JOÃO PAULO PACHECO RODRIGUES

SOYBEAN OIL SUPPLEMENTATION FOR CATTLE: EFFECTS ON IN VITRO DIGESTION, DAIRY CATTLE PERFORMANCE, AND MILK FAT COMPOSITION

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

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APPROVED: February 21, 2017.

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BIOGRAPHY

João Paulo Pacheco Rodrigues, son of Wanderley Rodrigues and Dora Valéria Pacheco Rodrigues was born in Juiz de Fora/MG, Brazil on November 5, 1988.

Started his undergraduate degree in Animal Science at the *Universidade Federal de Viçosa* in 2007 and obtained a Bachelor degree in Animal Science in 2011. In the same year, he started the *Magister Scientiae* course at *Universidade Federal de Viçosa*. In March of 2013 he obtained the *Magister Scientiae* degree in Animal Science.

In April of 2013 he started his *Doctor Scientiae* course in Animal Science at Universidade Federal de Viçosa.

From December 2015 until September 2016 he was a scholar at the Department of Agricultural Research for Northern Sweden of the Swedish University of Agricultural Sciences, Umeå (Sweden) where he did a part of this thesis.

On February 21th of 2017 he submitted his thesis to the Animal Science Graduate program of the *Universidade Federal de Viçosa* to obtain the *Doctor Scientiae* degree in Animal Science.

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ABSTRACT

RODRIGUES, João Paulo Pacheco, D.Sc., Universidade Federal de Viçosa, February, 2017. **Soybean oil supplementation for cattle: effects on in vitro digestion, dairy cattle performance, and milk fat composition.** Adviser: Marcos Inácio Marcondes. Co-advisers: Luciana Navajas Rennó and Edenio Detmann.

The present work was a compilation of three studies evaluating soybean oil (SBO) as fat supplement for dairy cows. The first study aimed to to evaluate the effects of SBO supplementation with different forages on *in vitro* gas production kinetics, methane (CH_4) emissions and potentially digestible neutral detergent fibre (pdNDF) digestibility (IVpdNDFD). Samples of whole-crop maize silage (MS), sugarcane (SC), ryegrass (RG), brachiaria grass (BG) and guinea grass (GG) were incubated for 72h with three concentrations of SBO (0, 30 and 60 g/kg of dry matter (DM)) in a fully automated *in vitro* gas system. Three runs were conducted using buffered rumen fluid from three cows. The SBO increase affected acetate proportion in VFA, acetate to propionate ratio, and total gas production at 72h differently according to the forage. Soybean oil inclusion did not affect total gas production for RG and BG; however, decreased quadratically for MS and SC and increased quadratically for BG. The SBO quadratically decreased CH_4 emission both as mL/g of OM and % of total gas production. The IVpdNDFD decreased 21.2 and 12.9% when SBO level was increased for MS and SC, respectively, without showing any effects for GG, BG and RG. In conclusion MS and SC are more sensitive to adverse effects of SBO addition from 30 to 60 g kg^{-1} MS on rumen digestion of pdNDF than RG, GG and BG. The second study aimed to quantify the productive and metabolic responses, and digestive changes in dairy cows fed various concentrations of SBO in high concentrate, sugarcane-based diets. Eight rumen-cannulated multiparous Holstein cows (body weight (BW) = 574 ± 19.1 kg and 122 ± 6.9 days in milk), averaging 22.5 ± 1.22 kg/d of milk were assigned to replicated 4 × 4 Latin squares. The experimental period lasted 21 d as follows: 14 d for adaptation, followed by a sampling period from d 15 to 21. The diets were formulated with increasing concentrations of SBO (% of DM): control (0%), low (LSBO; 1.57%), medium (MSBO; 4.43%) and high (HSBO; 7.34%). Dry matter intake (DMI) decreased quadratically in response to SBO addition. The greatest decrease in DMI was observed in MSBO and HSBO diets. Both milk and energy corrected milk (ECM) yield were quadratically affected by the SBO inclusion, with a slight decrease up to MSBO and substantial decrease in the HSBO diet. The milk fat concentration linearly decreased from 3.78% in the control to 3.50% in the HSBO diet. The potentially digestible neutral detergent fiber digestibility in the rumen decreased from

55.7% in the control to 35.2% in the HSBO diet. The fractional rate of digestion of potentially digestible neutral detergent fiber in the rumen decreased linearly from 3.13 to 1.39%/h from the control to HSBO diet. The fractional rate of indigestible neutral detergent fiber passage in the rumen was quadratically affected, with the lowest value (2.25%/h) for the HSBO diet. Rumen pH increased from 6.42 to 6.67, and ammonia nitrogen decreased from 28.1 to 21.4 mg/dL, in the control and HSBO diets, respectively. Rumen volatile fatty acids decreased quadratically, with the greatest decrease observed in MSBO and HSBO diets. Serum concentrations of glucose, non-esterified fatty acids and beta-hydroxybutyrate were unaffected by SBO inclusion. However, serum concentrations of total cholesterol and high-density and low-density lipoproteins linearly increased with increasing concentrations of SBO in the diet. Soybean oil supplementation at 1.57% of the diet DM proved to be a safe concentration for dairy cows fed high concentrate diets with sugarcane as the sole forage. Inclusion of SBO at concentrations from 4.43 to 7.34% of the diet DM decreased DMI, ECM production, fiber digestibility and rumen fermentation being not recommended. The third study aimed to investigate the effects of increasing concentrations of SBO in sugarcane-based diets on omasal digesta and milk fatty acids (FA) profile, focusing on conjugated linoleic acid (CLA) in milk. The diets were the same reported for the second study (control, LSBO, MSBO and HSBO) Experiment 1 (EXPI) was designed to quantify the flow of FA to the omasum and milk FA profile. In EXPI, eight rumen-cannulated multiparous Holstein cows averaging 574 ± 19·1 kg BW and 122 ± 6·9 days in milk were used in a replicated 4 × 4 Latin square design. Experiment 2 (EXPII) was designed to quantify the milk FA profile in cows after calving. In EXPII 14 primiparous (545 ± 17·2 kg BW) and eight multiparous (629 ± 26·7 kg BW) Holstein cows were used after calving. The concentration of 11*trans*-18:1, 9*cis*,11*trans*-18:2 CLA, and 9*cis*,11*trans*-18:2 CLA in omasal digesta increased linearly with the addition of SBO. Soybean oil increased quadratically the biohydrogenation rate of polyunsaturated FA (PUFA), with a slightly increase from MSBO to HSBO. Milk fat concentration in both experiments decreased with SBO supply; however, fat yield was not affected in EXPII. In both experiments, the concentration (g/kg) of 9*cis*,11*trans*-18:2 CLA in milk increased linearly in despite of the yield (g/d) increased quadratically. 10*trans*,12*cis*-18:2 CLA in milk was detected in MSBO and HSBO diets. The addition of SBO proved to be a feasible alternative to increase the rumen outflow of 11*trans*-18:1 and milk 9*cis*,11*trans*-18:2 CLA concentration; however, SBO supplementation greater than 15.7 g SBO/kg DM decreases milk and fat yield without additive response on 9*cis*,11*trans*-18:2 CLA yield.

RESUMO

RODRIGUES, João Paulo Pacheco, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Suplementação com óleo de soja para bovinos: Efeitos sobre a digestão in vitro, desempenho de gado de leite e composição da gordura do leite.** Orientador: Marcos Inácio Marcondes. Coorientadores: Luciana Navajas Rennó e Edenio Detmann.

O presente trabalho foi desenvolvido com base em três estudos avaliando o óleo de soja (OS) como suplemento lipídico para vacas leiteiras. O primeiro estudo objetivou avaliar os efeitos da suplementação com OS em diferentes forragens sobre a produção de gás, emissão de metano (CH_4) e digestibilidade da fibra em detergente neutro potencialmente digestível (IVFDNpdD) *in vitro*. Amostras de silagem de milho de planta inteira (SM), cana-de-açúcar (CA), Azevem (AZ), capim brachiaria (CB) e capim mombaça (CM) foram incubadas por 72 horas com três concentrações de OS (0, 30 e 60 g/kg de matéria seca (MS)) em um sistema *in vitro* totalmente automatizado. O aumento do OS afetou a proporção de acetato nos ácidos graxos voláteis (AGV), a relação acetato:propionato e a produção total de gás as 72 horas diferentemente de acordo com a forragem. A inclusão de OS não afetou a produção de gás para AZ e CM, no entanto a mesma decresceu quadraticamente para SM e CA e aumentou quadraticamente para BG. O OS diminuiu a emissão de CH_4 tanto em mL/g de matéria orgânica (MO) quanto em % da produção de gás total. A IVFDNpdD diminuiu 21,2 e 12,9% quando o OS foi aumentado para SM e CA, respectivamente, sem efeitos significativos para AZ, CB e CM. Conclui-se que SM e CA são mais susceptíveis aos efeitos adversos da suplementação com OS de 30 a 60 g/kg de MS sobre a IVFDNpdD do que AZ, CM e CB. O segundo estudo objetivou quantificar as alterações no desempenho, digestão e metabolismo de vacas leiteiras alimentadas com concentrações de OS e dietas com cana-de-açúcar e alto nível de concentrado. Foram utilizadas oito vacas multíparas da raça Holandês (peso vivo (PV) = 574 ± 19,1 kg e 122 ± 6,9 dias em lactação) fistuladas no rúmen. Os animais tinham produção de leite inicial de 22,5 ± 1,22 kg/dia e foram distribuídas em dois quadrados latinos 4 × 4 paralelos. O período experimental durou 21 dias, sendo os 14 primeiros para adaptação, seguidos de um período de coletas entre os dias 15 e 21. As dietas foram formuladas com concentrações crescentes de OS (% da MS): controle, baixo (BOS; 1,57%), médio (MOS; 4,43%) e alto (AOS; 7,34%). O consumo de matéria seca (CMS) diminuiu quadraticamente em resposta ao aumento de OS. O maior decréscimo no CMS foi observado entre as dietas MOS e AOS. Tanto a produção de leite e produção de leite corrigida para energia foram quadraticamente afetadas pela inclusão de OS, com um menor decréscimo até o MOS e maior decréscimo na dieta AOS. A concentração de

gordura no leite diminuiu de 3,78% no controle para 3,50% na dieta AOS. A digestibilidade ruminal da fibra em detergente neutro potencialmente digestível (FDNpd) diminuiu de 55,7% no controle para 35,2% na dieta AOS. A taxa de digestão da FDNpd no rúmen diminuiu de 3,13 para 1,39%/h do controle para a dieta AOS. A taxa de passagem da fibra em detergente neutro indigestível foi afetada quadraticamente, com valor menor (2,25 %/h) na dieta AOS. O pH ruminal aumentou de 6,42 para 6,67 e o nitrogênio amoniacal ruminal diminuiu de 28,1 para 21,4 mg/dL do controle para a dieta AOS, respectivamente. A concentração ruminal de AGV diminuiu quadraticamente com o maior decréscimo observado entre as dietas MOS e AOS. As concentrações séricas de glicose, ácidos graxos não esterificados e beta-hidroxi-butirato não foram afetadas pela inclusão de OS. No entanto as concentrações séricas de colesterol total, HDL e LSL diminuíram linearmente com o aumento da concentração de OS na dieta. A suplementação com OS até 1,57% da MS demonstrou ser uma concentração segura para vacas alimentadas com alto nível de concentrado e cana-de-açúcar como volumoso. A inclusão de óleo de soja entre 4,43 a 7,34% da MS da dieta diminuiu o CMS, produção de leite, a digestibilidade da fibra e a fermentação ruminal, não sendo recomendada. O terceiro estudo objetivou avaliar os efeitos do aumento das concentrações de OS sobre o perfil de ácidos graxos (AG) da digesta omasal e no leite de vacas alimentadas com com cana-de-açúcar, com foco sobre a concentração de ácido linoleico conjugado (CLA) no leite. Foram realizados dois experimentos com as mesmas dietas anteriormente descritas para o segundo estudo (controle, BOS, MOS e AOS). O experimento 1 (EXPI) foi delineado para quantificar o fluxo omasal de AG e o perfil de AG no leite. No EXPI foram utilizadas oito vacas multíparas da raça Holandês (PV = 574 ± 19,1 kg e 122 ± 6,9 dias em lactação) fistuladas no rúmen. Os animais tinham produção de leite inicial de 22,5 ± 1,22 kg/dia e foram distribuídas em dois quadrados latinos 4 × 4 paralelos. O experimento 2 (EXPII) foi delineado para quantificar o perfil de AG no leite de vacas no pós-parto. No EXPII foram utilizadas 14 vacas da raça Holandês primíparas (PV= 545 ± 17,2 kg) e oito multíparas (PV=629 ± 26,7 kg) após o parto. As concentrações de AG 11*trans*-18:1, 9*cis*,11*trans*-18:2 CLA e 9*cis*,11*trans*-18:2 CLA na digesta omasal aumentaram linearmente com o aumento de OS. O aumento no OS aumentou quadraticamente a taxa de biohidrogenação de AG poli-insaturados (PUFA), com um aumento pequeno da dieta MOS para AOS. A concentração de gordura no leite em ambos experimentos diminuiu com o aumento de OS, no entanto a produção de gordura não foi afetada no EXPII. Em ambos experimentos a concentração (g/kg) de 9*cis*,11*trans*-18:2 CLA no leite aumentou linearmente, diferentemente do aumento quadrático da produção (g/d) que aumentou

quadraticamente. A presença de 10*trans*,12*cis*-18:2 CLA no leite foi detectada nas dietas MOS e AOS. A adição de OS demonstrou ser uma alternativa viável para aumentar o fluxo omasal de 11*trans*-18:1 e a concentração de 9*cis*,11*trans*-18:2 CLA no leite. No entanto a suplementação com OS acima de 1,57% da MS da dieta diminuiu a concentração de gordura no leite e a produção de gordura, sem resposta aditiva na produção de 9*cis*,11*trans*-18:2 CLA.

GENERAL INTRODUCTION

The effects of fat supplementation on rumen metabolism in dairy cows have been widely studied (Lerch *et al.*, 2012; Piantoni *et al.*, 2015). Vegetable oils are rich in unsaturated fatty acids (UFA), that are highly hydrolyzed in the rumen and usually decrease ruminal methane (CH_4) emissions which is a source of energy waste during the digestion process and has a greenhouse gas effect (Takahashi, 2001; Jenkins & Harvatine, 2014). Due to its high concentration of UFA, soybean oil (SBO) has direct effects on rumen protozoa and cellulolytic bacteria and consequently decrease in CH_4 emissions and digestibility of fibre fractions (Yang *et al*., 2009; Waldo *et al.*, 2016; Weld & Armentano, 2017). In this way diets are formulated based on recommendations of maximum supplementary fat concentration of 4–5% DM (Lewis *et al.*, 1999) or 6–7% ether extract (EE) in diet DM (NRC, 2001). However, previous research has indicated that the forage source can determine the response of fat supplementation on fibre digestion. For instance, Salem *et al.* (1993) adding rapeseed oil at 6.6% of diet dry matter (DM) observed a lower reduction in grass hay NDF digestibility when compared with corn silage. Incorporating linseed oil at 4% DM, Benchaar *et al.* (2015) observed a reduction in neutral detergent fibre (NDF) digestibility in corn silage diets but no in red clover silage diets. Bateman II & Jenkins (1998) included SBO up to 8% DM in diets with Bermuda grass hay and did not observe any effects on fibre digestibility.

Anatomical and chemical differences in cell wall structure between forage species are the main factors controlling the digestion kinetics of cell wall fractions in the rumen (Akin, 1993; Kasuya *et al.*, 2008). The availability, rate of digestion (k_d) and digestibility of potentially digestible neutral detergent fibre (pdNDF) in fat supplemented diets are totally dependent on characteristics of the forage specie. These factors are related to the capacity of biohydrogenation rate, which can decrease both the CH_4 emissions by the consumption of hydrogen and the drop in methanogenic microorganisms' populations by

interaction of UFA and its membranes (Bateman II & Jenkins, 1998; Machmüller *et al.*, 1998). Despite the indications that the forage has a main role in the effects of oil supplementation on fibre digestibility, few studies have been conducted aiming to investigate the magnitude of this response in different forages (Benchaar *et al.*, 2015; Weld & Armentano, 2017).

Sugarcane is a forage commonly fed to beef and dairy cattle in tropical environments such as Brazil and often used as the main dietary fibre source (Gomes, 2006; Oliveira and Millen, 2014). It has excellent feeding potential for dairy cows because of its high productivity and wide harvesting window (Daniel *et al.*, 2014). However, its use for high yielding dairy cows is usually associated with feeding high amounts of concentrates, leading to a high proportion of non-fibre carbohydrates (NFC) in the diet (Oliveira *et al.*, 2011). Furthermore, sugarcane fibre contains a high proportion of indigestible neutral detergent fibre (iNDF) and low pdNDF at about 25 and 27% DM, respectively (Daniel *et al.*, 2014); that is associated with a low dry matter intake (DMI). Consequently, more concentrate is needed compared to corn silage-based diets (Oliveira *et al.*, 2011). Under these feeding situations, fat supplementation can be an alternative to decrease diet NFC and potentially increase energy intake. However, the optimal inclusion rate of added fat, in diets with sugarcane and other tropical forages, needs to be defined.

Studies evaluating changes in milk FA profile encompassing alterations in dairy cows' diets have been made in recent years and the supplementation with oils rich in UFA such as SBO proved to be a feasible strategy to increase milk conjugated linolenic acid (CLA) concentration (Shingfield & Wallace, 2014). The CLA isomers have shown effects on human metabolism and health. Studies demonstrated that the intake of 9*cis*,11*trans*-18:2 CLA by humans is beneficial and decreases the risk of diseases such as cancer, obesity, and immune disorders (Salter, 2013; Kanwar *et al*., 2016). Dairy products are an

important source of CLA in human nutrition and a better understanding of rumen biohydrogenation of UFA is key to increase CLA concentration in cow's milk.

Based on indications that the effects of fat supplementation on ruminal digestion can be dependent on the forage specie the Chapter 1 was designed to quantify the effects of SBO supplementation on *in vitro* gas production kinetics, CH_4 emissions and fibre digestibility of whole crop maize silage, sugarcane, ryegrass, guinea grass and brachiaria grass.

Based on the lack of data about fat supplementation of sugarcane based diets with vegetable oils on performance, digestion and milk FA in dairy cows the objectives of the chapters 1 and 2 were:

Chapter 2 - Quantify the productive, nutritional and metabolic responses of dairy cows fed different concentrations of SBO in high concentrate diets with sugarcane as the sole forage.

Chapter 3 - Investigate the inclusion of SBO at concentrations higher than conventional on ruminal lipid metabolism and milk FA profile of cows fed sugarcane-based diets.

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CHAPTER 1

Running Head: Forage and soybean oil interactions *in vitro*

Effect of soybean oil supplementation and forage type on methane production and fibre digestibility using the *in vitro* gas production system

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Keywords: palisadegrass, whole-crop maize silage, guinea grass, ryegrass, sugarcane, volatile fatty acids

ABSTRACT

We aimed to evaluate the effect of soybean oil (SBO) supplementation with different forages on *in vitro* gas production kinetics, methane (CH_4) emissions and potentially digestible neutral detergent fibre (pdNDF) digestibility (IVpdNDFD). Samples of whole-crop maize silage (MS; *Zea mays*), sugarcane (SC; *Saccharum sp.*), ryegrass (RG; (RG; *Lolium perenne*), guinea grass (GG; *Panicum maximum*), and palisadegrass (PG; *Brachiaria brizantha*) were incubated for 72h with three concentrations of SBO (0, 30 and 60 g kg^{-1} of dry matter) in a fully automated *in vitro* gas system. The interaction between forage specie and SBO inclusion affected molar proportion of acetate, acetate to propionate ratio, asymptotic gas and IVpdNDFD. Molar proportion of acetate decreased linearly for MS; increased quadratically for RG; and increased linearly for GG when SBO was added. Soybean oil inclusion decreased quadratically the asymptotic gas for MS and increased linearly for GG. Soybean oil inclusion decreased quadratically IVpdNDFD for MS and SC, without effects on RG, GG and PG. Methane emission was quadratically affected by SBO in all forages. In conclusion, MS and SC are more sensitive to adverse effects of SBO addition from 30 to 60 g kg^{-1} DM on rumen digestion when compared to RG, GG and PG.

Keywords: guinea grass, neutral detergent fibre, palisadegrass, ryegrass, sugarcane, whole-crop maize silage

1. INTRODUCTION

Methane (CH_4) emissions from ruminants are both a source of energy waste during the digestion and a prominent greenhouse gas (Takahashi, 2001). The effects of unprotected fat inclusion in ruminant diets such as soybean oil (SBO) has been known to decrease ruminal CH_4 emissions (Beauchemin et al., 2009; Mao et al., 2010) and fibre digestion (Weld & Armentano, 2017). Soybean oil is a fat source rich in α-linolenic acid (LNA; C 18:3 *cis*-9, *cis*-12, *cis*-15) which along with its main first biohydrogenation product, linoleic acid (LA; C18:2 *cis*-9, *cis*-12), has a known inhibitory effect on ruminal fibrolytic bacteria and protozoa populations (Maia et al., 2007; Yang et al., 2009; Wang et al., 2016). Despite fat characteristics and concentration in the diet dry mater (DM) are greatly determinant in effects on rumen fermentation and fibre digestion (Palmquist, 1988; Weld & Armentano, 2017). Indications that the forage specie has a relevant role in fat supplementation effects on rumen digestion can be found in the literature (Salem et al., 1993; Benchaar et al., 2015).

Anatomical and chemical differences in cell wall structure among forage species seem to be main factors controlling the digestion kinetics of cell wall fractions in the rumen (Akin, 1993; Kasuya et al., 2008). The extent and rate of digestion of potentially digestible neutral detergent fibre (pdNDF) in fat supplemented diets are totally dependent on the characteristics of the forage species (Akin, 1993). These forages' fibre characteristics directly determine the capacity of biohydrogenation rate, which can be directly related to the effects of fat supplementation both in decreasing CH_4 emissions and fibre digestion (Palmquist, 1988; Machmüller et al., 1998; Beauchemin et al., 2009). However, few studies were conducted to investigate effects of interaction between forage specie and fat supplementation on rumen digestion (Weld & Armentano, 2017).

Aiming to avoid adverse effects on fibre digestion, usually diet formulation for cattle

do not reach fat concentrations greater than supplementary fat at 40 to 50 g kg^{-1} of DM (Lewis et al. 1999) or total fat from 50 to 60 g kg^{-1} of DM (NRC, 2001), regardless the forage specie. However, studies have shown evidence of different fat supplementation effects on fibre digestion according to the forage species, which will likely affect rumen metabolism (Salem et al., 1993; Benchaar et al., 2015). Thus, studies aiming to clarify if fat supplementation interact with forage specie concerning digestion, fermentation and CH_4 emissions are needed.

We hypothesized that changes in kinetics parameters in the *in vitro* gas production system, and decreases in CH_4 emissions and fibre digestion by SBO addition are different according to the forage specie. Our objective was to quantify the effects of SBO supplementation on fermentation, gas production kinetics, CH_4 emissions and fibre digestibility of five forages using a fully automated *in vitro* gas production system.

2. MATERIALS AND METHODS

2.1.Forage samples and treatments

Four samples of monocot forages from Brazil and one from Sweden were chosen to achieve a wide range of forages.

Samples of whole-crop maize silage (MS; *Zea mays* var. SHS-4070) and sugarcane (SC; *Saccharum sp.* var. RB-867515) were obtained from the Dairy Cattle Research Station of the *Universidade Federal de Viçosa* (20°46'46.60"S, 42°51'42.66"W; altitude 745 m, Viçosa, Brazil). A composite sample of guinea grass (GG; *Panicum maximum* Jacq. var. Mombasa) averaging 70 to 80 cm was obtained from three pens in an irrigated pasture (20°40'16.38"S, 43°04'07.26"W altitude 700 m, Viçosa, Brazil) grazed previously for eight years by dairy cows in rotational stocking. Palisadegrass (PG; *Brachiaria brizantha* Stapf var. Marandu) averaging 30 to 40 cm was collected from three pens in a pasture (20°36'52.64"S, 43°07'09.13"W; altitude 713 m, Viçosa, Brazil) grazed previously for four

years by dairy cows in rotational stocking. Both GG and PG were collected in October 2015 and were cut at 50% of total height, which was the management preconized for residual grazing height in both farms. After sampling, the aliquots were immediately transported to the Dairy Cattle Research Station of the *Universidade Federal de Viçosa*, chopped in approximately 4 cm particle size, and oven dried. A standard sample of perennial ryegrass (RG; *Lolium perenne* L.) was obtained from Röbäcksdalen Research Farm (63°48'37.00"N, 63°48'36.75"E; altitude 10 m, Umeå, Sweden). All samples were oven dried at 55°C in a forced air oven and milled though a 1-mm screen.

To evaluate the interaction of forage species and SBO supplementation (Table 1) the factorial scheme was obtained considering five forages (GG; PG, RG, MS and SC) and three amounts of SBO (0; 30 and 60 g kg^{-1} of dry matter (DM)).

2.2.Samples composition

The samples' DM was quantified by AOAC (2005) method 930.15 and organic matter (OM) by method 942.05. Nitrogen content was quantified by AOAC (2005) method 984.13, considering 6.25 as the constant factor to convert nitrogen values to crude protein (CP). Neutral detergent fibre (NDF) was quantified with a heat stable amylase and sodium sulphite (Mertens, 2002). The indigestible neutral detergent fibre (iNDF) content of feeds was quantified by 288 h of in situ incubation using Sefar Petex 07-11/5 bags (Krizsan et al. 2015). The NDF and iNDF concentrations were expressed exclusive of residual ash corrected by incinerating at 500°C for 3 h. The pdNDF concentration was calculated as *NDF – iNDF*. The neutral detergent solubles (NDS) fraction was calculated as *OM – NDF*.

2.3.*In vitro* incubations and gas samplings

All managements involving the donor animals were conducted with the permission of the Swedish Ethical Committee on Animal Research.

Prior to each run, 500 mg of each forage were weighted into six serum bottles (Schott, Mainz, Germany, 250 mL). Three ethanol (96%) stock solutions with SBO at 0, 15 and 30 g L^{-1} were prepared one day before the first run and were kept at 2°C to be used in the following runs. To ensure the absence of ethanol residues, 1 mL of each stock solutions was added in two bottles of each forage twenty-four hours before the beginning of each run. In each run, three serum bottles were assigned as blanks without substrate, in which 1 mL of the ethanol stock solution without SBO was added. A total of three runs were conducted totalling 6 replicates for each treatment.

Rumen fluid averaging 6.3 ±0.10 of pH and 9.3 ±0.91 mg dL^{-1} of ammonia nitrogen (NH_3-N) was collected after morning milking from three cannulated Swedish Red cows fed a grass silage-based diet with a forage-to-concentrate ratio of 60:40. The fluid was kept in pre-warmed (39°C) thermos flasks that were previously flushed with CO_2. Rumen fluid was transported to the laboratory within 15 min, pooled, filtered through four layers of cheesecloth and flushed with CO_2. Filtered rumen fluid was then mixed with buffered mineral solution (Menke & Steingass, 1988) (20:80 v v^{-1}) supplemented with peptone (pancreatic digested casein) (Merck, Darmstadt, Germany) at 39°C with constant stirring and continuous flushing with CO_2. All bottles were then filled with 60 mL of buffered rumen fluid and placed in a water bath at 39°C whilst gently and continuously agitated. The gas production measurement was conducted by a fully automated system, readings were done every 12 min and corrected to the normal air pressure (101.3 kPa) (Cone et al., 1996). More details of the system were described by Ramin & Huhtanen (2012).

2.4.Methane measurements

To estimate CH_4 production, the procedure described by Ramin & Huhtanen (2012) was followed. Briefly, gas samples were taken from each bottle at 24, 48 and 72 h of

incubation by a gas tight syringe (Hamilton, Bonaduz, Switzerland). Methane was quantified by injecting 0.2 mL of gas into a star 3400 (CX series) gas chromatograph (Varian Chromatography, USA) equipped with a thermal conductivity detector (TCD). Separation was achieved using a 1.8 m long stainless steel column packed with Haysept T (80–100 mesh), argon as the carrier gas with a flow rate of 32 mL min^{-1} and an isothermal oven temperature of 32°C. The injector and detector temperatures were set to 110°C and 135°C, respectively. Calibration gas was completed using a standard mixture of CO_2 and CH_4 (100 mmol mol^{-1}) prepared by AGA Gas (AGA Gas AB, Sundbyberg, Sweden). Peaks were identified by comparison with the standard gas.

2.5.Ammonia nitrogen, pH and volatile fatty acids samplings

Samples for NH_3-N analysis (1.2 mL) were taken at 8 and 24 hours after incubation, acidified with 0.32 µL of sulfuric acid (98%) and sored at -20°C until analysis. The NH_3-N was quantified by the colorimetric method described by Chaney & Marbach (1962).

At 72 h of incubation, the bottles were removed from the water bath and pH was measured immediately. After pH measurement, a composite sample of 0.6 mL of each replicate (total 1.2 mL) of fluid were mixed with 0.3 mL of meta-phosphoric acid solution (20%), and stored at -20°C until volatile fatty acids (VFA) analysis. The samples for VFA were centrifuged (12000 × g, 10 min) and the cell-free supernatants were treated as described by Siegfried et al. (1984). Volatile fatty acids were quantified by HPLC (Dionex Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA) coupled to a refractive index (RI) detector (Shodex RI-101) maintained at 40°C and an ion exchange column (Phenomenex Rezex ROA, 300×7.8 mm maintained at 45°C. The mobile phase was prepared with 5 mmol L^{-1} sulfuric acid (H_2SO_4) and the flow rate was 0.7 mL min^{-1}. The following VFA were used for the calibration of the standard curve: acetic, propionic, valeric, isovaleric, isobutyric and butyric

acid. All acids were prepared with a final concentration of 10 mmol L^{-1}, except isovaleric acid (5 mmol L^{-1}) and acetic acid (20 mmol L^{-1}).

2.6.*In vitro* pdNDF digestibility

To evaluate pdNDF digestion, the bottles were put on ice immediately after the end of incubation. To estimate *in vitro* pdNDF digestibility (IVpdNDFD), bottles contents were filtered through Sefar Petex 07-11/5 bags and freezed at -20°C until NDF analysis. The IVpdNDFD was calculated following the equation (Eq. 1):

$$\text{IVpdNDFD (g kg}^{-1}) = \frac{incubated \text{ pdNDF (g)} - residual \text{ pdNDF (g)}}{1000 \times incubated \text{ pdNDF (g)}} \quad (1)$$

2.7.Gas production kinetics and first order digestion rate

Cumulative CH_4 production at each time point was calculated according to the equation (Eq. 2) (Ramin & Huhtanen, 2012):

$$TM = HSVol \times HSmet + GP \times A \times HSmet \quad (2)$$

Where TM = total CH_4 (mL); HSVol = headspace volume (mL); HSmet = headspace CH_4 concentration; GP = gas production (mL) and A is the ratio (0.55) of CH_4 concentration in outflow gas to headspace. The total headspace in the system was 265 mL. The total gas volume was automatically recorded by the system and corrected for the normal air pressure. The CH_4 concentration was quantified with the GC as explained above.

For each treatment, a mean cumulative curve of GP was estimated according to the 2-pool Gompertz function (Eq. 3) (Schofield et al., 1994):

$$GP_{(t)} = V_1 \times e^{\{-e^{[1+k_1 \times e \times (L_1 - t)]}\}} + V_2 \times e^{\{-e^{[1+k_2 \times e \times (L_2 - t)]}\}} \quad (3)$$

where $GP_{(t)}$ = the measured gas volume at time *t*; V_1, k_1, and L_1 = the asymptotic cumulative gas volume, rate and lag parameters for the first pool (rapid); and V_2, k_2, and L_2 = the asymptotic cumulative gas volume, rate and lag parameters for the second pool (slow) and *t* = incubation time (hours). The asymptotic gas production was calculated as the sum of V_1

and V_2. The parameters fitted to the previously described 2-pool Gompertz model were estimated using the NLIN procedure (SAS Institute Inc., Cary, NC, version 9.4) for each bottle.

The first order kinetics digestion rate (k_d) was estimated by dividing the derivative of the cumulative GP function ($GP'_{(t)} = dGP/dt$) by the cumulative gas (Huhtanen et al., 2008). A mean retention time (MRT) of 50 h (20 h in the first compartment and 30 h in the second compartment) corresponding to the maintenance level of feed intake was used (Ramin & Huhtanen, 2012).

2.8.Statistical analyses

The statistical analysis was done using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC, version 9.4), following the model (Eq. 5):

$$Y_{ijkl} = \mu + F_i + O_j + (F \times O)_{ij} + R_k + \varepsilon_{ijkl} \quad (5)$$

where Y_{ijk} is the dependent variable; μ is the overall mean; F_i is the effect of forage; O_j is the effect of SBO level; $(F \times O)_{ij}$ is the effect of interaction of forage and SBO; R_k is the random effect of run; and ε_{ijk} is the random error term.

The effects of SBO levels were evaluated by polynomial linear (Lin.) and quadratic (Quad.) contrasts and results are presented as least squares means. Significance was declared at $p < 0.05$. When the interaction "$(F \times O)_{ij}$" was significant, linear and quadratic effects of SBO were estimated for each forage. When the interaction "$(F \times O)_{ij}$" was not significant and forage specie effect was significant, means were compared using Tukey-Kramer method.

3. RESULTS

3.1.Ammonia nitrogen, pH and volatile fatty acids

No significant interactions of forage species and SBO concentration were observed for NH_3-N (p = .72) and pH (p = .65) (Table 2). Soybean oil did not affect NH_3-N (p = .07) and

pH (p = 0.52; Table 2). The greatest NH_3-N concentrations were observed for RG and GG at 8 and 24 h (p < .05). The lowest NH_3-N was observed for SC at 8 h (p < .05) and SC and for PG at 24 h (p < .05; Table 2). The greatest pH values were observed for MS and GG (p < .05; Table 2).

The VFA concentration was not affected by the interaction of forage species and SBO (p = 0.06; Table 2). The greatest VFA concentrations were 94.7, 89.1 and 89.0 mM L^{-1} observed for PG, MS and SC, respectively (p < .05; Table 2). The interaction between forage species and SBO affected molar proportion of acetate (p = .03) and acetate to propionate ratio (p = .03; Table 2). Molar proportion of acetate decreased linearly for MS (p = .03); increased quadratically for RG (p = .02); and increased linearly for GG (p = .02) when SBO was added (Figure 1). Increased level of SBO did not affect molar proportion of acetate for SC (p = 0.51) and PG (p = 0.56; Figure 1). Acetate to propionate ratio increased linearly for GG (p < .01) when SBO was added; and it was not affected by SBO inclusion for MS (p = .88), RG (p = .56), SC (p = .10), and PG (p = .85; Figure 1). Soybean oil inclusion did not affect molar proportions of propionate (p = .35), butyrate (p = .29), isobutyrate (p = .54), valerate (p = .65) and isovalerate (p = .33). The forage specie affected VFA concentration (p < .01) and molar proportions of butyrate (p < .01), isobutyrate (p < .01), valerate (p < .01), and isovalerate (p < .01; Table 2). Molar proportion of propionate was not affected by forage specie (p = .50; Table 2).

3.2.Gas kinetics, CH_4 and *in vitro* potentially digestible neutral detergent fibre digestibility

The effect of interaction between forage species and SBO affected asymptotic gas production (p < .01) and IVpdNDFD (p < .01), without effects on V_1 (p = .98), V_2 (p = .44), k_1 (p = .99), k_2 (p = .96), L_1 (p = .97), L_2 (p = .57), CH_4 (p = .39) and, first-order k_d (p = .54;

Table 3). Soybean oil inclusion decreased asymptotic gas quadratically for MS ($p < .01$) and linearly for PG ($p < .01$; Figure 2). Asymptotic gas was not affected by SBO inclusion for SC ($p = .22$), RG ($p = .90$), and GG ($p = .28$; Figure 2). The IVpdNDFD decreased quadratically when SBO increased for MS ($p < .01$) and SC ($p < .01$), without effects on GG ($p = .61$), PG ($p = .55$) and RG ($p = .93$; Figure 2). The forage specie affected all gas kinetics parameters ($p <.01$), CH_4 production ($p < .01$), and first-order k_d ($p < .01$; Table 3). Soybean oil inclusion decreased quadratically CH_4 production ($p = .02$; Table 3). The first order k_d was affected by forage specie ($p = <.01$) with greatest value of 0.097 h^{-1} for SC ($p < .05$) followed by 0.079 h^{-1} for MS ($p < .05$; Table 3).

4. DISCUSSION

We hypothesized that the effects of SBO supplementation on gas production, CH_4 emissions and IVpdNDFD are dependent on the forage specie. The results of this study do not reject our hypothesis due to the significant interaction between forage and SBO concentration observed for asymptotic gas production and IVpdNDFD.

4.1. Ammonia nitrogen

The NH_3-N values followed the forages' CP concentration without significant effects of SBO inclusion. In the study by Yang et al. (2009) adding SBO at 40 g kg^{-1} DM in alfalfa (100 g kg^{-1} DM) Chinese wildrye (280 g kg^{-1} DM) and maize silage (220 g kg^{-1} DM) based diets *in vivo* increased rumen NH_3-N concentrations from 6.2 to 7.7 mg dL^{-1} without decrease in ruminal populations of proteolytic bacteria. In our study, the absence of SBO effect on NH_3-N concentrations is probably related to the maintenance of proteolytic bacteria populations, even at 60 g kg^{-1} DM of SBO. The inclusion of vegetable oils rich in LNA have a known effect on decreasing protozoal populations, which can increase NH_3-N concentrations by decreasing bacterial predation by protozoa (Hristov et al., 2009; Yang et

al., 2009). In our study, probably this effect was marginal and does not resulted in significant increases in NH_3-N concentrations, being the forage effect more relevant.

4.2. Volatile fatty acids

Acetate and butyrate are the main VFA products from NDF digestion (Doane et al., 1997). The expected effect of fat supplementation on rumen fermentation pattern is likely related to inhibition of rumen cellulolytic bacteria and fibre digestion and consequently decrease in acetate production (McAllister et al., 1996). The increase in acetate to propionate ratio when adding SBO to GG is directly associated to the increase in acetate proportion in total VFA and was an unexpected result, since IVpdNDFD was not affected. Controversial results of fat supplementation on VFA can be found in the literature. Fiorentini et al. (2013) comparing rumen protected fat and SBO inclusion at 26 g kg^{-1} DM in corn silage (600 g kg^{-1} DM) based diets observed lower molar proportions of acetate without differences for propionate. In contrast, Yang et al. (2009) reported increased molar proportions of propionate and butyrate whereas the acetate was not affected by SBO and linseed oil inclusion for dairy cows fed a mixture of alfalfa, Chinese wildrye and maize silage diet. Ruppert et al. (2003) supplementing tallow at 0, 20 and 40 g kg^{-1} DM observed a linear decrease in ruminal proportions of acetate for maize silage and no effects for alfalfa silage based diets. The same authors observed a linear increase in rumen propionate for both forages. In a meta-analysis evaluating sheep data with diverse forages, Patra (2014) observed that fat supplementation does not significantly affects total VFA concentrations and proportions of acetate. The controversial results of fat supplementation in different forages observed in our study and in the literature, indicates that there is no standard response to fat supplementation on proportions of acetate or other VFA such as propionate. It can be also dependent on the forage specie as hypothesized in this study. It is important to note that NDF is a mixture of polymers

with variable sugar composition among forage species, which may change the digestion process (Doane et al., 1997). For instance, if xylose is fastest digested than arabinose, the proportions of these polymers in a forage will change VFA molar proportion during the digestion. Thus, the effect of SBO in each forage species will also depend on the effect of SBO on digestion of each polymer present in pdNDF. Beside this, it is also known that fat supplementation may have different effects on different populations of ruminal fibrolytic bacteria species (Wang et al., 2016). Thus, the combinations between NDF composition, digestion rate and extent, and ruminal bacteria species allows numerous possibilities of fermentation pathways. Studies with deep characterization of NDF physio-chemical composition, microbial populations, and fermentation steps are needed to clarify those interactions. In our study, probably the different carbohydrate composition of the forages explains the significance of forage effect on VFA concentration and molar proportions of VFA.

4.3.*In vitro* gas production kinetics

It can be conceptually interpreted that forages' NDS and pdNDF fractions are closely related to the rapidly and slowly degraded pools, respectively. However due to the high variability between the forages compositions those interpretations should be done carefully. The greater V_1 and k_1 for SC probably are a consequence of the high sucrose concentration in this forage, which is highly fermentable in the rumen (Hall and Weimer, 2007). The greater V_1 values for MS and SC are closely related to the high NDS concentration of these forages, however GG presented values of V_1 not different from those of MS and SC. It's possible that due to the early vegetative stage of GG, fractions of gas released from pdNDF digestion were accounted in parameters of the first pool in the model. The observed negative values of L_1 are not biologically consistent, however the original values were kept for a better model fit

to the gas data (Ramin & Huhtanen, 2012). The asymptotic gas decrease for MS is most likely related to the decrease in IVpdNDFD, which was not affected by SBO for GG, PG, and RG. Wang et al. (2016) also observed a decrease in total gas production with 36h of incubation of an artificial diet (starch, casein and cellulose) when SBO was added. The absence of effect of SBO inclusion in asymptotic gas for SC even with decrease in IVpdNDFD probably is due to the lower pdNDF concentration, and the gas produced by pdNDF digestion was less representative in SC. The linear increase in asymptotic gas for PG was unexpected since IVpdNDFD and kinetics parameters were not affected by SBO. The effect of interaction between forage specie and SBO was not significant for total VFA, however numeric values of total VFA for PG increased similarly to asymptotic gas as 85.0, 101.5 and 97.5 mmol L^{-1} for SBO at 0, 30 and 60 g kg^{-1} DM, respectively. Yang et al. (2009) observed decreased ruminal populations of *Ruminococcus albus* and *Fibrobacter succinogenes* feeding SBO at 40 g kg^{-1} DM without effects on amount of *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens*. The numerical increase in total VFA and asymptotic gas for PG probably can be consequence of two factors. Primarily, the selectively effect of SBO on bacterial populations can be different according to the substrate characteristics such as structural polymers composition, amino acids proportions, and non-fibre carboydrates composition (Doane et al., 1997; Tedeschi et al., 2001; Moraes et al., 2013). Secondly, considering that IVpdNDFD was not affected for PG, the SBO selective effects on microbial populations promoted different interactions between bacterial groups although cross-feeding, enhancing non-fibre carbohydrates digestion and/or changing fermentation pathways (Russell, 2002).

The first order k_d was higher at forages with higher NDS concentration and it can be consequence of the higher fast fermentable content in OM and estimation using the same

MRT for all forages, which can be different under *in vivo* conditions. Considering that the ether extract concentration in forages are generally less than 40 g kg^{-1}, the major components of NDS in forages are fast-digesting carbohydrates such as starch and sugars and pectic substances (Schofield & Pell, 1995).

4.4.Methane

Archaea are the exclusive methanogens in the rumen and its communities are relatively constant across diets changes (Lillis et al., 2011; Henderson et al., 2015). Supplementary fat can inhibit CH_4 emission by reducing the activity of rumen methanogens and number of bacteria and protozoa (Beauchemin et al., 2009; Lillis et al., 2011), decreasing the amount of fermentable substrate and through biohydrogenation of unsaturated fatty acids (UFA) (Johnson & Johnson, 1995). However, the biohydrogenation of UFA is not quantitatively relevant and has a low potential of CH_4 reduction when compared to the decrease microbial activity and reduction of fermentable substrate (Ramin & Huhtanen, 2013). In our study, the SBO was supplementary and the amount of incubated forage was the same in all SBO concentrations to keep the same amount of fermentable substrate between SBO levels. Thus, changes in bacterial and protozoa populations and archaea activity should be considered as main driven effects of our results.

Lillis et al. (2011) comparing SBO supplementation at 60g kg^{-1} DM in high concentrate (500 g kg^{-1} DM) and low concentrate (100 g kg^{-1} DM) diets observed that SBO inclusion promoted greater effects on bacteria rather than in archaea diversity, however SBO effects markedly decreased the population of both in the high concentrate diet. Wang et al., (2016) observed that SBO inclusion at 40 g kg^{-1} DM decreased the activity of coenzyme F_{420} hydrogenase *in vitro*, which can be used as an indicator of methanogen activity. In our study, the effects of SBO on CH_4 were more prominent with SBO at 60 g kg^{-1} DM. Considering

that our treatments were exclusively of concentrate, the results observed by Lillis et al. (2011) for low concentrate diets suggest that in our study CH_4 decrease was more related to changes in methanogen activity. Beside this, no major effects of interaction between forage and SBO were observed for molar proportion of most VFA and changes in acetate proportions were of minor magnitude, except for GG (Figure 2). In this sense, the similar decrease in CH_4 emissions for all forages were more likely due to decrease in methanogen activity with SBO at 60 g kg^{-1} DM. Although we did not observe any significant interaction between forage specie and SBO inclusion for CH_4 production, this effect was expected due to the decrease in IVpdNDFD for MS and SC (Getachew et al., 2005). It can be consequence of differences between our donor cows' diets and the evaluated forages composition, which can significantly underestimate CH_4 emissions (Hattew et al., 2015). Thus, further investigation integrating diet dependent factors of rumen digestion dynamics such as microbial population, fibre kinetics and variation in pH should be evaluated to better clarify how fat supplementation and forage specie interacts affecting CH_4 emissions.

4.5.*In vitro* potentially digestible neutral detergent fibre digestibility

We expected a reduction in pdNDF digestibility for all forages supplementing SBO at 60 g kg^{-1} DM, however with different magnitudes. The absence of reduction in digestibility of pdNDF of GG, PG and RG with supplementing SBO at 60 g kg^{-1} DM was a highlight aspect in this study (Figure 2), and it demonstrates relevant different responses according to the forage specie, as hypothesized.

Fat supplementation has a known effect of decreasing rumen population of fibrolytic bacteria and protozoa and activity of fibre degrading enzymes (Hristov et al., 2009; Yang et al., 2009; Patra & Yu, 2013). Palmquist (1988) suggested that the negative effect of UFA on ruminal fibre digestion would be minimized by increasing the proportion of forage due to its

ability to promote normal rumen function for maximum biohydrogenation. Bateman II & Jenkins (1998) observed that SBO inclusion up to 80 g kg^{-1} of DM in Bermuda grass diet did not decrease total tract NDF digestibility. The authors suggested that the high concentration of digestible NDF promoted the biohydrogenation of dietary UFA and consequent rapid growth of fibrolytic bacteria. In contrast, Rodrigues et al. (2017) observed a 11% decrease in ruminal pdNDF digestibility *in vivo* when supplementing SBO at 44.3 g kg^{-1} DM in sugarcane (400 g kg^{-1} DM) based diets. In our study, adding SBO from 0 to 60 g kg^{-1} DM in diets without concentrate ingredients decreased IVpdNDFD in 21.2% and 12.9% for MS and SC, respectively. Corroborating to our results, Salem et al. (1993) observed greater decrease in fibre digestibility in maize silage diets when compared to grass hay diet supplemented with rapeseed oil at 66 g kg^{-1} DM. Even with greater pdNDF concentration, the MS is likely more sensible to SBO supplementation and it indicates that not only the NDF level determines the SBO effects but also other characteristics such as non-fibre carbohydrates (Lillis et al., 2011).

Fibre digestion in ruminants requires a complex symbiotic collaboration of several fibrolytic microbes and rumen protozoa demonstrated an important role in fibre colonization and digestion through xylanases and carboxymethylcellulases activities (Newbold et al., 2015). Ueda et al. (2003) supplemented linseed oil in low (350 g kg^{-1}) and high concentrate (650 g kg^{-1}) diets and observed reduction in rumen NDF digestibility only in high concentrate diets. The interaction of rapidly fermentable carbohydrates and SBO has been observed altering bacterial populations in the rumen and reducing CH_4 emissions (Lillis et al., 2011). Corroborating the forage and fat interaction, Benchaar et al. (2015) supplemented linseed oil in red clover silage and maize silage diets with starch concentrations of 100 and 220 g kg^{-1} DM, respectively and observed that NDF digestibility decreased only in MS based diets.

Although not evaluated with different forage:concentrate ratios, the SBO is known to have adverse effects on protozoa numbers (Yang et al., 2009; Fiorentini et al., 2013) and probably interacts with increased availability of rapidly fermentable carbohydrates. erProbably the reduction in fibre digestibility only for MS and SC diets in our study indicates that the higher starch and sucrose content interacts with SBO reducing rumen fibrolytic bacteria and protozoa. Based in our results, and those of Salem et al. (1993) and Benchaar et al. (2015), these interactions are more likely to happen when supplementing vegetable oils. Weld & Armentano (2017) also demonstrated that vegetable oils and medium chain fatty acids are the most effective fat sources on pdNDF digestibility. The same authors did not observe significant interactions between forage specie and fat concentration, however the authors considered this as a possible effect present in the diet characteristics to explain differences in fibre digestibility between dry and lactating cows fed supplementary fat.

In summary, our results support the inference that safety levels of SBO supplementation to avoid effects on fibre digestion should be determined for similar groups of forage species rather than the use of general limits and needs further investigation *in vivo*. The better understanding of biological factors such as different plant cell wall components and linkages, interactions between forage specie, rapidly fermentable carbohydrates and fat source with microbial populations in the rumen are needed.

In conclusion, whole-crop maize silage and sugarcane are more sensitive to adverse effects of SBO supplementation on fibre digestion when compared to ryegrass, guinea grass and palisadegrass.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this study.

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TABLES

Table 1. Chemical composition of the forages and main fatty acids profile of soybean oil (SBO).

Item	Forage†					SBO
	MS	SC	RG	GG	PG	
g kg^{-1} DM						
Organic matter	965	960	945	889	922	99.8
Crude protein	64	36	144	223	100	-
NDF‡	490	413	654	604	596	-
iNDF§	81	183	116	78	111	-
pdNDF††	409	230	538	526	485	-
NDS‡‡	475	547	291	285	326	-
Fatty acids, g $100g^{-1}$ of fat						
16:0	-	-	-	-	-	10.6
18:0	-	-	-	-	-	3.9
18:1 *cis*-9	-	-	-	-	-	26.5
18:2n-6	-	-	-	-	-	50.0
18:3n-3	-	-	-	-	-	3.6

†Forage: GG = guinea grass; PG = palisadegrass; RG = ryegrass; MS = whole-crop maize silage; SC = sugarcane;
‡NDF = Neutral detergent fibre;
§iNDF = indigestible neutral detergent fibre;
††pdNDF = potentially digestible neutral detergent fibre (*NDF – iNDF*);
‡‡NDS = Neutral detergent solubes (organic matter – NDF).

Table 2. Mean values of ammonia (NH_3-N) concentration, pH and volatile fatty acids (VFA) according to the sampling time (h) of different forages supplemented with soybean oil levels.

	Forage†					Soybean oil (g kg^{-1} of DM)			SEM	P-value‡			
											Oil		
Item	**MS**	**SC**	**RG**	**GG**	**PG**	**0**	**30**	**60**		**F×O**	**F**	**Lin**	**Quad**
NH_3-N, mg dL^{-1}													
8 h	20.2^{b}	14.3^{c}	25.3^{a}	25.0^{a}	21.3^{b}	20.9	21.3	21.4	3.2	0.72	<0.01	0.07	0.48
24 h	27.4^{b}	24.9^{c}	32.7^{a}	36.2^{a}	25.2^{c}	29.1	29.5	29.2	0.55	0.83	<0.01	0.90	0.30
End of incubation (72 h)													
pH	6.62^{a}	6.55^{b}	6.57^{b}	6.62^{a}	6.55^{b}	6.59	6.57	6.59	0.57	0.65	0.01	0.87	0.52
VFA, m*M* L^{-1}	89.1^{a}	89.0^{a}	73.3^{b}	77.9^{b}	94.7^{a}	85.5	86.8	82.0	0.12	0.06	<0.01	0.31	0.29
Acetate (A), m*M* mol^{-1}	59.1	61.6	60.3	60.8	63.6	60.7	61.2	61.3	0.19	0.03	-	-	-
Propionate (P), m*M* mol^{-1}	17.0	16.5	16.4	16.6	16.9	16.8	16.7	16.5	0.31	0.43	0.50	0.35	0.79
Butyrate (B), m*M* mol^{-1}	12.2^{a}	11.6^{b}	10.3^{c}	9.2^{d}	9.3^{d}	10.6	10.5	10.4	0.07	0.15	<0.01	0.29	0.85
Isobutyrate, m*M* mol^{-1}	2.1^{b}	1.9^{c}	2.6^{a}	2.6^{a}	2.0^{bc}	2.2	2.3	2.3	0.1	0.58	<0.01	0.64	0.54
Valerate, m*M* mol^{-1}	4.4^{ab}	4.2^{bc}	4.8^{a}	4.8^{a}	3.7^{c}	4.4	4.3	4.4	3.2	0.58	<0.01	0.71	0.65
Isovalerate, m*M* mol^{-1}	5.2^{ab}	4.2^{c}	5.6^{a}	6.0^{a}	4.5^{bc}	5.3	5.0	5.1	0.55	0.21	<0.01	0.82	0.33
A P^{-1}	3.5	3.7	3.7	3.7	3.8	3.6	3.7	3.7	0.35	0.03	-	-	-
(A+B) P^{-1}	4.2	4.4	4.3	4.2	4.3	4.2	4.3	4.3	0.57	0.07	0.19	0.40	0.06

†Forage: GG = guinea grass; PG = palisadegrass; RG = ryegrass; MS = whole-crop maize silage; SC = sugarcane;
‡P-value: F×O = Interaction between forage and soybean oil level; F = effect of forage; Lin = linear effect of soybean oil level; Quad = quadratic effect of soybean oil level;
[a,b,c,d]Different letters at the same line indicate significant differences at $p < .05$ (Tukey-Kramer) among forage specie.

Table 3. *In vitro* gas production kinetics parameters, methane (CH_4) production and potentially digestible neutral detergent fibre (pdNDF) digestibility of different forages and soybean oil concentrations (unit are mL g^{-1} of OM unless otherwise stated).

	Forage†					Soybean oil (g kg^{-1} of DM)			SEM	P-value‡			
												Oil	
Item	**MS**	**SC**	**RG**	**GG**	**PG**	**0**	**30**	**60**		**F×O**	**F**	**Lin**	**Quad**
Gas kinetics††													
V_1	154^{ab}	172^{a}	137^{b}	164^{a}	101^{c}	142	146	189	14.4	0.98	<0.01	0.46	0.68
V_2	134^{bc}	147^{b}	147^{b}	118^{c}	181^{a}	145	152	140	15.7	0.44	<0.01	0.47	0.30
k_1, h^{-1}	0.095^{b}	0.247^{a}	0.066^{c}	0.063^{c}	0.101^{b}	0.116	0.114	0.114	0.0074	0.99	<0.01	0.69	0.81
k_2, h^{-1}	0.030^{a}	0.031^{a}	0.026^{c}	0.027^{bc}	0.029^{ab}	0.030	0.028	0.028	0.0026	0.96	<0.01	0.35	0.11
L_1, h	0.08^{b}	0.46^{a}	-1.36^{d}	-0.14^{bc}	-0.33^{c}	-0.28	-0.23	-0.25	0.177	0.97	<0.01	0.79	0.65
L_2, h	4.55^{b}	1.18^{d}	5.23^{b}	7.52^{a}	3.17^{c}	4.43	4.39	4.17	0.844	0.57	<0.01	0.60	0.66
Asymptotic gas	288	320	284	282	283	288	298	288	6.9	<0.01	-	-	-
CH_4													
24h	29.7^{b}	33.9^{a}	22.3^{e}	26.7^{c}	24.1^{d}	27.1	28.2	26.7	1.63	0.75	<0.01	0.50	0.02
48h	36.9^{b}	41.4^{a}	32.3^{c}	37.3^{b}	33.5^{c}	36.6	37.2	35.0	1.14	0.39	<0.01	0.20	0.02
72h	38.8^{bc}	43.6^{a}	35.9^{d}	40.3^{b}	37.8^{c}	39.0	40.2	38.6	1.76	0.41	<0.01	0.58	0.02
IVpdNDFD‡‡, g kg^{-1}	617	698	848	899	881	818	799	748	8.9	<0.01	-	-	-
First-order k_d, h^{-1}	0.079^{b}	0.097^{a}	0.059^{c}	0.065^{c}	0.064^{c}	0.073	0.071	0.075	0.0042	0.54	<0.01	0.64	0.07

†Forage: GG = guinea grass; PG = palisadegrass; RG = ryegrass; MS = whole-crop maize silage; SC = sugarcane;

‡P-value: F×O = Interaction between forage and soybean oil level; F = effect of forage; Lin = linear effect of soybean oil level; Quad = quadratic effect of soybean oil level;

††Gas kinetics: V_1, k_1, and L_1 = the asymptotic cumulative gas volume, rate and lag parameters for the first pool (rapid); and V_2, k_2, and L_2 = the asymptotic cumulative gas volume, rate and lag parameters for the second pool (slow);

‡‡IVpdNDFD = pdNDF digestibility after 72 h of *in vitro* incubation;

^{a,b,c,d}Different letters at the same line indicate significant differences at $p < .05$ (Tukey-Kramer) among forage specie.

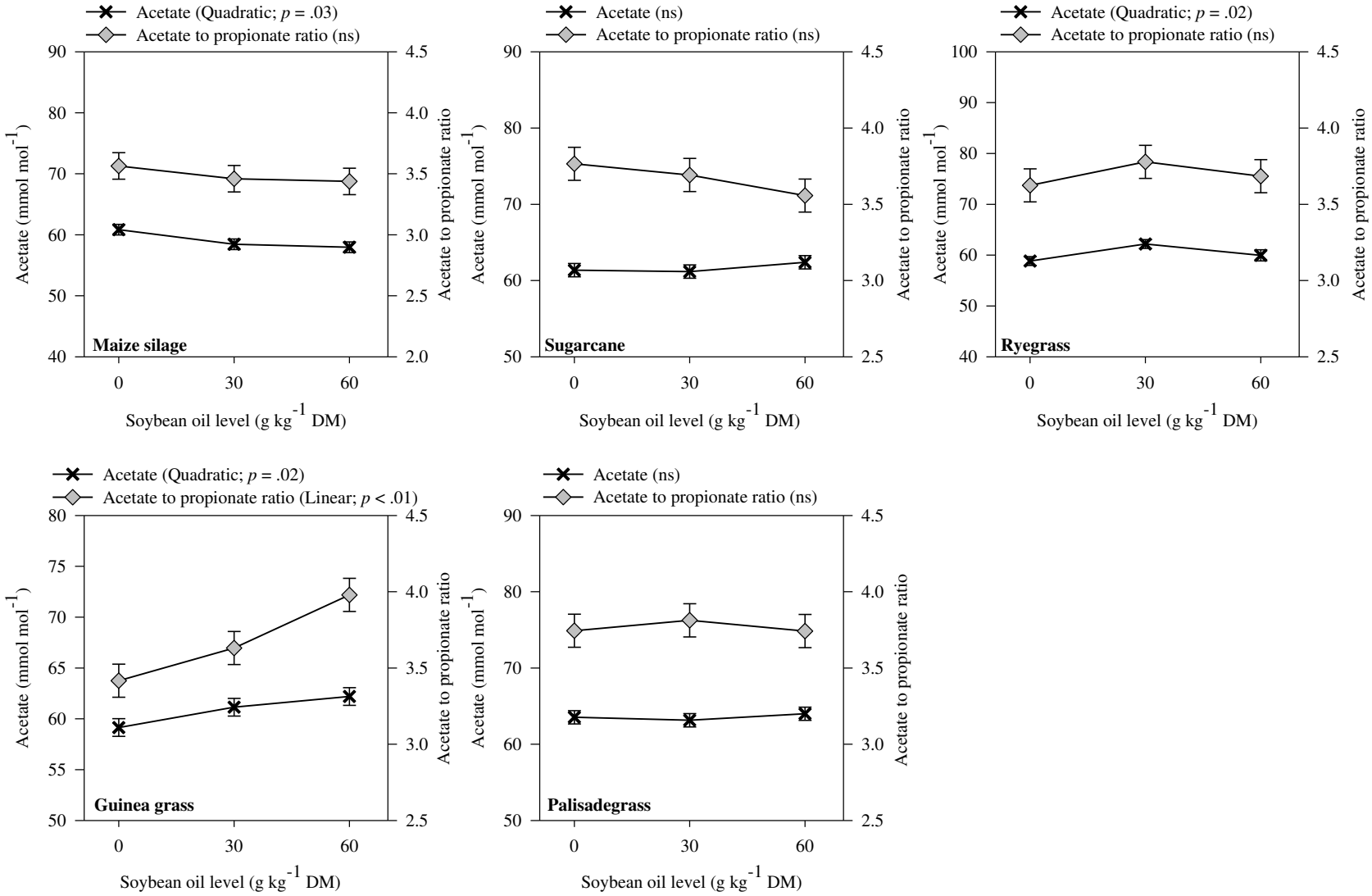

Figure 1. Effects of soybean oil inclusion on *in vitro* molar proportion of acetate on total volatile fatty acids, acetate to propionate ratio after 72 h of incubation of different forages.

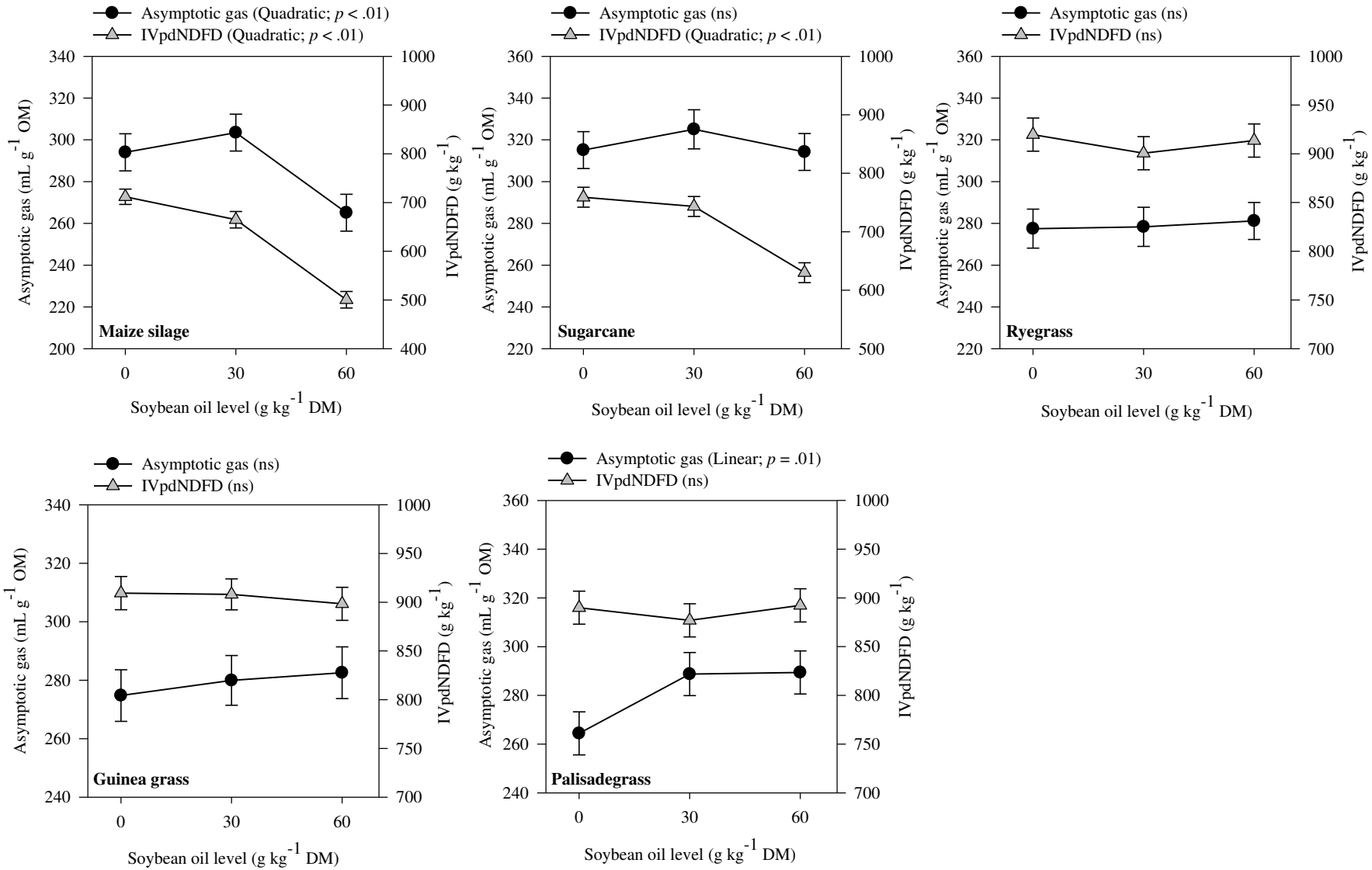

Figure 2. Effects of soybean oil inclusion on asymptotic gas and *in vitro* potentially digestible neutral detergent fibre digestibility (IVpdNDFD) of different forages.

CHAPTER 2

Interpretive Summary

Sugarcane is an important forage for dairy cows in tropical countries. However, studies evaluating fat supplementation as a strategy to increase energy intake using this forage are scarce. Four concentrations of soybean oil (SBO) were supplemented at 0, 1.57, 4.43, and 7.34% of the diet DM content. We observed that SBO inclusion greater than 4.43% DM decreased intake and milk production. The digestibility and rate of fiber digestion decreased as SBO increased. Soybean oil supplementation at 1.57% of the diet DM proved to be a safe concentration. However, none of the evaluated SBO concentrations increased energy intake and performance.

Short-term effects of soybean oil supplementation on performance, digestion and metabolism in dairy cows fed sugarcane-based diets

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ABSTRACT

We aimed to quantify the productive and metabolic responses, and digestive changes in dairy cows fed various concentrations of soybean oil (SBO) in high concentrate, sugarcane-based diets. Eight rumen-cannulated multiparous Holstein cows in mid-lactation (body weight = 574 ± 19.1 kg and 122 ± 6.9 days in milk), averaging 22.5 ± 1.22 kg/d of milk were assigned to replicated 4 × 4 Latin squares. The experimental period lasted 21 d as follows: 14 d for adaptation, followed by a sampling period from d 15 to 21. The diets were formulated with increasing concentrations of SBO (% of dry matter (DM)): control (0%), low (LSBO; 1.57%), medium (MSBO; 4.43%) and high (HSBO; 7.34%). Dry matter intake decreased quadratically in response to SBO addition. The greatest decrease in dry matter intake was observed in MSBO and HSBO diets. Both milk and energy corrected milk yield were quadratically affected by the SBO inclusion, with a slight decrease up to MSBO and substantial decrease in the HSBO diet. The milk fat concentration linearly decreased from 3.78% in the control to 3.50% in the HSBO diet. The potentially digestible neutral detergent fiber digestibility in the rumen decreased from 55.7% in the control to 35.2% in the HSBO diet. The fractional rate of digestion of potentially digestible neutral detergent fiber in the rumen decreased linearly from 3.13 to 1.39%/h from the control to HSBO diet. The fractional rate of indigestible neutral detergent fiber passage in the rumen was quadratically affected, with the lowest value (2.25%/h) for the HSBO diet. Rumen pH increased from 6.42 to 6.67, and ammonia nitrogen decreased from 28.1 to 21.4 mg/dL, in the control and HSBO diets, respectively. Rumen volatile fatty acids decreased quadratically, with the greatest decrease observed in MSBO and HSBO diets. Serum concentrations of glucose, non-esterified fatty acids and beta-hydroxybutyrate were unaffected by SBO inclusion. However, serum concentrations of total cholesterol and high-density and low-density lipoproteins linearly increased with increasing concentrations of SBO in the diet. Soybean oil supplementation

at 1.57% of the diet DM proved to be a safe concentration for dairy cows fed high concentrate diets with sugarcane as the sole forage. Inclusion of SBO at concentrations from 4.43 to 7.34% of the diet DM decreased DMI, ECM production, fiber digestibility and rumen fermentation being not recommended.

Keywords: fat supplementation; microbial protein synthesis; rumen kinetics; omasal flow

INTRODUCTION

Sugarcane is a forage commonly fed to cattle in tropical environments and often used as the main dietary fiber source. It has an excellent feeding potential for dairy cows because of its high productivity and wide harvesting window (Daniel et al., 2014). However, its use for high yielding dairy cows is usually associated with feeding high amounts of concentrates, leading to a high proportion of NFC in the diet (Oliveira et al., 2011). Furthermore, sugarcane fiber contains a high proportion of indigestible neutral detergent fiber (**iNDF**) and low potentially digestible neutral detergent fiber (**pdNDF**) at about 25 and 27% DM, respectively (Daniel et al., 2014) that is associated with a low DMI. Consequently, more concentrate is needed compared to corn silage-based diets (Oliveira et al., 2011).

Under these feeding situations, fat supplementation could be an alternative to increasing energy intake. However, the optimal inclusion rate of added fat, in diets with sugarcane as the sole forage needs to be defined. Previous research has indicated that the forage source can affect the response of fat supplementation on fiber digestion (Ueda et al., 2003). For instance, adding rapeseed oil at 6.6% DM, Salem et al. (1993) observed a lower decrease in grass hay NDF digestibility when compared with corn silage. Incorporating linseed oil at 4% DM, Benchaar et al. (2015) observed a decrease in NDF digestibility in corn silage diets but not in red clover silage diets. Bateman and Jenkins (1998) included soybean oil (**SBO**) up to 8% DM in diets with Bermuda grass hay and did not observe any effects on fiber digestibility. Studies evaluating fat supplementation in diets with sugarcane and other tropical forages are scarce. In this way, the diets are usually formulated based on recommendations of maximum supplementary fat concentration of 4–5% DM (Lewis et al., 1999) or 6–7% ether extract (**EE**) in diet DM (NRC, 2001).

The effects of fat supplementation on rumen metabolism in dairy cows have been widely studied (Lerch et al., 2012; Piantoni et al., 2015). In spite of advances in knowledge of the effects of fat supplementation on rumen digestion, metabolism, and milk quality (Shingfield et al., 2008), few attention has been paid to the effects of fat supplementation in dairy diets based on tropical forages. In addition, vegetable oils are rich in unsaturated fatty acids (**UFA**) and highly hydrolyzed in the rumen (Jenkins and Harvatine, 2014). A better understanding of production responses in dairy cows to increasing dietary fat concentrations is warranted particularly when tropical forages such as sugarcane are fed.

We hypothesized that SBO supplementation would increase energy intake and milk production in cows fed high concentrate diets with sugarcane. Based on this hypothesis, our objective was to quantify the productive, nutritional and metabolic responses of dairy cows fed different concentrations of SBO in high concentrate diets with sugarcane as the sole forage.

MATERIALS AND METHODS

The Committee of Production Animal Care and Use of the Federal University of Viçosa approved all animal feeding, management and procedures involved in this study (Protocol no. 072/2014).

Animals, Experimental Design and Diets

Eight, rumen-cannulated, multiparous, Holstein cows (574 ± 19.1 kg of BW and 122 ± 6.9 DIM) were used. Milk production was 22.5 ± 1.22 kg/d at the beginning of the study. The animals were housed in individual stalls (17.5 m^2) with rubber powder beds, water and feed bunks. Cows were grouped in a replicated 4 × 4 Latin square design

balanced for residual effects. The experimental period lasted 21 d, comprising of 14 d for adaptation (Machado et al., 2016) followed by a sampling period from d 15 to 21.

Four diets were formulated with increasing concentrations of SBO (%DM): control (0%), low (**LSBO**; 1.57%), medium (**MSBO**; 4.43%) and high (**HSBO**; 7.34%) (Table 2). The SBO concentrations were selected to achieve 4, 7 and 10% of EE in diet DM in LSBO, MSBO and HSBO diets, respectively. The diets were formulated to be isonitrogenous (16% CP) by changing the proportions of corn and soybean meal, and a fixed forage-to-concentrate ratio of 40:60 was used. The sugarcane (variety RB-867515) used was a third-cut crop (Table 1). It was harvested daily in the afternoon and chopped before the morning feeding. The sugarcane stems had an average soluble content of 18.0±1.68 °Brix. Oxidation was prevented by preparing a single batch of concentrate at the beginning of each experimental period and adding antioxidant butyl hydroxy toluene. The refined SBO (Cocamar, Maringá, Brazil) was first mixed with the soybean meal and corn meal, then the minerals, using a horizontal concentrate mixer. The cows were milked twice daily at 05:50 and 14:50 h, and fed diets as a TMR individually after each milking session. Intake was calculated by manually weighing the offered TMR and orts. Diets were offered on an ad libitum basis (target 100 g refusal/kg fed) and the amount offered was adjusted daily.

Samplings

The diets compositions were estimated by sampling each ingredient at the time of concentrate mixing. Samples of soybean meal, corn meal and soybean oil were taken in each batch of concentrate and stored at -20°C until analysis. Samples of sugarcane and diet refusals were taken from d 15 to 19 of each experimental period, stored at -20°C and pooled fresh based on the period for chemical analysis.

Milk production was measured and samples were collected from d 16 to 18 of each experimental period. A pooled sample was made proportionally to production in each milking session and stored as duplicates at -20ºC until analysis. For MUN analysis, 10 mL of milk was deproteinized with 5 mL of trichloroacetic acid (250 g/L), filtered and stored at -20°C until analysis.

Digestibility and microbial protein synthesis (**MPS**) were estimated by collecting a total of eight spot fecal and urine samples at 9 h intervals starting at 09:00 h on d 16. Fecal samples (300 mL) were collected from the rectum and immediately oven-dried at 55°C and stored. Urine samples (200 mL) were collected by stimulating the vulva and two aliquots were made; the first 10 mL was acidified with 40 mL of sulfuric acid (H_2SO_4, 1 mL/L) for allantoin analysis and the second was stored as obtained for N determination. Both urine samples were kept frozen at -20°C. Both urine and feces were pooled by cow and period before analysis.

From d 13 to 18 of each period, infusions of 8 g/d of Co-EDTA (766 mg of Co/d) were performed four times daily at 00:00, 06:00, 12:00 and 18:00 h. The Co-EDTA was prepared according to Udén et al. (1980). The omasal flow of nutrients was estimated by collecting eight omasal samples at 9 h intervals starting at 09:00 h on d 16. The omasal fluid was collected according to the technique described by Huhtanen et al. (1997). At each sampling time, approximately 1.2 L of digesta was obtained and divided into subsamples. To estimate markers concentrations and omasal flow, 500 mL of digesta was filtered through a 100 µm nylon mesh filter with 44% open area (Sefar Nitex 100/44; Sefar, Thal, Switzerland) to provide particle and fluid phase samples. Samples were immediately dried at 55°C and pooled separately by cow and period. The omasal flow was estimated using Co and iNDF as markers. To estimate the bacterial RNA-N to total N ratio (**RNA-N:N**) and the rumen fermented OM (**RFOM**), 300 mL was stored at -20°C for bacterial isolation according to Reynal et al. (2005) with modifications suggested by

Krizsan et al. (2010). Omasal samples for bacterial isolation were unfrozen (2°C) and pooled by cow and period immediately before the isolation procedure.

After each omasal sampling, ruminal contents (300 mL) were collected manually from the cranial, ventral and caudal areas of the rumen and filtered through two layers of cheesecloth. The pH of the rumen fluid was measured immediately (TEC-3P-MP; Tecnal, Piracicaba, SP, Brazil). Then, an aliquot (8 mL) of ruminal fluid was combined with 2 mL of metaphosphoric acid (250 g/L) and frozen for subsequent VFA analysis. Another aliquot (40 mL) was combined with 1 mL of H_2SO_4 (500 g/L) and frozen for later analysis of ammonia N (**NH_3-N**). Both samples were stored at -20°C until analysis. For VFA analysis, a pooled sample by period was made for each cow.

For the measurements of rumen pool size and calculations of digesta kinetics, two rumen evacuations were carried out on d 19 (4 h after feeding) and 21 (2 h before feeding). Whole rumen contents were collected into plastic containers and separated by filtering through cheesecloth into primarily liquid and particle fractions. Approximately 2 kg of each fraction were sampled and immediately oven-dried at 55°C.

Blood samples were taken on d 17 of each period after the morning milking. The samples were collected from the coccygeal vein using vacuum tubes (BD Vacutainer SST II Advance®; 8.5 mL) and immediately centrifuged (3000 × g, 15 min). Serum was separated and stored at -20°C until analysis.

Laboratory Analysis

Samples of feed, orts and feces were oven-dried at 55°C and milled through 2 and 1-mm screens for further analyses. Samples of milk and isolated bacteria were lyophilized and ground using a mortar and pestle. The DM content was evaluated by the AOAC (2005) method 930.15, and OM by method 942.05. Nitrogen content was analyzed according to the AOAC (2005) method 984.13, considering 6.25 and 6.38 as the factors

to convert N values to CP for feed and milk samples, respectively. The NDF concentration was evaluated using heat stable α-amylase with an Ankom 220 fiber analyzer (Ankom Technology, Fairport, USA), sodium sulfite was not used and the results were expressed without residual ash. The iNDF content of feed and feces was analyzed from samples ground and sieved through a 2-mm screen sieve, followed by 288 h of *in situ* incubation using F57 filter bags (Ankom Technology, Fairport, USA) (Valente et al., 2011). The milk fat concentration was quantified according to the AOCS (2004) method Am 5-04.

Cobalt was analyzed by an atomic absorption spectrophotometer (Spctr AA-800, Harbour City, CA).

Due to the high content of fat in the diets and the formation of calcium fatty acids (**FA**) salts in the rumen (AbuGhazaleh et al., 2002), acid hydrolysis with hydrochloric acid (ISO, 1973) was performed on all feed, digesta and fecal samples before ether extraction. Approximately 4 g of ground sample was poured in an Erlenmeyer flask and 40 mL of hydrochloric acid solution (333 mL/L) was added. The Erlenmeyer flask was covered with a watch glass and heated (110°C) for 30 min. After that, the contents were filtered through a filter paper (Whatman®, #54) and washed with hot distilled water until pH neutralization. The filtered contents were oven-dried at 105°C for 16 h and EE analysis was performed according to the AOAC (2005) method 2003.05.

Frozen aliquots of feeds were lyophilized and milled through a 1-mm screen. The FA profile of the feeds was evaluated by the extraction method described by Folch et al. (1957). The separated fats were methylated according to Kramer et al. (1997) and quantified by GC (Shimadzu GC-2010 with automatic injection) using a capillary column (SP-2560; 100 × 0.25 mm diameter, 0.02-mm thickness, Supelco, Bellefonte, PA, USA). The oven temperature was 70°C for 4 min, then increased at 13°C/min to 175°C, and held for 27 min, before a further increase at 4 °C/min until reaching 215°C, which was maintained for 31 min. Hydrogen (H_2) was used as the carrier gas with a flow of 40 cm^3/s.

A standard C4–C24 FA mix was used (TM 37; Supelco Sigma-Aldrich Group, Bellefonte, PA, USA).

Rumen NH_3-N was evaluated by a colorimetric method (Chaney and Marbach, 1962). For VFA analysis, the rumen fluid samples (2 mL) were centrifuged (12000 × g, 10 min) and the cell-free supernatants were treated as described by Siegfried et al. (1984). The VFA contents were evaluated by HPLC (Dionex Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA) coupled to a refractive index detector (Shodex RI-101) maintained at 40°C, and an ion exchange column (Phenomenex Rezex ROA, 300 × 7.8 mm) maintained at 45°C. The mobile phase was 5 mmol/L H_2SO_4 and the flow rate was 0.7 mL/min.

The analysis of allantoin in urine and deproteinized milk was performed by colorimetry according to Chen and Gomes (1992), and the concentrations of uric acid and creatinine in urine were evaluated by HPLC according to George et al. (2006). The RNA-N:N ratio in the isolated bacteria was estimated by analyzing the purine bases method according to Ushida et al. (1985).

Serum concentrations of glucose (K082), triglycerides (K117), cholesterol (K083), high-density lipoprotein (**HDL**; K071), total protein (**TP**; K031), albumin (K040) and calcium (K051) were measured using kits from Bioclin Diagnostics (Belo Horizonte, Brazil). Low-density lipoprotein (**LPL**) and very low-density lipoprotein (**VLDL**) concentrations were estimated according to Friedewald et al. (1972). Serum concentrations of non-esterified fatty acids (**NEFA**) was quantified by a colorimetric method (FA115, Randox Laboratories Ltd., São Paulo, Brazil) and BHB was evaluated by a kinetic enzymatic method based on oxidation of D-3-hydroxybutyrate to acetoacetate (RB1007; Randox Laboratories Ltd., Antrim, UK). The MUN, BUN and urinary urea N (**UUN**) were measured using kits (K056) from Bioclin Diagnostics (Belo Horizonte,

Brazil). An automatic biochemical apparatus (Mindray BS-200E; Shenzhen Mindray Bio-Medical Electronics Co. Ltd.) was used for all analyses.

Markers and Calculations

The DMI was calculated based on the DM weight of TMR offered and orts. The iNDF was used as an internal marker to estimate the fecal excretion. Total tract apparent digestibility was calculated as: 100 × (intake (g) – excretion (g))/intake (g). The digestible organic matter intake (**dOMI**) was estimated by multiplying the OM intake by the OM digestibility.

The NFC concentration was calculated as NFC = OM – (CP + NDF + EE). The pdNDF was calculated as NDF – iNDF. The concentrations of digestible energy (**DE**) were calculated considering the digested nutrients and their caloric values, as DE (Mcal/kg) = (dNFC × 0.042) + (dNDF × 0.042) + (dCP × 0.056) + (dEE × 0.094), where dNFC=digestible NFC, dNDF=digestible NDF, dCP=digestible CP and dEE=digestible EE (NRC, 2001).

The ECM (kg/d) was calculated as: MP × [(38.3 × fat) + (24.2 × protein) + (16.54 × lactose + 20.7]/3140, where MP = milk production (kg/d) and fat, protein and lactose in milk units are g/kg (Sjauna et al., 1990).

Digesta flow to the omasum was calculated based on the double marker method proposed by Faichney (1975) using Co as a fluid phase marker and iNDF as a particle phase marker.

Total urine excretion was estimated based on the daily urinary creatinine excretion estimate of 1.06 mmol/kg $BW^{0.75}$ (Chizzotti et al., 2008). Total excretion of purine derivatives (**PD**) was calculated as the sum of the allantoin and uric acid excreted in the urine and milk. Absorbed purines (**AP**) were calculated from the PD excretion (mmol/day) and BW (kg) using the equation AP (mmol/d) = (PD + 0.385 × $BW^{0.75}$)/0.85,

where 0.85 is the recovery of AP as PD and $0.385 \times BW^{0.75}$ is the endogenous contribution to the purine excretion (Verbic et al., 1990). Microbial protein synthesis in the rumen was calculated based on the AP as MPS (g N/d) = [(70 × AP)/(0.83 × RNA-N:N × 1000)]/6.25, where RNA-N:N is the ratio between purine-N:bacterial-N obtained in the isolated bacteria from the omasum and was considered for each cow by period (average = 0.169 ± 0.0073) (Chen and Gomes, 1992).

The RFOM was calculated as RFOM = (OM intake (kg) – ((OM flow (kg) – bacterial OM flow (kg))). The bacterial OM flow was estimated using the ratio of N and OM on the isolated bacteria in the omasal digesta. The omasal flow of RUP at the omasal canal was calculated as RUP flow (kg) = CP flow (kg) – bacterial CP flow (kg). The RDP was calculated as RDP (kg) = CP intake (kg) – RUP flow (kg) (Reynal and Broderick, 2005).

Digestion kinetics were calculated according to Robinson et al. (1987) as the fractional rate of ingestion ($\mathbf{k_i}$; %/h) = (100/24) × (intake/rumen pool); fractional rate of passage ($\mathbf{k_p}$; %/h) = (100/24) × (omasal flow/rumen pool); fractional rate of digestion ($\mathbf{k_d}$; %/h) = $k_i - k_p$.

Statistical Analysis

The statistical analysis was performed using the SAS MIXED procedure (SAS Institute Inc., Cary, NC, version 9.3) according to the model:

$$Y_{ijkl} = \mu + T_i + S_j + P(S)_k + C(S)_l + \varepsilon_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, T_i is the fixed effect of a treatment, S_j is the fixed effect of the square, $P(S)_k$ is the fixed effect of period within square, $C(S)_l$ is the random effect of cow within square, and ε_{ijkl} is the model error. The effect of interaction between treatment and Latin square was not significant and removed from the model. Denominators degrees of freedom (df) were calculated using the

Kenward–Roger method. Results are expressed as the least squares mean (LSM) with the standard error of the mean (SEM). The effects of SBO concentrations were evaluated by polynomial linear and quadratic contrasts. The unequal spacing of the treatments was corrected by using the ORPOL function of PROC IML to obtain the appropriate coefficients for the CONTRAST statements. The final coefficients were -0.594, -0.315, 0.195, 0.714 and 0.503, -0.307, -0.661, 0.465, for linear and quadratic effects, respectively. The significance was declared at $P < 0.05$ and P-values from 0.05–0.10 were considered to indicate a trend.

RESULTS

Intake and Performance

Soybean oil inclusion quadratically influenced ($P < 0.01$) DMI, dOM and DE intake (Table 3). The HSBO diet corresponded to the lowest DE intake. The addition of SBO quadratically affected milk and ECM production ($P < 0.01$ and $P = 0.04$); both decreasing slightly from the LSBO to MSBO diet, then decreasing considerably in the HSBO diet (Table 3). Increasing SBO resulted in a linear decrease in milk fat concentration and yield ($P = 0.04$ and $P < 0.01$). Milk protein concentration had a quadratic trend ($P = 0.06$), with the lowest concentration (3.05%) in the MSBO diet (Table 3). The addition of SBO did not affect ($P > 0.10$) MUN and feed efficiency.

Digestibility

The data on intake, omasal flow and digestibility of OM, NDF, pdNDF, EE and NFC are shown in Table 4. Supplementation of SBO quadratically affected ($P < 0.01$) the intake of OM, NDF and pdNDF, with a considerable decrease observed from MSBO to HSBO diet. Comparing the control to the HSBO diet, the total tract apparent digestibility of OM, NDF and pdNDF decreased by 5.4, 35.4 and 34.6%, respectively. The RFOM

decreased linearly ($P < 0.01$) by 21.8% from the control to HSBO diet. The rumen apparent digestibility of EE tended ($P = 0.08$) to increase linearly when SBO increased. The SBO tended ($P = 0.06$) to linearly increase the total tract apparent digestibility of NFC.

Nitrogen Balance and Microbial Protein Synthesis

The data on N digestion, MPS and N balance are shown in Table 5. Soybean oil inclusion did not affect rumen N disappearance ($P = 0.16$), which ranged from 29.5–36.9% of the N intake. The SBO quadratically affected RDP ($P = 0.04$) such that similar intake was observed for cows fed control and MSBO but it decreased considerably in the HSBO diet. The RUP supply tended to be quadratically affected by SBO ($P = 0.06$), with the lowest value in the MSBO diet. Soybean oil tended to quadratically affect ($P = 0.09$) the MPS, with the highest and lowest values at MSBO (307 g N/d) and HSBO (246 g N/d) diet, respectively. The efficiency of MPS as a function of RFOM increased linearly ($P = 0.02$) as SBO increased. Soybean oil supplementation quadratically decreased ($P = 0.03$) the fecal N excretion. The partition of excreted N quadratically decreased ($P < 0.01$) in feces and quadratically increased ($P < 0.01$) in urine as SBO increased. Soybean oil supplementation quadratically affected ($P = 0.01$) the N balance, with the lowest value occurring in the HSBO diet.

Rumen Digesta Kinetics

The results regarding to rumen pool and kinetics are shown in Table 6. We found a linear decrease ($P < 0.01$) in the rumen pool of DM and iNDF, as SBO increased. The SBO did not affect ($P = 0.62$) the k_p of pdNDF but quadratically affected ($P = 0.02$) the k_p of iNDF, with the highest value (2.60%/h) in the MSBO diet. The k_d of pdNDF decreased linearly ($P < 0.01$) with increasing SBO concentration in the diet.

Fermentation characteristics

We observed a linear increase ($P < 0.01$) in the rumen pH and decreasing concentrations of NH_3-N ($P < 0.01$) and lactate ($P < 0.05$; Table 7) with SBO inclusion. Soybean oil quadratically affected ($P < 0.01$) rumen VFA concentration, with 2.6 and 20.3% decrease, comparing the control diet to MSBO and HSBO, respectively (Table 7). We found no effects of SBO on molar proportions of acetate ($P = 0.90$), propionate ($P = 0.64$) and butyrate ($P = 0.91$).

Blood Metabolites

Soybean oil inclusion did not affect serum concentrations of glucose, NEFA, BHB, calcium, total proteins and albumins ($P > 0.10$) (Table 8). However, we observed a linear increase in total cholesterol ($P < 0.01$), LDL ($P < 0.01$), VLDL ($P = 0.02$) and triglycerides ($P = 0.02$) as SBO increased. Soybean oil quadratically effected ($P = 0.04$) the serum HDL concentration, with the highest value of 114 mg/dL observed in the LSBO diet (Table 8). The BUN concentrations decreased linearly ($P = 0.02$) with SBO supplementation (Table 8).

DISCUSSION

This study aimed to quantify the productive and digestive changes in dairy cows fed different concentrations of SBO in high concentrate diets with sugarcane as the main forage.

Intake and Performance

As previously reported, SBO inclusion up to 8% DM in Bermuda grass diets negatively affects DMI (Bateman and Jenkins, 1998). The uptake of UFA in the gut can decrease DMI, either by increasing hepatic oxidation or activity of proximal gut peptides,

like cholecystokinin (Choi and Palmquist, 1996; Allen et al., 2009). The quadratic decrease observed in this study in DMI, dOM and DE intake indicates that the adverse effects of SBO on intake are greatest in MSBO and HSBO diets. Beyond the metabolic factors regulating intake, the effects of SBO on fiber digestion in the rumen are manifested by a linear decrease in digestibility and k_d of pdNDF. The high SEM in DMI, milk production and ECM can be a consequence of the greater variation between cows fed the HSBO diet. Another likely factor for high SEM in these variables can be the possible different carryover effects of the HSBO diet on the other treatments which can be significant when associated with the short adaptation periods used in this study. Huhtanen and Nousiainen (2012) observed that increments in diet fat concentration up to 3.5% DM, can improve ECM production by increasing energy intake. However, in our study, the increase in dietary fat did not increase ECM, even at the LSBO diet.

The effects of fat supplementation on milk fat depression have been widely described in the literature. In our study, it may be mostly derived from the increased omasal flow of *trans-10*, *cis-12* conjugated linolenic acid from the rumen (data not shown) (Shingfield et al., 2010). The reduction in milk protein (Table 3) up to the MSBO diet is probably associated with the decrease in CP flow to the omasum and RUP supply (Table 5).

Digestibility

The substitution of a fermentable substrate (NFC) by an unfermentable substrate (EE) and the decrease in rumen digestibility of pdNDF are the main reasons that cause a decrease in RFOM. Bateman and Jenkins (1998) observed that SBO inclusion up to 8% of the diet DM in Bermuda grass diets, did not decrease NDF digestibility. The authors suggested that the high concentration of digestible NDF promoted the rapid growth of cellulolytic bacteria, which consequently increased the biohydrogenation of dietary fat.

However, the results of Bateman and Jenkins (1998) should be compared with caution because they restricted DMI to 85% of the ad libitum intake, which can decrease k_p of pdNDF, favoring its digestion (Van Soest, 1994). Supplementing linseed oil at 4% of the diet DM, Benchaar et al. (2015) observed a reduction in NDF digestibility in corn silage-based diets unlike in red clover silage diets. Although the authors did not report the diets pdNDF concentrations, this result indicates that characteristics of forage fiber can determine the effects of fat supplementation on fiber digestion. In our study, the low pdNDF concentration in the diets may have limited the extent of biohydrogenation, leading to a decrease in fiber digestibility, even at the LSBO diet. Studies identifying fiber characteristics, such as pdNDF concentration, k_d of pdNDF and chemical linkages between fiber compounds in different forages, should be conducted to clarify the forage × fat interactions in fiber digestion. The compensatory postruminal digestion of rumen non-degraded pdNDF was observed by Harvatine and Allen (2006) in cows fed supplementary fat. In our study the compensatory digestion of pdNDF in the hindgut was not observed. Thus, explaining the linear reduction in total tract pdNDF digestibility.

Nitrogen Balance and MPS

The experimental diets resulted in greater CP and RDP concentrations and rumen NH_3-N than expected mainly due to the high CP of soybean meal (Table 1). The decrease in rumen NH_3-N with SBO supplementation might be a consequence of the reduction in protozoal counts and microbial activity (Doreau and Ferlay, 1995; Yang et al., 2009). The observed high rumen N-NH_3 concentrations resulted in high MUN, BUN and UUN (Kauffman and St-Pierre, 2001; Broderick et al., 2010). Considering that the bacteria that ferment structural carbohydrates mainly utilize NH_3-N as the N source (Russell et al., 1992), the high rumen NH_3-N and N disappearance observed are probably associated with the high RDP content, low pdNDF in the diet and low k_d of pdNDF. The negative N

balance in HSBO diet indicates that the N intake and metabolism of the cows were impaired by the high fat concentration. The N excretion in feces and urine, from the control until MSBO diet are similar to the observations by Hristov and Ropp (2003), evaluating high CP diets (19.5% DM) with high NFC. The authors demonstrated that ruminal digestible fiber can improve the transfer of ruminal NH_3-N to N in milk. Considering the low pdNDF and high content of sucrose in fresh sugarcane, diets with this forage given to dairy cows should be formulated using other sources of pdNDF with relatively lower starch concentrations (e.g. whole cottonseed, citrus pulp or soybean hulls). However, data about the use of by-products with high pdNDF concentration in high concentrate sugarcane based diets are scarce in the literature (Lima et al., 2014).

The UFA have little influence on rumen MPS and changes in the efficiency of microbial N synthesis as a consequence of fat supplementation are mainly associated with the decrease in rumen protozoa populations or RFOM (Doreau and Ferlay, 1995). Fiorentini et al. (2013) did not observe differences in MPS in heifers adding SBO up to 5.8% EE in the diet DM. In our study, the high concentrations of NFC in the diets and high rumen NH_3-N did not restricted microbial growth. However, the great adverse effects of the HSBO diet on DMI and rumen pdNDF digestion resulted in a quadratic trend in MPS. Thus, the linear increase in efficiency of MPS seems to be associated with the linear decrease in RFOM.

Rumen Digesta Kinetics

Studies evaluating rumen pools and digestion kinetics in response to fat supplementation are scarce and absent with sugarcane-based diets. The decrease in rumen DM pool as a consequence of fat supplementation was not observed when corn oil was fed at 2% of the diet DM (Ramirez-Ramirez et al., 2016). The linear decrease in the rumen

pool of DM and iNDF is probably related to the lower DM intake, which was not observed by Ramirez-Ramirez et al. (2016).

Similar to our results, Tesfa (1993) did not observe a significant effect on the k_p of NDF, but the k_d of NDF decreased markedly when evaluating the inclusion of rapeseed oil to reach 11.7% EE on the diet OM, which is close to our HSBO diet. Harvatine and Allen (2006) observed that 2.5% of supplemental saturated fat on diet DM increase k_p and decrease k_d of pdNDF, while unsaturated fat at the same concentration had no impact on these parameters. A decrease from 3.42 to 2.86%/h in k_p of DM was observed by Ramirez-Ramirez et al. (2016) supplementing 2% corn oil in alfalfa hay and corn silage-based diets. Although the alteration in the k_p of DM cannot be directly associated with the k_p of the fiber fraction, this decrease observed by Ramirez-Ramirez et al. (2016) may indicate a lower turnover rate in the rumen, which is likely due to a decrease in the k_d of pdNDF. The pdNDF digestibility was severely affected by the HSBO diet, and this decrease may be explained by the reduction in the k_d of pdNDF. Supplementing SBO and linseed oil mixture (1:1 g/g) in dairy cow diets, Yang et al. (2009) observed a decrease in rumen populations of cellulolytic bacteria and protozoa. According to the authors, this decrease was both a consequence of direct antimicrobial activity and coating (Jenkins, 1993). Tesfa (1992) demonstrated that rapeseed oil supplementation affects fiber degradability more by direct effects of the oil on protozoa and bacteria populations and activity than by coating effects. It is likely that this effect is the main mechanism reducing the k_d of pdNDF in our study. The decrease in the k_d of pdNDF and the absence of an effect on the k_p of this fraction indicates that the reduction in fiber digestibility is more likely associated with the activity of fiber-digesting bacteria rather than the k_p of pdNDF

Rumen Parameters

Despite the high iNDF concentration, sugarcane is rich in sucrose, which can explain the observed high rumen VFA concentration. The considerable decrease in rumen VFA at concentrations greater than MSBO demonstrates that the adverse effects of SBO supplementation on carbohydrates digestion were greater at the HSBO diet. The magnitude of the SBO effect on rumen VFA was similar to the effect on RFOM and MPS. Corroborating to our results, Yang et al. (2009) observed that feeding SBO at 4% of the diet DM, did not affect the ruminal populations of amylolytic bacteria. This bacterial tolerance to SBO observed by Yang et al. (2009) may explain the reduction on rumen VFA only at concentrations greater than MSBO. The absence of changes in proportions of VFA is probably due to the absence of marked changes in the diet carbohydrates when SBO was included.

Blood Metabolites

In agreement with our results, Ye et al. (2009) observed an increase in total cholesterol, LDL and triglycerides when supplementing SBO to dairy cows at 2% of the diet DM. Blood LDL and VLDL coupled with chylomicrons are responsible for transporting triglycerides, and hypercholesterolemia is a common physiological state in ruminants fed high amounts of fat (Bauchart, 1993). The increase in serum LDL and VLDL shows that the cow's cholesterol metabolism was altered to improve the transport of FA from the gut.

CONCLUSIONS

We concluded that SBO supplementation at 1.57% of the diet DM proved to be a safe concentration for dairy cows fed high concentrate diets with sugarcane as the sole forage. Inclusion of SBO at concentrations from 4.43 to 7.34% of the diet DM decreased DMI, ECM production, fiber digestibility and rumen fermentation being not

recommended concentrations. Feeding SBO at 7.43% of the diet DM greatly decreased DMI, extent of digestion and k_d of pdNDF and nitrogen balance of dairy cows and should not be used. None of the evaluated SBO concentrations increased energy intake and milk production and other feeding strategies should be evaluated for dairy cows fed sugarcane based diets. Diets for cows fed sugarcane and high concentrate supplementation can be supplemented with vegetable oils until 4% of EE in diet DM.

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TABLES

Table 1

Analyzed composition and fatty acids profile of the main feeds used in the experimental diets.

	Feed			
	Sugarcane	Corn meal	Soybean meal	Soybean oil
Composition, % of DM				
OM	96.5	98.6	93.4	99.8
CP	3.0	8.9	53.4	-
Ether extract	1.0	3.5	1.2	99.8
NDF	46.6	11.1	12.7	-
iNDF[1]	21.5	1.5	1.4	-
pdNDF[2]	25.1	9.6	11.3	-
NFC[3]	45.9	75.1	26.1	-
Fatty acids, g/100g of fat				
16:0	26.3	13.6	19.0	10.6
16:1 *cis*-9	-	0.16	-	0.07
18:0	4.42	2.25	5.17	3.85
18:1 cis-9	13.5	34.1	16.9	26.5
18:2n-6	30.7	47.2	48.0	50.0
18:3n-3	18.3	0.95	5.28	3.55

[1]iNDF: Indigestible neutral detergent fiber evaluated by 288h in situ incubation.
[2]pdNDF: Potential digestible neutral detergent fiber (NDF – iNDF).
[3]NFC: Non-fiber carbohydrates.

Table 2

Ingredients and analyzed chemical composition of the experimental diets with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)			
	0	1.57	4.43	7.34
Item	Control	LSBO	MSBO	HSBO
Ingredient, % of DM				
Sugarcane	40.0	40.0	40.0	40.0
Corn meal	32.96	30.88	27.46	23.78
Soybean meal	21.58	22.09	22.65	23.42
Soybean oil	-	1.57	4.43	7.34
Sodium bicarbonate	0.91	0.91	0.91	0.91
Dicalcium phosphate	0.86	0.86	0.86	0.86
Urea	0.83	0.83	0.83	0.83
Limestone	0.65	0.65	0.65	0.65
Potassium chloride	0.57	0.57	0.57	0.57
Salt	0.55	0.55	0.55	0.55
Magnesium sulphate	0.43	0.43	0.43	0.43
Magnesium oxide	0.34	0.34	0.34	0.34
Sulphur	0.15	0.15	0.15	0.15
Ammonium sulphate	0.09	0.09	0.09	0.09
Vitamins[2]	0.06	0.06	0.06	0.06
Zinc sulphate	0.01	0.01	0.01	0.01
Antioxidant BHT[3]	0.01	0.01	0.01	0.01
Composition, % of DM				
OM	92.2	92.2	92.2	92.2
CP	18.1	18.2	18.2	18.3
Ether extract	1.8	3.3	6.1	8.9
NDF	25.1	24.9	24.6	24.4
iNDF[4]	9.4	9.4	9.3	9.3
pdNDF[5]	15.7	15.5	15.3	15.1
NFC[6]	47.2	45.8	43.3	40.6
DE[7] (Mcal/kg DM)	2.98	2.97	3.09	3.12
NE_L[8] (Mcal/kg DM)	1.60	1.60	1.71	1.78
Fatty acids, g/100g of fat				
16:0	17.3	14.2	12.4	11.8
16:1 *cis*-9	0.10	0.09	0.08	0.07
18:0	3.17	3.52	3.67	3.74
18:1 cis-9	27.3	26.7	26.2	26.1
18:2n-6	44.0	46.8	47.8	48.5
18:3n-3	5.44	4.64	4.16	4.00

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]Vitamins: 13.570 kUI/kg of vitamin A, 3.520 kUI/kg of vitamin D3, 9.500 UI/kg of vitamin E; 500 mg/kg of butyl hydroxy toluene.
[3]BHT: Antioxidant butyl hydroxy toluene.
[4]iNDF: Indigestible neutral detergent fiber evaluated by 288h in situ incubation.
[5]pdNDF: Potential digestible neutral detergent fiber (*NDF – iNDF*).
[6]NFC: Non-fiber carbohydrates.
[7]DE: Digestible energy based on apparent digestibility.
[8]NE_L: Net energy for lactation according to NRC (2001) equation 2-12.

Table 3

Nutrient intake, performance and milk composition in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
Intake, kg/d							
DM	21.1	20.1	19.6	14.7	0.73	<0.01	<0.01
dOM[2]	13.5	12.6	12.3	8.9	0.68	<0.01	<0.01
DE[3] (Mcal/d)	62.9	59.5	60.5	45.9	2.32	<0.01	<0.01
Production, kg/d							
Milk	22.7	22.1	22.6	17.9	1.17	<0.01	<0.01
ECM[4]	21.7	21.4	19.8	16.2	1.01	<0.01	0.04
Fat	0.87	0.88	0.75	0.61	0.054	<0.01	0.26
Protein	0.74	0.69	0.68	0.59	0.040	<0.01	0.43
Lactose	1.04	1.01	1.03	0.79	0.053	<0.01	<0.01
Milk Composition, %							
Fat	3.87	4.01	3.33	3.50	0.232	0.04	0.44
Protein	3.24	3.15	3.05	3.29	0.194	0.75	0.06
Lactose	4.56	4.58	4.54	4.41	0.056	0.04	0.21
MUN[5], mg/dL	11.6	11.1	11.1	12.0	0.54	0.41	0.10
Feed Efficiency							
kg ECM/kg DMI	1.03	1.06	1.01	1.10	0.049	0.55	0.54

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]dOM: Digestible OM.
[3]DE: Digestible energy based on apparent digestibility.
[4]ECM: Energy corrected milk according to Sjauna et al. (1990).
[5]MUN: Milk urea nitrogen.

Table 4

Intake, omasal flow and digestibility of organic matter (OM), potentially digestible neutral detergent fiber (pdNDF), ether extract (EE) and non-fiber carbohydrates (NFC) in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
OM							
Intake, kg/d	19.4	18.5	18.0	13.6	0.67	<0.01	<0.01
Omasal flow, kg/d	8.76	8.76	8.88	7.52	0.519	0.01	0.01
RFOM[2], kg/d	13.8	13.4	13.3	10.6	0.61	<0.01	0.06
Apparent digestibility, %							
Rumen	54.9	52.8	50.5	45.0	1.96	<0.01	0.31
Total tract	69.9	68.1	68.6	66.1	1.73	0.01	0.70
MFOM[3], % DMI	145	149	136	134	15.5	0.13	0.90
NDF							
Intake, kg/d	5.00	4.83	4.71	3.65	0.171	<0.01	<0.01
Omasal flow, kg/d	3.19	3.04	3.21	2.82	0.218	0.07	0.21
Apparent digestibility, %							
Rumen	35.7	36.9	31.9	22.8	4.48	<0.01	0.18
Total tract	43.5	39.9	36.7	28.1	3.39	<0.01	0.36
pdNDF[4]							
Intake, kg/d	3.20	3.08	2.98	2.27	0.139	<0.01	<0.01
Omasal flow, kg/d	1.39	1.29	1.49	1.45	0.159	0.42	0.95
Apparent digestibility, %							
Rumen	55.7	57.6	49.6	35.2	6.45	<0.01	0.19
Total tract	67.9	62.5	57.2	44.4	4.41	<0.01	0.44

EE							
Intake, kg/d	0.46	0.71	1.24	1.39	0.057	<0.01	<0.01
Omasal flow, kg/d	0.48	0.75	1.14	1.37	0.075	<0.01	0.13
Apparent digestibility, %							
Rumen	-6.6	-6.5	7.0	2.3	5.37	0.08	0.33
Total tract	76.7	76.6	74.3	81.9	2.73	0.19	0.12
NFC							
Intake, kg/d	9.93	9.14	8.37	5.78	0.324	<0.01	<0.01
Omasal flow, kg/d	2.55	2.49	2.22	1.39	0.192	<0.01	0.09
Apparent digestibility, %							
Rumen	74.4	72.9	73.3	76.3	2.15	0.33	0.21
Total tract	81.1	81.5	83.9	83.8	1.43	0.06	0.56

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]RFOM: rumen fermented organic matter = *OM intake* – (*OM flow – bacterial OM flow*).
[3]MFOM = metabolic fecal OM.
[4]pdNDF: Potential digestible neutral detergent fiber (*NDF – iNDF*).

Table 5

Intake, omasal flow and digestibility of N, microbial protein synthesis (MPS) and N balance in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
Intake, g N/d	645	613	590	437	23.7	<0.01	<0.01
Omasal flow, g N/d	406	397	370	310	24.2	<0.01	0.37
Rumen N disappearence[2], % of N intake	36.8	35.2	36.9	29.5	3.59	0.16	0.36
Intake, kg CP/d	4.03	3.83	3.69	2.73	0.148	<0.01	<0.01
RDP[3], kg CP/d	3.23	3.15	3.30	2.33	0.205	0.02	0.04
% of CP intake	80.1	82.1	89.5	85.3	5.62	0.17	0.32
RUP[4], kg CP/d	0.80	0.68	0.39	0.40	0.126	0.06	0.16
% of CP intake	19.9	17.9	10.5	14.7	5.62	0.17	0.32
Total tract apparent digestibility, %	73.7	70.1	72.7	71.4	15.32	0.63	0.64
MPS, as g N/d	278	288	307	246	25.6	0.06	0.09
% of N flow	68.4	72.4	83.2	79.4	8.46	0.04	0.37
Microbial efficiency, g N/kg RFOM[5]	20.0	21.4	23.2	23.4	1.25	0.02	0.29
N balance							
Fecal excretion, g/d	170	183	161	125	11.0	<0.01	0.03
% of N intake	26.3	29.9	27.3	28.6	1.72	0.63	0.64
% of total N excreted	27.1	30.6	28.1	23.9	1.29	0.02	<0.01
Urine N, g/d	338	305	304	303	25.1	0.17	0.23
% of N intake	52.5	49.7	51.5	69.3	3.32	<0.01	<0.01
% of total N excreted	54.0	50.9	53.0	58.0	1.79	0.07	<0.01

UUN[6], g/d	289	278	273	243	20.5	0.09	0.71
% of urine N	85.4	91.2	89.7	80.2	7.44	0.68	0.46
Milk N, g/d	118.4	110.4	108.8	94.4	6.40	<0.01	0.43
% of N intake	18.4	18.0	18.4	21.6	1.02	0.03	0.20
% of total N excreted	18.9	18.5	18.9	18.1	1.01	0.92	0.43
N balance[7], g/d	18.5	14.5	16.2	-85.4	19.8	<0.01	0.01

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]Rumen N disappearance = (*N intake – N flow to omasum*)/*N intake*.
[3]RDP: Rumen-degraded protein = *CP intake* – (*Omasal flow of CP – microbial CP flow*).
[4]RUP: Rumen-undegraded protein = *Omasal flow of CP – microbial CP flow.*
[5]RFOM: Rumen fermented organic matter = *OM intake* – (*OM flow – bacterial OM flow*).
[6]UUN: Urinary urea nitrogen.
[7]N balance: Calculated as: *N intake* – (*N in feces* + *N in urine* + *N in milk)*.

Table 6

Rumen pool charachteristics and rumen kinetics of dry matter (DM) and fiber fractions in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
Pool, kg							
Total	93.6	87.9	85.7	77.6	3.32	<0.01	0.87
DM	10.9	10.6	9.9	9.4	0.59	<0.01	0.92
NDF[2]	5.75	5.71	5.63	5.26	0.355	0.09	0.50
iNDF[3]	3.18	3.18	2.96	2.64	0.212	<0.01	0.22
pdNDF[4]	2.57	2.53	2.66	2.62	0.197	0.68	0.86
Kinetics, %/h							
pdNDF							
k_i[5]	5.43	5.20	5.21	3.74	0.386	<0.01	0.10
k_p[6]	2.30	2.16	2.63	2.35	0.321	0.62	0.63
k_d[7]	3.13	3.04	2.58	1.39	0.243	<0.01	0.10
iNDF							
k_p	2.41	2.36	2.60	2.25	0.159	0.39	0.02

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]NDF = Neutral detergent fiber.
[3]iNDF = indigestible neutral detergent fiber evaluated by 288h in situ incubation.
[4]pdNDF = potentially digestible neutral detergent fiber (*NDF – iNDF*).
[5]k_i: Fractional rate of ingestion = (100/24) × (*intake* (kg)/*rumen pool* (kg)).
[6]k_p: Fractional rate of passage = (100/24) × (*omasal flow* (kg)/*rumen pool* (kg)).
[7]k_d: Fractional rate of digestion = k_i - k_p.

Table 7

Rumen pH, ammonia nitrogen (NH_3-N), lactate and volatile fatty acids (VFA) concentration in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
pH	6.42	6.48	6.48	6.67	0.110	<0.01	0.13
NH_3-N, mg/dL	28.1	24.2	23.9	21.4	1.44	<0.01	0.52
Lactate, m*M*	2.98	2.78	1.82	0.87	0.872	0.03	0.84
Total VFA, m*M*	153	147	149	122	4.2	<0.01	<0.01
VFA, mmol/mol							
Acetate	510	552	506	532	13.1	0.90	0.99
Propionate	284	252	281	264	11.2	0.64	0.83
Butyrate	154	147	156	151	5.7	0.95	0.91
Isobutyrate	13.1	12.2	13.9	12.2	0.63	0.76	0.28
Valerate	20.8	19.1	22.8	20.1	1.30	0.71	0.35
Isovalerate	18.3	17.9	20.3	21.2	1.33	0.05	0.93
A/PR[2]	1.84	2.24	1.83	2.04	0.129	0.92	0.86
(A+B)/PR[3]	2.40	2.82	2.40	2.62	0.156	0.89	0.87

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]A/PR = *Acetate/Propionate*;
[3](A+B)/PR = (*Acetate* + *Butyrate*)/*Propionate*.

Table 8

Serum concentrations of metabolites in dairy cows fed sugarcane based diets supplemented with varying concentrations soybean oil (SBO).

	SBO concentration[1] (% of DM)				SEM	Contrast	
	0	1.57	4.43	7.34		Lin.	Quad.
Item	Control	LSBO	MSBO	HSBO			
mmol/L							
NEFA[2]	0.04	0.03	0.04	0.03	0.01	0.67	0.98
BHB[3]	0.44	0.44	0.47	0.43	0.04	0.89	0.62
mg/dL							
Glucose	62.3	64.2	63.2	63.3	1.10	0.76	0.42
TC[4]	129	159	179	208	11.8	<0.01	0.34
HDL[5]	99.7	113.5	109.9	105.1	7.45	0.70	0.04
LDL[6]	27.1	42.6	66.1	99.6	11.6	<0.01	0.64
VLDL[7]	2.06	2.61	2.55	2.79	0.17	0.02	0.39
Triglycerides	10.3	13.1	12.8	14.0	0.86	0.02	0.40
TP[8]	7.90	8.11	7.90	7.89	0.04	0.49	0.51
Albumin	3.34	3.41	3.39	3.39	0.11	0.36	0.28
BUN[9]	20.0	18.6	17.2	17.1	1.19	0.02	0.27
Calcium	9.28	9.21	8.88	9.08	0.16	0.16	0.17

[1]SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
[2]NEFA: Non-esterified fatty acids.
[3]BHB: Beta-hydroxy butyrate.
[4]TC: Total cholesterol.
[5]HDL: High-density lipoproteins.
[6]LDL: Low-density lipoproteins.
[7]VLDL: Very low-density lipoproteins.
[8]TP: Total proteins.
[9]BUN: Blood urea nitrogen.

CHAPTER 3

Effects of soybean oil supplementation on omasal digesta and milk fatty acids in dairy cows fed sugarcane-based diets

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Short title: **Digesta and milk fatty acids changes due to dietary soybean oil**

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ABSTRACT

The experiments reported in this paper aimed to investigate the effects of increasing concentrations of soybean oil (SBO) in sugarcane-based diets on omasal digesta and milk fatty acids (FA) profile, focusing on conjugated linoleic acid (CLA). The diets were formulated with increasing concentrations of SBO (g/kg of dry matter (DM)): control (0 g/kg), low (LSBO; 15.7 g/kg), medium (MSBO; 44.3 g/kg) and high (HSBO; 73.4 g/kg). Experiment 1 (EXPI) was designed to quantify the flow of FA to the omasum and milk FA profile. In EXPI, eight rumen-cannulated multiparous Holstein-Friesian cows averaging 574 ± 19·1 kg body weight (BW) and 122 ± 6·9 d in milk (DIM) were used in a replicated 4 × 4 Latin square design balanced for residual effects. Experiment 2 (EXPII) was designed to quantify the milk FA profile in cows after calving. In EXPII 14 primiparous (545 ± 17·2 kg BW) and eight multiparous (629 ± 26·7 kg BW) Holstein-Friesian cows were used after calving. The concentration of 11*trans*-18:1, 9*cis*,11*trans*-18:2 CLA, and 9*cis*,11*trans*-18:2 CLA in omasal digesta increased linearly with the addition of SBO. Soybean oil increased quadratically the biohydrogenation rate of polyunsaturated FA (PUFA), with a slightly increase from MSBO to HSBO. Milk fat concentration in both experiments decreased with SBO supply; however, fat yield was not affected in EXPII. In both experiments, the concentration (g/kg) of 9*cis*,11*trans*-18:2 CLA in milk increased linearly in despite of the yield (g/d) increased quadratically. 10*trans*,12*cis*-18:2 CLA in milk was detected in MSBO and HSBO diets. The addition of SBO proved to be a feasible alternative to increase the rumen outflow of 11*trans*-18:1 and milk 9*cis*,11*trans*-18:2 CLA concentration; however, SBO supplementation greater than 15.7 g SBO/kg DM decreases milk and fat yield without additive response on 9*cis*,11*trans*-18:2 CLA yield.

Keywords: biohydrogenation, conjugated linoleic acids, desaturase index, fat supplementation, fatty acid profile

INTRODUCTION

Due to the potential nutraceutical properties of milk fatty acids (FA), studies evaluating changes in milk FA profile encompassing alterations in dairy cows' diets have been made in recent years (Shingfield & Wallace, 2014). The CLA isomers have shown effects on human metabolism and health. Studies demonstrated that the intake of 9*cis*,11*trans*-18:2 CLA by humans is beneficial and decreases the risk of diseases such as cancer, obesity, and immune disorders (Salter, 2013; Kanwar *et al*., 2016). Dairy products are an important source of CLA in human nutrition and a better understanding of rumen biohydrogenation of polyunsaturated FA (PUFA) is key to increase CLA concentration in cow's milk. The use of plant oils such as SBO to feed cows generally is constrained to concentrations up to 60 or 70 g of ether extract (EE) per kg of DM to avoid deleterious effects on fibre digestibility (Lewis *et al*., 1999; NRC, 2001). In general, that kind of studies for evaluating the effects of plant oils, do not cross the concentrations of fat higher than recommended (Glasser *et al*., 2008; Rennó *et al*., 2013; do Prado *et al*., 2015). It is well known that the forage type and concentration in the diet affects the response to fat supplementation in ruminal lipid metabolism and milk FA composition (Sterk *et al*., 2012; Vazirigohar *et al*., 2014). Diets with sugarcane have limitations in energy intake as consequence of the high indigestible fraction in neutral detergent fibre (NDF) (Oliveira *et al*., 2011). Therefore, the supplementation with vegetable oils could be an alternative to increase the energy intake of dairy cows fed sugarcane. However, there is a lack of research about strategies to alter rumen lipid metabolism and milk FA using SBO with different type of forage and feeding systems (Rennó *et al*., 2013). Indeed, there is a limited data in the literature about ruminal lipid metabolism and milk FA from cows fed tropical forages such as sugarcane and fat concentrations in the diet.

Based on the hypothesis that SBO supplementation increases the flow of biohydrogenation intermediates to the omasum and increases milk CLA, two studies were conducted aiming to investigate the inclusion of SBO at concentrations higher than

conventional on ruminal lipid metabolism and milk FA profile of cows fed sugarcane-based diets. The objective was to find the optimum concentration of SBO inclusion to increase milk CLA in cows fed sugarcane-based diet.

MATERIALS AND METHODS

The Committee of Production Animal Care and Use of the Federal University of Viçosa approved all animal feeding, management and procedures involved in this study (Protocol nº 072/2014).

Diets

Diets were formulated with increasing concentrations of SBO (g/kg of DM): control (0 g/kg), low (LSBO; 15·7 g/kg), medium (MSBO; 44·3 g/kg) and high (HSBO; 73·4 g/kg) (Table 1). The diets were formulated to be iso-nitrogenous by changing the proportions of maize and soybean meal. A fixed forage-to-concentrate ratio of 40:60 was used. The sugarcane (variety RB-867515) was harvested daily in the afternoon and chopped before the morning feeding. The sugarcane stems had an average soluble content of 18·0±1·68 °Brix. The cows were milked twice daily at 05·50 and 14·50 h, and fed total mixed ration (TMR) individually after each milking. Intake was calculated by manually weighing the offered TMR and collected orts. Diets were offered on an *ad libitum* basis (target 100 g refusal/kg fed) and the amount offered was adjusted daily.

Experiment I

EXPI aimed to study the effects of SBO supplementation on FA flow to the omasum and milk FA profile in cows during the second third of lactation.

Animals and experimental design

Eight, rumen-cannulated, multiparous, Holstein-Friesian cows in mid-lactation (574 ± 19·1 kg BW and 122 ± 6·9 DIM) were used. Milk yield was 22·5 ± 1·22 kg/d at the beginning of the study. Cows were grouped in a replicated 4 × 4 Latin square design balanced for residual effects. The experimental period lasted 21 d, comprising of 14 d for adaptation followed by a sampling period from d 15 to 21.

Samplings

The diet composition and nutrient intakes were estimated by sampling each ingredient at the time of concentrate mixing. Samples of sugarcane and orts were collected from d 15 to 19 of each period and pooled fresh for chemical analysis. All feed samples were stored at -20°C for FA analysis.

From d 13 to 18 of each period, infusions of 8 g/d of Co-EDTA (766 mg of Co/d) were performed four times daily at 00.00, 06.00, 12.00 and 18.00 h. The omasal flow of nutrients was estimated by collecting eight omasal samples at 9 h intervals starting at 09.00 h on d 16 of each period. The omasal fluid was collected according to the technique described by Huhtanen et al. (1997) and adapted by Leão (2002). At each sampling time, approximately 1.2 L of digesta was obtained and divided into subsamples. The markers concentrations and omasal flow were estimated by filtering 500 mL through a 100 µm nylon mesh filter with 44% open area (Sefar Nitex 100/44; Sefar, Thal, Switzerland), providing particle and fluid phase samples. Samples were immediately dried at 55°C and the particle and fluid phases were pooled separately by cow and period. For FA analysis aliquots of omasal digesta (100 mL) were taken and stored at -20°C until analysis. Aliquots for FA analysis were pooled per cow and period.

Milk yield was measured daily at the every milking, and samples were collected for analyses from d 16 to 18 of each period. A pooled sample was made proportionally to production in each milk session and stored at -20°C until analysis.

Experiment II

EXPII aimed to study the effects of SBO supplementation on milk FA profile of cows after calving.

Animals and experimental design

Fourteen primiparous (545 ± 17·2 kg BW) and eight multiparous (629 ± 26·7 kg BW) Holstein-Friesian dairy cows were used. For 21 d before expected calving, all animals were fed with a sugarcane-based diet supplemented with refined SBO (150 g of crude protein (CP) and 20 g SBO/kg of DM). After calving, cows were randomly assigned within the two parity groups to the diets (Table 1). The experiment was designed to be analysed using 24 animals; however, the data of two primiparous cows were omitted for the experimental period due to babesiosis and dystocia followed by caesarean.

Samplings

The study was performed from calving until 84 DIM, divided in three periods of 28 d each. Dietary composition and intake was calculated in the same manner as for EXPI. Samples of sugarcane and orts were collected from d 13 to 16 of each period and pooled for chemical analysis. Aliquots of all feed samples were stored at -20°C for FA analysis.

Milk samples were taken on d 14 and 15 of each period during each milk session. After sampling, the milk was stored at -20ºC and pooled per cow and period according to the yield in each milking session.

Laboratory analysis and calculations

Samples of feed, orts and omasal digesta were oven-dried at 55°C for 72 h and grounded through 2 and 1 mm screens (Wiley mill; A. H. Thomas, Philadelphia, PA) before analyses. The DM, organic matter (OM) and CP content of feeds, orts, omasal

digesta and milk were analysed using established methods (AOAC 2005) methods number 930·15, 942·05 and 984·13, respectively. The concentration of NDF was evaluated using heat stable α-amylase with a fibre analyser ANKOM 220 (ANKOM Technology, Fairport, USA), using the AOAC (2005) method 2002·04, expressing the results without residual ash. The iNDF content of feeds and omasal digesta was analyzed according to Valente *et al*. (2011). The milk fat content was evaluated according to AOCS (2004) method Am 5-04.

Due to the high fat content of the experimental diets and the formation of FA-salts in the rumen (AbuGhazaleh *et al*., 2002), acid hydrolysis with HCl (ISO, 1973) was performed in all feeds, orts and omasal digesta samples before ether extraction according to AOAC (2005) method 2003·05.

Cobalt was analysed by an atomic absorption spectrophotometer (Spctr AA-800, Varian spectrometer, Harbour City, CA).

Digesta flow to the omasum was calculated based on the double marker method proposed by Faichney (1975) using Co as a fluid phase marker and iNDF as a particle phase marker.

Frozen aliquots of feeds were lyophilized and milled through a 1 mm screen. The FA profile of the feeds was analyzed by the extraction method described by Folch *et al*. (1957). The separated fats were methylated and the FA methyl esters, which were formed according to Kramer *et al*. (1997), were quantified by GC (Shimadzu GC-2010 with automatic injection) using a capillary column (SP-2560; 100×0.25 mm diameter, 0.02 mm thickness, Supelco, Bellefonte, PA, USA). The oven temperature was 70°C for 4 min, then increased at 13°C/min to 175°C, and held for 27 min, before a further increase at 4°C/min until reaching 215°C, which was maintained for 31 min. Hydrogen (H_2) was used as the carrier gas with a flow of 40 cm^3/s. During the identification process, four standards were used to identify the FA that were formed during the biohydrogenation of

unsaturated fatty acids: standard C4-C24 of FA (Supelco® TM 37), vacenic acid 11*trans*-18:1 (V038- 1G, Sigma®), 10*trans*,12*cis*-18:2 CLA (UC-61M 100 mg), 9*cis*,11*trans*-18:2 CLA (UC-60M 100 mg), (NU-CHEK-PREP, USA®). The FA profile was reported as in units of g/100g of fat.

The PUFA biohydrogenation rate (BHR) was calculated as $BHR = 100 \times (\Sigma PUFAi - \Sigma PUFAf)/\Sigma PUFAi$, where ΣPUFAi = intake of PUFA (g/d) and ΣPUFAf = omasal flow of PUFA (g/d) (Fievez *et al*. 2007). The desaturase index (**DI**) was calculated for pairs of FA as: *product of Δ9-desaturase/(product of Δ9-desaturase + substrate of Δ9-desaturase)* (Kelsey *et al*., 2003).

Statistical Analysis

The statistical analyses were performed using the SAS MIXED procedure (SAS Institute Inc., Cary, NC, version 9.4). Data of the EXPI was analysed using the model of the form:

$$Y_{ijkl} = \mu + T_i + SQ_j + P(SQ)_k + C(SQ)_l + \varepsilon_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, T_i is the fixed effect of a treatment, SQ_j is the fixed effect of the square, $P(SQ)_k$ is the fixed effect of period within square, $C(SQ)_l$ is the random effect of cow within square, and ε_{ijkl} is the model error.

Data from EXPII was analysed using the model:

$$Y_{(i)jkl} = \mu + T_i + B_j + P_l + (TB)_{ij} + (TP)_{il} + e_{ijkl}$$

where μ is the overall mean, T_i is the fixed effect of treatment, B_j is the random effect of parity, P_l is the fixed effect of period, $(TB)_{ij}$ is the effect of interaction between treatment and block $(TP)_{il}$, is the effect of interaction between treatment and period, and $e_{(i)jkl}$ is the model error. The effect of period was interpreted as repeated measures considering cow within treatment as subject. The best fitting covariance structures of the repeated

measures were chosen according to the lower Akaike's Information Criterion with correction (AICc).

Denominator degrees of freedom were calculated using the Kenward–Roger approximation. Results were expressed as least square means with standard error of the means and the effects of SBO concentrations were tested with polynomial linear and quadratic contrasts. To correct the unequally space of the treatments, the PROC IML's ORPOL function was used to obtain the appropriate coefficients to the CONTRAST statements. Significance of the effect was declared at $P < 0{\cdot}05$.

RESULTS

The data for intake and omasal flow of OM, EE and FA in EXPI and EXPII are shown on Table 2 and 4, respectively. In both experiments the SBO quadratically decreased ($P < 0{\cdot}05$) OM and EE intake, with greatest decrease in MSBO and HSBO diets. Mostly FA with 18 carbons flowing to the omasum increased linearly ($P < 0{\cdot}05$) when SBO increased except for C18:0 and 9,12*cis*-18:2 CLA which were quadratically ($P < 0{\cdot}05$) affected and unaffected ($P > 0.10$), respectively. The SBO quadratically increased ($P < 0{\cdot}05$) BHR of PUFA with lower increases at MSBO and HSBO diets. Soybean oil decreased ($P < 0{\cdot}05$) milk fat concentration in both experiments (Tables 3 and 4); however, due to the greater increase in milk yield, fat yield was not affected ($P > 0{\cdot}05$) in EXPII (Table 4).

In both experiments, the concentration of 9*cis*,11*trans*-18:2 CLA in milk increased linearly ($P < 0{\cdot}05$) (Tables 3 and 4). The SBO increased quadratically the 9*cis*,11*trans*-18:2 CLA yield ($P < 0{\cdot}05$) in both experiments (Tables 3 and 4). The presence of 10*trans*,12*cis*-18:2 CLA in milk was observed only in MSBO and HSBO diets (Tables 3 and 4). The concentration of both 9*cis*,11*trans*-18:2 and 10*trans*,12*cis*-18:2 CLA in milk fat were affected ($P < 0{\cdot}05$) by period (Table 4). The SBO addition linearly decreased (P

< 0·05) the DI of 9*cis*,11*trans*-18:2 CLA in both experiments (Tables 3 and 4). The DI of *cis*9-18:1 was quadratically decreased ($P < 0·05$) in EXPI (Table 3) and linearly decreased ($P<0·05$) in EXPII (Table 4) by SBO increase.

Fatty acids derived from *de novo* synthesis (up to 14 carbons) were quadratically decreased ($P < 0·05$) in both experiments (Tables 3 and 4). In both experiments the concentration of FA greater than 18 carbons increased quadratically ($P<0·05$) with the SBO inclusion (Tables 3 and 4). In both experiments the MUFA concentration in milk fat increased linearly ($P < 0·05$) until HSBO diet (Tables 3 and 4). The PUFA concentration in milk fat increased linearly ($P < 0·05$) in the EXPII (Table 4).

DISCUSSION

Cellulolytic bacteria are the most active bacteria in the ruminal biohydrogenation of PUFA, and SBO has an inhibitory effect on ruminal population of this bacterial group (Yang *et al*., 2009; Maia *et al*., 2010). The low concentration of digestible NDF in the diets could decrease the activity of cellulolytic bacteria (Harvatine & Allen, 2006). In our study, the increase in BHR of PUFA may be related to other mechanisms, such as the decrease in fractional rate of passage of iNDF (Rodrigues *et al*., 2016). The decrease on rate of passage in the rumen due to the decrease in feed intake could increase the BHR of PUFA (Harvatine & Allen, 2006).

The uptake of 9*cis*,11*trans*-18:2 CLA and 11*trans*-18:1 in the mammary gland are primarily sources to the content of 9*cis*,11*trans*-18:2 CLA in milk (Shingfield & Wallace, 2014). The 11*trans*-18:1 formed in the rumen is the main precursor of 9*cis*,11*trans*-18:2 CLA isomer synthesized by the Δ9-desaturase enzyme in the mammary gland (Corl *et al*., 2001). Thus, the increase in 11*trans*-18:1 flow to the omasum is the main objective when supplementing fat to increase CLA contents in ruminant products (Shingfield & Wallace, 2014). The additive effects of SBO inclusion were linear and increased about 8-times the

flow of 11*trans*-18:1 to the omasum when comparing the control and HSBO diets. Nevertheless, the increase in 9*cis*,11*trans*-18:2 CLA in milk fat did not follow the response of 11*trans*-18:1 flow to the omasum. It is important to note that the use of Co-EDTA as a fluid marker in the EXPI leads to a lower activity of Δ9-desaturase activity as observed by Shingfield *et al*. (2008). The lower response in increasing 9*cis*,11*trans*-18:2 CLA in milk fat was also observed in EXPII. The effect of period on 9*cis*,11*trans*-18:2 CLA concentration in milk probably is a consequence of two effects: the increase in mammary gland Δ9-desaturase activity increase as a function of DIM (Kelsey *et al*., 2003) and the increase in fat intake (Kay *et al*., 2005). However, the DI of 9*cis*,11*trans*-18:2 CLA do not show increase when DIM increased which could indicates greater response to the increase in fat intake. Also, Rennó *et al*. (2013) observed that SBO increased 9*cis*,11*trans*-18:2 CLA in milk of cows after calving. The decrease in DI 9*cis*-18:1 and 9*cis*,11*trans*-18:2 CLA in both studies was consequence of increase in flow of substrates to the omasum without increase in products. Perhaps the higher Δ9-desaturase activity in increased DIM explain the greater concentration of 9*cis*,11*trans*-18:2 CLA and DI of 9*cis*,11*trans*-18:2 CLA in milk observed in EXPI. However, the DI was higher in EXPI only for 9*cis*,11*trans*-18:2 CLA and lower for 9*cis*-18:1. When comparing the results observed in both experiments it seems that the inclusion of SBO to increase 9*cis*,11*trans*-18:2 CLA concentration in milk fat is more effective in cows after 84 DIM, and its response can be higher in farm conditions than in EXPI due to the absence of Co-EDTA on Δ9-desaturase activity (Shingfield *et al*., 2008). Wang *et al*. (2015) observed increase in 9*cis*,11*trans*-18:2 CLA in milk and Δ9-desaturase mRNA expression in mammary gland supplementing SBO at similar concentration (25 g/kg of diet DM) to LSBO diet. In our study, the LSBO diet promote the CLA production without greater milk fat depression (**MFD**) and decrease in DI of 9*cis*,11*trans*-18:2 CLA.

The absence of response in CLA production in the MSBO and HSBO diets probably are associated to the omasal flow and production of 10*trans*,12*cis*-18:2 CLA. The HSBO diet increased the concentration of 10*trans*,12*cis*-18:2 CLA level in omasal digesta and milk, which leads to an important decrease in milk fat with no increase in CLA yield. Bauman & Griinari (2003) demonstrated the direct effect of 10*trans*,12*cis*-18:2 CLA increase in milk and the occurrence of MFD and decrease in the *de novo* synthesis of FA in the mammary gland in cows fed low fibre diets. The similar effect was observed by AlZahal *et al*. (2008) supplementing SBO in corn silage and haylage based diets. Keating *et al*. (2006) observed that 10*trans*,12*cis*-18:2 CLA decrease the Δ9-desaturase enzyme activity in the mammary gland, decreasing the desaturation of 11*trans*-18:1 to 9*cis*,11*trans*-18:2 CLA. In current study, the 10*trans*,12*cis*-18:2 CLA flow to the omasum was only observed in HSBO diet in the EXPI and close to zero in control and LSBO diets in EXPII. Kim *et al*. (2002) showed that in high concentrate and low fibre diets the ruminal population of *Megasphaera elsdenii* YJ-4 produce 10*trans*,12*cis*-18:2 CLA using lactate as substrate. In the present study, rumen concentration of lactate was linearly decreased as SBO increased (data not shown). Probably the MSBO and HSBO diets favoured bacterial populations with similar roles in the rumen biohydrogenation of FA.

The concentration of 9*cis*,11*trans*-18:2 CLA in milk fat and daily yield of that isomer together may indicate that the increase in milk CLA concentration is more associated with MFD than the increase in its synthesis in the mammary gland. Based on the low increase in CLA in milk fat concentration greater than MSBO and the absence of an increase in CLA produced (g/d), the use of HSBO diet should be not recommended. Furthermore, the decrease in OM intake and milk yield in both studies indicate potential adverse effects of feeding cows at the HSBO diet. Despite the linear increase in the concentration of 9*cis*,11*trans*-18:2 CLA in milk in both studies, the presence of 10*trans*,12*cis*-18:2 CLA in milk fat at MSBO and HSBO represents another reason to

avoid high SBO supplementation. The amount of 9*cis*,11*trans*-18:2 CLA produced did not show additive response at concentrations greater than MSBO in either studies.

CONCLUSIONS

In conclusion, the addition of SBO proved to be a feasible alternative to increase rumen outflow of 11*trans*-18:1. However, to increase the 9*cis*,11*trans*-18:2 CLA contents in milk the SBO supplementation to concentrations between 4.43 and 7.34 g SBO/kg DM provided low efficiency of use of substrates to produce 9*cis*,11*trans*-18:2 CLA in the mammary gland. In order to increase the yield of 9*cis*,11*trans*-18:2 CLA without great decrease in intake and milk and fat production of dairy cows fed sugarcane-based diets, SBO supplementation at 1.57 g SBO/kg DM is recommended.

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TABLES

Table 1

Ingredients and chemical composition of the experimental diets

	SBO concentration†			
	Control	LSBO	MSBO	HSBO
Ingredient, g/kg of dry matter				
Sugarcane	400	400	400	400
Maize meal	330	309	275	238
Soybean meal	216	221	227	234
Soybean oil	-	15·7	44·3	73·4
Urea	8·3	8·3	8·3	8·3
Ammonium sulphate	0·9	0·9	0·9	0·9
Minerals and vitamins	45·4	45·4	45·4	45·4
Composition, g/kg of DM				
Experiment I				
Organic matter	922	922	922	922
Crude protein	181	182	182	183
Ether extract	18·3	33·3	60·8	88·6
NDF‡	251	249	246	244
NFC§	472	458	433	406
Fatty acids, g/100g of fat				
16:0	17·3	14·2	12·4	11·8
9*cis*-16:1	0·10	0·09	0·08	0·07
18:0	3·17	3·52	3·67	3·74
9*trans*-18:1	27·3	26·7	26·2	26·1
11*trans*-18:1	44·0	46·8	47·8	48·5
9*cis*-18:1	5·44	4·64	4·16	4·00
Experiment II				
Organic matter	920	920	920	919
Crude protein	162	163	163	163
Ether extract	17·4	32·4	59·9	87·7
NDF	231	229	226	224
NFC	510	496	471	444
Fatty acids, g/100g of fat				
16:0	17·5	14·4	12·5	11·9
9*cis*-16:1	0·10	0·08	0·07	0·07
18:0	3·24	3·56	3·69	3·76
9*trans*-18:1	26·9	26·6	26·2	26·1
11*trans*-18:1	45·2	47·4	48·2	48·7
9*cis*-18:1	5·34	4·56	4·09	3·94

† SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
‡NDF = Neutral detergent fibre
§NFC = Non-fibrous carbohydrates

Table 2

Intake and omasal flow of organic matter (OM), ether extract (EE) and fatty acids to the omasum of cows fed by sugarcane and concentrations of soybean oil (SBO) in the EXPI

	SBO concentration†				SEM	Contrast‡	
	0	1.57	4.43	7.34		L	Q
	Control	LSBO	MSBO	HSBO			
Intake, kg/d							
OM	19·4	18·5	18·0	13·6	0·67	<0·01	<0·01
EE	0·46	0·71	1·24	1·39	0·057	<0·01	<0·01
Fatty acids, g/d							
16:0	79	101	153	164	7·1	<0·01	<0·01
9cis-16:1	0·47	0·61	0·94	1·01	0·043	<0·01	0·01
18:0	14·4	25·0	45·3	52·2	2·07	<0·01	<0·01
9cis-18:1	124	190	323	365	14·9	<0·01	<0·01
18:2 n-6	200	333	590	676	27·0	<0·01	<0·01
18:3 n-3	24·7	33·0	51·3	55·8	2·38	<0·01	<0·01
Σ SFA§	101	136	216	235	9·0	<0·01	<0·01
Σ MUFA††	123	190	327	369	15·0	<0·01	<0·01
Σ PUFA‡‡	201	334	597	682	27·3	<0·01	<0·01
Omasal flow, kg/d							
OM	8·76	8·76	8·88	7·52	0·607	0·01	0·02
EE	0·48	0·75	1·14	1·37	0·075	<0·01	0·14
Fatty acids, g/d							
16:0	84·3	108·6	131·4	165·3	1·00	<0·01	0·90
9*cis*-16:1	1·10	0·98	0·22	0·13	0·198	<0·01	0·22
18:0	260	425	670	749	44·1	<0·01	0·04
9*trans*-18:1	3·2	5·2	10·9	18·8	1·65	<0·01	0·23
11*trans*-18:1	15·6	45·0	76·5	121·8	11·79	<0·01	0·99
9*cis*-18:1	36·1	37·8	44·3	49·5	5·49	<0·01	0·92

9,12*cis*-18:2	35·0	38·0	43·6	44·6	5·91	0·11	0·63
9,12,15*cis*-18:3	1·29	2·34	2·67	2·94	0·414	0·03	0·29
9*cis*,11*trans*-18:2	0·01	0·27	1·20	1·80	0·243	<0·01	0·88
10*trans*,12*cis*-18:2	0·02	0·00	0·25	1·49	0·354	<0·01	0·11
Σ SFA	370	561	832	946	53·8	<0·01	0·08
Σ MUFA	55·8	89·0	132·1	190·4	14·29	<0·01	0·88
Σ PUFA	36·5	40·6	47·7	50·9	6·41	0·04	0·70
BHR§§ (%)	81·8	87·8	92·0	92·5	1·56	<0·01	<0·01

† SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
‡Contrast: effect of SBO inclusion: L = Linear; Q = quadratic
§SFA: Saturated fatty acids
††MUFA: Mono-unsaturated fatty acids
‡‡PUFA: Polyunsaturated fatty acids
§§BHR = Biohydrogenation rate of PUFA

Table 3

Milk yield and composition and fatty acids (FA) profile in milk of cows fed sugarcane and different concentrations of soybean oil (SBO) in the EXPI

	SBO concentration†				SEM	Contrast‡	
	0	1.57	4.43	7.34		L	Q
	Control	LSBO	MSBO	HSBO			
Yield							
Milk, kg/d	21·7	22·1	22·6	17·8	1·19	<0·01	0·02
Fat, kg/d	0·83	0·88	0·75	0·61	0·056	<0·01	0·21
Fat, g/kg	37·8	40·1	33·3	35·0	2·07	0·05	0·56
Fatty acids, g/100 g FA							
Σ FA up to 14 C	24·9	20·1	14·9	10·7	0·82	<0·01	0·04
16:0	36·1	28·5	24·1	23·1	1·02	<0·01	<0·01
9*cis*-16:1	1·40	0·96	0·96	0·97	0·091	<0·01	<0·01
18:0	9·7	16·0	18·1	18·8	0·91	<0·01	<0·01
9*trans*-18:1	0·24	0·36	0·75	1·07	0·072	<0·01	0·86
11*trans*-18:1	0·79	1·54	3·12	5·31	0·480	<0·01	0·44
9*cis*-18:1	18·5	23·1	27·1	28·3	1·23	<0·01	<0·01
9,12*cis*-18:2	2·41	2·22	2·22	2·13	0·26	0·24	0·71
9*cis*,11*trans*-18:2	0·59	0·92	1·07	1·33	0·18	<0·01	0·56
g/d	4·51	7·94	7·69	7·57	1·029	0·06	0·05
10*trans*,12*cis*-18:2	nd§§	Nd	nd	0·018	0·0070	-	-
g/d	nd	Nd	nd	0·069	0·0345	-	-
9,12,15*cis*-18:3	0·110	0·094	0·091	0·089	0·02	0·33	0·59
Σ FA greater than 18C	33·4	45·1	53·1	57·7	1·10	<0·01	<0·01
Σ SFA§	70·6	64·7	57·1	52·7	1·54	<0·01	0·03
Σ MUFA††	21·8	26·5	32·5	36·1	1·16	<0·01	0·07
Σ PUFA‡‡	3·28	3·30	3·38	3·58	0·380	0·27	0·75
Desaturase index							

9*cis*-16:1	0·038	0·033	0·038	0·041	0·004	0·12	0·25
9*cis*-18:1	0·66	0·59	0·60	0·60	0·020	0·03	0·03
9*cis*,11*trans*-18:2	0·44	0·42	0·28	0·22	0·055	<0·01	0·93

† SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
‡Contrast: effect of SBO inclusion: L = Linear; Q = quadratic
§SFA: Saturated fatty acids
††MUFA: Mono-unsaturated fatty acids
‡‡PUFA: Polyunsaturated fatty acids
§§nd: not detected

Table 4

Milk yield and composition and fatty acids (FA) profile in milk of cows fed sugarcane and different concentrations of soybean oil (SBO) in the EXPII

	SBO concentration†				Period			SEM	*P-value*‡			
											Contrast§	
	0	1.57	4.43	7.34	1	2	3		P×T	P	L	Q
	Control	LSBO	MSBO	HSBO								
Intake, kg/d												
OM	17·1	17·4	16·8	14·7	14·5	17·0	17·9	1·62	0·29	<0·01	<0·01	0·02
EE	0·35	0·68	1·16	1·47	0·81	0·94	1·00	0·095	0·57	<0·01	<0·01	<0·01
Fatty acids, g/d												
16:0	61	92	146	175	106	122	131	1·26	0·60	<0·01	<0·01	<0·01
9cis-16:1	0·34	0·56	0·87	1·05	0·63	0·72	0·77	0·001	0·60	<0·01	<0·01	<0·01
18:0	11·5	24·3	43·0	55·1	29·8	34·3	36·4	3·57	0·55	<0·01	<0·01	0·01
9cis-18:1	95	181	305	383	214	247	263	25·7	0·57	<0·01	<0·01	0·01
18:2 n-6	160	323	562	714	391	450	479	46·5	0·56	<0·01	<0·01	0·01
18:3 n-3	18·7	31·0	47·7	57·9	34·5	39·7	42·4	4·07	0·60	<0·01	<0·01	0·01
Σ SFA§	79	131	202	246	146	168	179	17·4	0·60	<0·01	<0·01	0·01
Σ MUFA††	96	182	308	387	216	249	265	26·0	0·57	<0·01	<0·01	0·01
Σ PUFA‡‡	179	355	611	774	426	491	522	51·2	0·56	<0·01	<0·01	0·01
Yield												
Milk, kg/d	23·9	28·7	31·3	25·2	25·0	28·4	28·4	0·74	0·06	<0·01	0·65	<0·01
Fat, kg/d	1·02	1·16	0·94	0·91	0·94	1·07	1·01	0·032	0·02	0·03	0·13	0·65
Fat, g/kg	41·8	41·4	33·6	36·3	38·9	39·0	37·0	0·80	0·46	0·32	<0·01	0·11
Fatty acids, g/100 g FA												
Σ FA up to 14 C	23·1	21·3	12·6	10·9	15·0	16·5	19·3	1·76	0·47	<0·01	<0·01	0·18
16:0	34·0	28·8	23·6	23·5	27·0	26·6	28·9	1·38	<0·01	<0·01	<0·01	<0·01
9*cis*-16:1	1·46	1·17	1·30	1·07	1·35	1·26	1·15	0·120	0·15	0·06	0·07	0·90
18:0	9·91	13·65	16·86	16·28	15·65	14·27	12·60	0·48	0·58	<0·01	<0·01	<0·01

9*trans*-18:1	0·21	0·30	0·81	1·31	0·65	0·64	0·68	0·06	0·92	0·78	<0·01	0·31
11*trans*-18:1	0·69	1·26	3·32	6·17	2·91	2·61	3·08	0·31	0·98	0·39	<0·01	0·08
9*cis*-18:1	22·8	24·8	30·4	29·0	28·0	28·09	24·3	0·70	0·30	<0·01	<0·01	0·09
9,12*cis*-18:2	2·19	2·36	2·52	2·99	2·27	2·54	2·75	0·081	0·36	<0·01	<0·01	0·54
9*cis*,11*trans*-18:2	0·35	0·56	0·71	0·68	0·49	0·55	0·68	0·04	0·37	0·01	<0·01	0·08
g/d	3·46	5·81	6·96	5·43	4·43	5·41	6·41	0·837	0·64	0·02	0·13	0·03
10*trans*,12*cis*-18:2	0·000	0·000	0·007	0·037	0·000	0·024	0·009	0·004	<0·01	<0·01	<0·01	0·02
g/d	0·00	0·00	0·10	0·32	0·00	0·24	0·32	0·050	0·01	<0·01	<0·01	0·14
9,12,15*cis*-18:3	0·116	0·125	0·135	0·171	0·131	0·138	0·142	0·006	0·71	0·62	<0·01	0·43
Σ FA greater than 18C	37·4	44·2	55·4	57·4	51·1	49·8	45·1	2·33	0·11	<0·01	<0·01	0·03
Σ SFA††	66·1	64·1	53·2	50·8	58·0	57·6	60·8	1·05	0·03	<0·01	<0·01	0·14
Σ MUFA‡‡	26·1	28·4	36·4	38·1	33·4	33·3	30·0	0·86	0·19	<0·01	<0·01	0·25
Σ PUFA§§	2·86	3·26	3·44	3·92	3·04	3·37	3·69	0·10	0·22	<0·01	<0·01	0·94
Desaturase index												
9*cis*-16:1	0·041	0·038	0·052	0·043	0·048	0·045	0·038	0·0049	0·30	<0·01	0·25	0·18
9*cis*-18:1	0·70	0·65	0·64	0·64	0·64	0·67	0·67	0·019	0·75	0·01	0·08	0·22
9*cis*,11*trans*-18:2	0·35	0·35	0·21	0·10	0·22	0·28	0·24	0·029	0·70	0·11	<0·01	0·39

† SBO concentration: LSBO = low soybean oil; MSBO = medium soybean oil; HSBO = high soybean oil.
‡P-value: P×T = effect of interaction of period and concentrations of SBO; P = effect of period
§Contrast: effect of SBO inclusion: L = Linear; Q = quadratic·
††SFA: Saturated fatty acids
‡‡MUFA: Mono-unsaturated fatty acids
§§PUFA: Polyunsaturated fatty acids