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Effect of In Vitro Docosahexaenoic Acid Supplementation to Marine Algae-Adapted and Unadapted Rumen Inoculum on the Biohydrogenation of Unsaturated Fatty Acids in Freeze-Dried Grass

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ABSTRACT

The objective of this study was to examine the ruminal biohydrogenation of linoleic (18:2n-6) and linolenic (18:3n-3) acid during in vitro incubations with rumen inoculum from dairy cattle adapted or not to marine algae and with or without additional in vitro docosahexaenoic acid (DHA, 22:6n-3) supplementation. Treatments were incubated in 100-mL flasks containing 400 mg of freeze-dried grass, 5 mL of strained ruminal fluid, and 20 mL of phosphate buffer. Ruminal fluid was collected just before the morning feeding from 3 cows receiving a control diet (49% ryegrass silage, 39% corn silage, 1% straw, and 11% concentrate, fresh-weight basis) supplemented with marine algae for 21 d (adapted rumen fluid, aRF) or from the same cows receiving the control diet only for 14 d after marine algae supplementation was stopped (unadapted rumen fluid, uRF). In half of the incubation flasks, pure DHA (5 mg) was added as an oil-ethanol solution (100 mL). Incubations were carried out during 0, 0.5, 1, 2, 4, 6, and 24 h. After 24 h, in vitro addition of DHA resulted in greater amounts (mg/incubation) of 18:3n-3 (0.23, 0.43, 0.26, and 0.34 for aRF, aRF+DHA, uRF, and uRF+DHA), 18:2n-6 (0.14, 0.22, 0.15, and 0.20 for aRF, aRF+DHA, uRF, and uRF+DHA) and *trans*-11, *cis*-15-18:2 (0.27, 2.40, 0.06, and 2.21 for aRF, aRF+DHA, uRF, and uRF+DHA), whereas no effect of inoculum source was observed. *Trans*-11-18:1 accumulated after 24 h when aRF was incubated irrespective of in vitro DHA supplementation, whereas in incubations with uRF, accumulation of *trans*-11-18:1 only occurred when DHA was added (6.40, 4.35, 1.06, and 3.91 for aRF, aRF+DHA, uRF, and uRF+DHA). The increased amounts of *trans*-11-18:1 were due to the strong inhibition of the reduction to 18:0 because no 18:0 was formed when *trans*-11-18:1 accumulated after 24 h. The results of the current experiment shows hydrogenation of *trans*-11, *cis*-15-18:2 occurred in the absence of in vitro DHA only, whereas substantial hydrogenation of *trans*-11-18:1 to 18:0 only took place in incubations without DHA and with unadapted rumen inoculum, confirming the higher sensitivity of the latter process to DHA.
Key words: biohydrogenation, docosahexaenoic acid, rumen

INTRODUCTION

Trans-18:1 fatty acids and conjugated linoleic acid are produced by the incomplete biohydrogenation of long chain unsaturated fatty acids in the rumen (Harfoot and Hazlewood, 1997) and are subsequently incorporated into milk and meat of ruminant animals. The beneficial effects of *trans*-11-18:1 and *cis*-9, *trans*-11-18:2 (Belury, 2002; Lock et al., 2004) have encouraged research efforts to identify methods to increase levels of these fatty acids in ruminant products. Dietary means to reach this objective have been reviewed extensively (Chilliard et al., 2001; Palmquist et al., 2005) and include, amongst others, supplementation with vegetable oils, marine oils, and marine algae. Palmquist et al. (2005) suggested that the most important aspect in enhancing *cis*-9, *trans*-11-18:2 in ruminant products relates to dietary effects that regulate the synthesis and biohydrogenation of *trans*-11-18:1. Previous in vitro (Chow et al., 2004; Wasowska et al., 2006; Boeckaert et al., 2007b) and in vivo (Wonsil et al., 1994; Scollan et al., 2001; Shingfield et al., 2003) research showed a dramatic increase of *trans*-18:1 in the rumen when fish oil or marine algae were included in the diet. Subsequent studies suggested the highly polyunsaturated unesterified fatty acids were responsible for the inhibitory effects of fish oil and marine algae on ruminal fatty acid biohydrogenation (AbuGhazaleh and Jenkins, 2004; Boeckaert et al., 2007b), although its mode of action is still unknown. Boeckaert et al. (2007b) ex-

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cluded docosahexaenoic acid (**DHA**) and *trans*-11-18:1 as direct competitors for hydrogen. Wasowska et al. (2006) showed addition of DHA inhibited the growth and isomerase activity of *Butyrivibrio fibrisolvens* and Maia et al. (2007) extended the list of rumen bacteria sensitive to DHA, including the stearate-forming *Clostridium proteoclasticum* (Wallace et al., 2006). Although the latter might explain accumulation of *trans*-18:1 upon addition of DHA, it does not explain the accumulation of other biohydrogenation intermediates, such as *trans*-11, *cis*-15-18:2, when DHA is added (e.g., Shingfield et al., 2003; Wasowska et al., 2006; Boeckaert et al., 2007b). Furthermore, in none of the previous in vitro experiments reporting the effect of fish oil or marine algae, an element of adaptation was examined although changes in the rumen microbial population upon addition of fish oil may take several days to manifest themselves (Shingfield et al., 2006). The objective of this experiment was to investigate in vitro the effects of DHA supplementation to marine algae-adapted and unadapted rumen inoculum on rumen biohydrogenation of unsaturated fatty acids from freeze-dried grass. Previous in vitro experiments evaluating the effect of additives on rumen biohydrogenation generally involved the addition of 18:2n-6, 18:3n-3, or both as seed, oil, or nonesterified fatty acids in amounts often exceeding 0.5 mg/mL (Fievez et al., 2007b). Because of the high sensitivity of group B bacteria to polyunsaturated fatty acids (Harfoot and Hazlewood, 1997; Palmquist et al., 2005), no additional 18:2n-6 or 18:3n-3 source was added to the incubations flasks in the current experiment.

MATERIALS AND METHODS

Animals and Diets

Three lactating dairy cows (193 ± 45 d in milk for the first incubation series), each fitted with a ruminal cannula, were fed ad libitum a mixed diet comprising 49% ryegrass silage, 39% corn silage, 4% wheat, 3% soybean meal, 3% rapeseed meal, and 1% straw (fresh-weight basis) supplemented with 2 kg of concentrate containing marine algae (11% on fresh-weight basis of the concentrate) for 21 d after which supplementation of marine algae was stopped. Levels of fatty acids in the concentrate containing marine algae were (g/kg of DM): 14:0: 8.6; 16:0: 26.0; 18:0: 1.2; *cis*-9-18:1: 6.4; 18:2n-6: 14.9, 18:3n-3: 5.3, and 22:6n-3: 29.5. Samples of ruminal digesta were collected from each animal just before the morning feeding after 21 d of marine algae feeding (adapted rumen fluid, **aRF**) and 14 d after stopping supplementation of marine algae (unadapted rumen fluid, **uRF**) and immediately transferred into prewarmed thermos flasks. The rumen fluid was mixed and filtered through 2 layers of cheesecloth and flushed with CO_2.

In Vitro Incubations

Strained rumen fluid (5 mL) was added to the incubation flasks containing phosphate buffer (20 mL) and freeze-dried grass (0.4 g). The DHA (5 mg; Larodan Fine Chemicals, Malmö, Sweden) in 100 μL of ethanol was added directly into the cultures receiving DHA, whereas 100 μL of ethanol was added to the cultures receiving no DHA. Cultures were run at 39°C under anaerobic conditions. In vitro incubations were run on 2 separate days, one series using the marine algae-adapted inoculum and the second series, 14 d later, using the unadapted inoculum. Each series of incubations was composed of 2 incubators per donor animal for each incubation time: the aRF and uRF with or without DHA. This resulted in a total of 3 in vitro flasks per treatment and time point. After 0, 0.5, 1, 2, 4, 6, and 24 h of incubation, an incubation flask of each treatment was removed from the water bath and placed immediately in an ice bath, 750 μL of fluid was taken for VFA analysis with the remainder for fatty acid analysis. Both samples were stored at –20°C.

Analysis

Samples for fatty acid analysis were freeze dried, and fatty acids were extracted and methylated as described by Lourenço et al. (2005) and analyzed for fatty acids by GLC (HP 6890, Brussels, Belgium). Fatty acids in extracted lipids were methylated with NaOH in methanol (0.5 mol/L; 30 min, 50°C) followed by HCl in methanol (1/1; vol/vol; 10 min, 50°C). Methylated fatty acids were separated using a fused silica capillary column (100 m × 0.25 mm, i.d. × 0.20 μm thickness, CP-SiL88, Chrompack, Middelburg, the Netherlands) as described by Vlaeminck et al. (2005). Tridecanoic acid (13:0, 1 mg) was added to all samples as an internal standard prior to extraction. Fatty acid methyl esters were identified from external standards (S37, Supelco, Poole, Dorset, UK; ME61, *cis*-9, *trans*-11-18:2, *trans*-10, *cis*-12-18:2, odd- and branched-chain fatty acids, Larodan Fine Chemicals AB, Malmö, Sweden) and quantified using the internal standard. The concentration of VFA was determined using gas chromatography (GC type Fisons HRGC MEGA2, Fisons Instruments, Milano, Italy) as described by Chilibroste et al. (1998).

Calculations

Net production of VFA and odd- and branched-chain fatty acids was calculated by subtracting the amounts