Docosahexaenoic acid elevates *trans*-18:1 isomers but is not directly converted into *trans*-18:1 isomers in ruminal batch cultures

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ABSTRACT

Pathways of docosahexaenoic (DHA) biohydrogenation are not known; however, DHA is metabolized by ruminal microorganisms. The addition of DHA to the rumen alters the fatty acid profile of the rumen and milk and leads to increased *trans*-18:1 isomers, particularly *trans*-11 18:1. This study included 2 in vitro experiments to identify if the increase in *trans*-11 C18:1 was due to DHA being converted into *trans*-11 18:1 or if DHA stimulated *trans*-11 products from biohydrogenation of other fatty acids. In each experiment, ruminal microorganisms collected from a lactating Holstein cow were incubated in 10-mL batch cultures for 0, 6, 24, and 48 h and a uniformly ¹³C-labeled DHA was added to the cultures at 0 h as a metabolic tracer. Experiment 1 tested 0.5% DHA supplementation and experiment 2 examined 1, 2, and 3% DHA supplementation to determine if the level of DHA effected its conversion into *trans*-11 18:1. In both experiments, any fatty acid that was enriched with the ¹³C label was determined to arise from DHA. Palmitic (C16:0), stearic (C18:0), all *trans*-18:1, eicosanoic (C20:0), and docosanoic (C22:0) acids were examined for enrichment. In experiment 1, the amount of *trans*-18:1 isomers increased 0.415 mg from 0 to 48 h; however, no label was found in *trans*-18:1 at any time. Docosanoic acid was highly enriched at 24 h and 48 h to 20.2 and 16.3%. Low levels of enrichment were found in palmitic and stearic acids. In experiment 2, *trans*-18:1 isomers increased 185, 256, and 272% from 0 to 48 h when DHA was supplemented at 1, 2, and 3%, respectively; however, as in experiment 1, no enrichment occurred of any *trans*-18:1 isomer. In experiment 2, low levels of label were found in palmitic and stearic acids. Enrichment of docosanoic acid decreased linearly with increased DHA supplementation. These studies showed that *trans*-18:1 fatty acids are not produced from DHA, supporting that DHA elevates *trans*-18:1 by modifying biohydrogenation pathways of other polyunsaturated fatty acids.

Key words: docosahexaenoic acid (DHA), ruminal batch culture, *trans* fatty acid

INTRODUCTION

Fish oil contains a large amount of unsaturated, bioactive n-3 fatty acids including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Supplementation of DHA has been associated with physiological benefits in many species. In dairy cows, DHA supplementation has been shown to alter the fatty acid composition of some parts of the reproductive system and PGF_{2α} secretions, which may, in turn, improve reproductive efficiencies (R. Mattos et al., 2004). To increase DHA intake, DHA can be included in dairy rations, either as a component of fish meal or as a fat supplement. When DHA is injected into the duodenum more DHA is transferred to milk than when DHA is injected into the rumen, indicating that DHA is hydrogenated in the rumen (Lor et al., 2004). Feeding DHA can modify the ruminal environment and decrease DMI, milk production, and milk fat production as well as alter fatty acid profiles (Donovan et al., 2000; Boeckaert et al., 2008).

It is widely known that ruminal microorganisms eliminate unsaturated 18-carbon fatty acids such as linoleic (C18:2) and linolenic (C18:3) acids through biohydrogenation; however, it is not well documented if the same processes occur for longer-chain fatty acids such as DHA (Jenkins et al., 2008). Supplementation of DHA results in increased levels of *trans*-18:1, particularly *trans*-11 18:1, in the rumen (Donovan et al., 2000; Cruz-Hernandez et al., 2007; Boeckaert et al., 2008). Because of the biological implications of *trans*-18:1 isomers, it is important to understand when and how they are produced. *Trans*-18:1 isomers are important intermediates of linoleic acid and linolenic acid biohydrogenation. *Trans*-10 18:1 is an indicator of milk fat depression in dairy cows, which is a concern in the dairy industry (Shingfield et al., 2009). *Trans*-11 18:1 can be converted to *cis*-9,*trans*-11 conjugated linoleic acid (CLA) by stearyl-coenzyme A (CoA) desaturase in tissues (Pariza et al., 2001). Conjugated linoleic acid isomers, including CLA, are potent anticarcinogens (Pariza et al., 2001).

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The mechanism for the increase of *trans*-18:1 isomers in the rumen when DHA is supplemented has not been clearly documented. Possibly, DHA is converted into *trans*-18:1 isomers or DHA supplementation enhances *trans*-18:1 production from other 18-carbon unsaturated fatty acid biohydrogenation, or both. Our goal was to determine if DHA is converted into *trans*-18:1 isomers or if the increase in *trans*-18:1 is due to DHA altering other pathways. To determine this, we used a uniformly ^{13}C -labeled DHA in ruminal batch cultures and looked for the presence of the ^{13}C label in *trans*-11:18:1 isomers. If label appeared in a fatty acid, it was regarded as an intermediate of DHA biohydrogenation.

MATERIALS AND METHODS

The rumen in vitro design was adapted from Sayre and Van Soest (1972) and AbuGhazaleh and Jenkins (2004b). For each set of batch cultures, a novel collection of rumen fluid was taken from 1 of 2 lactating, ruminally fistulated Holstein cows being fed 36.5 kg/d of TMR (56.5% corn silage, 3.7% alfalfa hay, 3.7% rye grass and oat haylage mix, and 36% grain mix by weight). Experiment 1 was completed with a single collection of rumen fluid, whereas experiment 2 included 3 separate collections of rumen fluid, with a novel collection for each replicate. In all cases, the rumen fluid was strained through 2 layers of cheesecloth, and then transported to the laboratory in a sealed and insulated container. At 0 h, 8 mL of media, 0.4 mL of reducing solution, and 2 mL of rumen fluid were added to screw-capped culture tubes [15 cm $\times$ 2 cm (experiment 1) or 15 cm $\times$ 2.5 cm (experiment 2)] containing 100 mg of substrate. Acids are produced by ruminal microorganisms and accumulate in batch cultures over time, which can interfere with microbial activities including decreased biohydrogenation (Martin and Jenkins, 2002). In this study, the media used was buffered with 1 g of ammonium bicarbonate/L and 8.75 g of sodium bicarbonate/L in an effort to maintain the pH and prevent any negative effects of low pH, including preventing a decrease in *trans*-18:1 production AbuGhazaleh and Jenkins (2004b). The base substrate was composed of alfalfa pellets (50%), ground corn (48%), soybean meal (22%), soybean hulls (24%), dicalcium phosphate (2.7%), trace mineral salt (1.2%), and sodium bicarbonate (1.6%), all ground through a 0.5-mm screen. In both experiments, DHA treatments were added to the substrate in an injection of ethanol (0.5 mg of DHA in 15 μL of ethanol in experiment 1 and 1, 2, or 3 mg of DHA in 90 μL of ethanol in experiment 2). For both experiments, DHA treatments were a uniformly ^{13}C -labeled DHA methyl ester purchased from Cambridge Isotope Laboratories Inc. (Andover, MA) or

a not-labeled DHA purchased from Nu-Chek Prep Inc. (Elysian, MN). The 0-h cultures were acidified with 6 *N* HCl immediately before the addition of ruminal microorganisms, and then stored at -15°C to prevent biohydrogenation. The remaining cultures were flushed with CO_2 , capped, and incubated at 39°C . At 6-, 24-, and 48-h sampling times, the appropriate cultures were acidified with 6 *N* HCl and stored at -15°C .

Chemical Analysis

Whole cultures were analyzed for VFA or long-chain fatty acids (**LCFA**). Cultures designated for VFA analysis were thawed and centrifuged at $15,780 \times g$, and then 1 mL of supernatant was collected and 100 μL of 2-ethylbutyric acid (86 $\mu\text{mol}/100 \text{ mL}$) was added as an internal standard. The samples were analyzed by GC on a Supelco 30 m $\times$ 0.25 mm $\times$ 0.25 μm fused silica capillary column (Supelco Inc., Bellefonte, PA; Fellner et al., 1997).

Cultures designated for LCFA analysis were lyophilized, then fatty acids were converted to fatty acid methyl esters (**FAME**) using a 2-step methylation procedure (Jenkins, 2010). Methyl esters were separated by GC on a 100 m $\times$ 0.25 mm $\times$ 0.2 μm column (Supelco SP-2560; Supelco Inc.) and detected by a flame ionization detector. Individual FAME were quantified by comparison to a C17:0 or C19:0 internal standard.

Enrichment of ^{13}C labeling was measured by GC-MS using chemical ionization with methane as the reagent gas. The FAME samples were separated on a 100 m $\times$ 0.25 mm $\times$ 0.2 μm column (Varian CP7489; Agilent Technologies, Santa Clara, CA). The mass selective detector was set to scan mode, scanning masses between 200 and 420 with a threshold count level of 300. The amount of ^{13}C in each fatty acid was reported as enrichment, the ratio of ^{13}C to total carbon in the fatty acid. Enrichment was calculated as $\{[(M + n)/[M + (M + n)]] \times 100\}_{\text{Labeled}} - \{[(M + n)/[M + (M + n)]] \times 100\}_{\text{Not labeled}}$, where *M* is the molecular ion and *n* is the number of carbons in the fatty acid (Mosley et al., 2002). The molecular ion represents the naturally abundant ^{12}C and *M* + *n* represents the ^{13}C label. The enrichment of not labeled cultures was subtracted from the labeled cultures to account for any background levels of ^{13}C .

Experiment 1

In experiment 1, treatment groups were 0.5% ^{13}C -labeled DHA (**L**), 0.5% not-labeled DHA (**NL**), and nonfat-added control (**CTL**). Duplicate cultures were maintained for each treatment at each time and analysis. Half of the cultures were analyzed for VFA and the other half for LCFA.

Experiment 2

In experiment 2, we examined the effects of increasing levels of DHA in batch cultures. The treatment groups were 1% ^{13}C -labeled DHA (**1L**), 2% ^{13}C -labeled DHA (**2L**), 3% ^{13}C -labeled DHA (**3L**), 1% not-labeled DHA (**1NL**), 2% not-labeled DHA (**2NL**), 3% not-labeled DHA (**3NL**), and CTL. All cultures were analyzed for LCFA. This experiment was replicated 3 times in a randomized complete block design, with blocks being each in vitro run that started with a fresh collection of rumen inocula.

Statistical Methods

All statistical analysis was completed using SAS version 9.2 (SAS Institute Inc., Cary, NC). Fatty acids were determined to arise from DHA if enrichments differed from zero by *t*-test ($P < 0.01$). A least squares means ANOVA in the GLM procedure using incubation time and DHA level as sources of variation was used to test time and treatment effects. Experiment 1 was a completely randomized design with sources of variation being treatment and time, and experiment 2 was a randomized complete block design with sources of variation being treatment, time, and replicate (i.e., novel collection of rumen fluid inocula). In experiment 2, orthogonal contrast statements were used to determine if linear or quadratic effects of DHA supplementation level existed. Throughout, $P \leq 0.05$ was considered to be significant unless otherwise noted.

RESULTS AND DISCUSSION

Experiment 1

Ruminal batch cultures are used as a model of the rumen environment and, thus, should reasonably duplicate in vivo fermentation conditions. In experiment 1, an accumulation of VFA occurred from an average of 23 $\mu\text{mol/mL}$ at 0 h to 88 $\mu\text{mol/mL}$ at 48 h (Figure 1). The increase in VFA in the batch cultures indicates that fermentation was occurring and that the microorganisms were viable. No effect of either the labeled or not-labeled DHA was found on either the concentrations or proportions of VFA, indicating that the cultures were viable with and without the DHA treatments. Figure 2A shows that unsaturated fatty acids, including linoleic acid, linolenic acid, and DHA decreased over time in experiment 1. This was accompanied by an increase in saturated fatty acids, including 18:0 and 22:0 (Figure 2A), and monounsaturated fatty acids (Figure 2B). The shift from unsaturated to saturated fatty acids indicates that biohydrogenation was occurring in the

Figure 1. The accumulation of total VFA in experiment 1 cultures over time when either no fat (control; CTL), 0.5% not-labeled docosa-hexaenoic acid (DHA; NL), or 0.5% ^{13}C -labeled DHA (L) was added to cultures of mixed ruminal microorganisms. Each point is the mean of 2 replicates and pooled standard errors are shown.

batch cultures. Combined, the VFA accumulation and biohydrogenation signify that the microorganisms were active and responded similar to in vivo response.

Previous studies have shown that adding DHA to the rumen alters the fatty acid profile in the rumen and in milk, including increased *trans*-18:1 isomers (AbuGhazaleh and Jenkins, 2004b; Boeckert et al., 2008). As expected, accumulation of *trans*-18:1 fatty acids occurred in the cultures in experiment 1, particularly *trans*-11 18:1 (Figure 2B). The disappearance of DHA and accumulation of *trans*-18:1 isomers indicate that these cultures are good models for a ruminant being fed DHA and can be used to show if DHA is converted into *trans*-18:1 isomers.

Although an increase in *trans*-18 isomers occurred in the L and NL cultures, no enrichment occurred of any *trans*-18:1 isomer at any time (Figure 3). This indicates that DHA was not a direct contributor to the increase in *trans*-18:1 isomers in experiment 1. Additionally, no enrichment of oleic (18:1) or eicosanoic (20:0) acids occurred. These fatty acids are not known to be produced from DHA and the absence of label is an indicator that the label was not indiscriminately appearing in fatty acids that are not produced from DHA. Figure 3 shows the fatty acid that was found to be substantially enriched was docosanoic acid (C22:0), which was 16.3% labeled at 48 h. Known pathways of 18-carbon linoleic and linolenic acid biohydrogenation terminate in saturated stearic acid (Lock and Bauman, 2004). The

Figure 2. (A) The experiment 1 changes in amount of stearic, oleic, linoleic, docosanoic, and docosahexaenoic fatty acids (DHA) at 0 and 48 h when 0.5% ^{13}C -labeled DHA was added to cultures of mixed ruminal microorganisms. Each bar is the mean of 2 replicates and pooled standard errors are shown. (B) The changes in amount of *trans*-6/8, *trans*-9, *trans*-10, *trans*-11, and *trans*-12 C18:1 fatty acids at 0 and 48 h when 0.5% labeled DHA was added to cultures of mixed ruminal microorganisms. Each bar is the mean of 2 replicates and pooled standard errors are shown.

appearance of label in docosanoic acid may indicate that similar pathways of isomerization and saturation occur in 22 carbon fatty acids, resulting in docosanoic acid. In addition to the label in docosanoic acid, less

Figure 3. The percentages of ^{13}C enrichment of palmitic, stearic, *trans*-18:1, oleic, eicosanoic, and docosanoic acids at 0 and 48 h in experiment 1 when 0.5% ^{13}C -labeled docosahexaenoic acid (DHA) was added to cultures of mixed ruminal microorganisms. Enrichments were detected in palmitic, stearic, and docosanoic acids (P -value < 0.05). Each bar is the mean of 2 replicates and pooled standard errors are indicated.

than 0.1% enrichment occurred in palmitic and stearic acids (Figure 3). Experiment 1 results indicate that 0.5% DHA supplementation results in an increase in the amount of *trans*-18:1, but not conversion of DHA into *trans*-18:1 isomers. Labeled DHA was saturated to docosanoic acid in experiment 1, which may indicate that DHA biohydrogenation parallels known biohydrogenation pathways of linoleic and linolenic acids where double bonds are isomerized, and then eliminated, to produce a saturated fatty acid of the same chain length as the initial unsaturated fatty acid.

Experiment 2

In experiment 2, 3 levels of DHA supplementation were tested to determine if the level of supplementation altered the intermediates produced from DHA, including the formation of *trans*-18:1 isomers. Table 1 shows the major changes in fatty acid contents for CTL and 1, 2, and 3% DHA at 0 and 48 h in experiment 2. In all cultures, accumulations of saturated fatty acids and decreasing levels of unsaturated fatty acids occurred in the cultures over time (Table 1). The shift in fatty acid profile indicates that the batch cultures were viable and the biohydrogenation of fatty acids was comparable to what would occur in the rumen (Jenkins et al. 2008). In this experiment, DHA disappeared from cultures at each level of supplementation, so DHA products ap-

peared labeled in these cultures. No increase occurred in *trans*-10 18:1 in this study, which is consistent with the findings of Vlaeminck et al. (2008), but differs from the findings of Or-Rashid et al. (2008). The variation in *trans*-10 18:1 could be due to feeding pure DHA rather than fish oil, the effect of other confounding dietary components, or a time lag in the effect of DHA on *trans*-10 18:1. Vlaeminck et al. (2008) saw differences in *trans*-10 18:1 in rumen fluid that was adapted to marine algae for 21 d but not when pure DHA was added to cultures for 24 h. This supports either a lag in effect on *trans*-10 18:1 or confounding effects of other marine algae components. The changes in the fatty acid profile are typical of ruminal contents and support that these cultures were an adequate model of rumen activity and were capable of determining products of DHA in the rumen.

Trans-11 18:1 accumulated in all cultures over time with higher accumulations associated with higher levels of DHA supplementation (Figure 4). AbuGhazaleh and Jenkins (2004b) previously reported a correlation between DHA supplementation and *trans*-11 18:1; however, were not able to identify the source of the *trans*-11 18:1. If DHA is a source of *trans*-11 18:1 then enrichment of *trans*-11 18:1 should increase with level of DHA in this study.

Although a positive linear correlation existed between *trans*-11 18:1 accumulation and increased DHA supplementation, no enrichment of *trans*-11 18:1 occurred at

any time or level of DHA supplementation (Figure 5). Additionally, as seen in experiment 1, no enrichment of *t6*, *t8*, *t9*, *t10*, or *t12* 18:1 isomers occurred. The batch cultures were maintained for 48 h, which exceeds the amount of time that a fatty acid would remain in the rumen and ensures that the lack of conversion of DHA into *trans*-18:1 was not due to time constraints. Whenever a 0 result is recorded, it is important to understand the sensitivity of the techniques and instruments used and determine if they are sufficient to make conclusions about the biology or if they indicate insufficient measurement techniques. In experiment 2, an average of 0.531 mg of *trans*-11 was found per culture, which was equivalent to an average of 2377669 M and M + 18 isotope counts by the mass selective detector. The detector was programmed to record all counts above 300, which would be theoretically equivalent to 0.0126% enrichment or 0.067 µg of labeled *trans*-11 18:1 in the average *trans*-11 18:1 peak. Based on this level of sensitivity, it seems reasonable to conclude that, as no label was detected, DHA was not converted into *trans*-11 18:1 in this experiment. This confirms the results of experiment 1, that DHA causes an increase in *trans*-11 accumulation in ruminal batch cultures, but it is not a direct contributor to the increase. Instead, DHA is altering the rumen processes, perhaps by altering the microorganisms that are present, or altering the reaction pathways, or both, ultimately resulting in an increase in *trans*-11 18:1.

Table 1. The changes in amount of fatty acid (mg/culture) when no fat was added (control; CTL) and when 1, 2, or 3% docosahexaenoic acid (DHA) was added to cultures of ruminal microorganisms in experiment 2¹

Fatty acid ²	DHA Level (%)								<i>P</i> -value ³		
	0		1		2		3		Diet	Time	Diet × time
	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h			
C14:0	0.05	0.77	0.06	0.73	0.06	0.72	0.06	0.75	NS	<0.0001	NS
C14:1	0.05	0.11	0.06	0.11	0.05	0.12	0.06	0.12	NS	<0.0001	NS
C15:0	0.17	0.69	0.21	0.65	0.21	0.75	0.21	0.63	NS	<0.0001	NS
C16:0	1.38	1.81	1.63	1.75	1.60	1.71	1.66	1.72	NS	0.005	NS
C16:1	0.04	0.07	0.05	0.07	0.05	0.07	0.05	0.07	NS	0.001	NS
C18:0	2.34	4.49	2.87	3.56	2.76	3.13	2.91	3.03	<0.0001	<0.0001	<0.0001
<i>t6, t8</i> C18:1	0.03	0.05	0.04	0.09	0.04	0.10	0.04	0.10	0.0004	<0.0001	NS
<i>t9</i> C18:1	0.02	0.03	0.02	0.07	0.02	0.08	0.03	0.08	<0.0001	<0.0001	<0.0001
<i>t10</i> C18:1	0.05	0.02	0.06	0.10	0.06	0.07	0.05	0.07	NS	NS	NS
<i>t11</i> C18:1	0.15	0.38	0.18	0.59	0.18	0.81	0.18	0.90	<0.0001	<0.0001	NS
<i>t12</i> C18:1	0.04	0.06	0.05	0.12	0.05	0.13	0.05	0.13	<0.0001	<0.0001	<0.0001
C18:1	0.71	0.33	0.84	0.48	0.81	0.57	0.84	0.62	<0.0001	<0.0001	NS
C18:2	0.94	0.15	1.07	0.14	1.04	0.12	1.08	0.14	NS	<0.0001	NS
C20:0	0.04	0.07	0.05	0.06	0.05	0.05	0.05	0.05	NS	0.0284	0.0144
C18:3	0.19	0.05	0.22	0.04	0.23	0.05	0.23	0.04	NS	<0.0001	NS
C22:0	0.03	0.04	0.04	0.05	0.03	0.04	0.04	0.04	0.0116	0.0072	NS
DHA	0.00	0.00	0.71	0.03	1.41	0.20	2.19	0.89	<0.0001	<0.0001	0.0007

¹CTL values are means of 3 replicates and DHA values are means of 3 replicates where not-labeled DHA was added and 3 replicates where ¹³C-labeled DHA was added to the cultures.

²*t* = *trans*.

³*P*-values indicate an effect of diet, time, or an interaction between diet and time (*P*-value < 0.05).

Figure 4. The amount of *trans*-11 18:1 fatty acid that accumulated in ruminal batch cultures over time in experiment 2 when no fat (control; CTL), 1% docosahexaenoic acid (DHA), 2% DHA, or 3% DHA was added. The CTL data points are means of 3 replicates and DHA data points are means of 3 replicates where not-labeled DHA was added and 3 replicates where ^{13}C -labeled DHA was added. Pooled standard errors are shown.

In addition to *trans*-18:1 fatty acids, oleic, linoleic, linolenic, and eicosanoic acids were examined and were not enriched at any time or level of DHA (data not shown). As in experiment 1, palmitic and stearic acids were enriched less than 0.1% (Figure 6). Although the enrichment of stearic acid was statistically significant,

the biological implications are limited. The highest enrichments were found at 48 h, when 3.03 mg of stearic acid was in the 3% DHA cultures and stearic acid was 0.09% enriched. In these cultures a potential existed for stearic acid to become 43% enriched at 48 h if all of the DHA that disappeared had been converted to stearic acid. Multiplying the amount of stearic acid by the enrichment, <0.003 mg of the stearic acid was calculated to be derived from DHA, which would be equivalent to $<0.10\%$ of DHA being converted to stearic acid. The authors are not aware of any known pathways where ruminal microorganisms shorten the carbon chain length of fatty acids and do not believe that the slight levels of enrichment of stearic and palmitic acids found in these experiments are substantial enough to conclude that these pathways exist.

Docosanoic acid was 12.7% labeled at 48 h in the 1% DHA-supplemented cultures; however, a linear decrease in docosanoic acid enrichment occurred as DHA supplementation level increased (Figure 6). At high levels, Kim et al. (2000) found that linoleic acid inhibits its own biohydrogenation, and AbuGhazaleh and Jenkins (2004a) noted decreased percent disappearance of DHA and eicosapentaenoic acid with increased supplementation. Although DHA was lost at each supplementation level in this experiment, the amount of DHA that disappeared by 48 h decreased from 95% at 1% DHA to 63% at 3% DHA (Figure 7). The decrease in docosanoic acid enrichment, combined with the decreased percent loss of DHA, indicates that DHA biohydrogenation is inhibited when DHA levels are high.

Figure 5. Experiment 2 enrichments of *trans*-11 18:1 at 0 and 48 h by level of docosahexaenoic acid (DHA) supplementation in experiment 2. No significant enrichment occurred at any time (P -value < 0.05). Each data point is the mean of 3 replicates and pooled standard errors are indicated.

Figure 6. Experiment 2 enrichments of palmitic (C16:0), stearic (C18:0), and docosanoic acids (C22:0) at 48 h as level of docosahexaenoic acid (DHA) increased from 1 to 3%. Each point is the mean of 3 replicates and pooled standard errors are shown.

Figure 7. The amount of docosahexaenoic acid (DHA) that was present in experiment 2 cultures at 0 and 48 h by level of DHA supplementation. Each bar is the mean of 3 replicates where not-labeled DHA was added and 3 replicates where ^{13}C -labeled DHA was added. Pooled standard errors are shown.

These studies examined several levels of DHA supplementation at extended time points and conclusively determined that under these conditions, ruminal bacteria do not convert DHA to *trans*-18:1 isomers. The batch culture design is ideal to measure the culture contents precisely, ensuring true measures of inputs and products; however, some constraints exist. The cultures accumulate products, which could contribute to feedback inhibition. The cultures are highly buffered to enable the microorganisms to grow for extended periods of time without waste product removal. These cultures only looked at the effects of time and level of DHA and did not examine confounding effects of other fat or carbohydrate sources. As such, these cultures are conclusive that under these conditions, the microorganisms do not convert DHA to *trans*-18:1, but cannot conclude that under other circumstances ruminal microorganisms are not capable of this conversion.

Although differences exist between batch cultures and the rumen, several inferences can be drawn from this study about the effects of DHA on a ruminant animal. Although DHA is not converted into *trans*-18:1 isomers, it does result in an increase in these isomers. An increase in *trans*-11 18:1 will lead to increases in *cis*-9 *trans*-11 CLA in tissues. This study found no conversion of DHA into *trans*-10 18:1, which may indicate that DHA directly does not cause milk fat depression, but instead, other components of fish oil or interactions between DHA and other feed components are respon-

sible for the decrease in milk fat production, such as those reported by Whitlock et al. (2002). Understanding the mechanisms and pathways involved in ruminal biohydrogenation of fatty acids is essential to developing and implementing feeding strategies that produce the desired fatty acid composition and beneficially affect milk production. That DHA is not directly converted into *trans*-11 18:1 and, instead, is otherwise affecting its synthesis is an important step in elucidating the pathways of *trans*-11 18:1 formation and the pathways of DHA biohydrogenation.

CONCLUSIONS

Although DHA supplementation results in increased levels of *trans*-18:1 fatty acids, DHA is not directly converted into *trans*-18:1 isomers. Docosanoic acid is produced directly from DHA. As DHA is converted into docosanoic acid, it may be hydrogenated in a manner that resembles known biohydrogenation pathways of linoleic and linolenic acids. If this is the case, a myriad of 22-carbon unsaturated fatty acids with unknown biological activity likely are produced from DHA in the rumen.

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