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Glycerol combined with oils did not limit biohydrogenation of unsaturated fatty acid but reduced methane production *in vitro*

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ABSTRACT

Three runs of *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 at 850 g/kg DM without oil seeds (HWO), Tifton 85 hay + 80 g 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 16S 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, g/kg (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 $2 \times 3 \times 4$, glycerol, diets and time, respectively. Microbial quantification, IVTD and methane were evaluated with the same design but without time as a factor. The pH value and ammonia concentration were lower in HWO compared with HSO and HLO diets, independent of glycerol addition. *Ruminococcus albus, Ruminococcus flavefaciens, Butyrivrio* vaccenic acid and stearic acid 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. Methane production decreased in HSO diet combined with glycerol and HLO diets with or without glycerol addition. Lag time (h) from the gas production kinetics decreased in HWO and HSO diets combined with glycerol. The C2:C3 ratio decreased in diets with glycerol addition. Glycerol inclusion did not reduce biohydrogenation of unsaturated fatty acids *in vitro*. 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.

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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. Ruminants can ferment glycerol to propionate in the rumen and utilize its molecule at gluconeogenesis via hepatic metabolism. Currently, its use is under evaluation in several livestock diets as an energy source to replace cereal grain (e.g., corn) due to high availability and disposal costs of crude glycerin (Abo El-Nor et al., 2010).

Previous research has suggested that the association of glycerol with fat sources in the diet can partially inhibit bacterial lipases in the rumen (Krueger et al., 2010; Edwards et al., 2012). Once the biohydrogenation of unsaturated fatty acids is dependent of lipolysis to proceed (Harfoot and Hazlewood, 1997) and that triglycerides cause less impairment in uptake of nutrients by bacteria than free fatty acids (Chalupa et al., 1984). The addition of chemical compounds, such as glycerol, that inhibit lipolysis may increase the escape of unsaturated fatty acid to duodenum in diets rich in lipids without disturbing ruminal fermentation.

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 et al., 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).

The *in vitro* system show the potential to appropriately simulate biohydrogenantion of linoleic and linolenic acid and production of 18:0 unprotected fatty acid sources with possibility of assessing the inhibition or stimulation of specific intermediate biohydrogenation steps (Fievez et al., 2007). Association between kinetics of gas production *in vitro* and microbial profile can provide an improvement in our understanding of feed fermentability and microbial growth.

Several studies 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. 2014; Lage et al., 2014). To our knowledge, however, there has been no study to date that described the effects of glycerol on fatty acid composition, ruminal fermentation parameters and microbial changes when combined with vegetable oils. We hypothesize that the association of glycerol with vegetable oils could limit the intense biohydrogenation of unsaturated 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) combined with soybean and linseed oil on rumen fatty acid profile and ruminal fermentation parameters.

2. Materials and methods

The protocol used in this experiment was in accordance with the Brazilian College of Animal Experimentation (COBEA–Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA – Comissão de Ética e Bem Estar Animal) of the FCAV–UNESP–Jaboticabal campus (protocol number 021119/11).

2.1. Diets and incubation procedures

Three runs of *in vitro* ruminal incubations were conducted to evaluate the effect of glycerol (0 or 150 g/kg) combined with three different diets: Tifton 85 hay at 850 g/kg DM without oil seeds (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.

Rumen fluid from three canulated Nellore steers fed *Brachiaria brizantha* cv. Xaraes was used as inoculum for each run of incubation. Approximately 1 g of different proportions of ingredients (Table 1) was weighed into 160 ml flasks. For that, samples of 0.855 g of Tifton 85 hay plus 0.145 g of glycerol or 0.145 g of starch were weighed for the HWO diets with or without glycerol addition, respectively. In the HSO and HLO diets 0.075 g of soybean or linseed oil were weighed with 0.78 g of Tifton 85 hay plus 0.145 g of starch or 0.145 g of glycerol. Flasks were weighed in triplicates and divided into two portions: the first one was exclusively for quantification of long chain fatty acid composition and the second for quantification of ruminal fermentation parameters (volatile fatty acids [VFA], ammonia nitrogen [NH_3-N] and pH) and microorganism proportion.

2.2. Sample collections

The ruminal fluid collection was conducted at 7:00 h 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 et al., 1970).

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