PEDRO DEL BIANCO BENEDETI

GLYCERIN AS ALTERNATIVE ENERGY SOURCE IN FINISHING BEEF CATTLE DIETS

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

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This dissertation is dedicated to my grandparents Humberto Benedetti and Mario Del Bianco, who first inspired me to begin this long journey. Love you grandpas!

BIOGRAPHY

Pedro Del Bianco Benedeti, son of João Benedeti Neto and Maria Olivina Rodrigues Del Bainco Benedeti, was born in Franca/SP-Brazil on September 07, 1985.

He started the undergrad in Animal Science at *Universidade Federal de Viçosa* in 2005 and obtained his Bachelor of Science degree in 2010. At the same year he started the *Magister Scientiae* program with major on ruminant nutrition and beef cattle production and became a M.S. in Animal Science in February of 2012.

In March of 2012 he started his D.S. program in Animal Science with major on ruminant nutrition and beef cattle production. Thanks to the PDSE program of the Coordination for the Improvement of Higher Education Personnel (CAPES), from January of 2014 to August of 2015 he was a visiting scholar at the Department of Agriculture, Nutrition and Veterinary Science of the University of Nevada, Reno/NV – USA where part of his research was developed under supervision of Dr. Antonio Pinheiro Faciola. On February 18th of 2016 Mr. Benedeti defended his dissertation to obtain the *Doctor Scientiae* degree in Animal Science.

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ABSTRACT

BENEDETI, Pedro Del Bianco, D.Sc., Universidade Federal de Viçosa, February, 2016. **Glycerin as alternative energy source in finishing beef cattle diets** Adviser: Pedro Veiga Rodrigues Paulino. Co-Advisers: Antonio Pinheiro Faciola, Marcos Inácio Marcondes, and Mario Luiz Chizzotti.

The present work was developed based on three studies. The objective of the firs study was to evaluate the effects of partially replacing dry ground corn with glycerin on ruminal fermentation using a dual-flow continuous culture system. Six fermenters (1,223 ± 21 ml) were used in a replicated 3x3 Latin square arrangement with three periods of 10 d each, with 7 d for diet adaptation and 3 d for sample collections. All diets contained 75% concentrate and three dietary glycerin levels (0, 15, and 30% on dry matter basis), totaling six replicates per treatment. Glycerin levels did not affect apparent digestibility of DM ($P_{Lin.} = 0.13$; $P_{Quad.} = 0.40$), OM ($P_{Lin.} = 0.72$; $P_{Quad.} = 0.15$), NDF ($P_{Lin.} = 0.38$; $P_{Quad.} = 0.50$) and ADF ($P_{Lin.} = 0.91$; $P_{Quad.} = 0.18$). Also, glycerin inclusion did not affect true digestibility of DM ($P_{Lin.} = 0.35$; $P_{Quad.} = 0.48$), and OM ($P_{Lin.} = 0.08$; $P_{Quad.} = 0.19$). Concentrations of propionate ($P < 0.01$) and total volatile fatty acids ($P < 0.01$) increased linearly and concentrations of acetate ($P < 0.01$), butyrate ($P = 0.01$), iso-valerate ($P < 0.01$), and total branched-chain volatile fatty acids, as well as the acetate: propionate ratio ($P < 0.01$) decreased with glycerin inclusion. Linear increases on NH_3-N concentration in digesta effluent ($P < 0.01$) and on NH_3-N flow ($P < 0.01$) were observed due to glycerin inclusion in the diets. Crude protein digestibility ($P = 0.04$) and microbial N flow ($P = 0.04$) were greater in the control treatment compared with the other treatments and responded quadratically with glycerin inclusion. Furthermore, the inclusion of glycerin linearly decreased ($P = 0.02$) non- ammonia N flow. Glycerin levels did not affect the flows of total N ($P_{Lin.} = 0.79$; $P_{Quad.} = 0.35$), and dietary N ($P_{Lin.} = 0.99$; $P_{Quad.} = 0.07$), as well as microbial efficiency ($P_{Lin.} = 0.09$; $P_{Quad.} = 0.07$). These results suggest that partially replacing dry ground corn with glycerin may change ruminal fermentation, by increasing total volatile fatty acids, and propionate concentration without affecting microbial efficiency, which may improve glucogenic potential of beef cattle diets. The second study was developed aiming to evaluate the effects of replacing dry ground corn with crude glycerin on intake, apparent digestibility, performance, and carcass characteristics of finishing beef bulls. A completely randomized block design experiment with 25 d for adaptation and 100 d for data collection was conducted, in which 3,640 Nellore bulls (367 ±

36.8 kg; 18 ± 3 mo) were blocked by body weight and assigned to 20 pens. Bulls were randomly assigned to one of four treatments: 0, 5, 10, and 15% (dry matter basis) of crude glycerin in the diet. Intake of dry matter, organic matter, and neutral detergent fiber decreased linearly ($P < 0.05$) with crude glycerin inclusion. However, crude glycerin levels did not affect ($P > 0.05$) intakes of crude protein, non-fiber carbohydrates, and total digestible nutrients. Digestibility of dry matter, organic matter, neutral detergent fiber, and total digestible nutrients increased quadratically ($P < 0.05$) with the inclusion of crude glycerin in the diet. Crude glycerin inclusion did not change the intake of digestible dry matter, average daily gain, final body weight, carcass gain, carcass dressing, gain-to-feed ratio, *Longissimus thoracis* muscle area, and back and rump fat thicknesses ($P > 0.05$). These results suggest that crude glycerin may be included in finishing beef diets at levels up to 15% without impairing performance and carcass characteristics. For the third study, five *in vitro* experiments were conducted to evaluate the metabolizable energy and changes on ruminal fermentation, total gas production and greenhouse gases concentration of glycerin compared to corn and starch, as well as when glycerin was added in finishing beef diets. For Exp. 1, a 24 bottles system (Ankom^{RF} Gas Production System, Ankom technology, NY, USA) was used in 4 consecutive runs of 48 h. The treatments were three different feedstuffs: corn, glycerin, and starch. The 24 h total gas production, acetate concentration, and acetate: propionate ratio decreased ($P < 0.01$) only with feeding glycerin. The 48 h total gas production was highest ($P < 0.01$) for corn, and similar between glycerin and starch. The starch treatment presented the lowest ($P = 0.01$) total VFA concentration. Corn presented the lowest propionate concentration ($P < 0.01$). The metabolizable energy was highest ($P < 0.01$) for corn, and similar between glycerin and starch. For Exp. 2, the same system of Exp. 1 was used in 4 consecutive runs of 48h. The treatments were: inclusion of 0, 100, 200, and 300g/kg DM of glycerin replacing corn in finishing beef diets. Glycerin levels did not affect ($P > 0.05$) 24 h and 48 h total gas production, final pH, NH_3-N, total VFA, propionate, and butyrate concentrations. The inclusion of glycerin linearly decreased acetate concentration ($P = 0.03$) and acetate: propionate ratio ($P = 0.04$). For Exp. 3, a total of 20-serum bottles (155 mL) were used in 4 consecutive runs of 48h. The treatments were the same of Exp. 1. The CH_4 concentration increased ($P < 0.01$) only with feeding glycerin. The CO_2 in ml/g was higher ($P < 0.01$) for corn, but similar for glycerin and starch. The pH decreased ($P < 0.01$) only with feeding starch. Different feedstuffs had no effect ($P > 0.05$) on total VFA, and

propionate concentration. Compared with glycerin treatment, acetate concentration ($P < 0.01$) and acetate: propionate ratio ($P = 0.01$) were higher for corn and starch, whereas butyrate and valerate concentrations were higher ($P = 0.01$) for glycerin. For Exp. 4, a total of 25-serum bottles (155 mL) were used in 4 consecutive runs of 48h. The treatments were the same of Exp. 2. The glycerin inclusion did not affect ($P > 0.05$) the concentrations of CH_4, CO_2, final pH, and total VFA, propionate, butyrate, and acetate: propionate ratio. A linear decrease of acetate concentration ($P = 0.04$) was observed due to inclusion of glycerin in the diets. For Exp. 5, two systems of four 4-L digestion vessels (Daisy^{II} system, Ankom technology, NY, USA) were used in two consecutive runs of 48 h. The treatments were: orchard hay (0.4 g/bag), corn (0.4 g/bag), orchard hay (0.4 g/bag) + glycerin (0.2 g/bag) and corn (0.4 g/bag) + glycerin (0.2 g/bag). Orchard hay with glycerin inclusion presented lowest ($P < 0.01$) *in vitro* dry matter digestibility followed by orchard hay without glycerin, and corn treatments. There was a lack of effects for *in vitro* dry matter digestibility ($P > 0.05$) between corn with or without glycerin. We concluded that, under these experimental conditions, glycerin contributed more to the enhancement of methanogenesis than carbohydrates, but effectively replaced dietary corn as energy source at up to 300g/kg of the diet.

RESUMO

BENEDETI, Pedro Del Bianco, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Glicerina como fonte alternativa de energia em dietas de terminação para bovinos de corte.** Orientador: Pedro Veiga Rodrigues Paulino. Coorientadores: Antonio Pinheiro Faciola, Marcos Inácio Marcondes e Mario Luiz Chizzotti.

O presente trabalho foi desenvolvido baseado em três estudos. O objetivo do primeiro estudo foi avaliar os efeitos da substituição parcial do milho por glicerina sobre a fermentação ruminal utilizando um sistema de cultura contínua de fluxo duplo. Seis fermentadores (1,223 ± 21 ml) foram utilizados em um delineamento em quadrado latino 3x3 replicado, com três períodos de 10 d cada, 7 d para adaptação à dieta e 3 d para coletas de amostras. Todas as dietas continham 75% de concentrado e três níveis de glicerina na dieta (0, 15, e 30% com base na matéria seca), totalizando seis repetições por tratamento. Os níveis de glicerina não afetaram a digestibilidade aparente da MS ($P_{Lin.}$ = 0,13; $P_{Quad.}$ = 0,40), MO ($P_{Lin.}$ = 0,72; $P_{Quad.}$ = 0,15), FDN ($P_{Lin.}$ = 0,38; $P_{Quad.}$ = 0,50) e FDA ($P_{Lin.}$ = 0,91; $P_{Quad.}$ = 0,18). Além disso, a inclusão de glicerina não afetou a digestibilidade verdadeira da MS ($P_{Lin.}$ = 0,35; $P_{Quad.}$ = 0,48), e MO ($P_{Lin.}$ = 0,08; $P_{Quad.}$ = 0,19). As concentrações de propionato de (P < 0,01) e ácidos graxos voláteis totais (P < 0,01) aumentaram de forma linear. Já as concentrações de acetato (P < 0,01), butirato (P = 0,01), iso-valerato (P < 0,01), e ácidos graxos voláteis de cadeia ramificada, assim como a relação acetato: propionato (P < 0,01) diminuíram com a inclusão de glicerina. Foi observado aumento linear na concentração de N-NH_3 dos efluentes (P < 0,01) e no fluxo de N-NH_3 (P < 0,01), devido à inclusão de glicerina nas dietas. A digestibilidade da proteína bruta (P = 0,04) e o fluxo de N microbiano (P = 0,04) foram maiores no tratamento controle em comparação com os outros tratamentos e responderam de forma quadrática com a inclusão de glicerina. Além disso, a inclusão de glicerina diminuiu linearmente (P = 0,02) o fluxo de N não amoniacal. Os níveis de glicerina não afetaram os fluxos de N total ($P_{Lin.}$ = 0,79; $P_{Quad.}$ = 0,35), e N dietético ($P_{Lin.}$ = 0,99; $P_{Quad.}$ = 0,07), assim como a eficiência microbiana (P_{Lin} = 0,09; $P_{Quad.}$ = 0,07). Estes resultados sugerem que substituição ao milho seco por glicerina pode mudar a fermentação ruminal, aumentando a concentração de ácidos graxos voláteis totais e propionato, sem afetar a eficiência microbiana, o que pode melhorar o potencial gliconeogênico em dietas para bovinos de corte**.** O objetivo do segundo estudo foi avaliar os efeitos da substituição do milho seco por glicerina bruto sobre o consumo, digestibilidade aparente, desempenho e

características de carcaça de novilhos em terminação. O experimento foi delineado em blocos casualizados, com com 25 d para adaptação e 100 d para a coleta de dados, onde 3.640 touros da raça Nelore (367 ± 36,8 kg; 18 ± 3 meses) foram blocados por peso corporal e alocados em 20 baias. Os animais receberam, aleatoriamente, um dos quatro tratamentos: 0, 5, 10, e 15% (com base na matéria seca) de inclusão de glicerina bruto na dieta. Os consumos de matéria seca, matéria orgânica e fibra em detergente neutro diminuíram linearmente ($P < 0,05$) com a inclusão de glicerina bruto. No entanto, os níveis de glicerina bruto não afetaram ($P > 0,05$) o consumo de proteína bruta, carboidratos não fibrosos e nutrientes digestíveis totais. As digestibilidades da matéria seca, matéria orgânica, fibra em detergente neutro e nutrientes digestíveis totais aumentaram de forma quadrática ($P < 0,05$) com a inclusão de glicerina bruto na dieta. Inclusão de glicerina bruto não alterou o consumo de matéria seca digestível, ganho médio diário, peso corporal final, ganho de carcaça, rendimento de carcaça, eficiência alimentar, área muscular do *Longissimus thoracis*, espessuras de gordura subcutânea e espessuras de gordura subcutânea da garupa ($P > 0,05$). Estes resultados sugerem que o glicerina bruto pode ser incluído em dietas de acabamento de bovinos de corte em níveis de até 15%, sem prejudicar o desempenho e as características de carcaça. Para o terceiro estudo, cinco experimentos *in vitro* foram conduzidos para avaliar a energia metabolizável e as mudanças na fermentação ruminal, produção total de gases e concentração de gases do efeito estufa da glicerina, comparada ao milho e ao amido, bem como sua inclusão em dietas de terminação para gado de corte. Para o Exp. 1, um sistema de 24 garrafas (AnkomRF Gas Production System, Ankom technology, NY, USA) foi usado em 4 corridas consecutivas de 48 h. Os tratamentos foram três diferentes alimentos: milho, glicerina e amido. A produção total de gases às 24 h, a concentração de acetato e a relação acetato: propionato diminuiu ($P < 0,01$) somente para glicerina. A produção total de gases às 48 h foi maior ($P < 0,01$) para o milho e semelhante entre glicerina e amido. O tratamento com amido teve a menor ($P < 0,01$) concentração total de AGV. O milho apresentou a menor concentração de propionato ($P < 0,01$). A energia metabolizável foi maior ($P < 0,01$) para o milho e semelhante entre glicerina e amido. Para o Exp. 2, um total de 20 garrafas (155ml) foram usadas em 4 corridas consecutivas de 48 h. Os tratamentos foram os mesmos do Exp. 1. A concentração de CH4 aumentou ($P < 0,01$) somente para a glicerina. A produção de CO2 em ml/g foi maior ($P < 0,01$) para o milho e semelhante para glicerina e amido. Comparado com a glicerina, a concentração de acetato ($P < 0,01$) e a relação acetato:

propionato ($P < 0,01$) foram maiores para amido e milho, enquanto a as concentrações de butirato e valerato foram maiores ($P < 0,01$) para a glicerina. Para o Exp. 3, o mesmo sistema do Exp. 1 foi usado em 4 corridas consecutivas de 48 h. Os tratamentos foram: inclusão de 0, 100, 200 e 300g/kg de MS de glicerina substituindo o milho em dietas de terminação de bovinos de corte. A inclusão de glicerina não afetou ($P > 0,05$) as produções de gases às 24 e 48 h, o pH final, $N\text{-}NH_3$, AGV total, e as concentrações de propionato e butirato. A inclusão de glicerina diminuiu linearmente a concentração de acetato ($P = 0,03$) e a relação acetato: propionato ($P = 0,04$). Para o Exp. 4, um total de 25 garrafas (155ml) foram usadas em 4 corridas consecutivas de 48 h. Os tratamentos foram os mesmos do Exp. 3. A inclusão de glicerina não afetou ($P > 0,05$) as concentrações de CH4, CO2, AGV totais, propionato, butirato, relação acetato: propionato e pH final. Foi observada diminuição linear ($P = 0,04$) na concentração de acetato devido à inclusão de glicerina nas dietas. Para o Exp. 5, dois sistemas de quatro vasos de digestão de 4 L (Daisy^{II} system, Ankom technology, NY, USA) foram usados em duas corridas consecutivas de 48 h. Os tratamentos foram: feno de orchard (0,4 g/saco), milho (0,4 g/saco), feno de orchard (0,4 g/saco) + glicerina (0,2 g/saco) e milho (0,4 g/saco) + glicerina (0,2 g/saco). O feno de orchard com glicerina apresentou a menor ($P < 0,01$) digestibilidade in vitro da MS, seguido pelo feno de orchard sem adição de glicerina e pelos tratamentos com milho. A digestibilidade in vitro da MS não diferiu ($P > 0,05$) entre os tratamentos com milho sem ou com adição de glicerina. Como conclusão, sob essas condições experimentais, a glicerina contribuiu mais para o aumento da metanogenese do que carboidratos, mas efetivamente substituiu parcialmente o milho como fonte de energia em dietas para bovinos de corte.

INTRODUCTION

Corn is recognized as the main energy source for feeding cattle, being the primary source of grain used in feedlot diets. However, increased demands for corn in recent years due to ethanol production and human utilization have contributed to increased corn prices. Furthermore, as corn is a commodity, its market prices might be subject to large fluctuations depending on global supply and demand, which may increase beef cattle production costs. Therefore, because corn has been traditionally used as the main energy source in cattle diets, alternative energy sources are needed.

Some studies in the last decades have observed good performance results of energetic agro-industrial byproducts, such as citrus pulp (Prado et al., 2000), soybean hulls (Ezequiel et al., 2006), cassava residues (Marques et al., 2000), among others. Such research focus is important not only for improving performance, but also from sustainable perspectives since it shows that agriculture residues could partially replace corn as an energy source for finishing beef cattle diets. Therefore, the glycerin, an energetic residue from biodiesel production, becomes great option to be evaluated.

Biodiesel is a biofuel produced from oleaginous plants and some animal fat sources that can be considered an environmentally friendly and biodegradable fuel. It comes out ahead in the search for renewable energy sources, by reducing the emission of greenhouse gases (ANP, 2014). Because of these ecological advantages of the biodiesel utilization, the Brazilian government created in 2004 a national program that establishes the inclusion of biofuel in the diesel oil sold in Brazil. Thereby, the biodiesel production has had a high growth. The program started with the requirement of 3% of biodiesel inclusion in the diesel oil, however, due to the success of the program this required level have increased to 7% since November 2014 (ANP, 2014).

According to Brazilian National Agency of Petroleum, Natural Gas and Biofuels, Brazil produced 3.42 billion liters of biodiesel in 2014, having increased 112% in the last five years. Each 100 litters of biodiesel can yield approximately 10 litters of glycerin; thereby 341 million liters of glycerin were produced in 2014. Thus, the traditional glycerin consumers markets, such as cosmetics industry, medicine, food and chemistry, are not able to absorb all this production, which might lead to lower prices. Faced with these prospects of lowering prices, glycerin comes up as great alternative to corn as an energy source in beef cattle diets. Furthermore, livestock production can use the non purified (crude) glycerin,

which is cheaper than the purified one and cannot be used by traditional markets. Therefore, the growth of the biodiesel industry has increased the availability of low cost crude glycerin, which may position it as a viable alternative feed source.

Due to glycerin high potential use in animals' diets, the Ministry of Agriculture, Livestock and Food Supply (MAPA) has approved the product as an energy ingredient for animal feed. However, the composition of the glycerin commercialized in Brazil may vary according to the biodiesel extraction method utilized.

The glycerin (or glycerol) is an organic compound belonging to the alcohol function (IUPAC, 1993), being fluid at room temperature, odorless, hygroscopic, viscous, and having sweet taste. It is usually found in ruminant feed as a component of triglycerides and phospholipids, and it has an estimated metabolic energy of 4.03 Mcal/kg (Mach et al., 2009), which is higher than corn starch (Monnerat et al., 2013). In the rumen, microorganisms ferment glycerin to propionate (Rémond et al., 1993; Del Bianco Benedeti et al., 2015), main gluconeogenic precursor for ruminant animals (Bergman et al., 1968). Looking from a sustainable perspective, previous research has demonstrated that propionate decreases methane (CH4) production because pathways to propionate production act as a hydrogen sink (Moss et al., 2000; Boadi et al., 2004), and would reduce the availability of hydrogen for CH4 formation by changing the overall electron balance in the rumen (Lee et al., 2011). Thereby, the partial replacement of corn with a biofuel product such as glycerin might reduce CH4 production in beef cattle. Furthermore, glycerin may also increase ruminal starch digestibility when included in diets for beef cattle (Hales et al., 2013), which could increase total volatile fatty acid concentration and improve beef cattle energy efficiency.

The glycerin that escapes rumen fermentation may be absorbed by the rumen wall or small intestine, reaching the liver by bloodstream (Rémond et al., 1993). In the liver and adipocytes, glycerin is a precursor for the synthesis of triglycerides and phospholipids, which may increase the fat deposition. Moreover, the use of glycerin may increase muscle deposition, since glycerin is preferably used than protein for ATP production. It can be also converted to glucose in the liver (Bergman et al., 1968), providing energy for cell metabolism. Glucose is an important carbon source used for fatty acid synthesis (Schoonmaker et al., 2004), which may lead to an increase in marbling of meat (Versemann et al., 2008). Therefore, partial replacement of corn with a biodiesel by-product such as glycerin may improve energy efficiency in beef cattle, which is desirable from both energetic

and environmental perspectives.

Studies have reported the effects of glycerin inclusion on ruminal fermentation (Rico et al., 2012) and performance (Ramos and Kerley, 2012) when glycerin was included as a corn alternative in beef cattle diets; however, the effects of high glycerin inclusion in high concentrate diets for finishing cattle have not been evaluated and the maximum levels of glycerin use have not been established

Therefore, this study was developed based on three independently experiments with the objective to:

1- Evaluate the effects of partially replacing dry ground corn with glycerin on ruminal parameters using a dual-flow continuous culture system;

2- Assess the effects of partial replacing corn with crude glycerin on performance and carcass characteristics of Nellore bulls finished in feedlot;

3- Identify the influence of glycerin inclusion on *in vitro* rumen fermentation, total gas and methane productions in finishing beef diets.

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Chapter I

Effects of Partial Replacement of Corn with Glycerin on Ruminal Fermentation in a Dual- Flow Continuous Culture System

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Abbreviations: ADF, acid detergent fiber; BCVFA, branched-chain volatile fatty acids; CP, crude protein; DM, dry matter; NAN, non-ammonia nitrogen; NH3-N, ammonia nitrogen; NDF, neutral detergent fiber; OM, organic matter; VFA, volatile fatty acids.

Abstract

The objective of this study was to evaluate the effects of partially replacing dry ground corn with glycerin on ruminal fermentation using a dual-flow continuous culture system. Six fermenters (1,223 ± 21 ml) were used in a replicated 3x3 Latin square arrangement with three periods of 10 d each, with 7 d for diet adaptation and 3 d for sample collections. All diets contained 75% concentrate and three dietary glycerin levels (0, 15, and 30% on dry matter basis), totaling six replicates per treatment. Fermenters were fed 72 g of dry matter/d equally divided in two meals/d, at 0800 and 2000 h. Solid and liquid dilution rates were adjusted daily to 5.5 and 11%/h, respectively. On d 8, 9, and 10, samples of 500 ml of solid and liquid digesta effluent were mixed, homogenized, and stored at -20°C. Subsamples of 10 ml were collected and preserved with 0.2 mL of a 50% H_2SO_4 solution for later determination of NH_3-N and volatile fatty acids. Microbial biomass was isolated from fermenters for chemical analysis at the end of each experimental period. Data were analyzed using the MIXED procedure in SAS with $\alpha = 0.05$. Glycerin levels did not affect apparent digestibility of DM ($P > 0.05$), OM ($P > 0.05$), NDF ($P > 0.05$) and ADF ($P > 0.05$). Also, glycerin inclusion did not affect true digestibility of DM ($P > 0.05$), and OM ($P > 0.05$). Concentrations of propionate ($P < 0.01$) and total volatile fatty acids ($P < 0.01$) increased linearly and concentrations of acetate ($P < 0.01$), butyrate ($P = 0.01$), iso-valerate ($P < 0.01$), and total branched-chain volatile fatty acids, as well as the acetate: propionate ratio ($P < 0.01$) decreased with glycerin inclusion. Linear increases on NH3-N concentration in digesta effluent ($P < 0.01$) and on NH_3-N flow ($P < 0.01$) were observed due to glycerin inclusion in the diets. Crude protein digestibility ($P = 0.04$) and microbial N flow ($P = 0.04$) were greater in the control treatment compared with the other treatments and responded quadratically with glycerin inclusion. Furthermore, the inclusion of glycerin linearly decreased ($P = 0.02$) non-ammonia N flow. Glycerin levels did not affect the flows of total N ($P > 0.05$), and dietary N ($P > 0.05$), as well as microbial efficiency ($P > 0.05$). These results suggest that partially replacing dry ground corn with glycerin may change ruminal fermentation, by increasing total volatile fatty acids, and propionate concentration without affecting microbial efficiency, which may improve glucogenic potential of beef cattle diets.

Resumo

O objetivo deste estudo foi avaliar os efeitos da substituição parcial do milho seco por glicerina sobre a fermentação ruminal utilizando um sistema de cultura contínua de fluxo duplo. Seis fermentadores (1,223 ± 21 ml) foram utilizados em um delineamento em quadrado latino 3x3 replicado, com três períodos de 10 d cada, 7 d para adaptação à dieta e 3 d para coletas de amostras. Todas as dietas continham 75% de concentrado e três níveis de glicerina na dieta (0, 15, e 30% com base na matéria seca), totalizando seis repetições por tratamento. Os fermentadores foram alimentados com 72 g de matéria seca/d igualmente divididos em duas refeições/d, às 08:00 e 20:00 h. A taxas de diluição de sólido e líquido foram ajustadas diariamente a 5,5 e 11 %/h, respectivamente. Nos d 8, 9, e 10, amostras de 500 ml de efluente digesta sólido e líquido foram misturadas, homogeneizadas, e armazenadas a -20 ° C. Sub-amostras de 10 ml foram recolhidas e conservadas com 0,2 ml de solução de H_2SO_4 a 50% para posterior determinação de N-NH_3 e ácidos graxos voláteis. A biomassa microbiana foi isolada do liquido ruminal dos fermentadores para a análise química, no final de cada período experimental. Os dados foram analisados o procedimento MIXED do SAS com $\alpha = 0,05$. Os níveis de glicerina não afetaram a digestibilidade aparente da MS ($P > 0.05$), MO ($P > 0.05$), FDN ($P > 0.05$) e FDA ($P > 0.05$). Além disso, a inclusão de glicerina não afetou a digestibilidade verdadeira da MS ($P > 0.05$), e MO ($P > 0.05$). As concentrações de propionato de ($P < 0,01$) e ácidos graxos voláteis totais ($P < 0,01$) aumentaram de forma linear. Já as concentrações de acetato ($P < 0,01$), butirato ($P = 0,01$), iso-valerato ($P < 0,01$), e ácidos graxos voláteis de cadeia ramificada, assim como a relação acetato: propionato ($P < 0,01$) diminuíram com a inclusão de glicerina. Foi observado aumento linear na concentração de N-NH_3 dos efluentes ($P < 0,01$) e no fluxo de N-NH_3 ($P < 0,01$), devido à inclusão de glicerina nas dietas. A digestibilidade da proteína bruta ($P = 0,04$) e o fluxo de N microbiano ($P = 0,04$) foram maiores no tratamento controle em comparação com os outros tratamentos e responderam de forma quadrática com a inclusão de glicerina. Além disso, a inclusão de glicerina diminuiu linearmente ($P = 0,02$) o fluxo de N não amoniacal. Os níveis de glicerina não afetaram os fluxos de N total ($P > 0.05$), e N dietético ($P > 0.05$), assim como a eficiência microbiana ($P > 0.05$). Estes resultados sugerem que substituição ao milho seco por glicerina pode mudar a fermentação ruminal, aumentando a concentração de ácidos graxos voláteis totais e propionato, sem afetar a eficiência microbiana, o que pode melhorar o potencial gliconeogenico em dietas para bovinos de corte.

Introduction

Increased demands for corn in recent years due to ethanol production and human utilization have contributed to increased corn prices. Because corn has been traditionally used as the main energy source in cattle diets, alternative energy sources are needed. Glycerin is an important by-product of biodiesel production and can be converted to propionate in the rumen [1], which is the main glucogenic precursor in ruminants [2]. Glucose is the main carbon source used for fatty acid synthesis [3], which may lead to an increase in marbling of meat [4]. Furthermore, glycerin may also increase starch digestibility when included in diets for beef cattle [1], which could increase total volatile fatty acid (VFA) concentration and improve beef cattle energy efficiency. Therefore, partial replacement of corn with a biodiesel by-product such as glycerin may improve energy efficiency in beef cattle, which is desirable from both energetic and environmental perspectives.

Studies have reported the effects of glycerin inclusion on ruminal fermentation when glycerin was included at up to 20% [5,6] in beef cattle diets; however, the effects of higher inclusion levels in high concentrate diets for finishing cattle have not been evaluated and the maximum levels of glycerin use have not been established. The objective of this study was to evaluate the effects of partially replacing dry ground corn with glycerin on ruminal parameters using a dual-flow continuous culture system. We hypothesized that glycerin may partially replace corn as an energy source in finishing beef cattle diets and may be included at concentrations up to 30% [dry matter (DM) basis], increasing propionate concentration without compromising ruminal fermentation, digestibility, microbial N flow and microbial efficiency in a dual-flow continuous culture system.

Materials and methods

Ethical approval

The care and handling of all experimental animals, including ruminal cannulation were con- ducted under protocols approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (IACUC protocol number 00588). Ruminal cannulations were conducted 12 months prior to the experiment by Dr. Walter Mandeville, a clinical veterinarian from the University of Nevada, Reno. Standard surgical preparation was used on the left paralumbar fossa and sterile drapes and gowns were utilized, hair on the surgery area was clipped using a #40 blade and the area was scrubbed three times alternating povidone-

iodine and 70% alcohol. Local anesthesia was provided using an inverted L block with 150 cc 2% lidocaine hydrochloride and butorphanol tartrate was administered intravenously at a dosage of 0.05 mg/kg. Animals were restrained in a stanchion and a 10 cm incision was made through the skin and subcutaneous tissue and the peritoneum was incised to open the abdominal cavity. Stay sutures were placed in an avascular area of the rumen and the rumen was sutured to the peritoneum and the skin. A section of the rumen was excised and a ruminal cannula (7.5 cm in diameter, Bar Diamond Inc., Parma, ID) was inserted into the rumen fistula. To control post-operative infection, 30 cc penicillin was given once a day for seven days and post-operative discomfort was alleviated by administering flunixin meglumine at dosage of 1.6 mg/kg intravenously once daily for seven days.

Experiment Design and Diets

Six 1,223 ± 21 ml dual-flow continuous-culture fermenters, similar to that described by Hoover et al. [7], were used in three consecutive 10 d periods with 7 d for adaptation and 3 d for sampling. The experimental design included two 3 x 3 Latin squares, which were run simultaneously with six replicates per treatment. The treatments were: inclusion of 0, 15, and 30% (DM basis) of glycerin (99.7% of purity; Nature's Oil, Streetsboro, OH, USA) replacing corn in finishing beef diets. Experimental diets were composed of 25% wheat straw and 75% concentrate (DM basis) and were formulated to meet the nutrient requirements of beef cattle recommendations [8]. Dietary ingredients were ground to pass a 2 mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA) and diets were formulated to be isonitrogenous and urea was used to equalize nitrogen levels. Ingredient proportion and chemical composition of the experimental diets are presented in Table 1.

Dual-flow Continuous Culture System Operation and Sample Collection

On d 1, fermenters were inoculated with rumen fluid from two rumen cannulated Aberdeen Angus steers (Average BW of 770 kg). Steers were maintained on a total mix diet of 40% grass hay, 49% dry ground corn, 8% soybean meal, and 3% mineralized salt. Two hours after feeding, 10 L of rumen contents were collected, immediately filtered through 4 layers of cheesecloth and placed into pre-warmed thermal containers. Equal amounts of rumen content from each animal were homogenized thoroughly by agitation, infused with N_2 to maintain the anaerobic environment and adjusted to 39°C by submerging a 5,000 mL

Erlenmeyer flask in a pre-heated water bath. The rumen fluid was poured into each of the pre-warmed fermenters until it cleared the overflow spout.

In accordance with Hoover et al. [7], fermentation conditions were maintained constant with a temperature of 39°C and N_2 (40 ml/min) was infused into the fermenters to maintain the anaerobic conditions of the system. However, the pH was not controlled and urea was added to the artificial saliva to simulate recycled N [9]. Individual Cole-Parmer pH controllers (Model 5997–20) were used to monitor the pH of each fermenter. A central propeller apparatus driven by magnets was used to continuously agitate the fermenters contents. Artificial saliva infusion and filtered liquid flows were set to maintain solids and liquids rates at 5.5 and 11%/h, respectively, by adjusting buffer input and solid and liquid removal [10], to mimic real passage rates in beef cattle [11].

Fermenters were fed 72 g of DM/d of diet equally divided in two meals per d, at 0800 and 2000 h. Digesta effluent (solid and liquid) were collected in 4 L plastic containers. At 0730 h of each day of the adaptation period, the containers were weighed and contents were discarded. Twenty-four hours prior to the first collection and during the 3 d sampling period (d 8, 9, and 10), each container received 20 mL of 50% H_2SO_4 and were immersed halfway in a chilled (2– 4°C) water bath to stop microbial and enzymatic activities. On d 8, 9, and 10, samples of 500 ml of solid and liquid digesta effluent were mixed, homogenized (T25 basics, IKA Works, Inc, Wilmington, NC 28405), and stored at -20°C. A subsample of 10 ml was filtered through two layers of cheesecloth. Then, 0.2 mL of a 50% H_2SO_4 solution was added for later determination of ammonia nitrogen (NH_3-N) [12] and VFA. At the end of each period, digesta effluent from the three sampling days were composited by fermenter and freeze dried for further analysis.

On d 5, digesta effluent (solid and liquid) was homogenized by vigorous hand shaking and samples were collected to determine the digesta effluent background ^{15}N abundance [13]. Then, 0.077 g of 10.2% excess $^{15}(NH_4)_2SO_4$ (Sigma-Aldrich Co., St. Louis, MO) was infused into each fermenter to instantaneously label the NH_3-N Pool. Saliva was reformulated and 0.077 g/L of enriched $^{15}(NH_4)_2SO_4$ (Sigma-Aldrich Co., St. Louis, MO) were added in replacement of isonitrogenous amounts of urea to maintain a steady-state concentration of ^{15}N enrichment in fermenters.

Ruminal liquid from fermenters were sampled on d 8 to determine NH_3-N concentration in different time-points; 10 mL of liquid samples were collected at different

time points: 0, 1, 2, 4, 6, 8, and 10 h after feeding, filtered through a double layer of cheesecloth. Then, the samples were analyzed for NH_3-N [12]. At the same time-points, pH was measured with an Accumet portable AP61 pH meter (Fisher Scientific, Atlanta, GA). At the end of each 10 d experimental period, the fermenter contents were strained through 2 layers of cheesecloth, and centrifuged at 1,000 x g for 10 min to remove undigested substrate and possibly some attached microbial biomass [14]. Then, the supernatant was centrifuged at 10,000 x g for 20 min to isolate pure microbial biomass pellets [14]. Supernatant was discarded and microbial pellets were freeze dried and stored for further ^{15}N enrichment analysis [13].

Chemical Analyses and Calculations

Feed and digesta effluent samples were analyzed for DM (method 934.01), ash (method 938.08), crude protein (CP; method 984.13), and ether extract (method 920.85) according to AOAC [15]. The organic matter (OM) was calculated as the difference between DM and ash contents. For neutral detergent fiber (NDF) and acid detergent fiber (ADF), samples were sequentially analyzed, treated with alpha thermo-stable amylase without sodium sulfite according to Van Soest et al. [16] and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). Samples of microbial pellet and digesta effluent background were analyzed for DM, CP, and ash as detailed previously for feed samples.

Volatile fatty acid concentrations in the digesta effluent were determined using gas chromatography (Varian Model 3800; Varian, Inc, Walnut Creek, CA; equipped with a glass column [180 cm x 4 mm i.d.]) packed with GP 10% SP-1200/1% H3PO4 on 80/100 Chromosorb WAW [Supelco, Bellefonte, PA]), and N was used as a carrier gas at a flow rate of 85 mL/min-1. The NH_3-N concentrations (on fermenter and effluent digesta) were determined by colorimetric as described by Chaney and Marbach [12].

Background, digesta effluent and microbial pellets samples were analyzed for total N and ^{15}N enrichment according to Werner et al. [17]. Isotope analyses were performed using an Eurovector model 3028 elemental analyzer interfaced to a Micromass Isoprime stable isotope ratio mass spectrometer. Microbial N flow and microbial efficiency were calculated as follows: Microbial N flow (expressed in g/d) = [non-ammonia N (NAN) flow x percentage of ^{15}N atom excess of digesta effluent]/(percentage of ^{15}N atom of microbial pellet), with ^{15}N

digesta effluent background subtracted from ^{15}N enrichment. Microbial efficiency = Microbial N flow (g) / OM truly digestible (kg) [13]. According to Soder et al. [18], apparent and true digestibilities were calculated as follows (using DM as an example): DM apparently digested (%) = [(g of DM intake - g of effluent flow DM) / g of DM intake] x 100; DM truly digested (%) = {[g of DM intake - (g of effluent flow DM - g of microbial DM)] / g of DM intake} x 100. Effluent was corrected for grams of buffer in both equations.

Statistical Analysis

All results were tested for normality [19], and they followed normal distribution ($P > 0.05$). All statistical procedures were carried out using SAS 9.2 for Windows (Statistical Analysis System Institute, Inc., Cary, NC, USA) with $\alpha = 0.05$. Variance Components was used as the covariance structure in the two models used. Nutrient flow and digestibility, VFA, N metabolism (including NH3-N from digesta effluent), and microbial efficiency were analyzed for linear and quadratic responses using the following model:

$$Y_{ijk} = B_0 + B_1X_i + B_2X_i^2 + P_j + A_k + e_{ijk},$$

where:

Y_{ijk} is the observed measurement of the i^{th} level of glycerin inclusion in the diet of the j^{th} period, and of the k^{th} experimental unit; i = 1, 2, 3 (levels of inclusion of glycerin as a replacement of corn), B_0, B_1, B_2 = regression parameters of the model; X_i = effect of i^{th} level of fixed quantitative factor (replacement of glycerin with corn); P_j = effect of level of random factor period; A_k = effect of level of random factor fermenter; e_{ijkl} = residual error, assuming $e_{ijkl} \sim N(0, s^2)$.

Only pH and NH_3-N data collected from fermenters over time were analyzed as repeated measurements and the subject of the repeated statement was the fermenter within period. All one-way, two-way and three-way interactions were tested. Outliers were identified when the Studentized residue was greater than 2.5 or smaller than -2.5. All non-significant effects ($P > 0.05$) were removed from the model to determine the final equation. Therefore, pH and NH_3-N were analyzed for linear, quadratic, and cubic responses of time using the following model:

$$Y_{ijklm} = B_0 + B_1X_i + B_2X_i^2 + B_3T_k + B_4X_iT_k + B_5X_i^2T_k + B_6T_k^2 + B_7X_iT_k^2 + B_8X_i^2T_k^2 + B_9T_k^3 + B_{10}X_iT_k^3 + B_{11}X_i^2T_k^3 + P_j + A_l + e_{ijklm},$$

where:

Y_{ijklm} is the observed measurement of the i^{th} level of glycerin inclusion in the diet of the j^{th} period, of the k^{th} time, of the m^{th} experimental unit replication; i = 1, 2, 3 (levels of inclusion of glycerin as a replacement of corn), B_0, B_1, . . . B_{11} = regression parameters of the model; X_i = effect of i^{th} level of fixed quantitative factor (replacement of glycerin with corn); T_k = effect of k^{th} level of time; P_j = effect of level of random factor period; A_l = effect of level of random factor fermenter; e_{ijklm} = residual error, assuming $e_{ijklm} \sim N(0, s^2)$.

Results

Apparent and True Digestibility

Glycerin levels did not affect apparent digestibility of DM ($P_{Lin.}$ = 0.13; $P_{Quad.}$ = 0.40), OM ($P_{Lin.}$ = 0.72; $P_{Quad.}$ = 0.15), NDF ($P_{Lin.}$ = 0.38; $P_{Quad.}$ = 0.50) and ADF ($P_{Lin.}$ = 0.91; $P_{Quad.}$ = 0.18), which averaged 33.5 ± 2.63, 41.6 ± 2.56, 75.6 ± 4.07, and 70.6 ± 3.17%, respectively (Table 2). Also, glycerin inclusion did not affect true digestibility of DM ($P_{Lin.}$ = 0.35; $P_{Quad.}$ = 0.48), and OM ($P_{Lin.}$ = 0.08; $P_{Quad.}$ = 0.19), which averaged 57.7 ± 6.00%, and 59.6 ± 3.92%, respectively.

Volatile fatty acids

The inclusion of glycerin linearly increased total VFA ($P < 0.01$) and propionate concentrations ($P < 0.01$; Table 3). Compared to the control (0% glycerin), total VFA and propionate concentrations increased 11 and 105%, respectively, when 30% glycerin was fed. Concentrations of acetate ($P < 0.01$), butyrate ($P = 0.01$), iso-valerate ($P < 0.01$), and total BCVFA ($P < 0.01$), as well as the acetate: propionate ratio ($P < 0.01$) decreased linearly as glycerin replaced corn. Iso-butyrate concentration was greater for control compared with the other treatments and responded quadratically ($P = 0.03$) as dietary glycerin increased. Glycerin levels did not affect valerate concentration ($P_{Lin.}$ = 0.45; $P_{Quad.}$ = 0.53), which averaged 1.06 ± 0.21%.

Ruminal pH and NH_3-N over Time

Two equations were generated to describe ruminal pH and NH_3-N over time (Fig 1). No differences were observed ($P = 0.09$) on pH among different levels of glycerin. However, sampling time had a quadratic effect ($P < 0.01$) on ruminal pH, with the critical point (5.85) being reached 3 h after feeding. There was an interaction between inclusion of glycerin in the

diet and sampling time for ruminal NH_3-N ($P = 0.02$). Sampling time had a cubic effect ($P = 0.04$) on concentrations of ruminal NH_3-N, which were higher throughout the day for fermenters fed 30% glycerin.

Nitrogen Metabolism and Microbial Efficiency

A linear increase of NH_3-N concentration in digesta effluent ($P < 0.01$), and NH_3-N flow ($P < 0.01$) were observed due to inclusion of glycerin in the diets (Table 4). The digesta effluent NH_3-N concentration of 30% glycerin inclusion was about 61% greater than the control treatment. The CP digestibility ($P = 0.04$) and microbial N flow ($P = 0.04$) were greater for control (0% glycerin) compared to the other treatments and responded quadratically as glycerin replaced corn. Furthermore, the inclusion of glycerin linearly decreased NAN flow ($P = 0.02$). Glycerin levels did not affect the flows of total N ($P_{Lin.} = 0.79$; $P_{Quad.} = 0.35$), and dietary N ($P_{Lin.} = 0.99$; $P_{Quad.} = 0.07$), as well as microbial efficiency ($P_{Lin.} = 0.09$; $P_{Quad.} = 0.07$), which averaged 1.53 ± 0.14 g/d, 0.23 ± 0.11 g/d, and 22.3 ± 3.23 g of microbial N/kg of OM truly digested, respectively.

Discussion

Apparent and True Digestibility

We hypothesized that glycerin could replace corn and included at up to 30% in beef cattle diets without compromising apparent and true digestibility in a dual-flow continuous culture system. As expected, the replacement of corn with glycerin did not change any digestibility parameter. Lack of effects in ruminal DM and OM digestibility has been observed when glycerin was included in cattle diets, both in vitro [20] and in vivo [6,21]. These authors suggest that quick microbial adaptation and fast glycerin ruminal turnover [22,23,24] as well as high VFA production [23,25,26] may be possible reasons for this observation. According to Van Soest [27], the rates of digestion and passage are the two main factors that affect the digestibility. As the passage rate was set to be the same for all treatments, it seems that microbial adaptation played a role at maintaining adequate nutrient digestion among treatments. The increase in VFA concentration and the lack of differences for microbial efficiency observed in this study help explaining the results observed for digestibility. Therefore, these results suggest that glycerin inclusion at up to 30% in high concentrate diets has no detrimental effects on ruminal digestibility.

Volatile fatty acids

The findings in this study contradict our hypothesis that glycerin inclusion would not modify total VFA concentration in the rumen. The increase on total VFA concentration is probably related to the fact that glycerin has an additive relationship with corn-starch digestion [1]. Hales et al. [1] observed increase on starch digestibility when glycerin was included in diets of beef steers. According to these authors, glycerin stimulates amylolitic bacteria, such as *Selenomonas ruminantium* [28] and *Streptococcus bovis* [29], which makes glycerin an ingredient with potential to increase energy availability in finishing diets, which are rich in starch. Also, increases in total VFA concentration might be related to higher ruminal NH_3-N concentration [30]. Other studies have found increases in VFA concentration due to higher NH_3-N levels even when NH_3-N levels in the control diets were not limiting [31,32,33]. Thus, higher ruminal NH_3-N observed in glycerin treatments may also explain the increase on total VFA concentration for these treatments in this study. Results for total VFA have been conflicting with previous studies that observed no effects [5,34,35,36,37] or an increase [22,23,24,38] in total VFA concentration in the rumen when glycerin was fed.

The shift on VFA profile, with an increase in propionate concentration at the expense of acetate concentration and the decrease on acetate: propionate ratio were expected. However, the increase in propionate was also associated with a decrease in butyrate and iso-acids concentrations. Glycerin is preferentially fermented to propionate rather than acetate [5,20]. As mentioned before, glycerin stimulates *Selenomonas ruminantium* subsp. *lactilyca* [28], which are propionate producing bacteria [29]. Moreover, one of the main glycerin-fermenting bacteria, Anaerovibrio lipolytica does not have acetate as the main fermentation product when glycerin is the substrate [29]. Similar results were observed by Avila et al. [34] when the shift on VFA profile occurred, with increase of propionate at the expense of acetate and butyrate concentrations in an in vitro experiment when glycerin (99.5% purity) was included at up to 21% (DM basis). Rico et al. [35] also noted an increase in propionate and a decrease in acetate concentrations when fermenters in a continuous culture system were fed diets containing up to 8% glycerin (DM basis). Moreover, Shin et al. [36] observed an increase in propionate molar proportions at the expense of acetate in rumen cannulated Holstein cows when glycerin (75.8% purity) was included in the diet at up to 10% of DM. Furthermore, Avila-Stagno et al. [38] observed increases in total VFA and propionate

concentrations and decrease in acetate: propionate ratio in a semi-continuous culture system when glycerin (99.5% purity) was included at up to 15% (DM basis) in forage-based diets. Other studies indicated that dietary glycerin decreases acetate: propionate ratio when included at up to 10% [1], 17.5% [21] and 20% [5] in beef cattle diets. The reduction in iso-acids in treatments with glycerin may be explained by the decrease on CP digestibility that was observed in this study. Iso-acids are produced mainly by the amino acid fermentation in the rumen [39]. Nevertheless, the increase in propionate concentrations in this study indicates higher glucogenic potential when glycerin replaces corn in beef cattle diets. This might be important for finishing animals since glucose is the main precursor of intramuscular fatty acid synthesis [40].

Ruminal pH and NH_3-N over Time

Partial dietary replacement of corn with glycerin did not affect ruminal pH over time in this study. As no variation was observed in digestibility parameters and microbial efficiency, the lack of effect on pH is justified. Ruminal pH is associated to ruminal VFA fermentation [41]. Ruminal pH had similar pattern for all treatments, reaching the critical point (peak of minimum values) 3 h after feeding. The glycerin has a fast fermentation in the rumen [22,23,24], such that 50 to 80% of glycerin disappears within 4 h [42], which could decrease ruminal pH faster than corn. However, corn was ground in this study, allowing faster access to starch-fermenting bacteria, which might have caused similar fermentation rate than glycerin. Similar results were observed by Rico et al. [35], when glycerin inclusion did not affected pH in a continuous culture experiment in which diet components were ground through a 4-mm mesh screen. Moreover, pH values observed in this study were within the optimal recommended values for cattle fed high concentrate diets [43,44]. However, the rumen might be more buffered in this study than in in vivo situations, since continuous culture system utilizes artificial saliva with constant rate of infusion.

The higher ruminal NH_3-N concentration in fermenters fed diets with glycerin are likely related to the higher dietary urea levels in the glycerin diets. Urea contributed with 0, 8.6, and 17.3% of the total dietary CP for the treatments with 0, 15, and 30% glycerin; respectively. Urea is quickly converted to NH_3-N in the rumen, which can increase NH_3-N levels faster than its use by microorganisms [44]. A fast release of nitrogen may cause an asynchrony of NH_3-N and energy in the ruminal environment. Thus, the low ruminal NH_3-N

concentration observed in the control treatment (0% glycerin) in this study may be related to better nitrogen: energy synchrony.

Nitrogen Metabolism and Microbial Efficiency

We hypothesized that glycerin inclusion would not modify nitrogen metabolism in a dual-flow continuous culture system. However, compared to corn, glycerin increased NH_3-N flow and decreased CP digestibility, and the flows of NAN and microbial-N. Nevertheless, there were no differences among treatments for total N flow and microbial efficiency. As mentioned before, glycerin increased NH_3-N concentration in the rumen, likely due to dietary urea. Thus, the rapid accumulation of ruminal NH_3-N could not be matched by the microorganism's rate of N incorporation. This may have reduced microbial N flow, and CP digestion in glycerin diets. Wang et al. [24] also observed that the in situ ruminal CP digestibility was reduced with increasing doses of glycerin in beef steers diets. However, in the same study it was observed an increase in total tract DM, OM, NDF, and CP digestibilities. The authors concluded that glycerin inclusion improved post ruminal nutrient digestibility. Furthermore, Hales et al. [1] observed a linear increase trend in post ruminal N digestibility when glycerin was included at up to 10% in diets of beef steers. Furthermore, in our study the lower microbial N flow in fermenters fed glycerin was not capable of affecting microbial efficiency, which did not differ among treatments. Ramos and Kerley [5] observed a linear increase in microbial efficiency in fermenters fed diets with glycerin at up to 20% of DM. According to these authors, this increase in microbial efficiency occurred mainly due to the decrease on OM digestibility found in their study. In the current study, glycerin inclusion did not affect ruminal OM digestibility as well as microbial efficiency. Therefore, these results lead us to conclude that glycerin inclusion in beef cattle diets may change N flow partitioning, reducing microbial-N, despite not changing total dietary nitrogen flow as well as microbial efficiency in a dual-flow continuous culture system.

Conclusion

These results suggest that partially replacing corn with glycerin may change ruminal fermentation; increasing total VFA, and propionate concentration without affecting rumen microbial efficiency in a dual-flow continuous culture system. From a glucogenic perspective, these findings suggest that the inclusion of glycerin would increase dietary

glucogenic potential, which is especially important for marbling in finishing animals. However, glycerin inclusion caused a decrease in microbial N flow, which might or might not be desirable depending on the animals' requirements. Therefore, under these experimental conditions, glycerin inclusion increased glucogenic precursors and effectively replaced dietary corn at up to 30% of the diet.

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Table 1. Ingredient and chemical composition of experimental diets

Item[1]	Glycerin, %		
	0	15	30
Ingredient, % DM			
Wheat straw	25.0	25.0	25.0
Dry ground corn	53.3	37.9	22.4
Glycerin[2]	0.0	15.0	30.0
Soybean meal	18.7	18.7	18.7
Urea	0.00	0.44	0.89
Mineralized salt[3]	3.00	3.00	3.00
Composition, % DM			
DM, %	88.9	90.0	91.1
OM	92.7	93.0	93.2
NDF	25.6	23.7	22.2
CP	14.5	14.5	14.5
Ether extract	3.43	2.67	1.91

[1]DM = dry matter; OM = organic matter; NDF = neutral detergent fiber; CP = crude protein.

[2]Purity of 99.7% (Nature's Oil, Streetsboro, OH, USA).

[3]Provided (per kg of DM): 955 g of NaCl, 3,500 ppm of Zn, 2,000 ppm of Fe, 1,800 ppm of Mn, 280 ppm of Cu, 100 ppm of I, and 60 ppm of Co.

Table 2. Effect of glycerin inclusion on apparent and true digestibility of dietary nutrients in dual-flow continuous culture system

Item[1]	Glycerin, %			SEM	*P*-value	
	0	15	30		Linear	Quadratic
Apparent Digestibility, %						
DM	32.7	35.0	35.1	0.64	0.13	0.40
OM	40.9	42.7	41.3	0.60	0.72	0.15
NDF	76.1	76.5	74.3	0.96	0.38	0.50
ADF	70.0	72.1	69.7	0.75	0.91	0.18
True Digestibility, %						
DM	60.1	56.3	56.8	1.42	0.35	0.48
OM	60.8	57.0	57.3	0.92	0.08	0.19

[1]DM = dry matter; OM = organic matter; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Table 3. Effect of glycerin inclusion on total and individual VFA concentrations in dual-flow continuous culture system

Item[1]	Glycerin, %			SEM	*P*-value	
	0	15	30		Linear	Quadratic
Total VFA, mM	113	123	125	1.81	< 0.01	0.12
VFA, mM						
Acetate	66.5	59.9	48.8	2.17	< 0.01	0.42
Propionate	25.3	43.5	58.6	3.55	< 0.01	0.47
Butyrate	17.7	16.4	13.3	0.82	0.01	0.52
Valerate	1.33	1.18	1.21	0.07	0.45	0.53
Iso-Butyrate	0.42	0.24	0.22	0.03	< 0.01	0.03
Iso-Valerate	1.61	1.19	0.69	0.11	< 0.01	0.74
BCVFA, mM	2.98	2.62	2.03	0.31	< 0.01	0.07
Acetate: propionate	2.65	1.41	0.84	0.19	< 0.01	0.22

[1]VFA = volatile fatty acids; BCVFA = Branched-chain VFA.

Table 4. Effect of glycerin inclusion on nitrogen metabolism of rumen microorganisms in dual-flow continuous culture system

Item[1]	Glycerin, %			SEM	*P*-value	
	0	15	30		Linear	Quadratic
NH_3-N, mg/100ml	11.0	13.1	17.8	0.90	< 0.01	0.24
CP digestibility, %	79.4	67.6	65.9	1.82	< 0.01	0.04
Nitrogen flow, g/d						
Total N	1.57	1.49	1.54	0.03	0.79	0.35
NH_3-N	0.35	0.41	0.56	0.03	< 0.01	0.22
NAN	1.24	1.11	0.98	0.05	0.02	0.95
Microbial N	0.99	0.75	0.79	0.04	0.01	0.04
Dietary N	0.20	0.30	0.20	0.03	0.99	0.07
Microbial efficiency[2]	23.9	19.4	20.4	0.78	0.09	0.07

[1]NH_3-N = ammonia nitrogen; CP = crude protein; NAN = non-ammonia nitrogen; OM = organic matter.

[2]Microbial efficiency = g of microbial N/kg of OM truly digested.

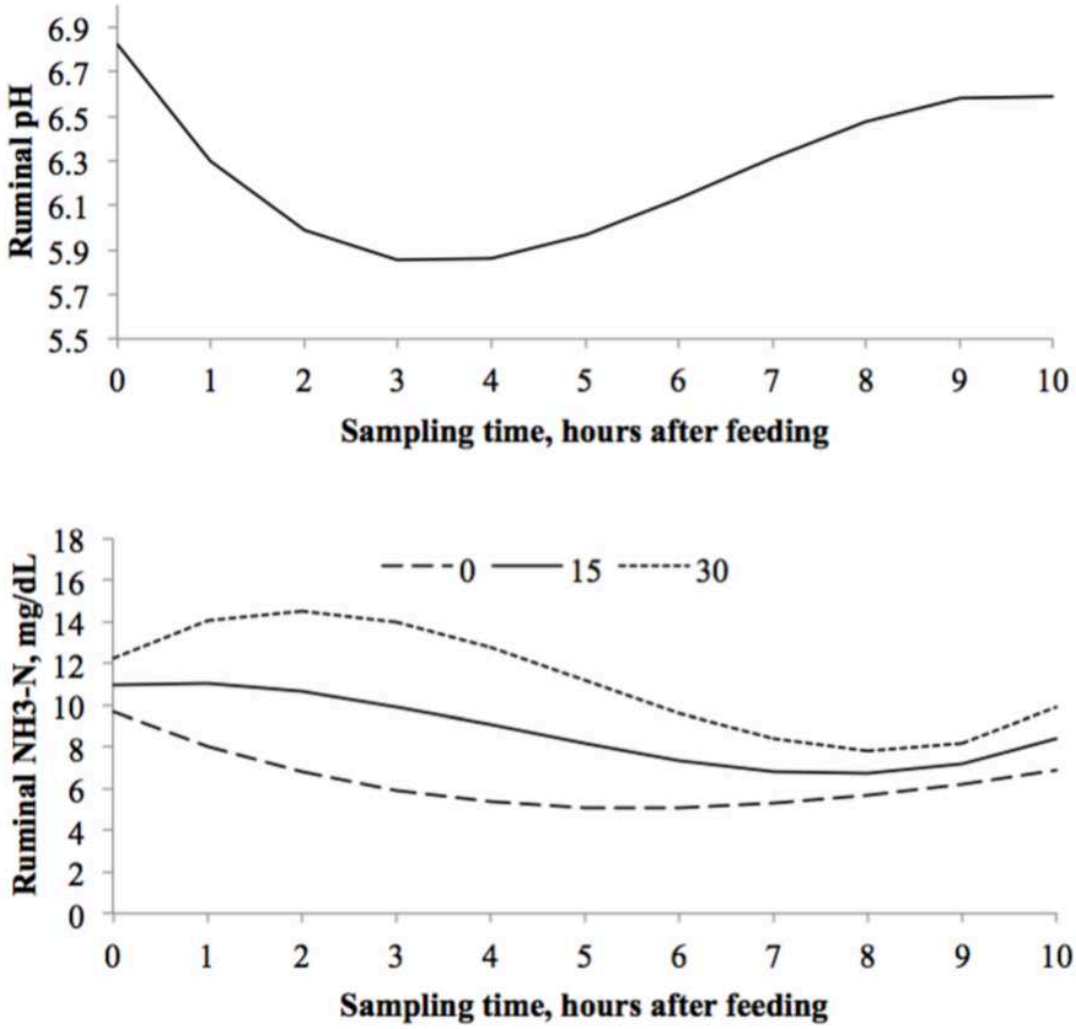

Figure 1. Effect of glycerin inclusion on ruminal pH and NH_3-N over time in dual-flow continuous culture system. pH = 6.825 ± 0.1727 - (TIME x 0.6487 ± 0.07149) + ($TIME^2$ x 0.1281± 0.01751) - ($TIME^3$ x 0.00656 ± 0.001143); R^2 = 0.999, MSE = 0.139. NH_3-N = 9.6399 ± 1.4148 + (glycerin x 0.08728 ± 0.05779) + (glycerin x TIME x 0.146 ± 0.06041) - (TIME x 1.8245 ± 1.1698) - (glycerin x $TIME^2$ x 0.03459 ± 0.0148) + ($TIME^2$ x 0.2095 ± 0.2866) + (glycerin x $TIME^3$ x 0.002012 ± 0.000966) - ($TIME^3$ x 0.00546 ± 0.01871); R^2 = 0.381, MSE = 11.75.

Chapter II

Partial Replacement of Ground Corn with Glycerol in Beef Cattle Diets: Intake, Digestibility, Performance, and Carcass Characteristics

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Abbreviations: ADG, average daily gain; BW, body weight; BFT, back fat thickness, CG = crude glycerol; CP, crude protein; DGC, dry ground corn; DM, dry matter; EE, ether extract; G:F, gain-to-feed ratio; LMA, *Longissimus thoracis* muscle area; NDF, neutral detergent fiber; NFC, non-fiber carbohydrate; OM, organic matter; RFT, rump fat thickness; TDN, total digestible nutrients.

Abstract

The objective of this study was to evaluate the effects of replacing dry ground corn with crude glycerol on intake, apparent digestibility, performance, and carcass characteristics of finishing beef bulls. A completely randomized block design experiment with 25 d for adaptation and 100 d for data collection was conducted, in which 3,640 Nellore bulls (367 ± 36.8 kg; 18 ± 3 mo) were blocked by body weight and assigned to 20 pens. Bulls were randomly assigned to one of four treatments: 0, 5, 10, and 15% (dry matter basis) of crude glycerol in the diet. Initially, 20 bulls were slaughtered to serve as a reference to estimate initial empty body weight, which allowed for carcass gain calculation. Bulls were weighed at the beginning, at two-thirds, and at the end of the experiment for performance calculations. Carcass measurements were obtained by ultrasound. Fecal output was estimated using indigestible neutral detergent fiber as an internal marker. Data were analyzed using the mixed procedures in SAS 9.2 (SAS Institute Inc., Cary, NC). Intake of dry matter, organic matter, and neutral detergent fiber decreased linearly ($P < 0.05$) with crude glycerol inclusion. However, crude glycerol levels did not affect ($P > 0.05$) intakes of crude protein, non-fiber carbohydrates, and total digestible nutrients. Digestibility of dry matter, organic matter, neutral detergent fiber, and total digestible nutrients increased quadratically ($P < 0.05$) with the inclusion of crude glycerol in the diet. Crude glycerol inclusion did not change the intake of digestible dry matter, average daily gain, final body weight, carcass gain, carcass dressing, gain-to-feed ratio, *Longissimus thoracis* muscle area, and back and rump fat thicknesses ($P > 0.05$). These results suggest that crude glycerol may be included in finishing beef diets at levels up to 15% without impairing performance and carcass characteristics.

Resumo

O objetivo deste estudo foi avaliar os efeitos da substituição do milho seco por glicerina bruto sobre o consumo, digestibilidade aparente, desempenho e características de carcaça de novilhos em terminação. O experimento foi delineado em blocos casualizados, com com 25 d para adaptação e 100 d para a coleta de dados, onde 3.640 touros da raça Nelore (367 ± 36,8 kg; 18 ± 3 meses) foram blocados por peso corporal e alocados em 20 baias. Os animais receberam, aleatoriamente, um dos quatro tratamentos: 0, 5, 10, e 15% (com base na matéria seca) de inclusão de glicerina bruto na dieta. Inicialmente, 20 animais foram abatidos para servir como referência para estimar o peso corporal vazio inicial, o que permitiu o cálculo de ganho de carcaça. Os animais foram pesados no início, a dois terços, e no final do experimento para os cálculos de desempenho. As medidas de carcaça foram obtidas por ultrassom. Produção fecal foi estimada utilizando fibra em detergente neutro indigerível como marcador interno. Os dados foram analisados utilizando os proc. mix. do SAS 9,2 (SAS Institute Inc., Cary, NC). Os consumos de matéria seca, matéria orgânica e fibra em detergente neutro diminuíram linearmente ($P < 0,05$) com a inclusão de glicerina bruto. No entanto, os níveis de glicerina bruto não afetaram ($P > 0,05$) o consumo de proteína bruta, carboidratos não fibrosos e nutrientes digestíveis totais. As digestibilidades da matéria seca, matéria orgânica, fibra em detergente neutro e nutrientes digestíveis totais aumentaram de forma quadrática ($P < 0,05$) com a inclusão de glicerina bruto na dieta. Inclusão de glicerina bruto não alterou o consumo de matéria seca digestível, ganho médio diário, peso corporal final, ganho de carcaça, rendimento de carcaça, eficiência alimentar, área muscular do *Longissimus thoracis*, espessuras de gordura subcutânea e espessuras de gordura subcutânea da garupa (P> 0,05). Estes resultados sugerem que o glicerina bruto pode ser incluído em dietas de acabamento de bovinos de corte em níveis de até 15%, sem prejudicar o desempenho e as características de carcaça.

Introduction

Corn is typically the main feed ingredient used for finishing cattle in feedlots [1]. However, due to its high cost, alternative energy sources may have the potential to improve livestock profitability. The growth of the biodiesel industry worldwide has increased the availability of low cost crude glycerol (CG), in Brazil alone, it has been estimated that in 2014 the country produced 3.42 billion liters of biodiesel, yielding 341 million liters of CG [2]. This may position CG as a viable alternative feed source for finishing cattle. The CG primary component is glycerol, which has an estimated metabolic energy of 4.03 Mcal/kg [3], a higher value than corn starch [4]. In the rumen, glycerol is fermented to propionate [5,6], main gluconeogenic precursor for ruminant animals [7]. Furthermore, the glycerol that escapes rumen fermentation may be converted to glucose in the liver [7]. Therefore, from both economic and energy perspectives, CG has the potential to partially replace corn as an alternative energy source for beef cattle finishing diets.

Some research has been done on the use of CG by ruminants [8,9]; however, the effects of CG have been conflicting and the maximum levels of CG in the diet of finishing cattle have not been established. Discrepancies across experiments may be due to the limited number of experimental units used among other aspects; therefore, it is relevant to evaluate the effects of CG using a large number of animals.

The objective of this study was to evaluate the effects of replacing corn with CG in the diets of 3,640 Nellore bulls finished in feedlot. We hypothesized that CG may partially replace dry ground corn (DGC) as dietary energy source in the diet of finishing cattle diets and may be included at concentrations up to 15% [dry matter (DM) basis] without compromising intake, apparent digestibility, performance, and carcass characteristics.

Materials and methods

Ethics Statement

Care and handling of all experimental animals were conducted under protocols approved by the Institutional Animal Care and Use Committee of the Animal Science Department of the Federal University of Viçosa, protocol number 84/2013.

Animals, Experimental Design, and Diet Composition

A total of 3,640 Nellore bulls averaging [body weight (BW) = 367.0 ± 36.8 kg and 18 ± 3 mo] were allocated to 20 pens (182 animals/pen; 16.3 m2/animal) in a commercial feedlot located at Ribas do Rio Pardo, MS, Brazil (21° 9'16.15"S, 53°16'46.85"W, elev. 348 m); all pens were equipped with water and feed troughs. Before the beginning of the experiment, all bulls were weighed, vaccinated, dewormed, and received individual numbered tags. Cattle were adapted to the diets, facilities, and management for 25 d. Then, 20 bulls (one per pen) were randomly selected and slaughtered to serve as a reference for initial empty BW and initial carcass dressing. After the adaptation period the animals remained for an additional 100 d in the trial.

Bulls were blocked by BW into four blocks of 905 animals with similar BW and then within each block, bulls were randomly assigned to one of four experimental treatments in a completely randomized block design resulting in 905 animals per treatment. Animals were allocated to 20 pens (181 animals per pen and five pens per treatment).

Experimental treatments consisted of four dietary levels of CG; 0, 5, 10, and 15% (DM basis) as a substitute of DGC. Experimental diets were composed of 15.3% of corn silage and 84.6% of concentrate (DM basis) and were formulated to meet nutritional requirements of beef cattle [10]. Ingredient and chemical composition of the experimental diets are presented in Table 1. The CG chemical analyses were conducted using the esterification process of vegetable oils with subsequent purification according to the standards established by the Ministry of Agriculture, Livestock and Food Supply [11], CG was composed of 82.8% glycerol, 8.4% water, 6.3% ash, 1.4% fatty acids, 1.1% crude protein (CP), and 0.01% methanol.

Experimental Procedures and Sample Collections

Bulls were fed four times daily at 0700, 1000, 1300, and 1600 h. Diets were mixed in two mixer feeder wagons (3142 Reel Auggie, Kuhn, Passo Fundo, RS, Brazil). The wagons were checked for residual feed between each dietary mix to avoid cross-contamination. Feed bunkers were evaluated at 0530h each day to quantify orts and to adjust daily feed allowance to a maximum of 5% orts. Samples of feed and orts were collected daily from each pen and then composited every 14 d. The samples were frozen at −18 °C until further laboratory analysis.

Bulls were observed at least once daily during the experimental period to record the presence of anything abnormal (loss of tags, bloat, or injury) that may compromise the study and bulls that presented these conditions (n = 61) were removed from the experiment. Individual DM intake was calculated by the ratio between the amount of diet offered minus the orts per pen and the number of bulls per pen. Bulls were individually weighed after a 16-h solid-feed fasting at the beginning, at two-thirds, and at the end of the experiment. The average daily gain (ADG) was determined as the slope of the regression of BW. On the same day, measurements of the *Longissimus thoracis* muscle area (LMA), back fat thickness (BFT), and rump fat thickness (RFT) were obtained by ultrasound (Aloka Echo Camera Model SSD-500, Campinas, SP, Brazil). The LMA was measured on a transversal section in the 12th rib, BFT was measured on a longitudinal section in the 12th rib, and RFT was measured on a longitudinal section on the rump [13]. Ultrasound images were collected and analyzed by a certified technician (Ultrasound Guidelines Council) from the Aval Serviços Tecnológicos S/S, Goiania, GO, Brazil.

Apparent digestibility of nutrients was determined in two periods of three consecutive day, during the sampling collections (d 43-45 and d 87-89). Because collecting fecal samples from all 3,620 bulls would be unfeasible, on each sampling day, fecal samples from 50 animals per pen were taken, from the pen floor immediately after defecation and any dirt was carefully removed to avoid contamination. To ensure sample representation and homogeneity, all 50 samples were collected in the morning of the first day of collection, in the afternoon of the second day, and at night of the third day. This process was performed twice (total of 6 d); therefore, 300 fecal samples were collected per pen corresponding to 6,000 samples. Samples were composited by pen, homogenized, and 300 g (wet basis) were weighed and stored in plastic bags and frozen at −18 °C. Fecal excretion was estimated using indigestible neutral detergent fiber (NDF) as an internal marker, which was obtained after a 12-d in situ incubation according to Huhtanen et al. [14]. The digestible DM intake was calculated as follow: [DM intake (kg) x DM digestibility (%)] / 100.

After the experimental period, animals were transported to a commercial abattoir (JBS – Friboi, Campo Grande, MS, Brazil) for slaughter. Pre-harvest handling was conducted in accordance with good animal welfare practices, and slaughtering procedures followed strict guidelines stablished and regulated by the Sanitary and Industrial Inspection Regulation for Animal Origin Products [15]. At the abattoir, hot carcasses were weighed individually

and received scores by a certified technician for fat deposition according to the Brazilian system for classification of cattle carcasses [16], briefly, the carcass fatness was classified into five fat categories: thin (< 1 mm), scarce (1-3 mm), medium (3-6 mm), uniform (6-10 mm), and excessive (> 10 mm). This data was compared with ultrasonography evaluation to determine the accuracy between the fatness classification (from abattoir technician) and the ultrasound measurements. Carcass dressing was calculated based on the final carcass weight and BW ratio after fasting. Initial carcass weight was calculated using the value of 54.3% of live BW, which was obtained in the reference slaughter at beginning of the trial. The carcass ADG was determined considering the difference between final and initial carcass weights.

Economic Analysis

Economic values included: feed cost ($/kg DM), diet cost ($/animal/day) and cost of gain ($/kg). Cost data was obtained from the prices of feedstuffs practiced in the state of Mato Grosso do Sul, Brazil during the period of July-November 2012. Cost of gain per animal was calculated by dividing finishing diet cost by carcass gain. Because cost data can vary substantially over time, a sensitivity analysis was performed, according to Diniz et al. [49], to determine which diet was more economical according to the current CG prices as a function of DGC prices.

Chemical Analysis

Feed, orts, and fecal samples were thawed, oven dried at 55°C for 48 h, and then ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). After that, samples were analyzed for DM [17], ash (method 942.05; AOAC, 2005), CP (method 984.13; AOAC, 2005), and ether extract (EE; method 920.39; AOAC, 2005). The organic matter (OM) was calculated as the difference between DM and ash contents. For NDF analysis, samples were treated with alpha thermo-stable amylase without sodium sulfite according to Van Soest et al. [18] and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). Total digestible nutrients (TDN) were calculated by the following equation: TDN(%)=DCP+DNDF+DNFC+(2.25×EE)TDN (%) = DCP + DNDF + DNFC + (2.25 × DEE), where DCP = apparent digestible CP, DNDF = apparent digestible NDF, DNFC = apparent digestible non-fiber carbohydrates (NFC), and DEE = apparent digestible EE. The NFC were calculated as NFC=100-[(CP-CP_(from

urea)+Urea)+NDF+EE+Ash]NFC = 100 − [(CP − CP from urea + Urea) + NDF + EE + Ash] [12].

Statistical Analysis

Experimental units, defined as the smallest unit upon which a measure was made, were selected according to Robinson *et al.* (Robinson et al., 2006). For parameters measured at the animal level (performance and carcass characteristics), animal was used as the experimental unit and pen was included as a random effect in the model. For parameters measured at the pen level (intake, digestibility, and G: F), pen was used as the experimental unit and no random effect was added in the model. All parameters were analyzed using the following model:

$$Y_{ij} = B_0 + B_1 X_i + B_1 X_i^2 + e_{ij},$$

where:

Y_{ij} is the observed measurement of the i^{th} level of CG inclusion in the diet and of the j^{th} experimental unit; i = 1, 2, 3, 4 (levels of inclusion of CG as a replacement of DGC), B_0, B_1 = regression parameters of the model; X_j = effect of i^h level of fixed quantitative factor (replacement of CG with DGC); e_{ij} = residual error, assuming $e_{ij} \sim N(0, s^2)$. All statistical procedures were carried out using the mixed procedure of SAS 9.2 (SAS Institute Inc., Cary, NC) and significance was established at $\alpha = 0.05$.

Results

Intake and digestibility

The inclusion of CG linearly decreased ($P < 0.05$) DM, OM, and NDF intake (Table 2). The DM intake of the control treatment (0% of CG) was about 10% greater than the treatment with 15% CG inclusion. However, CG levels did not affect ($P > 0.05$) intakes of CP, NFC, and TDN, which averaged 1.54 ± 0.09, 4.80 ± 0.40 and 7.04 ± 0.46 kg/d, respectively. The DM intake variation during the fecal collection period is presented in Fig. 1.

A quadratic effect was observed ($P < 0.05$) for digestibility traits (Fig. 2). The digestibility of DM, OM, and TDN decreased when 5% CG was included in the diet compared with the control diet and increased when 10 and 15% CG was included in the diet (Fig. 2). Digestibility of NDF decreased when 10% CG was included in the diet compared

with 5% CG inclusion and increased when 15% CG was included in the diet compared with 10% CG inclusion. However, the inclusion of CG did not affect ($P > 0.05$) the intake of digestible DM (Fig. 3), which averaged 6.48 ± 0.48 kg/d.

Performance and Carcass Characteristics

No differences were observed ($P > 0.05$) for initial BW (367.0 ± 36.8 kg), final BW (502.3 ± 38.5 kg), ADG (1.37 ± 0.29 kg/d), carcass ADG (0.85 ± 0.23 kg/d), gain-to-feed ratio (G:F; 0.136 ± 0.014 kg/kg), and carcass G:F (0.857 ± 0.076 kg/kg) (Table 3).

Carcass characteristics are presented in Table 4. Initial carcass weight (199.2 ± 19.9 kg), initial LMA (53.9 ± 6.17 cm2), initial BFT (1.45 ± 0.29 mm), and initial RFT (1.56 ± 0.61 mm) were not different among treatments ($P > 0.05$). Inclusion of CG did not affect ($P > 0.05$) final carcass weight (284.5 ± 24.7 kg), carcass dressing (56.7 ± 3.23 %), final LMA (81.9 ± 7.99 cm2), LMA gain (27.9 ± 8.04 cm2), final BFT (4.48 ± 1.53 mm), BFT gain (3.02 ± 1.51 mm), final RFT (6.44 ± 1.90 mm), and RFT gain (4.90 ± 1.76 mm).

Comparison Between the Two Types of Carcass Classification: Abattoir vs. Ultrasound

The comparison between the two types of carcass classification (abattoir vs. ultrasound) is presented in Fig. 4. The abattoir scores classified 0.8% of the carcasses as thin, 42.3% scarce, and 56.9% medium fat. The ultrasound analysis classified 15% of the carcasses as scarce, 68.7% medium fat, 15.8% uniform fat, and 0.5% excessive fat. Comparing the two methods of carcass evaluation, it was found that generally, ultrasound analysis overestimated fat deposition when compared to the abattoir classification, 65% of the carcasses classified as scarce fat by the abattoir were classified as medium fat in the ultrasound analysis, as well as 28% of the carcasses classified as medium fat by the abattoir were classified as uniform fat in the ultrasound analysis.

Economic Analysis

Treatment without CG had the lowest diet cost, followed by treatments with 5%, 15%, and 10% of CG inclusion, respectively (Table 5). Sensitive analysis (Fig. 5) indicated that the lowest treatment cost per kg of carcass produced changed depending on the CG: DGC price relationship. When CG price was up to 10% higher than DGC price, the treatment that provided the lowest price per kg of carcass produced was 15% CG inclusion. However,

when CG price was starting from 20% higher than DGC price, the diet without CG was the most economical treatment.

Discussion

Intake and Digestibility

We hypothesized that partial replacement of DGC with CG would not compromise DM intake and apparent digestibility. However, compared to DGC, CG decreased intakes of DM, OM, and NDF in finishing beef cattle diets. Nevertheless, the intakes of CP, NFC, and TDN were not affected by the inclusion of CG. The lack of effect observed on TDN intake indicates an increase in energy density of the diets as CG replaced DGC. Mach et al. [3] estimated glycerol metabolic energy at 4.03 Mcal/kg. In this study the estimated CG (90.38 % of glycerol, DM basis) metabolic energy was 3.64 Mcal/kg, which is 12% greater than the 3.25 Mcal/kg of corn [10]. Monnerat et al. [4] also observed higher energy levels for CG compared to corn, suggesting that CG contributes with more energy per unit of DM than corn. That would explain why in this study the intake of DM decreased with CG inclusion. Also, the inclusion of CG did not affect ($P > 0.05$) the intake of TDN and digestible DM in this study, showing that energy intake was not influenced by CG inclusion. Diets with greater CG levels had lower NDF levels and this may explain the reduction on NDF intake with CG inclusion.

Reduction in DM intake has been observed in cattle fed CG and the literature suggests possible reasons for this observation ranging from effects on ruminal metabolism up to effects on intermediary metabolism. Parsons et al. [20] suggest that concentrations above 5% in the diets may impair rumen function, resulting in reduced DM intake. These authors observed a linear decrease on DM intake in crossbred heifers fed finishing diets containing 0, 2, 4, 8, 12, or 16% CG (DM basis). Pyatt et al. [21] replaced cracked corn with CG up to 10 % in finishing diets of Angus-crossbred steers resulting in a decrease of 10% on DM intake and increase of 11.4 and 19.2% on ADG and feed efficiency, respectively. Also, Hales et al. [22] observed a linear reduction in DM intake of steers when CG replaced roughage at 0, 2.5, 5, and 10% of the diet. Trabue et al. [23], using an in vitro system, reported that lactic acid concentration increased rapidly through the first 8 h of incubation and according to these authors, this may have slowed microbial activity and glycerol fermentation, which affected DM intake. It is well known that CG has the capacity of increasing propionate concentration

in the rumen [5,9,24]. When infused in isocaloric amounts in lactating cows, propionate resulted in lower DM intake than acetate [25]. Conversely, other studies have reported that DM intake was not affected by CG inclusion. Mach et al. [3] observed no effect on DM intake, ADG or G:F in Holstein bulls. Daily intake was not affected when four CG levels (0, 4, 8, and 12% of concentrate DM) were fed to the animals. Likewise, Eiras et al. [26] did not observe a reduction on DM intake when CG was added up to 17.8% in finishing diets for Purunã bulls.

The quadratic increase in DM apparent digestibility observed in animals fed diets with higher levels of CG was not expected, and the three main factors that could explain these results are: 1) The quick microbial adaptation and fast disappearance of CG in the rumen [5,27,28]. According to Donkin [9], glycerol is fermented to short chain fatty acids inside the rumen and may also be integrally absorbed through the ruminal epithelium such that 50 to 80% of the glycerol disappears within 4 hours. Furthermore, other studies observed higher volatile fatty acid concentrations in the rumen of animals fed CG [3,27,29]. Moreover, Hales et al. [22] observed an increase in starch digestibility when steers were fed diets containing 0, 2.5, 5, and 10% CG. This could also explain the higher values of OM and NFC digestibility in diets with the highest levels of CG; 2) The inclusion of CG decreases the acetate: propionate ratio in the rumen [6,23,28,29]. Compared to acetate, propionate synthesis in the rumen is more energy efficient [30]; therefore, this could potentially increase energy efficiency due to an increased energy availability; 3) Diets with higher levels of CG may have longer retention times in the rumen. Digestibility is the result of the competition between the rates of digestion and passage, and passage rate positively correlates with DM intake [31]. Although DM intake was lower as CG was included in the diet, feed could have remained longer in the rumen and consequently allowed greater microbial and enzymatic activity, thereby increasing DM digestibility. The increase on DM digestibility affected the intake of digestible DM, which did not differ between treatments in the present study.

Effects of CG on nutrient digestibility in ruminants have been conflicting and the variation in the chemical composition as well as the amount of CG used in different studies may be an explanation for this inconsistency. Hales et al. [32] also reported a quadratic effect in DM digestibility when CG (82.7 % glycerol) was included at up to 15% (DM basis) in finishing diets. Eiras et al. [26] observed an increase in digestibility of DM, OM, NDF, and CP when CG (81.2% glycerol, 2.3% water, 4.8% ash, and 3.3% methanol) was added at 60,

120, and 178 g/kg of DM in beef cattle finishing diets. Moreover, Wang et al. [28] observed an increase in digestibility of OM, NDF, and CP when Simmental steers were fed 100, 200, and 300 g/d of CG (99% glycerol). Hess et al. [33] observed that DM and NDF digestibility were not affected when CG was added at up to 15% of DM in an in vitro system. Others have observed a reduction in digestibility when feeding CG. Shin et al. [34] noted a decrease in NDF digestibility when Holstein cows were fed diets containing 0, 5, or 10% CG (80.3% glycerol, 12.4% water, and 0.46% methanol). Furthermore, van Cleef et al. [35] noted a decrease in NDF digestibility when Nellore bulls were fed diets containing 0, 7.5, 15, 22.5, and 30% CG (86% glycerol, 5% water, and >0.01% methanol). These results suggest suppression in fiber digestibility when CG is included in cattle diets. Roger et al. [36] observed inhibition in growth and activity of two cellulolytic bacteria, Ruminococcus flavefaciens and Fibrobacter succinogenes, and of an anaerobic fungal species, Neocallimastix frontalis inoculated with glycerol at a concentration of 5%. Therefore, the inhibition of cellulolytic activity could affect fiber digestion, passage rate and consequently decrease intake. However, in the present study an increase ($P < 0.05$) was observed in NDF digestibility for treatments with 5 and 15% CG inclusion. Therefore, it is likely that other aspects such as grain processing methods, roughage source and amount, and feed additives may play a role in glycerol utilization and its effects on intake and digestibility.

Cattle Performance and Carcass Characteristics

We hypothesized that CG may partially replace DGC and may be included at concentrations up to 15% (DM basis) without compromising performance and carcass characteristics. As expected, the replacement of DGC with CG did not change ADG, G:F, and final BW. These results are consistent with findings in previous reports and are usually related with the quality of the CG [3,35,37]. The CG used in this study had a high purity (above 80%) with low fatty acid and methanol concentration, which allowed similar performance when compared to DGC. Furthermore, the diets with higher levels of CG presented higher digestibility. In this study, the increase in digestibility observed when animals were fed higher levels of CG appeared to offset the decrease in intake and as a consequence, bulls fed higher levels of CG achieved the similar performance as bulls fed DGC. Similar results were observed by Parsons et al. [20] when DM intake decreased without affecting ADG in an experiment that heifers were fed up to 12% CG (DM basis).

Bartoň et al. [38] also noted no differences in ADG and feed conversion ratio when beef bulls were fed diets containing 0, 4.7, or 9.3% of CG (DM basis). Moreover, Ramos and Kerley [37] observed no differences in growth performance of crossbreed steer calves when CG was included in the diet at up to 20% of DM. Other studies indicated that CG inclusion in the diet improved animal performance when included at up to 7.5% [39], 10% [21] and 14.9% [40] in beef cattle diets. The lack of effect observed for carcass ADG and carcass G:F demonstrates that diets containing up to 15% CG may promote the same efficiency of diet utilization than DGC diets.

Partial dietary replacement of DGC with CG did not affect hot carcass weight, carcass gain, carcass percentage dressing, LMA, BFT, and RFT traits in this study. These results were expected and as no significant differences were observed between energy intake, protein intake, and performance in the treatments, lack of effect on these variables is justified. Similarly to our results, no effects on carcass characteristics have been reported when CG was included at up to 10% in diets of finishing bulls [38,41,42]. Eiras et al. [43] also observed a lack of effects in fat thickness and LMA when CG (81.2% glycerol) was included at up to 18% (DM basis) in diets of young Purunã bulls finished in feedlot. Moreover, improved hot carcass weight, LMA, and BFT were observed when diets containing 12 and 15% CG were fed to bulls [44] and beef calves [40], respectively. As previously discussed, the glycerol can be converted to propionate in the rumen or be directly absorbed through the rumen wall [5,9], providing greater available energy to the animals. The LMA has a positive correlation with the carcass edible portion [45]. The lack of difference in LMA in this study suggests that the inclusion of CG at up to 15% in the diet has the same efficiency of DGC to support muscle growth in feedlot finishing cattle. Furthermore, the lack of difference for BFT and RFT traits suggests that CG allows a satisfactory carcass thickness for finishing bulls.

Comparison Between the Two Types of Carcass Classification: Abattoir vs. Ultrasound

Accuracy differences in abattoir and ultrasound classifications may explain the differences observed in this study. Ultrasound evaluations give an accurate and repeatable measurement of carcass fat in beef cattle [46,47], and are an adequate method to determining carcass merit [48]. On the other hand, the abattoir system, which is the major carcass grading system used in Brazil, is subjective and less accurate. In this study, 46% of all abattoir scores

were lower than ultrasound scores, which may lead to unfair financial compensation to beef producers. In situations that carcasses are awarded or penalized according to fat deposition, ultrasound analysis should be used for a more accurate evaluation of beef cattle carcasses.

Economic Analysis

We hypothesized that CG could partially replace DGC at up to 15% in finishing beef cattle diets without compromising production cost. However, the treatment without CG had the lowest diet cost ($/kg of carcass) which could be explained by the high values paid for CG in relation to DGC. The CG used in this study was imported from Rio Grande do Sul state, which is over 300 km away from the experimental site. The shipping cost substantially increased overall CG cost, which was about 81% higher than DGC cost. This resulted in the higher cost per kg of produced carcass. In this study, the relationship between the marketing values of CG and DGC was the major factor defining the most economical diet. Therefore, the sensitivity analysis indicated that CG inclusion at 15% of DM might promote a lower cost per kg of carcass produced only when CG prices were up to 10% higher than DGC price. Data from the economic analysis indicate that CG utilization may be an economical alternative to corn when CG cost is up to 10% higher than corn cost.

Conclusion

Results from this large-scale study indicate that CG may partially replace corn and may be included at up to 15% in finishing beef cattle diets without affecting performance and carcass characteristics. The information presented here has direct practical implications in the field. It is important not only from the performance perspective but also from a sustainable perspective since glycerol is a biofuel residue and could potentially partially replace corn as an energy source for finishing beef cattle diets.

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Table 1. Ingredient and chemical composition of experimental diets

Item	Crude glycerol, %			
	0	5	10	15
Ingredient, % DM[1]				
Corn silage	15.35	15.35	15.35	15.35
Dry ground corn	38.89	33.72	28.56	23.40
Crude glycerol[2]	0.00	5.00	10.00	15.00
Citrus pulp	25.00	25.00	25.00	25.00
Cottonseed cake	16.58	16.58	16.58	16.58
Urea	0.80	0.97	1.13	1.29
Slow release urea[3]	0.45	0.45	0.45	0.45
Vitamin-mineral premix[4]	2.93	2.93	2.93	2.93
Composition, % DM				
Dry matter, %	80.2	80.3	80.3	80.3
Non-fiber carbohydrates[5]	48.0	48.8	49.6	50.4
Neutral detergent fiber	28.4	27.7	27.0	26.2
Crude protein	14.7	14.8	14.8	14.9
Ash	6.8	7.0	7.3	7.6
Ether extract	4.4	4.2	4.1	3.9

[1]DM = dry matter.

[2]The crude glycerol used in this study was analyzed and contained 82.8% glycerol, 8.4% water, 6.3% ash, 1.4% fatty acids, 1.1% crude protein, and 0.01% methanol. It was obtained from an esterification process of vegetable oils with subsequent purification. It was provided by Granol Indústria Comércio e Exportação S.A (Cachoeira do Sul, RS, Brazil) and met the standards established by the Ministry of Agriculture, Livestock and Supply (Brasil, 2010).

[3]Optigen 1200 controlled-release nitrogen, Alltech, Araucária, PR, Brazil.

[4]Provided (per kg of DM): 195 g of Ca, 50 g of Na, 26.7 g of S, 20 g of P, 17 g of Mg, 2,000 mg of Zn, 1,000 mg of monensin, 840 mg of Mn, 490 mg of Fe, 420 mg of Cu, 25 mg of Co, 25 mg of I, 7 mg of Se, *Saccharomyces cerevisiae* 100 x 10^9 CFU, 100,000 IU of vitamin A, 10,400 IU of vitamin D3, 242 IU of vitamin E.

[5]Non-fiber carbohydrates = 100 − [(crude protein – crude protein from urea + urea) + neutral detergent fiber + ether extract + Ash] (Detmann and Valadares Filho, 2010).

Table 2. Effect of crude glycerol inclusion on daily intake of dietary nutrients in finishing beef cattle

	Crude glycerol, %					*P*-value	
Intake[1]	0	5	10	15	SEM	Linear	Quadratic
DM, kg/d	10.5	10.2	10.2	9.6	0.15	<0.01	0.60
DM, % BW	2.40	2.36	2.33	2.23	0.14	<0.01	0.42
OM, kg/d	9.77	9.38	9.39	8.78	0.15	<0.01	0.54
CP, kg/d	1.59	1.57	1.52	1.50	0.02	0.09	0.98
NDF, kg/d	2.85	2.55	2.47	2.42	0.05	<0.01	0.09
NFC[2], kg/d	4.97	4.80	4.99	4.46	0.09	0.10	0.31
TDN[3], kg/d	7.35	6.82	7.06	7.04	0.12	0.63	0.21

[1]DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; NFC = non-fiber carbohydrate; TDN = total digestible nutrients.

[2]NFC = 100 − [(CP − CP from urea + urea) + NDF + EE + Ash] (Detmann and Valadares Filho, 2010).

[3]TDN = DCP + DNDF + DNFC + (2.25 × DEE), where DCP = apparent digestible CP, DNDF = apparent digestible NDF, DNFC = apparent digestible non-fiber carbohydrates, and DEE = apparent digestible EE.

Table 3. Effect of crude glycerol inclusion on performance of finishing beef cattle

	Crude glycerol, %					*P*-value	
Item[1]	0	5	10	15	SEM	Linear	Quadratic
Body weight, kg							
Initial	370	363	372	363	0.636	0.89	0.96
Final	510	499	504	497	2.178	0.20	0.45
Performance							
ADG, kg/d	1.41	1.37	1.35	1.34	0.025	0.20	0.78
G:F, kg/kg	0.13	0.14	0.13	0.14	0.002	0.46	0.56
Carcass ADG, kg	0.89	0.85	0.84	0.83	0.019	0.23	0.67
Carcass G:F, kg/kg	0.09	0.08	0.08	0.09	0.001	0.56	0.15

[1]ADG = average daily gain; G:F = gain-to-feed ratio

Table 4. Effect of crude glycerol inclusion on carcass characteristics of finishing beef cattle

	Crude glycerol, %					*P*-value	
Item[1]	0	5	10	15	SEM	Linear	Quadratic
HCW, kg	290	282	285	281	1.44	0.07	0.35
Dressing, %	56.9	56.7	56.6	56.5	0.33	0.64	0.92
LMA, cm x cm							
Initial	54.0	53.7	53.6	54.9	0.46	0.58	0.37
Final	83.0	82.2	82.1	80.9	0.40	0.06	0.88
LMA gain	28.8	28.4	28.5	25.9	0.61	0.11	0.34
Back fat, mm							
Initial	1.44	1.44	1.43	1.48	0.01	0.31	0.39
Final	4.62	4.37	4.44	4.51	0.09	0.14	0.12
Back fat gain	3.18	2.92	3.00	3.02	0.05	0.45	0.10
Rump fat, mm							
Initial	1.53	1.56	1.55	1.60	0.02	0.29	0.39
Final	6.65	6.39	6.42	6.39	0.06	0.09	0.25
Rump fat gain	5.11	4.83	4.86	4.80	0.09	0.08	0.30

[1]HCW = hot carcass weight; LMA = *Longissimus thoracis* muscle area

Table 5. Effect of crude glycerin inclusion on economic analysis.

Item[1]	Crude glycerin, %				
	0	5	10	15	$/kg DM
Corn silage	15.4	15.4	15.4	15.4	0.09
Dried ground corn	38.9	33.7	28.6	23.4	0.21
Crude glycerin	0.0	5.0	10.0	15.0	0.38
Citrus pulp	25.0	25.0	25.0	25.0	0.13
Cottonseed cake	16.6	16.6	16.6	16.6	0.23
Urea	0.80	0.97	1.13	1.29	0.55
Slow release urea[2]	0.45	0.45	0.45	0.45	1.78
Vitamin-mineral premix	2.93	2.93	2.93	2.93	1.10
Diet cost, $/kg DM	0.21	0.22	0.23	0.24	
DMI, kg/d	10.5	10.2	10.2	9.60	
Diet cost, $/animal/d	2.22	2.23	2.32	2.27	
Carcass gain, kg/animal/d	0.89	0.85	0.84	0.83	
Diet cost, $/kg carcass	2.49	2.62	2.76	2.72	

[1]Corn silage = $ 38.31/ton NM; Crude glycerin = $ 328.91/ton NM; Dry ground corn = $ 186.21/ton NM; Citrus pulp = $ 114.20/ton; Cottonseed cake = $216.01/ton NM; Urea = $535.30/ton NM; Slow release urea = $1,673.53/ton NM; Mineral mix. = $ 1078.70/ton NM. Market values for all feed ingredients were obtained from August to November of 2012.

[2]Optigen 1200 controlled-release nitrogen, Alltech, Araucária, PR, Brazil.

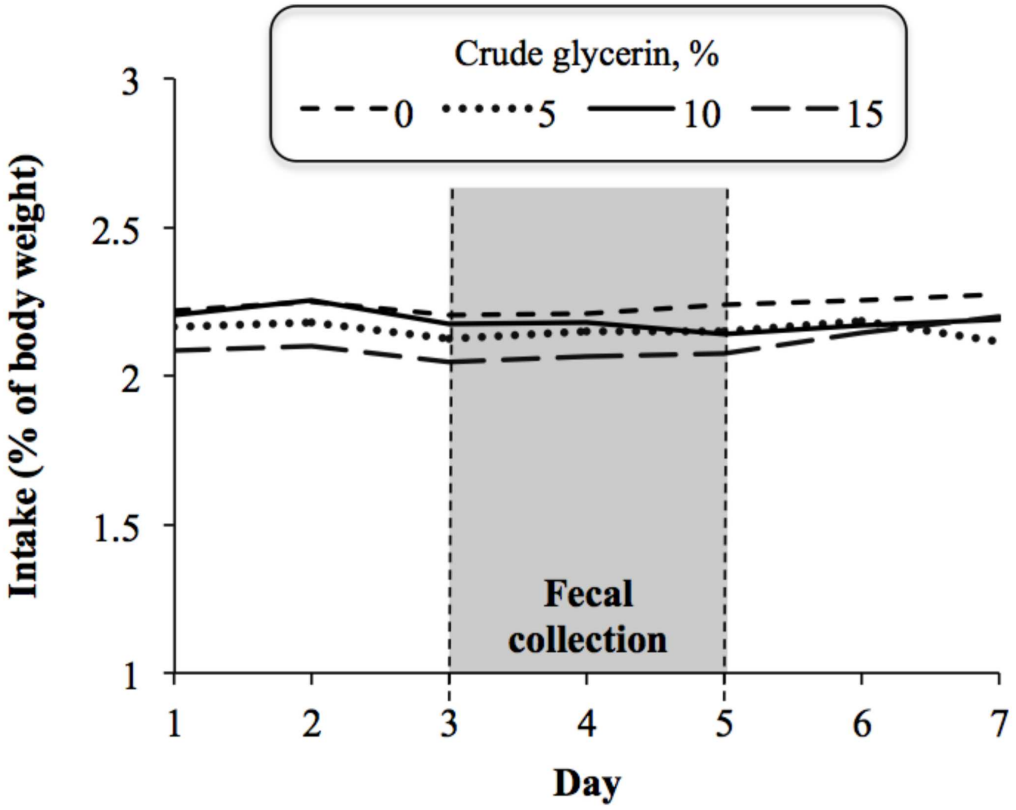

Figure 1. Effect of crude glycerol inclusion on average dry matter intake during fecal collection.

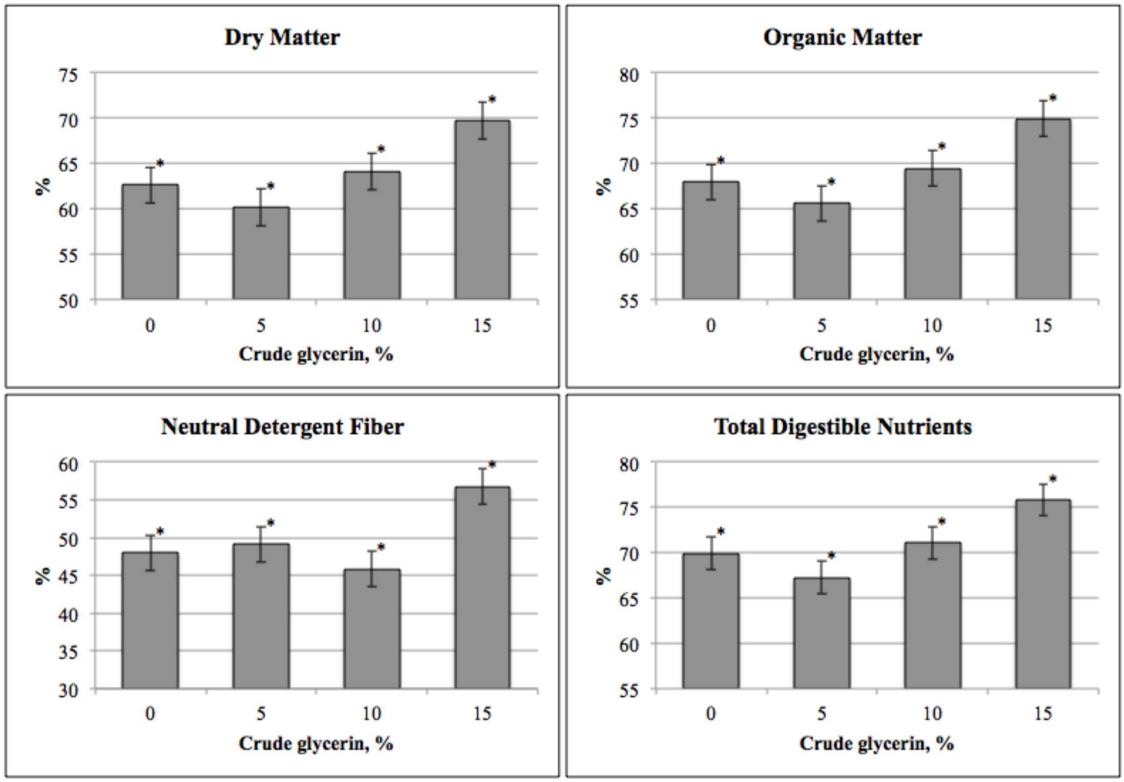

Figure 2. Effect of crude glycerol inclusion on apparent digestibility of dietary nutrients in finishing beef cattle. *Quadratic effect ($P < 0.01$)

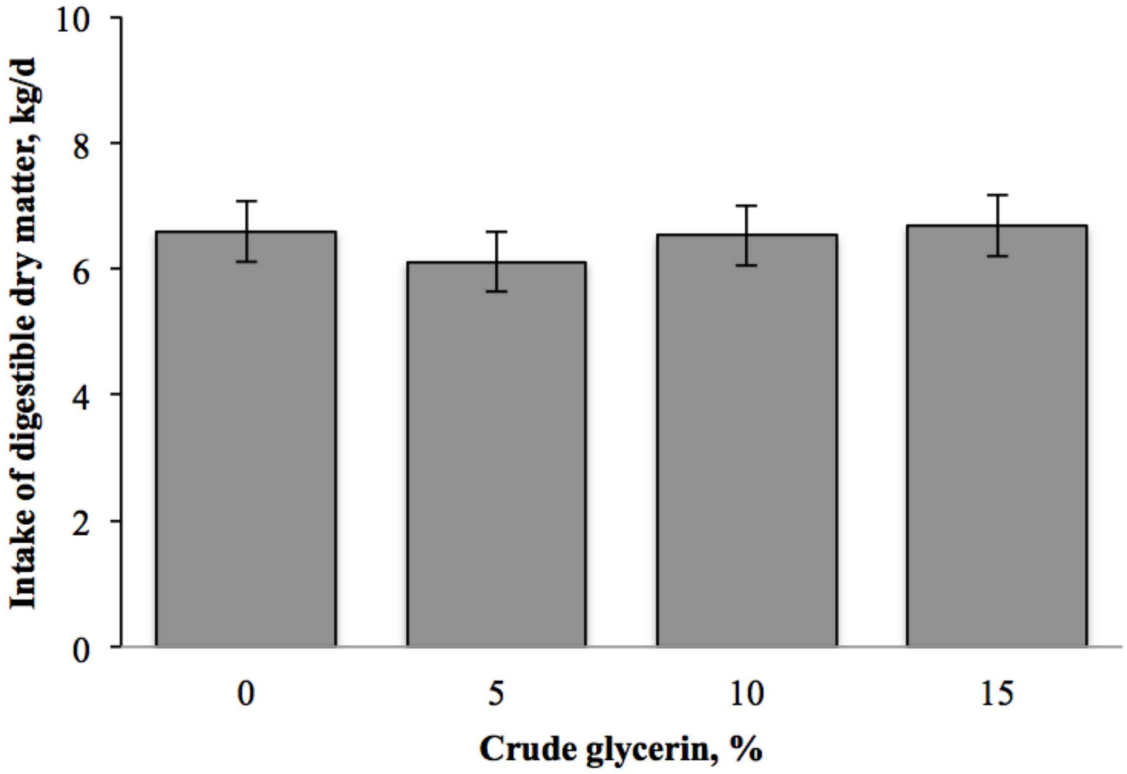

Figure 3. Effect of crude glycerol inclusion on intake of digestible DM in finishing beef cattle.

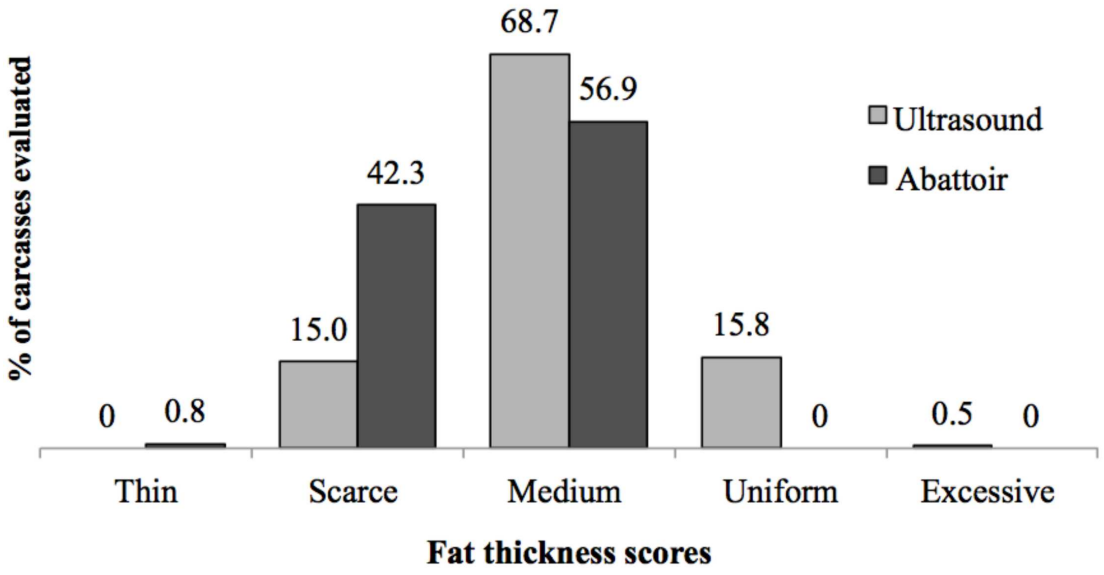

Figure 4. Beef cattle carcass classification by two different methods (ultrasound and abattoir). Fat cover classified as thin (less than 1 mm), scarce (1-3 mm), medium (3-6 mm), uniform (6-10 mm) and excessive (over 10 mm).

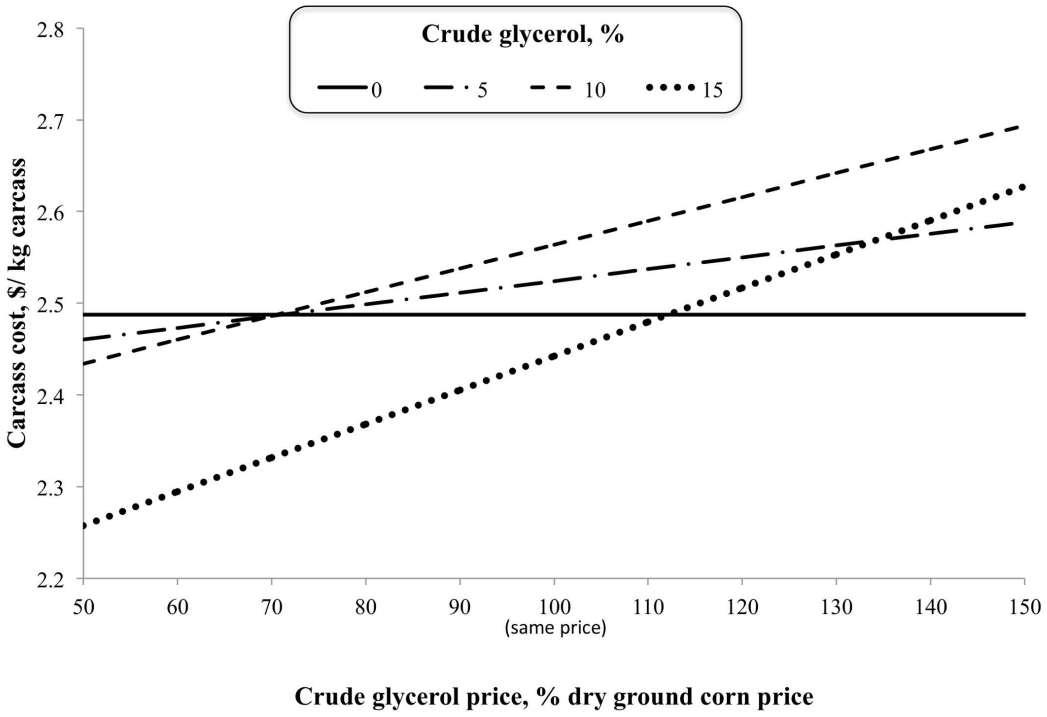

Figure 5. Effect of crude glycerin inclusion on sensitive analysis of the cost per carcass gain as a function of crude glycerin prices in relation to ground corn prices.

Chapter III

Glycerin as alternative energy source in finishing beef diets: metabolizable energy, *in vitro* fermentation, total gas production, and greenhouse gases emission

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Abstract

Five *in vitro* experiments were conducted to evaluate the metabolizable energy and changes on ruminal fermentation, total gas production, and greenhouse gases production of glycerin compared to corn and starch, as well as when glycerin was added in finishing beef diets. For Exp. 1, a 24 bottles system (620 mL each) equipped with wireless pressure sensors (AnkomRF Gas Production System) were used in 4 consecutive 48-h runs. Three ingredients were tested (corn, glycerin, and starch) at 0.5 g per bottle. The experimental design was: 4 incubation runs x 3 ingredients x 7 bottles per treatment, plus 12 blank bottles (3 per run), totaling 96 observations. The 24 h total gas production, acetate concentration, and acetate: propionate ratio decreased ($P < 0.01$) only with feeding glycerin. The 48 h total gas production was highest ($P < 0.01$) for corn, and similar between glycerin and starch. The starch treatment presented the lowest ($P = 0.01$) total VFA concentration. Corn presented the lowest propionate concentration ($P < 0.01$). The metabolizable energy was highest ($P < 0.01$) for corn, and similar between glycerin and starch. For Exp. 2, a total of 20-serum bottles (155 mL) equipped with stoppers and sealers were used in four consecutive 48-h runs. Treatments were the same of Exp. 1 and the experimental design was: 4 incubation runs x 3 ingredients x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 80 observations. The CH4 production increased ($P < 0.01$) only with feeding glycerin. The CO2 in mL/g was higher ($P < 0.01$) for corn, but similar for glycerin and starch. The pH decreased ($P < 0.01$) only with feeding starch. Different ingredients had no effect ($P > 0.05$) on total VFA, and propionate concentration. Compared with glycerin treatment, acetate concentration ($P < 0.01$) and acetate: propionate ratio ($P = 0.01$) were higher for corn and starch, whereas butyrate and valerate concentrations were higher ($P = 0.01$) for glycerin. For Exp. 3, the same system of Exp. 1 was used in four consecutive 48-h runs. The treatments were: inclusion of 0, 10, 20, and 30% DM of glycerin replacing corn in finishing beef diets. The experimental design was: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 16 blank bottles (4 per run), totaling 96 observations. Glycerin levels did not affect ($P > 0.05$) 24 h and 48 h total gas production, final pH, NH3-N, total VFA, propionate, and butyrate concentrations. The inclusion of glycerin linearly decreased acetate concentration ($P = 0.03$) and acetate: propionate ratio ($P = 0.04$). For Exp. 4, a total of 25-serum bottles (155 mL) were used in four consecutive runs of 48h. Treatments were the same of Exp. 3 and the experimental design was: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 100

observations. The glycerin inclusion did not affect ($P > 0.05$) the productions of CH_4, CO_2, final pH, and total VFA, propionate, butyrate, and acetate: propionate ratio. A linear decrease of acetate concentration ($P = 0.04$) was observed due to inclusion of glycerin in the diets. For Exp. 5, two systems of four 4-L digestion vessels (DaisyII system, Ankom technology, NY, USA) were used in four consecutive runs of 48 h. The treatments were: orchard hay (0.4 g/bag), corn (0.4 g/bag), orchard hay (0.4 g/bag) + glycerin (0.2 g/bag) and corn (0.4 g/bag) + glycerin (0.2 g/bag). Compared with the orchard hay treatment, the in vitro DM digestibility decreased ($P < 0.01$) with including glycerin. However, the glycerin inclusion had no effects ($P > 0.05$) on corn's in vitro dry matter digestibility. We concluded that, under these experimental conditions, glycerin contributed more to the enhancement of methanogenesis than carbohydrates, but effectively replaced dietary corn as energy source at up to 30% (DM basis) of the diet.

Resumo

Cinco experimentos *in vitro* foram conduzidos para avaliar a energia metabolizável e as mudanças na fermentação ruminal, produção total de gases e emissão de gases do efeito estufa da glicerina, comparada ao milho e ao amido, bem como sua inclusão em dietas de terminação para gado de corte. Para o Exp. 1, um sistema de 24 garrafas (Ankom^{RF} Gas Production System, Ankom technology, NY, USA) foi usado em 4 corridas consecutivas de 48 h. Os tratamentos foram três diferentes alimentos: milho, glicerina e amido. A produção total de gases às 24 h, a concentração de acetato e a relação acetato: propionato diminuiu ($P < 0,01$) somente para glicerina. A produção total de gases às 48 h foi maior ($P < 0,01$) para o milho e semelhante entre glicerina e amido. O tratamento com amido teve a menor ($P < 0,01$) concentração total de AGV. O milho apresentou a menor concentração de propionato ($P < 0,01$). A energia metabolizável foi maior ($P < 0,01$) para o milho e semelhante entre glicerina e amido. Para o Exp. 2, um total de 20 garrafas (155ml) foram usadas em 4 corridas consecutivas de 48 h. Os tratamentos forma os mesmos do Exp. 1. A concentração de CH_4 aumentou ($P < 0,01$) somente para a glicerina. A produção de CO_2 em ml/g foi maior ($P < 0,01$) para o milho e semelhante para glicerina e amido. Comparado com a glicerina, a concentração de acetato ($P < 0,01$) e a relação acetato: propionato ($P < 0,01$) foram maiores para amido e milho, enquanto a as concentrações de butirato e valerato foram maiores ($P < 0,01$) para a glicerina. Para o Exp. 3, o mesmo sistema do Exp. 1 foi usado em 4 corridas consecutivas de 48 h. Os tratamentos foram: inclusão de 0, 100, 200 e 300g/kg de MS de glicerina substituindo o milho em dietas de terminação de bovinos de corte. A inclusão de glicerina não afetou ($P > 0,05$) as produções de gases às 24 e 48 h, o pH final, N-NH_3, AGV total, e as concentrações de propionato e butirato. A inclusão de glicerina diminuiu linearmente a concentração de acetato ($P = 0,03$) e a relação acetato: propionato ($P = 0,04$). Para o Exp. 4, um total de 25 garrafas (155ml) foram usadas em 4 corridas consecutivas de 48 h. Os tratamentos foram os mesmos do Exp. 3. A inclusão de glicerina não afetou ($P > 0,05$) as concentrações de CH_4, CO_2, AGV totais, propionato, butirato, relação acetato: propionato e pH final. Foi observada diminuição linear ($P = 0,04$) na concentração de acetato devido à inclusão de glicerina nas dietas. Para o Exp. 5, dois sistemas de quatro vasos de digestão de 4 L (Daisy^{II} system, Ankom technology, NY, USA) foram usados em duas corridas consecutivas de 48 h. Os tratamentos foram: feno de orchard (0,4 g/saco), milho (0,4 g/saco), feno de orchard (0,4 g/saco) + glicerina (0,2 g/saco) e milho (0,4 g/saco) + glicerina

(0,2 g/saco). O feno de orchard com glicerina apresentou a menor ($P < 0,01$) digestibilidade in vitro da MS, seguido pelo feno de orchard sem adição de glicerina e pelos tratamentos com milho. A digestibilidade in vitro da MS não diferiu ($P > 0,05$) entre os tratamentos com milho sem ou com adição de glicerina. Como conclusão, sob essas condições experimentais, a glicerina contribuiu mais para o aumento da metanogenese do que carboidratos, mas efetivamente substituiu parcialmente o milho como fonte de energia em dietas para bovinos de corte.

Introduction

The expansion of the biodiesel industry has increased glycerin supply (Hales et al., 2013), which may position it as a viable alternative for feeding cattle. Glycerin is an organic compound belonging to the alcohol group that can increase the glycogenic potential of beef cattle diets (Del Bianco Benedeti et al., 2015). Glucose is an important carbon source used for fatty acid synthesis, being especially significant for marbling in finishing animals (Versemann et al., 2008). Thus, glycerin could be included in finishing beef cattle diets as energy source and replace other traditional feed ingredients such as corn, and hence, reduce feeding costs. The gas production technique is an efficient method to estimate the nutritional value of feeds (Tagliapietra et al., 2011), and can be used as a tool for ranking feeds (Valentin et al., 1999; Hall and Mertens, 2008). Therefore, estimates of the glycerin metabolizable energy, as well comparing it with other well-known energy sources such as corn and starch would be important to help nutritionists to correctly include this ingredient in diets. Previous research showed that glycerin may reduce methane (CH_4) production (Lee et al., 2011), possibly due to an increase in propionate concentration. Because pathways to propionate formation acts as a hydrogen sink (Moss et al., 2000; Boadi et al., 2004), it may reduce the availability of hydrogen for CH_4 formation. Therefore, glycerin has the potential to be a sustainable alternative to corn as energy source for beef cattle finishing diets.

Previous *in vitro* (Rico et al., 2012; Del Bianco Benedeti et al., 2015) and *in vivo* (van Cleef et al., 2015; Del Bianco Benedeti et al., 2016) studies have reported the effects of glycerin inclusion on ruminal fermentation when glycerin was included in beef cattle diets; however, there is some controversy regarding to the effects of glycerin inclusion on total gas production and greenhouse gases production (Avila-Stagno et al., 2014). We hypothesized that glycerin fermentation would be similar to starch, and could partially replace corn as dietary energy source in the diet of finishing cattle diets at up to 30% (DM basis) without compromising ruminal fermentation, total gas production, greenhouse gases production, and *in vitro* DM digestibility. Therefore, the objective of this research was to evaluate the metabolizable energy and changes in ruminal fermentation of glycerin compared to corn and starch, as well as when glycerin was added in finishing beef diets.

Materials and Methods

Location and Ethical approval

All experiments were conducted at the University of Nevada, Reno. Care and handling of all experimental animals were conducted under protocols approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (IACUC protocol number 00588). Ruminal cannulations were conducted as described in (Del Bianco Benedeti et al., 2015).

Ingredients evaluation: corn, glycerin and starch (Exp. 1 and Exp. 2)

For **Exp. 1**, a 24 bottles system (Ankom^{RF} Gas Production System, Ankom technology, NY, USA) equipped with pressure sensors wireless connected to a computer was used in four consecutive 48-h runs. The treatments were three different ingredients: corn, glycerin (99.7% w/w; Nature's Oil, Streetsboro, OH, USA), and starch. The experimental design was: 4 incubation runs x 3 ingredients x 7 bottles per treatment, plus 12 blank bottles (3 per run), totaling 96 observations. Corn was ground through a 2-mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA) and analyzed for chemical composition, which was: Dry matter (DM) = 88%, OM = 98.5% DM, NDF = 11.5% DM, CP = 8.1% DM, and EE = 4.9% DM.

Each bottle (620 mL) was filled with 0.5 g of each ingredient. Samples were hydrated with deionized water to avoid particle dispersion. The buffer mineral solution was prepared following Menke and Steingass (1988), except for the addition of sodium sulfite and L-cysteine. The buffer solution was kept in a water bath at 39◦C and purged continuously with N_2 for 30 min. Resazurin indicator was used to control the buffer pH and N_2 saturation. Rumen fluid was collected from two Aberdeen Angus steers cannulated in the rumen (Average BW of 500 kg). Steers were maintained on a total mix diet composed of 40% grass hay, 47% dry ground corn, 10% soybean meal, and 3% mineralized salt (DM basis). Two h after feeding, 2 L of rumen fluid were collected, immediately filtered through 4 layers of cheesecloth and kept into pre-warmed thermal containers. The rumen fluid was mixed with the buffer solution (1:2 v/v) in water bath at 39◦C under anaerobic conditions by flushing N_2 (Menke and Steingass, 1988). Bottles were inoculated with 75 mL of rumen/buffer solution keeping the headspace of bottle continuously flushing with N_2. Bottles without feed samples, but with rumen/buffer solution were used as blanks to correct fermentation of remaining

substrates content in the rumen inoculum. After inoculation, bottles were closed and placed in the air-ventilated shaker incubator (Innova 4400 incubator shaker; New Brunswick Scientific, Edison, NJ, USA) under controlled temperature and agitation (39◦C and 80 RPM). The data collector software (Gas Pressure Monitor, Ankom technology, NY, USA) was set to record cumulative pressure every 5 minutes for 48 h. Valves were set to automatic release the gas when the pressures reached 3.4kPa (Tagliapietra et al., 2011). At the beginning and at the end of the incubation (48 h), the solution pH was measured with an Accumet portable AP61 pH meter (Fisher Scientific, Atlanta, GA). Subsamples of 10 mL were filtered through two layers of cheesecloth from the rumen/buffer solution before incubation and from each bottle at 48 h. Then, 0.2 mL of a 50% H_2SO_4 solution was added for later determination of NH_3-N (Chaney and Marbach, 1962) and VFA.

For **Exp. 2**, a total of 20-serum bottles (155 mL) equipped with stoppers and sealers were used in four consecutive 48-h runs. The treatments were three different ingredients: corn, glycerin, and starch (same of **Exp. 1**). The experimental design was: 4 incubation runs x 3 ingredients x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 80 observations. Each bottle was filled with 0.2 g of each ingredient. Ingredients composition, animals feeding, rumen samplings, as well as the preparation of samples and rumen/buffer solution were performed according **Exp. 1** procedures. Bottles were inoculated with 20 mL of rumen/buffer solution keeping the headspace of bottle continuously flushing with N_2. After inoculation, bottles were sealed with butyl rubber stoppers and aluminum caps, and then placed in the air-ventilated shaker incubator (39◦C and 80 RPM). After 48 h of fermentation, the productions of CO_2 and CH_4 gases in the bottles were determined using a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a Porapak Q (Supelco) column (60°C, 30 mL/min of helium (99.99%) carrier gas). The pH measurements and samplings of rumen/buffer solution for NH_3-N and VFA analysis were performed according **Exp. 1**.

Diets evaluation: different glycerin levels in beef cattle diets (Exp. 3 and Exp. 4)

For **Exp. 3**, the same 24 bottles system of **Exp. 1** was used in four consecutive 48-h runs. The treatments were: inclusion of 0, 10, 20, and 30% (DM basis) of glycerin replacing corn in finishing beef diets. The experimental design was: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 16 blank bottles (4 per run), totaling 96 observations.

Experimental diets were composed of 20% orchard hay and 80% concentrate (DM basis) and were formulated to meet the nutrient requirements of beef cattle recommendations (NRC, 2000). Diet ingredients were ground through a 2 mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA) for further analysis. Ingredient proportion and chemical composition of the experimental diets are presented in Table 1. Evaluated parameters (Total gas production, pH, NH_3-N, and VFA) and experimental procedures were the same of **Exp. 1**.

For **Exp. 4**, a total of 25-serum bottles (155 mL) equipped with stoppers and sealers were used in four consecutive 48-h runs. The treatments and diets composition were the same of **Exp. 3**: inclusion of 0, 10, 20, and 30% (DM basis) of glycerin replacing corn in finishing beef diets. The experimental design was: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 100 observations. Evaluated parameters (CH_4 and CO_2 productions, pH, NH_3-N, and VFA) and experimental procedures were the same of **Exp. 2**.

In vitro dry matter digestibility (Exp. 5)

For **Exp. 5**, two systems of four 4-L digestion vessels (DaisyII system, Ankom technology, NY, USA), equipped with slow rotation and temperature controller were used in four consecutive 48-h runs. The experimental design included two 4x4 Latin squares, which were run simultaneously. Within each Latin square, four treatments were applied to the jars totaling 8 replicates each. The treatments were: orchard hay (0.4 g/bag), corn (0.4 g/bag), orchard hay (0.4 g/bag) + glycerin (0.2 g/bag), and corn (0.4 g/bag) + glycerin (0.2 g/bag). Orchard hay and corn were ground through a 2 mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA), and analyzed for chemical composition: Orchard hay (DM = 89.9%, OM = 88.7% DM, NDF = 45.1% DM, CP = 16.8% DM, and EE = 6.0% DM), and corn (DM = 88%, OM = 98.5% DM, NDF = 11.5% DM, CP = 8.1% DM, and EE = 4.9% DM).

Each of the four treatments was weighed into filter bags (F57, Ankom technology, Macedon, NY, USA), which were heat-sealed and placed into the digestion vessels. According with Holden (1999), each vessel received 6 bags of each treatment plus 2 bags with no samples (blanks). The buffer mineral solution was prepared following the equipment manual and the pH was adjusted to 6.8, if needed (Holden, 1999). The buffer solution was kept in a water bath at 39.5◦C and purged continuously with N_2. Then, 1600 mL of buffer solution was added in each vessel. Vessels were placed into DaisyII incubator and kept at

39◦C for 30 min. Rumen fluid was collected from two Aberdeen Angus steers cannulated in the rumen (Average BW of 500 kg). Steers were maintained on the same diet of **Exp. 1**. Two h after feeding, 2 L of rumen fluid were collected, immediately filtered through 4 layers of cheesecloth and kept into pre-warmed thermal containers. Approximately 300 g of rumen solid particles were added to the containers and transported to the lab. Then, material of the containers was blended for 2 min, followed by filtering through 4 layers of cheesecloth. The rumen inoculum was added to the vessels (400 mL in each one) under anaerobic conditions by flushing N_2. After inoculation, vessels were closed and then placed into the incubator with temperature at 39.5◦C for 48 h. At completion of incubation, bags were rinsed with cold water and analyzed for neutral detergent fiber (Holden, 1999).

Chemical analysis and calculations

Ingredients samples were analyzed for DM (method 934.01), ash (method 938.08), crude protein (Leco CN-628 Series Determinator), and ether extract (method 920.85) according to AOAC (2006). The organic matter (OM) was calculated as the difference between DM and ash contents. For NDF, samples were treated with alpha thermo-stable amylase without sodium sulfite according to Van Soest et al. (1991) and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). Volatile fatty acid concentrations were determined using gas chromatography (Varian Model 3800; Varian, Inc, Walnut Creek, CA; equipped with a glass column [180 cm x 4 mm i.d.]) packed with GP 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb WAW [Supelco, Bellefonte, PA]), and N_2 was used as a carrier gas at a flow rate of 85 mL/min-1. The NH_3-N concentration was determined by colorimetry as described by Chaney and Marbach (1962). The total VFA and NH_3-N concentration were calculated subtracting the values measured on the initial content of the components in the rumen/buffer solution from the final concentrations of each bottle (Tagliapietra et al., 2013). The cumulated gas pressures at 24 and 48 h were converted to milliliters according to Tagliapietra et al. (2011) as GP, mL = (P_c/P_o) x V_o, where P_c is the cumulated pressure change (kPa) in the bottle headspace; V_o is the bottle headspace volume (545 mL), P_o is the atmospheric pressure read by the equipment at the beginning of the measurement. The bottles final gas production volumes were corrected by subtracting the final gas production of the blank bottles. Metabolizable energy was calculated according to Menke and Steingass (1988), with lipid content ignored (Grings et al., 2005), as ME (MJ/kg

DM) = 2.20 + (0.1357 × GP200) + (0.0057 × CP) where GP200 (mL/200 mg of DM incubated) is the gas production measured at 48h. The *in vitro* dry matter digestibility (IVD) was calculated as IVD, % DM = 100 - (W_3 - (W_1 x C_1)) x 100 / (W_2 x DM), where: W_1 = Bag tare weight, W_2 = Sample weight, W_3 = Final bag weight after *in vitro* and sequential NDF determination, C_1 = Blank bag correction (final oven-dried weight/original blank bag weight), DM = % dry matter.

Statistical analysis

All results were tested for normality (Davis and Stephens, 1989), and they followed normal distribution ($P > 0.05$). All statistical procedures were carried out using SAS 9.2 for Windows (Statistical Analysis System Institute, Inc., Cary, NC, USA) with $\alpha = 0.05$. Variance Components was used as the covariance structure in the models used. For data from **Exp. 1**, and **Exp. 2** (ingredients evaluation), the model contained the effect of bottle and run as random effects, and feed was considered fixed effect. When an interaction between main effects occurred, multiple comparisons between treatments were completed with Tukey's test. Data from **Exp. 3** and **Exp. 4** (diets with different levels of glycerin) were analyzed for linear and quadratic responses in a regression analysis. The model contained the effect of glycerin inclusion as fixed quantitative effect. Run and bottle were considered random effects. For **Exp. 5** (*in vitro* DM digestibility) the model contained the effect of vessel and period as random effects, and treatment was considered fixed effect. When an interaction between main effects occurred, multiple comparisons between treatments were completed with Tukey's test.

Results

Ingredients evaluation: corn, glycerin and starch (Exp. 1 and Exp. 2)

For **Exp. 1**, the 24 h total gas production decreased ($P < 0.01$) with feeding glycerin, relative to corn and starch (Table 2). However, the 48 h total gas production was highest ($P < 0.01$) for corn, and similar between glycerin and starch. Compared with glycerin, the pH and NH_3-N decreased ($P < 0.01$) with feeding starch but were similar with corn. The starch treatment presented the lowest ($P = 0.01$) total VFA concentration relative to glycerin and corn. Volatile fatty acids patterns differed among substrate types. Acetate concentration decreased ($P < 0.01$) with feeding glycerin relative to the corn and starch. On the other hand,

corn presented the lowest propionate concentration ($P < 0.01$) compared with glycerin and starch. Glycerin treatment presented higher ($P < 0.01$) butyrate and valerate concentrations than corn and starch. Bottles fed with glycerin presented lowest ($P < 0.01$) acetate: propionate ratio, *iso*-butyrate and *iso*-valerate molar proportions, followed by starch and corn, respectively. The metabolizable energy was highest ($P < 0.01$) for corn, and similar between glycerin and starch.

For **Exp. 2**, the CH_4 (% and mL/g) production increased ($P < 0.01$) with feeding glycerin relative to corn and starch (Table 3). However, the CO_2 in % was lowest ($P < 0.01$) for glycerin, and similar between corn and starch. The CO_2 in mL/g was highest ($P < 0.01$) for corn, and similar between glycerin and starch. The pH decreased ($P < 0.01$) only with feeding starch. Different ingredients had no effect on total VFA ($P = 0.50$), propionate ($P > 0.86$), *iso*-butyrate ($P = 0.10$), and *iso*-valerate ($P = 0.08$) concentrations. Compared with glycerin treatment, acetate concentration ($P < 0.01$) and acetate: propionate ratio ($P = 0.01$) were higher for corn and starch, whereas butyrate and valerate concentrations were higher ($P = 0.01$) for glycerin than corn and starch treatments.

Diets evaluation: different glycerin levels in beef cattle diets (Exp. 3 and Exp. 4)

For **Exp. 3**, glycerin levels did not affect 24 h ($P_{Lin.} = 0.82$; $P_{Quad.} = 0.88$), and 48 h ($P_{Lin.} = 0.67$; $P_{Quad.} = 0.84$) total gas production, final pH ($P_{Lin.} = 0.74$; $P_{Quad.} = 0.92$), NH_3-N ($P_{Lin.} = 0.65$; $P_{Quad.} = 0.75$), and total VFA ($P_{Lin.} = 0.61$; $P_{Quad.} = 0.58$), which averaged 270 ± 60.9 mL/g DM, 321 ± 76.6 mL/g DM, 5.85 ± 0.21, 28.8 ± 4.33 mg/100mL, and 6.64 ± 1.84 mM/g DM respectively (Table 4). The inclusion of glycerin linearly decreased acetate concentration ($P = 0.03$) and acetate: propionate ratio ($P = 0.04$). Linear increase of valerate concentration ($P < 0.01$) was observed due to inclusion of glycerin in the diets. Also, glycerin inclusion did not affect propionate ($P_{Lin.} = 0.13$; $P_{Quad.} = 0.99$), butyrate ($P_{Lin.} = 0.93$; $P_{Quad.} = 0.96$), *iso*-butyrate ($P_{Lin.} = 0.30$; $P_{Quad.} = 0.83$), and *iso*-valerate ($P_{Lin.} = 0.40$; $P_{Quad.} = 0.95$), which averaged 27.1 ± 2.09, 23.32 ± 2.41, 2.98 ± 0.36, and 7.27 ± 0.68 mol/100mol, respectively.

For **Exp. 4**, the glycerin inclusion did not affect the productions of CH_4 in % ($P_{Lin.} = 0.11$; $P_{Quad.} = 0.83$) or mL/g ($P_{Lin.} = 0.44$; $P_{Quad.} = 0.92$), and CO_2 in % ($P_{Lin.} = 0.60$; $P_{Quad.} = 0.63$) or mL/g ($P_{Lin.} = 0.75$; $P_{Quad.} = 0.71$), which averaged 6.01 ± 0.97 %, 9.65 ± 3.73 mL/g DM, 30.0 ± 3.31 %, 48.0 ± 9.64 mL/g DM, respectively (Table 5). Glycerin levels also did

not affect final pH ($P_{Lin.}$ = 0.49; $P_{Quad.}$ = 0.79), and total VFA ($P_{Lin.}$ = 0.64; $P_{Quad.}$ = 0.34), which averaged 5.33 ± 0.13, and 20.54 ± 9.06 mL/g DM respectively. A linear decrease of acetate concentration (P = 0.04) was observed due to inclusion of glycerin in the diets. The inclusion of glycerin linearly increased valerate concentration (P < 0.01). Glycerin levels did not affect the concentrations of propionate ($P_{Lin.}$ = 0.71; $P_{Quad.}$ = 0.84), butyrate ($P_{Lin.}$ = 0.17; $P_{Quad.}$ = 0.52), *iso*-butyrate ($P_{Lin.}$ = 0.44; $P_{Quad.}$ = 0.91), *iso*-valerate ($P_{Lin.}$ = 0.83; $P_{Quad.}$ = 0.96), as well as acetate: propionate ratio ($P_{Lin.}$ = 0.31; $P_{Quad.}$ = 0.96), which averaged 27.4 ± 3.95 mol/100 mol, 29.0 ± 2.80 mol/100 mol, 2.24 ± 0.34 mol/100 mol, 5.37 ± 0.87 mol/100 mol, and 1.13 ± 0.29, respectively.

In vitro dry matter digestibility (Exp. 5)

Orchard hay with glycerin inclusion presented lowest (P < 0.01) *in vitro* dry matter digestibility followed by orchard hay without glycerin, and corn treatments (Figure 1). There was a lack of effects for *in vitro* dry matter digestibility (P > 0.05) between corn with or without glycerin.

Discussion

Ingredients evaluation: corn, glycerin and starch (Exp. 1 and Exp. 2)

The findings in this study confirm our hypothesis that glycerin presents similar 48 h total gas production than starch, but lower than corn. However, compared to starch, glycerin had lower 24 h total gas production. Moreover, starch had all gas produced until 24 h of fermentation, which might be also an explanation for the lower pH and NH_3-N observed for starch compared to the others treatments. It seems that the quick starch fermentation and mainly microbial adaptation to glycerin played a role in the ruminal parameters and gas production rate. The microbial adaptation to glycerin is slow and may affect the ruminal fermentation kinetics, essentially gas production rate and lag time (Ferraro et al., 2009; Lee et al., 2011). According to (Van Cleef et al., 2013) prior adaptation might be required to optimize glycerin utilization by ruminal microorganisms. These authors observed higher digestibility when glycerin was added to *in vitro* cultures containing ruminal inoculum from adapted animals, compare to those utilizing inoculum from unadapted animals. In this study the donor animals were not fed with glycerin. Changes in VFA profile may affect the ruminal gas production, and fermentation to acetate yields more gas than that to propionate (Blümmel

et al., 1997). Thus, the higher total gas production in corn treatment may be explained by the shift in VFA profile, with higher acetate concentration at the expense of lower propionate concentration. Lee et al. (2011) observed that glycerin presented lower total gas production than corn and alfalfa using an *in vitro* fermentation system. Others indicated higher total *in vitro* gas production and slowest rate of gas production for glycerin when compared to alfalfa, corn silage, propylene glycol, and molasses (Ferraro et al., 2009). Therefore, our results suggest that compare to starch, glycerin has a slower rate of degradation, but the same fermentation pattern at 48 h.

With regards to total VFA concentration, the lack of differences observed between treatments for **Exp. 2** are in agreement with our hypothesis that glycerin could have the same ruminal fermentation potential than starch or corn. However, for the **Exp. 1**, starch presented lower total VFA than other treatments. The quickly fermentation of starch could increase the lactic acid concentration, which also could decrease the total VFA concentration and reduce the ruminal pH (Ørskov, 1986). In this study the ruminal pH was lower for starch than for corn and glycerin. As expected, compare to corn and starch, glycerin has decreased acetate concentration and acetate: propionate ratio. However, the decrease in acetate was also associated with an increase in butyrate concentration. Acetate is on the pathway of butyrate production (Rémond et al., 1993) with intense interconversion between them, thereby the increase in butyrate may be associated with the decrease in acetate concentration when glycerin was fed. Moreover, butyrate increases in ruminal fluid has been related with Megasphaera elsdenii, an importing glycerin-fermenting bacteria (Shin et al., 2012). Furthermore, the similar results between starch and glycerin for propionate concentration are in agreement with our hypothesis that both ingredients have the same potential of gluconeogenic precursors production in the rumen. The lower propionate concentration for corn relative to corn and starch on **Exp. 1** probably is associated with the fermentation of its fibrous components that lead to acetate production. Nevertheless, no differences were observed in propionate concentration for **Exp. 2**. Previous studies have observed no differences in total VFA and lower acetate concentration and acetate: propionate ratio with feeding glycerin relative to corn (Lee et al., 2011), and corn silage (Ferraro et al., 2009).

Agreeing with our hypothesis, glycerin *in vitro* fermentation showed similar metabolizable energy than starch. These results suggest that glycerin has the same energetic potential in the rumen than starch, probably because both are mainly fermented to

propionate. Also, in this study corn presented a metabolizable energy 7.3 and 9.7% higher than glycerin and starch, respectively, which may also suggest that glycerin has lower energy content per kg of DM than corn. This fact might be a problem especially for finishing animals, which have high-energy requirements. However, the equation utilized to estimate the metabolizable energy takes into account the crude protein content of the ingredient evaluated. Thus, the higher metabolizable energy observed in bottles fed with corn also might be related to the crude protein presence in corn chemical composition. The other two ingredients tested (glycerin and starch) do not present this nutrient in their composition. Furthermore, the metabolizable energy observed in this study was calculated considering that 100% of glycerin was fermented in the rumen. Rémond et al. (1993) observed that glycerin may escapes rumen fermentation, being absorbed by the rumen wall (up to 43%) or small intestine (up to 13%), which may provide more energetic contribution (Mach et al., 2009). The glycerin that escapes ruminal fermentation reaches the intestine with an energy content of 18.0 MJ/kg, a value 32.4% higher than corn energy content, which is 13.6 MJ/kg (NRC, 2001). Mach et al. (2009) calculated glycerin metabolic energy (16.9 MJ/kg), considering that 50% of glycerin is fermented to propionate (1.54 MJ of ME per mol (Baldwin, 1968)) in the rumen, and the other 50% escapes ruminal fermentation, being absorbed by the intestine. Working with cannulated crossbreed steers, Monnerat et al. (2013) also observed higher energy levels for glycerin (15.2 MJ/kg) compared to corn, suggesting that glycerin contributes with more energy per unit of DM than corn.

We hypothesized that glycerin would reduce the greenhouse gases production because its fermentations leads to propionate production more than starch (Del Bianco Benedeti et al., 2015). Pathways to propionate formation from hexoses act as a hydrogen sink (Moss et al., 2000; Boadi et al., 2004), reducing the availability of hydrogen for CH_4 formation by changing the overall electron balance in the rumen. Furthermore, there is no CO_2 formation on pathways to propionate production (Figure 2). As expected, glycerin treatment reduced the CO_2 (%) production compared to corn and starch. However, the higher CH_4 production for glycerin, compared to the other treatments, contradicts our hypothesis. An explanation for these results is the fact that propionate formation from glycerin does not act as a hydrogen sink (Avila-Stagno et al., 2014). Its pathway formation from glycerin releases two more hydrogen ions than that from hexoses (Zhang and Yang, 2009). Figure 2 shows glycerin and glucose fermentation pathways to acetate, butyrate, and propionate, as

well as the ATP, NADH, H_2, and CO_2 balance of each one. Each mol of glycerin enters on the glycolysis pathway as D-glyceraldehyde 3-P, releasing one mol of NADH + H^+ and consuming one ATP. Therefore to produce VFA, glycerin releases more hydrogen than hexoses and contribute more than corn or starch to increasing CH_4 production. The higher butyrate concentration for glycerin also may explain the higher CH_4 production for this treatment. Butyrate formation releases more reducing equivalents than acetate or propionate, and contribute to the increase in CH_4 production (Figure 2). Avila-Stagno et al. (2014) observed increases in total CH_4 production, propionate and butyrate concentrations, and decrease in acetate: propionate ratio in a semi-continuous culture system when glycerin (99.5% purity) was included at up to 15% (DM basis) in forage-based diets. According to these authors, the butyrate and acetate formation from glycerin instead of from carbohydrates would further contribute to the enhancement of methanogenesis.

Diets evaluation: different glycerin levels in beef cattle diets (Exp. 3 and Exp. 4)

We hypothesized that glycerin could replace corn and be included at up to 30% in beef cattle diets without compromising total gas production in an *in vitro* fermentation system. As expected, the replacement of corn with glycerin did not change the 24 h and 48 h total gas production. Avila et al. (2011) also observed lack of differences for 48 h total gas production occurred in an *in vitro* experiment when glycerin (99.5% purity) was included at up to 21% (DM basis) in diets. Despite **Exp. 1** has indicated that glycerin has slower gas production rate than corn and starch, the lack of effects observed for 24h total gas production with glycerin inclusion on **Exp. 3** indicate an additive relationship between glycerin and cornstarch, which may have provided same fermentation patter. Hales et al. (2013) observed increase on starch digestibility when glycerin was included at up to 10% in diets of beef steers. The lack of effects for total VFA, NH_3-N and final pH with glycerin inclusion may also support our hypothesis.

The lack of effects for ruminal parameters also may be the reasons for partial dietary replacement of corn with glycerin does not have affected the greenhouse gases production in this study. As discussed before, glycerin pathways to VFA release more hydrogen than that from hexoses. There was a decrease in acetate: propionate ratio with glycerin inclusion in this study. Acetate production from both glycerin and hexoses would result in higher release of reducing equivalents than propionate production (Avila-Stagno et al., 2014). Thus, the

decreasing in acetate concentration for glycerin treatments seems to be compensated by the higher hydrogen releasing when acetate is produced from glycerin, thereby causing similar CH_4 production among treatments. Effects of glycerin on greenhouse gases production in ruminants have been conflicting by *in vitro* and *in vivo* experiments. According to Avila-Stagno et al. (2014), the pre adaptation of donor animals as well as the absorption through rumen and intestine walls on *in vivo* experiments may be an explanation for this inconsistency. These authors observed an increase in CH_4 production in a pre adapted semi-continuous culture system when glycerin (99.5% purity) was included at up to 15% (DM basis) in forage-based diets. However, the *in vitro* production of greenhouse gases (CH_4 and CO_2) was unaffected by glycerin inclusion using ruminal inoculum from adapted and unadapted donor animals (Van Cleef et al., 2013). Similar to this study, Avila et al. (2011) observed that CH_4 production was not affected when glycerin was added at up to 21% of DM in an *in vitro* system. Avila-Stagno et al. (2013) also noted a lack of effects in CH_4 production when lambs were fed diets containing up to 21% (DM basis) of glycerin. Others have observed a reduction in CH_4 and CO_2 productions in Nelore steers when crude glycerin was included up to 30% (DM basis) in the diet (van Cleef et al., 2015). As discussed before, part of the glycerin may escape rumen fermentation, being absorbed by rumen and intestine walls and consequently decreasing ruminal CH_4 production.

As expected, the replacement of corn with glycerin changed the VFA profile, with decreasing acetate concentration and acetate: propionate ratio. The main explanation for these findings is that glycerin is preferentially fermented to propionate rather than acetate (Abo El-Nor et al., 2010; Ramos and Kerley, 2012) and the fact that one of the main glycerin-fermenting bacteria, Anaerovibrio lipolytica does not have acetate as the main fermentation product when glycerin is the substrate (Stewart et al., 1997). However, despite a numerical decrease for propionate concentration in this study, a lack of differences was observed for this parameter with glycerin inclusion. Previous *in vivo* (van Cleef et al., 2015) and *in vitro* (Rico et al., 2012; Del Bianco Benedeti et al., 2015) studies have reported decrease on acetate: propionate ratio with glycerin inclusion. Therefore, the lack of differences in VFA concentration and total gas production and greenhouse gases production in this study indicate same energy efficiency when glycerin replaces corn in beef cattle diets.

In vitro dry matter digestibility (Exp. 5)

Results confirm our hypothesis that glycerin adition would not impact the corn *in vitro* DM digestibility. However, adding glycerin reduced the *in vitro* DM digestibility of orchard hay. These results suggest suppression in fiber digestibility when glycerin is added in forage feedstuffs. In the study by Roger et al. (1992) an inhibition in growth and activity of cellulolytic bacteria and anaerobic fungal species were observed when glycerin was added at a concentration of 5% DM. Therefore, the inhibition of cellulolytic activity could affect fiber digestion, and consequently decrease fiber digestibility in forage based diets (Paggi et al., 2004). However, previous studies have presented lack of effects in ruminal DM and NDF digestibility on *in vitro* studies (Abo El-Nor et al., 2010; Del Bianco Benedeti et al., 2015), or even a quadratic increase in apparent DM and NDF digestibility when glycerin was included in high concentrate diets for finishing steers (Del Bianco Benedeti et al., 2015). According with these authors, glycerin fermentation characteristics as microbial adaptation, fast ruminal turnover (Del Bianco Benedeti et al., 2016), glycerin additive relationship with starch digestion (Bergner et al., 1995; Wang et al., 2009), as well as high VFA production (Hales et al., 2013) may stimulates DM digestion.

Conclusion

Results for ingredients evaluation studies indicate that glycerin present slower rate of degradation but similar 48 h total gas production than starch. Furthermore, glycerin and starch presented similar metabolizable energy, which suggesting that glycerin may be used as energy source in finishing beef cattle diets. However, glycerin presented higher CH_4 production than corn and starch indicating that glycerin would contribute more to the enhancement of methanogenesis than carbohydrates.

The lack of effects for ruminal parameters, total gas production and greenhouse gases production for glycerin on diets evaluation studies suggest same energy efficiency when glycerin replaces corn in beef cattle diets. Therefore, under these experimental conditions, glycerin effectively replaced dietary corn at up to 30% of the diet. However, results for **Exp. 5** suggest suppression in fiber digestibility when glycerin is added in forage feedstuffs.

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Table 1. Ingredients and chemical composition of experimental diets used on Exp.3 and Exp.4

Item	Glycerin, %			
	0	10	20	30
Ingredient, % DM				
Orchard hay	20.0	20.0	20.0	20.0
Dry ground corn	72.4	60.4	48.4	36.4
Glycerin[1]	0.00	10.0	20.0	30.0
Soybean meal	7.63	9.63	11.6	13.6
Composition, % DM				
Dry matter, %	89.4	90.0	90.6	91.2
Organic matter	97.4	97.4	97.4	97.4
Neutral detergent fiber	20.5	19.1	17.7	16.3
Crude protein	13.5	13.5	13.5	13.5
Ether extract	2.81	2.43	2.04	1.65

[1]Purity of 99.7% (Nature's Oil, Streetsboro, OH, USA)

Table 2. Effects of different ingredients on total gas production and ruminal parameters in gas production system in Exp. 1[1]

Item	Treatments			SEM	*P*-value
	Corn	Starch	Glycerin		
24h total gas production, mL/g DM	324[a]	324[a]	287[b]	19.1	< 0.01
48h total gas production, mL/g DM	384[a]	344[b]	354[b]	24.1	< 0.01
Final pH	5.89[a]	5.67[b]	5.93[a]	0.13	< 0.01
N-NH_3, mg/100mL	18.9[a]	10.5[b]	16.6[a]	2.11	< 0.01
Total volatile fatty acids, mM/g DM	15.3[ab]	14.4[b]	17.4[a]	3.67	0.01
Volatile fatty acids profile, mol/100mol					
Acetate	35.8[a]	35.2[a]	24.8[b]	0.42	< 0.01
Propionate	24.8[b]	29.5[a]	30.0[a]	1.43	< 0.01
Butyrate	23.0[b]	21.4[b]	28.9[a]	1.27	< 0.01
Valerate	6.26[b]	5.67[c]	8.34[a]	0.41	< 0.01
Iso-butyrate	3.13[a]	2.62[b]	2.40[c]	0.09	< 0.01
Iso-valerate	7.09[a]	5.61[b]	5.63[b]	0.18	< 0.01
Acetate: propionate	1.46[a]	1.23[b]	0.86[c]	0.07	< 0.01
Metabolizable energy[2], MJ/kg DM	12.7[a]	11.5[b]	11.8[b]	0.65	< 0.01

[a,b,c] Means with different superscripts in the same row are different ($P < 0.05$).

[1]Twenty-four bottles (620 mL) equipped with wireless pressure sensors (Ankom[RF] Gas Production System) used in four consecutive 48-h runs; Experimental design: 4 incubation runs x 3 ingredients x 7 bottles per treatment, plus 12 blank bottles (3 per run), totaling 96 observations.

[2]Metabolizable energy (MJ/kg DM) = 2.20 + (0.1357 × GP200) + (0.0057 × CP) where GP200 (mL/200 mg of DM incubated).

Table 3. Effects of different ingredients on enteric greenhouse gases production and ruminal parameters in gas production system in Exp. 2[1]

Item	Treatments			SEM	*P*-value
	Corn	Starch	Glycerin		
CH_4, %	5.22[b]	5.16[b]	8.92[a]	0.42	< 0.01
CH_4, mL/g DM	9.71[b]	8.49[b]	13.6[a]	1.67	< 0.01
CO_2, %	30.9[a]	30.1[a]	26.5[b]	1.29	< 0.01
CO_2, mL/g DM	57.1[a]	48.5[b]	40.8[b]	6.85	< 0.01
Final pH	5.22[a]	5.00[b]	5.37[a]	0.07	< 0.01
Total volatile fatty acids, mM/g DM	19.8	16.7	19.3	3.16	0.50
Volatile fatty acids profile, mol/100mol					
Acetate	30.7[a]	35.1[a]	18.3[b]	1.29	< 0.01
Propionate	27.5	29.2	28.3	2.37	0.86
Butyrate	29.1[b]	25.4[b]	39.6[a]	1.96	0.01
Valerate	5.32[b]	4.46[b]	7.94[a]	0.62	0.01
Iso-butyrate	2.26	1.92	1.80	0.16	0.10
Iso-valerate	5.15	4.00	4.09	0.44	0.08
Acetate: propionate	1.17[a]	1.23[a]	0.65[b]	0.12	0.01

[a,b] Means with different superscripts in the same row are different ($P < 0.05$).

[1]Twenty serum bottles (155 mL) equipped with stoppers and sealers used in four consecutive 48-h runs; Experimental design: 4 incubation runs x ingredients x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 80 observations.

Table 4. Effects of glycerin inclusion on total gas production and ruminal parameters in gas production system in Exp. 3[1]

Item	Glycerin, %				SEM	*P*-value	
	0	10	20	30		Linear	Quadratic
24h total gas production, mL/g DM	280	262	274	266	29.9	0.82	0.88
48h total gas production, mL/g DM	340	312	324	311	37.5	0.67	0.84
Final pH	5.84	5.83	5.87	5.88	0.12	0.74	0.92
N-NH_3, mg/100mL	29.4	29.2	27.7	28.7	1.79	0.65	0.75
Total volatile fatty acids, mM/g DM	6.7	6.8	6.9	6.2	0.66	0.61	0.58
Volatile fatty acids profile, mol/100mol							
Acetate	34.2	33.5	32.5	31.7	0.79	0.03	0.89
Propionate	25.9	26.9	27.3	28.2	1.01	0.13	0.99
Butyrate	23.3	23.2	23.4	23.4	1.32	0.93	0.96
Valerate	5.94	6.22	6.52	6.78	0.11	< 0.01	0.91
Iso-butyrate	3.14	2.94	2.98	2.85	0.17	0.30	0.83
Iso-valerate	7.51	7.29	7.24	7.06	0.36	0.40	0.95
Acetate: propionate	1.33	1.26	1.20	1.13	0.06	0.04	0.99

[1]Twenty-four bottles (620 mL) equipped with wireless pressure sensors (Ankom[RF] Gas Production System) used in four consecutive 48-h runs; Experimental design: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 16 blank bottles (4 per run), totaling 96 observations.

Table 5. Effects of glycerin inclusion on enteric greenhouse gases production and ruminal parameters in gas production system in Exp. 4[1]

Item	Glycerin, %				SEM	*P*-value	
	0	10	20	30		Linear	Quadratic
CH_4, %	5.52	5.91	6.03	6.61	0.44	0.11	0.83
CH_4, mL/g DM	8.39	10.6	8.37	10.9	1.49	0.44	0.92
CO_2, %	29.9	30.8	29.6	29.3	1.25	0.60	0.63
CO_2, mL/g DM	45.1	55.3	40.9	47.5	4.93	0.75	0.71
Final pH	5.31	5.31	5.33	5.38	0.07	0.49	0.79
Total volatile fatty acids mM/g DM	20.8	18.2	19.4	22.4	2.85	0.64	0.34
Volatile fatty acids profile, mol/100mol							
Acetate	32.3	30.0	29.2	27.6	1.40	0.04	0.73
Propionate	26.9	27.1	27.0	28.1	2.12	0.71	0.84
Butyrate	27.5	29.2	30.1	30.0	1.28	0.17	0.52
Valerate	5.51	6.00	6.24	6.59	0.25	0.01	0.77
Iso-Butyrate	2.33	2.26	2.17	2.14	0.19	0.44	0.91
Iso-Valerate	5.41	5.34	5.37	5.25	0.46	0.83	0.96
Acetate: propionate	1.26	1.15	1.11	1.02	0.15	0.31	0.96

[1]Twenty-five serum bottles (155 mL) equipped with stoppers and sealers used in four consecutive 48-h runs; Experimental design: 4 incubation runs x 4 diets x 5 bottles per treatment, plus 20 blank bottles (5 per run), totaling 100 observations.

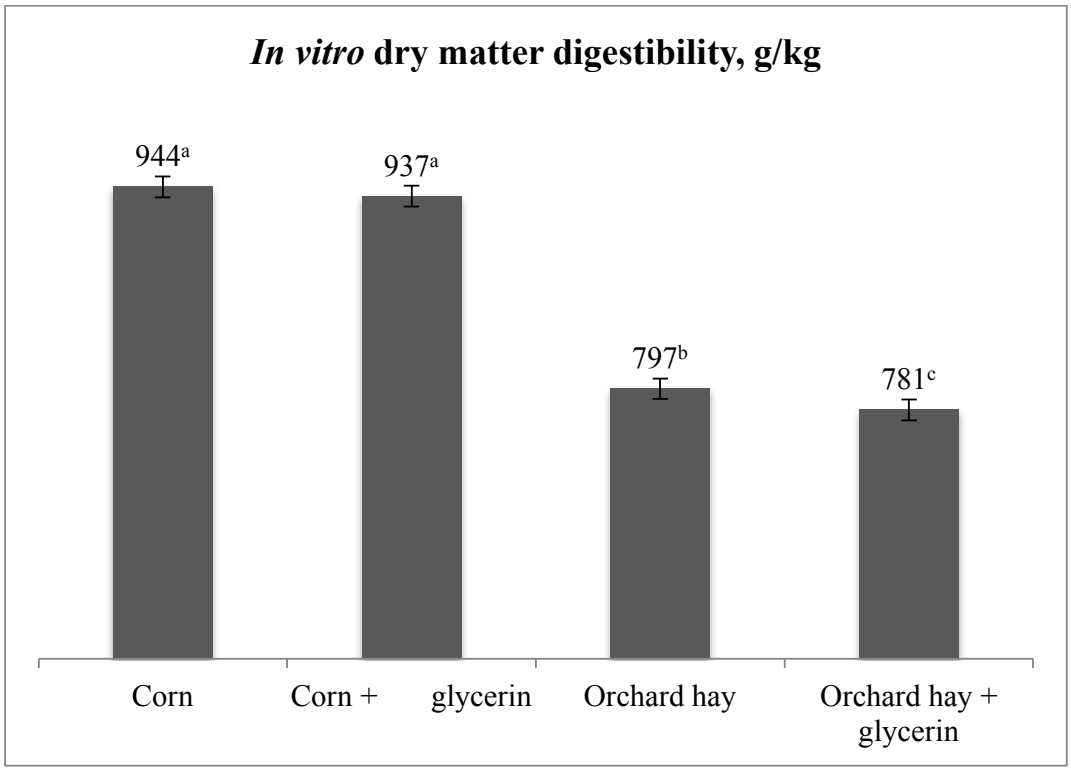

Figure 1. In vitro dry matter digestibility of individual ingredients (corn and orchard hay) and co-incubation with glycerin in Exp. 5 using two systems of four 4-L digestion vessels (DaisyII system, Ankom technology, NY, USA) in four consecutive 48-h runs. The experimental design included two 4x4 Latin squares ran simultaneously.

Substrate	Products				
	Propionate	ATP	NADH	H	CO_2
Glucose	1	1	-1	-2	0
Glycerin	1	1	0	0	0
Acetate					
Glucose	1	2	2	4	1
Glycerin	1	2	3	6	1
Butyrate					
Glucose	1	3	2	4	2
Glycerin	1	3	4	8	2

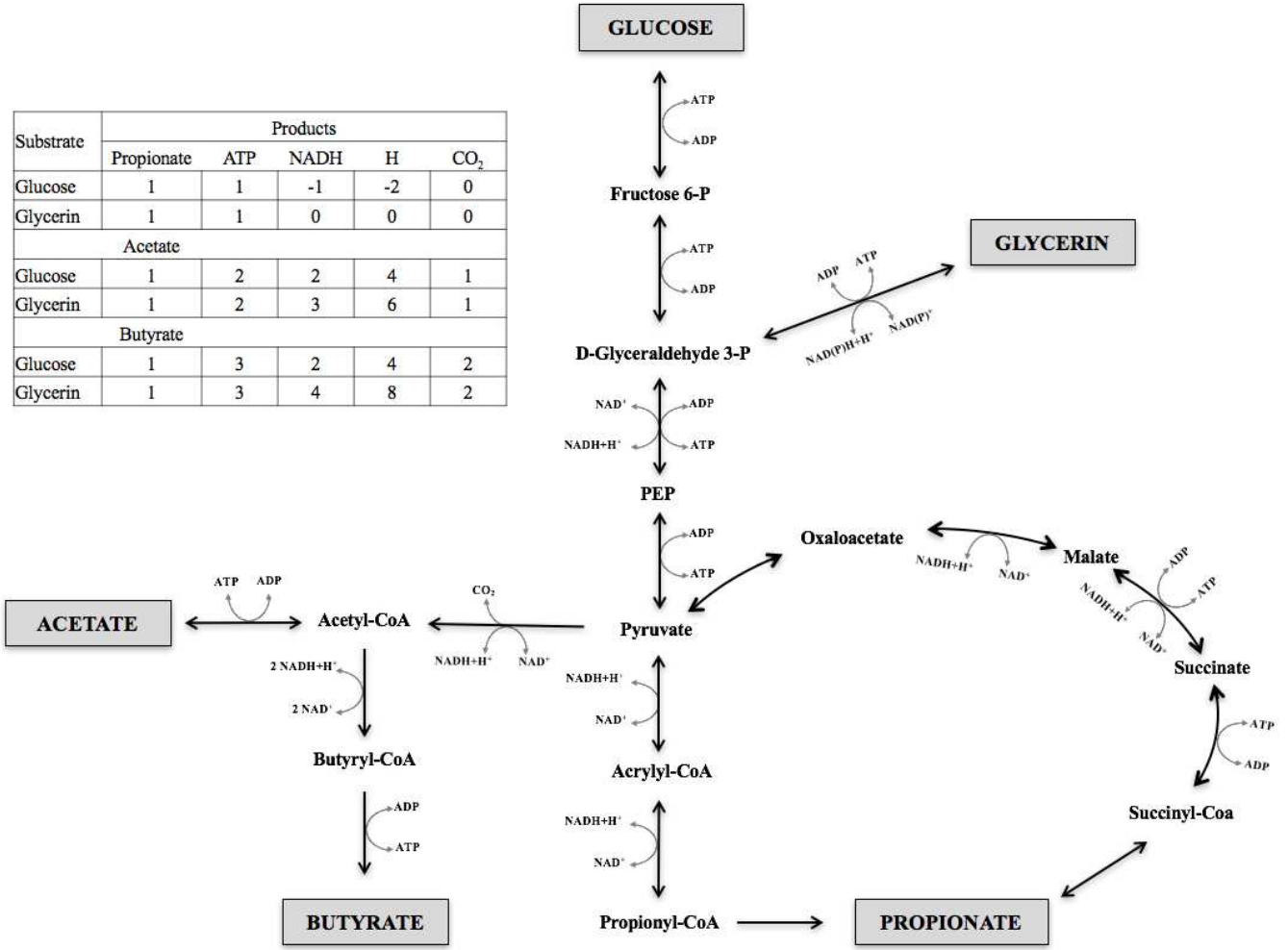

Figure 2. Main products of glucose and glycerin pathways to acetate, butyrate, and propionate formation (adapted from Nelson and Cox (2009)).