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Strategies to modify the ruminal biohydrogenation of polyunsaturated fatty acids and the production of *trans*-10, *cis*-12 C18:2 *in vitro*

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ABSTRACT

The production of *trans*-10, *cis*-12 C18:2 isomer in the rumen is associated with milk fat depression. Two experiments were conducted to determine the effects of some additives on rumen fermentation and apparent biohydrogenation of linoleic (LA) and linolenic (LNA) acids, and the production of the *trans*-10, *cis*-12 C18:2 isomer associated with milk fat depression. In experiment 1, a 1:1 forage:concentrate diet containing linseed oil (84 g/kg of DM) was incubated in a 2 h or 6 h batch culture with rumen fluid in 2 replicated periods. Treatments in the 2 h incubations were: control; lipase 1 and 2 (4 and 40 µl/l) and a lipase inhibitor (4 and 20 mg/l); and in the 6 h incubations were: Oxy-propyl-thiosulfate (PTSO; 60 and 120 mg/l); Eugenol (EUG; 150 and 500 mg/l) and Cinnamaldehyde (CIN; 150 and 500 mg/l). After incubation, samples were collected to analyze ammonia-N, volatile fatty acids (VFA) and the fatty acid (FA) profile. In experiment 2, 8 continuous culture fermenters (1320 ml) were used in 3 replicated periods (5 d of adaptation and 3 d of sampling). Fermenters were fed 95 g/d of DM of a 60:40 forage:concentrate diet containing 50 g/kg DM of linseed oil. Treatments were control, lipase 1 (4 µl/l), PTSO (90 mg/l) and CIN (250 mg/l), and were tested at 2 pH levels (6.4 and 5.6). During the last 3 d of each period, samples were taken to analyze VFA, ammonia-N and the FA profile. In experiment 1, Lipase 1 increased the apparent biohydrogenation of LNA, but these results were not observed in experiment 2. Both CIN and PTSO seem to inhibit the apparent biohydrogenation of LNA, but only the PTSO inhibited the biohydrogenation of LA in a 6 h incubation compared with control. Cinnamaldehyde in Experiment 1, and PTSO in Experiment 1 and 2 decreased total VFA concentrations. Although the short-term batch incubation suggested some potential benefits of use of some additives for modifying rumen biohydrogenation pathways, effects observed in the long-term fermentation were not relevant for the purpose of decreasing the production of *trans*-10, *cis*-12 C18:2 isomer involved in milk fat depression.

1. Introduction

There is commercial interest in producing milk with a healthier fatty acid (FA) profile. One strategy for this objective is to

Abbreviations: ADFom, acid detergent fiber; BCVFA, branched-chain volatile fatty acid CIN cinnamaldehyde; CLA, conjugated linoleic acid; CP, crude protein; CTR, control; DM, dry matter; EE, ether extract; EO, essential oils; EUG, eugenol; FA, fatty acids; LIN, lipase inhibitor; LA, linoleic acid; LIP1, lipase 1; LIP2, lipase 2; LNA, linolenic acid; aNDFom, neutral detergent fiber; OM, organic matter; PUFA, polyunsaturated fatty acids; PTSO, oxy-propyl-thiosulphate; VFA, volatile fatty acids

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supplement the diet of dairy cows with plant oils rich in linoleic (LA) and linolenic (LNA) acids. This strategy results in the production of vaccenic acid (*trans*-11 C18:1) in the rumen and its subsequent desaturation to *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in the mammary gland by the Δ-9 desaturase enzyme (Griinari and Bauman, 1999). However, under certain dietary conditions, the biohydrogenation pathways are altered and other FA intermediaries are produced, of which some are potent inhibitors of milk fat synthesis (Bauman and Griinari, 2001). The *trans*-10, *cis*-12 CLA is known to be the major rumen biohydrogenation intermediate that has been associated to milk fat depression (Shingfield et al., 2010), which has important economic implications in dairy farms that produce milk enriched with polyunsaturated FA (PUFA) omega-3 or CLA.

Inhibiting lipolysis and (or) biohydrogenation may increase the flow of PUFA to the small intestine (Van Nevel and Demeyer, 1996; Krueger et al., 2009). Several factors affect the rate of lipolysis and biohydrogenation such as dietary concentrate proportion, ruminal pH, dietary level of unprotected lipids, ionophores and marine products, among others (Van Nevel and Demeyer, 1996; Demeyer and Doreau, 1999; Beam et al., 2000; Fuentes et al., 2009, 2011). In contrast, increasing lipolysis increases the concentration of non-esterified PUFA in the rumen and could inhibit the biohydrogenation process in the rumen and increased the production of *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA (Lourenço et al., 2010).

Essential oils (EO) have been shown to affect ruminal fermentation, changing volatile fatty acids (VFA) production, protein degradation, or both (Calsamiglia et al., 2007). However, the effect of EO on ruminal biohydrogenation has been less studied, although some indications of modified rumen lipolysis and biohydrogenation have been reported. Lourenço et al. (2009) reported that EO rich in monoterpenes such as limonene and carvone resulted in the ruminal accumulation of *cis*-9, *trans*-11 CLA, indicating the potential of plant secondary metabolites to affect ruminal biohydrogenation. Durmic et al. (2008) reported the ability of some plants extracts and EO to inhibit the growth and/or activity of important ruminal biohydrogenating bacteria such as *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus*. Gunal et al. (2013) observed a reduction in the concentration of C18:0 and *trans* C18:1 FA with some EO (Siberian fir needle oil, citronella oil, rosemary oil, sage oil) suggesting a shift in the biohydrogenation pathways. Ramos-Morales et al. (2013) also observed changes in the rumen FA profile when diets were supplemented with diallyl disulfide and propyl propane thiosulfinate, two soluble organosulfur compounds derived from garlic EO. While these strategies may be used to increase the supply of PUFA to the small intestine, they may also be used to modify the biohydrogenation processes that lead to the production of intermediaries associated with milk fat depression as *trans*-10, *cis*-12 C18:2. Industry is interested in developing feeding strategies to produce omega-3 and CLA enriched milk without the negative effect of these strategies on milk fat content.

The objective of this study was to determine the *in vitro* effects of several additives on rumen fermentation and apparent biohydrogenation of linoleic (LA) and linolenic (LNA) acids, and the production of the *trans*-10, *cis*-12 C18:2 isomer associated with milk fat depression.

2. Material and methods

Two experiments were conducted to determine the best strategies to aim the objectives of this study. In Experiment 1, the effect of six additives each at two doses were screened using a short term *in vitro* incubations to choose the most relevant treatments for the second experiment. In Experiment 2, dual-flow continuous culture fermenters were used to determine the long term effects and potential adaptations of the rumen microbial ecosystem to treatments selected in Experiment 1.

2.1. Experiment 1: short term in vitro batch fermentation

The effects of several commercial lipases, lipase inhibitor and EO on rumen fermentation and apparent biohydrogenation of LA (C18:2n-6) and LNA (C18:3n-3) were evaluated using *in vitro* batch incubations in two replicated periods. A 1:1 forage:concentrate diet containing (per kg of DM): corn grain (293 g), soybean (156 g), dehydrated alfalfa (183 g), corn silage (275 g), linseed oil (84 g) and a vitamin and mineral mixture (9.2 g) was used. The vitamin and mineral mixture contained, per kg of DM: 7 mg Co, 167 mg Cu, 33 mg I, 2660 mg Mn, 27 mg Se, 4660 mg Zn, 1000 kIU of vitamin A, 200 kIU of vitamin D3, 1330 mg of vitamin E, 267 g of urea, 67 g of NaCl, 33 g of sulphur and 300 g of MgO. The diet was formulated to meet or exceed current nutrient recommendations for lactating dairy cows (NRC, 2001). The chemical composition (per kg DM) of the diet was: 155 g CP, 290 g aNDFom, 156 g ADFom, and 111 g EE. The FA profile of the diet (per kg of total FA) was: 80.7 g C16:0, 34.6 g C18:0, 98.6 g *cis*-9 C18:1, 7.3 g *cis*-11 C18:1, 240 g *cis*-9, *cis*-12 C18:2 (LA), and 426 g *cis*-9, *cis*-12, *cis*-15 C18:3 (LNA).

Doses of each treatment were selected based on literature for enzymes (Eun and Beauchemin, 2007) and EO (Lourenço et al., 2008; Busquet et al., 2005, 2006; Foskolos et al., 2015). Also, different incubations times were selected based on the literature, one for lipases and one for EO. Moate et al. (2008) and Lee et al. (2008) reported that maximum lipolysis activity occurred at 2–6 h of incubation. In contrast, biohydrogenation of PUFA was almost complete at 8 h of incubation (Boufäied et al., 2003; Akraim et al., 2006; Enjalbert et al., 2003). Therefore, two *in vitro* incubations were conducted. Lipases and the lipase inhibitor were incubated for 2 h, and treatments were: control (CTR); lipase 1 (LIP1) and 2 (LIP2) at 4 (LIP1L and LIP2L, respectively) and 40 (LIP1H and LIP2H, respectively) µl/l; and a lipase inhibitor (Orlistat, Sigma-Aldrich, Saint Louis, MO, USA) (LIN) at 4 (LINL) and 20 (LINH) mg/l. The EO were incubated for 6 h and treatments were control (CTR), Oxy-propyl-thiosulphate, a stable form of garlic (PTSO) at 60 (PTSOL) and 120 (PTSOH) mg/l; Eugenol (EUG) and Cinnamaldehyde (CIN) at 150 (EUGL and CINL, respectively) and 500 mg/l (EUGH and CINH, respectively). Treatments were tested in triplicate at each dose and in two replicated periods. Incubations were conducted using rumen fluid from 2 fistulated, lactating dairy cows fed a 60:40 forage:concentrate diet. Rumen fluid was strained through 2 layers of cheesecloth, and mixed in a 1:1 proportion with phosphate-bicarbonate buffer (McDougall, 1948). The incubation was conducted in 90 ml tubes containing 50 ml of diluted fluid and 0.5 g of diet per tube. Initial pH of each tube was 6.4 ± 0.05 and was gassed with