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 docosaheptaenoic 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 pre-warmed thermos flasks. The rumen fluid was mixed

and filtered through 2 layers of cheesecloth and flushed with CO₂.

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 Chilibraste et al. (1998).

Calculations

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

Table 1. Effect of marine algae-adapted and unadapted rumen fluid (RF) and in vitro DHA-supplementation on net production of total VFA (mmol/L) and fermentation pattern (mmol/mol) after 24-h incubations

Item	Marine algae-unadapted RF		Marine algae-adapted RF		SEM	<i>P</i> -value		
	– DHA	+ DHA	– DHA	+ DHA		RF	DHA	RF × DHA
Total VFA	103.9	87.6	78.4	74.0	2.93	0.001	0.012	0.089
Acetate	656	489	626	504	9.3	NS	0.001	0.052
Propionate	215	419	247	400	3.8	NS	0.001	NS
Butyrate	105.3	63.1	95.7	61.1	5.63	NS	0.001	NS
Iso-butyrate	4.72	5.31	5.30	5.63	0.546	NS	NS	NS
Valerate	13.7	16.6	18.8	22.3	2.21	0.050	NS	NS
Iso-valerate	5.39	6.72	6.34	6.90	0.929	NS	NS	NS
NGR ¹	3.86	1.48	3.14	1.55	0.161	0.091	0.001	0.048

¹NGR = nonglucogenic to glucogenic VFA ratio calculated as [(acetate + 2 × (butyrate + isobutyrate) + valerate)]/(propionate + valerate).

at the 0-h time point from amounts found after 0.5, 1, 2, 4, 6, and 24 h of incubation. The ratio of nonglucogenic to glucogenic VFA was calculated as [(acetate + 2 × (butyrate + isobutyrate) + valerate)]/[propionate + valerate] (Orskov, 1975). Apparent biohydrogenation values for 18:2n-6, 18:3n-3, and 22:6n-3 were calculated based on the proportional loss of these fatty acids after 24 h of in vitro incubation, i.e., $(FA_{0h} - FA_{24h})/FA_{0h}$ with FA_{0h} and FA_{24h} the amount of 18:2n-6, 18:3n-3, or 22:6n-3 in the incubation flask (mg/incubation) at the start of the incubation and after 24 h.

Statistical Analysis

The individual values measured at the different sampling times were analyzed as repeated measures using the mixed procedure of SAS (SAS Institute, 2004). The statistical model included the fixed effects of inoculum source (aRF vs. uRF), in vitro DHA supplementation, incubation time and cow and the interaction terms inoculum × DHA, incubation time × inoculum, incubation time × DHA and incubation time × inoculum×DHA, assuming an autoregressive order one covariance structure fitted on the basis of Akaike information and Schwarz Bayesian model fit criteria. In addition, values obtained after 24 h of incubations were analyzed separately using the same model but without the variable time. Least squares means are reported, and significance was declared at $P < 0.05$.

Principal component analysis was used to identify correlated variables. Variables included in this analysis were the net production of acetate, propionate, and butyrate; the net production of the odd- and branched-chain fatty acids; and the amounts of 18:0, *trans*-11-18:1, and *trans*-11, *cis*-15-18:2 after 24 h of incubation. A hierarchical cluster analysis was conducted to find fatty acids that showed a similar behavior during the 24-h incubations. Fatty acids with 18-carbon atoms of

all time points were included in the analysis. Multivariate statistical analyses (principal component analysis and hierarchical cluster analysis) were performed using SPSS 12.0 (SPSS software for Windows, release 12.0., SPSS Inc., Chicago, IL).

RESULTS

Rumen Fermentation Pattern

Irrespective of inoculum source, DHA decreased molar proportions of acetate and butyrate, whereas molar propionate proportions increased (Table 1). These changes were reflected in a decreased ratio of nonglucogenic to glucogenic VFA when DHA was added to the incubation flasks, with a greater decrease for uRF. The total production of volatile fatty acids was inhibited by DHA supplementation when added to uRF only (Table 1). This decreased production was due to a decrease in acetate and butyrate production, whereas propionate production increased.

C18-Fatty Acids

The amounts of fatty acids in ruminal cultures at the start (0 h incubation of the nonDHA-supplemented incubation flasks) and after 24 h of in vitro incubation are presented in Tables 2 and 3, respectively. Fatty acid composition of the 0 h incubation was largely influenced by inoculum source (Table 2). Initial amounts of 18:0 were greater with the uRF, whereas most 18:1- and nonconjugated 18:2-isomers were greater in the marine algae-aRF. In vitro supplementation of DHA decreased the disappearance of dietary unsaturated fatty acids after 24 h of incubation resulting in lower apparent biohydrogenation values for 18:2n-6 (0.879 vs. 0.831; SEM = 0.014; $P = 0.055$) and 18:3n-3 (0.947 vs. 0.915; SEM = 0.007; $P = 0.024$), whereas inoculum source showed no effect on the disappearance of 18:2n-

Table 2. Amounts of fatty acids (mg/incubation) at the beginning of the in vitro incubations [rumen fluid (RF) + freeze dried grass + buffer]¹

Item	Marine algae-unadapted RF	Marine algae-adapted RF	SEM	P-value
<i>iso</i> -14:0	0.009	0.014	0.001	NS
14:0	0.123	0.119	0.008	NS
<i>iso</i> -15:0	0.035	0.038	0.003	NS
<i>anteiso</i> -15:0	0.077	0.093	0.011	NS
15:0	0.066	0.062	0.008	NS
16:0	2.169	2.323	0.233	NS
<i>iso</i> -17:0	0.031	0.020	0.009	NS
<i>anteiso</i> -17:0	0.031	0.035	0.007	NS
17:0	0.039	0.039	0.005	NS
18:0	2.126	0.245	0.333	0.057
<i>trans</i> -5-18:1	0.003	0.006	0.001	0.057
<i>trans</i> -6-, 7-, 8-18:1	0.029	0.039	0.005	NS
<i>trans</i> -9-18:1	0.020	0.076	0.003	0.008
<i>trans</i> -10-18:1	0.048	0.517	0.048	0.020
<i>trans</i> -11-18:1	0.203	0.558	0.023	0.008
<i>trans</i> -12-18:1	0.042	0.107	0.004	0.007
<i>trans</i> -13, 14-18:1	0.065	0.144	0.008	0.019
<i>trans</i> -15-18:1	0.047	0.080	0.006	0.053
<i>cis</i> -9-18:1	0.468	0.488	0.084	NS
<i>cis</i> -11-18:1	0.081	0.120	0.003	0.011
<i>cis</i> -12-18:1	0.036	0.014	0.002	0.024
<i>cis</i> -13-18:1	0.007	0.015	0.001	0.010
<i>cis</i> -14+ <i>trans</i> -16-18:1	0.042	0.042	0.008	NS
<i>cis</i> -15-18:1	0.040	0.050	0.010	NS
<i>trans</i> -11, <i>trans</i> -15-18:2	0.004	0.022	0.001	0.001
<i>trans</i> -9, <i>trans</i> -12-18:2	0.009	0.015	0.003	NS
<i>cis</i> -9, <i>trans</i> -13-18:2	0.015	0.023	0.004	NS
<i>trans</i> -11, <i>cis</i> -15-18:2	0.042	0.059	0.010	NS
<i>cis</i> -9, <i>trans</i> -11-18:2	0.087	0.075	0.012	NS
<i>trans</i> -10, <i>cis</i> -12-18:2	0.007	0.009	0.001	NS
<i>trans</i> -11, <i>cis</i> -13-18:2	ND	ND		
<i>trans</i> -11, <i>trans</i> -13-18:2	0.004	ND	0.001	NS
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:3	0.353	0.371	0.044	NS
18:2n-6	1.255	1.234	0.105	NS
18:3n-3	4.546	4.669	0.290	NS
22:6n-3	ND	0.030	0.004	0.039

¹NS = not significant ($P > 0.1$). ND = not detected.

6 and 18:3n-3 ($P > 0.5$). Disappearance of 22:6n-3 was low and independent of inoculum source (0.303 and 0.293 for aRF and uRF, respectively; SEM = 0.035; $P = 0.853$).

Addition of DHA to the incubation flasks resulted in a large increase in *trans*-11, *cis*-15-18:2, which accounted for 49.9% of the initial 18:3n-3, whereas only 3.6% of the initial 18:3n-3 accumulated as *trans*-11, *cis*-15-18:2 in the nonDHA-supplemented incubation flasks. Similar effects of DHA were observed for other 18:2-intermediates of biohydrogenation, but the effect was less pronounced compared with *trans*-11, *cis*-15-18:2 (Table 3). The amounts of most 18:1-isomers, with the exception of *trans*-10-, *trans*-11-, *cis*-9, *cis*-11- and *cis*-12-18:1, decreased when DHA was added. Amounts of *trans*-10- and *cis*-12-18:1 were not affected by in vitro DHA supplementation, whereas *cis*-9-18:1 increased. The effect of in vitro DHA supplementation on *trans*-5-, *trans*-11-, *trans*-12-, and *cis*-11-18:1 was dependent

of the inoculum source. In vitro supplementation of DHA to aRF decreased amounts of *trans*-5-, *trans*-11-, *trans*-12-, and *cis*-11-18:1, whereas in vitro DHA supplementation to uRF had no effect on *trans*-5-, *trans*-12-, and *cis*-11-18:1, whereas *trans*-11-18:1 increased (Table 3). A DHA $\times$ inoculum interaction was also detected for 18:0 with no effect of DHA supplementation with aRF and a decrease in 18:0 with uRF.

Figure 1 depicts the time course of some characteristic fatty acids during the 24-h incubations. Selection of fatty acids which are depicted was based on a hierarchical cluster analysis (Figure 2). In hierarchical cluster analysis, the similarity between variables is calculated using the distance concept. In a successive procedure, each variable is linked to the closest variable or group of variables and a characteristic similarity is used to describe this union. The results are represented in a dendrogram, which shows at which normalized or re-scaled distance a group of variables is differentiated

Table 3. Effect of marine algae-adapted and unadapted rumen fluid (RF) and in vitro DHA-supplementation on amounts of fatty acids after 24-h incubations (mg/incubation flask)¹

Item	Marine algae-unadapted RF		Marine algae-adapted RF		SEM	P-value		
	– DHA	+ DHA	– DHA	+ DHA		RF	DHA	RF × DHA
18:0	8.945	2.144	0.486	0.339	0.288	0.001	0.001	0.001
<i>trans</i> -5-18:1	0.009	0.004	0.022	0.008	0.001	0.001	0.001	0.023
<i>trans</i> -6-, 7-, 8-18:1	0.096	0.032	0.178	0.041	0.013	0.014	0.001	0.033
<i>trans</i> -9-18:1	0.064	0.026	0.188	0.074	0.012	0.001	0.001	0.022
<i>trans</i> -10-18:1	0.097	0.106	0.614	0.679	0.072	0.001	NS	NS
<i>trans</i> -11-18:1	1.061	3.905	6.403	4.348	0.344	0.001	NS	0.001
<i>trans</i> -12-18:1	0.148	0.099	0.331	0.161	0.025	0.003	0.004	0.049
<i>trans</i> -13-, 14-18:1	0.351	0.168	0.437	0.222	0.049	NS	0.007	NS
<i>trans</i> -15-18:1	0.182	0.056	0.195	0.085	0.019	NS	0.001	NS
<i>cis</i> -9-18:1	0.181	0.428	0.296	0.451	0.053	NS	0.009	NS
<i>cis</i> -11-18:1	0.080	0.112	0.250	0.163	0.016	0.001	NS	0.009
<i>cis</i> -12-18:1	0.037	0.036	0.039	0.020	0.006	NS	NS	NS
<i>cis</i> -13-18:1	0.016	0.010	0.033	0.019	0.004	0.008	0.028	NS
<i>cis</i> -14+ <i>trans</i> -16-18:1	0.200	0.043	0.092	0.037	0.015	0.010	0.001	0.017
<i>cis</i> -15-18:1	0.063	0.023	0.110	0.072	0.011	0.006	0.014	NS
<i>trans</i> -11, <i>trans</i> -15-18:2	0.027	0.374	0.108	0.363	0.025	NS	0.001	NS
<i>trans</i> -9, <i>trans</i> -12-18:2	ND	0.023	0.066	0.084	0.008	0.001	0.044	NS
<i>cis</i> -9, <i>trans</i> -13-18:2	0.046	0.182	0.101	0.202	0.012	0.018	0.001	NS
<i>trans</i> -11, <i>cis</i> -15-18:2	0.062	2.205	0.268	2.395	0.245	NS	0.001	NS
<i>cis</i> -9, <i>trans</i> -11-18:2	0.026	0.031	0.023	0.023	0.005	NS	NS	NS
<i>trans</i> -10, <i>cis</i> -12-18:2	0.009	0.007	0.010	0.011	0.001	0.015	NS	0.064
<i>trans</i> -11, <i>cis</i> -13-18:2	0.004	0.010	0.007	0.015	0.003	NS	0.080	NS
<i>trans</i> -11, <i>trans</i> -13-18:2	0.006	0.009	0.008	0.007	0.008	NS	NS	NS
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:3	0.049	0.069	0.044	0.070	0.007	NS	0.014	NS
18:2n-6	0.153	0.195	0.144	0.220	0.021	NS	0.031	NS
18:3n-3	0.256	0.341	0.234	0.432	0.039	NS	0.011	NS
22:6n-3	ND	3.536	0.010	3.504	0.140	NS	0.001	NS

¹NS = not significant ($P < 0.1$). ND = not detected.

from others. From the figure, 3 main clusters were apparent with dietary unsaturated fatty acids (*cis*-9-18:1, 18:3n-3, and 18:2n-6) and their conjugated isomerisation products (*cis*-9, *trans*-11, *cis*-15-18:3, and *cis*-9, *trans*-11-18:2) in cluster I, stearic acid appearing in cluster II, and the majority of biohydrogenation intermediates in cluster III. The latter cluster could be further divided in a cluster with nonconjugated- (cluster IIIa, *trans*-11, *trans*-15-18:2, *cis*-9, *trans*-13-18:2, and *trans*-11, *cis*-15-18:2) and conjugated-18:2 intermediates (cluster IIIb, *trans*-11, *cis*-13-18:2, and *trans*-11, *trans*-13-18:2) and monoenic C18-fatty acids (cluster IIIc). As expected, fatty acids belonging to cluster I and II were negatively related ($r = -0.789$) with biohydrogenation intermediates in cluster III indicating that when the amounts of biohydrogenation intermediates were high, the remaining dietary unsaturated fatty acids and stearic acid were low. Similarly, cluster I and II were negatively related ($r = -0.509$). Fatty acids belonging to the same cluster were positively related and showed a similar behavior during the 24-h incubations. The dynamic features of a characteristic fatty acids of each cluster is depicted in Figure 1 (18:3n-3, 18:0, *trans*-11, *cis*-13-18:2, *trans*-11, *cis*-15-18:2, and *trans*-11-18:1 for cluster I to IIIc, respectively). To aid

in the visualisation of the data, the different time points are connected by a straight line.

The lower disappearance of 18:3n-3 when DHA was added to the incubation flasks was due to an increase in lag-time (0.055 vs. 0.444 h; SEM = 0.085; $P = 0.018$) as well as a decrease in biohydrogenation rate (0.188 vs. 0.101; SEM = 0.010; $P < 0.001$; Figure 1A). The disappearance of 18:3n-3 resulted in the transient accumulation of *cis*-9, *trans*-11, *cis*-15-18:3 (data not shown), followed by formation of *trans*-11, *cis*-15-18:2 (Figure 1B). *trans*-11, *cis*-15-18:2 peaked at 4 h (1.07 mg) and 6 h (1.60 mg) of incubation for uRF and aRF when no DHA was added, whereas peak values (2.20 and 2.37 mg for uRF and aRF, respectively) were reached after 24 h of incubation when DHA was added. Hence, DHA increased the lag-time prior to *trans*-11, *cis*-15-18:2 formation and slowed down the disappearance resulting in accumulation of *trans*-11, *cis*-15-18:2. A representative fatty acid of cluster IIIb is *trans*-11, *cis*-13-18:2. Peak levels of *trans*-11, *cis*-13-18:2 (0.15 mg) were observed after 4 h of incubation when no DHA was added, whereas addition of DHA resulted in lower peak amounts after 6 h (0.05 and 0.02 for aRF and uRF, respectively; Figure 1B). The DHA increased the lag-time prior to *trans*-11, *cis*-13-18:2 formation but appar-

Figure 1. Changes in the amounts of some characteristic fatty acids during the 24-h incubations. Incubations with marine algae-adapted and -unadapted rumen fluid are represented by triangles and circles, respectively. In vitro incubations without additional in vitro DHA are represented by the empty symbols and incubations with 0.2 mg/mL DHA by full symbols. (SEM for 18:3n-3 = 0.223; *trans*-11, *cis*-15-18:2 = 0.181; *trans*-11, *cis*-13-18:2 = 0.014; *trans*-11-18:1 = 0.165; 18:0 = 0.337).

ently did not affect the reduction of *trans*-11, *cis*-13-18:2, resulting in similar amounts after 24-h incubations. Reduction of *trans*-11, *cis*-15-18:2 resulted in peak amounts of *trans*-11-18:1 after 6 h (2.37 mg) for uRF without DHA, whereas *trans*-11-18:1 was not reduced further to 18:0 for the other treatments, resulting in the highest amounts after 24 h of incubation (Figure 1C). During the 24-h incubation, an increase of 18:0 was only observed for uRF without DHA (Figure 1D).

Odd- and Branched-Chain Fatty Acids

Bacterial synthesis of total odd- and branched-chain fatty acids increased with DHA-supplementation. Of the individual odd- and branched-chain fatty acids, only *anteiso*-15:0 increased with DHA addition (Table 4). In contrast, synthesis of odd-chain *iso*-fatty acids (*iso*-15:0 and *iso*-17:0) decreased with DHA. Irrespective of DHA supplementation, synthesis of *iso*-14:0 and 15:0 was higher with uRF compared with aRF.

Figure 2. Hierarchical cluster diagram of fatty acids with 18-carbon atoms to identify fatty acids with a similar behavior during the 24-h in vitro incubations.

Principal Component Analysis

Principal component analysis was used to identify correlated variables. The first principal component explained 44.9% of the variation and was mainly determined by a contrast between, on the one hand, *trans*-11-18:1 and, on the other hand, the linear odd-chain fatty acids (15:0 and 17:0), *iso*-14:0 and 18:0 (Figure 3). The second component accounted for a further 37.0% of the variation and was mainly due to a contrast between, on the one hand, acetate and butyrate and, on the other hand, propionate, *anteiso*-15:0, and *trans*-11, *cis*-15-18:2.

DISCUSSION

Ruminal biohydrogenation of 18:2n-6 and 18:3n-3 involves the formation of conjugated intermediates that are subsequently reduced to yield 18:0 as the major end product (Harfoot and Hazlewood, 1997). Based on observations with pure strains of rumen bacteria, bacteria involved in hydrogenation have been classified into 2 groups (Harfoot and Hazlewood, 1997) with group A bacteria hydrogenating polyunsaturated fatty acids to *trans*-11-18:1 and group B bacteria hydrogenating *trans*-11-18:1 to 18:0 and *trans*-11, *cis*-15-18:2 to *trans*-15- and *cis*-15-18:1. In addition to the main known bio-

Table 4. Effect of marine algae-adapted and unadapted rumen fluid (RF) and in vitro DHA-supplementation on net production of odd- and branched-chain fatty acids after 24-h incubations

Item	Marine algae-unadapted RF		Marine algae-adapted RF		SEM	<i>P</i> -value ¹		
	– DHA	+ DHA	– DHA	+ DHA		RF	DHA	RF × DHA
<i>iso</i> -14:0	0.051	0.043	0.029	0.024	0.005	0.004	NS	NS
<i>iso</i> -15:0	0.069	0.037	0.048	0.035	0.007	NS	0.020	NS
<i>anteiso</i> -15:0	0.185	0.299	0.183	0.306	0.025	NS	0.004	NS
15:0	0.191	0.178	0.107	0.117	0.022	0.017	NS	NS
<i>iso</i> -17:0	0.035	0.018	0.035	0.026	0.005	NS	0.059	NS
<i>anteiso</i> -17:0	0.021	0.024	0.029	0.028	0.003	NS	NS	NS
17:0	0.047	0.028	0.029	0.029	0.005	NS	NS	NS

¹NS = not significant (*P* > 0.1).

Figure 3. Loading plot, describing the relationship between net production of volatile fatty acids (mmol/L), net production of odd- and branched-chain fatty acids (mg/incubation), and amounts of 18:0, *trans*-11-18:1, and *trans*-11, *cis*-15-18:2 (mg/incubation).

hydrogenation pathways of 18:2n-6 and 18:3n-3, other pathways appear to exist (Palmquist et al., 2005; Jouany et al., 2007).

The objective of the current experiment was to investigate in vitro the effects of DHA supplementation to marine algae-adapted and algae-unadapted rumen inoculum on rumen biohydrogenation of unsaturated fatty acids from freeze-dried grass, which contains mainly 18:3n-3. For this, 2 series of in vitro incubations were run 14 d apart. Use of this experimental approach can be criticized due to the confounding effect of inoculum source and day of incubation. However, changes in the rumen microbial population were most likely due to dietary treatment (i.e., removal of marine algae from the diet) rather than advances in stage of lactation because animals were in late lactation or management or environmental factors, such as weather, because those were stable during the trial. Obviously, differences in the amount of various fatty acids (e.g., 18:0, *trans*-11-18:1) in the uRF and aRF inoculum were inevitable because they resulted from an altered rumen biohydrogenation in response to marine algae. However, these differences are considered as inherent characteristics of the uRF vs. aRF and are part of the experimental design.

Addition of marine oils or algae to the diet is well known to inhibit the reduction of *trans*-18:1 to 18:0, resulting in a dramatic increase of *trans*-18:1 in rumen digesta (e.g., Looor et al., 2004; Boeckaert et al., 2007a), duodenal (Wonsil et al., 1994), and omasal lipids

(Shingfield et al., 2003). In vitro addition of DHA to uRF increased *trans*-11-18:1 with 284% after 24 h of incubation with a concomitant decrease of 18:0 with 76% in line with changes observed in vivo in dairy cows (75 to 612% increase of *trans*-11-18:1 and 31 to 93% decrease in 18:0; AbuGhazaleh et al., 2002; Shingfield et al., 2003; Qiu et al., 2004; Boeckaert et al., 2007a). Surprisingly, in vitro addition of DHA to aRF did not result in a further increase in *trans*-11-18:1. This is related to the strong accumulation of *trans*-11-18:1 in aRF without DHA and the accumulation of intermediates in the preceding steps of biohydrogenation in aRF with DHA. Indeed, the accumulation of *trans*-11-18:1 and absence of 18:0 formation in incubations with aRF without in vitro DHA suggest stearate-forming bacteria were not present in aRF inoculum at the start of the incubation. The high sensitivity of 18:0-forming bacteria to DHA was recently illustrated by Maia et al. (2007), who showed that the stearate-forming *Clostridium proteoclasticum* did not reach a stationary growth phase within 96 h when 50 µg/mL of DHA was added to the growth medium.

Shingfield et al. (2003) observed an increase in most 18:1-isomers in omasal digesta with fish oil supplementation. Similar results were obtained in the current in vitro experiment when comparing aRF and uRF without in vitro DHA supplementation. However, when DHA was added in vitro, most 18:1-isomers decreased, but the effect with aRF was generally stronger. This decrease of 18:1-isomers reflects the inhibitory effect of

DHA on the preceding steps of biohydrogenation rather than an increased removal of 18:1 fatty acids through biohydrogenation to 18:0 because no additional 18:0 was formed. Indeed, several 18:2-isomers increased upon addition of DHA with *trans*-11, *cis*-15-18:2, increasing from 1.5 to 21.7% of total C18-fatty acids. *Trans*-11, *cis*-15-18:2 is formed through the saturation of *cis*-9, *trans*-11, *cis*-15-18:3 catalyzed by a conjugated linoleic acid reductase (Kepler and Tove, 1967; Fukuda et al., 2007) and further reduced to *trans*-11-18:1, *trans*-15-18:1 or *cis*-15-18:1 (Harfoot and Hazlewood, 1997; Palmquist et al., 2005) with the biohydrogenation of the double bond at carbon atom 11 or 15 depending on the initial 18:3n-3 concentration (Body, 1976). The limited formation of *trans*-15-18:1 and *cis*-15-18:1 in the current in vitro experiment might reflect the preferential saturation of the double bond at carbon atom 15 when initial 18:3n-3 concentrations are low (Body, 1976).

Conjugated fatty acids only transiently accumulated when DHA was added to the incubation flasks with similar amounts after 24 h of incubation for all treatments. In contrast, biohydrogenation of nonconjugated-18:2-isomers was reduced with addition of DHA resulting in accumulation of these intermediates. Hence, DHA is a more potent inhibitor of bacteria, enzymes, or both, involved in the biohydrogenation of nonconjugated 18:2-isomers compared with those responsible for the biohydrogenation of conjugated fatty acids. The accumulation after 24 h of incubation of these intermediates is the result of a delay in the formation as well as a decreased reduction. Because aRF without DHA did not result in accumulation of nonconjugated 18:2 isomers, bacteria capable of hydrogenating nonconjugated 18:2-isomers should have been present in aRF inoculum at the start of the incubation. Indeed, in the absence or at low concentrations of DHA in the medium (i.e., during in vitro incubations with rumen inoculum adapted or not adapted to micro algae and without additional in vitro supplementation of unesterified DHA), accumulation of *trans*-11, *cis*-15-18:2 is transient only. Results from previous in vitro incubations suggest the occurrence of a transient accumulation of nonconjugated-18:2 fatty acids during 24-h in vitro incubations is dose dependent (Chow et al., 2004; Boeckaert et al., 2007b). This could partially explain the discrepancy observed in the extent of *trans*-11, *cis*-15-18:2 accumulation observed in vivo [16.7 and 30.4% of 18:3n-3 accumulated as *trans*-11, *cis*-15-18:2 in the experiment of Shingfield et al. (2003) and Looor et al. (2005), respectively] and in vitro [46.0 and 49.9% of 18:3n-3 accumulated as *trans*-11, *cis*-15-18:2 in the experiment of Boeckaert et al. (2007b) and the current experiment, respectively]. Indeed, ruminal concentrations of DHA

rapidly decrease in vivo, through a combination of both biohydrogenation and rumen outflow, whereas in vitro biohydrogenation of DHA is limited (Fievez et al., 2007b). Assuming a biohydrogenation rate of 0.20 and passage rate of 0.05, ruminal concentrations of DHA would be 3.5 times lower 5 h after intake, resulting in a reduced inhibitory effect on the hydrogenation of *trans*-11, *cis*-15-18:2.

The loading plot of the principal component analysis suggests the mechanism of *trans*-11, *cis*-15-18:2 and *trans*-11-18:1 accumulation is independent. Accumulation of *trans*-11, *cis*-15-18:2 was associated with increased synthesis of propionate and *anteiso*-15:0 and only occurred when DHA was present in the incubation flasks. In contrast, the additional in vitro supplementation of DHA was not necessary for *trans*-11-18:1 to accumulate, and a similar increase in *trans*-11-18:1 was observed for the aRF compared with the treatments receiving DHA in vitro.

The study by Maia et al. (2007) illustrates that, besides the stearate forming bacteria, all the main species that comprise the ruminal cellulolytic flora appear vulnerable to polyunsaturated fatty acids, highlighting the need for a mutual evaluation of both biohydrogenation and rumen digestion. The sensitivity of the ruminal cellulolytic flora to DHA is illustrated by the decreased synthesis of the branched-chain *iso*-fatty acids, which are characteristic fatty acids of cellulolytic bacteria (Vlaeminck et al., 2006). Supplementation of DHA decreased rumen production of volatile fatty acids in line with previous research (Fievez et al., 2003, 2007a). Quantitative and qualitative assessment of rumen volatile fatty acid production is important with the type of volatile fatty acids formed determining partitioning of nutrients within the host animal. The decreased acetate and increased propionate proportions upon addition of DHA was observed before (Fievez et al., 2003, 2007a). The shift from lipogenic (acetate and butyrate) toward glucogenic (propionate) precursors is interesting, because the latter is often limiting, mainly in the beginning of the lactation. Hence, the beneficial effect of an increased supply of glucogenic precursors is counteracted by the decrease in rumen production of volatile fatty acids.

CONCLUSIONS

The results of the current experiment showed hydrogenation of *trans*-11, *cis*-15-18:2 only occurred in the absence of in vitro DHA, 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 and DHA-enriched marine algae. In

addition, DHA is a more potent inhibitor of bacteria, enzymes, or both involved in the biohydrogenation of nonconjugated 18:2-isomers compared with those responsible for the biohydrogenation of conjugated fatty acids because the latter only transiently accumulated with similar amounts after 24-h incubations. The delay in the formation of hydrogenation intermediates as well as a decreased reduction rate when DHA was added suggest this might be a useful model for detailed characterization of rumen biohydrogenation pathways.

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