OZANA DE FÁTIMA ZACARONI

CRUDE GLYCERIN AS AN ENERGY FEED FOR DAIRY COWS

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Zootecnia, área de concentração em Produção Animal, para a obtenção do título de Doutor.

Orientador

Dr. Marcos Neves Pereira

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2014

Aos meus pais, Israel e Aparecida,
Ao meu irmão, Israel,
A minha irmã, Ana Beatriz,

DEDICO.

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“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

Charles Chaplin

RESUMO GERAL

Com o crescimento mundial na produção de biodiesel existe maior disponibilidade do co-produto glicerina bruta. Glicerol é o principal componente da glicerina bruta, tendo valor energético próximo ao do milho, podendo ser usado na alimentação animal. A inclusão de glicerina bruta em dietas contendo silagem de cana-de-açúcar pode ser uma forma para compensar a perda de energia que ocorre na ensilagem desta forrageira. No entanto, o maior contaminante da glicerina é o metanol, que pode causar danos a saúde animal. O objetivo deste estudo foi avaliar a inclusão de glicerina bruta com alto teor de metanol em dietas de vacas leiteiras. No primeiro experiemento testou-se a substituição parcial de silagem de milho por uma mistura isofibrosa de silagem de cana e glicerina bruta (7,2% de metanol). Simultaneamente foi avaliada a incorporação de flavorizantes às forragens, em arranjo fatorial 2x2 de tratamentos. Foi avaliado o desempenho, a digestibilidade, a seletividade e o equilíbrio ácido-básico venoso. A adição de flavorizantes à dieta com silagem de milho reduziu a produção de leite (32,2 vs 31.1 kg/d), mas induziu aumento da produção das vacas consumindo silagem de cana e glicerina bruta (30.3 vs 31.7 kg/d). Silagem de cana e glicerina aumentaram o teor de gordura e proteina no leite. Flavorizantes reduziram a concentração de glicose no sangue quando adicionados a dietas contendo silagem de milho, mas aumentaram quando foram adicionados a dietas contendo silagem de cana. Houve rejeição de particulas longas e o consumo preferencial por particulas pequenas quando palatabilizantes foram adicionados a silagem de milho, no entanto ocorreu uma reduçãona rejeição de partículas longas quando adicionados a silagem de cana. Não houve efeito dos tratamentos sobre a digestibilidade aparente de nutrientes no trato digestivo total. O tipo de forragem não determinou o equilíbrio ácido-básico venoso, no entanto, antes da alimentação matinal, flavorizantes reduziram a pressão parcial de gás carbônico e a saturação de hemáceas e aumentaram a pressão parcial de oxigênio e a saturação de oxigênio. No segundo experimento foi avaliado a sustituição parcial milho finamente moído por 0, 5 e 10% de glicerina bruta. Foi avaliado o desempenho, a digestibilidade e o equilíbrio ácido-básico. A inclusão de glicerina reduziu linearmente a produção de leite (22,2; 21,2; 20,0 kg/d) e a secreção de lactose, sem afetar o consumo de matéria seca, reduzindo a eficiência alimentar. O teor de gordura (4,11; 4,33; 4,37%) e de proteína (3,47; 3,64; 3,73%) aumentou linearmente com a suplementação de glicerina. Os tratamentos com 5 e 10% de glicerina induziram a redução da pressão parcial de gás carbônico e aumento na saturação da hemoglobina com oxigênio, 6 horas após a alimentação matinal. A inclusão de glicerina bruta contendo 7.2 % de metanol não causou efeito negativo na saúde de vacas leiteiras.

Palavras-chave: Glicerol. Glicerina bruta. Metanol. Cana-de-açucar. Palatabilizantes.

GENERAL ABSTRACT

The worldwide growth in biodiesel production there is greater availability of crude glycerin co-product. Glycerol is the main component of crude glycerin, with energy value close to that of corn, and can be used as animal feed. The inclusion of crude glycerin in diets containing sugarcane silage can be a way to compensate the energy loss that occurs in the silage. However, the most important contaminant of glycerin is methanol, which can cause damage to animal health. The objective of this study was to evaluate the inclusion of crude glycerin with high content of methanol in diets of dairy cows. In the first experiment was tested the partial replacement of corn silage with a mixture of sugarcane silage and crude glycerin (7.2% methanol). At the same time was evaluated the addition of sensorial feed additives, in 2 x 2 factorial arrangement, was evaluated performance, digestibility, the selectivity and the acid/base balance. The addition of sensorial feed additives to the diet with corn silage reduced milk production (32.2 vs. 31.1 kg/d), but induced increase in production of cows consuming sugarcane silage and crude glycerin (30.3 vs 31.7 kg/d). Sugarcane silage and Glycerin increased fat content and protein in milk. Flavoring reduced the concentration of glucose in the blood when added to diets containing corn silage, but increased when added to diets containing sugarcane silage. There was rejection of long particles and small particles by preferential consumption when sensorial feed additives were added to corn silage; however, there was a decrease long particle rejection when added to sugarcane silage. There was no effect of the treatments on the apparent digestibility tract total of nutrients. The kind of roughage has not determined the vein acid/base balance, however, before the morning feeding, flavoring reduced partial pressure of carbon dioxide and the hemoglobin saturation, and increased partial pressure of oxygen and oxygen saturation. In the second experiment was to evaluate the finely ground corn for partial replacement 0, 5 and 10% of crude glycerin. Evaluating the performance, digestibility and the acid/base balance. The inclusion of glycerin reduced linearly milk production (22.2; 21.2; 20.0 kg/d) and the secretion of lactose, without affecting the dry matter intake. Which reduced the feed efficiency. The fat content (4.11; 4.33; 4.37%) and protein (3.73; 3.47; 3.64%) increased linearly with the supplementation of glycerin. The treatments with 5 and 10% glycerin induced a reduction in partial pressure of carbon dioxide and increase in hemoglobin saturation with oxygen, 6 hours after the morning feeding. The inclusion of crude glycerin containing 7.2% methanol caused no negative effect on the health of dairy cows.

Key words: glycerol, crude glycerin, methanol, sugarcane, sensorial feed additives

SUMMARY

FIRST PART 1
1 INTRODUCTION 1
2 LITERATURE REVIEW 2
2.1 Glycerol 2
2.2 Crude glycerin 5
2.3 Methanol 6
2.4 Glycerin as feed ingredient 8
2.5 *In vitro* studies 10
2.6 Absorption of glycerol 13
2.7 Sugarcane silage 14
2.8 Sensory feeds additives 15
2.9 Feed sorting 17
REFERENCES 19
SECOND PART - PAPERS 1
PAPER 1 The partial replacement of corn silage with sugarcane silage plus crude glycerin and the effect of sensorial feed additives for dairy cows 1
PAPER 2 Performance, digestibility, and blood acid-base balance of dairy cows in response to the replacement of corn by crude glycerin 1

FIRST PART

1 INTRODUCTION

Biodiesel is a renewable fuel, mainly produced by esterifying vegetable oil or animal fat with methanol, having alkali as catalyzer (HU et al., 2012). Brazil produced 2.5 billion liters of biodiesel in 2013. It is estimated that each liter of biodiesel generates approximately 100 mL of the by-product crude glycerin (DOSARI et al., 2005), containing variable glycerol content (WILBERT et al., 2013). Glycerol has energy content similar to corn starch and can be used as animal feed (DONKIN et al., 2009, WILBERT et al., 2013). However, crude glycerin contains impurities, such as methanol, sodium hydroxide, fat, esters, and low amounts of sulfur, protein, and minerals (CELIK; OZBAY; CALK, 2008). Crude glycerin may contain up to 14% methanol (HANSEN et al., 2009), which has been shown to be potentially toxic to animals (CHALMERS, 1986). Refining crude glycerol to pure glycerin (>98% glycerol content) would make it a more desirable feed source, however it may not be economically feasible (HU et al., 2012).

Two strategies evaluated crude glycerin as a feed for dairy cows. The first experiment evaluated the possibility of using crude glycerin to compensate for the inevitable energy loss in sugarcane forage as a consequence of ensilaging. The objective was to evaluated dairy cow performance, digestion, and blood acid-base balance in response to the partial replacement of corn silage with an iso-NDF mixture of sugarcane silage and crude glycerin, with or without the addition of sensorial feed additives to the forages, aimed at reducing forage sorting. The second experiment evaluated the substitution of finely ground corn grain with increasing levels of crude glycerin.

2 LITERATURE REVIEW

2.1 Glycerol

Glycerol is a carbohydrate molecule ($C_3H_8O_3$), odorless, hydroscopic, sweet-tasting liquid that has the potential to replace corn in the diet. Schroder and Sudekum (1999) estimated that glycerol has net energy value of 1.98-2.29 Mcal/kg, which is approximately equal to the energy contained in corn starch. The net energy content of corn grain is approximately 2.0 Mcal/kg (NATIONAL RESEARCH COUNCIL - NRC, 2001). According to the FDA (2007, 21 C.F.R. 582.1320), glycerol is recognized as a safe ingredient for use as animal feed. Glycerol is present in crude glycerin from biodiesel production or can be purchased as pure glycerol, with more than 99% purity.

According to Linke et al. (2004), in order to be glucogenic, glycerol must be delivered in water to associate with the liquid fraction of the rumen contents or be able to bypass the rumen in a form to be absorbed as glycerol. In the rumen, glycerol may be converted to propionic and butyric acids. Glycerol converted to butyrate will be metabolized to β-hydroxybutyrate (BHBA) by the rumen epithelium. Therefore, glycerol metabolized to butyrate is ketogenic, rather than glucogenic.

Glycerol that bypasses ruminal fermentation may be a highly efficient glucogenic substrate, because it can enter the gluconeogenesis pathway in the liver at the triose phosphate level and is not dependent on the rate limiting enzymes pyruvate carboxylase and phosphenolpyruvate carboxykinase for its conversion to glucose by glycerol kinase (Leng, 1970). Glycerol kinase converts glycerol (Km=3 to 10 µM) (LIN, 1977) and ATP to glycerol-3-phosphate and ADP, an intermediate step where glycerol is directed to glycolysis or gluconeogenesis. Dairy cows in negative energy balance have pathways

activated for the utilization of glycerol released by the mobilization and hydrolysis of triglycerides from body fat. This activity depends on the absorption of glycerol rather than the fermentation of glycerol to propionate and butyrate (HIPPEN; DEFRAIN; LINKE, 2008).

The single most important nutrient required for milk synthesis is glucose, although nearly all glucose consumed by the dairy cow is degraded in the rumen to volatile fatty acids, which are absorbed and metabolized by the liver (HIPPEN; DEFRAIN; LINKE, 2008). During lactation, over 70% of the synthesized glucose is used for milk production (ELLIOT, 1976). The liver is responsible for converting propionate from starch fermentation in the rumen, glucogenic amino acids, and glycerol from adipose triglycerides into glucose (HIPPEN; DEFRAIN; LINKE, 2008).

Harzia et al. (2013) evaluated the replacement of starch with crude glycerin. Eight primiparous mid-lactation dairy cows were used in replicated 4 x 4 Latin Square experiment with 21-d periods, having one square of rumen cannulated cows. Four iso-energetic diets were evaluated. Control cows were fed a barley based TMR (T0), and other treatments were formulated by replacing 1 kg (T1), 2 kg (T2), and 3 kg (T3) of barley with crude glycerin. The crude glycerin had 82.6% glycerol, 9.3% salts, 7.1% water, 0.6 ether extract, and 0.4% methanol. Treatments T2 and T3 increased the molar proportion of propionate and butyrate in rumen VFA. Treatment T3 increased rumen valerate proportion. Glycerol increased milk protein and lactose contents. Milk coagulation was increased as barley was replaced with glycerin.

Donkin et al. (2009) evaluated the effect of feeding glycerol as a replacement to corn grain on intake, milk yield, milk composition, and total-tract nutrient digestibility in lactating cows. Sixty Holsteins were blocked based on parity and milk yield and randomly assigned for 56 days to diets containing 0, 5, 10 or 15% pure glycerol. Daily milk yield was around 37 kg and was not

affected by treatment. In the last week of the experiment, a modest decrease in DMI of cows fed 15% glycerol was observed. Milk urea-N content was reduced by glycerol feeding. Milk solids content did not differ. Total tract NDF digestibility was lower for cows fed 5% glycerol. There was a linear increase in blood glucose content in response to glycerol feeding. Replacement of corn with up to 15% glycerol in the diet had no adverse effect on milk yield or composition.

Boyd, West e Bernard (2011) evaluated the effect of direct-fed microbial and glycerol supplementation (2x2 factorial) on milk yield, feed efficiency, and nutrient digestibility during hot weather. Sixty mid-lactation Holsteins were fed for 10 weeks after a 2-week standardization period: Cows received 400 g/d of glycerol. There was no detectable interaction between factors, except for total tract nutrient digestibility. Apparent digestion of forage DM, CP, ADF in the rumen was increased in cows supplemented with glycerol. There was no effect of glycerol on DMI, milk yield, body temperature, or blood glucose content.

Carvalho et al. (2011) replaced corn with pure glycerol in diets for transition dairy cows. Twenty-six multiparous Holsteins were paired blocked based on expected calving date and randomly assigned to a diet containing high-moisture corn or glycerol plus soybean meal. Treatments were fed from 28 days before the expected calving date to 56 d postpartum. Glycerol was included at 11.5 and 10.8% of diet DM during the pre-partum and post-partum periods, respectively. There was no treatment effect on pre- and post-partum DMI, milk yield, milk composition, milk urea-N, and energy balance. Pre-partum blood glucose content was decreased in cows fed glycerol, and this same trend was observed post-partum. Blood BHBA concentration was increased by glycerol feeding. Glycerol increased the ruminal concentrations of propionate, butyrate, and valerate, and decreased acetate, isobutyrate, and the acetate to propionate ratio.

2.2 Crude glycerin

Crude glycerin is a major byproduct from the biodiesel production process. It is estimated that approximately 1 kg of crude glycerin is generated for every 10 kg of biodiesel produced (HU et al., 2012). With the rapid growth of the world's biodiesel production in recent years, a large surplus of glycerin has been created (JONHSON; TACONI, 2007). The production of biodiesel in the world in 2013 was 25 billion liters, Brazil produced around 11% (BIOFUEL DIGEST, 2013). It was projected that the world biodiesel market would reach 140 billion liters by 2016, which implied 15 billion liters of crude glycerin (ANAND; SAXENA, 2011).

According to the "Agência Nacional de Vigilância Sanitária" (ANVISA) (Resolution 386/1999), glycerin use as an allowed humectant for human and animal consumption. However, glycerin quality standards were not defined when it was used as animal feed, nor the obligation of prior registration of the glycerin (ANVISA, 1999). In May 2010, the "Ministério da Agricultura, Pecuária e Abastecimento" (MAPA) regulated the use of glycerin (crude and blonde) as a feed ingredient for animals and defined quality standards, such as the contents of glycerol (minimum 800 g/kg), moisture (maximum 130 g/kg), methanol (maximum 159 mg/kg), sodium, and minerals.

The glycerol content of glycerin (Purity) is variable and reflects the different stages of biodiesel production. Low purity glycerin has high content of water and methanol. High purity glycerin (>99% of glycerol) is the most valuable product, however the identification of alternative uses for low purity glycerin may make biodiesel production more competitive in the growing global biofuel market (OMAZIC et al,. 2013).

Crude glycerin has little economic value due to the presence of various impurities such as methanol, soap, fatty acid methyl esters, and alkaline catalyst residues (HU et al., 2012; MCCOY et al., 2006; SANTIBANEZ; VARNERO; BUSTAMANTE, 2011). Crude glycerin available has become a serious issue for the biodiesel industry (JOHNSON; TACONI, 2007). Considerable research has been conducted on potential uses of crude glycerin. Impurities present in crude glycerin significantly affect its proprieties and its conversion to value-added products (HU et al., 2012). Soap and methanol can have negative impacts on algae production of docosahexaenoic acid from crude glycerin (PYLE; GARCIA; WEN, 2008). The high salinity (Na or K) of crude glycerin can inhibit microbial activity when crude glycerin is anaerobically digested (SANTIBANEZ; VARNERO; BUSTAMANTE, 2011).

Hu et al. (2012) described the physical and chemical properties of five crude glycerin samples from biodiesel production. Density ranged from 1.01 to 1.20 g/cm³ and was lower than the density of pure glycerin (1.31 g/cm³). Sample pH ranged from 6.4 to 10, the pH value of pure glycerin was 6.4. However, in the study of Hansen et al. (2009), pH of 11 crude glycerin samples ranged from 2.0 to 10.8.

2.3 Methanol

The rumen commonly produces methanol as a product of hydrolysis of methyl esters from pectin driven by bacteria and protozoa. The ruminal concentration of methanol is around 28 µg/mL (POL; DEMEYER, 1988; VANTCHEVA; PRADHAM.; HEMKEN, 1970). Methanol is not likely accumulated in ruminal fluid since it can be used by methylotropic organisms and converted to acetate or butyrate (NEUMANN; WEIGAND; MOST, 1999). It has also been reported that methanol in excess of what can be metabolized in

the rumen, has a severe effects in ruminants, causing inhibition of milk synthesis, anorexia, dullness, and death (CHALMERS, 1986).

Methanol is metabolized to formaldehyde by the liver enzyme alcohol dehydrogenase (ADH) (BARCELOUX et al., 2002; KRAUT; KURTZ, 2008). Formaldehyde is metabolized by the enzyme formaldehyde dehydrogenase to formic acid. Formate is metabolized to CO_2 and H_2O, a process that is dependent on liver tetrahydrofolate concentration (BARCELOUX et al., 2002; KERNS et al., 2002). This pathway is easily saturable, leading to accumulation of formic acid in the blood (KRAUT; KURTZ, 2008). Formic acid can cause metabolic acidosis, hyperosmolality, retinal damage with blindness, putaminal damage with neurologic dysfunction (KRAUT; KURTZ, 2008).

Winsco et al. (2011) evaluated the effect of methanol on intake and digestion in beef cattle. Four ruminally cannulated Holstein steers, in a 4 x 4 Latin Square, had *ad libitum* access to a grain-based diet (48.9% corn, 10% molasses, 16% cottonseed meal, 15.6% cottonseed hulls, 7.5% rice bran, 31.1% starch, and 14.7% crude protein). Treatments consisted of four levels of methanol (0. 70, 140 and 210 g/d) infused directly into the rumen. Experimental periods were 16 day long, with 10 for adaptation and 6 of sampling. Infusions of increasing levels of methanol increased the ruminal concentration from 0 to 6,563, 13,356, and 19,831 ppm. Daily DMI, and ruminal pH, total VFA concentration, and the molar proportion of acetate did not differ among treatments. A quadratic trend for a reduction in propionate proportion was observed, and was likely the result of a quadratic increase in butyrate. No adverse health or well-being effects were observed when methanol was infused into the rumen.

2.4 Glycerin as feed ingredient

Zacaroni (2010) evaluated the response of lactating cows to the complete replacement of finely ground mature corn by crude glycerin in a crossover design experiment with 21-d periods. An iso-nitrogenous mixture of crude glycerin plus soybean meal replaced finely ground mature corn in the diet. The crude glycerin contained 6.29% moisture, 76.2% glycerol, 1.33% ether extract, 2.93% ash, and 0.88% methanol. The dietary content of glycerin was 12.3% of DM, the content of corn was 14.8% in the Control diet. The replacement of corn with crude glycerin depressed milk yield by 10%, without affecting intake, and reduced feed efficiency. Glycerin feeding reduced the daily secretion of lactose, and there was a trend for reduced milk protein secretion. Total tract apparent digestibility of OM was increased when glycerol replaced starch. Glycerin increased the molar proportion of butyrate and decreased the proportion of acetate in rumen fluid, but had no effect on ruminal propionate. Glycerin reduced the content of glucose in blood plasma.

Shin et al. (2012) evaluated the replacement of ground corn, corn gluten feed, and citrus pulp with crude glycerin for dairy cows. Twenty four Holsteins, in a 2x3 factorial arrangement of treatments, were fed two roughage sources (cottonseed hulls or corn silage) and three dietary concentrations of glycerin (0, 5, or 10% of DM). Crude glycerin contained 12% water, 5% fat, 6.8% sodium chloride, and 0.4% methanol. Crude glycerin at 5% of diet DM increased DMI without affecting milk yield. For these diets with low fiber content (24.4% NDF), the content of milk fat (3.12% for 0% glycerin) was reduced when 10% glycerin was fed (3.03%). Total tract NDF digestibility was also 30% lower for the 10% glycerin diet compared to Control. Diets with 5 and 10% crude glycerin improved 4% fat-correct milk when corn silage was fed, but decreased it when cottonseed hulls replaced corn silage.

Omazic et al. (2013) evaluated the effect of low and high purity glycerin on intake, lactation performance, blood metabolites and BCS of dairy cows. Forty-two cows were allocated to 14 blocks based on parity and expected day of parturition and were randomly assigned to a treatment. Treatments were: Control, and low or high purity glycerin, both at 0.5 kg/d, starting at day 2 post-partum for 28 days. The low purity glycerin contained 88.1% glycerol, 9.3% moisture, 0.9% ash, and 0.8% methanol. High purity glycerin contained 99.5% glycerol. Grass silage and concentrates were fed separately four times per day. Glycerin was top dressed to the concentrate at 9 AM and 5 PM, in equal amounts. Low and high purity glycerin had no effect on BCS and silage and total intakes. There were trends for increased yield of milk and contents of fat and protein for cows fed high compared to low purity glycerin, but milk lactose content responded in the opposite direction to the same treatments. Treatments had no effect on the content in plasma of glucose, insulin, NEFA, and BHBA.

Boyd, Bernard e West (2013) evaluated the effect of replacing a portion of ground corn with crude glycerin on rumen fermentation profile, blood metabolites, and nutrient digestibility in lactating cows. Six rumen cannulated Hosteins (56 ± 18 DIM) producing 38±8.2 kg of milk/d were used. The design was a replicated 3x3 Latin Square with 4-week periods. Treatments were: Control, 200 g of glycerin/d (G2), or 400 g of glycerin/d (G4). Glycerin contained 80 to 85% glycerol, 14% moisture, 7% sodium chloride and 18 ppm of methanol. There was a decrease in DMI with increasing amounts of glycerin. Milk yield was reduced by 1.8 kg/d and 2.4 kg/d for G4 compared with Control and G2. Treatment G2 reduced milk fat content and yield compared to Control. Blood glucose and urea-N did not differ among treatments. The molar proportions in rumen fluid of acetate and valerate and the acetate to propionate decreased, and the proportion of propionate and butyrate increased with increased glycerin feeding.

Eight Jerseys received diets containing 0, 4, 8, and 12% crude glycerin (WILBERT et al., 2013). The design was a replicated 4x4 Latin Square with 17-d periods. The crude glycerin contained 81.4% glycerol, 14% moisture, 1.1% CP, and greater than 50 ppm methanol. Crude glycerin had no effect on milk and energy correct milk yield, and on fat, lactose, and total milk solids content and yield. Milk protein content was increased with 12% and 8% crude glycerin in the diet. There was no treatment effect on intake, digestibility of DM, OM and NDF, and serum concentrations of NEFA and urea-N. The response in plasma glucose to glycerol feeding was quadratic, with a reduction at the lower levels (4 and 8% crude glycerin) and subsequent increase (12% crude glycerin).

2.5 *In vitro* studies

Early studies on glycerol metabolism suggests that it is rapidly fermented in the rumen. Garton, Lough and Vioque (1961) observed that the disappearance of glycerol after 2 h of incubation in rumen fluid *in vitro* was 25%, and that 90% disappeared when incubations were performed for 8 h. Remond, Souday and Jouany (1993) added glycerol to continuous fermentors containing starch or cellulose. Glycerol reduced fluid pH more when starch was the substrate than with cellulose. The molar proportion of butyrate was increased only when glycerol was added to fermentors containing starch. The authors concluded that glycerol is rapidly fermented in the rumen and the response in ruminal propionate and butyrate to glycerol feeding is diet dependent.

Continuous fermenters were used by Abo El-Nor et al. (2010) to investigate the effect of substituting corn with glycerol at different levels on fermentation profile and DNA concentration of selected rumen bacteria. Four dual-flow continuous culture systems were used in 4x4 Latin Square, with 10-day periods. Diets were formulated with glycerol (grade: 995 mL/L) at 0 (Control), 36, 72, and 108 g/kg of DM. Substituting corn for glycerol had no

effect on DM digestibility, however, feeding glycerol at 72 and 108g/kg of DM reduced NDF and tended to reduce ADF digestibility compared to Control. The molar proportion of acetate decreased with glycerol feeding and was lowest with 108 g/kg DM. The acetate to propionate ratio decreased with the 72 e 108g/kg of DM and the molar proportion of butyrate and isovalerate were increasedby glycerol compared to Control, but were similar among glycerol levels. Glycerol did not determine the concentration of DNA for*Ruminococcus albus* and*Succinivibrio dextinosolvens*. Relative to Control, the DNA concentration for *Selenomonas ruminantium* and *Butyrivibrio fibrisolvens*were decreasedon diets containing 72 and 108 g/kg of glycerol. The DNA concentration for*Clostridium proteoclasticum*was decreased byglycerol feeding, but did not differ amongglycerol levels. These results suggest that substituting corn with glycerol at low level has no adverse effect on fermentation, digestion, and ruminal bacteria. Higher substitution levels may have negative impact on fiber digestion and reduce acetate production.

Krueger et al. (2010) evaluated theeffect of glycerol on ruminal fat lipolysis *in vitro*. Three levels were evaluated: 0, 2 and 20% of glycerolin tubes containing 10% of olive oil. Both levels of glycerol inhibited lipolysis, inducing reductions of 48% and 77% in free fatty acid accumulation in rumen fluid as compared to Control. The effect of glycerol on fermentation kinetics of alfalfa hay was also evaluated. Five levels of glycerol were used: 0, 5, 10, 20 or 40%. Gas production was measured using a computerized gas monitoring apparatus. The fast and slow degrading pools were assumed to represent glycerol and fiber, respectively. Gas accumulation of the first pool increased linearly as the amount of glycerol was increased. Higher levels of glycerol induced a quadratic decrease in first pool fractional rate of fermentation, the fractional rate of fermentation was slower at 20% and 40% compared to 0, 5, and 10% glycerol. Glycerol reduced the fractional degradation rate of the second pool. Increasing levels of

glycerol induceda linear decrease in acetate accumulation, and a quadratic increase in propionate, reducing the acetate to propionate ratio. Data suggested that long term feeding glycerol might ultimately select and enrich the populations of glycerol fermenting microbes such as *Megasphaera elsdenii* and *Selenomonas rumiantium*.

Avila et al. (2011), evaluating the impact of increasing dietary levels of (0, 7, 14, 21%glycerol) on the*in vitro* ruminal fermentation and methane production of a barley based high concentrate diet. Methane production did not differ among treatments. However, Avila-Stagno et al. (2013), using a semi-continuous fermentation system to evaluate the inclusion of glycerol at 0, 5, 10 and 15% DM replacing corn silage, observed a linear increase in methane in response to increased glycerol levels, resulting in a linear increase in the methane do digested DM ratio. Glycerol decreased acetate and increased butyrate and propionate production.

The effects of substituting corn with glycerol as a feed alternative were investigated using continuous fermenters Abo El-Nor et al. (2010). Four fermenters were used in a 4×4 Latin square design with four 10 days consecutive periods. Treatments diets contained 0 (T1), 36 (T2), 72 (T3) and 108 (T4) gglycerol/kg dry matter (DM). Diets consisted of 600g/kg alfalfahay, 400g/kg concentrate (DM basis), and glycerol replaced the corn in the concentrate. Results show edthatneutral detergent fiber digestibility decreased ($P<0.05$) with the T3 and T4 diets compared with the T1 diet. Glycerol substitution had no effects on fermenters pH, NH3-N concentration, and digestibility coefficients of DMandacid detergent fiber. The molar proportion for acetate decreased ($P<0.05$) while the molar proportions for butyrate, valerate and isovalerate increased ($P<0.05$) with the glycerol diets compared with the T1 diet. The DNA concentrations for *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium* decreased ($P<0.05$) with the T3 and T4 diets compared with the T1 diet. The

DNA concentration for *Clostridium proteoclasticum* also decreased ($P<0.05$) with glycerol substitution. No differences in the DNA concentrations for *Ruminococcus albus* and *Succinivibrio dextrinosolvens* among diets were observed. Results from this study suggest that substituting corn with glycerol at low level had no adverse effects on fermentation, digestion or ruminal bacteria.

2.6 Absorption of glycerol

Homologous water channel proteins (ROJEK et al., 2008) mediate glycerol transport across epithelia. Aquaporins are channels that facilitate the transport of water across the cell membrane (KING; KOZONO; AGRE, 2004). These channels possess two highly conserved asparagines-proline-alanine boxes, which is essential to the formation of a water-transporting pore (MAEDA; FUNAHASHI; SHIMOMURA, 2008). Aquaporins form a simple pore that enables water to pass through the cell membrane bidirectionally according to osmosis; they are not pumps or exchangers (HUB; GROOT, 2008). Thirteen aquaporin subtypes have been identified in mammals (CAMPOS et al., 2011; MAEDA; FUNAHASHI; SHIMOMURA, 2008). It can be divided into two major groups: those selective for water and functioning as water channels (called orthodox aquaporins) and those permeable to small solutes including glycerol (called aquaglyceroporins) (CAMPOS et al., 2011). Among them, types 3, 7, 9 and 10 are subcategorized as well as water (MAEDA; FUNAHASHI; SHIMOMURA, 2008). Two subtypes (7 and 9) are highly expressed in adipocytes and the liver and are important parts in the homeostasis of metabolism. The expression and physiological function of aquaporins are less investigated in ruminants (RØJEN, et al., 2011)

Glycerol channels were presumed to prevent acute rises in intracellular osmotic pressure while glycerol production was increased during lipolysis in

adipocytes. The identification of aquaglyceroporins, however indicated a mechanism of glycerol metabolism, especially in adipocytes and hepatocytes (MAEDA; FUNAHASHI; SHIMOMURA, 2008).

AQP3 expression has been reported in several mammalian tissues including kidney, epidermis, urinary, respiratory and digestive tracts (TAKATA; MATSUZAKI; TAJIKA, 2004), and human erythrocyte (ROUDIER et al., 1998). AQP3 is moderately permeable to water, but highly permeable to glycerol and possibly to urea (CAMPOS et al., 2011). Rojen et al. (2011) observed the AQP3 expression in ruminal papillae and mRNA expression and protein abundance are affected by diet. AQP7 is highly expressed in white and brown adipose tissues from rats and humans, and a weak expression is also observed in cardiac and skeletal muscle and the kidney (MAEDA; FUNAHASHI; SHIMOMURA, 2008).

2.7 Sugarcane silage

During the ensilaging of sugarcane, the alcoholic fermentation of sugars to ethanol, driven by yeast, can induce as much as 30% of dry matter loss (ALLI; BAKER; GARCIA, 1982; FREITAS et al., 2006; KUNG JÚNIOR; STANLEY 1992; SANTOS; NÚSSIO; MOURÃO, 2008). Silage additives have not been capable of reducing the loss of non-fiber carbohydrates and resulting increase in forage NDF content (MIRANDA et al., 2011). Ethanol formation can reduce the amount of sugars available to lactic acid producing bacteria, and can also reduce silage palatability (BUCHANAN-SMITH, 1990), increase in acetate and caproate in rumen fluid (DURIX et al., 1991), and alters the organoleptic properties of milk (RANDBY; SELMER-OLSEN; BAEVRE, 1991).

Pedroso et al. (2010) evaluated the performance of dairy cows fed fresh sugarcane or sugarcane silage treated with urea plus sodium benzoate or

Lactobacillus buchneri. Twenty-four Holsteins (150 DIM) were used in replicated 3x3 Latin Squares. Cows fed both sugarcane silages had lower DMI (18.4 vs. 21.4 kg/d) and milk yield (17.5 vs 18.5 kg/d) than cows fed fresh sugarcane.

2.8 Sensory feeds additives

Sensorial feed additives (flavors and odors) area group of products capable of enhancing taste and smell of animal feedstuffs, aiming at stimulating feed intake or reducing feed sorting at the feed bunk. Ruminant feed intake is determined by the physical and chemical characteristics of the diet. Diet particle size and forage source and content determine animal response to sensorial feed additives (BAUMONT, 1996; GALEAN; DEFOOR, 2003). Flavor and odor are important chemical signals in feed selection (SCHLEGEL, 2005). Chiy and Phillips (1999) evaluated the effect of sweet, salty, or bitter taste on dairy cow feeding behavior. Bitter and salty concentrates were consumed at a slower rate than sweet. Nombrekela et al. (1994) observed a similar trend, DMI of dairy cows was increased with a sweetened TMR compared to diet without artificial flavor. The hypothesis that the preference for sweeteners may be due to the nutritional benefits they provide to the animal and not just due to the sweet flavor. Rapidly fermentable sugars may contribute to the synchrony between the nitrogen and carbohydrate fermentation in the rumen (BRODERICK; RADLOFF, 2004; FIRKINS et al., 2008).

Saccharin is one of the oldest artificial sweeteners, being discovered in 1879. Saccharin is 300 to 500 times sweeter than sucrose for humans (HOLLINGSWORTH, 2002). Saccharin has been added to cattle diets in an attempt to increase intake. Brown et al. (2004) fed male calves with concentrates containing 0, 88, 176, or 264 g/ton of Sucram, an additive containing 97%

sodium saccharin. Feeding 176 g/ton of Sucram increased DMI and daily gain. McMeinman et al. (2006) also found a trend for increased body weight of beef calves when 200 mg of Sucram/kg of diet DM was fed. Since saccharin has no caloric value, the improved sweet flavor caused the increasein intake and animal performance.

The evaluation of flavors fordairy cows have focused on the postpartum period (MURPHY et al., 1997; NOMBEKELA; MURPHY, 1995). Shah et al. (2004) conducted a study to evaluate the effects of a liquid feed flavor on dairy cows during the transition period. Twenty-four Holsteins, from three weeks prepartum to six weeks postpartum, were assigned to either a Control orto a liquid-flavored TMR (0.52 ml/kg). The flavor product did not determine DMI and milk yield.

Merrill et al. (2013) evaluated the effect of improving forage palatability on intake, milk production and composition, rumen pH, and sorting behavior of lactating cows. Twenty-eight Holsteins (54 DIM), were fed a TMR containing (% of DM) 45% corn silage, 10% alfalfa haylage, and 45% concentrates for 10 weeks. Half of the cows had the forage portion of the diet treated with a palatability enhancer (Luctarom ProEfficient, Lucta S.A., Spain). The sensorial additive was mixed in water to achieve a dose of 12 mL/cow/d prior to be mixed to the TMR. In the Control treatment, only water was added to the forage portion of the diet. For all cows, there was no treatment effect on DMI and milk yield and composition. However, when the data from multiparous cows was analyzed separately, there were trends for increased DMI (+1.5 kg/d) and milk production (+3.9 kg/d) in response to flavors. Cows fed flavors had higher rumen pH. There was no difference in the particle size distribution of the TMR throughout the day.

2.9 Feed Sorting

In a total mixed ration (TMR) feeding system, forage and concentrate feed components are combined into a single feed mixture. The objective of this feeding method is to deliver to each cow, a well-balanced ration that is formulated to maintain health and maximize milk production. However, there are indications that the composition of what an individual cow consumes is not the same as what was initially delivered (DEVRIES et al., 2012). Cows fed TMR will often preferentially select (sort) for the grain component and discriminate against the longer forage components (LEONARDI; ARMENTANO, 2003).

A further complication exists when cows sort, and do not ingest feeds in proportion to dietary concentration. In particular, when diets are formulated close to minimum recommendations, sorting could reduce intake of long particles and thereby possibly decrease chewing activity, rumen pH, and milk fat test (LEONARDI; ARMENTANO, 2003).

Carvalho et al. (2012) conducted a study to evaluate the effect of replacing high moisture corn with pure glycerol (around 10% of diet DM) on feed sorting and the feed intake pattern of transition dairy cows. Since glycerol is a sweet-tasting feed, it could increase the selective consumption of long particles in the TMR, either as consequence of coating of particles or through minimizing particle separation. Feed intake pattern and sorting wasevaluated on days -16, -9, 9, 16 and 51 relative to calving, at 4, 8, 12, and 24hourspost-feeding. Feed intake did not differ. During the prepartum period, glycerol reduced the amount of feed consumed during the first 4 hours post-feeding, but increased feed consumption from hours 12 to 24 post-feeding. Glycerol increased the proportion of long particles in the pre-partum diet, and reduced the proportion of small feed particles. Glycerol did not change the distribution of

feed particles in the postpartum diet. Glycerol reduced sorting against long feed particles by close-up dry cows, increased sorting in favor of medium particles, and reduced sorting in favor of short feed particles.

DeVries et al. (2012) evaluated the effects of adding a liquid molasses-based supplement, at 4.1% of DM, to a TMR on feed sorting behavior and production of lactating dairy cows. Addition of molasses liquid feed did not change the nutrient composition of the diet, with the exception of an expected increase in dietary sugar concentration (from 4.0 to 5.4%).

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SECOND PART - PAPERS

PAPER 1

DIETS FORMULATED FOR COWS

The partial replacement of corn silage with sugarcane silage plus crude glycerin and the effect of sensorial feed additives for dairy cows

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ABSTRACT

This experiment evaluated diets formulated by the partial replacement of corn silage with an iso-NDF amount of sugarcane silage plus crude glycerin, added or not of sensorial feed additives (flavor and odor). Thirty-two Holsteins (182 DIM) received a standardization diet for two

weeks and a treatment for 44 days, in a covariate adjusted randomized block design with repeated measures over time. Treatments (2x2 factorial) were (% of diet DM): Forages CS (30.2% corn silage) or SG (15% corn silage, 10% sugarcane silage, and 3.3% crude glycerin); with or without sensorial additives (Luctarom SFS-R 3386-Z and 1353-Z). Sensorial additives were added to ground corn grain and then mixed to the forages in aTMR mixer, other feeds were added in sequence. Diets also contained 9.2% sorghum silage, 4.4% Tifton hay, and 24.5±0.5 forage NDF. The as fed proportion of the diets below an 8 mm screen was around 70%. Milk yield was reduced when sensorial feed additives were added to CS (31.1 vs. 32.2 kg/d) and increased it when added to SG (31.7 vs. 30.3 kg/d); daily yields of lactose and total solids followed the same trend. The ratio of milk to DMI had greater positive response to sensorial additives in SG (1.43 vs. 1.34) than in CS (1.46 vs. 1.44). Forage SG increased the contents of fat and protein in milk, improving total solids content. Sensorial additives reduced blood plasma glucose content when added to CS and increased it when added to SG. Total tract apparent digestibility was not determined by treatments, neither the intake of digestible OM. From 2PM to 7PM, sensorial additives induced rejection

of long particles and preferential consumption of small particles when added to forage CS, but reduced the rejection of long particles when added to SG. The rate of intake from 7AM to 1PM was faster in SG, and tended to reduce when sensorial additiveswere added to CS and to increase when they were added to SG. Chewing activity was similar across treatments, as well as the daily excretion of urinary allantoin, ruminal fluid pH, and protozoa count. When added of sensorial feed additives, the partial replacement of corn silage by sugarcane silage plus crude glycerin was a plausible alternative for feeding dairy cows. The effect of sensorial feed additives on feed sorting and lactation performance interacted with forage source.

Key words: glycerol, crude glycerin, methanol, sugarcane, sensorial feed additives

INTRODUCTION

Sugarcane (*Saccharum* spp.) is a forage crop used as animal feed because of its high DM production and energy content, due to the high concentration of sugars, mainly sucrose. However, the complete

replacement of corn silage with fresh sugarcane has shown to decrease dairy cow intake and lactation performance, in consequence of its low fiber digestibility (Correa et al., 2003).

The strategic use of sugarcane forage for dairy cows in late lactation and/or its partial substitution for corn silage may increase the agronomic efficiency of dairy farming in tropical regions, without the negative sugarcane effect on cow productivity. However, harvesting fresh sugarcane is labor demanding, being a frequent rationale to ensile the crop. The ensiling of sugarcane often results in overgrowth of yeasts, which leads to high DM loss throughout the fermentative process (Kung and Stanley, 1982). Epiphytic bacterial inoculum can improve the alcoholic fermentation profile of sugarcane silage (Ávila et al., 2014), but silage additives have not prevented the increase in forage NDF content (Miranda et al., 2011). Sugarcane silage has high content of low-digestibility NDF.

Glycerin, a byproduct of biodiesel production, is a high energy feed for ruminants (Donkin et al., 2009). The world's biodiesel production in 2013 was estimated to be 25 billion liters; Brazil produced about 11% of that (Biofuel Digest, 2013). Each 10 L of biodiesel generates about 1 L of

crude glycerin (Thompson and He, 2006). Crude glycerin contains variable amounts of glycerol, water, catalysts, salts, and methanol (Dasari et al., 2005; Hansen et al., 2009). Methanol can be metabolized to formaldehyde by the liver and then to formic acid. Formic acid is capable of inducing visual disorders, central nervous system depression, respiratory dysfunction, and metabolic acidosis in animals (Black et al., 1985; Nie et al., 2007). The addition of methanol-rich crude glycerin to sugarcane silage diets may be a way to compensate for the energy loss during fermentation of the forage, but the effect on animal health needs evaluation.

Sensorial feed additives, such as aromas and odors, may determine feeding behavior of dairy cows, affecting sorting of feed ingredients, the rate and pattern of intake along the day, and ultimately the physical and chemical properties of the consumed diet. Altering forage palatability with flavors may stabilize rumen pH and increase DMI and milk yield in dairy cows (Merrill et al., 2013). However, the adhesiveness of sensorial feed additives to distinct feed ingredients can vary, and in consequence the response in feeding behavior. The response of dairy cows to sensorial feed additives may depend on type of forage.

The objective of this experiment was to evaluate the response of dairy cows in mid to late lactation to the partial replacement of corn silage with sugarcane silage plus crude glycerin, and the interaction between forage type and sensorial feed additives.

MATERIALS AND METHODS

Experimental procedures were approved by the Federal University of Lavras Bioethic Committee. Thirty-two Holstein cows (182±109 DIM, 12 primiparous) were fed one of four diets for 6 weeks, in sequence to a common diet fed for a 2-week standardization period (Table 1). Cows formed 8 blocks based on parity and milk yield and were assigned to a treatment within block. Treatments were a 2 x 2 factorial arrangement of factors: Forage and sensorial feed additives. Forages were corn silage (CS) or an iso-NDF mixture of sugarcane silage plus crude glycerin (SG). Soybean meal was added to the SG diets to achieve the same CP content as diets CS. Sugarcane silage was inoculated with anepiphytic strain of *Lactobacillus hilgardii* (Ávila et al., 2014), and was stored for 24 d before feeding. The sugarcane silage had 27.1% DM (as fed basis), and 4.0% CP, 69.2% NDF, 0.8% EE, and 5.7% ash on a DM basis. Crude glycerin

from beef tallow (Tecno-Oil Indústria e Comércio, Mombuca, Brazil) contained 29.8% moisture and 7.3% methanol on an as fed basis, and 0.92% CP, 7.1% EE, 7.9% ash, 0.52% Na, 0.25% S, 0.06% K, 0.05% P, 0,03% Ca, and 0.01% Mg on a DM basis. Glycerin pH was 1.89.

Sensorial feed additives (SA) were Luctarom SFS-R 3386-Z (30g/cow/d) and 1353-Z (333g/ton of TMR) (Lucta, Barcelona, Spain). Feed additives were mixed to ground corn (755 g corn, 30 g SFS-R 3386-Z, and 15g 1353-Z). This mixture or pure corn (Control) were mixed to the forage portion of the diets in a stationary vertical TMR mixer (Unimix 1200. Casale Equipamentos, São Carlos, Brazil). Concentrate feedstuffs were added to mixer in sequence. Feeding was performed twice daily at approximately 6 a.m. and 2 p.m.

Cows were individually fed in sand bedded tie stalls and milked twice per day. Feed offered and refusals were recorded daily. Refusals from each cow and feed ingredients were sampled daily and composite samples were formed by week. Weekly composites of feeds and refusals were dried in a forced air oven at 55^{o}C for 72 h and ground through a 1-mm mesh screen. The DM content was determined by drying at 100^{o}C for 24 h and CP was by micro-Kjeldahl analysis. The EE was analyzed after

hydrolysis with hydrochloric acid. Ash was analyzed by incineration at 550^{o}C for 8 h. The NDF was analyzed using a TE–149 fiber analyzer (Tecnal Equipamentos para Laboratórios, Piracicaba, Brazil) with amylase and sodium sulfide.

Milk yield was recorded daily. Milk samples were collected from four consecutive milkings on days 6 and 7 of each week. Solids and MUN content were analyzed (Laboratório Centralizado da Associação Paranaense de Criadores de Bovinos da Raça Holandesa, Curitiba, Brazil) by infrared analysis Bentley 2000. Bentley Instruments Inc., Chaska, MN). Milk energy secretion (**Milk E**, Mcal/d) was calculated as: [(0.0929 x %fat) + (0.0547 x % protein) + (0.0395 x % lactose)] x kg of milk (NRC, 2001). Energy-corrected milk yield (**ECM**, kg/d) was calculated as Milk E/0.70, assuming that the energy content of milk with 3.7% fat, 3.2% protein and 4.6% lactose is 0,70 Mcal/kg (NRC, 2001). The yield of 4% fat corrected milk (**FCM**, kg/d) was (0.4 + 15 x % fat/100) x kg of milk. After the morning milking, BW was determined at 7-day intervals, and three independent appraisers evaluated BCS.

Jugular blood acid-base balance was measured weekly at 0, 6, and 12 h after feeding. Blood was collected in heparinized tubes and analyzed

within one hour of sampling (AGS22 blood pH and gas analyzer. Drake, São José do Rio Preto, SP). At the same sampling days, blood samples from the coccygeal vessels were collected 12 h after feeding in tubes containing potassium fluoride for glucose analysis (Doles Reagentes e Equipamentos para Laboratório, Goiânia, Brazil).

Blood samples from the coccygeal vessels were obtained on day 41 to determine plasma urea-N (**PUN**). Samples were obtained immediately before the first daily feeding and 1, 2, 3, 6, 9, 12, and 18 h after feeding. The blood, collected with EDTA, was immediately refrigerated, centrifuged at 1,000 x g for 15 min, and the plasma was frozen at -20°C. The PUN content was analyzed with a laboratory kit (Urea 500. Doles Reagentes e Equipamentos para Laboratório, Goiânia, Brazil). On day 41, blood samples were also collected 12 h after feeding in heparinized tubes for analysis of aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) (Doles Reagentes e Equipamentos para Laboratório, Goiânia, Brazil).

Ruminal fluid was collected by gentle aspiration through a tube extending through the esophagus into the rumen on day 42. Samples were obtained12.3± 0.8 h after feeding. The pH was measured immediately and

10 mL of formaldehyde was added to 10 mL of rumen fluid for protozoa count (Dehority, 1984). Protozoa was enumerated in 1mL formalized samples in 0.1 mm depth Newbauer chambers (Warner, 1962).

Feed sorting was determined on d 21 as suggested by Leonardi and Armentano (2003) to represent no selection (sorting index 100), preferential consumption (sorting index >100), or rejection (sorting index <100). Samples were size separated using the Penn State Particle Separator (Lammers et al., 1996) at 0 (7 a.m.), 6, 12, and 24 h relative to the morning feeding.

Total tract apparent digestibility of DM, OM, NDF, and non-NDF OM was determined on days 38 to 40 by total collection of feces by trained personal. Feces were collected concurrent to defecation during three 8-hour sampling periods and weighed. The second and third sampling periods were each delayed by 8 h to avoid a major disturbance to the animals, while still representing a 24-h collection period. Fecal aliquots (equal fresh weight basis) were immediately frozen along the collection period and a composite sample was formed. Total urinary output was collected, simultaneously to fecal sampling, to estimate rumen microbial synthesis based on purine derivate excretion. A 10% sulfuric

194 acid solution was immediately added to the urine samples (1:9) before
195 refrigeration at 4°C. Composite urine samples were diluted 1:3 with
196 distilled water and frozen at -20°C. Allantoin was analyzed as in Young
197 and Conway (1942).

198 Chewing activity was evaluated o days 38 to 40 by visual
199 observation of the oral activity of each cow at 5-minute intervals,
200 simultaneously to fecal and urine sampling. Activities considered were
201 feed consumption, water ingestion, rumination and idle. Chewing time
202 was the sum of ingestion and rumination times. Chewing, ingestion, and
203 rumination per unit of DMI used the intake measured during the day of
204 chewing evaluation.

205

206 ***Statistical Analysis***

207 Data obtained over time used the repeated measures approach of the
208 MIXED procedure of SAS (1999). Variables measured at the end of the
209 standardization period (DMI, milk yield, milk solids, BW, BCS, liver
210 enzymes, plasma glucose, blood acid-base balance) were analyzed with a
211 model containing a continuous covariate effect, the random effect of
212 block (1 to 8), and the fixed effects of forage (CS or SG), sensorial

213 additive (Control or SA), interaction of forage and sensorial additive, time
214 (days or weeks), and the two and three term interactions among time,
215 forage, and sensorial additive. Cow nested within the interaction of forage
216 and sensorial additive was defined as random. The most suited covariance
217 structure was defined by the Akaike's information criterion. Other
218 variables used variations of the previous model, depending on availability
219 of a covariate measurement and repeated sampling over time.
220

RESULTS

The nutrient and ingredient composition of diets is presented in Table 1. Diets had similar contents of NDF and CP, as predicted. The NDF content of the sugarcane silage inoculated with epiphytic microorganisms was high (67.1% of DM). Sugarcane NDF in diets SG replaced roughly 50% of the NDF from corn silage. A similar proportion of dietary forage NDF originated from sorghum silage and Tifton hay in all diets. Sugarcane NDF represented about 1/3 of the forage NDF content of diets SG. Sorghum silage had the smallest particle size, and sugarcane silage had greater proportion of short feed particles than corn silage (Figure 1).

Forage SG increased DMI compared to CS, and there was a trend (P = 0.07) for reduced intake in response to SA (Table 2). However, the response in milk yield to SA addition to forage CS was negative, while milk yield response was positive when flavors and odors were added to SG. The response in milk yield to SG-SA and CS diverged positively from CS-SA and SG at the 4^{th} and 5^{th} experimental weeks (Figure 2), similar response patterns over time were observed for ECM and 4% FCM (Table 2). Daily lactose and total solids secretion and feed efficiency

241 (Milk/DMI) responded to treatments similarly to milk yield. Forage SG
242 increased milk fat content, and tended to increase milk protein content
243 compared to CS.

244 There was no major feedsorting behavior during the first period of
245 the day (from 7 a.m. to 1 p.m.) (Table 3), however, rejection of long feed
246 particles was marked in the period from 7 p.m to the next morning, even
247 with morning orts at around 13% of the daily feed offer (Table 4). From 2
248 p.m. to 7 p.m., SA induced rejection of long particles and preferential
249 consumption of short particles in forage CS, while this feeding behavior
250 was not observed when SA was added to SG. In the afternoon, cows on
251 SG preferentially selected in favor of long feed particles. Feed sorting
252 evaluated from the second daily feeding (2 p.m.) until the next morning (7
253 a.m.) followed the same pattern of response as the milk yield response
254 (Table 2). The addition of SA to CS increased the rejection of long
255 particles (decreased milk yield) and the addition of SA to SG avoided
256 selective sorting against long particles (increased milk yield). Cows on
257 SG consumed more feed in the interval from 7 a.m. to 1 p.m. (Table 4). In
258 the morning period, there was also a trend ($P = 0.08$) for SA to reduce the
259 rate of feed intake when added to CS and to increase it when added to SG.

Sensorial feed additives interacted with forage type and sorting behavior differed markedly among periods of the day, even with diets that had about 70% of particles below the 8 mm screen (Table 4).

Plasma glucose content was lowest in SG, but SA addition to this forage increased glucose content, resulting in content value similar to the CS diets (Table 5, Figure 3). There was a trend for SG to reduce GGT activity, without affecting AST (Table 5). There was no treatment effect on chewing activity (Table 6), rumen pH and protozoa content (Table 5), as well as on total tract apparent digestibility of nutrients (Table 7). The content of PUN varied along the day (Figure 4), but treatment effects were not detected (Table 5). Crude glycerin feeding did not determine venous acid-base balance (Tables 8-10), however, immediately before the 7 a.m. feeding, SA reduced the partial pressure of CO_2 and increased the partial pressure of O_2, and erythrocytes and oxygen saturation (Table 8).

DISCUSSION

The high NDF content of the sugarcane silage suggests that the epiphytic strain of *Lactobacillus hilgardii* could not significantly reduce sugar loss during fermentation in the silo. This same silage inoculum reduced the loss of dry matter when sugarcane was ensiled in laboratory mini-silos (Miranda et al., 2011). Experimental horizontal-type silos were 3 m wide and 1.5 m high, allowing for at least 15 cm removal each day. A beneficial effect of the inoculum on nutrient density of sugarcane silage could not be demonstrated in this experiment.

The partial replacement of corn silage with sugarcane silage increased DMI, in contrast to the usual depressing effect of sugarcane forage on intake, suggesting that this nutritional strategy was more desirable than the complete substitution of sugarcane for corn silage (Correa et al., 2003). All diets had a high proportion of short feed particles (<8 mm) and milk fat content was low, suggesting that some degree of ruminal acidosis may have occurred, although rumen motility disturbance or off-feed cows were not observed along the experiment. The association of unsaturated fatty acids from raw soybeans with rapidly fermentable starch from ensiled corn grain may have interacted with

295 forage particle size to reduce milk fat secretion (Bauman and Lock,
296 2010). Rumen pH was not low (>6.50), but measurements were done in
297 fluid samples obtained from the reticulum, aiming more at describing
298 treatment effects than indeed representing total rumen digest fermentation
299 profile. At the dietary inclusion of sugarcane adopted in this experiment,
300 some low digestibility fiber favored DMI of cows in mid to late lactation.
301 The dietary content of sugarcane was not high enough to reduce total tract
302 NDF digestibility or the synthesis of microbial protein in the rumen, and
303 diet SG increased milk fat content.

304 Treatment SG reduced milk yield, but SA compensated for the
305 negative impact of SG on lactation performance. However, the response
306 in milk yield to SA had an opposite direction in diet CS. Feed sorting
307 behavior may be involved in the dissimilar response to flavors and odors
308 on each forage type. Sorting behavior was not pronounced in the
309 morning, when feed availability was plenty. During the night period, SA
310 induced rejection of long particles and preferential consumption of short
311 particles in CS, but reduced selective sorting against long particles in SG.
312 Flavors and odors mixed with corn grain may have migrated to the
313 concentrate portions of the diet during TMR mixing, inducing selection in

favor of concentrate feedstuffs in diet CS. Alternatively, the adhesion of SA to forage SG may have been more effective than to CS, acting favorably on selective sorting against long particles in this treatment. Sensorial feed additives determined feeding behavior of each forage differently, even when the diet did not favor feed sorting, since it had low particle size distribution and the proportion of daily orts per cow was larger than 12% of the amount offered. There was a weak trend ($P < 0.15$) for diets CS and SGSA to require less rumination, suggesting that they may have resulted in rumen environment more suitable to forage fiber digestion.

Forage type affected the amount of diet consumed from 7 a.m. to 1 p.m. As the amount of diet offered to each cow at 7 a.m. was the same (25 kg/d), feed intake rate was increased in SG compared to CS. Diet carbohydrate profile or organoleptic properties of the diets determined intake pattern. Sugars, alcohols, and glycerol in SG were apparently less inhibitory the morning feed intake than CS starch and organic acids. Sensorial feed additives reduced the morning feed intake in diet CS, and tended to increase it in diet SG. The highest rate of morning intake of SG induced by SA may have altered the metabolism of glycerol, reducing the

proportion of the carbohydrate fermented in the rumen. Faster intake rate in the morning could have had a “drench like” action on glycerol consumption (Goff and Horst, 2001; Osman et al., 2008), consistent with the increase in plasma glucose content of SG-SA compared to SG. Increased glucose availability to the mammary gland apparently increased milk lactose secretion, a reasonable explanation for the positive response in lactation performance when SA was added to SG. The plasma glucose content of cows fed SG without SA was the lowest at and beyond the 21st experimental day (Figure 3). The larger intake of long feed particles on SGSA compared to SG, may also have increased ruminal motility and glycerol passage rate, plausibly increasing its absorption.

The lower activity of GGT on cows consuming SG suggests that a treatment effect on liver function occurred. The GGT activity increases in alcoholic induced hepatitis (Nishimura and Tescheke, 1983). However, decreased GGT activity indicates less liver damage in response to crude glycerol feeding. Similarly, Lima (2014) with heifers observed reductions in plasma AST in response to crude glycerin feeding. Crude glycerin intake also had no impact on venous blood acid-base balance, suggesting no occurrence of metabolic acidosis. The intake of methanol was around

70 g/d, liver enzyme activity and blood parameter data suggest that toxicity was not an issue in this experiment. However, immediately before the morning feeding, sensorial feed additives reduced the partial pressure of carbon dioxide and increased oxygen in blood, suggesting an increase in respiratory frequency (hyperventilation).

CONCLUSIONS

When added of sensorial feed additives, the partial replacement of corn silage with sugarcane silage plus crude glycerin was a plausible alternative for feeding mid- to late lactation dairy cows.

The effect of sensorial feed additives on feed sorting and lactation performance interacted with forage source.

At low inclusion in the diet, methanol rich crude glycerin did not have detectable negative effects on cow health.

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446 **Table 1.** Ingredient and nutrient composition of the experimental diets (%
447 of DM)

	Treatment[1]			
Item	CS	CSSA	SG	SGSA
Ingredients, % of DM				
Corn silage (31.8% DM, 56.5% NDF)	30.2	30.2	15.0	15.0
Sugarcane silage (28.5% DM, 67.1% NDF)			10.0	10.0
Crude glycerin			3.3	3.3
Tifton hay (92.5% DM, 65.9% NDF)	4.4	4.4	4.4	4.4
Sorghum silage (35.3% DM, 50.5% NDF)	9.2	9.2	9.1	9.1
Finely ground corn hydrated and ensiled (63.6% MS)	5.3	5.3	5.4	5.4
Finely ground corn	9.8	9.8	9.9	9.9
Citrus pulp	12.3	12.3	12.4	12.4
Soybean meal (53.9% CP)	16.2	16.2	17.9	17.9
Raw soybeans	4.6	4.6	4.6	4.6
Corn with sensorial additives orcorn	3.1	3.1	3.1	3.1
Premix[2]	4.9	4.9	4.9	4.9
DM, % of as fed	52.2	52.3	54.4	54.0
CP	17.6	17.6	17.6	17.7
NDF	34.5	34.3	33.8	33.8
Forage NDF	24.9	24.8	24.2	23.9
Corn silage NDF	17.0	16.9	8.5	8.4
Sugarcane silage NDF			7.7	7.7
EE	4.2	4.2	3.8	4.0
Ash	6.3	6.4	6.4	6.5
NFC[3]	37.5	37.5	38.4	38.1

448 [1]CS = corn silage, CSSA = corn silage + sensorial additive, SG =
449 sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
450 [2]20% limestone, 18% sodium bicarbonate, 7% magnesium oxide, 4%
451 NaCl, 8% minerals and vitaminas (18,5% Ca; 15,0% P; 3,0% Mg; 3,0%
452 S; 240ppm Co; 3,000ppm Cu; 8.000ppm Mn; 12.000ppm Zn; 90ppm Se;
453 180ppm I; 8.000.000 UI/kg Vit.A; 2,000,000 UI/kg Vit.D; 50,000
454 UI/kgVit.E).
455 [3]100 – (CP + EE + NDF+ Ash).
456

Table 2. Effect of forage type and sensorial feed additives on intake, milk yield, BW, and BCS of dairy cows

	Treatment[1]					*P*-values[2]						
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S	T	F*T	S*T	F*S*T
DMI,kg/d	22.2	21.6	22.7	22.4	0.38	<0.01	0.07	0.52	<0.01	0.94	0.68	0.84
Milk, kg/d	32.2	31.1	30.3	31.7	0.38	0.10	0.80	<0.01	<0.01	0.53	0.98	0.07
4% FCM,kg/d	27.1	26.2	26.6	26.9	0.07	0.90	0.63	0.38	<0.01	0.27	0.91	0.05
ECM,kg/d	28.4	27.6	28.1	28.6	0.79	0.69	0.89	0.44	<0.01	0.32	0.95	0.05
Fat, kg/d	0.940	0.913	0.970	0.961	0.027	0.17	0.52	0.73	<0.01	0.18	0.84	0.13
Protein, kg/d	0.982	0.977	0.936	0.988	0.030	0.57	0.46	0.37	<0.01	0.68	0.99	0.26
Lactose, kg/d	1.508	1.429	1.370	1.451	0.038	0.14	0.98	0.05	<0.01	0.72	0.99	0.06
Solids, kg/d	3.778	3.578	3.531	3.691	0.084	0.42	0.82	0.04	<0.01	0.45	0.99	0.05
Fat, %	3.00	2.96	3.23	3.12	0.072	0.01	0.31	0.61	<0.01	0.38	0.92	0.59
Protein, %	3.08	3.14	3.20	3.16	0.035	0.08	0.76	0.20	<0.01	0.05	0.88	0.47
Lactose, %	4.66	4.61	4.60	4.64	0.020	0.40	0.63	0.03	<0.01	0.52	0.37	0.10
Solids, %	11.71	11.62	11.92	11.73	0.091	0.03	0.35	0.97	<0.01	0.10	0.96	0.29
MUN, mg/dL	19.1	19.2	19.4	20.1	0.06	0.29	0.50	0.60	<0.01	0.91	0.06	0.22
Milk/DMI, kg/kg	1.44	1.46	1.34	1.43	0.015	<0.01	<0.01	0.03	<0.01	0.72	0.90	0.67
ECM/DMI, kg/kg	1.07	1.03	1.09	1.07	0.031	0.32	0.31	0.67	<0.01	0.20	0.94	0.50
BW, kg	622	625	625	630	2.8	0.21	0.15	0.74	<0.01	0.29	0.25	0.48
Daily gain,g/d	256	232	168	321	82.3	0.99	0.44	0.29	0.14	0.45	0.38	0.66
BCS, 1 to 5	3.38	3.35	3.31	3.22	0.56	0.10	0.31	0.63	<0.01	0.99	0.99	0.92

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.

[2]Probability values for the effects and interaction, F = forage, S = sensorial additive, and T = time

Table 3. Effect of forage type and sensorial feed additives on sorting behavior

	Treatment[1]					*P*-values[2]		
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S
Observed / Predicted[3], %								
From 7a.m.to 1p.m.								
>19mm	109	100	104	102	6.22	0.84	0.38	0.57
8-19mm	96	94	97	98	2.38	0.59	0.90	0.32
<8mm	100	102	101	100	1.35	0.68	0.68	0.39
From 2p.m.to 7p.m.								
>19mm	106	88	115	124	10.64	0.05	0.70	0.23
8-19mm	107	91	100	101	3.15	0.61	0.03	0.01
<8mm	96	104	99	96	1.78	0.23	0.13	<0.01
From 7p.m.to 7a.m.								
>19mm	90	73	55	59	17.09	0.17	0.68	0.54
8-19mm	102	87	100	93	6.71	0.85	0.11	0.54
<8mm	96	109	104	105	4.14	0.61	0.09	0.17
From 2p.m. to 7a.m.								
>19mm	99	82	89	99	6.20	0.56	0.55	0.03
8-19mm	102	95	100	98	1.36	0.86	<0.01	0.12
<8mm	99	103	101	101	0.86	0.67	0.02	0.02

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
[2]Probability values for the effects and interaction, F = forage, S = sensorial additive.
[3]100 = no selection, >100 = preferential consumption, <100 = rejection.

475 **Table 4.** Diets offered, refusals and particle size distribution of diets and
476 refusals

	Treatment[1]					*P*-values[1]		
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S
	fresh weight, kg							
7a.m. offered	25.0	25.0	25.0	25.0				
Daily offered	48.6	47.9	50.9	45.4	1.82	0.95	0.10	0.20
1p.m. orts	6.3	8.3	4.8	4.1	0.67	<0.01	0.36	0.06
7p.m. orts	13.0	13.7	12.9	12.3	1.53	0.62	0.96	0.71
7a.m. orts	5.8	7.0	6.9	5.4	1.09	0.82	0.90	0.23
	% of offered							
1 p.m.orts	25.2	31.8	19.4	16.5	2.54	<0.01	0.47	0.08
7 p.m. orts	43.3	45.8	47.7	41.8	5.15	0.96	0.75	0.43
7 a.m. orts	12.9	13.4	13.8	12.5	2.29	0.99	0.86	0.70
	% of fresh weight							
7 a.m. TMR								
>19mm	7.6	7.4	8.6	8.8				
8-19mm	21.6	22.4	19.6	19.0				
<8mm	70.6	69.0	71.0	72.2				
2 p.m. TMR								
>19mm	6.0	4.0	6.0	8.0				
8-19mm	21.4	23.0	20.0	22.8				
<8mm	71.4	72.0	73.0	68.0				
1 p.m. orts								
>19mm	6.0	8.3	6.2	7.6	1.39	0.99	0.27	0.63
8-19mm	23.6	26.3	22.0	21.1	1.47	0.03	0.56	0.24
<8mm	69.3	64.0	70.3	70.2	2.62	0.18	0.32	0.33
7 p.m. orts								
>19mm	5.4	7.1	4.5	6.0	0.92	0.29	0.08	0.92
8-19mm	28.6	26.7	21.2	22.4	1.17	<0.01	0.73	0.20
<8mm	65.3	65.4	73.9	71.0	1.91	<0.01	0.47	0.45
7 a.m. orts								
>19mm	6.9	10.4	9.1	8.8	1.68	0.84	0.35	0.27
8-19mm	28.5	26.7	21.7	23.2	1.12	<0.01	0.87	0.15
<8mm	63.9	60.5	68.5	67.2	2.71	0.05	0.38	0.73

477 [1]CS = corn silage, CSSA = corn silage + sensorial additive, SG =
478 sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
479 [2]Probability values for the effects and interaction, F = forage, S =
480 sensorial additive.

Table 5. Effect of forage type and sensorial feed additives on glucose, urea-N, and liver enzymes AST (Aspartate aminotransferase) and GGT(Gamma-glutamyl transpeptidase) in plasma, urinary allantoin excretion, rumen pH and total protozoa content.

	Treatment[1]					*P*-values[2]						
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S	T	F*T	S*T	F*S*T
Glucose, mg/dL	57.4	55.2	48.7	54.2	1.33	<0.01	0.23	0.01	<0.01	0.90	0.90	0.19
PUN,mg/dL	15.9	15.5	16.2	16.8	0.85	0.39	0.93	0.58	<0.01	0.38	0.07	0.34
AST,mol/min/L	41.3	40.4	40.1	40.3	2.07	0.75	0.86	0.79				
GGT,mol/min/L	36.1	34.9	29.2	31.5	2.87	0.09	0.84	0.55				
Allantoin, mmoles/d	46.3	34.6	37.4	32.0	5.07	0.28	0.11	0.54				
Rumen pH	6.70	6.61	6.73	6.50	0.137	0.75	0.24	0.58				
Protozoa, $x10^4$/mL	25.5	33.5	28.5	27.5	4.85	0.76	0.48	0.36				

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.

[2]Probability values for the effects and interaction, F = forage, S = sensorial additive, and T = time.

Table 6. Effect of forage type and sensorial feed additives on chewing activity of dairy cows

	Treatment[1]					*P*-values[1]		
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S
Ingestion, min/d	317	323	325	327	24.3	0.81	0.87	0.93
Rumination, min/d	426	462	467	432	23.8	0.82	0.99	0.15
Chewing[3],min/d	743	785	792	759	37.9	0.77	0.91	0.33
Ingestion, min/DMI	14.0	15.0	15.7	15.3	1.38	0.49	0.82	0.62
Rumination, min/DMI	19.1	21.7	21.9	20.4	1.37	0.60	0.68	0.14
Chewing,min/d	33.1	36.7	37.6	35.6	2.38	0.49	0.72	0.25

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
[2]Probability values for the effects and interaction, F = forage, S = sensorial additive.
[3]Chewing = Rumination+Ingestion.

Table 7. Effect of forage type and sensorial feed additives on total tract apparent digestibility of nutrients and energetic efficiency

	Treatment[1]					*P*-values[2]		
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S
Digestibility, % of intake								
DM	64.0	66.0	65.1	64.6	2.95	0.85	0.69	0.54
OM	68.2	70.7	69.0	69.7	2.62	0.96	0.49	0.68
NDF	41.6	47.1	43.4	36.6	5.01	0.39	0.90	0.24
Non-NDF MO	84.1	85.9	84.9	83.4	2.59	0.75	0.95	0.51
Digestible OM intake, kg/d	13.8	14.6	14.5	14.2	0.81	0.85	0.72	0.51
Efficiency[3], Mcal/kg	1.44	1.31	1.22	1.33	0.066	0.14	0.97	0.09

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive. [2]Probability values for the effects and interaction, F = forage, S = sensorial additive[3]Efficiency = Milk energy secretion (Mcal/d)/Digestible OM intake.

519 **Table 8.** Effect of forage type and sensorial feed additives on acid-base balance of the jugular blood of dairy
520 cows immediately before the morning feeding

	Treatment[1]					*P*-values[2]						
Item	CS	CSSA	SG	SGSA	SEM	F	S	S*S	T	F*T	S*T	F*S*T
pH	7.40	7.40	7.41	7.41	0.006	0.15	0.55	0.50	<0.01	0.40	0.33	0.63
pCO_2[3], mmHg	36.69	35.23	36.20	34.73	0.634	0.44	0.03	0.99	<0.01	0.07	0.31	0.61
pO_2[4], mmHg	32.98	33.84	31.72	34.12	0.662	0.47	0.02	0.26	<0.01	0.58	0.77	0.90
HCO_3^-[5], mEq/L	23.10	22.19	23.29	22.58	0.562	0.67	0.20	0.79	<0.01	0.60	0.18	0.22
TCO_2[6], mEq/L	24.25	23.26	24.29	23.66	0.602	0.72	0.20	0.77	<0.01	0.66	0.15	0.18
BE[7], mEq/L	-0.49	-1.36	-0.53	-0.66	0.653	0.61	0.47	0.27	<0.01	0.45	0.40	0.27
$SatO_2$[8],% of hemoglobin	63.17	64.74	59.29	65.81	1.414	0.34	0.01	0.10	<0.01	0.30	0.40	0.88
O_2ct[9],% of hemoglobin	14.15	14.72	13.35	14.72	0.332	0.26	<0.01	0.23	<0.01	0.13	0.24	0.99

521 [1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane +
522 glycerin + sensorial additive.
523 [2]Probability values for the effects and interaction, F = forage, S = sensorial additive, and T = time.
524 [3]pCO_2 = Partial pressure of carbon dioxide.
525 [4]pO_2 = Partial pressure of oxygen.
526 [5]HCO_3^- = Bicarbonate ion.
527 [6]TCO_2 = Total carbon dioxide.
528 [7]BE = Excess bases.
529 [8]$SatO_2$ = Oxygen saturation.
530 [9]O_2ct = Erythrocytes saturation.
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Table 9. Effect of forage type and sensorial feed additives on acid-base balance of the jugular blood of dairy cows 6 hours post morning feeding

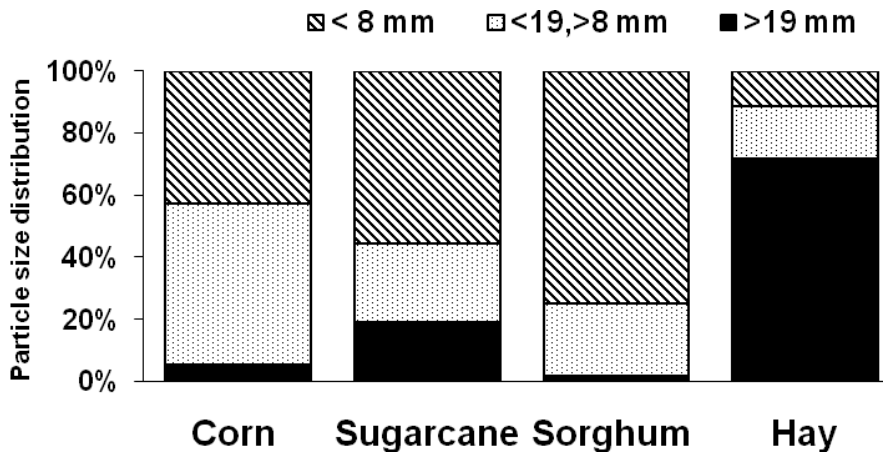
	Treatment[1]					*P*-values[2]						
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S	T	F*T	S*T	F*S*T
pH	7.41	7.40	7.40	7.40	0.007	0.86	0.48	0.69	<0.01	0.06	<0.01	0.68
pCO_2[3], mmHg	35.77	35.00	35.09	35.11	0.656	0.67	0.58	0.55	<0.01	0.10	0.72	0.98
pO_2[4], mmHg	32.57	33.13	32.59	31.96	0.774	0.46	0.97	0.45	<0.01	0.13	0.30	0.88
HCO_3^-[5], mEq/L	22.81	21.92	21.98	21.67	0.485	0.30	0.21	0.55	<0.01	0.76	0.04	0.74
TCO_2[6], mEq/L	23.88	22.73	22.90	22.90	0.432	0.36	0.20	0.19	<0.01	0.45	0.01	0.39
BE[7], mEq/L	-0.70	-1.58	-1.59	-1.73	0.495	0.32	0.31	0.45	<0.01	0.69	<0.01	0.62
$SatO_2$[8],% of hemoglobin	62.63	62.74	60.50	60.91	1.123	0.09	0.82	0.90	<0.01	0.12	0.14	0.94
O_2ct[9],% of hemoglobin	14.03	14.05	13.56	13.65	0.251	0.10	0.82	0.90	<0.01	0.12	0.14	0.94

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
[2]Probability values for the effects and interaction, F = forage, S = sensorial additive, and T = time.
[3]pCO_2 = Partial pressure of carbon dioxide.
[4]pO_2 = Partial pressure of oxygen.
[5]HCO_3^- = Bicarbonate ion.
[6]TCO_2 = Total carbon dioxide.
[7]BE = Excess bases.
[8]$SatO_2$ = Oxygen saturation.
[9]O_2ct = Erythrocytes saturation.

Table 10. Effect of forage type and sensorial feed additives on acid-base balance of the jugular blood of dairy cows 12 hours post morning feeding

	Treatment[1]					*P*-value[2]						
Item	CS	CSSA	SG	SGSA	SEM	F	S	F*S	T	F*T	S*T	F*S*T
pH	7.39	7.38	7.34	7.39	0.409	0.31	0.37	0.30	<0.01	0.64	0.76	0.71
pCO_2[3], mmHg	40.57	40.34	40.24	40.36	0.447	0.73	0.91	0.71	<0.01	0.51	0.94	0.76
pO_2[4], mmHg	32.94	32.84	32.08	33.08	0.869	0.71	0.64	0.53	<0.01	0.60	0.94	0.69
HCO_3^-[5], mEq/L	24.08	24.36	24.48	24.61	0.513	0.53	0.70	0.88	<0.01	0.95	0.56	0.72
TCO_2[6], mEq/L	26.12	25.25	25.72	25.64	0.743	0.99	0.53	0.60	<0.01	0.83	0.50	0.75
BE[7], mEq/L	0.69	0.34	0.11	0.48	0.692	0.86	0.63	0.32	<0.01	0.84	0.19	0.41
$SatO_2$[8],% of hemoglobin	62.00	60.80	59.15	60.84	1.642	0.38	0.89	0.37	<0.01	0.92	0.85	0.10
O_2ct[9],% of hemoglobin	13.90	13.62	13.26	13.62	0.365	0.38	0.92	0.38	<0.01	0.92	0.84	0.10

[1]CS = corn silage, CSSA = corn silage + sensorial additive, SG = sugarcane + glycerin, SGSA = sugarcane + glycerin + sensorial additive.
[2]Probability values for the effects and interaction, F = forage, S = sensorial additive, and T = time.
[3]pCO_2 = Partial pressure of carbon dioxide.
[4]pO_2 = Partial pressure of oxygen.
[5]HCO_3^- = Bicarbonate ion.
[6]TCO_2 = Total carbon dioxide.
[7]BE = Excess bases.
[8]$SatO_2$ = Oxygen saturation.
[9]O_2ct = Erythrocytes saturation.



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565 **Figure 1**. Particle size distribution (% of feed fresh weight) of corn
566 silage, sugarcane silage, sorghum silage, and Tifton hay

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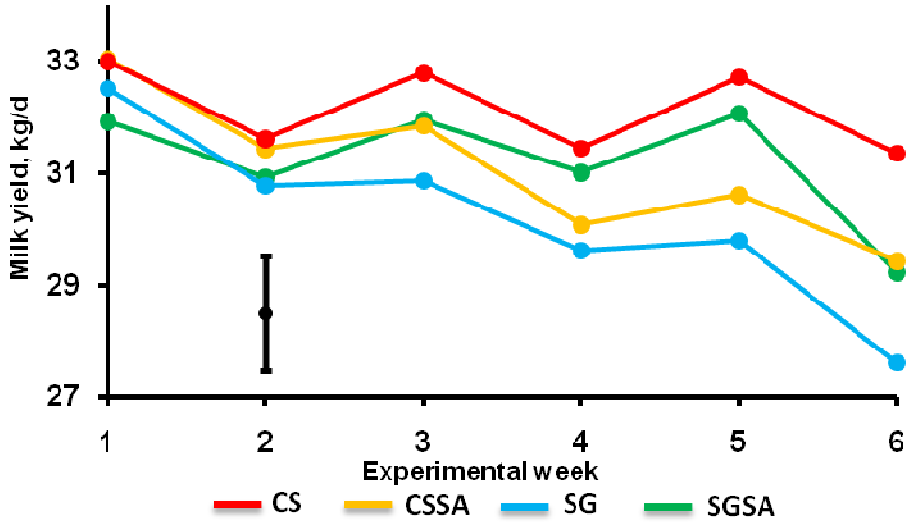

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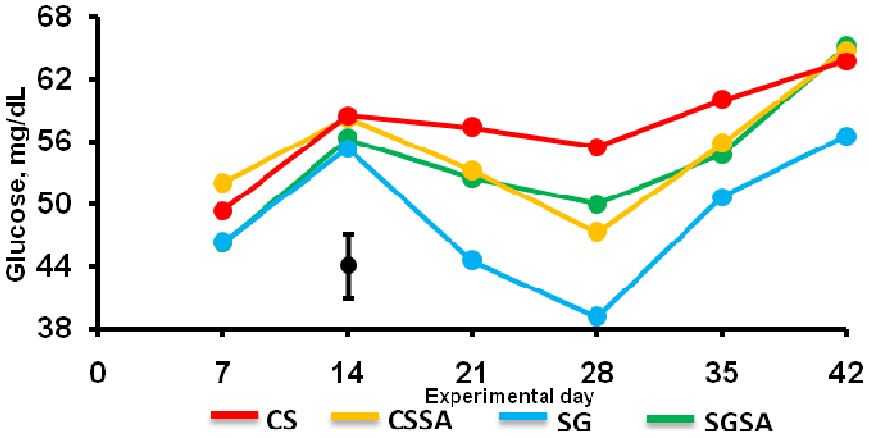
571 **Figure 2.** Milk yield along the 6-week comparison period on treatments
572 corn silage (CS), corn silage + sensoral additive, sugarcane silage + crude
573 glycerin (SG), sugarcane silage + crude glycerin + sensorial additive
574 (SGSA). The interaction effect (P = 0.07)



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577 **Figure 3.** Plasma glucose 12 h post feeding on treatments corn silage
578 (CS), corn silage + sensorial additive, sugarcane silage + crude glycerin
579 (SG), sugarcane silage + crude glycerin + sensorial additive (SGSA). The
580 forage effect ($P < 0.01$) and interaction between forage and sensorial
581 additive ($P < 0.01$)
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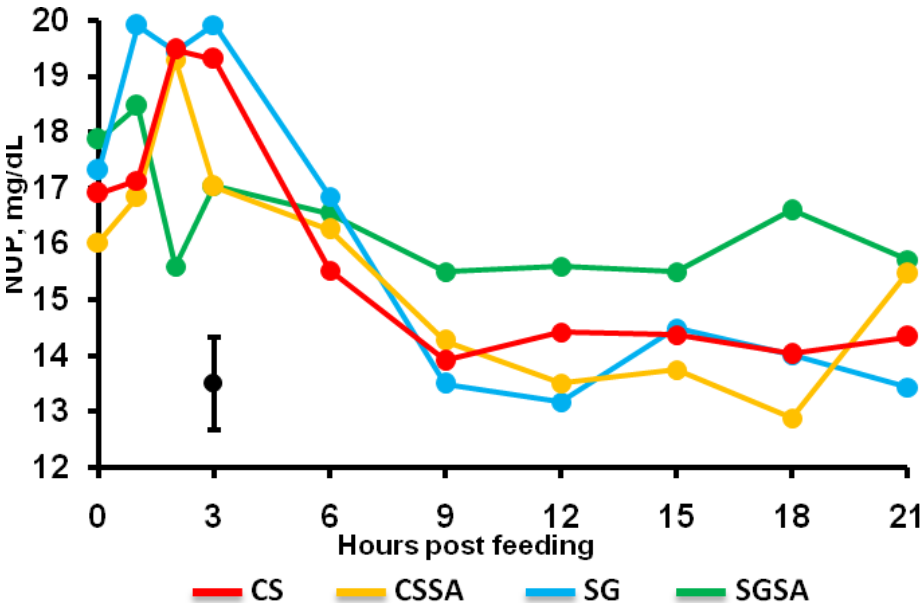

Figure 4. Plasma urea nitrogen (NUP) on treatments corn silage (CS), corn silage + sensorial additive, sugarcane silage + crude glycerin (SG), sugarcane silage + crude glycerin + sensorial additive (SGSA). Interaction of sensorial additive and time (P = 0.07).

PAPER 2

REPLACEMENT OF CORN BY GLYCERIN FOR COWS

Performance, digestibility, and blood acid-base balance of dairy cows in response to the replacement of corn by crude glycerin

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ABSTRACT

This experiment evaluated the response of late lactation dairy cows to the partial replacement of corn by methanol-rich, crude glycerin. The tallow derived glycerin contained 70.2% DM and 7.3% methanol on an as fed basis. Twelve Holstein cows (219±57 DIM) were assigned to treatment

sequences of 3 diets within four 3x3 Latin Squares consisting of 35-day periods. Diets were isonitogenous (15.8% CP) and contained either: 11.8% finely ground mature corn and 17.2% soybean meal (0% glycerin); 4.9% glycerin, 5.9% corn, and 18.3% soybean meal (5% glycerin); or 9.7% glycerin and 19.4% soybean meal (10% glycerin). Other ingredients were: 31.9% corn silage, 28.2% sugarcane silage, and 6.2% high moisture corn. Statistical analysis was performed using the SAS, with a model containing the random effects of cow and period and the fixed effect of treatment. Were evaluated contrasts: Linear = 0 vs. 10, and Quadratic = 5 vs. (0 + 10). The replacement of corn by glycerin induced a linear decrease in milk (22.2, 21.1, 20.0 kg/d for 0, 5, and 10% glycerin) and lactose yield (kg/d), without affecting DMI (17.8 kg/d) and consequently there was a reduction in feed efficiency. Milkfat (4.11, 4.33, 4.37%) and protein (3.47, 3.64, 3.73%) were linearly increased by glycerin, but daily yiled was not different among treatments. Milk urea nitrogen was similar (13.8 mg/dL), as well as chewing activity, except the daily ingestion time, reduced by glycerin. Total tract apparent digestibility of the non-NDF organic matter was linearly increased by glycerin (90.3, 91.4, 93.2% of intake), but the intake of digestible organic matter was similar (10.6

kg/d). The ratio of the daily milk energy secretion to the intake of digestible organic matter was linearly reduced by glycerin. Rumen pH 12 hours post feeding was similar (5.67). The intake of crude glycerin was 1.24 kg/d in 5% and 2.5 kg/d in 10%, methanol intake was 134 mg/kg of BW in T5 and 269 mg/kg of BW in 10%. There were no adverse health events observed during the study. Glycerin reduced the partial pressure of CO_2 and increased the saturation of hemoglobin with O_2 in jugular blood samples obtained 6 hours post feeding, suggesting an induction of hyperventilation. Venous blood pH, bicarbonate level, base excess, and the partial pressure of O_2 were not affected by treatment. The replacement of corn with crude glycerin for cows resulted in a reduction in milk yield and feed efficiency and a reduction in daily milk lactose yield.

Keywords: methanol, crude glycerin, glycerol, energy, biofuel

INTRODUCTION

Biodiesel is a promising renewable fuel that is mainly produced from the transesterification of vegetable oils or animal fats with methanol catalyzed by alkali (Hu et al., 2012). Industry growth is expected to

increased availability and promote favorable pricing of glycerin, which is a by-product in the production of biodiesel (Thompson and He, 2006). Brazil produced 2.5 billion liters of biodiesel in 2013 (Biofuel Digest, 2013). It is estimated that each litter of biodiesel produced generates about 100 mL of crude glycerin (Dasari et al., 2005), which contains variable glycerol content (Wilbert et al., 2013). Crude glycerin contains several impurities including residual methanol, sodium hydroxy, fat, esters, and low amounts of sulfur compounds, proteins, and minerals (Celik et al., 2008). Glycerol, the main component of crude glycerin, has high energy content, which is approximately the same as that of corn starch and can be used for animal feeding (Donkin et al., 2009; Wilbert et al., 2013). One of the major challenges for the utilization of crude glycerin is the inconsistency of its composition since it varies with the feedstocks, production process, and post-treatments involved in biodiesel production. Upgrading or refining crude glycerol to technical grade glycerin (>98% glycerol content) makes its composition more consistent, but currently this is not economically viable (Hu et al., 2012). One concern is about the methanol content of crude glycerin, in a range of <0.01 to 13.94% (Hansen et al., 2009). Methanol is metabolized to

formaldehyde by the liver enzyme alcohol dehydrogenase (ADH) (Barceloux et al., 2002; Kraut et al., 2008). Formaldehyde is then metabolized via enzyme formaldehyde dehydrogenase to formic acid, formate then being metabolized to CO_2 and H_2O, a processe tha depends on liver tetrahydrofolate concentrations (Barceloux et al., 2002; Kerns et al., 2002). This pathway is easily saturable, contributing to accumulation of formic acid in the blood (Kraut et al., 2008). Formic acid can cause metabolic acidosis, hyperosmolality, retinal damage with blindness, putaminal damage with neurologic dysfunction (Kraut et al., 2008).

However, the use of crude glycerin in animal feed can be financially and nutritionally efficient, requires prior assessment of response in animal performance and health. The objective of this experiment was evaluated the performance, diet digestibility and venous acid -base balance of dairy cows in late lactation to increasing dietary levels of methanol rich-crude glycerin as a replacement to corn.

MATERIALS AND METHODS

Cows and Management

Twelve lactating (4 primiparous and 8 multiparous) Holstein cows with an average DIM of 219 ± 57 were housed in invidual tie stalls with sand beds. Cows were fed individually at 0700 and 1400 hours. The amount of feed offered was adjusted each day to achieve at least 5% refusal. The amount of silage on an as-fed basis was adjusted weekly according to the DM content of fresh silage, as determined by drying for 60 minutes using Koster (Koster Moisture Tester, Medina, USA). Cows were milked twice daily at 0500 and 0400 during the study. Cows formed four groups of three animals based on daily milk production. Animals within each group were randomly allocated to one of three possible sequences of three treatments.

Experimental Treatments and Design

The experimental design for this study was a replicated 3 x 3 Latin square. Each period consisted of 28 days for treatment adjustment followed by 7 days for data collection. Treatments were either the control diet, a diet formulated by replacing mature finely ground corn by isonitrogenous mixture of crude glycerin and soybean meal (Table 1). The composition of crude glycerin, derived from beef tallow (Tecno-Oil

Indústria e Comércio Ltda, Mombuca, SP), was: 29.8% moisture and 7.3% methanol (as fed basis),0.92% crude protein (CP), 7.1% ether extract (EE), 7.9% ash, 0.52% sodium, 0.25% sulfur, 0.06% potassium, 0.05% phosphorus, 0,03% calcium, 0.01% magnesium (DM basis), and pH = 1.89.

Data Collection

The amount of feed offered and refused was recorded daily during 5-d collection week. Composite samples of feed and refusal per animal per period were formed by mixing equal quantities as feed of the daily samples. The DM content was determined by drying in a forced-air oven at 55°C for 72 h. Samples were ground to pass through a 1-mm screen using a Willey mill (Thomas Scientific, Swedesboro, NJ) for analysis of DM, CP, ether extract, ash (AOAC, 1990), NDF (Van Soest et al., 1991).

Milk yield was recorded at each milking (2X) during days 29 through 35 of each period collection week. Milk samples were collected from 6 consecutive milkings each period collection, preserved with 2-bromo-2-nitropropane-1,3diol, and analysed for concentrations of protein, fat, lactose, total solids, and milk urea nitrogen (MUN). (Laboratório

Centralizado da Associação Paranaense de Criadores de Bovinos da Raça Holandesa - APCBRH, Curitiba, PR). The daily secretion of energy in milk (NEL) was calculated as NE=[(0.0929 x % fat) + (0.0547 x % protein) + (0.0395 x % lactose)] x kg of milk (NRC, 2001). Energy-corrected milk yield as ECM=NEL/0.70, assuming that the energy content in milk with 3.7 % fat, 3.2% protein and 4.6% lactose is 0.70 Mcal/kg. The milk yield corrected to 4% fat as FCM = (0.4 + 15 x % of milk fat/100) x kg of milk. Body weights and condition cores were obtained at the day 5 of each collection week. Body weight was measured after the morning milking and body condition was scored by 3 trained individuals based on a 5-point scale (Wildman et al., 1982).

Blood samples from the coccygeal vessel were collected at day 34 of each period and used for analysis of plasma urea nitrogen (PUN). Blood was collected into vacutainers containing EDTA at 0, 1, 2, 3, 6, 9, 12 and 18 h after the morning feeding. The plasma were analysed by enzymatic colorimetric method (Urea 500. Doles Reagentes e Equipamentos Laboratorios Ltda, Goiania, GO). At the time 12 h after morning feed, were collected blood samples in vacutainers containing potassium fluoride and vacutainers with heparin for glucose analysis (Glicose

enzimática líquida. Doles Reagentes e Equipamentos para Laboratório Ltda, Goiânia, GO), and beta-hydroxybutyrate based on the method of Williamson et al. (1962)

The acid-base balance was measured in blood samples obtained from the jugular vein on day 6. Sampling times were zero, prior the morning feeding and six hours after. Blood was collected in vacutainers containing heparin and analyzed within one hour after collection (Avaliador de pH e gases sanguineos AGS22 - Drake , São José do Rio Preto , SP) .

Ruminal fluid from individual cows was collected by gentle aspiration through a tube extending through the esophagus into the rumen. Samples were obtained from all cows between 1100 to 1200 h. The pH of ruminal fluid wasimmediately measured and 10 mL of formaldehyde was added to 10 mL of rumen fluid and stored for protozoa (Dehority, 1984). The number of protozoa was counted under a light microscope using samples of 1mL of fluid formalized allocated in Newbauer chamber with 0.1 mm depth (Warner , 1962).

Digestibility Study, Urine Sampling and Chewing Activity

Fecal samples were collected by total fecal collection during days 31 to 34 of each period. Feces were collected concurrent to defecation during three 8-hour sampling periods and weighed. The second and third sampling periods were each delayed by 8 h to avoid a major disturbance to the animals, while still representing a 24-h collection period. Samples were frozen at the time of collection and a composite sample was formed for each cow for each period. The fecal samples were dried in a forced-air oven at 55°C for 72 h and ground to pass thorough a 1-mm screen, and analyzed for DM, NDF, ash as described above for feed samples. Daily intake of digestible organic matter intake (DOMI) was calculated to estimate the energy intake. The energy efficiency was calculated by the ratio ECM/DOMI, as an indirect measure of the energy lost as methane.

Total urine was collected from all animals and used to the synthesis of microbial protein in the rumen. The volume of urine collected was immediately acidified with sulfuric acid and stored at 4°C pending analysis for allantoin content. A composite sample was obtained for each cow at the end of week collection, diluted with 4% solution sulfuric acid in the ratio 1:3, and frozen at -20°C until measurement of allantoin content (Chen and Gomes, 1992).

Ingestion time and ruminating time was determined by visual observation of oral activity every five minutes, during the total fecal collection on days 31 to 34 of each period. Time spent chewing was the calculated sum of time eating and ruminating. All times are reported per 24 h interval. The corresponding DMI on day of observation visual observation was used to calculate the rate of ingestion and chewing in min per unit DMI.

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS (1999). The model accounted for the fixed effect of treatment (0, 5, or 10% glycerin), random effect of period (1 to 3), random effect of cow (1 to 12). Pre-planned contrasts, linear (0 vs. 10) and (5 vs. 0 + 10) were used to test the glycerin inclusion. NUP content was analyzed as repeated measures over time, at the model above were added sampling time (0, 1, 2, 3, 6, 9, 12 and 18h) and interaction with treatment. The covariance structure used was defined by the Akaike information criterion, auto regressive of order 1, unstructured and compound symmetry. Significance was defined as $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$.

RESULTS

There was no treatment effect of partial replacement of finely ground corn with increasing quantities of crude glycerin in diets fed to dairy cows on DMI (Table 2) but there was a linear decrease in milk yield ($P < 0.01$) and reduced feed efficiency ($P < 0.01$). Milk yield was reduced ($P < 0.01$) by 1.1 and 2.2 kg/d with the substitution of corn grain with 5 and 10% glycerin respectively. The inclusion of glycerin in the diet resulted in a linear reduction in milk lactose content ($P < 0.01$) and yield ($P < 0.01$), and milk protein yield ($P = 0.03$). In contrast, milk protein and milk fat percentage increased linearly with glycerin. A significant linear effect of increasing inclusion of glycerin was detected on the BW ($P = 0.03$).

Time spent eating showed a quadratic response to increasing glycerol in the diet, being lower for the 5% glycerin ($P = 0.03$) (Table 3). There was no effect of treatment on the rumination time. Apparent total tract digestibility of OM non-NDF showed a linear increased with higher as glycerin content in the diet (Table 4). The content of protozoa in the rumen fluid was higher on treatment 5% glycerin ($P = 0.03$) (Table 4),

however no effect was observed in ruminal pH (Table 4) and MUN (Table 2). There was no effect of treatments on the venous acid-base balance (Tables 5 and 6) but plasma glucose decreased ($P = 0.01$), and plasma BHBA concentration increased ($P < 0.01$) linearly with glycerin inclusion (Table 7). However, in venous blood taken six hours post morning feeding (Table 6), glycerin supplementation reduced ($P = 0.02$) the partial pCO_2 and tended to increase ($P = 0.07$) the oxygen saturation of hemoglobin.

DISCUSSION

There was no effect increasing dietary crude glycerin containing 7.containing 7.3% methanol on DMI but there was a linear decrease in milk yield, reducing feed efficiency. Milk was reduced ($P < 0.01$) by 1.1 and 2.2 kg/d for 5 and 10% glycerin respectively. These data are in contrast to earlier studies demonstrating a lack of effect of replacement of corn with pure glycerol on feed intake and milk production in mid-lactation (Donkin et al., 2009) or transition dairy cows (Carvalho et al., 2011), however results from feeding crude glycerol are equivocal (Shin et al., 2012).

The decrease in the mammary secretion of lactose was a plausible explanation for the lower performance on diets containing glycerin. Plasma glucose decreased as the glycerin increased on diet. When administered as a drench (Osman et al., 2008; Goff and Horst, 2001) glycerol may bypass rumen metabolism and be absorbed into portal blood and metabolized by liver for gluconeogenesis. When glycerol is used as feed ingredient, it is likely metabolized propionate in the rumen and used for gluconeogenesis and therefore is subject to greater regulation by insulin, glucagon, other hormones and allosteric regulators of gluconeogenesis (Donkin and Armentano, 1994). Alternatively the inclusion of methanol as a contaminant of crude glycerol may limit gluconeogenesis from as alcohols in liver favor the synthesis of NADH and the reduction of oxaloacetate to malate to render less oxalacetate available for gluconeogenesis from propionate and lactate.

The differences in body weight the animals indicates an effect of glycerol feeding although these data should be interpreted with caution since the design of the Latin square not be adequate to assess the effect of treatments on weight gain despite a significant linear effect of increasing glycerin. The data suggest that the substitution of corn by glycerin

directed nutrients for weight gain instead lactose synthesis mammary gland. These data are consistent with previous observations in mid lactations cows fed glycerol and may point to the need for better assessment of the energy content of glycerol in formulating diets for lactating cows. The linear increase in apparent total tract digestibility of OM non-NDF has been with glycerin content in the diet suggests that additional interactions with diet components may also alter the feeding value of the ration to increase glycerol apparent digestibility and energy value along the digestive tract.

The daily ingestion time had a quadratic response to treatments, being lower in 5% glycerin but there was no effect of treatment on the activity and rumination, resulting in no effect on total chewing activity. These data suggest that replacing corn glycerin did not alter the physical effectiveness of diets or their palatability.

There was a decrease in efficiency with inclusion of glycerin to the diet. This might have resulted from the conversion of methanol to methane by microbial metabolism in the rumen (Czerkawski and Breckenridge, 1972; Pol and Demeyer, 1988) or the direction of digestible energy intake in body gain rather this be secreted into milk as

287 energy. The daily intake of crude glycerin with 29.8% moisture content
288 was 1.24 and 2.50 kg on treatments 5 and 10% glycerin. These values are
289 equivalent to daily intake of 91 and 183g of methanol respectively, and
290 had no effect on intake. The results are in agreement with Winsco et al.
291 (2011) that infused 210g of methanol into the rumen and intake was not
292 affected. Although, the venous acid-base balance was linearly decresed by
293 inclusion of glycerin, suggesting that hyperventilation happened.
294 Methanol is metabolized to formaldehyde by the liver enzyme alcohol
295 dehydrogenase (ADH) (Barceloux et al., 2002; Kraut et al., 2008).
296 Formaldehyde is then metabolized via enzyme formaldehyde
297 dehydrogenase to formic acid, formate then being metabolized to CO_2 and
298 H_2O (Barceloux et al., 2002; Kerns et al., 2002). This pathway is easily
299 saturable, contributing to accumation of formic acid in the blood, and
300 formic acid can cause metabolic acidosis (Kraut et al., 2008). These data
301 showed, even the amount of methanol was high, there was adequate
302 capacity of liver to metabolize methanol to CO_2. Changes in respiratory
303 activity would suggest that the CO_2accumulated induced a
304 hyperventilation, featuring respiratory alkalosis and is supported by the

numerical increase in partial $pO_2$with greater with glycerin inclusion in the diet.

Plasma BHBA concentration increased when cows were fed with glycerin. It is known that glycerin is fermented in the rumen to propionate, acetate and butyrate (Remond et al., 1993; Defrain et al., 2004; Bodarski et al., 2005). Furthermore, the omasal and ruminal epithelium convert butyrate to BHBA to provide energy and lessen toxic effect of butyrate on digestive mucosa (Schroder and Sudekum, 1999). Greater BHBA concentration in glycerin fed cows may indicate an increased ruminal fermentation of glycerin.

CONCLUSION

The results of this study indicate that the substitution of finely ground mature corn to more than 10% of crude glycerin containing 7.3% methanol as DM, reduced milk production, feed efficiency, and lactose of dairy cows in late lactation. Although glycerol has been effective in replacing corn these data point to a need for consideration of the negative effects of inclusion of methanol and other contaminants in crude glycerol and the potential negative impact on milk production.

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Table 1. Ingredient and nutrient composition of the experimental diets

	Glycerin, % of diet DM		
Item	0	5	10
Ingredients, % of DM			
Corn silage (58.9% NDF)	32.3	31.7	31.7
Sorghum silage (60.7% NDF)	27.9	28.4	28.4
Soybean meal (53.2% CP)	17.2	18.3	19.4
High moisture corn (66.2% DM)	6.2	6.2	6.2
Ground corn	11.8	5.9	
Crude glycerin		4.9	9.7
Premix[1]	4.6	4.6	4.6
DM, %	40.8	41.3	41.5
Chemical compositon, % of DM			
CP	15.7	15.8	15.8
NDF	38.2	37.5	37.6
NDFF	34.0	33.8	34.2
NDF corn silage	18.0	17.6	17.8
NDF sorghum silage	16.0	16.2	16.4
Ether extract	3.8	3.4	3.0
Ash	5.3	5.5	5.7
NFC[2]	37.0	37.8	37.9

[1]Premix = 15% limestone, 15% sodium bicarbonate, 7% % magnesium oxide, 4% NaCl, 8% minerals and vitaminas(18,5% Ca; 15,0% P; 3,0% Mg; 3,0% S; 240ppm Co; 3,000ppm Cu; 8.000ppm Mn; 12.000ppm Zn; 90ppm Se; 180ppm I; 8.000.000 UI/kg Vit.A; 2,000,000 UI/kg Vit.D; 50,000 UI/kgVit.E).

445 [2]Non-fiber carbohydrates = 100 - (CP + NDF + EE + Ash).

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449 **Table 2.** Performance of lactating Holstein cows fed diets supplemented

450 with different amounts of supplemental crude glycerin

	Glycerin, % of diet DM				*P*-values		
	0	5	10	SEM	Treat	Linear	Quadratic
DMI, kg/d	17.6	17.8	18.1	0.57	0.53	0.27	0.88
Milk, kg/d	22.2	21.1	20.0	1.30	<0.01	<0.01	0.91
4% FCM,kg/d	22.4	21.9	20.9	1.25	0.20	0.08	0.71
ECM, kg/d	23.7	23.2	22.1	1.41	0.22	0.09	0.74
Fat, kg/d	0.903	0.899	0.866	0.051	0.36	0.20	0.54
Protein, kg/d	0.772	0.757	0.738	0.042	0.11	0.03	0.88
Lactose, kg/d	1.000	0.941	0.874	0.075	<0.01	<0.01	0.88
Solids, kg/d	2.894	2.804	2.671	0.176	<0.01	<0.01	0.69
Fat, %	4.10	4.32	4.36	0.169	0.02	0.01	0.27
Protein, %	3.49	3.64	3.72	0.108	<0.01	<0.01	0.25
Lactose, %	4.48	4.37	4.28	0.136	<0.01	<0.01	0.78
Solids, %	13.04	13.29	13.31	0.277	0.02	0.01	0.20
MUN,mg/dL	13.5	14.0	13.8	1.53	0.27	0.36	0.18
Milk energy, Mcal/d	16.6	16.2	15.5	0.99	0.22	0.09	0.74
Milk/DMI, kg/kg	1.26	1.19	1.10	0.076	<0.01	<0.01	0.75
ECM/DMI kg/kg	1.35	1.31	1.21	0.074	0.02	<0.01	0.52
BW, kg	670	676	680	26.2	0.10	0.03	0.80
BCS, 1 to 5	3.51	3.56	3.55	0.173	0.62	0.46	0.53

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453 **Table 3.** Chewing activity of dairy cows fed diets supplemented with

454 different amounts of supplemental crude glycerin

	Glycerol, % of diet DM				*P*-values		
Item	0	5	10	SEM	Treat	Linear	Quadratic
Ingestion, min/d	329	287	303	21.81	0.04	0.11	0.04
Rumination, min/d	430	434	430	24.37	0.98	0.99	0.85
Chewing[1], min/d	726	721	717	43.32	0.96	0.79	0.99
Ingestion, min/DMI	18.9	17.3	18.5	1.49	0.30	0.71	0.13
Rumination, min/DMI	24.9	26.1	26.6	1.89	0.62	0.35	0.80
Chewing[1], min/DMI	41.4	43.5	44.1	2.95	0.40	0.20	0.70

455 [1]Chewing = Rumination + ingestion.

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Table 4. Total tract apparent digestibility of nutrients, efficiency and allantoin on rumenof dairy cows fed diets supplemented with different amounts of supplemental crude glycerin

Item	Glycerin, % of diet DM			SEM	*P*-values		
	0	5	10		Treat	Linear	Quadratic
DM digestibility, % of intake	60.8	59.0	60.8	1.99	0.69	0.99	0.39
OM digestibility, % of intake	63.4	61.9	64.0	1.73	0.64	0.79	0.37
NDF digestibility, % of intake	26.1	28.7	30.4	3.24	0.25	0.22	0.26
Non-NDF OM digestibility, % of intake	90.3	91.4	93.3	1.25	0.14	0.05	0.73
Digestible OM intake, kg/d	10.5	10.4	10.9	0.47	0.66	0.46	0.60
Efficiency3[1] Mcal/kg	1.58	1.55	1.42	0.09	0.08	0.05	0.24
Allantoin, mmoles/d	31.3	33.1	35.6	11.36	0.26	0.22	0.23

[1]Efficiency 3 = Milk energy/Digestible OM Intake.

Table 5. Acid-base balance in the jugular blood of dairy cows immediately before the morning feed

	Glycerin, % of diet DM				*P*-values		
Item	0	5	10	SEM	Treat	Linear	Quadratic
pH	7.37	7.40	7.37	0.033	0.19	0.67	0.08
pCO_2[1] mmHg	46.04	44.70	45.48	2.170	0.78	0.77	0.52
pO_2[2] mmHg	33.06	34.05	32.70	1.724	0.75	0.84	0.47
HCO_3^-[3] mEq/L	26.77	27.52	25.80	2.769	0.25	0.35	0.17
TCO_2[4] mEq/L	28.25	28.99	27.43	2.867	0.32	0.42	0.20
BE[5] mEq/L	1.25	1.94	0.43	2.796	0.38	0.45	0.24
$SatO_2$[6] % of hemoglobin	60.86	63.08	60.22	4.023	0.65	0.84	0.37

[1]pCO_2 = partial pressure of carbon dioxide.

[2]pO_2 = partial pressure of oxygen.

[3]HCO_3^- = bicarbonate ion.

[4]TCO_2 = total carbon dioxide.

[5]BE = excess bases.

[6]$SatO_2$ = oxygen saturation.

Table 6. Acid-base balance in the jugular blood of dairy cows six hours after morning feed

	Glycerin, % of diet DM				*P*-values		
Item	0	5	10	SEM	Treat	Linear	Quadratic
pH	7.38	7.39	7.40	0.030	0.92	0.68	0.96
pCO_2[1] mmHg	42.28	40.10	37.13	5.231	0.06	0.02	0.82
pO_2[2] mmHg	36.67	38.15	39.08	5.301	0.65	0.36	0.90
HCO_3^-[3] mEq/L	22.35	23.50	21.52	5.985	0.61	0.69	0.37
TCO_2[4] mEq/L	23.48	24.65	22.82	6.125	0.67	0.74	0.40
BE[5] mEq/L	-4.94	-2.25	-3.33	7.220	0.69	0.61	0.49
$SatO_2$[6] % of hemoglobin	55.76	66.91	66.94	4.177	0.12	0.07	0.29

[1]pCO_2 = partial pressure of carbon dioxide.

[2]pO_2 = partial pressure of oxygen.

[3]HCO_3^- = bicarbonate ion.

[4]TCO_2 = total carbon dioxide.

[5]BE = excess bases.

[6]$SatO_2$ = oxygen saturation.

Table 7. Allantoin, plasma B-hidroxybutirate (BHBA), glucose and urea nitrogen, protozoa and ruminal pH ofdairy cows fed diets supplemented with different amounts of supplemental crude glycerin twelve hours after the morning feed

	Glycerin, % of diet DM				*P*-values		
Item	0	5	10	SEM	Treat	Linear	Quadradic
Allantoin, mmol/dL	31.3	33.1	35.6	11.36	0.26	0.22	0.23
BHBA, mmol/L	0.13	0.17	0.19	0.015	<0.01	<0.01	0.54
Glucose, mg/dL	72.6	65.4	64.4	7.11	0.02	0.01	0.23
PUN, mg/dL	17.5	18.2	17.8	0.75	0.66	0.63	0.45
Protozoa, $x10^4$/mL	31.7	43.0	29.7	5.12	0.09	0.75	0.03
Ruminal pH	5.73	5.63	5.65	0.080	0.69	0.51	0.61