Dilution Rate and pH Effects on the Conversion of Oleic Acid to *Trans* C_{18:1} Positional Isomers in Continuous Culture

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ABSTRACT

In a previous in vitro study, mixed ruminal microorganisms converted oleic acid to a variety of *trans* monoenes when grown in batch cultures under constant environmental conditions. To determine whether a similar conversion occurs under environmental conditions more typical of the rumen, conversion of ¹³C-labeled oleic acid to biohydrogenation intermediates was determined in ruminal microorganisms grown in continuous culture at two pH (5.5 and 6.5) and liquid dilution rates (0.05 and 0.10/h) arranged factorially. After each morning feeding of the dual-flow continuous cultures, 250 mg of oleic acid in 5 mL of ethanol were injected into each culture. On d 10, 250 mg of oleic-1-¹³C replaced the unlabelled oleic acid in ethanol. *Trans* fatty acids were isolated from culture samples by solid phase extraction, and ¹³C enrichment and identity of double bond position was determined by gas chromatography-mass spectroscopy. At pH 6.5 and 0.10/h dilution rate, ¹³C enrichment was detected in all *trans*-C_{18:1} isomers having double bond positions from C₆ through C₁₆ in the acyl chain. However, when pH or dilution rate in fermentors was lowered, no ¹³C enrichment was detected in any *trans* isomer with a double bond position beyond C₁₀. Enrichment in stearic acid increased by reducing culture pH from 6.5 to 5.5, but decreased when dilution rate dropped from 0.10 to 0.05/h. The stearic acid carbons that originated from oleic acid biohydrogenation increased from 30 to 72% when pH dropped from 6.5 to 5.5. The ¹³C enrichment of *trans*-10 was reduced under low pH and dilution rate conditions. The results of this study confirm that ruminal microorganisms are capable of converting oleic acid to a wide variety of *trans*-C_{18:1} positional isomers when ruminal conditions are favorable (such as the pH 6.5 and 0.10/h dilution rate treatment). However, at low pH and dilu-

tion rate, the conversion of oleic acid to *trans*-C_{18:1} still occurs, but positional isomers produced are restricted to double bond positions from C₆ to C₁₀. Low pH conditions also increased the conversion of oleic acid to stearic acid. (**Key words:** biohydrogenation, oleic acid, pH, dilution rate)

Abbreviation key: APE = atom percent excess, CLA = conjugated linoleic acid, DMDS = dimethyl disulfide, FAME = fatty acid methyl ester, GC-MS = gas chromatography-mass spectroscopy.

INTRODUCTION

Trans fatty acids and conjugated linoleic acid (CLA) are produced as intermediates during microbial biohydrogenation of unsaturated fatty acids. *Trans* fatty acids have been shown to act as metabolic modifiers of lipid metabolism in humans (Blankson et al., 2000) and animals (Ostrowska et al., 1999). When *trans* fat, composed of 93% shortening and 7% corn oil, and *cis* fat, composed of 65% high oleic sunflower oil and 35% cocoa butter, were infused into the abomasums of lactating dairy cows, milk fat percentage was reduced only with *trans* fat (Gaynor et al., 1994). Recent studies have targeted specific *trans* C_{18:1} and CLA isomers as the cause of milk fat reduction. Post ruminal infusion of *trans*-10, *cis*-12 CLA, not *cis*-9, *trans*-11 CLA resulted in milk fat depression in dairy cows (Baumgard et al., 2000). Furthermore, Griinari et al. (1998) showed that diet-induced milk fat depression corresponds with increased *trans*-10 C_{18:1}.

Amounts of *trans* fatty acids and CLA produced in the rumen influence their concentrations in tissues and milk. Concentration of *trans*-11 C_{18:1} and *cis*-9, *trans*-11 CLA are greater in milk from cows fed fish oil and a linoleic acid fat source (AbuGhazaleh et al., 2002) or grazed on pasture (Lor et al., 2003). *Trans*-10 C_{18:1} and *trans*-10, *cis*-12 CLA concentration in milk fat increases when high grain diets are fed (Piperova et al., 2002). Other factors such as unsaturated fatty acid concentration and source, rumen pH, dilution rate, ionophores, and carbohydrate source have been shown to influence *trans* fatty acid production in the rumen (Bessa et al., 2000; Martin et al., 2002; Jenkins et al., 2003).

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Oleic acid ($C_{18:1}$ *cis*-9), a typical fatty acid in ruminant diets, is usually presented as being hydrogenated directly to stearic acid without the formation of *trans* fatty acid intermediates (Harfoot and Hazlewood, 1988). However, a recent in vitro batch culture study in our laboratory (Mosley et al., 2002) demonstrated that oleic acid is also a precursor for several *trans* $C_{18:1}$ isomers, including *trans*-10 and *trans*-11 $C_{18:1}$. A follow up study showed that once a *trans*- $C_{18:1}$ isomer is formed, it is converted into other *trans* monoenes by microbial isomerases (Proell et al., 2002). This study was conducted to further confirm the conversion of oleic acid to *trans* monoenes by ruminal microorganisms and to get a better understanding of how two important environmental variables, namely rumen pH and dilution rate, affect the type and amount of *trans* monoenes produced during oleic acid biohydrogenation.

MATERIALS AND METHODS

Reagents

Labeled oleic acid (oleic acid-1- ^{13}C) was purchased from Aldrich Chemical Company (Milwaukee, WI) and Medical Isotopes (Pelham, NH). Oleic acid ($C_{18:1}$ *cis*-9; 90% pure) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All solvents were HPLC grade. Dimethyl disulfide (**DMDS**), silver nitrate crystal, anhydrous ethyl ether, iodine, and sodium thiosulfate were purchased from Fisher Scientific (Pittsburgh, PA).

Experimental Design

Four dual-flow continuous culture systems, as described by Teather and Sauer (1988), were used in a 4×4 Latin square with 4 periods of 10 d each (9 d for adaptation and d 10 for sampling). Treatments were arranged in a 2×2 factorial as follows: 1) 6.5 pH and 0.10/h dilution rate, 2) 6.5 pH and 0.05/h dilution rate, 3) 5.5 pH and 0.10/h dilution rate, and 4) 5.5 pH and 0.05/h dilution rate. A total of 22 g of feed (Table 1) consisting of alfalfa pellets and concentrate mix (55:45; DM basis) were placed into each fermentor daily in 2 equal portions at 0830 and 1630 h. Because oleic acid was added to the concentrate mix, it accounted for 55% of the total fatty acids. Linoleic ($C_{18:2}$ *cis*-9, *cis*-12) and linolenic ($C_{18:3}$ *cis*-9, *cis*-12, *cis*-15) acids were also present in the diet, but at much lower proportions (9 and 4%, respectively).

Along with oleic acid from the diet, an additional 250 mg of oleic acid in 5 mL of ethanol were added directly into the fermentors each day just after the a.m. feeding. On d 10, 250 mg of oleic acid-1- ^{13}C replaced the unlabelled oleic acid in the 5 mL of ethanol. Thus, oleic acid

Table 1. Ingredient and fatty acid composition of experimental diet.

Ingredient	(%, DM basis)
Alfalfa, pellets	55.0
Corn, ground	19.9
Soybean meal	9.0
Soy hulls	10.7
Oleic acid ¹	2.9
Calcium phosphorus	1.2
Trace mineral salt ²	0.56
Sodium bicarbonate	0.74

¹Oleic acid (90% pure) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

²Contained (g/kg): NaCl, 955 to 9.8; Zn, 10.0; Mn, 7.5; Fe, 6.0; Mg, 0.5; Cu, 0.32; I, 0.28; and Co, 0.11.

entering the cultures from both sources, i.e., diet plus injection, was the same on all days. Day 10 only differed in that the source of injected oleic acid was the stable isotope rather than the unlabelled oleic acid injected on d 1 to 9.

Whole ruminal contents were taken from a ruminally fistulated dry Holstein cow fed a predominantly forage diet. At each collection time, ruminal contents were strained through a double-layer of cheesecloth and transferred to the laboratory in a sealed container. Approximately 900 mL of the strained ruminal fluid were added to each of the 4 fermentors. Anaerobic conditions were maintained by infusion of CO_2 at a rate of 20 mL/min. Using a precision pump with 2 different tubing sizes, buffer (Slyter et al., 1966) was delivered continuously at a flow rate of 1.5 and 0.75 mL/min resulting in a fractional dilution rate of 0.10 and 0.05/h, respectively. Buffer pH was titrated each day with sufficient 3 N NaOH or 3 N HCl to maintain treatment pH values (6.5 or 5.5). The pH was measured daily at 0830, 1200, and 1640 h using a portable pH meter. The temperature of ruminal cultures was maintained at 39°C by a circulating water bath, and cultures were stirred continuously at 25 rpm.

Sample Collection and Analysis

On d 10 of each period, two 5-mL samples were taken from each fermentor at 0 (prior to addition of feed or oleic acid-1- ^{13}C) and 24 h after adding the oleic acid-1- ^{13}C . Prior to sampling, the contents of the fermentor were thoroughly mixed by increasing agitation to 250 rpm, and samples were withdrawn with a pipette into 16- $\times$ 125-mm glass culture tubes and immediately placed in an ice bath. Samples were freeze-dried and fatty acid methyl esters (**FAME**) were prepared according to Kramer et al. (1997). The FAME were then separated into saturated, *trans* monoene, *cis* monoene,

and diene fractions using solid phase extraction as described by Mosley et al. (2002). The FAME in the *trans* and *cis* monoene fractions were converted to DMDS adducts as described by Mosley et al. (2002) to identify double bond position from mass spectral data.

The FAME in the saturated fraction were analyzed on a gas chromatograph equipped with a Saturn II ion trap mass spectrometer (Varian Instruments Inc., Walnut Creek, CA) using a 30-m $\times$ 0.25-mm with 0.2- μ m film Supelco 2380 column (Supelco, Inc., Bellefonte, PA). Column temperature was programmed at 140°C for 3 min, then increased to 220°C at 3°C/min, and finally held at 220°C for 5.5 min. The carrier gas was He (20 cm/s) with an injector split of 100:1. The gas chromatography-mass spectroscopy (GC-MS) analysis of FAME-DMDS adducts were performed using the same GC-MS conditions described previously except that the column temperature was programmed at 185°C for 31.6 min, then increased to 189°C at 0.2°C/min, and finally increased to 215°C at 4°C/min and held at this temperature for 6.8 min.

Additional FAME samples were analyzed by gas chromatography (Shimadzu GC-14A, Columbia, MD) to determine fatty acid profile. The gas chromatograph was equipped with a flame ionization detector and a 100-m $\times$ 0.25-mm (0.2- μ m film) capillary column coated with CP-Sil 88 (Chrompack, Raritan, NJ). The injector and detector temperatures were held at 250°C. The carrier gas was H₂ (33 cm/s) with an inlet pressure of 250 kPa. The column temperature was programmed for 140 °C for 3 min, then increased to 220°C at 2°C/min, and held at 220°C for 2 min.

Calculations and Statistics

The DMDS derivatives of FAME produce 2 distinctive spectral fragments that are indicative of the double bond position in monoenes when analyzed by GC-MS. The F fragment is the methyl thio adduct of the methyl end of the FAME. The G fragment is the methyl thio adduct of the carboxyl end of the FAME. The atom percent excess (APE) is calculated from the mass abundance of the G and G + 1 fragments using the following equation $\{APE = [(G + 1)/G (G + 1)]\}$. To eliminate the natural levels of ¹³C, the APE for unlabelled cultures (0-h sample; before adding oleic acid-1-¹³C) was subtracted from the APE of labeled cultures (24-h sample). Therefore, fatty acid enrichments were expressed on a percentage basis and calculated as $(APE_{24h} - APE_{0h}) \times 100$. Zero enrichment values indicate that an isomer was detected, but its $(APE_{24h} - APE_{0h}) \times 100$ did not differ from 0 by *t*-test ($P < 0.05$). The select ion mode in GC-MS was used to measure ¹³C enrichment in all fatty acids.

Figure 1. pH in continuous culture fermentors for pH 6.5 and 0.10/h dilution rate (■), pH 6.5 and 0.05/h dilution rate (□), pH 5.5 and 0.10/h dilution rate (●), and pH 5.5 and 0.05/h dilution rate (○). Standard errors of the means were 0.09, 0.07, 0.08, and 0.08, respectively.

Data were analyzed as a Latin square with a 2×2 factorial arrangement of treatments using the mixed procedure of SAS (2000). Fixed effects were period, pH, dilution, and the pH $\times$ dilution interaction, while random effects were fermentors. The only significant interactions detected were for enrichments in the *trans*-12 through *trans*-15 C_{18:1} isomers. Interactions for these isomers were caused by the failure to observe enrichment for any treatment except for the pH 6.5 and 0.10/h dilution rate treatment. Therefore, only the main effects of pH and dilution rate are presented in tables. Main effects were considered significant if $P \leq 0.05$.

RESULTS AND DISCUSSION

The experimental approach used in the present study to control pH and dilution rate successfully maintained cultures at the targeted values (Figures 1 and 2). Culture pH was adjusted by varying buffer pH, but without affecting its composition. Other researchers have altered culture pH by changing buffer composition (Yang et al., 2002) or by adding concentrated acids and bases directly into fermentors (De Veth and Kolver, 2001). It is unknown whether the abrupt changes in pH brought about by the direct addition of acids (e.g., from 6.4 to 5.5 in 4 min) have a direct effect on the microbial population. Some may argue that constant ruminal pH and dilution rate are not biologically meaningful because ruminal pH and dilution rate show diurnal variation in vivo. A valid counterargument is that when the main objectives are to study pH and dilution rate effects, these factors should be isolated and kept as constant as possible.

Figure 2. Dilution rate in continuous culture fermentors for pH 6.5 and 0.10/h dilution rate (■), pH 6.5 and 0.05/h dilution rate (□), pH 5.5 and 0.10/h dilution rate (●), and pH 5.5 and 0.05/h dilution rate (○). Standard errors of the means were 0.006, 0.004, 0.006, and 0.003, respectively.

In culture samples taken 24 h after introduction of the labeled oleic acid into cultures maintained at pH 6.5 and 0.10/h dilution rate, significant enrichment was observed in stearic acid and all *trans*-C_{18:1} positional isomers (Table 2). We did not find any significant ¹³C enrichment in palmitic, linoleic, and linolenic acids (data not shown). A closer examination of GC-MS results revealed ¹³C enrichment in 4 unknown peaks. Two of these unknown ¹³C enriched peaks appeared after C_{24:0} and were relatively large peaks, representing 4.3 to 14.3% of total fatty acids in fermentor contents; the other 2 unknown ¹³C enriched peaks appeared between C_{18:1} and C_{18:2} and were very small (<0.1% of total fatty

acids in culture; data not shown). Although the identity of the 4 unknown peaks was not determined in this study, their enrichment indicates that biohydrogenation of oleic acid by ruminal microorganisms leads to formation of intermediates other than just stearic acid or *trans*-C_{18:1} isomers.

Enrichment in stearic acid and the *trans* positional isomers confirms that biohydrogenation of oleic acid is not a one-step reduction to stearic acid, but also involves the production of various *trans* monoenes as described by Mosley et al. (2002). The results also show that the conversion of oleic acid to *trans*-C_{18:1} isomers is inhibited by lowering either culture pH or dilution rate. For instance, reducing pH from 6.5 to 5.5 increased ($P = 0.01$) the enrichment of stearic acid, but reduced ($P \leq 0.03$) or completely eliminated enrichment in most of the *trans*-C_{18:1} isomers examined. Reducing dilution rate from 0.10 to 0.05/h also affected enrichment (Table 2), causing a decline in the enrichment of stearic acid and several *trans*-C_{18:1} isomers (with double bond positions at C₆, C₁₀, C₁₄, and C₁₆). Among all *trans*-C_{18:1} isomers examined, only enrichment in the *trans*-7 positional isomer was not affected in some way by lowering pH or dilution rate. The presence of a *trans*-8 C_{18:1} isomer could not be verified; therefore, no determination of enrichment was possible.

Whether the inhibitory effect of low pH and dilution rate on ¹³C enrichment of *trans* monoenes was due to a shift on the bacterial population or due to direct interference with enzyme activity could not be deduced from this experiment. Low rumen pH and dilution rate were shown to have a negative effect on microbial growth (Hoover et al., 1984; Martin et al., 2002), particularly on growth of cellulolytic bacteria (Latham et al., 1972).

Table 2. ¹³C enrichment of fatty acids after 24 h of labeled oleic acid addition to fermentors.

Fatty acid	Treatment				SE	Main effect ¹	
	0.10/h dilution rate		0.05/h dilution rate			pH	Dilution rate
	pH 6.5	pH 5.5	pH 6.5	pH 5.5			
	(%)						
C18:1 <i>trans</i> -6	2.75	2.85	1.74	2.07	0.37	NS	*
C18:1 <i>trans</i> -7	3.34	2.97	2.84	3.01	0.58	NS	NS
C18:1 <i>trans</i> -9	3.96	2.92	3.44	2.27	0.39	**	NS
C18:1 <i>trans</i> -10	1.32	0.67	0.74	0.44	0.16	*	*
C18:1 <i>trans</i> -11	1.42	0 ²	0.68	0	0.21	*	NS
C18:1 <i>trans</i> -12	2.17	0	0	0	0.28	*	NS
C18:1 <i>trans</i> -13	1.63	0	0	0	0.26	**	NS
C18:1 <i>trans</i> -14	2.99	0	0	0	0.39	**	*
C18:1 <i>trans</i> -15	1.71	0	0	1.16	0.26	NS	NS
C18:1 <i>trans</i> -16	2.28	0	0	0	0.59	NS	*
C18:0	2.33	3.05	0.85	2.86	0.35	**	*

¹* $P < 0.05$, ** $P < 0.01$; NS = not significant.

²The isomer was detected but contained no significant enrichment by *t*-test ($P < 0.05$).

The acidification of ruminal contents and low dilution rate may give rise to a modification in the distribution (number, species) of cellulolytic bacteria colonizing the plant material. Cellulolytic bacteria, such as *Butyrivibrio fibrisolvens*, are indeed the main ruminal bacteria responsible for biohydrogenation (Harfoot and Hazlewood, 1988). When high concentrate/low forage (80:20) diets were fed to cows, promoting low pH conditions, the ratios of cellulolytic (primarily *Butyrivibrio fibrisolvens*) to propionogenic, lactogenic, or amylolytic bacteria in the rumen were severely reduced (Latham et al., 1972). Using continuous cultures, Hoover et al. (1984) showed that, at a 0.04/h dilution rate, lactic acid accounted for 53% of all organic acids, but as dilution rate increased (0.08/h), lactic acid was reduced to 28%. A further increase in dilution rate decreased lactic acid to $\leq 8\%$. The growth yield of *Butyrivibrio fibrisolvens* was 75% of its maximum yield when pH was 5.75; at pH 5.5, the organism was washed out in continuous cultures (Russell and Dombrowski, 1980).

In addition to affecting bacterial growth, low pH and dilution rate could also have influenced microbial attachment to feed particles. Low pH conditions alters microbial attachment to feed particles and binding affinity of fatty acids onto both bacterial membranes and feed particles (Martin et al., 2002). At low pH and dilution rate, the "balance" of microorganisms capable of carrying out various steps of biohydrogenation may be altered, leading to changes in production of biohydrogenation intermediates.

The reduction in ^{13}C enrichment of *trans* monoenes at low pH and dilution rate may also indicate a significant inhibition of oleic acid isomerization to *trans* monoenes. Previous work in our laboratory showed that during the biohydrogenation of oleic acid, *trans* monoenes are formed as transient intermediates via enzymatic isomerization (Mosley et al., 2002; Proell et al., 2002). Low rumen pH may negatively affect the physiology of ruminal bacteria, including the enzyme activity responsible for biohydrogenation (Kopečný et al., 1983). Biohydrogenation may be decreased if cell membrane integrity is disrupted at non-physiological pH, as the enzymes responsible for biohydrogenation are membrane-associated (Kepler and Tove, 1967).

It is not clear whether the same bacterial species can isomerize both oleic acid and *trans* monoenes (possess a multitude of *cis/trans* isomerases) or if different species are involved. Van de Vossenberg and Joblin (2003) investigated the monounsaturated fatty acid specificities of *Butyrivibrio hungatei* Su6. They showed that *B. hungatei* Su6 isomerized and hydrogenated *cis*-6-, *cis*-9-, and *cis*-11-octadecenoic acids yielding a mixture of *trans* monoenes and stearic acid. However, *B. hungatei* Su6 did not isomerize *trans*-6, *trans*-9, and *trans*-11-

Figure 3. The effects of A) pH 5.5 and 6.5 and B) 0.05 and 0.10/h liquid dilution rates on the positional isomers of *trans* monoenes expressed in grams per 100 g of total fatty acids in continuous cultures of mixed ruminal microorganisms (* $P \leq 0.05$).

octadecenoic acids, but only hydrogenated these *trans* monoenes to stearic acid.

We still cannot rule out the possibility that other ruminal bacteria have the ability to isomerize both *cis*- and *trans*-octadecenoic acids at the same time. Using pure cultures, Kemp et al. (1975) showed the ability of a single rumen isolate to isomerize and hydrogenate linoleic and linolenic acids. It is also not clear if these series of *trans* monoenes are substrate for one or a multitude of isomerases. The fact that pH 5.5 did not reduce the ^{13}C enrichment in some *trans* monoenes (*trans*-6 and *trans*-7) may indicate that more than one isomerase is involved in *trans* monoene production from oleic acid. Isomerization of linoleic acid to *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are thought to be carried out by 2 different enzymes (linoleate-*cis*-12, *trans*-11-isomerase and linoleate-*cis*-9, *trans*-10-isomerase, respectively) (Kepler and Tove, 1967).

Table 3. The percentage of each *trans* C_{18:1} positional isomer in the continuous culture samples that originated from oleic acid after 24 h of ¹³C-labeled oleic acid addition to fermentors.

Fatty acid	Treatment ¹				SE	Main effect ¹	
	0.10/h dilution rate		0.05/h dilution rate			pH	Dilution
	pH 6.5	pH 5.5	pH 6.5	pH 5.5			
C18:1 <i>trans</i> -6	49.70	66.20	22.00	52.90	7.73	*	*
C18:1 <i>trans</i> -7	60.50	69.10	35.80	77.00	11.01	NS	NS
C18:1 <i>trans</i> -9	71.70	67.90	43.40	58.10	6.11	NS	*
C18:1 <i>trans</i> -10	23.90	15.60	9.30	11.10	2.63	NS	**
C18:1 <i>trans</i> -11	25.70	ND ²	ND	ND	3.66	*	NS
C18:1 <i>trans</i> -12	39.40	ND	ND	ND	7.31	NS	NS
C18:1 <i>trans</i> -13	29.60	ND	ND	ND	4.48	*	NS
C18:1 <i>trans</i> -14	54.30	ND	ND	ND	7.31	**	NS
C18:1 <i>trans</i> -15	30.90	ND	ND	29.70	5.56	NS	NS
C18:1 <i>trans</i> -16	41.20	ND	ND	ND	11.37	NS	*
C18:0	42.20	71.00	17.70	73.20	8.22	**	NS

¹**P* < 0.05, ***P* < 0.01; NS = not significant.

²Values were not determined when enrichments shown in Table 2 were zero.

Among the *trans* monoenes, 2 specific isomers (*trans*-11 and *trans*-10) are of particular interest. Under most dietary conditions, *trans*-11 is the major *trans* isomer produced during the biohydrogenation of linoleic and linolenic acids (Griinari et al., 1998). *Trans*-11 can be used as a substrate for endogenous synthesis of *cis*-9, *trans*-11 CLA (anticarcinogenic) via Δ^9 desaturase (Bauman et al., 2003). It was estimated that at least 93% of *cis*-9, *trans*-11 CLA in cows milk was produced postruminally via Δ^9 desaturase (Bauman et al., 2003). *Trans*-10 replaced *trans*-11 as the predominant *trans* monoene isomer in the rumen when high concentrate-low fiber diets were fed to cows (Griinari et al., 1998) and steers (Beaulieu et al., 2002). Under such conditions, Bauman et al. (2003) proposed a putative pathway for the production of *trans*-10 where the *trans*-10, *cis*-12 CLA-producing bacteria become the predominant bacteria in the rumen, resulting in formation of *trans*-10, *cis*-12 CLA as the first intermediate during linoleic acid biohydrogenation. Hydrogenation of the *cis*-12 bond would then result in formation of *trans*-10, analogous to the production of *trans*-11 from *cis*-9, *trans*-11 CLA. *Trans*-10 has been associated with reduced mammary gland lipogenic enzyme activity and milk fat depression (Piperova et al., 2002). The presence of ¹³C enrichment in *trans*-11 and *trans*-10 in this study adds to previous evidence that these 2 isomers are also produced during isomerization of oleic acid (Mosley et al., 2002). In a follow up study, Proell et al. (2002) showed that once a single *trans* monoene isomer formed, it can be converted to many other positional isomers. Therefore, an alternate pathway for *trans*-10 synthesis, other than directly from oleic, linoleic, and linolenic acid biohydrogenation, might be from isomerization of their *trans* monoene intermediates.

In this study, the concentration (g/100 g of total fatty acids) of *trans*-10 in fermentors was higher than *trans*-11 at both pH levels (Figure 3). The presence of *trans*-10 as the predominant *trans* monoene at pH 6.5 may indicate that acidic conditions are not necessary for *trans*-10 formation from oleic acid and that *trans*-11 is not the major *trans* monoene intermediate during oleic acid biohydrogenation. After 24 h of incubation, the percentage of carbons in *trans*-10 and *trans*-11 derived from ¹³C oleic acid was 89 and 37%, respectively (Mosley et al., 2002). Additionally, when dairy cows were fed a 50:50 forage:concentrate diet high in oleic acid, the proportion of *trans*-10 and *trans*-11 in milk fat increased by 114 and 52%, respectively, compared with a control diet (AbuGhazaleh et al., 2003). As expected, lowering culture pH to 5.5 reduced the concentration of *trans*-11, but increased *trans*-10 concentration (Figure 3). The fact that ¹³C enrichment of *trans*-10 was lower at pH 5.5 compared with pH 6.5 (Table 2) indicates that more of the *trans*-10 at low pH originates from sources other than oleic acid.

Using stable isotope enrichments, the percentages of fatty acid intermediates originating from oleic acid were calculated (Table 3). The percentage of *trans* monoenes originating from oleic acid biohydrogenation ranged from 24 to 72% at pH 6.5 and 0.10/h dilution rate. Among the individual *trans* monoene isomers, only double bond positions at C₇, C₉, and C₁₄ were primarily (>50%) derived from oleic acid. The *trans*-10 and *trans*-11 isomers of greater interest and importance were ≤25% derived from oleic acid in this study. The percentage of *trans* monoenes originating from oleic acid were all affected by either pH or dilution rate, except for the isomers with double bond positions at carbons C₇, C₁₂, and C₁₅. The *trans*-6 isomer was the only isomer af-

affected by both pH and dilution rate. When significant, lowering pH and dilution rate reduced the percentage of *trans* monoenes originating from oleic acid, except for the *trans*-16 isomer. The percentage of stearic acid produced from oleic acid was not affected by dilution rate, and it was higher at pH 5.5 compared with pH 6.5.

The present study confirms the conversion of oleic acid by mixed ruminal microbes to stearic acid and a multitude of *trans*-C_{18:1} isomers having double bond positions from C₆ through C₁₆ when environmental conditions within the continuous cultures were favorable, i.e., pH 6.5 and 0.10/h liquid dilution rate. However, this study shows for the first time that the nature of *trans*-C_{18:1} isomers produced from oleic acid is greatly influenced by environmental conditions imposed on the microbial population. Departure from favorable rumen conditions, including reductions in pH (from 6.5 to 5.5) or dilution rate (from 0.10 to 0.05/h), will impede the conversion of oleic acid to positional *trans* isomers having double bond positions higher than C₁₀ in the fatty acyl chain. Thus, any model attempting to predict the nature of *trans* isomers produced from oleic acid biohydrogenation will need to account for changes in environmental conditions within the rumen.

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