72	0.71	0.05	0.1743	0.7818	0.8152

[1]Diet without crude glycerin (CG-) or CG at 100 g/kg DM (CG+); diet without VM (VM-) or VM at 25 mg/kg DM (VM+).
[2]SFA= saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; UFA = unsaturated fatty acids.
[3] Δ9 desaturase 16 = 100[(C16:1cis9)/(C16:1cis9 + C16:0)];
[4] Δ9 desaturase 18 = 100[(C18:1cis9)/(C18:1cis9 + C18:0)]
[5] Elongase= 100[(C18:0 + C18:1cis9)/(C16:0 + C16:1cis9 + C18:0 + C18:1cis9)]
[6] Atherogenicity = [C12:0 + 4(C14:0) + C16:0]/ΣUFA
[7]Standard error of mean

4. Discussion

Feeding feedlot beef cattle diets containing CG at 10% DM did not affect their DMI (Lage et al., 2014; Mach et al., 2009; Schieck, Shurson, Kerr, & Johnston, 2010). In the present study, the increase in the intake of DM, OM, and CP in CG+ diets may be attributed to the fast rate of glycerin fermentation and epithelium absorption (Remond, Souday, & Jouany, 1993). Another possible explanation for the increase in the DMI in cattle fed CG+ diets could be related to a reduction in non-esterified fatty acids (NEFA) concentration in the blood, as described in experiments with ewes during late gestation (Polizel et al., 2017), and primiparous dairy cows (Kass et al., 2012). The hepatic oxidation of fuels in liver (i.e., plasma NEFA) decreases the rate of hepatic vagal afferents, and results in satiety (Allen, Bradford, & Oba, 2009).

The increase in the DMI (7.25%) in cattle fed CG+ diets resulted in a greater CCW and ADG compared with cattle fed CG- diets. According to Waldo and Jorgensen (1981), DMI is a meaningful factor affecting animal performance, and a positive correlation between ADG and time spent ingesting feed can be expected (McGee et al., 2014).

The VM+ diets did not affect cattle DMI, but did tend to increase ADG and feed efficiency. (Rogers et al. (1995)) showed in a series of dose–response trials (19 mg to 27 mg VM/kg of DM) with steers and heifers, that VM enhanced average daily gain (4.6%), and gain to feed ratio (3.6%). These results demonstrated an increase in efficiency of energy utilization in cattle fed VM+ diets compared with cattle fed VM- diets. Possible mechanisms involved in growth-performance enhancement include: inhibition of lactate producing bacteria (Coe et al., 1999; Nagaraja, Taylor, Harmon, & Boyer, 1987); increases in retention time and intestinal digestion (Ravindran, Kornegay, and Webb (1984) in pigs; and reduction in acetogenic bacteria content in the rumen, and facilitating growth of propionate-producing bacteria (Nagaraja et al., 1987; Van Nevel, Demeyer, & Henderickx, 1984), which are more energetically efficient.

The VM+ diets did not modify LMA/100 kg of cold carcasses, or RFT, which is probably due to an indirect lack of effects on adjusted final BW and HCW (Salinas-Chavira et al., 2009). The CG+ diets also did not affect LMA/100 kg of cold carcasses, which can be attributed to the genetic similarity between the cattle used in the

experiment (Lage et al., 2014). The RFT (4.78 mm) did not differ significantly between cattle fed the different diets. The values of RFT (3 to 6 mm) met the requirements of the Brazilian beef industry. Other studies using CG in feedlot diets have reported similar RFT (Carvalho et al., 2014; Favaro et al., 2016; Ribeiro, Messana, Neto, Fiorentini, & Berchielli, 2016).

The use of dietary VM or CG did not affect WBSF, and the average value across our treatments (4.6) represents an overall consumer's satisfaction perception for tenderness (Platter et al., 2003). The slight decrease in CKL in cattle fed CG+ diets would not change consumers' acceptability of meat. This reduction in the CKL values may be explained by a high osmotic pressure in the muscle induced by glycerin, as demonstrated in pigs (Mourot, Aumaitre, Mounier, Peiniau, & François, 1994), and by the interaction of glycerin and protein during the cooking process (Lacroix & Castaigne, 1985). Although a large proportion of glycerin is fermented in the rumen (Claudia Kijora et al., 1998), some reports suggest that a portion may be absorbed in the epithelium, or flow to other parts of the body (Remond et al., 1993), which increases its concentration in the bloodstream. This reduction in the CKL in cattle fed CG+ would not

The mean carcass pH across the treatments with CG and VM was 5.53. Meat of high quality has a pH in the range of 5.4–5.6, and pH can affect meat quality traits, such as color (Weglarz, 2010). The diets used in the present study probably had the potential to maintain glycogen concentration at the time of slaughter, as insufficient glycogen at slaughter results in pH values above 5.5, which, in extreme cases, leads to a serious quality problem known as dark-cutting (Immonen, Ruusunen, Hissa, & Puolanne, 2000). Although diets containing glycerin would have the potential to increase glycogen concentration due to availability for ruminal and duodenal metabolism, CG+ diets did not change ultimate pH values of the carcasses.

Meat color is an indicator of freshness, and it represents the main quality factor affecting purchasing decisions of consumers (Mancini & Hunt, 2005). In the present study, meat from cattle fed CG+ and VM+ diets had mean values of 35.4, 17.2, and 8.25 for brightness, redness, and yellowness, respectively. The results of the present study are in accordance with Muchenje et al. (2009), who describe in a review of

biochemical aspects of meat quality a range of values from 33.2 to 41, 11.1 to 23.6, and 6.1 to 11.3, for brightness, redness, and yellowness, respectively.

Favaro et al. (2016) have shown a reduction in b* value in meat due to the inclusion of glycerin at 10 and 15% of DM, replacing corn grains. The authors reported a possible reduction in carotenoid intake due to replacement of corn with CG during the formulation of diets. The lack of effect in the present study can be attributed to the fact that we used sugarcane bagasse as a source of forage. During the production of sugarcane bagasse by-products, the higher temperatures and grinding process reduces the concentration of carotenoids (Dunne, Monahan, O'Mara, & Moloney, 2009), and the combination of sugarcane bagasse with CG+ diets (e.g., low corn inclusion) did not compensate for the reduction in b* indices. Lage et al. (2014) evaluated meat quality of Nellore cattle fed diets with 10% crude glycerin replacing corn or soybean hulls, and also did not find any difference in beef color when glycerin was replaced by corn grain. The authors suggested that inclusion of crude glycerin at 10% DM was not sufficient to promote changes in meat and fat color.

Experiments evaluating carcass traits in feedlot diets with VM addition are scarce, and they did not demonstrate effects on fat color (Boucque, Fiems, Cottyn, & Buysse, 1990; Salinas-Chavira et al., 2009). However, in in the present study, VM+ diets decreased b* values of fat, which can be explained by the rate of gain. The VM+ diets tended to increase ADG compared with VM- diets. Knight, Death, Lambert, and McDougall (2001) conducted an experiment in which three groups of steers were fed with the objective of gaining weight rapidly, gaining weight slowly, or losing weight, with a fourth group of steers slaughtered at the outset. The yellowness and carotenoid concentrations of subcutaneous fat of the steers gaining weight rapidly were significantly lower than the group slaughtered at the outset, or those losing weight.

The nutritional value of beef has become increasingly important, as consumers are increasingly aware of the relationship between the consumption of certain fatty acids and cardiovascular health, which likely affects purchasing decisions (Buchanan et al., 2015). The CG+ diets increased MUFA and UFA content of meat, and decreased the proportion of SFA. Studies have suggested that glycerin may increase UFA absorption in the duodenum, due to a possible inhibition of ruminal lipolysis (Edwards et al., 2012; Krueger et al., 2010), and that glycerin may increase the duodenal flow of

UFA (Granja-Salcedo et al., 2017), with enhancement of UFA deposition on meat (Carvalho et al., 2014; Eiras et al., 2014; Favaro et al., 2016)

Buchanan et al. (2015) evaluated genetic parameters for intramuscular fatty acids of Angus cattle, and found that the genes that cause increased levels of SFA do so at the expense of reduced levels of MUFA, or vice versa, which is in accordance with our results. These effects can be regulated by stearoyl-CoA desaturase (SCD), which introduces a double bond between carbon atoms 9 and 10 of a SFA to produce C14:1, C16:1, and C18:1 (Matsuhashi et al., 2011). Of the SFA, only margaric acid and stearic acid were lower in cattle fed CG+ diets in the present study. The reduction in stearic acid may be due to a reduction in the biohydrogenation of linoleic acid, which could limit the hydrogenation of vaccenic acid to stearic acid (Lage et al., 2014), a function of ruminal lipolysis inhibition (Edwards et al., 2012). On the other hand, the reduction in margaric acid concentration may be related to the low concentration in diets without CG. Both margaric and stearic acid have been demonstrated to have no net impact on serum cholesterol concentrations in humans (Daley, Abbott, Doyle, Nader, & Larson, 2010).

Heptadecenoic acid (C17:1) is synthesized by rumen bacteria from propionate (Berthelot, Bas, Schmidely, & Duvaux-Ponter, 2001), and its increase may be related to the ruminal fermentation of glycerin, which increases the proportions of propionate and butyrate at the expense of acetate (Bajramaj et al., 2017; Castagnino et al., 2015; C. Kijora et al., 1998). In our experiment , we quantified the rumen microbes (unpublished data), and found that the addition of glycerin increased the microbial N flow to the small intestine, which could explain the higher deposition of heptadecenoic acid in meat. The enhancement of heptadecenoic acids due to CG+ diets can provide healthy anti-carcinogenic effects for humans (Wongtangtintharn, Oku, Iwasaki, & Toda, 2004). Oleic acid deposition in the longissimus muscle was higher in cattle fed CG+ diets, probably due to the action of SCD, which corresponds with the higher index of Δ^9-desaturase enzyme activity for C18 fatty acids in CG+ diets. These results are in agreement with previous studies by (Lage et al., 2014), who observed an increase in the content of oleic acid in meat from feedlot Nellore cattle fed diets containing CG.

Overall, concentrations of UFA, and the UFA/SFA ratios were greater in meat from cattle fed CG+ diets, compared to meat from cattle fed VM+ diets. However, these

differences were small, and are not considered sufficiently significant to have positive effects on human health. The PUFA/SFA ratio in meat remained similar among treatments (0.19), and was lower than the recommend value (0.4) for red meat in human diet (Department of Health, 1994).

Human intake of UFA has been demonstrated to reduce the risk of cardiovascular disease, and possibly the incidence of some cancers, asthma, and diabetes, among other conditions (Milicevic et al., 2014). Diets with VM+ had a tendency to slightly increase ADG and feed efficiency when compared with VM- diets. However, VM+ did not enhance the unsaturated fatty acid content in the meat.

5. Conclusions

Crude glycerin at 100 g/kg DM could be a suitable replacement for VM, as it led to an increase in UFA deposition in meat, although this increase was probably too small to have significant health benefits to human consumers of the meat. Simultaneous administration of VM and CG did not positively affect performance and carcass traits of feedlot Nellore cattle.

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