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CÂMPUS DE JABOTICABAL

IN VITRO FERMENTATION PARAMETERS AND BIOHYDROGENATION OF VEGETABLE OILS WITH OR WITHOUT GLYCEROL

Pablo de Souza Castagnino

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DEDICO

Aos meus pais Círia e Angelo Castagnino;
meu irmão Douglas de Souza Castagnino;
e aos meus avós.

"Todos os homens consideram os limites do seu campo de visão como os limites do Mundo "

(Arthur Schopenhauer)

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AVALIAÇÃO *IN VITRO* DOS PARÂMETROS FERMENTATIVOS E DA BIOHIDROGENAÇÃO DE ÓLEOS VEGETAIS COM OU SEM A INCLUSÃO DE GLICEROL

RESUMO – O glicerol combinado com óleos vegetais poderia limitar a intensa biohidrogenação dos ácidos graxos de cadeia longa e a produção de metano sem causar efeitos deletérios sobre a população microbiana e digestibilidade**.** Três incubações *in vitro* foram conduzidas para avaliar o efeito do glicerol (0 or 150 g/kg MS) combinado com três diferentes dietas: Feno de Tifton 85 sem a inclusão de óleo (FSO), Feno de Tifton 85 + 80g de óleo de soja/kg MS (FOS) e Feno de Tifton 85 + 80 g de óleo de linhaça/kg MS (FOL) incubados por 0, 6, 12 e 24 h sobre a composição de ácidos graxos e os parâmetros de fermentação ruminal. A PCR em tempo-real (qPCR) foi utilizado para quantificação de microorganismos às 24 h. As arquéias metanogênicas ,bactérias celulolíticas e lipolíticas foram expressas em função das bactérias totais. Separadamente, a cinética de produção de gases foi avaliada nos tempos 2, 4, 6, 8, 10, 12, 14, 18, 22, 24, 36, 42 e 48 h. A digestibilidade verdadeira *in vitro* (DVIV) e a produção CH_4 (%/g DMS) foram avaliadas às 48 h. Os valores de pH e as concentrações de amônia (NH_3-N) foram menores nas dietas FSO comparado com FOS e FOL, independente da adição de glicerol (P<0,05). A proporção da *Anaerovibrio lipolytica* aumentou 84, 33 e 13 vezes nas dietas FSO, FOS e FOL com adição de glicerol, respectivamente, comparadas com as dietas sem glicerol (P<0,05). *Ruminococcus albus, Ruminococcus flavefaciens* e os subgrupos *Butyrivrio* VA e SA subgroup não foram alteradas com a inclusão dos óleos vegetais e de glicerol (P>0,05). As bactérias *Fibrobacter succinogenes* foram mais sensíveis a adição de óleos vegetais entre todas as bactérias celulolíticas (P<0,05). A DVIV e a produção de CH_4 foram inferiores nas dietas FOS com adição de glicerol e na dieta FOL independente da adição de glicerol (P<0,05). A redução na produção de gases ocorreu nas dietas FOS e FOL associadas com glicerol (P<0,05). A relação C2:C3 foi menor em todas as dietas com a inclusão de glicerol (P<0,05). A inclusão do glicerol não reduziu a biohidrogenação dos ácidos graxos insaturados *in vitro* (P>0.05). A dieta FOS associada com glicerol e as dietas FOL com ou sem glicerol reduziram a produção de metano e a proporção de metanogênicas, no entanto reduziram a DVIV comparada com a dieta FSO. O glicerol pode substituir o amido como fonte energética nas dietas de ruminantes e também reduzir metano quando associado com óleo de soja. O glicerol não reduziu os efeitos negativos dos óleos vegetais sobre os parâmetros fermentativos e digestibilidade *in vitro.* Além disso, as dietas associadas com glicerol aumentaram a proporção de *Anaerovibrio lipolytic*a e não reduziram a biohidrogenação dos ácidos graxos dos óleos vegetais *in vitro.*

Palavras-Chave: bactérias, biohidrogenação, metano, óleo de linhaça, óleo de soja

IN VITRO FERMENTATION PARAMETERS AND BIOHYDROGENATION OF VEGETABLE OILS WITH OR WITHOUT GLYCEROL

ABSTRACT – Glycerol combined with vegetable oils could limit biohydrogenation and enhance the reduction in methane production without depressing digestibility and microbial population. Three *in vitro* incubations were conducted to evaluate the effect of glycerol (0 or 150 g/kg DM) combined with three different diets: Tifton 85 hay without oil seeds (HWO), Tifton 85 hay + 80g of soybean oil/kg DM (HSO) and Tifton 85 hay + 80 g of linseed oil/kg DM (HLO) incubated for 0, 6, 12 and 24 h on fatty acid composition and ruminal fermentation parameters. Real-time PCR was used to quantify microbial population at 24 h. Methanogens, fibrolitic and lipolityc bacteria were expressed as a proportion of total rumen bacterial 16 S rDNA. Separately, kinetic of gas production was assessed at 2, 4, 6, 8, 10, 12, 14, 18, 22, 24, 36, 42 and 48 h. *In vitro* true digestibility (IVTD) and CH_4 (%/g DMD) production were evaluated at 48 h. The experimental design for fatty acid composition and ruminal parameters was a randomized block in a factorial arrangement 2 x 3 x 4, involving the following factors: glycerol (2), diets (3) and time (4). Microbial quantification, IVTD and CH_4 were evaluated with the same design but without time as a factor. The pH value and ammonia (NH_3-N) concentration were lower in HWO compared with HSO and HLO diets, regardless of glycerol addition ($P<0.05$). *Anaerovibrio lipolytica* proportion increased 84, 33 and 13 times in HWO, HSO and HLO diets with glycerol, respectively, compared with diets without glycerol ($P<0.05$). *Ruminococcus albus, Ruminococcus flavefaciens* and *Butyrivrio* VA and SA subgroup did not change with glycerol and oil addition ($P>0.05$). Among all cellulolytic bacteria, *Fibrobacter succinogenes* was the most sensitive to the addition of vegetable oil in the diet ($P<0.05$). CH_4 production decreased in HSO diet associated with glycerol and HLO diets with or without glycerol addition ($P<0.05$). Lag time decreased in HWO and HSO diets associated with glycerol ($P<0.05$). The C2:C3 ratio decreased in diets with glycerol addition ($P<0.05$). Glycerol inclusion did not reduce biohydrogenation of unsaturated fatty acids *in vitro* ($P>0.05$). Our findings indicate that glycerol may replace starch as an energy source in the diets of ruminants and also decrease methane production when associated with soybean oil. Glycerol was ineffective to reduce the potentially negative effects of vegetable oils on ruminal fermentation and digestibility *in vitro*. Indeed, the proportion of *Anaerovibrio lipolytica* increased in all diets with glycerol added and unsaturated fatty acids profile remained unchanged.

Keywords: bacteria, biohydrogenation, methane, linseed oil, soybean oil

CHAPTER 1- GENERAL CONSIDERATIONS

Brazilian beef cattle production uses mainly tropical grass pasture. However, seasonal changes reduce the quality and quantity of forage, decreasing animal performance during part of year. During these periods supplementation of energy and protein can be used to increase performance, reducing age of slaughter and first age calving (REIS et al., 2009). Cereal grain (e.g. corn) supplementation has higher market prices, and new energy by-products are being tested to reduce feeding cost.

Glycerol production from the Brazilian biodiesel industry has increased with governmental investment in renewable energy. The process of biodiesel production includes a transesterification phase, which consists of a chemical reaction between animal fat or vegetable oil and an alcohol (methanol or ethanol) in the presence of a catalyst, this reaction also yields the by-product glycerol, considered an unrefined raw product (LEONETI; ARAGÃO-LEONETI; OLIVEIRA, 2012).

Biodiesel production yields about 10% (w/w) glycerol as the main by-product. The high cost of purifying and the environmental impact caused by inadequate disposal has driven its use in other areas, such as animal production. Supplementation of dairy cattle with glycerol has been conducted since the early 1950s as a glucogenic additive to prevent ketosis (JOHNSON, 1951). Currently, due high availability and favorable prices, its use is being evaluated in several livestock diets (ABO EL-NOR et al., 2010).

After ingestion glycerol quickly disappears, it can be fermented in the rumen, suffer absorption through the rumen wall or pass from the rumen to the lower gut (REMOND; SOUDAY; JOUNAY, 1993). Ruminants can ferment glycerol to propionate in the rumen and utilizes its molecule at gluconeogenesis via hepatic metabolism. Propionate and others end products from glycerol metabolism depend on the metabolic pathway used by different ruminal microorganisms (Figure 1). According to Trabue et al., (2007) disappearance of glycerol occurs via ruminal fermentation after 24 hours *in vitro*.

Microbial population oxidizes glycerol to dihydroxyacetone by glycerol dehydrogenase and then phosphorylated to dihydroxyacetone phosphate by dihydroxyacetone kinase (WANG et al., 2001). Triose phosphate isomerase in the

glycolysis directs the dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. Each mole of glycerol converted to pyruvate would generate 2 moles of NADH, which must then go through the propionic acid synthesis pathway in order to completely convert the NADH back to NAD^+ and maintain the redox balance (ZHANG; YANG, 2009).

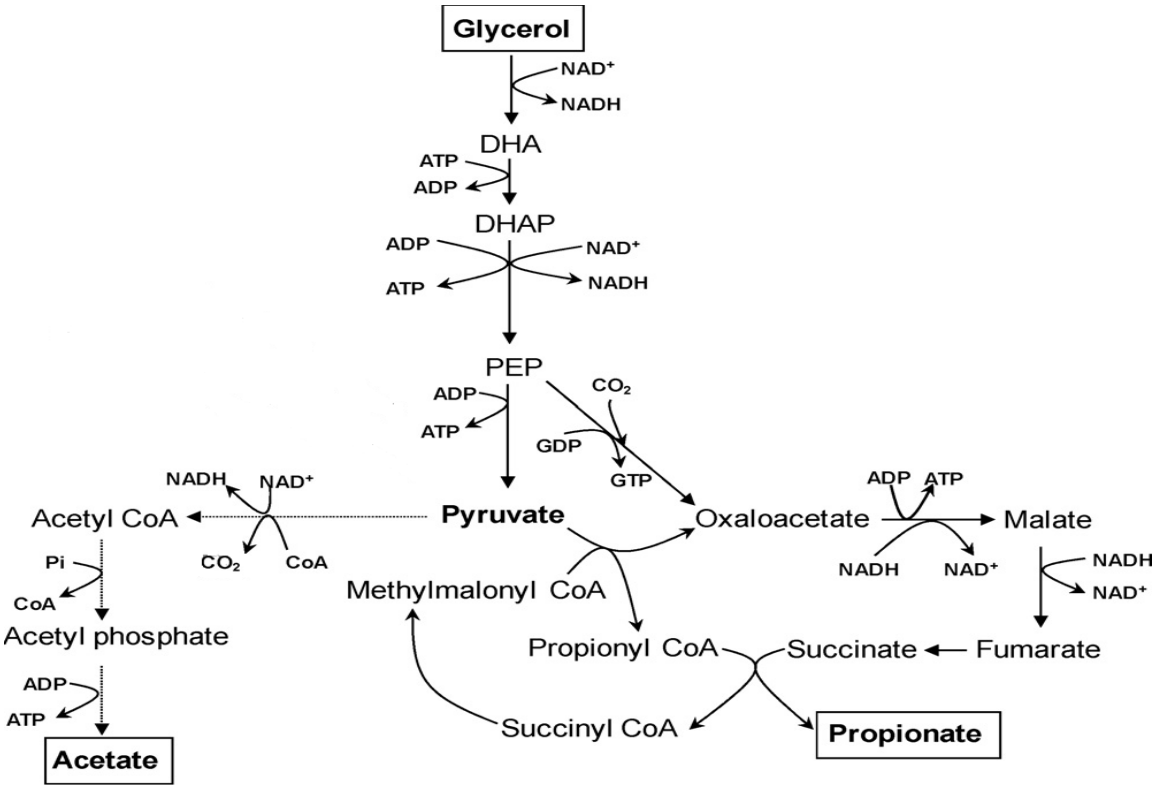

Figure 1. Some end-products of glycerol metabolism by microorganism (ZHANG; YANG, 2009).

Propiogenic properties of glycerol could provide a H_2 sink and reduce methane production. However, variable results of methane production are found in literature and these discrepancies can be attributed to feed interaction. Glycerol replacing high- starch diets cause low differences in methane emissions. Avila-Stagno et al. (2013) reported increases in methane production testing maize silage associated with glycerol *in vitro* and suggests that this increases in methane production occurred because glycerol

conversion to propionate did not allow for a net electron incorporation and causes increasing butyrate concentration resulting in high production of methane.

Supplementation of dairy cows with glycerol have demonstrated increased total volatile fatty acid concentration and lower acetate: propionate ratio (DEFRAIN et al., 2004) and no harmful problems on feed intake, milk production, or milk composition when glycerol substituted for up to 15% of ration DM (DONKIN et al., 2009). Feeding crude glycerin up to 10 % DM to beef cattle has shown a great potential to replace cereal grains as an energy source and may provide glycogenic precursors to increase marbling in meat (VERSEMANN et al., 2008; LAGE et al., 2014).

Glycerol has also been used in diets formulation with fat sources (SILVA et al., 2013), because it is believed to present the ability to partially inhibit the bacterial lipases in the rumen and possibly promote incorporation of unsaturated fats into milk and meat (EDWARDS et al., 2012).

Triglycerides, phospholipids, and galactolipids are the main types of lipids entering the rumen (JENKINS et al., 2007). Fat supplementation has been used to increase diet energy density and improve animal performance. Lipid source can influence directly or indirectly the volatile fatty acids production of ruminal fluid and composition of fatty acids leaving the rumen to be absorbed in the duodenum (HESS; MOSS; RULE, 2008). According to Doreau and Chilliard (1997) modifications on ruminal digestion are related mainly with: (a) the amount of fat; (b) the nature of fat; (c) composition of basal diet; (d) the amount of Ca and (e) lesser extent the animal.

Higher levels of lipid supplementation (more than 5% DM) disrupt fermentation and reduce digestibility of structural carbohydrates (JENKINS, 1993). Level of lipids higher than 50-60 g/kg DM can reduce intake (HESS; MOSS; RULE, 2008) while Harvatine and Allen (2006) account that up 30 g/kg DM diet can be used without relevant impact in ruminal fermentation.

These disturbance are attributed mainly to modifications in the rumen microbial ecosystem (DOREAU; CHILLIARD, 1997). Polyunsaturated fatty acids (PUFA) decreased protozoa and promote cellulolysis (DOREAU; FERLAY, 1995). The major

effect of PUFA occurs in the microbial membrane, causing metabolic disorders, mainly in cellulolytic flora (MAIA et al., 2007).

Since dietary lipids are not fermented in the rumen and they present anti-methanogenic proporperties, their use have been suggested as a strategy to reduce enteric methane emissions. Methane production from ruminants has received global attention in relation to its contribution to the greenhouse gas effect and global warming (MCALLISTER; NEWBOLD, 2008).

Rumen fermentation produces methane as a result of anaerobic digestion of feed components by microbial population (bacteria, protozoa and fungi) and results in volatile fatty acid production, mainly propionate, acetate and butyrate and production of gases (CO_2 and CH_4) (MARTIN; MORGAVI; DOREAU, 2010). During sugars oxidation via the Embden–Meyerhof–Parnas pathway, NAD^+ is reduced to NADH which has to be oxidize to NAD^+ to allow progress of fermentation, however in anaerobic conditions without presence of oxygen as a acceptor of electrons, the reduction of CO_2 to CH_4 by methanogens become the major H_2 sink (MCALLISTER; NEWBOLD, 2008). Methanogens are classified to the domain *Archae* and the phylum Euryarcheota, these *Archaes* have symbiotic relationships with hydrogen transfers microorganism, specially protozoa (HOOK; WRIGHT; BRIAN, 2010).

Many nutritional strategies have been suggested in livestock production to mitigate CH_4 emissions, such as ruminal defaunation, the use of ionophores, immunization, and the use of diets containing inorganic acids, lipids, tannins, and saponins (COTTLE; NOLAN; WIEDEMANN, 2011). Lipids are a promising alternative to reduce enteric methane emissions, since they have demonstrated reducing 5.6 % of methane production per percentage unit of lipid add to the diet (BEAUCHEMIN et al., 2008).

Lipid supplementation has also been utilized to improve meat quality (SCOLLAN et al., 2006). Ruminant products have been criticized for the higher content of saturated fatty acids (SFA) and negative effects on human health. SFA increase the concentration of serum low-density lipoprotein, a long used predictor of cardiovascular disease and coronary heart disease in specific (GIVENS, 2010).

Modifying the fatty acid profile of ruminant-derived foods provide the chance to adjust the consumption of fatty acids in human populations with public health policies without significant changes in eating habits (SHINGFIELD; BONNET; SCOLLAN, 2012). Dietary recommendation have promoted research into the modifications of animal diets to increase content of beneficial polyunsaturated fatty acids (PUFA), in specific PUFA of the omega-3 series (18:3 n-3, linolenic acid; eicosapentaenoic acid (EPA); 22:6n-3, docosahexaenoic acid (DHA) and conjugated linoleic acid (CLA) in meat products (MOLONEY et al., 2012) .

Milk and dairy products from ruminants have been manipulate using either whole seeds or extracted seed oils. Soybean and linseed are rich in linoleic acid (18:2 n-6) and linolenic acid (18:3 n-3) and supplementation of them, mostly result in increased level of these fatty acids in animal tissue (DEWHURST et al., 2003).

The major challenges to modify the polyunsaturated fatty acids composition of meat and milk are the intense process of lipolysis and biohydrogenation carried by ruminal microorganisms. *Anaerovibrio lipolytica* is a gram positive bacteria that realize hydrolyses of triacyglycerides releasing glycerol and ester of fatty acids in the rumen. This bacteria metabolize glycerol via dicarboxilic acid pathway producing propionate and succinate (PRINS et al., 1975).

Hydrolyses of esterified fatty acid is a critical process on biohydrogenation of unsaturated fatty acids. The *Butirivibrio fibrosolvens* only starts hydrogenation on free carboxyl fatty acids groups (LOURENÇO; MORALES; WALLACE, 2010), as a consequence, rates of biohydrogenation are always less than those of hydrolysis, and factors that affect hydrolysis also impact biohydrogenation (BAUMAN; LOCK, 2006).

During biohydrogenation of linoleic acid (18:2, n-6) and linolenic acid (18:3, n-3) several intermediate compounds are produced until reduction to stearic acid (18:0), Figure 2. Hydrogenation of double bonds is energetically costly, then unsaturated fatty acid isomers with *trans* double bonds are formed to reduce energy activation and favors redutases enzymes function (JENKINS; KLEIN; LEE, 2009).

Rumen bacteria involved in biohydrogenation were classified in previous studies in group A and B (HARFOOT; HAZLEWOOD, 1997). Group A bacteria carry hydrogenation of linoleic and linolenic acid to vaccenic acid and group B bacteria are able to hydrogenate a broad octadecenoic acid to stearic acid. Currently, according to Lourenço, Ramos-Morales and Wallace (2010) with technological advances in molecular biology, the classification of bacteria responsible by biohydrogenation can be made by correct taxonomy instead of divide in group A and B. The bacterial classification mainly consider sensitivity to unsaturated fatty acids and mechanism by which bacteria form butyrate (WALLACE et al., 2006; PAILLARD et al., 2007).

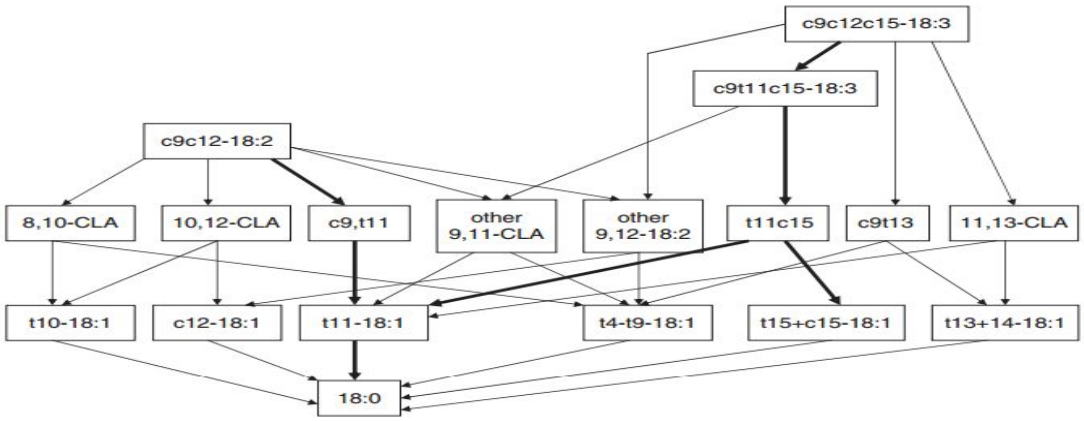

Figure 2. Biohydrogenation pathways of linoleic acid (18:2 n-6, Cis-9 Cis-12) and linolenic acid (18:3 n-3, Cis-9 Cis-12 Cis-15). Adapted from CHILLIARD et al. (2007).

Conjugated linoleic acid (CLA) Cis-9 trans-11 is the first intermediate formed in linoleic acid biohydrogenation and has showed potential benefits for human health, such as anticarcinogenic properties (SONG; KENELLY, 2003). The reduction in the biohydrogenation of linoleic acid can allow greater escape of CLA for absorption in the duodenum or limit hydrogenation of vaccenic acid to stearic. The vaccenic acid can be converted to CLA in the tissues via endogenous desaturation by Δ^9-desaturase (BAUMAN et al., 2000).

Several nutritional strategies have being used to limit extent of polyunsaturated fatty acid biohydrogenation and reduced the negative effects of lipids on microbial population (JENKINS, 1993). According to Fievez et al. (2007), the three main technological process of lipid protection can be categorized as follow: (a) chemical protection (e.g. encapsulation in a protein matrix followed by aldehyde treatment, (b) formation of calcium salts and amides of fatty acids (c) technological treatments of oil seeds, such as extrusion, roasting, cracking. However, the high cost of these sources can limit its utilization in the animal production in developing countries. In this manner, glycerol could be used as an inexpensive protect fat due its inhibition of ruminal lipolysis and reduce the intense biohydrogenation of unsaturated fatty acids.

There are several researches evaluating glycerol effects *in vitro* (FERRARO et al., 2009; LEE et al., 2011; ABO EL-NOR et al., 2010) and on animal performance (MACH; BACH; DEVANT, 2009; LAGE et al., 2014).To our knowledge, this is the first report describing the fatty acid composition and microbial changes when glycerol is combined with vegetable oils.

We hypothesize that association of glycerol with vegetable oils could limit the intense biohydrogenation of polyunsaturated fatty acid and enhance the reduction in methane production without depressing digestibility and microbial population.

Our objective was to evaluate *in vitro* the effect of glycerol (0 or 150 g/kg DM) associated with three different diets on fermentative parameters, microbial changes and fatty acid composition.

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CHAPTER 2 – *IN VITRO* PARAMETERS FERMENTATION AND BIOHYDROGENATION OF VEGETABLE OILS WITH OR WITHOUT GLYCEROL

1. INTRODUCTION

Glycerol production, a by-product of the biodiesel industry, has increased with increasing investments in renewable energy sources both on developed and developing countries. Currently, its use is under evaluation in several livestock diets as an energy source to replace cereal grain (e.g. corn) due to its high availability and disposal costs (ABO EL-NOR et al., 2010). Indeed, ruminants can ferment glycerol to propionate in the rumen and utilise its molecule at gluconeogenesis via hepatic metabolism.

The effect of glycerol on ruminal fermentation parameters is not, however, fully established. On the one hand, supplementation of dairy cows with glycerol has been shown to increase total volatile fatty acid concentration and reduce acetate:propionate ratio (DEFRAIN et al., 2004). In contrast, no modifications on feed intake and fermentative parameters of beef cattle fed with glycerol at levels up to 120 g/kg of concentrate DM were found (MACH; BACH; DEVANT, 2009). The propionic properties of glycerol could provide an H_2 sink and reduce methane production. However, variable effects of glycerol on methane production have been reported, which might be attributed to feed interactions (AVILA-STAGNO et al., 2013).

Previous research has also suggested that the association of glycerol with fat sources in the diet can partially inhibit bacterial lipases in the rumen and limit the intense biohydrogenation of polyunsaturated fatty acids (EDWARDS et al., 2012). This finding is in line with dietary recommendations for modifications of animal diets that promote increases in the content of beneficial polyunsaturated fatty acids (PUFA) and conjugated linoleic acid (CLA) in meat products (MOLONEY et al., 2012). Accordingly, supplementation with soybean and linseed oil, both rich in linoleic acid (18:2 n6) and

linolenic acid (18:3 n3), have been shown to result in increased levels of these fatty acids in animal tissue (DEWHURST, 2003).

Despite these potential benefits, these vegetable oils release a high quantity of unsaturated fatty acid in the rumen that can be harmful to microbial membranes and cause metabolic disorders, mainly in cellulolytic flora (MAIA et. al, 2007). In contrast, their use has been suggested as a strategy to reduce enteric methane emissions, as dietary lipids are not fermented in the rumen and present anti-methanogenic properties. Accordingly, lipid addition has been demonstrated to reduce 5.6 % of methane production per percentage unit of lipid added to the diet (BEAUCHEMIN et al., 2008).

Several studies have evaluated the effect of glycerol *in vitro* (FERRARO et al., 2009; ABO EL-NOR et al., 2010; LEE et al., 2011) and on animal performance (MACH et al., 2009; AVILA-STAGNO et al., 2013; LAGE et al., 2014). To our knowledge, however, there has been no study to date that has described the effects of glycerol on fatty acid composition, ruminal fermentation parameters and microbial changes when combined with vegetable oils.

We hypothesise that the association of glycerol with vegetable oils could limit the intense biohydrogenation of polyunsaturated fatty acid and enhance the reduction in methane production without depressing digestibility and microbial population. Our objective was therefore to evaluate *in vitro* the effect of glycerol (0 or 150 g/kg DM) associated with soybean and linseed oil on these parameters.

2. MATERIAL AND METHODS

2.1. Diets and incubation procedures

Three *in vitro* ruminal incubations were conducted to evaluate the effect of glycerol (0 or 150 g/kg) associated with three different diets: Tifton hay 85 without oil seed (HWO), Tifton hay 85 plus 80 g/kg DM of soybean oil (HSO) and Tifton hay 85 plus 80 g/kg DM of linseed oil (HLO) on fermentative parameters and fatty acid composition.

For each incubation, an individual fistulated Nellore steer fed *Brachiaria brizantha* cv. Xaraes was used as inoculum donor Samples (± 1 g) with different proportions of the ingredients (Table 1) were weighed into 160 mL flasks.

Table 1- Ingredients and nutrient composition of experimental diets

	Diets[1]					
	HWO		HSO		HLO	
	0	150	0	150	0	150
Ingredients, g/kg DM						
Tifton 85 hay	850.0	850.0	770.0	770.0	770.0	770.0
Soybean oil	0.0	0.0	80.0	80.0	0.0	0.0
Linseed oil	0.0	0.0	0.0	0.0	80.0	80.0
Glycerol[2]	0.0	150.0	0.0	150.0	0.0	150.0
Starch	150.0	0.0	150.0	0.0	150.0	0.0
Urea	0.0	0.0	3.0	3.0	3.0	3.0
Chemical composition, g/kg DM						
Dry matter	92.7	92.7	92.5	92.5	92.5	92.5
Crude protein	97.0	97.0	96.0	96.0	96.0	96.0
Acid detergent fiber	288.7	288.7	261.9	261.9	261.9	261.9
Neutral detergent fiber	610.2	610.2	520.5	520.5	520.5	520.5
Ether extract	13.5	13.5	92.2	92.2	92.2	92.2
Ash	57.3	57.4	52.0	52.0	52.0	52.0
Non fibrous carbohydrates	220.2	220.2	209.5	209.5	209.5	209.5

[1] HWO=Tifton 85 hay without oil seed with 0 or 150 g/kg DM of Glycerol; HSO=Tifton 85 hay + soybean oil (80g/kg DM) with 0 or 150 g/kg DM of Glycerol; HLO=Tifton 85 hay + linseed oil (80g/kg DM) with 0 or 150 g/kg DM of Glycerol.[2] Glycerol=99.5 % pure.

Flasks were weighed in triplicate and divided into two portions: the first one was exclusively for quantification of long chain fatty acids composition and the second for quantification of ruminal fermentation parameters (VFA, NH_3-N, pH) and microorganism.

2.2. Sample collections

The ruminal fluid collection was conducted at 7:00h in all runs, strained through a double layer of cheese cloth under continuous CO_2 injection and mixed with the buffer solution (THEODOROU et al., 1994). The ratio of buffer/ruminal fluid was 8:2 (GOERING; VAN SOEST, 1970).

Buffered solution (100 mL) was transferred to three flasks (160 mL) for each diet combined with glycerol and incubation time (0, 6, 12 and 24h). The flasks were sealed and incubated anaerobically in a water bath at 39°C. Every 6 hours, agitation and release of gas present inside the bottles was performed. For each incubation time, the bottles were removed from the incubator and the pH of culture fluid was measured. The bottles were immersed in ice water to inhibit microbial activity and immediately sampled for subsequent analysis.

Aliquots of 10mL were collected for determination of ammonia (NH_3-N) and volatile fatty acid (VFA) and subsequently stored at -20°C. To each NH_3-N samples was added 0.2mL of 50% (v/v) sulphuric acid and the samples were analyzed calorimetrically with a commercial kit (Bioclin/Quibasa kits). The VFA concentration was determined according to the methodology described by PALMQUIST AND CONRAD (1971).

2.3 Feed composition analysis

Tifton 85 hay was analysed to determine dry matter (DM; 934.01), mineral matter (MM; 942.05) and ether extract (EE; 920.39) according to AOAC (1995). Nitrogen was determined using an LECO FP-528 nitrogen analyser (LECO Corp., St. Joseph, MI, USA).

Neutral detergent fibre was determined using *α*-amylase and without the addition of sodium sulphite and of the according to Van Soest; Robertson and Lewis (1991), adapted for Ankom^{200} Fiber Analyzer (Ankom Technology, Fairport, NY). Acid detergent fibre was determined using the method described by Goering and Van Soest (1970), adapted for Ankom^{200} Fiber Analyzer (Ankom Technology, Fairport, NY).

2.4. Fatty acid quantification

Freeze dried samples were extracted following the protocol described by Shingfield et al. (2003). For this,100 mg of sample was mixed with 0.5mL of water and 1 mL of internal standard (2mg $_{C19:0}$\mL) dissolved in ethanol and acidified with 2-M HCl to pH 2. Then, the lipids were extracted twice with 4mL of a solution with 3:2 hexane to isopropanol (v/v). Hexane soluble extracts were combined, washed with Millipore water and dried with sodium sulphate.

After evaporation of the hexane stream of nitrogen, the lipid fraction was methylated with a basic solution of sodium methoxide following Christie (1982), with the adaptations of Chouinard, Girard and Brisson (1998). The profile of fatty acids was determined by gas chromatography using a capillary column of fused silica 200m (Varian CP -2571), hydrogen as a carrier gas (1.8 mL/ min) , flame ionisation detector (FID), temperature injector and detector 250°C and 300°C, respectively, and reason for injection of samples of 20:1. The protocol to run fatty acids started with 100°C and maintained for seven minutes, then raised 10°C/min to 210°C and maintained for another 10 minutes and finally a new increase of 5 °C/min to 240°C and maintained for five more minutes.

The identification of fatty acids was made by comparison of their retention times with those observed in commercial standards (C4-C22), as Supelco 18919-1AMP-, methyl ester mixture of 37 fatty acids, butter and a standard (CRM 164-the Commission of the European Community Bureau of Reference, Brussels, Belgium). The major fatty acids were identified by pure commercial standards: 05632-SIGMA, methyl ester mixture of the conjugated linoleic acid cis -9, trans-11 and trans-10, cis-12; V1381-SIGMA, methyl

ester vaccenic acid, SigmaD2656, methyl ester of docosahexaenoic acid cis-4, 7, 10, 13,16,19 (DHA); SIGMAE2012-, methyl ester of eicosapentaenoic acid cis-5, 8, 11, 14,17 (EPA).

2.5 DNA extraction and quantification

Samples (70 mL) collected at 24h were immediately treated with PBS buffer (1% Tween, pH 7.4). The buffered solution was centrifuged at 16.000x *g* for 10 minutes at a temperature of 10°C forming bacterial pellets. Pellets were resuspended in 10:1 TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), centrifuged at 16.000x*g* for 10 minutes, the supernatant discarded and the bacterial pellet frozen at -20°C until DNA extraction.

DNA extraction was performed using “Fast spin kit for soil” from MP Bio® according to the manufacturer´s instructions and quantified using NanoDrop instrument (Thermo Scientific, Wilmington, MA), based on UV spectroscopy. Wavelengths absorbance ratio of A260/280 and A260/230 were used to measure DNA purity. Samples with ratio between 1.8 and 2.0 were utilized in qPCR reactions.

2.5.1 Real-time PCR (qPCR)

Real-time PCR was performed with Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). Rox was used as a passive reference dye.

Four concentrations (200, 400, 600 and 800 nM) of forward and reverse primers were tested to determine minimum primer concentration giving the lowest threshold cycle (C_t) and to reduce nonspecific amplification before start the reaction. The primer sets used for qPCR are described in Table 2.

Table 2. Target primers used in the relative quantification of cellulolytic, lipolityc bacteria and methanogenic by q-PCR

Target bacterium	Forward primer	Reverse primer	Bp[1]
General bacteria[2]	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	130
F. succinogenes[2]	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121
R. flavefaciens[2]	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	132
R.albus[3]	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA	175
Anaerovibrio lipolityca[4]	TTGGGTGTTAGAAATGGATTCTAGTG	TCGAAATGT TGTCCCCAT CTG	82
Butyrivibryo SA subgroup[5]	TGAAAAACTCCGGTGGTATGAGAT	CCGTGTCTCAGTCCCAATGTG	126
Butyrivibryo VA subgroup[5]	TGCATTGGAAACTGTAGAACTAGAGTGT	GCGTCAGTAATCGTCCAGTAAGC	124
Methanogens[6]	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	140

[1]Amplicon size in base pairs; [2]DENMAN;MCSWEENEY (2006); [3]MOSONI et al. (2007); [4]FUENTES et al.(2009);[6] POTU (2011); [6]DENMAN;TOMKINS; MCSWEENEY (2007)

Conditions of PCR were 50°C for 2 min; 95°C for 10 min; 35 cycles of 95°C for 15 s; and 60°C for 1 min. Each conventional PCR mixture (25µL) contained (final concentrations) 1 × Power SYBR Green PCR Master Mix (Applied Biosystems), 400 or 600 nM of each primer, and 150 ng of metagenomic DNA.

Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplicon specificity was performed via dissociation curve analysis of PCR end products.

Relative quantification was used to determine species proportion. The results were expressed as a 16S rDNA ratio of general bacteria, following the equation:

Relative quantification = $2^{-(Ct\ target - Ct\ total\ bacteria)}$;

Where Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold.

2.6 Gas production and feed degradability

Three runs were conducted separately in the same period of the parameters trial, however on different days to evaluate the kinetics of gas production *in vitro* (THEODOROU et al., 1994) adapted to the semi-automatic system (MAURICIO et al., 2004).

The procedures used were similar those used to test fermentative parameters, except that the pressure caused in the bottles was measured at 2, 4, 6, 8, 10, 12, 14, 18, 22, 24, 36, 42 and 48 h after incubation using a pressure transducer. The pressure values were converted to volume of gas using equation previously determined for laboratory conditions:

$V = (4.25 \times P) - 0.1$

Where: V= gas volume (mL); *P*= measured pressure (psi).

At 48 h post inoculation 5 mL of gas was collected in syringes and immediately injected into the chromatograph for methane quantification. After that, bottles were immersed in ice water and filtered through pre-weight Gooch crucibles porosity 2 (50um). Next 50 mL of neutral detergent solution was added and autoclaved at temperature of 110 ˚C for 40 min (SENGER et al., 2007). Fibre residue was filtered and soaked in hot water (90–100˚C), oven-dried at 105 ˚C during 24 h and weighed. The *in vitro* true digestibility (IVTD) was calculated as follow:

$$IVTD = \frac{(DM\ incubated - NDF\ residue\ corrected\ for\ blank)\ X100}{DM\ feed} =$$

Where IVTD= *In vitro* true digestibility,(%)

2.7 Statistical analysis

The ruminal fermentation parameters and fatty acid profile during 24h were analysed as a completely randomised block design using the MIXED procedure of SAS (version 9.2). The model included fixed effect of glycerol (1degree of freedom, DF), oil (2DF), time (3DF) and all interactions (6DF). Random effect was block (2DF) and residual error. When significant, the means between treatments were compared using the Fisher's least significant difference (e.g, the option pdiff command LSMEANS). Significance was declared P ≤0.05. Orthogonal contrasts were used to assess linear and quadratic relationships between time and the response variable.

Microbial quantification, IVTD and CH_4 were evaluated in a randomised complete block design. In the model the variety levels of glycerol (1DF) and the vegetable oil (2 DF) were evaluated as a fixed effect . Random effect was block (2DF) and residual error kinetics of gas production was performed using the discrete exponential equation (Schofield et al., 1994). Curves were fitted for each treatment using PROC NLIM of SAS (SAS inst. Inc., Cary, NC).

3. RESULTS

The pH value and NH_3-N concentration were lower in HWO compared with HSO and HLO diets irrespective glycerol addition (P<0.05; Figure 3).

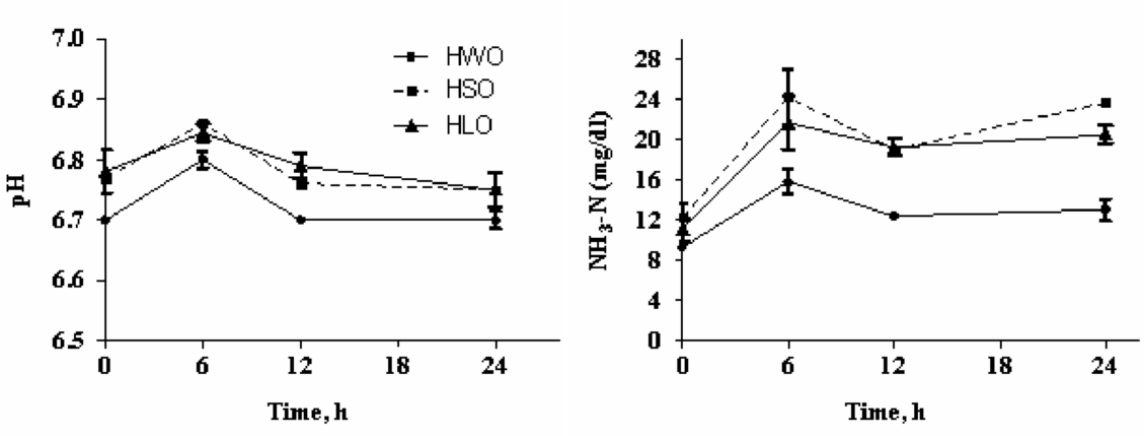

Figure 3. NH_3-N concentration (mg/dL) and pH values in Tifton 85 hay diets without oil (HWO), plus soybean oil (HSO) or plus linseed oil (HLO) evaluated *in vitro*. Error bars represent standard errors. Replacement of starch by glycerol in the diet did not affect either parameter (P>0.05).

Glycerol addition (150g/kg DM) changed the VFA profile at 0, 6, 12 and 24 h (Table 3; P<0.05). The effect of glycerol on VFA depended significantly on time (P<0.05). Production of acetate, propionate and total VFA increased linearly with glycerol addition over the course of 24 hours, regardless of diet (P<0.05). Propionate production increased twice in diets with glycerol compared with diets without glycerol at 24 h (P<0.05). Specifically, propionate production increased doubled in diets with glycerol compared with diets without glycerol at 24 h (P<0.05). Accordingly, C2:C3 ratio decreased in diets with glycerol addition (P<0.05). Butyrate concentration did not differ at 24 h with glycerol addition in all diets (P>0.05).

Table 3. Effect of glycerol addition (0 or 150 g/kg DM) in diets without oil (HWO), soybean oil (HSO) and linseed oil (HLO) on volatile fatty acids (VFA) composition at 0, 6, 12 and 24 hours

	Diets[1]						SEM	P-value[2]			
	HWO		HSO		HLO			Main Effect			I
	0	150	0	150	0	150		G	O	T	
VFA, mM											
Acetate											
0	16.5	17.7	17.6	17.50	17.50	17.70					
6	20.2	20.6	23.3	19.80	19.80	17.90	0.96	0.04	0.68	<0.01	G vs T
12	25.1	19.5	20.6	17.50	17.50	21.60					
24	29.0	26.1	27.0	25.60	25.70	23.70					
Contrast[3]	L	L	L	L	L	L					
Propionate											
0	2.7	3.70	3.60	3.60	3.44	2,90					
6	5.5	5.70	6.30	5.70	5.70	5.10	0.64	<0.01	0.97	<0.01	G vs T
12	7.0	9.70	5.60	9.40	6.50	11.30					
24	10.7	20.10	9.50	21.60	10.30	21.00					
Contrast	L	L	L	L	L	L					
Butyrate											
0	2.31	2.44	2.53	2.49	2.47	2.19					
6	3.57	3.87	3.99	3.44	3.43	3.67	0.59	0.194	0.015	<0.01	G vs T
12	6.01	5.99	4.61	4.38	4.83	5.04					
24	8.37	7.58	7.77	5.79	7.36	5.33					
Contrast	L	L	L	L	L	L					
Valerate											
0	0.15	0.16	0.96	0.18	0.61	0.16					
6	0.27	0.27	0.78	0.24	0.26	0.29	0.09	0.015	0.015	<0.01	G vs T
12	0.74	0.60	0.47	0.42	0.37	0.46					
24	0.58	0.80	0.59	0.64	0.52	0.51					
Contrast	Q	Q	Q	Q	Q	Q					
C2:C3[4]											
0	5.84	4.81	4.87	4.84	5.17	4.81					
6	3.64	3.62	3.7	3.46	3.77	3.46	0.07	<0.01	0.420	<0.01	G vs T
12	3.81	2.70	3.85	2.6	3.17	2.23					
24	2.74	1.30	2.84	1.19	2.72	1.17					
Contrast	L, Q	L, Q	L, Q	L, Q	L, Q	L, Q					
Total											
0	22.50	24.40	24.10	25.70	24.70	22.10					
6	30.00	31.10	34.90	29.60	31.80	27.50	3.13	0.045	0.56	<0.01	G vs T
12	40.50	37.00	32.60	33.00	33.60	40.30					
24	49.50	55.20	45.80	54.20	46.70	52.30					
Contrast	L	L	L	L	L	L					

[1] HWO=Tifton 85 hay without oil seed with (0g/kg or 150 g/kg DM of Glycerol; HSO=Tifton 85 hay + soybean oil (80g/kg DM) with 0 or 150 g/kg DM of Glycerol; HLO=Tifton 85 hay + linseed oil (80g/kg DM) with 0 or 150 g/kg DM of Glycerol); [2] Fixed effect of glycerol (G), Oil (O), Time (T),and I=Interaction, Glycerol vs Time interaction (G x T); NS=not significant (P>0.05); [3]Contrast= L or Q (P<0.05); [4]C2:C3= acetate:propionate ratio ;There is no oil effect, P>0.05)

Total gas production was significantly affected by the addition of glycerol to those diets combined with vegetable oils. Specifically, it decreased by 12 and 20 % in the HSO and HLO diets with glycerol addition (Table 4; P<0.05). However, it remained unchanged in HWO diets (P>0.05). Lag time decreased with glycerol addition in HWO (1.76 times) and HSO (1.15 times) diets (P<0.05), but was not affected in HLO diets (P>0.05).

Glycerol addition did not cause any significant change on IVTD (P>0.05). IVTD was higher in HWO compared with HSO and HLO diets (P<0.05). Methane concentration (%/g DMD) was significantly lower in the diet combined with linseed oil, even in the absence of glycerol. Accordingly, the addition of glycerol reduced methane concentration only for the HSO diet (P<0.05; Table 4).

Table 4. Effect of glycerol addition (0 or 150 g/Kg DM) in diets without oil (HWO), soybean oil (HSO) and linseed oil (HLO) on kinetics of gas production, *in vitro* true digestibility (IVTD) and methane concentration at 48 h.

	Diets[1]						SEM	P-value[2]		Interaction
	HWO		HSO		HLO			Main Effect		
	0	150	0	150	0	150		Glycerol	Oil	
Kinetic										
a[3]	169.58	162.82	131.8	116.20	122.01	97.36	3.34	<0.010	<0.010	G *vs* O
b[4]	6.62	3.76	10.51	9.11	12.50	12.52	0.37	<0.010	<0.010	G *vs* O
IVTD (%)[5]	66.2	68.4	61.00	63.00	62.80	63.00	1.05	0.260	0.011	NS
CH4 (%) / g DM[6]	10.13	10.02	8.42	7.78	7.57	7.77	0.37	0.097	0.879	G *vs* O
CH4 (%) / g DMD[7]	16.79	16.36	16.23	13.20	12.70	13.18	0.73	0.076	<0.010	G *vs* O

[1]HWO=Tifton 85 hay without oil seed with (0g/kg DM or 150 g/kg DM of Glycerol); HSO=Tifton 85 hay + soybean oil (80g/kg DM) with 0 g/kgDM or 150 g/kg DM of Glycerol; HLO=Tifton 85 hay + linseed oil (80 g/kg DM) with 0g/kg DM or 150 g/kg DM of Glycerol; [2] Fixed effect of glycerol (G), Oil (O) and Interaction = Glycerol vs Oil interaction (G vs O) (P<0.05); NS=Not significant (P>0.05);[3]*a*=Maximum volume, mL;[4]*b=lag time,* hours; [5]IVTD= *in vitro* true digestibility (%); [6]CH_4 (%)/g DM=CH_4 / g dry matter incubated;[7]CH_4 (%)/g DMD=CH_4 / g dry matter disappeared.

Glycerol did not affect the profile of long chain fatty acid when combined with HWO, HSO or HLO diets at 0, 6, 12 and 24 h (P>0.05; Figure 4). Linoleic acid concentration (18:2 n-6) was higher in HSO diets compared with the other two diets (P<0.05; Fig.4a). Specifically, it was 39% higher than HLO diets at 24 h (P<0.05). Linolenic acid (18:3 n-3) was higher in HLO than in HWO and HSO diets (P<0.05; Fig.2b). Cis-9 trans-11 conjugated linoleic acid (CLA) concentration was lower in HWO

than HSO and HLO diets ($P<0.05$) and did not change with time ($P>0.05$). CLA production was 56 % higher in HSO diets compared with HLO at 24 h ($P<0.05$; Fig.4c). Total saturated fatty acid (SFA; Fig.4g) was 53% higher in HWO diets compared with HSO and HLO diets at 24 h ($P<0.05$).

C18:1 isomer (*t*10, *t*11, *t*12) concentration was similar for HSO and HLO diets at 24 h ($P>0.05$), yet in both cases higher than in HWO diets ($P<0.05$; Fig.4e). Total monounsaturated fatty acid (MUFA) and total unsaturated fatty acid (UFA) were, as expected, lower for the HWO diet. While MUFA was higher in the HLO than HWO and HSO diets at 24 h ($P<0.05$; Fig.4d), PUFA was higher for both the HSO and HLO compared with the HWO diet ($P<0.05$; Fig.4f).

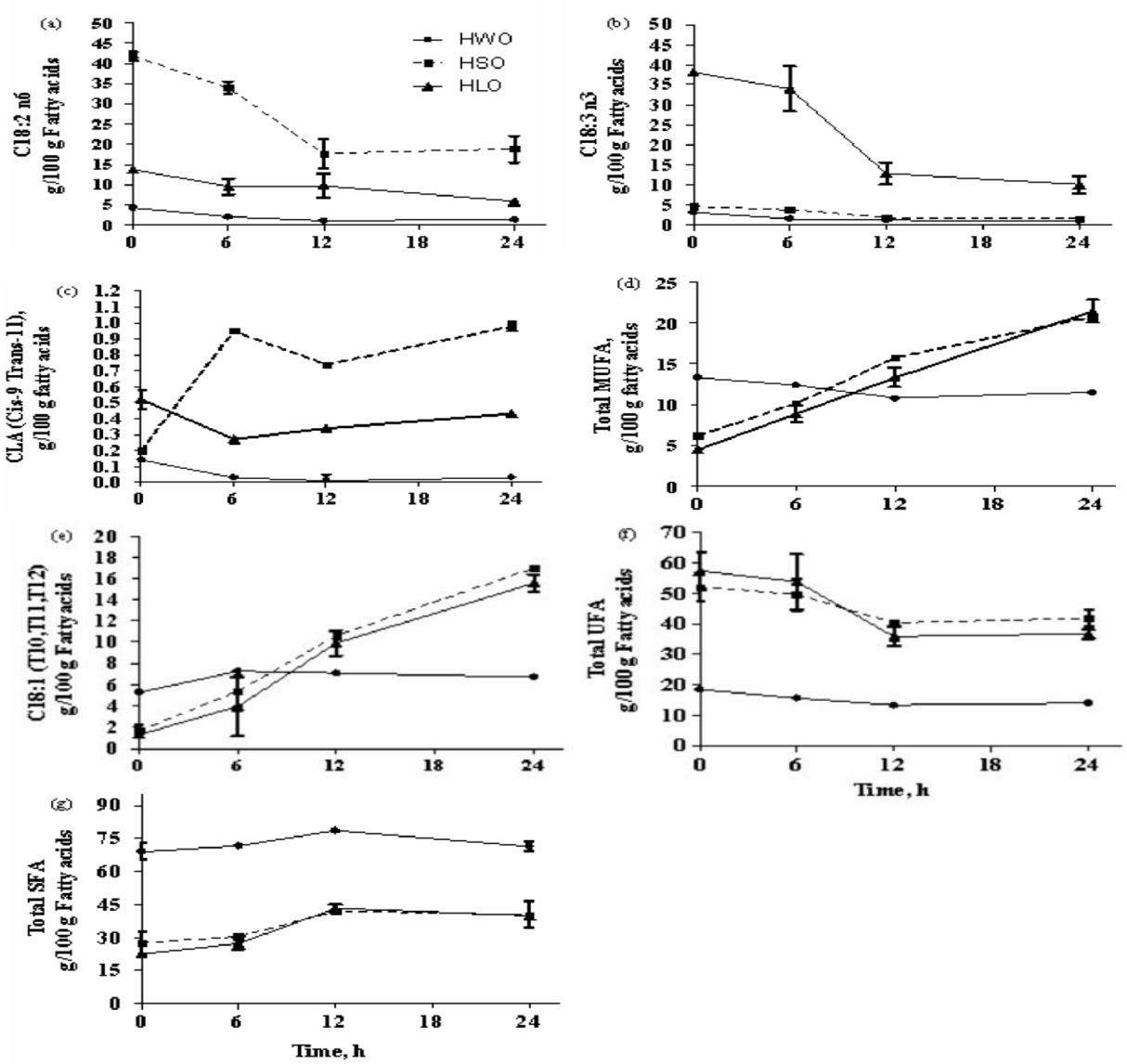

Figure 4. Fatty acid composition of rumen fluid incubated *in vitro* with HWO = Tifton 85 hay without oil seed; HSO = Tifton 85 hay + soybean oil (80 g/kg DM); HLO = Tifton 85 hay + linseed oil (80 g/kg DM); (A) Linoleic acid (18:2 n-6), SEM = 2.2; (B) Linolenic acid (18:3 n-3), SEM = 0.79; (C) CLA = Conjugated linoleic acid, SEM = 0.08; (D) MUFA= Total monounsaturated fatty acid (18:1C9, C16:1C9, C18:1C9, C18:1, T6, T7, T'8, T9 + C18:1T10 + T11+ T12, SEM = 0.54; (E) C18:1 (T10, T11, T12), SEM = 0.68; (F) UFA = total unsaturated fatty acid (C18:3 n3 + C18:2 n6 + C18:2 C9 T11 + MUFA), SEM = 1.52; (G) FA=Total saturated fatty acid (C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0), SEM = 0.72; Error bars represent standard errors. (Interaction = oil *vs* time; $P<0.05$). There was no effect of glycerol ($P>0.05$).

Anaerovibrio lipolytica proportion increased 84 , 33 and 13 times in HWO, HSO and HLO diets with glycerol, respectively, compared with diets without glycerol (P<0.05; Table 5). The proportion of *Anaerovibrio lipolytica* was lower in HLO than HWO and HSO diets when these diets were combined glycerol (P<0.05). *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivrio* VA and SA subgroup did not change with either glycerol or oil addition (P>0.05). The proportion of *Fibrobacter Succinogenes* and Methanogens decreased, respectively, by 70% and 90% in those diets combined with either vegetable oil compared with HWO (P<0.05).

Table 5. Effect of glycerol addition (0 or 150 g/Kg DM) in diets without oil (HWO), soybean oil (HSO) and linseed oil (HLO) on relative proportion of *Ruminococcus .albus, Fibrobacter succinogenes*, *Ruminococcus Flavefaciens,* methanogens, *Anaerovibrio lipolytica and Butyrivibrio fibrisolvens VA and SA subgroup* at 48h.

Target bacterium	Diets[1]						SEM	P-value[2]		Interaction
	HWO		HSO		HLO			Main Effect		
	0	150	0	150	0	150		Glycerol	Oil	
Anaerovibrio lipolytica	0.021	1.76	0.05	1.81	0.06	0.87	0.040	<0.010	<0.010	G *vs* O
Butyrivibrio VA subgroup	0.85	0.83	0.74	2.15	0.90	0.87	0.001	0.388	0.879	NS
Butyrivibrio SA subgroup	2.03	1.98	1.96	1.61	1.90	1.50	0.246	0.140	0.688	NS
Fibrobacter succinogenes	5.45	5.52	1.72	2.15	0.62	2.13	0.090	0.278	<0.010	NS
Ruminococcus albus	0.21	0.20	0.25	0.13	0.11	0.14	0.15	0.290	0.163	NS
Ruminococcus flavefaciens	0.02	0.02	0.02	0.03	0.05	0.01	0.006	0.140	0.688	NS
Methanogens	0.14	0.16	0.04	0.05	0.02	0.01	0.023	0.317	0.019	G *vs* O

[1]HWO=Tifton 85 hay without oil seed with 0 or 150 g/kg DM of Glycerol; HSO=Tifton 85 hay + soybean oil (80g/kg DM) with 0or 150 g/kg DM of Glycerol; HLO=Tifton 85 hay + linseed oil (80g/kg DM) with 0or 150 g/kg DM of Glycerol.

[2] Fixed effect of glycerol (G), Oil (O) and *Interaction*=Glycerol X Oil interaction (G vs O); NS=Not significant (P>0.05)

4. DISCUSSION

We hypothesised that the association of glycerol with vegetable oils could limit the intense biohydrogenation of polyunsaturated fatty acids and enhance the reduction of methane production, without depressing digestibility and microbial population. However, our results did not support this hypothesis.

The observed reduction of total gas production and IVTD in the HSO and HLO diets was likely due to the negative effect of double bonds in the unsaturated fatty acids (UFA) present in these diets. UFA can be harmful to microbial membrane and cause metabolic disorders, mainly in cellulolytic flora (MAIA et al., 2007). Although the proportion of cellulolitc bacteria *R. albus and R. flavefaciens* did not change in the HSO and HLO diets, there was a reduction in the proportion of *Fibrobacter succinogens,* the major fibre digesting species in the rumen (JUN et al., 2007), as observed elsewhere (RAMESH et al., 2011; PATRA; YU, 2013). This may have induced the observed IVTD reduction in these diets by decreasing fibre digestion.

Lag time has been shown to increase when pure glycerol is added *in vitro* with rumen fluid (LEE et al., 2011). However, a reduction in lag time was observed in HWO and HSO diets associated with glycerol, which can be explained by an interaction of glycerol with these diets. Tifton 85 hay can form an interface between bacteria adhering to fibre particle, increasing glycerol degradation. This can also explain the higher proportion of *Anaerovibrio lipolytica* when glycerol was added to HWO diets, which may have resulted from this increase in the surface available for microbial growth. In our experiment lag time was 3.76 h in the HWO diet associated with glycerol, similarly to that observed by Lee et al. (2011), who found a reduced lag time (3.4 h) in diets with alfalfa hay combined with glycerol.

In the diets containing vegetable oils rumen fermentation parameters were altered towards increased NH_3-N concentration. This higher concentration of NH_3-N can be explained by decreasing protozoa counts that result in the proliferation of proteolytic bacteria, hence intense protein degradation with NH_3-N release (YANG, 2009). There were no changes, however, in *Butyribrio* VA and SA subgroup bacteria, which are very

sensitive to pH variations (FUENTES et al., 2009). Mean pH was slightly lower (6.7) in HWO diets, a reduction that was probably not large enough to cause a disturbance in bacterial populations. Accordingly, no changes occurred on VFA production.

The higher digestibility of HWO than HSO and HLO diets should be accompanied by higher VFA production. A possible reason why VFA production was not increased might be related to the high content of triglycerides in the soybean and linseed oils. Glycerol released from triglyceride hydrolysis in HSO and HLO diets could be fermented (PATRA and YU, 2013) and ensure the maintenance of similar VFA concentration as compared to the HWO diet. Our results are in line with evidence showing few or no changes in VFA production with the addition of lipid sources to ruminant diets (BATEMAN AND JENKINS, 1998).

Although there were no differences in VFA production among diets, the addition of glycerol led to an increase in the molar proportion of propionate and reduction of the C2:C3 ratio. This may have been due to the fact that glycerol is a substrate that is more reduced than the glucose that is present in diets without glycerol. To maintain the oxidation-reduction balance, the metabolism is directed to formation of more reduced end-products, such as propionate. This is consistent with previous research (Defrain et al., 2004; Trabue et al., 2007; Krueger et al., 2010; LEE et al., 2011) showing a reduction in the acetate:propionate ratio in diets with added glycerol.

A reduction in both % CH_4/g DMD and total gas production was expected in HWO diets with glycerol addition, in line with our observation that glycerol fermentation produced more propionate than starch the 24 h time point. Because the propionate pathway acts as a H_2 sink, it should decrease the H_2 available to methanogens. However, this effect may depend on an interaction of glycerol with the substrate. For example, Avila-Stagno et al. (2013) reported an increase in methane production in diets with maize silage, suggesting that this process occurred because the glycerol conversion to propionate did not allow net electron incorporation, increasing butyrate concentration, which in turn resulted in a higher production of methane. In our experiment, however, butyrate concentration was unchanged in diets with added glycerol. The only difference in the HWO diet without glycerol was in the valerate and

propionate concentration. However, the increase in propionate with the addition of glycerol may not have been sufficient to cause a reduction in methane production.

Rumen ciliate protozoa is responsible for symbiotic transfers of H_2 with methanogens, which is used to reduce CO_2 to CH_4, producing over 25% of CH_4 in the rumen (NEWBOLD; LASSALAS; JOUANY, 1995). The observed decrease of CH_4 production in the HLO diets, which was independent of glycerol addition, probably occurred due the UFA profile of this vegetable source, which likely reduced protozoa counts, hence protozoa-associated methanogens, and may have also had a direct inhibitory effect on the membrane transport of methanogens (BEAUCHEMIN et al., 2008). Another possible effect of UFA is that associated with H_2 consumption by biohydrogenation, but this effect is known to be minor (1% to 2%) (CZERKAWSKI, 1972).

Similarly, there was a reduction in CH_4 production in HSO diets with added glycerol, yet no effect in the absence of glycerol. In the former case, this may have been due to the cumulative effect of the glycerol added and that released from hydrolyses of triglycerides through the propionate pathway. This differential effect of glycerol addition was not observed for HLO diets, possibly due to the higher toxicity of unsaturated fatty acids in linseed than in soybean oil (linolenic acid versus linoleic acid, respectively). These results indicate that the effect of glycerol might be highly dependent on its interaction with the specific dietary substrate. Conversely, substrate-specific metabolites that were not measured in this study may have interfered in other pathways acting as hydrogen consumption. Future research could focus sorting out, these possibilities.

Our results also show that the addition of glycerol to all diets did not alter the long-chain fatty acid profile of rumen fluid at 0, 6, 12 and 24 h of incubation. Recent evidence indicates that glycerol inhibits lipolysis *in vitro* when 6 or 20% (v/v) glycerol is added and decreases the ratio of free fatty acid production (nmol/ml) by 80 and 86 %, respectively, at 48 h of incubation (Edwards et. al. 2012). In this manner, the transfer of higher concentrations of UFA from the rumen is expected, as *Butirivibrio fibrisolvens* can only hydrogenate free carboxyl fatty acid groups (LOURENÇO; MORALES; WALLACE, 2010).

However, in our experiment the long-chain fatty acid profiles were unchanged with glycerol addition. This is consistent with the observed increase of *Anaerovibrio lipolytica* proportion, which can rapidly ferment glycerol and reduce its concentration in the medium. It also agrees with previous research that evaluated 15 to 25% glycerol (v/v) addition *in vitro*, and showed that up to 90% of glycerol disappeared within 6 hours (BERGNER, 1995). Therefore, lipolysis inhibition by glycerol during long-term incubation (48 h), as reported by Edwards et al. (2012), would be difficult to maintain. In that case, it is possible that end-products of the glycerol metabolism accumulated *in vitro* and inhibited lipolytic enzymes.

In this study no changes in SFA with glycerol were observed. This is in agreement with recent evidence (Avila-Stagno et al., 2012) showing no changes in total SFA and MUFA proportions in the subcutaneous fat of lambs supplemented with increasing concentrations of glycerol. However, Lage et al. (2014) found slightly increased MUFA and CLA concentrations in beef of Nellore cattle fed with crude glycerin (10% DM) in replacement of corn or soybean hulls. Although the authors attributed these results predominantly to lipolysis, they can be also linked to the stabilisation of ruminal pH in diets with glycerol. High concentrate diets can cause pH reduction and formation of 18:1 t-10 (BAUMAN; GRIINARI, 2003) rather than vaccenic acid. As a consequence, the isomer 18:1 t-10 cannot be desaturated to CLA in meat (Kramer et al., 2004), reducing its concentration in starch-rich diets compared with those supplemented with glycerol (AVILA-STAGNO et al., 2012).

The apparent biohydrogenation of linoleic acid in HSO and linolenic acid in HLO diets at 24 h was found to bef 55 and 73 %, respectively. These values were lower than those reported by a review of *in vivo* experiments of DOREAU and FERLAY (1995) which reported biohydrogenation ranging from 75 to 95% for linoleic acid and from 85% to 100% for linolenic acid. Here, the lower rate of biohydrogenation may have derived from a reduced concentration of rumen fluid and, consequently, low microbial activity. According to Getachew et al (2001), low biohydrogenation in diets with inclusion of fat in the form of triglycerides could be explained by reduced hydrolysis and release of free

fatty acids in *in vitro* systems or, alternatively, by a slow rate of fatty acid release for ruminal hydrogenation.

Overall, our findings suggest that glycerol can be used to replace up to 150 g/kg DM of starch without impacting negatively on ruminal fermentation. However, they also indicate that glycerol may not always be effective as a nutritional strategy to reduce methane production. Likewise, glycerol did not decrease lipolysis sufficiently to limit the intense biohydrogenation of unsaturated fatty acids in the rumen fluid, hence would possibly not increase the healthy UFA deposition in meat and milk. Our findings also indicate that the effect of glycerol on ruminal fermentation may depend on differences in the fatty profiles of different lipid sources. Therefore, they pave the way for further research using other sources of lipids, or alternative forms of lipid protection. Indeed, soybean and linseed oil have a high cost owing to the costly extraction process. Alternatively, feeding the animals with these vegetables oils in the form seeds could reduce costs while ensuring a lower ruminal biohydrogenation.

5. CONCLUSIONS

Our findings indicated that glycerol may replace starch as an energy source in the diets of ruminants and also decrease methane production when associated with soybean oil. Glycerol was ineffective to reduce the potentially negative effects of vegetable oils on ruminal fermentation and digestibility *in vitro*. Indeed, the proportion of *Anaerovibrio lipolytica* increased in all diets with glycerol added and unsaturated fatty acid profile remained unchanged.

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