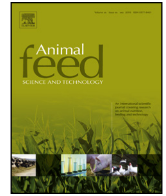
Animal Feed Science and Technology 225 (2017) 195–204

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Diet containing glycerine and soybean oil can reduce ruminal biohydrogenation in Nellore steers

Yury Tatiana Granja-Salcedo[a,*], Vinícius Carneiro de Souza[a], Ana Veronica Lino Dias[a], Arturo Samuel Gomez-Insuasti[a], Juliana Duarte Messana[a], Telma Teresinha Berchielli[a,b]

[a] Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Univ Estadual Paulista–UNESP, Via de Acesso Professor Paulo Donato Castellane, km 5, Rural, Jaboticabal, São Paulo CEP 14884-900, Brazil

[b] INCT/CA–UFV-Department of Animal Science, Av. Peter Henry Rolfs s/n, Campus Universitário, Viçosa, Minas Gerais CEP 36570-000, Brazil

ARTICLE INFO

Article history:
Received 24 June 2016
Accepted 29 January 2017

Keywords:
Unsaturated fatty acids
Ruminal bacteria
Glycerol
Lipolysis

ABSTRACT

This study was conducted to evaluate the effects of the addition of crude glycerine and soybean oil into the diet on the rate of ruminal biohydrogenation (BH), duodenal flow, and on the intestinal digestibility of fatty acids (FA), as well as on the major bacterial species that participate in the BH. Eight castrated Nellore steers were fitted with ruminal and duodenal cannulas. The diet ingredients used were: Tifton 85 hay (roughage source), and a concentrate composed of ground corn, soybean meal and urea, together with 100 g/kg of dry matter (DM) of crude glycerine (CG) or 60 g/kg DM of soybean oil (SO) or 100 g/kg DM crude glycerine plus 60 g/kg (DM) soybean oil (CGSO). Data were analyzed as a double Latin square 4×4 design (four treatments and four periods), balanced for residual effects, in a factorial arrangement ($A \times B$). The fixed effect of factor A corresponds to the provision of SO, and that of factor B to the provision of CG. The dry matter intake (DMI) suggested an interaction between CG and SO ($P = 0.045$); animals fed with the CG diet presented higher dry matter intake (DMI) than those fed the SO and CGSO diets ($P < 0.05$); however, the CGSO diet led to a DMI similar to that obtained with the CO diet, and higher than that obtained with the SO diet. A higher duodenal flow of monounsaturated FA (MUFA), poly-unsaturated FA (PUFA) and unsaturated FA (UFA) was observed with the CGSO diet ($P < 0.05$). There was an interaction between CG and SO on the ruminal BH rate of PUFA, UFA, and linolenic acid, the BH being lower with the CGSO diet than with the SO diet ($P < 0.05$). This interaction was also observed to affect the BH rate of MUFA, which was lower with the CGSO diet than with the SO and CG diets. The SO diet reduced the population of *Ruminococcus flavefaciens* ($P = 0.046$), *R. albus* ($P = 0.028$) and *Fibrobacter succinogenes* ($P = 0.041$). However, the relative proportion of *Anaerovibrio lipolytica* increased in the diets containing CG ($P = 0.043$). The association of CG and SO in the diet limited the BH of UFA, and increased the duodenal flow of these acids without influencing the cellulolytic bacteria of the rumen; therefore, this association may be a nutritional strategy to increase the deposition of healthy UFA in meat; however, this needs to be further investigated.

© 2017 Elsevier B.V. All rights reserved.

Abbreviations: aNDFom, NDF assayed with a heat stable amylase and expressed exclusive of residual ash; BH, biohydrogenation; DMI, dry matter intake; CG, crude glycerine; CGSO, crude glycerine and soybean oil association; CLA, conjugated linoleic acid; DM, dry matter; FA, fatty acids; NDFi, Indigestible neutral detergent fiber; SO, soybean oil; PUFA, poly-unsaturated fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids.

* Corresponding author.
E-mail address: yurygranjasalcedo@gmail.com (Y.T. Granja-Salcedo).

http://dx.doi.org/10.1016/j.anifeedsci.2017.01.021
0377-8401/© 2017 Elsevier B.V. All rights reserved.

1. Introduction

The nutritional quality of bovine meat has been criticized owing to its relatively high concentrations of saturated fatty acids (SFA), which are risk factors for cardiovascular diseases in humans (Salter, 2013). This high proportion of SFA in products derived from ruminants is partially based on the intense ruminal biohydrogenation (BH), which quickly saturates the unsaturated fatty acids (UFA) and poly-unsaturated fatty acids (PUFA) consumed by ruminants (Hartfoot and Hazlewood, 1997).

Lipolysis is indispensable for ruminal BH (Fievez et al., 2007), where several bacterial species are involved (Harfoot and Hazlewood, 1997). The bacterium *Anaerovibrio lipolytica* is considered the main lipolytic microorganism in the ruminal environment, particularly in animals fed with high proportions of concentrates or with supplements rich in triglycerides (Lourenço et al., 2010).

Ruminal BH is influenced by diet composition; *in vitro* studies have demonstrated that the inclusion of glycerol in the diet can inhibit lipolysis; thus, it limits the BH of ruminal UFA and PUFA (Krueger et al., 2010; Edwards et al., 2012). Meanwhile, crude glycerine, a by-product of biodiesel production that contains mainly glycerol, has become an alternative to replace grain as a source of energy in ruminant feed (Bartoň et al., 2013). Therefore, glycerine supplementation can be a strategy to increase the duodenal flow of UFA and PUFA, and consequently exert an immediate improvement on the fatty acid (FA) profile of meat and milk. This would be based on the effect of glycerine on lipids, which are used only in low quantities in ruminant diets, since levels of lipids above 70 g/kg of dry matter (DM) may negatively affect DM intake (Fiorentini et al., 2015), owing to the increase of the lag time of microorganisms and the antimicrobial effect exercised by lipids, largely by free PUFA (Harfoot and Hazlewood, 1997; Maia et al., 2007, 2010). Thus, by limiting ruminal lipolysis, and consequently the release of UFA in the rumen, the association of glycerine and lipids in the diet could reduce the bacteriostatic effect of UFA and PUFA towards ruminal microorganisms, increasing the flow of UFA and PUFA to the duodenum without harming the microbial activity in the rumen.

However, *in vitro* approaches have not yet completely elucidated the inhibition mechanism of glycerine on ruminal lipolysis (Krueger et al., 2010; Edwards et al., 2012; Castagnino et al., 2015). Castagnino et al. (2015) conducted an *in vitro* study to evaluate the association of glycerine with different vegetable oils, and observed that the addition of 150 g/kg (DM) of glycerine into the diet had no effect on the BH of UFA and PUFA; however, the proportion of *A. lipolytica* increased in animals fed diets containing glycerine.

In this context, the hypothesis of this study is that the addition of crude glycerine and soybean oil association to the diet may limit ruminal BH and increase the flow of duodenal UFA. Thus, this study aimed to evaluate the effect of crude glycerine, soybean oil or it association addition into the experimental diets on ruminal BH rate, FA duodenal flow, FA intestinal digestibility, and major bacterial species that participate in ruminal BH in Nellore steers.

2. Materials and methods

2.1. Animals and diets

The experimental procedures used in this study followed the Ethical Principles for Animal Experimentation adopted by the Brazilian College of Animal Experimentation, and approved by the Ethics Committee of Animal Experimentation of the Faculdade de ciências Agrarias e Veterinárias (FCAV) (07784/14).

We used eight castrated Nellore steers (initial weight 501.3 ± 18 kg; final weight 580.2 ± 28 kg), fitted with a ruminal (diameter: 10 cm) and duodenal (Type T) silicone cannula, distributed in two 4 × 4 balanced Latin square design for residual effects, with four treatments (diets) and four experimental periods.

Diets were formulated according to recommendations of the Agricultural and Food Research Council (AFRC, 1993), where the isonitrogenous had a roughage:concentrate ratio of 30:70. The ingredients used were (Table 1): Tifton 85 hay, as roughage source, and a concentrate, composed by ground corn, soybean meal and urea (Control diet), with the addition of 100 g/kg DM of crude glycerine (CG), or 60 g/kg DM of soybean oil (SO), or 100 g/kg DM of glycerine together with 60 g/kg DM of soybean oil (CGSO) (Table 2).

The crude glycerine was obtained from biodiesel production based on soybean oil (glycerine: 803.4 g/kg; ether extract: 15.9 g/kg; ash: 50.3 g/kg; water: 120.2 g/kg). Owing to the inclusion of oil and glycerine, concentrates were prepared to be consumed within a maximum of 4 d, in order to prevent the oxidation of dietary ingredients.

Animals were kept in individual stables (12 m^2) equipped with individual feed troughs and automatic drinkers. Each experimental period lasted 21 d, including a 14-d adaptation phase and a 7-d sampling phase. Experimental diets were given as total mixed ration at 0600 h and 16:00 h. Additionally, 100 g/animal/d of a mineral mixture was supplied (composition per kg of supplement: calcium, 146 g; phosphorus, 40 g; sulfur, 40 g; sodium, 130 g; copper, 1.35 g; manganese, 1.04 g; zinc, 5 g; iodine, 100 mg; cobalt, 80 mg; selenium, 26 mg; fluorine, ≤800 mg).

2.2. Intake and duodenal flow

At the beginning of each experimental period, the feed was sampled. Diets were supplied such that the leftovers were less than 5% of the total food offered. Between days 15–21 of each experimental period, the leftovers were collected and weighed

Table 1
Diet ingredients: bromatologic composition and fatty acid profile analyzed.

	Diet ingredient			
	Tifton 85 hay	Ground corn	Soybean meal	Soybean oil
Chemical composition				
Dry matter%	78.87	85.72	87.18	99.0
Organic matter, % DM	90.96	98.65	92.72	99.0
Crude protein, % DM	7.01	7.99	42.73	–
aNDFom, % DM	87.39	26.30	28.09	–
Ether extract, % DM	1.41	4.74	1.66	99.30
Gross energy, Mcal/kg	4.00	4.20	4.47	5.46
Fatty acid profile, g/kg of DM				
C14:0	0.03	0.17	0.11	3.17
C16:0	0.74	2.85	0.00	33.92
C17:0	0.04	0.17	0.00	2.97
C18:0	2.17	3.82	2.48	121.66
C18:1 *cis 9*	0.21	6.36	1.60	301.59
C18:2 *cis 9, 12*	2.04	13.75	5.15	347.83
C20:0	0.02	0.00	1.28	139.14
C18:3 *cis 9, 12, 15*	0.04	0.15	0.06	2.29
C22:0	0.13	0.24	0.00	29.33
C14:0	1.01	1.44	0.35	2.84
Total SFA	3.23	4.73	5.03	329.18
Total UFA	9.29	40.08	11.57	663.82

aNDFom, NDF assayed with a heat stable amylase and expressed exclusive of residual ash.; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0; C20:0 and C22:0); UFA, unsaturated fatty acids (sum of C14:1 *cis* 9, C16:1 *cis* 9, C18:1 *cis* 9, C18:2 *cis* 9 *cis* 12, C18:3 *cis* 9 *cis* 12 *cis* 15, C20:1 *cis* 11 and C22:1 *cis* 13).

Table 2
Experimental diets: proportion of ingredients, bromatologic composition, and fatty acid profiles.

	Experimental diets			
	CO	SO	CG	CGSO
Proportion of ingredients, % of DM				
Hay	30.00	30.00	30.00	30.00
Ground Corn	56.00	48.50	43.40	36.20
Soybean meal	13.00	14.50	15.60	16.80
Urea	1.00	1.00	1.00	1.00
Glycerine	0.00	0.00	10.00	10.00
Soybean oil	0.00	6.00	0.00	6.00
Bromatologic composition				
Dry matter%	83.99	89.64	84.95	85.83
Organic matter, % DM	94.60	87.26	92.49	86.69
Crude protein, % DM	15.12	15.24	15.35	15.37
aNDFom, % DM	44.60	43.05	42.01	40.46
Ether extract, DM%	3.30	8.92	3.01	8.64
ME, Mcal/kg	2.55	2.48	2.47	2.56
Fatty acid profile, g/kg of DM				
C14:0	0.10	0.22	0.18	0.30
C16:0	1.44	3.39	1.23	3.09
C17:0	0.09	0.26	0.07	0.24
C18:0	0.55	7.84	0.53	7.82
C18:1 *cis 9*	3.29	20.93	2.76	20.42
C18:2 *cis 9, 12*	7.47	27.55	7.52	27.62
C20:0	0.15	8.52	0.18	8.54
C18:3 *cis 9, 12, 15*	0.09	0.21	0.07	0.20
C22:0	0.15	1.89	0.12	1.87
Other	12.86	12.23	11.63	11.13
Total SFA	5.69	25.00	5.04	24.35
Total UFA	20.65	58.25	19.30	56.97

CO, control; CG, concentrate with 10% crude glycerine; SO, concentrate with 6% of soybean oil; CGSO, concentrate with 10% crude glycerine and 6% of soybean oil; aNDFom, NDF assayed with a heat stable amylase and expressed exclusive of residual ash; ME, metabolizable energy; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0; C20:0 and C22:0); UFA, unsaturated fatty acids (sum of C14:1 *cis* 9, C16:1 *cis* 9, C18:1 *cis* 9, C18:2 *cis* 9 *cis* 12, C18:3 *cis* 9 cis 12 *cis* 15, C20:1 *cis* 11 and C22:1 *cis* 13); Other, sum of C8:0, C10:0, C12:0, C13:0, C14:1 *cis* 9, C15:0, C16:1 *cis* 9, C18:2 *trans* 9, 12, C20:1 *cis* 11 and C22:1 *cis* 13.

daily before the morning feeding of animals. At the end of each experimental period, composite samples were collected for each animal based on the leftovers' weight, and stored at −20 °C.

Duodenal content was collected at 6-h intervals at days 19 and 20 of each experimental period. The sample collection during the second day was delayed for to ensure that every 3-h in a 24-h period was properly represented (Oliveira et al., 2007). The 200-mL duodenal content samples were obtained by means of a cannula, and divided into two aliquots; a 50-mL sample, stored at −20 °C and lyophilized, used to analyze the profile of long-chain fatty acids; and a 150-mL sample, dried in a forced-air-circulation oven at 55 °C for 72 h, used for DM and neutral detergent fiber (NDF) analysis.

In order to determine fecal production, stool samples were collected daily between days 16–20 of each experimental period, immediately after each spontaneous defecation, and stored in 20-L buckets. The total feces produced per animal/d were weighed and homogenized; from this, a sample (approximately 200 g) was removed, dried, and milled. For each animal, in each experimental period, we formed a fecal-composite sample.

Feed, leftovers and feces samples were dried in a forced-air-circulation oven at 55 °C for 72 h, and individually milled in a Willey-type mill (Thomas Scientific, Swedesboro, NJ, USA) with a 1-mm sieve; then, DM content was analyzed (DM; 934.01) in accordance with the methods prescribed by the Association of Official Analytical Chemists (AOAC, 1990). Feed samples were used to determined ether extract (EE) (Method 920.39) according to (AOAC, 1990). Nitrogen was determined using a LECO FP-528 nitrogen analyzer (LECO Corp., St. Joseph, MI) and the nitrogen values were converted to crude protein (CP) by multiplying by 6.25. Gross energy was obtained by the combustion of samples in an adiabatic bomb calorimeter IKA model 2000 Basic.

Indigestible neutral detergent fiber (NDFi), an indicator of the daily flow of DM in the duodenum (Harvatine and Allen, 2006), was assessed by *in situ* incubation of feeds offered, leftovers, feces, and duodenum samples for 288 h, following the methodology proposed by Valente et al. (2011). FDNi determination was conducted by using α-amylase without the addition of sodium sulfite, following the recommendations of Van Soest et al. (1991), and adapted for the ANKOM 200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA).

2.3. Ruminal biohydrogenation

Total FA were extracted from the feed ingredients, leftovers, and duodenum samples by means of the methylation method described by Palmquist and Jenkins (2003). During this process, 1 mL of nonadecanoic acid C19:0 at 2.0% was added to each sample, which was used as the internal standard for FA quantification. The FA profile was quantified by gas chromatography (GC Shimatzu model 20-10, with automatic injection; Shimatzu Corporation, Kyoto, Japan) using a SP-2560 capillary column (100 m × 0.25 mm diameter, 0.02 mm thick; Supelco, Bellefonte, PA, USA), and hexane as carrier gas. In order to identify each FA, we used external standards (Supelco, Bellefonte, PA, USA; Nu-Chek Prep, Elysian, MN, USA), whereas an internal standard (C19:0) was used for quantification.

The rates of ruminal BH were calculated by means of using the model described by Jenkins and Bridges (2007), which estimates the difference between the FA consumed (initial, g/d) and the FA observed in the duodenal flow (final, g/d), calculated according to the following formula: BH of PUFA (BPUFA) = $100 * (PUFA_{INITIAL} - PUFA_{FINAL})/PUFA_{INITIAL.}$

2.4. Ruminal bacteria

The quantitative polymerase chain reaction (qPCR) method was used to identify and quantify the main bacterial species that participate in the ruminal BH of UFA. For this, on day 21 of each experimental period, before the morning feeding, 50-g samples of solid and liquid fractions of ruminal content were collected from the medial region of the rumen through a ruminal cannula; samples collected were immediately frozen and lyophilized.

Metagenomic DNA extraction was performed by using the FastDNA SPIN Kit for Soil (MP Bio, Biomedicals, Illkirch, France) with 150 mg of the sample, following the manufacturer recommendations. The integrity and quantity of DNA was verified on a 0.8% agarose gel stained with ethidium bromide (5 mg/mL); size was estimated by comparison with 1-kb plus DNA ladder marker (Invitrogen, Waltham, MA, USA), and the yield and quality of DNA were evaluated by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, USA) and by fluorometry (Qubit 3.0, Life Technology, Waltham, MA, USA).

All forward and reverse primers used in this study (Table 3) were tested in four concentrations (200, 400, 600, and 800 nM) to determine the minimum concentration of primer with the lowest threshold cycle (Ct), and reduce nonspecific amplifications. We determined the slope value, and calculated the efficiency. The validation of the selected-primers concentrations was performed with different concentrations of DNA (150, 125, 100, 50, and 25 ng).

The qPCRs (final volume reactions 12.5 μL) contained 6.25 μL of SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 400 or 600 nM of each primer pair, ultrapure water (Milli-Q, Millipore Corporation, Merck, Billerica, MA, USA), and 100 ng of the metagenomic DNA. Reactions were performed in an Applied Biosystems 7500 Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). We conducted negative controls in the analysis, omitting the metagenomic DNA. ROX was used as a passive reference dye.

PCR conditions were as follows: amplification at 50 °C for 2 min, 95 °C for 10 min; 35 denaturing cycles at 95 °C for 15 s; annealing at 60 °C for 60 s; and extension at 78 °C for 1 min. After the amplification cycle, a step was added, increasing the temperature from 60 °C to 95 °C, in order to obtain a dissociation curve of the reaction products, and analyze the specificity of the amplification.

Table 3
Primers used for specific quantification of ruminal bacteria by qPCR.

	Primer (5′–3′)		Product(bp)	Efficiency (%)
	Forward	Reverse		
Total bacteria[a,b]	CGGCAACGACAACCC	CCATTGTAGCACCTGTGTAGCC	130	100
Ruminococcus flavefaciens[b]	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	132	95
Ruminococcus albus[c]	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA	175	96
Fibrobacter succinogenes[b]	GTTCGG AATTAC TGG GCGTAAA	CGCCTGCCCCTGAACTAT C	121	98
Anaerovibrio lipolytica[d]	TTGGGTGTTAGAAATGGATTCTAGTG	TCGAAATGTTGTCCCCATCTG	82	96
Butyrivibrio SA[d]	TGAAAAACTCCGGTGGTATGAGAT	CCGTGTCTCAGTCCCAATGTG	126	99
Butyrivibrio VA[d]	TGCATTGGAAACTGTAGAACTAGAGTGT	GCGTCAGTAATCGTCCAGTAAGC	124	94

[a] Primers used for the normalization of qPCR.
[b] Denman and McSweeney (2006).
[c] Koike and Kobayashi (2001).
[d] Fuentes et al. (2009); SA, subgroup producer of stearic acid; VA, subgroup producer of vaccenic acid.

Table 4
Mean dry matter intake (DMI) and fatty acids intake by Nellore steers fed with experimental diets with crude glycerine, soybean oil or crude glycerine and soybean oil association.

	Experimental diet				SEM	P-value		
	CO	SO	CG	CGSO		O	G	O × G
DMI, kg/d	9.61[ab]	8.25[c]	10.62[a]	9.38[b]	0.396	0.046	0.129	0.045
Fatty acids intake, g/d								
C14:0	0.32	0.83	1.07	1.89	0.151	0.006	<0.001	0.437
C16:0	13.49	25.30	12.04	28.04	1.557	<0.001	0.761	0.284
C17:0	0.82	1.83	0.72	2.11	0.131	<0.001	0.805	0.149
C18:0	4.97	60.55	5.28	70.46	5.782	<0.001	0.119	0.194
C18:1 *cis 9*	17.04	112.67	15.82	124.41	9.720	<0.001	0.731	0.672
C18:2 *cis 9, 12*	76.97	200.07	79.58	210.81	15.981	<0.001	0.311	0.989
C20:0	0.69	64.93	1.18	79.93	6.633	<0.001	0.111	0.804
C18:3 *cis 9, 12, 15*	6.38	15.36	5.83	17.70	0.109	<0.001	0.389	0.342
C22:0	1.31	14.55	1.18	14.48	1.385	<0.001	0.610	0.084
SFA	29.93	174.43	28.75	201.0	4.551	<0.001	0.376	0.157
MUFA	105.13	182.64	102.08	202.91	12.403	<0.001	0.664	0.557
PUFA	82.35	215.45	85.41	229.44	15.914	<0.001	0.313	0.980
UFA	187.49	398.10	187.50	432.36	29.110	<0.001	0.605	0.653
Total FA	217.39	572.53	216.26	633.20	43.143	<0.001	0.313	0.327

CO, control diet; CG, diet with 10% crude glycerine; SO, diet with 6% of soybean oil; CGSO, diet with 10% crude glycerine and 6% of soybean oil; SEM, standard error of mean, O, oil effect; G, glycerine effect; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0; C20:0 and C22:0); MUFA, mono-unsaturated fatty acids (sum of C14:1 *cis* 9, C16:1 *cis* 9, C18:1 *cis* 9, C20:1 *cis* 11 and C22:1 *cis* 13); PUFA, polyunsaturated fatty acids (sum of C18:2 *cis* 9 *cis* 12, C18:3 *cis* 9 *cis* 12 *cis* 15); UFA, unsaturated fatty acids (MUFA + PUFA); FA, total fatty acids (SFA + UFA). Values followed with different superscript letters on the same row represent a significant difference (P < 0.05), as obtained with Tukey's test.

The relative quantification was used to determine the proportion of target bacterial species. Results were expressed as a proportion of the 16S rRNA associated with total bacteria, according to the following equation: [relative quantification = $2^{-[Ct\ \text{target} - Ct\ \text{total bacterial}]}$], where *Ct* is defined as the number of cycles needed for the fluorescent signal to cross the threshold.

2.5. Experimental design and statistical analysis

Data of feed intake, duodenal flow, intestinal digestibility, and ruminal BH were analyzed as a double 4 × 4 Latin square design (four treatments and four periods), balanced for residual effects, in a factorial arrangement ($A \times B$). The fixed effect of factor *A* (degree of freedom, DF 1) corresponds to the soybean oil provision (yes/no), and factor *B* to the crude glycerine (DF 1) provision (yes/no), factors interactions (DF 3), treatments error, the random effects of Latin square (DF1), period (DF 6), animal (DF 6), and residues corresponding to the model. These analyzes of variance were performed using R Software version 3.2.2 (R Core Team, 2015) after verification of mathematical assumptions (Shapiro-Wilk test and Bartlett test). Tukey's post-hoc test was applied when ANOVA indicated a significant difference between means, considering statistical significance when $P \leq 0.05$. Bacterial proportions were compared between experimental groups (CO, SO, CG and CGSO) using Kruskal-Wallis and post-hoc Dunns's tests.

3. Results

The dry matter intake (DMI) presented an interaction between crude glycerine and soybean oil (Table 4; P = 0.045); animals fed with the diet containing crude glycerine associated with oil (CGSO) presented a reduction in the DMI when

Table 5
Duodenal flow of dry matter and fatty acids in Nellore steers fed with experimental diets with crude glycerine, soybean oil or crude glycerine and soybean oil association.

	Diet				SEM	P-value		
	CO	SO	CG	CGSO		O	G	O × G
Dry matter, kg/d	4.14^{a}	2.92^{b}	3.79^{a}	4.15^{a}	0.225	0.290	0.275	0.040
Duodenal flow of fatty acids, g/d								
C13:0	2.77^{b}	3.00^{b}	1.97^{b}	6.08^{a}	0.372	0.003	0.099	0.003
C14:0	1.61^{b}	1.75^{b}	1.07^{b}	3.81^{a}	0.251	<0.001	0.027	<0.001
C15:0	8.69^{b}	8.30^{b}	5.75^{b}	17.54 ^{a}	1.22	0.001	0.154	<0.001
C16:0	8.14^{b}	5.60^{b}	9.32^{ab}	15.60^{a}	0.985	0.007	0.487	0.004
C17:0	3.82^{b}	4.30^{b}	2.87^{b}	8.66^{a}	0.526	0.002	0.122	<0.001
C18:0	199.84	273.07	170.41	331.97	22.256	0.009	0.780	0.160
C18:1 *trans 6*	0.05	0.13	0.01	0.01	0.034	0.539	0.256	0.877
C18:1 *cis 6*	0.32	0.42	0.30	0.46	0.049	0.034	0.415	0.150
C18: 1 *trans 9*	2.88^{b}	2.82^{b}	2.06^{b}	5.82^{a}	0.408	0.025	0.141	0.009
C18:1 *cis 9*	14.04^{b}	16.91^{b}	13.12^{b}	29.31^{a}	4.558	0.004	0.050	0.016
C18:1 *trans 11*	0.20	0.23	0.21	0.26	0.009	0.172	0.406	0.135
C18:1 *cis 9, trans 11*	0.09	0.04	0.01	0.08	0.004	0.133	0.529	0.144
C18:2 *cis 9, 12*	0.66	0.42	0.44	0.67	0.074	0.638	0.965	0.415
C18:2 *trans 9, 12*	0.63^{b}	0.70^{b}	0.50^{b}	1.42^{a}	0.220	0.009	0.079	0.005
C18:3 *cis 9, 12, 15*	0.32^{b}	0.41^{b}	0.26^{b}	0.80^{a}	0.055	0.005	0.097	0.014
C20:0	0.79^{b}	1.01^{b}	0.64^{b}	1.97^{a}	0.136	0.004	0.108	0.007
C22:0	0.07^{b}	0.09^{b}	0.06^{b}	0.14^{a}	0.008	<0.001	0.128	0.009
SFA	228.25	306.78	189.78	331.73	25.241	0.020	0.847	0.191
MUFA	29.96^{b}	32.59^{b}	23.55^{b}	79.71^{a}	6.432	0.004	0.047	0.003
PUFA	1.53^{b}	1.52^{b}	1.20$_{b}$	4.73^{a}	0.271	0.006	0.114	0.002
UFA	31.50^{b}	34.11^{b}	24.75^{b}	84.44^{a}	6.697	0.019	0.113	0.013
Total FA	259.75^{b}	340.89^{ab}	214.53^{b}	416.17^{a}	27.597	0.004	0.785	0.048

CO, control diet; CG, diet with 10% crude glycerine; SO, diet with 6% of soybean oil; CGSO, diet with 10% crude glycerine and 6% of soybean oil; SEM, standard error of mean; O, oil effect; G, glycerine effect; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0; C20:0 and C22:0); MUFA, mono-unsaturated fatty acids (C14:1 *cis* 9, C16:1 *cis* 9, C18:1 *cis* 9, C20:1 *cis* 11 and C22:1 *cis* 13); PUFA, polyunsaturated fatty acids (C18:2 *cis* 9 *cis* 12, C18:3 *cis* 9 *cis* 12 *cis* 15); UFA, unsaturated fatty acids (MUFA + PUFA); FA, total fatty acids (SFA + UFA). Values followed with different superscript letters on the same row represent a significant difference ($P < 0.05$) as obtained using Tukey's test.

compared with animals supplemented only with glycerine (CG; $P < 0.05$); however, the DMI of animals the difference was not significant between the CGSO and CO groups. The animals fed with diets supplemented with oil (SO) had lower DMI than did the CGSO and CO groups ($P = 0.046$).

The addition of soybean oil to the diet was associated with a higher intake of total FA, SFA, mono-unsaturated FA (MUFA), PUFA, and UFA, and a higher individual intake of all individual FA evaluated ($P < 0.05$) (Table 4).

An interaction was observed between crude glycerine and soybean oil on the duodenal flow of DM (Table 5; $P = 0.040$). DM was lower with the SO diet. However, there was a higher duodenal flow of total FA in the CGSO group than in the CO and CG groups ($P < 0.05$). The association of crude glycerine and soybean oil in the CGSO diet led to higher duodenal flow of MUFA, PUFA, UFA, and the FA C13:0, C14:0, C15:0, C17:0, C18:1 *trans* 9, C18:1 *cis* 9, C18:2 *trans* 12, 9, C18:3, C20:0 and C22:0, than did the other diets ($P < 0.05$).

The association of crude glycerine and soybean oil in the CGSO diet led to higher duodenal flow of palmitic acid (C16:0) than did the CO ($P = 0.024$) and SO diets ($P = 0.003$). However, the use of soybean oil, crude glycerine, or their association in the diet had no effect on the duodenal flow of the FA C18:1 *trans 6,* C18:1 *trans 11,* C18:2 *cis 9, trans 11* and C18:2 *cis 9, 12* ($P > 0.05$).

An interaction between the crude glycerine and soybean oil was observed on the rate of ruminal BH of PUFA, UFA and linolenic acid (C18:3 *cis* 9, 12, 15), with a lower rate in the CGSO group than in the SO group ($P < 0.05$; Table 6). This interaction was also observed in the rate of BH of MUFA, being lower with the CGSO diet than with the SO and CG diets. In contrast, the rate of BH of oleic acid (C18:1 *cis* 9) and linoleic acid (C18:2 *cis* 9, 12) was higher in the diets with soybean oil.

The apparent intestinal digestibility coefficient of FA C18:1 *trans 6*, C18:1 *trans 11*, C18: 2 *cis 9*, *trans 11* and C18:3 was 100% in all diets; meanwhile the apparent intestinal digestibility of stearic acid (C18:0), linoleic acid (C18:2 *cis 9, 12*), total FA, total SFA, and total UFA, were not influenced by the diets ($P > 0.05$; Table 7).

Some FA, in particular C18:1 *cis 6*, C18:1 *trans 9* and C18:2 *trans 9, 12*, presented higher digestibility when the diet was supplemented with soybean oil ($P < 0.05$). In addition, there was an interaction between soybean oil and crude glycerine on the intestinal digestibility of oleic acid (C18:1 *cis 9*), being higher in the CGSO diet than in the CG diet ($P < 0.05$).

The SO diet affected negatively the proportion of cellulolytic bacteria assessed in this study ($P < 0.05$; Table 8). Thus, *R. flavefaciens* presented a threefold lower relative proportion in the SO diet than in the other diets; the SO diet also presented a fivefold lower population of *Ruminococcus albus* than in the other diets, and the relative proportion of *Fibrobacter succinogenes* was 8-, 9- and 7-fold lower in the SO diet than in the CO, CG and CGSO diets, respectively.

The relative proportion of *Anaerovibrio lipolytica* presented a 20-, 18- and 4-fold increase with the CG, CGSO, and SO diets, respectively, over that with the CO diet. In the SO and CGSO diets, the proportions of *Butyrivibrio fibrisolvens* subgroup

Table 6
Rate of ruminal biohydrogenation of fatty acids (% of total ingested) in Nellore steers fed with experimental diets with crude glycerine, soybean oil or crude glycerine and soybean oil association.

	Experimental diet				SEM	P-value		
	CO	SO	CG	CGSO		O	G	O × G
C18:1 *cis 9*	43.15	86.31	40.35	60.33	6.079	0.004	0.139	0.599
C18:2 *cis 9, 12*	88.17	99.21	92.83	98.68	3.181	<0.001	0.086	0.062
C18:3 *cis 9, 12, 15*	55.19[b]	78.50[a]	66.23[ab]	52.35[b]	5.140	0.579	0.450	0.044
MUFA	69.90[ab]	77.70[a]	77.60[a]	65.04[b]	4.799	0.348	0.274	0.041
PUFA	98.03[ab]	99.18[a]	98.65[ab]	97.71[b]	4.359	0.051	0.919	0.035
Total UFA	82.17[b]	90.61[a]	87.25[ab]	80.31[b]	9.552	0.192	0.502	0.005

CO, control diet; CG, diet with 10% crude glycerine; SO, diet with 6% of soybean oil; CGSO, diet with 10% crude glycerine and 6% of soybean oil; SEM, standard error of mean; O, oil effect; G, glycerine effect; MUFA, mono-unsaturated fatty acids (C14:1 *cis* 9, C16:1 *cis* 9, C18:1 *cis* 9, C20:1 *cis* 11 and C22:1 *cis* 13); PUFA, polyunsaturated fatty acids (C18:2 *cis* 9 *cis* 12, C18:3 *cis* 9 *cis* 12 *cis* 15); UFA, unsaturated fatty acids (MUFA + PUFA); Values followed with different superscript letters on the same row represent a significant difference (P < 0.05), as obtained using Tukey's test.

Table 7
Apparent intestinal digestibility of fatty acids (% of duodenal flow) in Nellore steers fed with experimental diets with crude glycerine, soybean oil or crude glycerine and soybean oil association.

	Experimental diet				SEM	P-value		
	CO	SO	CG	CGSO		O	G	O × G
C18:0	83.96	81.13	87.98	84.16	4.880	0.641	0.479	0.672
C18:1 *cis 6*	74.08	81.82	69.24	89.85	4.942	0.012	0.770	0.100
C18:1 *trans 9*	63.61	75.53	53.88	92.02	9.363	0.013	0.714	0.166
C18:1 *cis 9*	76.97[ab]	77.80[ab]	73.44[b]	90.67[a]	4.489	0.014	0.451	0.046
C18:2 *cis 9, 12*	92.04	97.26	99.19	97.83	8.318	0.413	0.216	0.180
C18:2 *trans 9, 12*	67.93	76.57	54.07	93.66	4.967	0.009	0.847	0.078
SFA	82.11	79.83	82.21	84.60	4.744	0.775	0.680	0.714
UFA	77.10	80.01	75.37	79.29	9.236	0.506	0.799	0.152
Total FA	81.37	78.99	79.27	83.82	3.928	0.828	0.731	0.385

CO, control diet; CG, diet with 10% crude glycerine; SO, diet with 6% of soybean oil; CGSO, diet with 10% crude glycerine and 6% of soybean oil; SEM, standard error of mean; O, oil effect; G, glycerine effect; SFA, saturated fatty acids; UFA, unsaturated fatty acids (C14:1, C16:1, C18:1, C18:2, C18:3, C20:1 and C22:1). Values followed with different superscript letters on the same row represent a significant difference (P < 0.05), as obtained using Tukey's test.

Table 8
Ruminal bacteria[a] proportions (medians and interquartiles) in Nellore steers fed with experimental diets composed by crude glycerine, soybean oil or crude glycerine and soybean oil association.

	Experimental diet				P-value
	CO	SO	CG	CGSO	
Ruminococcus flavefaciens	0.010 ± 0.002[a]	0.003 ± 0.001[b]	0.010 ± 0.003[a]	0.009 ± 0.003[a]	0.046
Ruminococcus albus	0.035 ± 0.011[a]	0.006 ± 0.002[b]	0.029 ± 0.007[a]	0.028 ± 0.004[a]	0.028
Fibrobacter succinogenes	0.097 ± 0.009[a]	0.011 ± 0.003[b]	0.105 ± 0.016[a]	0.086 ± 0.007[a]	0.041
Anaerovibrio lipolytica	0.019 ± 0.004[c]	0.092 ± 0.012[b]	0.381 ± 0.028[a]	0.345 ± 0.018[a]	0.043
Butyrivibrio VA	0.759 ± 0.107	0.911 ± 0.096	0.802 ± 0.074	0.935 ± 0.144	0.461
Butyrivibrio SA	1.068 ± 0.346[b]	3.449 ± 0.128[a]	0.797 ± 0.095[b]	3.510 ± 0.427[a]	0.037

[a] Measured based on the proportion of the specific 16S rRNA associated with total bacteria; CO, control diet; CG, diet with 10% crude glycerine; SO, diet with 6% of soybean oil; CGSO, diet with 10% crude glycerine and 6% of soybean oil; AS, subgroup producer of stearic acid; VA, subgroup producer of vaccenic acid. Values followed with different superscript letters on the same row represent a significant difference (P < 0.05), as obtained using Dunn's test.

producer of stearic acid (SA) displayed a 3- and 4-fold increase over that with the CO and CG diets, respectively. The population of *B. fibrisolvens* subgroup producer of vaccenic acid (VA) was not influenced by the addition of soybean oil, glycerine, or their association in the diet (P > 0.05; Table 8).

4. Discussion

This study evaluated the effect of the dietary association of crude glycerine and soybean oil on the ruminal BH rate, duodenal flow and intestinal digestibility of FA, as well as in the major bacterial species that participate in the BH in Nellore steers. Our results demonstrate that the association of crude glycerine and soybean oil reduced the BH of MUFA, PUFA and total UFA, and increased the duodenal flow of these acids without influencing the population of ruminal cellulolytic bacteria. Therefore, we confirmed the hypothesis that the association of soybean oil and crude glycerine in the diet may limit the ruminal BH and increase the flow of duodenal UFA.

The reduction of the DMI in the animals fed with SO diet is related with the high content of ether extract derived from the lipid supplementation, and its high proportion of UFA, which can reduce fiber digestibility and intake by filling, or by

activating the chemotactic factor in the satiety center (Allen, 2000; Yang et al., 2009). The degree of unsaturation of soybean oil and its elevated inclusion in the diet enabled a high intake of UFA. Several studies have reported that UFA may increase the *lag* time, and that PUFA can change the bi-layer lipid structure of the bacterial cells, mainly of cellulolytic bacteria (Maia et al., 2007, 2010; Yang et al., 2009). This could explain the reduction of the proportion of important cellulolytic bacterial species such as *R. flavefaciens, R. albus,* and *F. succinogenes* in the ruminal environment of animals fed with the SO diet in our study.

On the other hand, in cattle, lipid supplementation can affect the intake of DM, due to an increase of free FA serum concentration, which activates the receptors of the satiety center located in the hypothalamus, inhibiting the appetite (Allen, 2000). The results of the present study agree with Fiorentini et al. (2015), who observed in Nellore steers a marked reduction in the DMI in diets with high content of ether extract (70 g/kg of DM).

Steers fed with diets containing soybean oil showed more ingestion of SFA, MUFA, PUFA and UFA. These results were expected due to the higher content of SFA and UFA in soybean oil. Despite SO diet have induced a lower DMI, was observed an increase in intake of advisable FA, since it depends of the concentration of FA in the diet.

Despite the higher intake of MUFA, PUFA, and UFA in diets supplemented with soybean oil, the duodenal flow of these acids in the SO group was similar to that in the CO and CG groups, resulting from the intense BH rate observed with the SO diet. Nevertheless, this was not observed with the CGSO diet (*i.e.*, when crude glycerine was associated with soybean oil), where the BH rate of ruminal MUFA, PUFA and UFA was smaller than that in the SO diet. These results support previous *in vitro* studies, which suggested a plausible inhibitory effect of dietary glycerine on ruminal BH (Krueger et al., 2010; Edwards et al., 2012). The lower rate of ruminal BH of MUFA, PUFA and UFA when the steers were fed with the CGSO diet, allowed a higher duodenal flow of these acids.

The larger proportion of *A. lipolytica* in the rumen of animals fed with diets containing glycerine, may be a result of a microorganism preference in using glycerine as substrate (Prins et al., 1975), being consistent with previous *in vitro* results (Castagnino et al., 2015). The bacterium *A. lipolytica* produces two enzymes—a mainly intracellular esterase and a lipase that is secreted to the culture medium (Hobson and Summers 1967; Edwards et al., 2013). However, esterases preferentially catalyze the ester bonds of short-chain fatty acids and lipases of long-chain fatty acids (Fojan et al., 2000). Fay et al. (1990) demonstrated that, when cultivated in a medium containing glycerine, *A. lipolytica* presented higher microbial growth and the activity of the esterase doubled.

Coinciding with the *in vitro* findings of Fuentes et al. (2009), our results suggest that the higher concentration of *A. lipolytica* may not be necessarily linked to a higher lipolytic activity in the ruminal environment. Thus, it is plausible that the growth of this microorganism in the presence of glycerine is a key factor for the limiting effect on the ruminal lipolysis, and future studies are required to better understand this mechanism. This limiting effect, together with the rate of passage of the diet, would allow a higher proportion of MUFA and PUFA leaving the rumen without undergoing BH, and may have an impact on the improvement of the fatty acid profile of meat and milk, without harming the microbial ruminal activity.

The amount of CG used in this study can be considered normal in cattle feeding systems, once similar inclusion level were used in cattle nutrition studies without negative effect in DMI and diet digestibility (Shin et al., 2012; Paiva et al., 2016). Furthermore, CG use in cattle diets, may permit high levels of lipid supplementation in cattle (e.g. ether extract 80 g/ kg of DM) without negative effects in animal performance. Resulting, in the final product (milk or meat) a relative increase of PUFA concentration.

The increase in the BH rate of oleic acid (C18:1 *cis 9*) and linoleic acid (C18:2 *cis 9, 12*) in diets supplemented with soybean oil, can be explained by the increase in the availability of these acids in the ruminal environment due to lipid supplementation (Loor et al., 2002). The effect of the BH in modifying the profile of FA consumed can be observed by the increased flow of duodenal SFA and of FA derived from BH, such as C18:1 *trans* 6, C18:1 *cis* 6, C18: 1 *trans* 9, C18:1 *trans* 11 and C18:1 *cis* 9 *trans* 11.

Previous studies conducted by Fontes et al. (2009), suggested that the subgroup *Butyrivibrio* SA would have a marginal role in the production of C18:0, and that its metabolic activity may not be proportional to the microorganism concentration in the medium. However, in our study, the animals supplemented with soybean oil presented an increase in the population of *Butyrivibrio* SA. These diets also enabled a higher intake of linoleic and oleic acids, which were frequently saturated and converted into C18:0 during ruminal BH; this would entail a higher activity of part of this group of *Butyrivibrio*.

Although diets with soybean oil enabled a high intake of linoleic acid (>200 g/d), the duodenal flow of conjugated linoleic acid (CLA) (C18:1 *cis* 9, *trans* 11) was negligible (<0.10 g/d); this result may be due to the high proportion of concentrate in the diets, which can provide lower isomerization or higher reduction of FA during the final steps of the BH (Abughazaleh and Jacobson, 2007). Other studies in cattle have also reported that diets with high proportion of concentrate reduce the duodenal flow of CLA (Griinari and Bauman, 1999; Menezes et al., 2010; Fiorentini et al., 2015). Therefore, supplementation with soybean oil or crude glycerine would have no effect on the population of *Butyrivibrio* VA; this is supported by the findings of C18:1 *trans 11* in the duodenal flow with all experimental diets.

The odd-chain fatty acids are derived from the lipids of ruminal microorganisms' membranes; as such, they have been used as markers of duodenal flow of microbial proteins (Kim et al., 2002; Vlaeminck et al., 2005). The association of crude glycerine and soybean oil enabled a higher duodenal flow of the odd-chain fatty acids C13:0, C15:0 and C17:0, suggesting that these diets are probably associated with a higher microbial flow.

Intestinal digestibility is an important parameter to be considered in order to manipulate the FA composition that will be further deposited in meat and milk; and in general, depends on the chain length and in the degree of saturation of these

FA (Scollan et al., 2003). The supplementation with soybean oil enabled a higher intestinal digestibility of the FA C18:1 *cis* 6, C18:1 *trans* 9, and C18:2 *trans* 9, *trans* 12, demonstrating that the higher availability of FA may entail a higher intestinal absorption (Wang et al., 2013).

5. Conclusions

The association of crude glycerine and soybean oil in the diet of Nellore steers restricted the BH of UFA, and increased the duodenal flow of these FA. Therefore, this association may be a nutritional strategy that would increase the deposition of healthy UFA in the meat.

Conflict of interest

None.

Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP, grants 2013/23851-6 and 2015/17966-0) and National Counsel of Technological and Scientific Development (CNPq), for providing financial support.

References

AFRC, 1993. Agricultural and Food Research Council. Energy and Protein Requirements of Ruminants. An Advisory Manual Prepared by the AFRC Technical Committee on Responses to Nutrients. CAB Int., Wallingford, Oxfordshire, UK.
AOAC, 1990. Official Methods of Analysis, 15th. ed. Association of Official Analytical Chemists, Arlington, VA, USA.
Abughazaleh, A.A., Jacobson, B.N., 2007. The effect of pH and polyunsaturated C18 fatty acid source on the production of vaccenic acid and conjugated linoleic acids in ruminal cultures incubated with docosahexaenoic acid. Anim. Feed Sci. Technol. 136, 11–22.
Allen, M.S., 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. J. Dairy Sci. 83, 1598–1624.
Bartoň, N., Bureš, D., Homolka, P., Jančík, F., Marounek, M., Rehák, D., 2013. Effects of long-term feeding of crude glycerine on performance carcass traits, meat quality, and blood and rumen metabolites of finishing bulls. Livest. Prod. Sci. 155, 53–59.
Castagnino, P.S., Messana, J.D., Fiorentini, G., Jesus, R.B., San Vito, E., Carvalho, I.P.C., Berchielli, T.T., 2015. Glycerol combined with oils did not limit biohydrogenation of unsaturated fatty acid but reduced methane production in vitro. Anim. Feed Sci. Technol. 201, 14–24.
Denman, S.E., McSweeney, C.S., 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulotic bacterial population within the rumen. FEMS Microbiol. Ecol. 58, 572–582.
Edwards, H.D., Anderson, R.C., Miller, R.K., Taylor, T.M., Hardin, M.D., Smith, S.B., Krueger, N.A., Nisbet, D.J., 2012. Glycerol inhibition of ruminal lipolysis *in vitro*. J. Dairy Sci. 95, 5176–5181.
Edwards, H.D., Anderson, R.C., Taylor, T.M., Miller, R.K., Hardin, M.D., Nisbet, D.J., Krueger, N.A., Smith, S.B., 2013. Interactions between oil substrates and glucose on pure cultures of ruminal lipase-producing bacteria. Lipids 48, 749–755.
Fay, J.P., Jakobera, K.D., Cheng, K.J., Costerton, J.W., 1990. Esterase activity of pure cultures of rumen bacteria as expressed by the hydrolysis of p-nitrophenylpalmitate. Can. J. Microbiol. 36, 585–589.
Fievez, V., Vlaeminck, B., Jenkins, T., Enjalbert, F., Doreau, M., 2007. Assessing rumen biohydrogenation and its manipulation in vivo in vitro, in situ. Eur. J. Lipid Sci. Technol. 109, 740–756.
Fiorentini, G., Carvalho, I.P.C., Messana, J.D., Canesin, R.C., Castagnino, P.S., Lage, J.F., Arcuri, P.B., Berchielli, T.T., 2015. Effect of lipid sources with different fatty acid profiles on intake, nutrient digestion and ruminal fermentation of feedlot Nellore steers. Asian Aust. J. Anim. Sci. 28 (11), 1583–1591.
Fojan, P., Jonson, P.H., Petersen, M.T.N., Petersen, S.B., 2000. What distinguishes an esterase from a lipase: a novel structural approach. Biochimie 82 (11), 1033–1041.
Fuentes, M.C., Calsamiglia, S., Cardozo, P.W., Vlaeminck, B., 2009. Effect of pH and level of concentrate in the diet on the production of biohydrogenation intermediates in a dual-flow continuous culture. J. Dairy Sci. 92, 4456–4466.
Griinari, J.M., Bauman, D.E., 1999. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: Yurawecz, M.P., Mossoba, M., Kramer, J.K., Nelson, G., Pariza, M.W. (Eds.), Advances in Conjugated Linoleic Acid Research. AOCS Press, Champaign, IL, USA, pp. 180.
Harfoot, C.G., Hazlewood, G.P., 1997. Lipid metabolism in the rumen. In: Hobson, P.N., Stewart, C.S. (Eds.), The Rumen Microbial Ecosystem. Blackie Academic and Professional Press, London, UK, pp. 382–419.
Harvatine, K.J., Allen, M.S., 2006. Fat supplements affect fractional rates of ruminal fatty acid biohydrogenation and passage in dairy cows. J. Nutr. 136, 677–685.
Hobson, P.N., Summers, R., 1967. The continuous culture of anaerobic bacteria. J. Gen. Microbiol. 47, 53–65.
Jenkins, T.C., Bridges, W.C., 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. Eur. J. Lipid Sci. Technol. 109, 778–789.
Kim, E.J., Evans, R.T., Tweed, J.K.S., Davies, D.R., Merry, R.J., Dewhurst, R.J., 2002. Odd-chain fatty acids as markers of the microbial colonization of freshly-ingested grass and microbial colonization of Dacron bag residues. Brit. Soc. Anim. Sci., 50.
Koike, S., Kobayashi, Y., 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microb. Lett. 204, 361–366.
Krueger, N.A., Anderson, R.C., Tedeschi, L.O., Callaway, T.R., Edrington, T.S., Nisbet, D.J., 2010. Evaluation of feeding glycerol on free-fatty acid production and fermentation kinetics of mixed ruminal microbes in vitro. Bioresour. Technol. 101, 8469–8472.
Loor, J.J., Herbein, J.H., Jenkins, T.C., 2002. Nutrient digestion biohydrogenation, and fatty acid profiles in blood plasma and milk fat from lactating Holstein cows fed canola oil or canola. Anim. Feed Sci. Technol. 97, 65–82.
Lourenço, M., Ramos -Morales, E.R., Wallace, J., 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. Animal 4 (7), 1008–1023.
Maia, M.R., Chaudhary, L.C., Figueres, L., Wallace, R., 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Ant. Van Lee 91, 303–314.
Maia, M.R., Chaudhary, L.C., Bestwick, C.S., Richardson, A., McKain, N., Larson, T.R., Graham, I.A., Wallace, R., 2010. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. BMC Microbiol. 10, 52.
Menezes, L.F.G., Kozloski, G.V., Restle, J., Brondani, I.L., Pazdiora, R.D., Cattelam, J., 2010. Profile of ingested fatty acids and in the duodenal digest of steers fed different diets. Rev. Bras. Zootec. 39, 2502–2511.
Oliveira, S.G., Berchielli, T.T., Pedreira, M., Primavesi, O., Frighetto, R., Lima, M., 2007. Effect of tannin levels in sorghum silage and concentrate supplementation on apparent digestibility and methane emission in beef cattle. Anim. Feed Sci. Technol. 135, 236–248.

Palmquist, D.L., Jenkins, T.C., 2003. Challenges with fats and fatty acid methods. J. Anim Sci. 81 (12), 3250–3264.
Paiva, P.G., Del Valle, T.A., Jesus, E.F., Bettero, V.P., Almeida, G.F., Bueno, I.C.S., Bradford, B.J., Rennó, F.P., 2016. Effects of crude glycerin on milk composition, nutrient digestibility and ruminal fermentation of dairy cows fed corn silage-based diets. Anim. Feed Sci. Technol. 212, 136–142.
Prins, R.A., Lankhorst, A., Van Der Meer, P., Van Nevel, C.J., 1975. Some characteristics of *Anaerovibrio lipolytica:* a rumen lipolytic organism. Ant. Van Lee 41, 1–11.
R Core Team, 2015. R: A Language and Environment for Statistical. R Foundation for Statistical Computing, Vienna, Austria.
Salter, A.M., 2013. Dietary fatty acids and cardiovascular disease. Animal 7 (Suppl. S1), 163–171.
Shin, J.H., Wang, D., Kim, S.C., Adesogan, A.T., Staples, C.R., 2012. Effects of feeding crude glycerin on performance and ruminal kinetics of lactating Holstein cows fed corn silage or cottonseed hull-based, low-fiber diets. J. Dairy Sci. 95, 4006–4016.
Scollan, N.D., Lee, M.R.F., Enser, M., 2003. Biohydrogenation and digestion of long chain fatty acids in steers fed on *Lolium perenne* bred for elevated levels of water-soluble carbohydrate. Anim. Res. 52, 501–511.
Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.
Valente, T.N.P., Detmann, E., Valadares Filho, S.C., Cunha, M.D., Queiroz, A.C., Sampaio, C.B., 2011. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. Rev. Bras. Zootec. 40 (3), 666–675.
Vlaeminck, B., Dufour, C., Van Vuuren, A.M., Cabrita, A.R.J., Dewhurst, R.J., Demeyer, D., Fievez, V., 2005. Use of odd and branched-chain fatty acids in rumen contents and milk as a potential microbial marker. J. Dairy Sci. 88, 1031–1042.
Wang, T.Y., Liu, M., Portincasa, P., Wang, D.Q.-H., 2013. New insights into the molecular mechanism of intestinal fatty acid absorption. Eur. J. Clin. Invest. 43 (11), 1203–1223.
Yang, S.L., Bu, D.P., Wang, J.Q., Hu, Z.Y., Li, D., Wei, H.Y., Zhou, L.Y., Loor, J.J., 2009. Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows. Animal 3, 1562–1569.