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METABOLIC TRACER STUDIES OF LINOLEIC AND LINOLENIC ACIDS TO IDENTIFY BIOHYDROGENATION INTERMEDIATES PRODUCED BY RUMINAL MICROORGANISMS

Yong-jae Lee
Clemson University, ylee@clemson.edu

METABOLIC TRACER STUDIES OF LINOLEIC AND LINOLENIC ACIDS TO IDENTIFY BIOHYDROGENATION INTERMEDIATES PRODUCED BY RUMINAL MICROORGANISMS

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Yong-Jae Lee
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Accepted by:
Dr. Thomas, C. Jenkins, Committee Chair
Dr. Susan K. Duckett
Dr. Gary L. Powell
Dr. William C. Bridges, Jr.

ABSTRACT

Conjugated linoleic acids (CLA) have been shown to regulate many physiological functions affecting human health. These CLA can endogenously originate from *trans* fatty acids through the enzymatic activity of desaturase, or they are produced from dietary polyunsaturated fatty acids via the biohydrogenation process in the rumen. A minimum of seventeen CLA isomers have been identified in the intestinal contents of ruminants; however, the entire synthetic pathway for all isomers is not known. The objective of this study was to establish the number and identity of intermediates including CLA that originate from linoleic and linolenic acid biohydrogenation. To accomplish this objective, the carbon atom of these two fatty acids was tagged with a ^{13}C stable isotope to trace its appearance in CLA isomers in cultures of mixed ruminal microbes. It was found that ^{13}C migrated to seven CLA from linoleic and eight from linolenic acid, including *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA. These results indicate that the biohydrogenation of linoleic and linolenic acid by mixed ruminal microbes involves more complex biological pathways than formation of only one or two CLA as previously reported.

DEDICATION

I dedicate this work to my family and to my wife Yoon-Jung. This dissertation exists because of their love, support and prayers.

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I would like to thank Jesus Christ, whose character was impressed into my heart. He always protects and helps me. I am able to make this work with him.

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LITERATURE REVIEW

Dietary Lipids in Ruminant Animals

The diet of ruminant animals generally contains less than 10% lipid and 65 – 75% fiber (Varga et al., 1998). The ration is typically divided into forages and concentrates. Forages are primarily composed of plant material such as leaves, hay, silage, and pasture. The forage material provides 6 to 7% of the lipids, which are largely composed of glycolipids (up to 80% of the complex lipids) and phospholipids; the major fatty acids are linoleic (*cis*-9 *cis*-12 C18:2) and linolenic acid (*cis*-9 *cis*-12 *cis*-15 C18:3). Oleic acid (*cis*-9 C18:1) is found in relatively small amounts in forages (Harfoot, 1978; Van Soest, 1994). Galactolipids, consisting of glycerol, galactose, and fatty acids, are the major portion of glycolipids in forages (Van Soest, 1994). The concentrate portion of the rations refers to high-energy and low-fiber such as cereal seeds, and their milling by-products. These concentrates contain less than 18% crude fiber (Van Soest, 1994). Triglycerides, the major storage compounds in seed oils are the predominant lipid components in concentrates, and the major fatty acids are oleic and linoleic acid (Bauman et al., 1999), but linseed oil contains an unusually high amount of linolenic acid (57%) (Palmquist and Jenkins, 1980).

Ruminant animals gain metabolic energy and essential fatty acids through the metabolism of dietary lipids via microbial fermentation in the rumen. After dietary material enters the rumen, feed particles are mixed with liquid (water and saliva), gas (such as methane and carbon dioxide), and a vast

ruminal microbe population, called rumen contents. These ruminal microbes consist primarily of bacteria, fungi, and protozoa (Van Soest, 1994). During fermentation, dietary lipids undergo two major transformations in the rumen. Lipolysis: the process by which dietary lipids are hydrolyzed through microbial lipases to release free fatty acids, and biohydrogenation, where unsaturated fatty acids are isomerized and hydrogenated through microbial isomerases and reductases, to more becoming saturated fatty acid endproducts.

Lipid Metabolism in the Rumen

Lipolysis

In lipolysis, microbial lipases hydrolyze carboxyl ester bond, releasing free fatty acids from the glycerol backbone. Subsequently, the glycerol is rapidly fermented, yielding mostly propionic acid, which makes up between 25 and 44% of total volatile fatty acids (VFA). Ruminally produced propionic acid is then absorbed across the epithelium cell line and used as the main energy source for ruminants (Ward et al., 1961). The released unsaturated free fatty acids undergo biohydrogenation to remove double bonds that are toxic to ruminal microbes (Jenkins, 1993; Van Nevel and Demeyer, 1995).

Microbial lipases are primarily extracellular enzymes that can be divided into three categories: 1) Nonspecific lipases that randomly break down triacylglycerol releasing free fatty acids from glycerol, 2) Regiospecific lipases that react at the C1 and/or C3 of the glycerol backbone yielding 1,2(2,3)-diacylglyceride and 2-monoacylglyceride, and 3) Fatty acid-specific

lipases that hydrolyze lipids via a fatty acid based on chain length and degree of unsaturation (Gupta et al., 2004). Through the process of lipolysis, between 67 and 80% of linoleic and linoenic acids are released from soy oil triglycerides during the incubation of rumenial content. Low pH (less than 6) and antibiotics (such as ionophores and amoxicillin) are considered inhibitors of lipolysis. According to Van Nevel and Demeyer (1996), some of these antibiotics inhibit lipolysis by 10–20%.

A concentrated diet largely composed of triglycerides will rapidly hydrolyze in the rumen. For example, *Anaerovibrio lipolytica,* when isolated from the rumen, is well known to have the capability of hydrolyzing dietary triglycerides and produce two hydrolytic enzymes; a cell-bound esterase and a lipase. In the rumen, triglycerides are normally hydrolyzed to diglycerides and free fatty acids by lipase. Galactosidase converts the remaining diglycerides to galactose through hydrolysis (Harfoot, 1978).

Only a small amount of triglycerides are commonly found in forages. Glycolipids, especially galactolipids, make up the greatest concentration of lipids in most forage. Dawson et al. (1974) measured the hydrolysis of these galactolipids by feeding ^{14}C-labeled grass to sheep and discovered that they were rapidly hydrolyzed to free fatty acids and mono/di-galactosylglyceride.

Phospholipids, which make up between 20 and 30% of the lipids in the plant leaf, were also found to hydrolyze rapidly in the rumen. Dawson and Kemp (1969) observed that ruminal protozoa from sheep rapidly hydrolyzed phosphatidylcholine, and concluded that the phospholipase activity originated

in either the plant or the bacteria. Hazlewood and Dawson (1975) found that *Butyrivibrio fibrisolvens* hydrolyze phosphatidylcholine and phosphatidylethanolamine. The phospholipase A deacylatizes the phospholipids, which, in turn, releases lysophospholipids and unesterified fatty acids. Lysophospholipids are rapidly broken down to glycerylphosphorylated derivatives by lysophospholipase, producing unesterified fatty acid. Phospholipase A deacylation was increased by the presence of cysteine but decreased under aerobic conditions. In addition, calcium ions did not stimulate phospholipase A activity. Hazlewood and Dawson (1975) proposed that this enzyme required a low redox potential and a negative charge to increase activation.

In a study by Hespell and O'Bryan-Shah (1988), esterase activity of 30 strains of B. *fibrisolvens* were studied, and it was found that a few species could hydrolyze long chain fatty acids; however, all 30 strains had a wide range of esterase activity. More specifically, Fay et al. (1990) isolated and identified 74 strains of ruminal bacteria that could hydrolyze carboxyl ester bonds of ρ-nitrophenylpalmitate. Althought this hydrolyzing activity varied among the ruminal bacteria species, A. *lipolytica* and B. *fibrisolvens* exhibited low hydrolysis activity. Yadav et al. (2007) found that *Lactobacillus acidophilus* and L. *casei* were also able to produce free fatty acid and CLA through lipolysis during fermentation in milk fat.

Dawson and Hemington (1974) reported that microbial lipases contributed to the hydrolysis of glycolipids and phospholipids in the rumen.

However, Faruque et al. (1974) suggested that the hydrolysis of esterified fatty acids was primarily due to the plant lipases. In their study, the plant lipases were highly active, leading to the hydrolysis of triglyceride after 5 h incubation in the presence of ruminal microorganisms, while microbial lipase activity in the rumen was low. *In vivo* experiments showed similar results, the researchers finding that the lipolytic activity of rumen fluid after 0.5 to 5 h of feeding on grass was two times higher than the rumen fluid obtained after overnight fasting. However, the Dawson et al. (1977) study showed the opposite results. They inoculated a sheep with ^{14}C-labeled grass which was autoclaved to deactivate plant lipase. The results showed that the galactolipids were rapidly hydrolyzed without the accompaniment of functional plant lipases. In a second study, ^{14}C-labeled grass mixed with ruminal fluids was boiled to deactivate microbial lipases; in this case the galactolipids were not hydrolyzed. As these two studies shown, microbial lipase plays a key role in the hydrolysis of plant lipids in the rumen.

Metabolic Pathway of Biohydrogenation

Unsaturated fatty acids, released after lipolysis, undergo biohydrogenation, yielding saturated end products. This process is a detoxification mechanism for the survival of ruminal microorganisms. Reiser (1951) was the first to investigate the biohydrogenation of unsaturated fatty acids during incubation of linseed oil with sheep rumen contents *in vitro*, showing that linolenic acid content in the oil decreased from 30 to 5%.

Subsequently, Shorland et al. (1955) found that the linolenic acid in pasture grasses rapidly converted to dienoic, monoenoic, and stearic acid (C18:0). After this initial research, various *in vivo* and *in vitro* experiments were conducted, examining the biohydrogenation process of unsaturated fatty acid, specifically, oleic, linoleic, and linolenic acid.

Oleic Acid (*cis*-9 C18:1)

Biohydrogenation of oleic acid, a non-essential fatty acid synthesized *de novo*, was initially thought to produce only stearic acid without the formation of *trans* intermediates. However, several bacterial species have been reported to have the capability of *cis* to *trans* isomerization. Mortimer and Niehaus (1972) found that *Pseudomona*s strain NRRL, a non-ruminal bacteria, converted oleic acid to *trans*-10 C18:1 under low pH conditions. Kemp et al. (1975) found that *Fusocillus* T344, a ruminal bacteria, was capable of converting oleic acid to *trans*-11 C18:1. Okuyama et al. (1991) proposed that this *cis* to *trans* isomerization could change the plasma membrane fluidity and protect the bacteria itself against growth inhibitors or environmental insults. Moreover, catalytic hydrogenation also produces *trans* fatty acids by isomerization from unsaturated fatty acids and movement of the double bonds up and down the chain with the position changing from C2 to C12 (Zock and Katan, 1997).

More recently, Mosley et al. (2002) observed that mixed ruminal microbes rapidly converted ^{13}C-labeled oleic acid to several positional isomers

of *trans* monomers by mixed ruminal microbes including the formation of stearic acid *in vitro*. They found that all *trans*-C18:1 with double bond position from carbon number 9 to 16 was produced from labeled oleic acid. In a similar experiment using ^{13}C-labeled *trans*-9 C18:1, Proell et al. (2002) traced the intermediates of the biohydrogenation pathway of *trans*-9 C18:1 after being incubated with mixed ruminal microbes. After 48 h incubation, they found that labeled stearic acid, *trans*-C18:1 isomers with double bond at C6 to C16, and two *cis* monoenes (*cis*-9, and *cis*-11 C18:1) were produced from the labeled *trans*-9 C18:1. In addition, mixed ruminal microbes in continuous cultures have also been found to convert oleic acid to 10-hydroxystearic acid and 10-ketostearic acids. Up to 30% of the oleic acid in these cultures was used in the production of these two acids, while only 6-10% of it was used in a similar process *in vitro* with mixed ruminal microorganisms (Jenkins et al., 2006).

Figure 1.1 summarizes this previous research on oleic acid transformation by mixed ruminal microbes. The biohydrogenation of the double bond of oleic acid forms stearic acid. However, a wide range of positional *trans*-C18:1 isomers was also produced throught the *cis/trans* isomerization of oleic acid with double bond positions from C6 to C16. The hydration of oleic acid produced 10-hydroxystearic acid, which was then oxidized to 10-ketostaric acid.

Linoleic Acid (*cis*-9 *cis*-12 C18:2)

Most research on linoleic acid biohydrogenation discusses its

conversion to *cis*-9 *trans*-11 CLA and *trans*-11 C18:1 before the formation of stearic acid (Garton, 1977). However, Griinari and Bauman (1999) suggested that *trans*-10 *cis*-12 CLA also originated from linoleic acid, followed by the production of *trans*-10 C18:1 in the rumen (Figure 1.2). This hypothesis was supported by the study conducted by Kim et al. (2002), who found that some *Megasphaera elsdenii* strains produced *trans*-10 *cis*-12 CLA formed by linoleic acid.

A recent study identified a wide range of *trans*-C18:1 and CLA isomers, all of which had been identified in the intestinal content of cattle of these CLA isomers. At least 17 can be classified as the double bond geometric configurations of *cis/cis, trans/trans, cis/trans*, and *trans/cis* (Table 1.1). On the other hand, some biohydrogenation intermediates of linoleic acid are not well documented. In the study conducted by Coakley et al. (2006), linoleic acid was hydrogenated to produce *cis*-9 *trans*-11 CLA, *trans*-9 *trans*-11 CLA, and *trans*-10 *cis*-12 CLA via the *Bifidobacterium* species of human intestinal origin. They observed the effect of incubation on six selected *bifidobacteria* and these three CLA isomers, in a growth medium containing individual fatty acids. The results showed that the linoleic acid was converted to these three CLA, whereas *cis*-9 *trans*-11 CLA was converted to *trans*-9 *trans*-11 CLA. Subsequently, *trans*-10 *cis*-12 CLA was converted to *trans*-9 *trans*-11 CLA. Additionally, Wallace et al. (2007) demonstrated that *trans*-9 *trans*-11 CLA was also produced from linoleic acid by B. *fibrisolvens*, while Ogawa et al. (2001) showed an alternative route of biohydrogenation by which 10-hydroxy-

*cis*12-C18:1 and 10-hydroxy-*trans*12-C18:1 were produced as intermediates of linoleic acid biohydrogenation by *Lactobacillus acidophilus* AKU 1137 under microaerobic conditions. They found that these two intermediates were produced before the formation of *cis*-9 *trans*-11 CLA and *trans*-9 *trans*-11 CLA.

Linolenic Acid (*cis*-9 *cis*-12 *cis*-15 C18:3)

The biohydrogenation process of linolenic acid in the rumen has not been researched as extensively as oleic and linoleic acids. In the study conducted by Harfoot and Hazlewood (1988), linolenic acid initially is converted to *cis*-9 *trans*-11 *cis*-15 conjugated octadecatrienoic acid by ruminal biohydrogenation. Then, *trans*-11 *cis*-15 C18:2 is produced through the reduction of the *cis*-9 double bond. This is further hydrogenated to *trans*-11 C18:1 to produce stearic acid. *Trans*-11 *cis*-15 C18:2 can also hydrogenate to *trans*-15 or *cis*-15 C18:1 (Figure 1.3).

In the study conducted by Loor et al. (2004), linolenic acid was isomerized into three C18:3 intermediates: *cis*-9 *trans*-12 *cis*-15 C18:3, *cis*-9 *trans*-12 *trans*-15 C18:3, and *trans*-9 *trans*-12 *trans*-15 C18:3. Low concentrate diets (65:35 forage to concentrate) and the addition of linseed oil in cows increased the duodenal flow of these C18:3 isomers. In addition, Destaillats et al. (2005) reported that 0.3% of milk fat was the *cis*-9 *trans*-11 *cis*-15 C18:3 and the *cis*-9 *trans*-13 *cis*-15 C18:3 isomers, suggesting that these two isomers were the initial intermediates of linolenic acid

biohydrogenation. Subsequently, *cis*-9 *trans*-11 *cis*-15 C18:3 isomer was reduced to *cis*-9 *trans*-11 C18:2 and *tran*s-11 *cis*-15 C18:2, and *cis*-9 *trans*-13 *cis*-15 C18:3 isomer to *cis*-9 *trans*-13 C18:2 and *trans*-13 *cis*-15 C18:2. Both *cis*-9 *trans*-11 C18:2 and *trans*-11 *cis*-15 C18:2 were able to hydrogenate to *trans*-11 C18:1, reducing to stearic acid. The *cis*-9 *trans*-13 C18:2 and *trans*-13 *cis*-15 C18:2 was hydrogenated to *trans*-13 C18:1 and then subsequently to stearic acid. Destaillats et al. (2005) suggested that two CLA isomers (*cis*-9 *trans*-11 CLA; *trans*-13 *cis*-15 CLA) were produced from linolenic acid biohydrogenation. However, no evidence supporting this route was given in this study. Wąsowska et al. (2006) reported that *cis*-9 *trans*-11 *cis*-15 C18:3 and *trans*-9 *trans*-11 *cis*-15 C18:3 accumulated in strained rumen fluid from linolenic acid biohydrogenation. They also found that the *trans*-11 *cis*-15 C18:2 originated from linolenic acid, but *cis*-9 *trans*-11 CLA was not observed in their study.

DHA and EPA in Fish Oil

The biohydrogenation of polyunsaturated fatty acids having more than 3 double bonds such as docosahexaenoic acid (DHA; *cis*-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 C22:6) and eicosapentaenoic acid (EPA; *cis*-5 *cis*-8 *cis*-11 *cis*-14 *cis*-17 C20:5) in fish oil are even less understood in the ruminant animal. The research that has been conducted, however, has proposed that fish oil (DHA and EPA) inhibits the biohydrogenation of both linoleic and linolenic acid, causing an accumulation of *trans*-C18:1 and *trans*-C18:2 (Wachira et al., 2000;

Lee et al., 2005; Shingfield et al., 2003; Loor et al., 2005). For example, according to the study conducted by Gulati et al. (1999), DHA and EPA disappeared with a high degree of biohydrogenation when fish oil was inoculated into less than 1 mg/ml of rumen fluid. This disappearance was accompanied by the production of *trans*-C18:1. In addition, biohydrogenation of DHA and EPA decreased at high concentrations of fish oil. The same effect was observed by AbuGhazaleh and Jenkins (2004). They found that both DHA and EPA disappeared in cultures of mixed ruminal microbes over 24 h incubations, transforming to other fatty acids such as *trans*-C18:1. They suggested that the reason for the DHA and EPA disappearance was due to bacterial isomerization, hydrogenation, and/or carbon chain shortening. If DHA and EPA biohydrogenation followed the same process as that of linolenic acid, the initial intermediate of DHA and EPA would have at least one *trans* double bond resulting from bacterial isomerization. Subsequently, reductases reducing double bonds would have produced increased saturated end products. However, no evidence suggests ruminal biohydrogenation of DHA and EPA (Jenkins et al., 2008).

Enzymes

Biohydrogenation in the rumen is basically a two-step process involving microbial isomerases and reductases. Okuyama et al. (1998) purified and characterized the transformation of 9-hexadecenoic acid (*cis*-9 C16:1) to the *trans* isomerase using *Pseudomona*s sp. strain E-3. The purified isomerase,

which was a monomeric protein of 80 kDa, catalyzed this conversion of a double bond position at carbon 9, 10, or 11. However, fatty acids with a double bond position at 6 or 7 and carbon chain lengths between 14 to 17 were not catalyzed by this isomerase. Furthermore, the isomerase activity was strongly reduced by catecholic antioxidants such as α-tocopherol and nordihydroguaiaretic acid but was not affected by 1,10-phenanthroline, EDTA (ethylene-diamine-tetra-acetic acid) or anoxic conditions.

Kepler and Tove (1967) examined the isomerization reaction of linoleic and linolenic acid through incubation of B. *fibrisolvens*, finding that *cis*-9 *trans*-11 CLA and *cis*-9 *trans*-11 *cis*-15 C18:3 were the end products. The isomerases present in the cell envelope did not require a cofactor and a prosthetic group. Kepler et al. (1970) concluded that at least three parameters were involved in binding substrates to the enzyme of B. *fibrisolvens*; the π system of the substrate double bond, hydrophobic interaction, and hydrogen bonding of the substrate carboxyl group. In addition, Kepler et al. (1971) found that the isomerase from B. *fibriosolvens* was highly specific for a straight chain fatty acid such as linoleic acid with six carbon atoms from the methyl group end. Since hydrogen was found to be stereospecific when added to the C13 of linoleic acid in the D configuration, these researchers proposed that the hydrophobic pocket of this enzyme initially binds to a fatty acid in the form of a loop which involves π-electrons interaction between a substrate double bond and an electrophilic group on the enzyme. The undissociated carboxyl group of the substrate subsequently binds to an electronegative center

on the enzyme through hydrogen bonding, serving as the donor for the hydrogen added at C13. The product, *cis*-9 *trans*-11 CLA, is released from the enzyme which reverts to its initial states after completing its isomerization reaction. Unlike most enzymes, Kim et al. (2000) suggested that the isomerase does not recycle to catalyze more substrates although the reaction is very rapid.

Liavonchanka et al. (2006) found that the isomerases in *Propionibacterium acnes* catalyzed linoleic acid to *trans*-10 *cis*-12 CLA. Activity of the isomerase required a hydrogen radical from C11 to bind to the N5 of bound flavin adenine dinucleotide (FAD): subsequently, the double bond shifted through electron migration and then rehydrogenation at C9 of the fatty acid was also required. Wallace et al. (2007) concluded that this isomerase mechanism of P. *acnes* producing *trans*-10 *cis*-12 CLA from linoleic acid differed from B. *fibrisolvens* that produces *cis*-9 *trans*-11 CLA. Based on stereochemical considerations, the ionic reaction was caused by isomerization in P. *acnes*, a mechanism not requiring an exchange with water. However, the synthesis of *cis*-9 *trans*-11 CLA was initiated by hydrogen abstraction on C11 catalyzed by a radical intermediate enzyme. The radical was thermodynamically less favorable than the conjugated double bond system with the radical located on C13. These results suggest that the double bond migration from carbon 12 and 13 is due to thermodynamic stability.

The second step in biohydrogenation results from bacterial reductases. Hunter et al. (1976) demonstrated that reduced methyl viologen, NADH, and an endogenous electron donor could serve as reductants, concluding that at

least two hydrogenation systems existed, possibly involving a flavoprotein. Hughes et al. (1982) purified the reductase of B. *fibrisolvens* A38, converting *cis*-9 *trans*-11 CLA to *trans*-11 C18:1, which is a single subunit having a molecular weight of 60 kDa. This purified enzyme, which included the iron, phsphatidylethanolamine, and carbohydrates, can utilize α-tochpherolquinol alone as a reductant although the cell extract can reduce the double bond using NADH or α-tochpherolquinol.

Fukuda et al. (2007) also purified the CLA reductase from a B. *fibrisolvens* MDT-10 strain. This reductase was a monomeric protein having a molecular mass of 52.9 kDa. These researchers found that the relative expression of the intracellular reductase mRNA increased, accompanied by an increase in reductase protein in the presence of C-18 unsaturated fatty acids. Saturated fatty acids such as palmitoleic acid were less effective in increasing the reductase mRNA and protein. They concluded that unsaturated fatty acids enhanced reductase synthesis and that carbon chain length affected enzyme synthesis. Finally, they hypothesized that reductase synthesis might be regulated at the transcriptional level based on their result indicating the increase in the expression of both reductase mRNA and protein.

Rumen Microorganisms Involved in Biohydrogenation

The population of microorganisms in the rumen is primary composed of three types; bacteria, protozoa and fungi. The bacteria are prokaryotic cells, with no nuclear membrane but diverse subcellular organelles. The protozoa are

eukaryotic cells which have membrane-limited nuclei and membranous subcellular organelles such as the mitochondria, Golgi apparatus and endoplasmic reticulum (Harfoot, 1978). Fungi are heterotrophic organisms, characterized by a chitinous cell wall and filamentous growth such as multicellular hyphae forming a mycelium. Although several distinct morphological types of fungi exist, they are similar to one another (Li and Heath, 1992). These microorganisms contribute to biohydrogenation of unsaturated fatty acids in the rumen.

Bacteria

Hazelwood et al. (1976) divided ruminal bacteria into three groups. Groups 1 and 2 were able to hydrogenate the *cis*-9 double bond of linoleic and linolenic acid after isomerization. In addition, Group 2 bacteria also hydrogenated the *cis*-15 double bond of linolenic acid, producing a C18:2 intermediate after isomerization. Group 3 bacteria utilized oleic, *trans*-11 C18:1 and linoleic acid, with stearic acid being the end product. Kemp and Lander (1983) separated ruminal bacteria into two groups. Group A, which included B. *fibrisolvens, Micrococcus* sp., and *Eubacterium* sp., hydrogenated linoleic and linolenic acid yielding *trans*-11 C18:1 and *trans*-11 *cis*-15 C18:2, respectively, but could not reduce these to stearic acid. Group B bacteria, which included *Fusocillus* sp., reduced the *trans*-11 bond to produce stearic acid as the end product of biohydrogenation. In a later study, Kemp et al. (1984) examined the ability of *Fusocillus* sp. T 344 to hydrogenate *cis* to

trans-C18:1 isomers. Approximately 75% of *cis*-5 to 11 C18:1 isomers were converted to stearic acid, and 45% of *trans*-8 to 10 C18:1 isomers were converted to stearic acid.

Polan et al. (1964) examined biohydrogenation activity using more than 20 pure cultures of rumen bacteria. A culture of B. *fibrisolvens* strain 100 Bran 9 produced 46% monoenoic acid but no stearic acid from linoleic acid. However, microscopic examination of the same organism in another strain (A-38, 1, D-1) revealed that each was contaminated by a different organism. To test if these contaminating organisms contributed to the linoleic acid biohydrogenation, B. *fibrisolvenses* were inoculated in the presence of *Peptostreptococcus elsdenii* strain T-81 and an unnamed *Selenomonas* sp. Strain 233. They observed that these two microorganisms contribute to significant biohydrogenation activity while the pure bacterial cultures were found inactive.

Kim et al. (2002) isolated *Megasphaera elsdenii,* an important lactate-producing bacteria in cattle feed grain (Counotte et al., 1981) and more resistant to linoleic acid than B. *fibrisolvens* (Henderson, 1973), as the predominant ruminal bacteria that isomerase linoleic acid, producing *trans*-10 *cis*-12 CLA. In a previous study, Kim et al. (2000) found that *trans*-10 *cis*-12 CLA was produced quickly from linoleic acid by M.*elsdenii*, but smaller amounts of the linoleic acid were converted to *trans*-10 *cis*-12 CLA than to B. *fibrisolvens*. Only 7 μg *trans*-10 *cis*-12 CLA per mg protein was produced by M. *elsdenii* YJ-4 cultures, while 18 μg *cis*-9 *trans*-11 CLA per mg protein was

produced by B. *fibrisolvens*. More recently, van de Vossenberg and Joblin (2003) examined the isolation of the stearic acid-forming bacteria in bovine rumen based on the 16s rRNA gene sequence; they found that *Butyrivibrio hungatei* was able to convert oleic, linoleic and linolenic acid to stearic acid. Koppová et al. (2006) determined that the isomerization activity of CLA-producing bacteria from linoleic acid in the rumen. They identified that *Pseudobutyrivibrio ruminis* had the highest isomeriase activity, even higher than B. *hungatei* A38.

Hudson et al. (2000) suggested that lactic acid bacteria in the rumen such as *Streptococcus, Straphylococcus, Enterococcus, Lactobacillus* and *Pediococcus* were able to hydrate oleic and linoleic acid. They concluded that the lactic acid bacteria hydrated unsaturated fatty acids rather than their being biohydrogenation in the rumen. On the other hand, Van Nieuwenhove et al. (2007) found that seven dairy bacteria, including *Lactobacilli, Bifidobacteria*, and *Streptococci*, produced CLA from linoleic acid, this formation being between 17% and 36% after 24 h of incubation. L. *casei*, L. *rhamnosus*, B. *bifidum* and S. *thermophilus* showed the highest CLA formation accompanied with a decrease in linoleic acid concentration.

Protozoa

In the rumen, 40-80% of microbial biomass is protozoa (Harrison and McAllan, 1980). The protozoa are primarily ciliates (10^5-10^6 protozoa/ml) with a small number being flagellates such as *Monocercomonas* sp. and

Trichomonas sp. (Williams, 1986). The ciliate protozoa of the rumen require a wide range of phosphorous-containing lipids and a high proportion of unsaturated fatty acids as nutrients. Based on their morphological and physiological properties, they can be classified as either holotrich protozoa or entodiniomorph protozoa. These two groups can be easily distinguished by microscopic examination. The entire cell surface of holotrich protozoa is covered by cilia which ingest soluble carbohydrates and bacteria as their energy source. The entodiniomorph protozoa, which have cilia only at the anterior end of the cell, utilize food particles, chloroplasts and bacteria as food sources (Harfoot, 1978).

Write (1959) concluded that the biohydrogenation of unsaturated fatty acids by protozoa contained principally holotriches and *Epidinium* spp. However, Dawson and Kemp (1969) suggested that the presence of protozoa was not required for the biohydrogenation process to occur. Girard and Hawke (1978) and Singh and Hawke (1979) also proposed that the activity of ingested or associated bacteria contributed only slightly in the biohydrogenation by protozoa. The three prime holotrich species isolated in the rumen were *Isotricha intestinalis*, I. *prostoma*, and *Dasytricha ruminantium*. The lipid proportion of a mixed holotrich preparation consisted of 70% phospholipids and 30% nonphospholipids (Williams, 1986).

Most recently, Devillard et al. (2006) examined the role of ruminal protozoa in the formation of CLA and *trans*-11 C18:1 from linoleic acid. Mixed protozoa in sheep rumen contained at least two or three times higher

unsaturated fatty acids, including CLA and *trans*-11 C18:1, than bacteria. Different protozoa species exhibited different fatty acid composition, with large fibrolytic species such as *Epidinium ecaudatum caudatum* containing more than ten times higher CLA and *trans*-11 C18:1 than such small species as *Entodinium nanellum*. Compared to bacteria, protozoa exhibit lower linoleic acid biohydrogenation activity. In addition, an alternative route of CLA and *trans*-11 C18:1 formation via desaturation also did not occur in the protozoa based on studies using radioactivity and PCR-based methods. Therefore, although protozoa are rich in CLA and *trans*-11 C18:1, these two fatty acids biohydrogenated from linoleic acid did not synthesize to stearic acid, suggesting protozoa might preferentially incorporate CLA and *trans*-11 C18:1 formed by ruminal bacteria.

Fungi

Ruminal fungi, considered a minor part of the biomass, are not essential to the function of the rumen. However, they are probably important in the overall metabolisms because of their high fiber digestion activity (Wilson and Wood, 1992).

Kemp et al. (1984) found that *Piromyces communis* converted stearic acid to oleic acid by Δ9-desaturase. Moreover, P. *communis* converted linoleic and linolenic acid to conjugated products. Maia et al. (2006) examined two species of ruminal fungi, *Neocallimastix frontalis* and *Piromyces communis*, which were grown in a medium containing 50 μg/ml linoleic acid. After a 5%

inoculum for 96 h, only N. *frontalis* was grown in the linoleic acid-containing the medium. P. *communis* was not grown under the same conditions. N. *frontalis* had metabolized 50% linoleic acid to form *cis*-9 *trans*-11 CLA. Nam and Garnsworthy (2007) confirmed that rumen fungi could biohydrogenate linoleic acid to *cis*-9 *trans*-11 CLA, but the biohydrogenation rate was much slower than rumen bacteria. *Trans*-11 C18:1 was the end product of fungal biohydrogenation, similar to that of Group A bacteria. They observed that *Orpinomyces* was the highest active biohydrogenating fungus.

Influential Factors of Biohydrogenation

Changes in diets offered to ruminants affect the extent of biohydrogenation in the rumen. The rumen environmental factors such as pH, soluble carbohydrates, microbial growth factors, solid dilution rate, and fatty acids have differential effects on biohydrogenation in addition to diet supply (Hungate, 1966; Church, 1969; Martin and Jenkins, 2002; AbuGhazaleh and Buckles, 2005).

Martin and Jenkins (2002) examined continuous culture incubations that were conducted at dilution rate of 0.05 and 0.10/h with pH values of 5.5 and 6.5, and 0.5 and 1.0 g/L of mixed soluble carbohydrate. They found that the major environmental factor that influenced formation of CLA and *trans*-C18:1 from linoleic acid biohydrogenation was culture pH. At pH of 5.5, the concentration of *trans*-C18:1 and CLA were significantly reduced. It means that the CLA production also reduced at the tissue level because *trans*-C18:1 is

a precursor of CLA especially the formation of *cis*-9 *trans*-11 CLA from *trans*-11 C18:1 by Δ^9-desaturase. Similar effects were observed by Troegeler-Meynadier et al. (2003). They found that the amounts of biohydrogenation products were always lower at pH 6.0 than at pH 7.0 at 24 h *in vitro* incubations in ruminal fluids. Low amounts of CLA at pH 6.0 could be due to low isomerase activity or to high reductase activity. Moreover, they found that low pH (pH 6.0) resulted in lower amount of *trans*-11 C18:1 at all incubation times compared with higher pH (pH 7.0), but concentration of *trans*-10 C18:1 was higher at 16 to 24 h of incubation. Low pH inhibited initial isomerization and the second reduction (*trans*-11 C18:1 to stearic acid), leading to an accumulation of *trans*-11 C18:1 in ruminal cultures (Troegeler-Meynadier et al., 2006). Choi et al. (2005) reported that *cis*-9 *trans*-11 CLA are produced at pH's higher than 6.2 by rumen bacteria, but *trans*-10 *cis*-12 CLA are produced more than *cis*-9 *trans*-11 CLA at lower pH. They concluded that *trans*-10 *cis*-12 CLA producing bacteria may be more aero and acid-tolerant than *cis*-9 *trans*-11 CLA producing bacteria.

Oleic acid biohydrogenation was also affected by ruminal pH and dilution rate. AbuGhazaleh et al. (2005) reported that low pH and dilution rate are restricted to formation of *trans*-C18:1 and increased the concentration of stearic acid from oleic acid. They conducted the experiment using ^{13}C-labeled oleic acid to determine biohydrogenation intermediate in mixed ruminal microorganisms grown in continuous cultures at different pH and liquid dilution rate. At pH 6.5 and 0.10/h dilution rate, ^{13}C enrichment was detected

in *trans*-6 through 16 C18:1 isomers. However, at pH 5.5 and 0.05/h dilution rate, ^{13}C enrichment was not detected in any *trans* isomers with a double bond position over C10.

Qiu et al. (2004) reported that reduced ruminal pH can affect microbial populations, especially cellulolytic bacteria. Total cellulolytic bacteria numbers are reduced, accompanied by reduced acetate-to-propionate ratio and biohydrogenation when pH was low. The rumen pH also influenced fungal growth and metabolism. Culturing rumen fungi at pH 6.0 and pH 7.0 slowed biohydrogenation compared pH 6.5. CLA production was increased by pH 7.0 compared to pH 6.0 and pH 6.5. Therefore, optimum pH was 6.5 and 7.0 for biohydrogenation and CLA production, respectively, by ruminal fungi (Nam and Garnsworthy, 2007).

Particular types of volatile fatty acid (VFA) and vitamin are considered essential growth factors for ruminal microorganisms. Specifically, short chain carbon (2-5) lengths of VFAs have been found to be necessary to growth of fibrolytic microorganisms (Bryant, 1973). In addition, VFAs have the potentially to affect intermediary metabolism although supplemented VFAs did not influence fiber digestion or microbial protein production (Andries et al., 1987).

Vitamins also can increase the enzyme activity in ruminal bacteria, producing high propionic acid (Schussler et al., 1978). The study conducted by Chikunya et al. (2004) found that high levels of dietary vitamin E increased the plasma α-tocopherol level. Similarly, Pottier et al. (2006) determined that

this vitamin increased the amount of fat and decreased the *trans*-10 C18:1 content in milk while increasing the concentration of *cis*-9 *trans*-11 CLA and *trans*-11 C18:1. Not much is known, however, what vitamin E mechanisms is responsible for the biohydrogenation process. Researchers have suggested four possibilities: 1) Vitamin E might act as an electron donor for the reduction of the *cis* bond of the CLA. 2) Vitamin E might be metabolized to become electron donors by ruminal microorganisms 3) Vitamin E might act as an electron donor to restore α-tochopherolquinol and deoxy-α-tocopherolquinol. 4) Vitamin E could inhibit the growth and function of *trans*-10 C18:1 producing bacteria. However, Nam and Garnsworthy (2007) confirmed that the addition of VFA and vitamin did not affect the pattern or extent of biohydrogenation by mixed ruminal fungi in *in vitro* cultures.

Soluble carbohydrates such as glucose, cellobiose, maltose, and xylose have been found to influence the extent of the biohydrogenation process. According to Mountfort and Asher (1983), glucose was the preferred substrate over fructose and xylose, with cellobiose being more preferentially utilized than fructose by rumen fungi. Moreover, they reported that fungal zoospores rapidly developed when glucose was added to a culture medium, suggesting that biohydrogenation activity may be reduced during this stage of the fungal life cycle. Cellobiose also inhibited biohydrogenation, causing the primary products of its fermentation such as lactate, acetate, hydrogen and carbon dioxide by rumen fungi (Orpin and Munn, 1986). Their production might change the pH thereby affecting the biohydrogenation in the rumen. Martin

and Jenkins (2002) investigated the effect of various soluble carbohydrate mixtures on biohydrogenation by mixed ruminal bacteria, finding that an increase in the concentration from 0.5 to 1.0 g/L caused an increase in the proportion of such fatty acids as palmitic acid, stearic acid, linoleic acid, linolenic acid, *cis*-C18:1, *cis*-9 *trans*-11 CLA, and *trans*-10 *cis*-12 CLA. Therefore, soluble carbohydrates affected the metabolism of long-chain fatty acid in the rumen. Both glucose and cellobiose inhibited biohydrogenation by the mixed ruminal fungi, although the pattern of biohydrogenation was similar (Nam and Garnsworthy, 2007).

In addition, the concentration of fatty acid substrates affects the biohydrogenation process. Polan et al. (1964) found that the high amounts of stearic acid produced by biohydrogenation inoculated low concentrations of linoleic acid. As the linoleic acid concentration increased, the conversion of stearic acid decreased. Similarly, Harfoot et al. (1973) found that linoleic acid inoculated less than 1.0 mg/ml of ruminal contents, with stearic acid being the primary end product. When the concentration of linoleic acid increased to more than 1.0 mg/ml of ruminal contents, *trans*-11 C18:1 was the primary end products.

Kelly et al. (1998) examined dietary fatty acid sources such as peanut oil, sunflower oil, and linseed oil fed to lactating dairy cows, in addition to measuring the fatty acid composition in milk the resulting. The major dietary fatty acid in peanut oil is oleic acid (51.5%), in sunflower oil, linoleic acid (69.4%), and in linseed oil, linolenic acid (51.4%). The addition of peanut oil

to the diet increased the concentration of palmitic acid, while addition of sunflower and linseed oils increased the concentration of octadecenoic acid (C18:1), indicating that the high amount of long chain fatty acids irreversibly inhibit the final biohydrogenation step of C18:1 to stearic acid. A similar effect was observed by Troegeler-Meynadier et al. (2003 and 2006), who confirmed that the high concentration of linoleic acid inhibited the two subsequent reductions, the reduction of CLA to *trans*-C18:1 and the reduction of *trans*-C18:1 to stearic acid, thus increasing CLA production. The high amount of linolenic acid which inhibited linoleic acid isomerization led to the lower CLA production. Moreover, the high amount of linoleic and linolenic acid led to an accumulation of *trans*-11 C18:1.

AbuGhazaleh et al. (2002 and 2003) reported that lactating dairy cows which consumed an increased concentration of fish oil increased their production of *cis*-9 *trans*-11 CLA and *trans*-11 C18:1 in milk, by stimulating the conversion of linoleic and linolenic acid from other dietary sources. This increase may have been due to the inhibited reductase activity of ruminal microorganisms, causing the accumulation of CLA and *trans*-C18:1. Wąsowska et al. (2006) found that the major fatty acids in fish oil, EPA or DHA, only partially inhibited ruminal biohydrogenation.

Fatty amides and calcium salts have been found to resist ruminal biohydrogenation in the rumen. For example, Fotouhi and Jenkins (1992a, b) showed that fatty acyl amide could protect ruminants against ruminal biohydrogenation increasing the absorption of unsaturated fatty acids. In

addition, linoleoyl methionine was contributed to an increased duodenal flow of linoleic acid in sheep. In a similar study, Jenkins (1995) and Jenkins et al. (1996) examined the ability of butylsoyamide, a fatty acyl amide in soybean oil, to reduce biohydrogenation and to increase linoleic acid concentration in blood and milk. They found that the fatty acyl amide protected the double bonds of unsaturated fatty acids from biohydrogenation and increased absorption of selected unsaturated fatty acids.

Wu et al. (1991) reported that calcium salts in palm fatty acids also resisted biohydrogenation. CLA and oleic acid concentration in milk was increased after feeding a variety of fat sources as calcium salts to lactating cows. However, the concentrations of linoleic and linolenic acid did not increase in milk after soybean oil and linseed oil were added to the calcium salts (Chouinard et al., 1997).

Ju and Jung (2003) examined the effect of adding sulfur to the culture during the biohydrogenation of soybean oil with a nonselective type nickel catalyst. They found that addition of sulfur enhanced the CLA formation in soybean oil during hydrogenation. The optimal ratio of sulfur to nickel for increasing CLA formation was 0.06:1.

Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid (CLA) refers to a mixture of several isomers of linoleic acid, specifically positional and geometric isomers of octadecadienoic acid with conjugated double bonds. The double bonds in CLA

are primary found at carbon numbers between 9 and 12 either as *cis* or *trans* isomers. CLA is synthesized either by the ruminal biohydrogenation process or from *trans*-11 to yield *cis*-9 *trans*-11 CLA by delta-9-desaturase in the tissues (Donovan et al., 2000).

According to the Chin et al. (1992), *cis*-9 *trans*-11 CLA comprises more than 82% of the CLA in dairy products. CLA theoretically includes 28 positional and geometric isomers, of which only a few, especially *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA, have been reported to exhibit biological activities (Banni, 2002). CLA has potential health benefits, including anti-carcinogenesis, anti-atherogenesis, and the ability to prevent tumorigenesis and to reduce the immune stimulation (Pariza, 2004; Wahle et al., 2004). In addition, *trans*-10 *cis*-12 CLA has been reported to reduce body fat and alter the ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) (Pariza, 2004; Wang and Jones, 2004).

Anti-Carcinogenesis

An anti-carcinogenic effect of CLA was originally examined by Ha et al. (1987) in fried ground beef. More recently, Ha et al. (1990) reported that grilled ground beef contained 7,12-dimethylbenz(a)anthracene factors, inhibiting mouse epidermal carcinogenesis. They concluded that *cis*-9 *trans*-11 CLA, preferentially incorporated into forestomach membrane phospholipids might have operated as an *in situ* membrane defense mechanism, attacking free radicals as an effective antioxidant, explaining, in part, the anti-

carcinogenic properties of CLA. In a relative study, Ip et al. (1991) found that the maximal antioxidant activity occurred with 0.25% CLA in the diet, although the maximal tumor suppression was observed at 1.0% CLA. These results suggest other mechanisms may also function as antioxidants and anti-carcinogenics.

The molecular mechanism inducing this anti-carcinogenic effect of CLA has yet to be established. CLA might react to multiple mechanisms depending on the type of tissue, the transcriptional regulators, and the gene expressions (Ip et al., 2003). One possible mechanism involves the peroxisome proliferator activated receptor (PPAR) families, which are ligand-dependent transcription factors, and a reaction with the retinoid X receptors (RXR). Song et al. (2007) found that *trans*-10 *cis*-12 CLA was associated with PPARγ and an increase in the phagocytic activity of RAW macrophages by increasing the tumor necrosis factor (TNF)-α expression. Another mechanism involves the sterol-regulatory binding protein family (SREBPs), hepatic nuclear receptor-4 (HNF-4) and liver X receptor-α (LXR-α), which are inhibited or modulated by CLA.

CLA has been shown to inhibit HT-29 colon cancer cell growth and proliferation. For example, Kim et al. (2003) suggested that CLA might reduce the amount of insulin-like grown factor (IGF) II synthesis and inhibited the extracellular signal-regulated kinase-1/2 pathway and the IGF-I receptor signaling in colon cancer. Cho et al. (2003) found that *trans*-10 *cis*-12 CLA decreased cell proliferations, whereas *cis*-9 *trans*-11 CLA had no effect on HT-

29 colon cancer cells.

Research on MCF-7 breast cancer cells has shown that CLA inhibited the growth cancer cells in *in vitro* culture with Chujo et al. (2003) reporting that *trans*-10 *cis*-12 CLA inhibited cell proliferation induced by estrogen and insulin but not by the epidermal growth factor (EGF). Estrogen and insulin were not affected by *cis*-9 *trans*-11 CLA. In addition, CLA isomers depressed the estrogen receptor α expression at mRNA in protein, and capability of the nuclear binding protein to a canonical estrogen response (Tanmahasamut et al. 2004). In the study conducted by Wang et al. (2005), MCF-7 cancer cells were inoculated with human breast stromal cells in the presence of *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA isomers. These researchers found that both isomers depressed mRNA expression and protein levels in these cancer cells. However, *trans*-10 *cis*-12 CLA showed more efficiency than the *cis*-9 *trans*-11 CLA.

Ochoa et al. (2004) reported that two CLA isomers (*cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA) decreased PC-3 prostate carcinoma cell line proliferation *in vitr*o. The *trans*-10 *cis*-12 CLA was the most effective isomer (55% inhibition), decreasing the apoptosis gene expression and increasing the level of cell cycle control of mRNA, while *cis*-9 *trans*-11 CLA had an effect on both the 5-lipoxygenase expression and the cyclooxygenase-2 protein level. Therefore, both CLA isomers inhibit the proliferation of prostate cancer but through different pathways; *trans*-10 *cis*-12 CLA works preferentially through apoptosis and cell cycle control gene modulation. *Cis*-9 *trans*-11 CLA

regulates genes through the arachidonic acid metabolism, mechanisms in eicosanoid synthesis.

Anti-Atherogenesis

Lee et al. (1994) examined the effect of CLA on atherosclerosis, reporting rabbits fed a semi-synthetic diet and augmented with 0.5 g CLA / day. By 12 weeks feeding, exhibited significantly lower LDL cholesterol and triglycerides after 12 weeks. Moreover, the ratio of LDL-cholesterol to HDL-cholesterol and the ratio of total cholesterol to HDL-cholesterol were reduced in CLA-fed group. The aortas of CLA-fed rabbits also showed less atherosclerosis. Similarly, Lee et al. (2005) observed the anti-atherosclerotic activity of CLA in C57BL/6J mice. They found that the amount of serum total cholesterol and total triglyceride significantly decreased in a diet supplementation with CLA, although HDL-cholesterol levels increased.

In addition, the acyl CoA: cholesterol acyltransferase (ACAT) was found to play an important role in the development of atherosclerosis in the transformation of macrophages and/or smooth muscle by forth cells (Sakashita et al., 2000). A similar transformation was found in the liver of CLA-fed mice by Lee et al. (2005). In a study conducted by Valeille et al. (2006), hyperlipidemic hamsters were fed diets containing natural *cis*-9 *trans*-11 CLA in commercial milk. They found that these CLA-fed hamsters exhibited decreased aortic cholesterol levels, LDL-peroxidability indices, and gene expressions of proinflammatory IL-1β in the aorta, concluding that this CLA

significantly reduced the atherogenic potential of milk fat. In a recent study by Franczyk-Zarow et al. (2008), CLA-fed mice showed a decrease in the size of atherosclerotic plaque and total plasma cholesterol in their aortic roots. Moreover, these mice had a reduced number of atherogenic macrophages and an increased area occupied by smooth muscle cells in atherosclerotic lesions.

Immune System

CLA decreased the growth rate in chicks and rats after they were injected with endotoxin (lipopolysaccharide; LPS). This probably caused by release of cytokines and the prevention of the catabolic effects (Cook et al., 1993). Miller et al. (1994) examined the prevention of endotoxin-induced growth suppression in mice fed with 0.5% fish oil and CLA. The fish oil fed-group lost twice as much body weight after the inoculation with endotoxin than the CLA-fed groups. The researchers found that the CLA in the endotoxin injection inhibited anorexia (a decreased sensation of appetite) and increased splenocyte blastogenesis, concluding that it might inhibit arachidonic acid synthesis, thus preventing the catabolism of tissue by removing eicosanoid precursors. In addition, Bontempo et al. (2004) examined the effects of CLA on the immunological variables of lactating sows and piglets fed with a 0.5% CLA diet. They found that CLA-fed sows exhibited increased colostrum IgG and serum leptin, and IgG and lysozyme. Nursing piglets of CLA-fed sows exhibited higher levels of the latter than former. As these results shown, dietary CLA enhanced the effect of immunological variables in lactating sows

and piglets.

Effect on Body Weight and Fat

Park et al. (1997) found that CLA contributed to a reduction in body fat mass and an increase in lean body mass. Mice fed a 0.5% CLA augumented diet exhibited 57-60% lower body fat and 5-14% increased lean body mass than the controls. They also found that the total carnitine palmitoyltransferase activity was enhanced with dietary CLA supplementation. Moreover, in cultured 3T3-L1 adipocytes, CLA treatment reduced the intracellular heparin-releasable lipoprotein lipase activity and triacylglyceride and glycerol concentration. In addition, DeLany et al. (1999) suggested that CLA played a key role in reducing body fat content and increasing protein accumulation in mice. In this study, the diets of mice were supplemented with CLA (0.0, 0.25, 0.50, 0.75, and 1.0% by weight). In the 0.50, 0.75, and 1.0% CLA feeding groups, body fat was significantly lower than in the control, however, energy was not depressed by any CLA dose. As these results suggested, dietary CLA reduces fat deposition and increases lipolysis in adipocytes, coupled with enhanced fatty acid oxidation.

In a human trial, Blankson et al. (2000) conducted a double blind study with 60 overweight or obese volunteers (body mass index 25-35 kg/m^2). The CLA doses given varied from 1.7, 3.4, 5.1, or 6.8 g/day for 12 weeks. The reduction of body fat mass was significant for the 3.4, 5.1, and 6.8 g CLA groups. No significant differences in lean body mass, body mass index, blood

safety variables or blood lipids were observed in the groups. Long-term supplementation of CLA studied by Gaullier et al. (2007), who found that a six-month supplementation of CLA reduced body fat mass and increased lean body mass. They conducted a double blind placebo-controlled study with 118 healthy, overweight, and obese adults. The CLA dose was 3.4 g/day. During the six-month period of study, the CLA significantly decreased the body fat mass (-3.4%) located primary in the legs.

Two families of transcription factors can be in the intracellular signaling mechanisms of CLA, are affected by peroxisome proliferator activated receptors (PPAR) and sterol regulatory element binding-protein (SREBP) in adipose tissue (Tsuboyama-Kasaoka et al., 2000). However, the mechanisms by which CLA alters lipid metabolism in the human body are still unclear. Initially, CLA, especially *trans*-10 *cis*-12 CLA, enters the cell membrane through unknown transport mechanisms before being shuttled into various regulatory compartments. Brown and Mclntosh (2003) suggested that subsequently three mechanisms in adipose tissue depressed the triglyceride. The first esterified CLA into triglyceride-rich oil droplets. This mechanism did not have much effect on the regulation as a whole, even increasing triglyceride stores. The second mechanism esterified CLA into the membrane-bound phospholipids bilayer, where it changed the fluidity of the membranes associated with a signal transduction cascade. The last mechanism modified the activity of a currently unidentified transcription factor (TFX), resulting in less regulation of PPAR and the depression of lipoprotein lipase, an acyl-CoA-

binding protein, an adipocyte fatty acid binding protein, a glucose transporter, and leptin. In addition, CLA reduced the acitivity of steroyl-CoA desaturase and acetyl-CoA carboxylase by altering the activity of TFX. By inhibiting these factors, CLA attenuated insulin-stimulated glucose uptake, malonyl-CoA synthesis, and oleate synthesis, collectively decreasing de novo fatty acid synthesis. CLA also inhibited the expression of SREBP, by enhancing SREBP transcript decay as well as by inhibiting the proteolytic cleavage, releasing the activated nuclear SREBP fragment.

Effect of Milk Fat Synthesis

According to the study conducted by Baumgard et al. (2002), *trans*-10 *cis*-12 CLA decreased the lipogenic rate and milk fat synthesis of dairy cows, the results showing a 42% decrease in milk fat content and a 48% reduction in milk fat yield. These researchers also found that lipogenic activity decreased 82% using a radio-labeled acetate, and the activity of acetate oxidation to carbon dioxide was reduced to 61% in dairy cows inoculated with *trans*-10 *cis*-12 CLA. Additionally, the mRNA expression of all measured enzymes decreased from 39 to 54% after a dosing with *trans*-10 *cis*-12 CLA. The results suggested that the *trans*-10 *cis*-12 CLA inhibited milk fat synthesis by decreasing the enzyme activity through the inhibition of gene expression affecting *de novo* fatty acid synthesis, uptake, and transport. A similar effect depression of milk fat synthesis by *trans*-10 *cis*-12 CLA was observed in lactating sheep (Lock et al., 2006). These researchers found that the addition

of CLA reduced the milk fat content from 6.4 to 4.9% and decreased fat yield from 95 to 80 g/day. *Trans*-9 *cis*-11 CLA and *cis*-10 *trans*-12 CLA have also been reported as potential inhibitors of milk fat synthesis (Sæbø et al., 2005; Perfield II et al., 2007) with the former being associated with a 15% reduction in milk fat yield.

Stable Isotope Technique in Biohydrogenation

Research using a stable isotope to identify fatty acid metabolism in ruminants has increased over the last decade. The development of an analytical methodology and the commercial availability of labeled compounds are the reasons for this increase (Emken, 2001). As a result, the use of radio-isotopes, popular in early metabolic studies in animals, has decreased because of their potential risk of ionising radiation.

The stable isotope technique provides reliable information about intermediates originating from a particular labeled compound. The study may require gas chromatography-mass spectrometry (GC-MS) for isotopic analyses of an individual labeled compound in biohydrogenation. GC-MS has been used for decades to monitor the metabolism of stable isotope tracers with a high enrichment level. The primary advantage of using GC-MS to study stable isotopes is rapid acquisition and accurate measurement. Therefore, this technique can be used at low metabolic concentration levels and with small sample sizes, saving both time and money. In GC-MS, biohydrogenation intermediates, after separation, are ionised in the ion source and fragmented

with different masses to gain charge. A properly charged fragment is measured by its isotopomer distribution. The distribution consists of the intensity of the mass M, M+1, M+2, where M represents the entire mass as the most abundant lightest molecules (^{12}C, ^{1}H, ^{14}N, ^{16}O), and M+1 one mass higher than M such as ^{13}C, ^{2}H, ^{15}N and ^{17}O, and M+2 two masses higher than M such as ^{18}O . The natural abundance of ^{13}C is approximately 1.1%. A molecule containing 18 carbon atoms shows an M+1 contribution of approximately 19.8%. However, the commercial ^{13}C labeled compound approaches 100%. The isotope ratio of labeled to unlabeled intermediates provides the information about whether they originated from the initial compound (Stellaard and Elzinga, 2005).

The initial research in this area used deuterium (^{2}H), the hydrogen isotope ^{1}H, in biohydrogenation studies. However, several side effects and toxicities were observed in animal experiments. Deuterium affects the activation energy of chemical reactions, and metabolic and diffusion rates (Katz, 1969). Protozoa, worms and small fish did not survive after 3 h under 92% D_2O conditions (Taylor et al., 1933). Current studies primarily use carbon-13 (^{13}C). This stable isotope is considered nontoxic and fully compatible with living organisms, including humans and microorganisms, without side effects (Koletzko et al. 1997). However, stable isotope analyses do not reveal the entire metabolic pathway. They only provide which intermediates were produced from which original compound. Therefore, stable isotopic labeled intermediates must be examined under the same conditions as the original compound to reveal the entire pathway.

Table 1.1. Double bond position and geometry of identified CLA isomers present in intestinal content of cattle[1].

Double Bond Position	Double Bond Geometry			
	cis/cis	*trans/trans*	*cis/trans*	*trans/cis*
7/9		B,D		B
8/10		A, B, D		B, C
9/11	A, C	A, B, D	A, B, D	C
10/12		A, B, D	C	A, B, C, D
11/13	A	A, B, C, D	A, B, C, D	B, C, D
12/14		D	D	

[1]A = Duckett et al. (2002); B = Piperova et al. (2002); C = Loor et al. (2004); D = Shingfield et al. (2003).

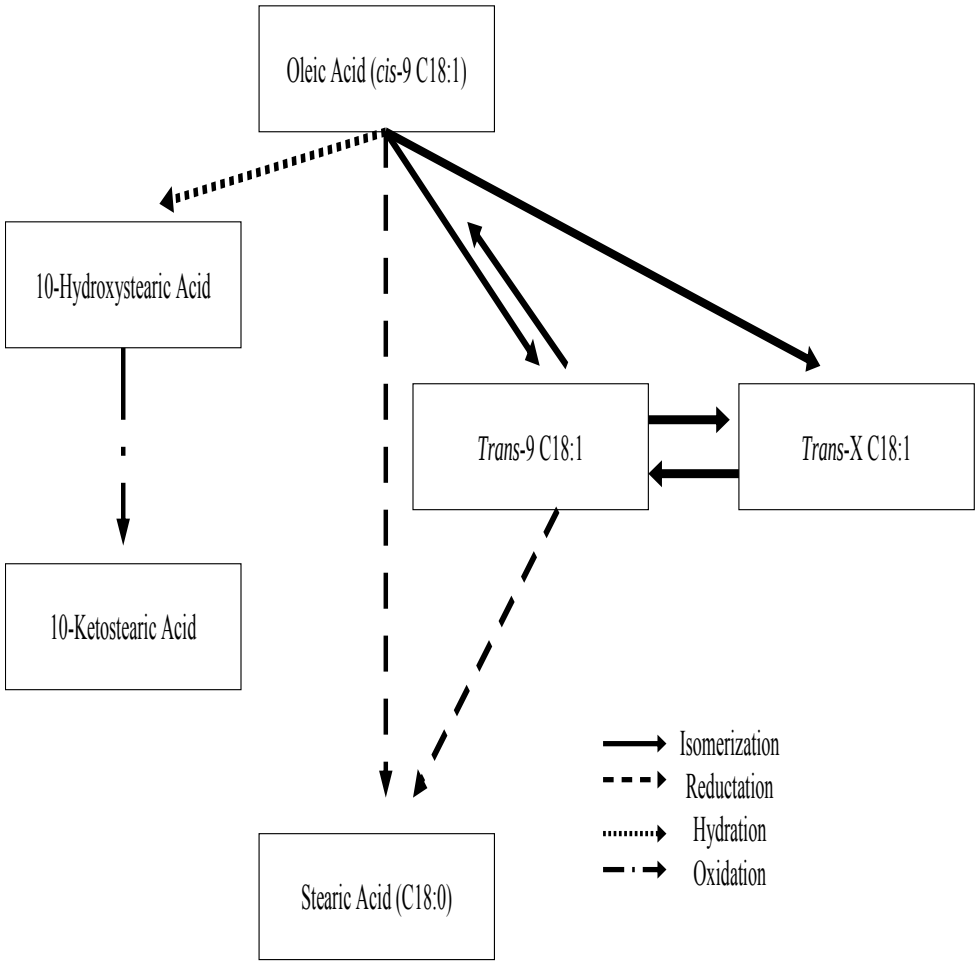

Figure 1.1. Oleic acid transformation by ruminal microbes. Modified from Mosley et al. (2002); Proell et al. (2002); Jenkins et al. (2006).

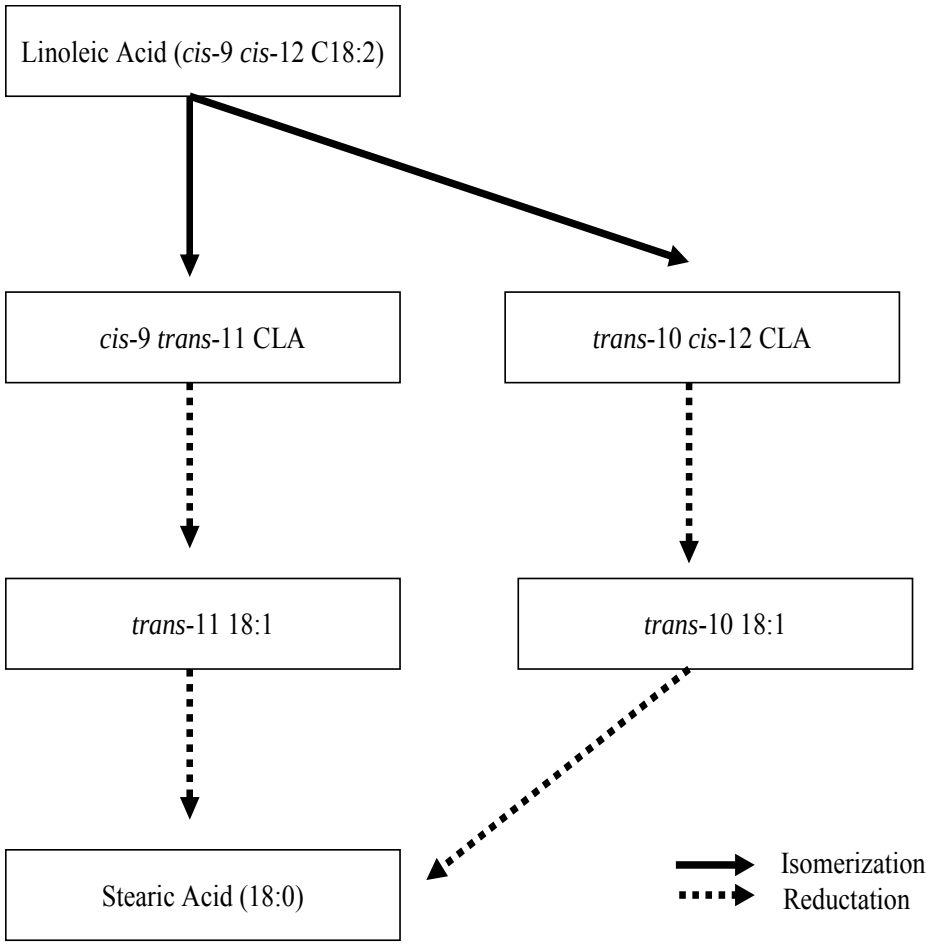

Figure 1.2. The pathway of linoleic acid biohydrogenation. Modified by Griinari and Bauman (1999).

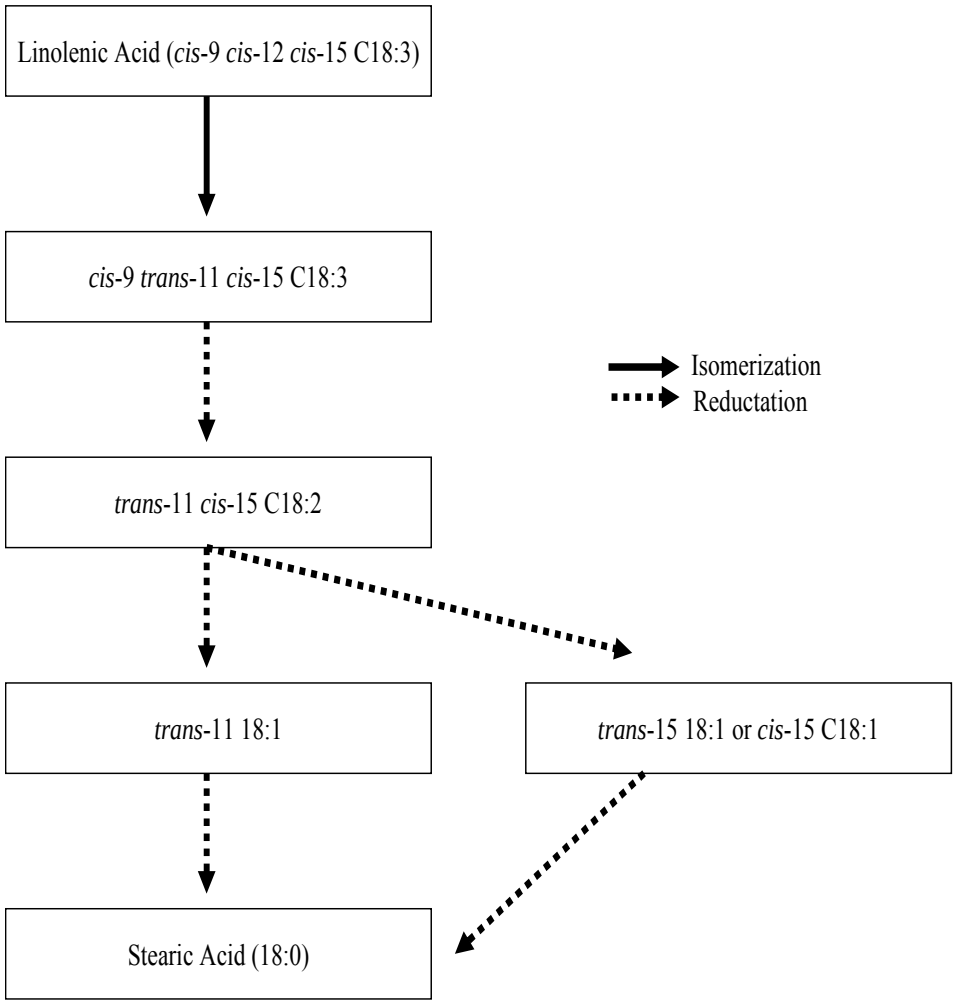

Figure 1.3. Ruminal biohydrogenation of linolenic acid (*cis*-9 *cis*-12 *cis*-15 C18:3). Pathway established using published data (Griinari and Bauman, 1999; Harfoot and Hazlewood, 1988).

IDENTIFICATION OF ENRICHED CONJUGATED LINOLEIC ACID ISOMERS IN CULTURES OF RUMINAL MICROORGANISMS AFTER DOSING WITH 1-^{13}C-LINOLEIC ACID

ABSTRACT

Most studies of linoleic acid biohydrogenation propose that it converts to stearic acid through the production of *cis*-9 *trans*-11 CLA and *trans*-11 C18:1. However, a several other CLA have been identified in ruminal contents, suggesting additional pathways may exist. To explore this possibility, this research investigated the linoleic acid biohydrogenation pathway to identify CLA isomers in cultures of ruminal microorganisms after dosing with a ^{13}C stable isotope. The ^{13}C enrichment was calculated as [(M+1/M)×100] in labeled minus unlabeled cultures. After 48h incubation, significant ^{13}C enrichment was observed in seven CLA isomers, indicating their formation from linoleic acid. All enriched CLA isomers had double bonds in either the 9,11 or 10,12 position except for *trans*-9 *cis*-11 CLA. The *cis*-9 *trans*-11 CLA exhibited the highest enrichment (30.65%), followed by enrichments from 21.06 to 23.08% for *trans*-10 *cis*-12, *cis*-10 *trans*-12, *trans*-9 *trans*-11, and *trans*-10 *trans*-12 CLA. The remaining two CLA (*cis*-9 *cis*-11 and *cis*-10 *cis*-12 CLA) exhibited enrichments of 18.38 and 19.29%, respectively. The results of this study verified the formation of *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA isomers from linoleic acid biohydrogenation. An additional five CLA isomers

also contained carbons originating from linoleic acid, indicating that pathways of linoleic acid biohydrogenation are more complex than previously described.

INTRODUCTION

Fatty acids with conjugated double bond arrangements have exhibited high biopotency compared to methylene-interrupted isomers. For instance, conjugated linoleic acid (CLA) was shown to modulate a multitude of biological functions affecting human health including lipid metabolism, carcinogenesis, immune function, obesity, diabetes, and atherogenesis (Ryder et al., 2001; DeLany and West, 2000; Pariza, 2004; Wahle et al., 2004). Particularly, the *cis*-9 *trans*-11 CLA isomer has been reported to function as an antioxidant and anticarcinogenic in animal models (Ip et al., 1999; Belury, 2002; Mosley et al., 2002). However, physiological functions appear to be isomer specific.

Conversion of linoleic acid to CLA is carried out naturally by anaerobic microorganisms that inhabit the gut of animals in a process called biohydrogenation. Biohydrogenation accomplishes the conversion of unsaturated fatty acids to stearic acid with accumulation of CLA and *trans* monoenes as intermediates. Biohydrogenation is particularly relevant in cattle and other ruminant species because it accounts for the high concentration of saturated fatty acids in meat and milk despite polyunsaturated fatty acids being the primary fatty acids consumed. Because of the large volume (up to 200 L) of anaerobic fermentation occurring in stomach compartments of cattle, the

microbial population in the rumen provides an ideal ecosystem for studying biohydrogenation and the synthesis of CLA.

Biohydrogenation is initiated by the action of an isomerase that maintains the same number of double bonds but produces positional and geometric isomers including CLA. The interaction of the *cis*-9 electrons of linoleic acid substrate with the electronegative enzyme region acting as the hydrogen binding site to produce the *cis*-9 *trans*-11 CLA isomer has been proposed by Harfoot and Hazlewood (1997). Also, the geometry of fatty acid binding to the isomerase and the involvement of specific amino acid residues in the formation of *trans*-10 *cis*-12 CLA by *Propionibacterium acnes* has been described by Liavonchanka et al. (2006). Despite detailed information being available on enzymatic action of the isomerase, little is known about the identity of CLA isomers produced from specific polyunsaturated fatty acid substrates. As many as 14 CLA isomers have been identified in ruminal contents taken from cattle (Jenkins et al., 2008). Yet, most published pathways of biohydrogenation account for the synthesis of only one or two CLA isomers. The ability to regulate CLA synthesis in ruminal contents to deliver specific isomers with specific physiological functions to animal tissues is dependent on delineating the exact synthetic pathways for all isomers.

Because linoleic acid is believed to be the parent compound for most of the CLA isomers found in digestive contents of cattle, and that only one or two CLA isomers are usually identified in pathways of linoleic acid biohydrogenation, this study was conducted to establish the number and

identity of CLA isomers that originate from linoleic acid biohydrogenation. To accomplish this objective, the carboxy carbon of linoleic acid was tagged with a ^{13}C stable isotope to trace its appearance in other C18:2 intermediates produced in cultures of mixed ruminal microorganisms. Once the ^{13}C label was identified in a C18:2 compound, the double bond position and *cis-trans* geometry was determined by tandem MS/MS. This approach allowed verification that CLA were produced from linoleic acid including its exact structural identity.

MATERIAL AND METHODS

Materials

TMR (Total mixed rations) were obtained from Clemson University Dairy Farm (including 54% corn silage, 2.9% grass hay, 4.9% alfalfa hay, 28.4% grain mix, and 9.8% moisture) and Emersol 315 (including 2.5% palmitic, 1% stearic, 27.2% oleic, 57.5% linoleic, 5.1% linolenic, and 6.7% other fatty acid) were purchased from Continental Industrial Chemicals (Charlotte, NC). Unlabeled linoleic acids (*cis*-9 *cis*-12 C18:2; >99% chemical purity) were purchased from Sigma-Aldrich (St, Louis, MO) and 1-^{13}C-linoleic acids (>99% chemical purity; 99% isotopic purity) were purchased from Larodan AB, Inc. (MALMÖ, Sweden).

In Vitro Microbial cultures

Whole digesta contents were collected from the rumen of a fistulated

Holstein cow two hours after the AM feeding. The contents were filtered through cheesecloth to remove large particles, and the filtrate containing mixed ruminal microorganisms was then transferred directly to the laboratory in a sealed container and used within 20 min. Six cultures in 125 mL Erlenmeyer flasks containing 0.5 g of diet (TMR + 5% Emersol 315), 10 mL of filtered rumen fluid, 40 mL of *in vitro* media, and 2 mL of reducing solution (Goering and Van Soest, 1970) were divided into two groups of three cultures each of mixed rumen microbial population. Cultures containing either 50 mg unlabeled linoleic acid or 35 mg unlabeled linoleic acid + 15 mg 1-^{13}C-linoleic acid in 1 mL of ethanol were run at 39^{o}C water bath under anaerobic conditions. Two 5 mL samples were taken from each culture flask at 0, 24 and 48 h while being stirred with a magnetic bar under CO_2, and then transported immediately in an ice bath, and then stored freezer at -5 ^{o}C.

Analysis of Fatty Acid Methyl Esters (FAME)

Culture samples were freeze-dried and transesterified to methyl esters in sodium methoxide followed by methanolic HCl procedure (Kramer et al., 1997). FAME contents were then quantified by gas chromatography (GC) using internal standard (C17:0, heptadecanoic acid). A Hewlett-Packard 5890 gas chromatograph equipped with P-2380, fused the silica capillary column (100 m × 0.25 mm) with 0.2 μm film thickness (Supelco Inc., PA). The conditions used were initially 140 ^{o}C for 3 min with a ramp of 3.7 ^{o}C per min up to 220 ^{o}C holding for 20 min. Helium was used as the carrier gas at 20 cm/s.

The ^{13}C enrichment in individual FAME was analyzed by GC-MS (Agilent 6890N gas chromatograph equipment with a model 5973 quadrapole mass selective detector). The GC-MS was equipped with a 100 m × 0.25 mm chrompack CP-Sil 88 column with 0.20 μm film thickness. The carrier gas was helium at 20 cm/s with splitless injection. Column temperature was programmed initially 140 ^{o}C for 5 min with a ramp of 4 ^{o}C per min up to 220 ^{o}C for 20 min. The ions chosen for fatty acid analysis in select ion mode was the quasimolecular ion [M], a [M + 1] ion to indicate the labeled compound, and [M + 29] ion that represented the [M + $C_2H_5^+$] ion.

FAME double bond positions and geometry were verified by gas chromatography (GC), followed by covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS) according to methods outlined in detail previously (VanPelt and Brenna, 1999; Michaud et al., 2002, 2003; Lawrence and Brenna, 2006). Briefly, FAME were separated with a CP-Sil 88 capillary column (100 m × 0.32 mm × 0.25 μm), and temperature programmed from 80 ^{o}C to 120 ^{o}C at a rate of 10 ^{o}C/min and then increased to 220 ^{o}C at a rate 2.5 ^{o}C/min. Total run time was 60 min. FAME eluting into the MS undergo an ion-molecule reaction to form an adduct with molecular weight 54 mass units above that of the parent FAME. Collisional dissociation of this adduct yields diagnostic ions that are characteristic of double bond position. In the case of CLA, relative diagnostic ion intensity is indicative of double bond geometry (Michaud et al., 2003), and can otherwise be discerned by GC relative retention time.

Calculations and Statistical Analysis

Fatty acid contents and ^{13}C enrichments were analyzed by analysis of variance (ANOVA) using the GLM (general linear model) procedure of SAS (2003). The fatty acid contents in the samples were calculated by comparing the fatty acid peak areas on the chromatograms with the internal standard. Data are shown to means ± SEM as mg of fatty acid contents per 5 ml microbial in vitro cultures. Abundances of select ions in samples were exhibited as [(M + 1 / M]. Enrichment was identified as $[(M + 1 / M]_{labeled}$ - $[(M + 1 / M]_{unlabeled}$ cultures to adjust for naturally occurrence of the ^{13}C isotope. Enrichment % means ± SEM taken from ANOVA were shown for each fatty acid. Least significant difference was conducted to identify the main effect and differences of fatty acid contents and enrichments of labeled fatty acids by treatment within incubation time. The fatty acid contents and enrichments data were subsequently tested for their differences from zero using Student's t-test ($P<0.05$).

RESULTS AND DISCUSSION

The fatty acid content (Table 2.1) in cultures investigated here supports the previous research in this area (Kellens et al., 1986; Griinari and Bauman, 1999; Nam and Garnsworthy, 2007ab). Linoleic acid decreased 84.29% (5.42 mg) over 48 h in a 5 mL microbial *in vitro* culture, accompanied by an

increase in *trans*-11 C18:1 (3.19 mg) and stearic acid (0.97 mg) of 58.86% and 17.90%, respectively. A previous *in vitro* study by Van Nevel and Demeyer (1996) found that more than 90% of the linoleic acid was hydrogenated at 6 h of incubation, while the *in vivo* experiment of Wu et al. (1991) determined that approximately 80% of the linoleic acid disappeared. In addition, this study found that the concentration of *cis*-12 C18:1 also increased (0.80 mg) over time, as the linoleic acid disappeared. According to Kemp et al. (1975), several *cis*-C18:1 isomers may originate from linoleic and linolenic acid. In total approximately 95% of the linoleic acid lost was accounted for by the increase in the sum of these three fatty acids: stearic, *trans*-11 and *cis*-12 C18:1.

The C18:2 isomers, including the CLA, were present in very small amounts or were not detected at 0 h. After 24 h, the *cis*-10 *trans*-12 CLA content was highest at 0.18 mg followed by the *trans*-9 *trans*-11 and *trans*-10 *trans*-12 CLA at 0.15 mg. Other CLA accounted for less than 0.05 mg at the end of incubation. These results do not agree with previous research which demonstrated that *cis*-9 *trans*-11 CLA is the predominant CLA isomer under similar conditions (Kellens et al., 1986; Griinari and Bauman, 1999; Griinari et al., 2000; Nam and Garnsworthy, 2007a). For example, Nam and Garnsworthy (2007a) reported that *cis*-9 *trans*-11 CLA comprised 15% of the fatty acid content after 10 min of incubation in the presence of mixed ruminal bacteria. The pattern of CLA production in the study reported here may be related to the rapid biohydrogenation rate of *cis*-9 *trans*-11 CLA to *trans*-11 C18:1, thereby quickly reducing the concentration of the former and

increasing the latter.

Previous research (Proell et al., 2002; Kellens et al., 1986) found a disappearance of linoleic acid simultaneous with increasing *trans* isomers and stearic acid concentration during 50 h of incubation with mixed ruminal microbes. More recently, Nam and Garnsworthy (2007ab) reported that the linoleic acid content decreased significantly within 60 min of incubation with mixed ruminal bacteria and within 12 h of incubation with mixed ruminal fungi. In our research, biohydrogenation proceeded more slowly than in previous studies (Kellens et al., 1986; Nam and Garnsworthy, 2007ab). This slower rate may be related to the injection of highly concentrated linoleic acid that may inhibit the biohydrogenation process. In this study, linoleic acid was inoculated 0.96 mg per mL, a dose five times higher than the 0.20 mg per mL of the Nam and Garnsworthy study. Based on the results of Maia et al. (2007), biohydrogenation and the growth of ruminal bacteria can be inhibited by inoculation of as little as 50 µg linoleic acid per mL.

The enrichment analysis found that linoleic acid was isomerized and hydrogenated to several intermediates at the end of 48 h after the injection of the 1-^{13}C-linoleic acid (Table 2.2, 2.3, 2.4). A total of 21 fatty acid peaks were identified including 6 C18:1 isomers and 11 C18:2 isomers of which 7 were CLA. This finding is inconsistent with currently accepted pathways (Griinari and Bauman, 1999) for the biohydrogenation of linoleic acid. Many researchers believe that ruminal microbes produce only *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA, which are converted to *trans*-11 C18:1 and *trans*-10

C18:1, respectively, before the formation of stearic acid (C18:0). However, since 7 CLA were identified in this study, including *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA, biohydrogenation appears to be more complicated than previously thought.

Enrichment of linoleic acid remained statistically constant from 0 to 24 h (22.20% to 28.15%, respectively), then increased from 24 to 48 h (Table 2.4). However, constant enrichment of linoleic acid during incubation was expected because the microbial system in the rumen cannot produce the linoleic acid, which originated from other fatty acid sources such as oleic and linolenic acid. Without the additional linoleic acid produced in the culture, it was expected that the ruminal microbes would utilize both isotope-labeled and unlabeled linoleic acid in the biohydrogenation process with equal efficiency. The increased enrichment of isotope-labeled linoleic acid from 24 h to 48 h, however, may be due to the method of sample collecting. In this study, 5 mL sample was collected at 0, 24, and 48 h from the same *in vitro* cultures. However, when samples were collected by individual cultures, i.e. without subsampling, the enrichment of the linolenic acid was almost constant - 0 h (33.47%), 3 h (33.26%), 24 h (33.14%), 48 h (35.49%). This result suggests a fatty acid by subsampling might be distributed evenly throughout in the cultures, causing an increase in the enrichment after 24 h during the biohydrogenation process.

The enrichment of stearic acid was increased at all incubation times from 0% at 0 h to a final enrichment of 2.69% at 48 h (Table 2.2). These

results suggest that the stearic acid originated during the biohydrogenation of linoleic acid at that time. Stearic acid accounted for only 17.90% of the C18:2 lost by 48 h; these results do not support the findings of Kellens et al. (1986), who determined that stearic acid concentration increased significantly at 50 h incubation with ruminal microbes while the linoleic acid disappeared. The study conducted by Nam and Garnsworthy (2007a) found that stearic acid comprised 100% of the identified fatty acids for linoleic acid biohydrogenation until 90 min of incubation in the presence of mixed ruminal bacteria. It is possible, as mentioned previously, that the high linoleic acid concentrations in the cultures inhibited the biohydrogenation process (Beam et al., 2000), especially from intermediates C18:1 isomer to stearic acid. Lower enrichment might also be attributed to the isotope introduction method. In addition, other isomers such as C18:3, C20:0, and C20:1n-9 were identified but not enriched. Because ruminal anaerobes do not have the ability to desaturate fatty acids, especially C18:2, with the addition of a double bond, no enrichment of C18:3 was found during the incubation time. Since ruminal anaerobes cannot add acetyl units to the fatty acid structure by reacting with elongases, C20:0 cannot be formed, accounting for the elongation of labeled C18:0. For that reason, C18:3, C20:0, and C20:1n-9 intermediates were not derived from the labeled linoleic acid by the rumen microorganisms. However, other possible sources of unlabeled unsaturated fatty acids were present in the diet substrate *in vitro*. It is likely that the isomerization and biohydrogenation products in the mixed rumen microorganism cultures affected the formation of

all intermediates of linoleic acid.

The enrichment of all C18:1 isomers was either negligible or not detected at 0 h incubation, indicating that none originated from linoleic acid biohydrogenation (Table 2.3). The *trans*-11, *trans*-12, *cis*-9, *cis*-11, *cis*-12, and unidentified positional C18:1 isomers were identified as intermediates of linoleic acid biohydrogenation. From 0 to 24 h, enrichment of *trans*-11 C18:1 increased to 14.51% but did not change from 24 to 48 h. *Trans*-11 C18:1 generally is considered the major intermediate of linoleic acid biohydrogenation in the rumen (Griinari et al., 2000). In addition, our results suggest those intermediates of *cis*-9, *cis*-11, *cis*-12, and unidentified C18:1 were enriched by 48 h. Proell et al. (2002) reported that 1-^{13}C-elaidic acid (*trans*-9 C18:1) was hydrogenated, becoming stearic acid and *trans*-C18:1 isomers by ruminal microorganisms. These researchers also found that 17.6% of the oleic acid (*cis*-9 C18:1) originated from elaidic acid (*trans*-9 C18:1). Similarly Harfoot and Hazlewood (1997) found that the *cis*-12 bond of linoleic acid is converted to *trans*-11 bond by isomerase. As all of these studies suggest, the formation of *cis*/*trans* C18:1 positional isomers is possible during the biohydrogenation of linoleic acid. Although *trans*-10 C18:1 has been suggested as an intermediate of the biohydrogenation of *trans*-10 *cis*-12 CLA by Griinari and Bauman (1999), the amount, if present in this research, was too small to detect.

The enrichment of C18:2 isomers, with the exception of C18:2n-7, detectable at 0 h incubation, even though it was theoretically impossible for

them to be formed at this point (Table 2.4). One possible explanation for this apparent contradiction is that C18:2n-7 might be converted to form CLA or other C18:2 isomers within a few seconds and, thus, have such a short half-life that it is undetectable using this assay. Similarly, the study conducted by Kim et al. (2002) found that the ruminal bacteria *Megasphaera elsdenii* YJ-4 produced *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA very quickly, reaching maximal levels after only two minutes of incubation.

The enrichment of *cis*-9 *trans*-11 CLA reached a maximum at 24 h (30.90%) and did not increase significantly from 24 to 48 h (30.65%), while the accumulation of other CLA continued to increase throughout incubation. These results indicate the ^{13}C label is transferred to other intermediates during biohydrogenation. Likewise, *cis*-9 *trans*-11 CLA may be a potential precursor of other CLA and/or C18:1 isomers. In addition, *trans*-10 *cis*-12, considered the second major isomer, and *trans*-9 *trans*-11 and *trans*-10 *trans*-12 CLA increased slightly over all incubation times, the final proportions of these CLA reaching 22.60% and 21.06% by 48 h. Three CLA isomers (*cis*-9 *trans*-11, *trans*-10 *cis*-12, and *trans*-9 *trans*-11 CLA) are produced by several species of bacteria in the rumen (Griinari and Bauman, 1999). According to Kim et al. (2000), *Butyrivibrio fibrisolvens* are important ruminal bacteria, normally used as models for producing *cis*-9 *trans*-11 CLA, but they do not produce *trans*-10 *cis*-12 CLA, which are produced by *Propionibacterium acnes* and P. *freudenreichii* (Jiang et al., 1998). One important finding of the study conducted by Coakley et al. (2006) was that linoleic acid was hydrogenated to

produce *cis*-9 *trans*-11 CLA and smaller amounts of *trans*-9 *trans*-11 CLA. Subsequently, *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA were converted to *trans*-9 *trans*-11 CLA via the *Bifidobacterium* species. Instead of direct formation of these CLA, Ogawa et al. (2001) suggested another route of biohydrogenation by which 10-hydroxy-*cis*12-C18:1 and 10-hydroxy-*trans*12-C18:1 became intermediates of linoleic acid before the formation of *cis*-9 *trans*-11 CLA and *trans*-9 *trans*-11 CLA by *Lactobacillus acidophilus* AKU 1137 under microaerobic conditions.

Cis-10 *trans*-12 CLA, and *cis*-10 *cis*-12 CLA were detected at all incubation times. The enrichment of these CLA increased from 0 to 24 h and from 24 to 48 h to final values of 23.08%, and 19.29% respectively. The enrichment of *cis*-9 *cis*-11 CLA was similar at 0 h (15.40%) but did not increase significantly from 0 to 48 h incubation. According to the calculations, all of these CLA originate from labeled linoleic acid derived by bacterial isomerases; however, which biochemical pathways are involved is still unclear.

As the CLA isomers that have been identified, at least 14 can be divided into the four double bond geometric configurations of *cis/cis, trans/trans, cis/trans,* and *trans/cis* (Duckett et al., 2002; Piperova et al., 2002; Shingfield et al., 2003). Furthermore, each of these CLA was identified in the intestinal contents of ruminants. However, only seven CLA originated from linoleic acid biohydrogenation in this study (Table 2.5). The remaining CLA isomers (>7) might be formed by biohydrogenation of other polyunsaturated fatty acids. All identified CLA carried a double bond at carbon 9 and 12, and

all possible double bond geometries (*cis/cis, trans/trans, cis/trans,* and *trans/cis*) were identified, except for *trans*-9 *cis*-11 CLA, by linoleic acid biohydrogenation. Liavonchanka et al. (2006) proposed that the isomerization of linoleic acid is initiated at carbon number 11 by hydrogen abstraction which then produces several CLA isomers of carbons 9 and 12 with differences in isomerization capacity by different ruminal bacteria species.

Although the results of the linoleic acid biohydrogenation pathway using stable isotope utilization by mixed ruminal microbes had not been evaluated previously (Griinari and Bauman, 1999), the current study verified the formation of *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA isomers from linoleic acid via biohydrogenation. An additional five CLA isomers contained carbons that originated from linoleic acid, indicating that the pathways of linoleic acid biohydrogenation are more complex than previously thought. Additional studies are necessary to identify the enteric biological pathway of lipid metabolism. The results of such an investigation may lead to benefits in the area of human health such as carcinogenesis, immune function, obesity, diabetes, and atherogenesis.

Table 2.1. Concentration (mg / 5 mL culture) of stearic acid (C18:0), C18:1 isomers, C18:2 isomers and other isomers detected at 0, 24, and 48 h.

	Fatty acid content (mg)			
Fatty acid	0h	24h	48h	SEM
C18:0	1.74 [a]	2.34 [ab]	2.71 [b]	0.23
C18:1 isomers				
trans-11 C18:1	0.13[a]	2.40[b]	3.32[c]	0.15
trans-12 C18:1	ND	<0.01	<0.01	<0.01
cis-9 C18:1	1.46 [a]	1.40 [a]	1.26[b]	0.03
cis-11 C18:1	ND	0.17	0.18	0.01
cis-12 C18:1	0.02[a]	0.81 [b]	0.82 [b]	0.03
unidentified C18:1	0.02 [a]	0.04 [ab]	0.05 [b]	0.01
C18:2 isomers				
unidentified C18:2 (I)	0.03[a]	0.07 [b]	0.07 [b]	<0.01
C18:2n-8	0.07[a]	0.16[b]	0.13[c]	0.01
C18:2n-7	ND	0.27	0.25	0.03
cis-9 *cis*-12 C18:2	6.43[a]	2.11[b]	1.01[c]	0.09
unidentified C18:2 (II)	<0.01[a]	0.01[b]	0.01[b]	<0.01
cis-9 *trans*-11 CLA	0.01 [a]	0.01 [a]	0.02[b]	<0.01
cis-10 *trans*-12 CLA	0.05 [a]	0.18[b]	0.09 [a]	0.01
trans-10 *cis*-12 CLA	<0.01[a]	0.02[b]	0.02[b]	<0.01
cis-9 *cis*-11 CLA	<0.01[a]	0.05[b]	0.03[c]	0.01
cis-10 *cis*-12 CLA	<0.01[a]	0.01[b]	0.02[c]	<0.01
trans-9 *trans*-11 and *trans*-10 *trans*-12 CLA	0.02[a]	0.15[b]	0.13[b]	0.01

Table 2.1. (Continued)

Other isomers				
C20:0	0.05[a]	0.04[b]	0.04[b]	<0.01
C18:3	0.02[a]	0.02 [a]	0.01[b]	<0.01
C18:3n-3 + C20:1n-9	0.17[a]	0.03[b]	0.02[b]	0.01

Values in a row are the least square means of fatty acid content for each treatment time followed by the pooled SEM (n=6).

[abc]Least squares means within a row with different superscript letters indicate significant difference (P<0.05).

ND, not detected.

Table 2.2. Enrichment of stearic acid (C18:0), linolenic acid (C18:3n-3) and other isomers.

	Enrichment (%)			
Fatty acid	0h	24h	48h	SEM
C18:0	-0.02[a]	1.93[b]	2.69[b]	0.96
C18:3n-3 + C20:1n-9	-0.11[a]	-0.81[b]	-0.01[c]	0.44
C18:3	0.89[a]	-1.77[b]	-3.30[c]	2.40
C20:0	6.98[a]	-3.59[b]	-3.34[c]	8.43

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).

[abc]Least squares means within a row with different superscript letters indicate significant difference ($P<0.05$).

Table 2.3. Enrichment of C18:1 isomers.

	Enrichment (%)			
Fatty acid	0h	24h	48h	SEM
trans-11 C18:1	NS	12.50 [a]	14.51[b]	1.09
trans-12 C18:1	ND	14.55[a]	2.90[b]	2.67
cis-9 C18:1	0.03[a]	5.60 [b]	14.07[c]	1.17
cis-11 C18:1	ND	16.99[a]	18.66[b]	1.88
cis-12 C18:1	NS	14.14	13.57	0.66
Unidentified C18:1	NS	9.92	11.47	1.31

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).

[abc]Least squares means within a row with different superscript letters indicate significant difference ($P<0.05$).

NS, not significant from zero

ND, not detected.

Table 2.4. Enrichment of C18:2 isomers.

Fatty acid	Enrichment (%)			SEM
	0h	24h	48h	
unidentified C18:2 (I)	5.88[a]	20.05[b]	22.09[c]	0.70
C18:2n-8	20.19[a]	24.80[b]	32.54[c]	2.27
C18:2n-7	ND	19.14[a]	23.71[b]	2.57
cis-9 *cis*-12 C18:2	22.20[a]	28.15[a]	37.21[b]	3.49
unidentified C18:2 (II)	11.24[a]	1.71[b]	3.60[b]	0.88
cis-9 *trans*-11 CLA	12.80[a]	30.90[b]	30.65[b]	3.82
cis-10 *trans*-12 CLA	13.14[a]	16.72[b]	23.08[c]	1.73
trans-10 *cis*-12 CLA	15.04[a]	18.99[b]	22.60[c]	1.02
cis-9 *cis*-11 CLA	15.40	16.74	18.38	1.24
cis-10 *cis*-12 CLA	9.51[a]	14.66[b]	19.29[c]	1.07
trans-9 *trans*-11 and *trans*-10 *trans*-12 CLA	14.25[a]	17.39[b]	21.06[c]	0.91

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=3).

[abc]Least squares means within a row with different superscript letters indicate significant difference ($P<0.05$).

ND, not detected.

Table 2.5. Double bond position and geometry of identified seven CLA.

	Double Bond Position	
Double Bond Geometry	9/11	10/12
cis/cis	☑	☑
trans/trans	☑	☑
cis/trans	☑	☑
trans/cis	ND	☑

☑, Identified CLA

ND, not detected

IDENTIFICATION OF INTERMEDIATES PRODUCED

BY RUMEN MICROBIAL BIOHYDROGENATION OF LINOLENIC ACID

ABSTRACT

Current literature suggests that linolenic acid biohydrogenation converts to stearic acid through the transformation of *cis*-9 *trans*-11 *cis*-15 C18:3 and *trans*-11 *cis*-15 C18:2, followed by the hydrogenation of *trans*-11/*cis*-15 double bond without the formation of conjugated linoleic acid (CLA). However, a multitude of monoenoic, dienoic, and trienoic unsaturated fatty acid isomers containing CLA were identified in the rumen. Some of these monoenoic and dienoic isomers originated from oleic and linoleic acid biohydrogenation, but the other isomers have yet to be verified. Many researchers believe that tentative metabolic pathways may exist during linolenic acid biohydrogenation.

This study utilized a stable isotope tracer to investigate the biohydrogenation intermediates of ^{13}C-linolenic acid including CLA. A continous culture fermenter was used to maintain a pH 6.5 condition for six days, the mixed culture contents were transferred to batch cultures containing labeled and/or unlabeled linolenic acid, which were used in triplicate at 0, 3, 24, and 48 h. The ^{13}C enrichment was calculated as the ^{13}C/^{12}C ratio of the labeled cultures minus the unlabeled cultures expressed as a percentage. After 48 h incubation, eight CLA isomers were significantly enriched, suggesting that these CLA isomers originated from ^{13}C-linolenic acid. The *cis*-10 *cis*-12

CLA isomer exhibited the highest enrichment (21.73%), followed by *cis*-9 *cis*-11 and *trans*-8 *trans*-10 CLA. The enrichment of these two CLA isomers ranged from 20.14% to 21.11%, and the remaining five CLA including *cis*-9 *trans*-11 CLA were below 15.00%. These results do not support traditional linoleic acid biohydrogenation because *cis*-9 *trans*-11 CLA is the major CLA isomer during the biohydrogenation of linoleic acid.

Fifteen C18:3 isomers were enriched during 48 h of incubation. The *cis*-7 *cis*-9 *cis*-13 C18:3 isomer exhibited the highest enrichment (28.15%) at 3 h incubation, suggesting that a variety of C18:3 isomers may contribute to the formation of C18:2 isomers by microbial reductases. The results of this study confirm that mixed ruminal microbes are capable of the formation of several CLA and C18:3 isomers from linolenic acid, indicating that linolenic acid biohydrogenation pathways are more complex than previously reported. Moreover, the formation of the identified CLA differed from linoleic acid biohydrogenation, which was a result of a different microbial enzyme system contributing to linoleic and linolenic acid biohydrogenation.

INTRODUCTION

Mammals cannot make linolenic acid (omega-3 polyunsaturated fatty acid; ω3-PUFA) because they do not have the enzyme fatty acyl-CoA desaturases (Δ^{12} and Δ^{15}). Linolenic acid can prevent the cardiovascular disease itself and the precursors to arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids serve a

multitude of biological functions concerning pain, inflammation, immune, and anticonvulsant management (Sealls et al., 2008; Porta et al., 2008).

Naturally, the metabolic conversion of linolenic acid is caused by ruminal microorganisms in the anaerobic rumen ecosystem through a process called biohydrogenation. Trienoic, dienoic, and monoenoic *trans* fatty acids are produced as intermediates during biodydrogenation of linolenic acid, and stearic acid is created as the final product. Linolenic acid (*cis*-9 *cis*-12 *cis*-15 C18:3) initially is transformed to partially conjugated trienoic fatty acid (*cis*-9 *trans*-11 *cis*-15 C18:3) by Δ^{12}-*cis*, Δ^{11}-*trans*-isomerase (Kepler and Tove, 1967). Next, *cis*-9 double bond is hydrogenated to produce non-conjugated dienoic fatty acid (*trans*-11 *cis*-15 C18:2), followed by hydrogenation of *trans*-11/*cis*-15 double bond, producing *cis/trans* monoenoic fatty acid. These C18:1 isomers are further hydrogenated to produce stearic acid (Harfoot and Hazlewood, 1988). This metabolic scheme, which has been confirmed by recent studies, has also identified several other intermediates produced during the linolenic acid biohydrogenation process. Three additional non-conjugated C18:3 isomers (*cis*-9 *trans*-12 *cis*-15, *cis*-9 *trans*-12 *trans*-15, and *trans*-9 *trans*-12 *trans*-15 C18:3) were investigated in duodenal fatty acid flow (Loor et al., 2004), and partially conjugated C18:3 isomer (*cis*-9 *trans*-13 *cis*-15 C18:3) was reported as the initial intermediate of the biohydrogenation of linolenic acid (Destillats et al., 2005).

Linolenic acid is usually considered as hydrogenated without the formation of conjugated linoleic acid (CLA) isomers. A recent study by

Destillats et al. (2005) suggested that two CLA isomers (*cis*-9 *trans*-11 and *trans*-13 *cis*-15 CLA) are produced through linolenic acid biohydrogenation, but no supproting evidence was provided. Due to its many reported physiological functions, determining whether CLA is formed from linolenic acid is important.

The main objective of this study was to establish the number and identity of intermediates containing CLA that originate from linolenic acid biohydrogenation. To complete this objective, an *in vitro c*ulture containing the ^{13}C stable isotope was used, which was expected to provide information about the intermediates that originate from linolenic acid during incubation.

MATERIAL AND METHODS

Reagents

U-^{13}C-linolenic acid (99% chemical purity; 96% ^{13}C isotopic purity) was purchased from Larodan AB, Inc. (MALMÖ, Sweden). 1-^{13}C-linolenic acid (>99% chemical purity; 99% ^{13}C isotopic purity) was purchased from Medical Isotopes, Inc. (Pelham, NH). Unlabeled linolenic acid (C18:3, *cis*-9 *cis*-12 *cis*-15; 99% chemical purity) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Reagents for general fatty acid analysis, including sodium methoxide, methanolic HCl, and hexane, were also purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

Continuous Culture

Whole ruminal contents were collected from a ruminally-fistulated Holstein cow two hours after being fed a 50% forage / 50% concentrate diet. Within 20 minutes of collection, the large particles of ruminal contents were removed by filtration through two-layers of cheesecloth, and then the filtrated ruminal fluid was transferred immediately to the laboratory in a sealed container. A dual-flow continuous culture fermenter, as described by Teather and Sauer (1988), was used with several modifications. Approximately 800 mL of the filtrated ruminal fluid were added to the fermenter. The culture was maintained for six days (five days for adaptation and the last day for sampling). A total of 60 g of diet (Table 3.1) were inoculated into the fermenter daily in two equal portions at 0800 and 1630 h. A buffer solution (Slyter et al., 1966) was delivered continuously at a flow rate of 1.5 mL/min using a precision pump, resulting in a 0.10/h fractional dilution rate. Buffer pH was titrated each day with sufficient 6N NaOH or 3N HCl to maintain a pH of 6.5. The pH was measured daily at 0800, 1100, and 1630 h using a portable pH meter. The fermenter was continuously infused with CO_2 at a rate of 20 mL/min to maintain an anaerobic condition, and the cultures were stirred continuously at 44 r.p.m. The temperature of the fermenter was held at 39 ^{o}C by a circulating water bath. Culture samples were taken for volatile fatty acid (VFA) analysis on the last day at 0 (before the AM feeding), and at 2 and 4 h after feeding.

In Vitro Cultures

On the sixth day (4 h after the AM feeding), the mixed culture contents from the continuous fermentor were transferred to *in vitro* batch cultures. The larger particles were removed by filtration through two layers of cheesecloth. The cultures were placed in a 32 mL culture tube that contained 100 mg of the diet, 2.0 mL of the mixed culture contents, 8.0 mL of the *in vitro* media, and 0.4 mL of the reducing solution (Goering and Van Soest, 1970). A mixed diet was obtained from Clemson University Dairy Farm (Table 3.1). Twenty-four batch cultures were run at 39 ^{o}C in a water bath containing either 1.50 mg unlabeled linolenic acid or 0.75 mg unlabeled linolenic acid + 0.75 mg U-^{13}C-linolenic acid in ethanol. Another set of batch cultures was also run containing either 1.80 mg unlabeled linolenic acid or 0.90 mg unlabeled linolenic acid + 0.90 mg 1-^{13}C-linolenic acid in ethanol. Samples were taken from the water bath at 0, 3, 24, and 48 h. To immediately stop the enzymatic biohydrogenation and kill the ruminal microorganisms at the 0 h sampling time, 0.4 mL 6N HCl was injected prior to inoculation of the mixed culture contents. The samples were transported immediately in an ice batch, and then stored at -5 ^{o}C.

Fatty Acid Methyl Esters (FAME) Sample Analysis

The culture samples were freeze-dried and converted to methyl esters in sodium methoxide, which was followed by the methanolic HCl procedure described by Kramer et al. (1997). The contents of these FAME samples were

analyzed using Hewlett-Packard 5890 gas chromatography (GC) equipment with heptadecanoic acid, C17:0 as the internal standard. The column was a 100 m × 0.25 mm silica capillary column with a 0.2 μm film thickness (Supelco Inc., PA). Helium was used as the carrier gas at 20 cm/s. The initial temperature of the column was 140 ^{o}C for 3 min with a ramp of 3.7 ^{o}C per min up to 220 ^{o}C, where it was maintained for 20 min.

The ^{13}C enrichment of an individual FAME was determined using Agilent 6890N gas chromatograph equipment, which included a Model 5973 quadrapole mass selective detector. This GC-MS was equipped with a 100 m × 0.25 mm Chrompack CP-Sil 88 column (0.20 μm film thickness). The carrier gas was helium at 20 cm/s. The initial temperature of the column was 140 ^{o}C, held for 5 min with a ramp of 4 ^{o}C per min up to 220 ^{o}C, where it was held for 20 min. The ion chosen for the FAME sample of 1-^{13}C-linolenic acid cultures in the select ion mode was the quasimolecular ion; [M]. A [M+1] ion indicated the labeled compound (Figure 3.1). The ion chosen for the U-^{13}C-linolenic acid cultures was the quasimolecular ion; [M]. A [M+18] ion indicated the labeled compound, and a [M+29] ion represented the $C_2H_5^+$ ion (Figure 3.2).

Initially, the FAME peaks were identified by the molecular mass and the retention times matched with the standard purchased from the chemical company. In addition, the FAME double bond position and geometry were characterized using a GC, followed by covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS) based on the methods in Michaud et al. (2003) and Lawrence and Brenna (2006). The FAME samples were

separated using a CP-Cil 88 Capillary Column (100 m × 0.32 mm) with a 0.25 μm film thickness. The temperature conditions were programmed to range from 80 ^{o}C to 120 ^{o}C at a rate of 10 ^{o}C/min and then increased to 220 ^{o}C at a rate of 2.5 ^{o}C/min. The total run time was 60 min. The FAME eluting into the MS undergo an ion-molecule reaction, forming an adduct at [M+54] above that of the parent FAME, [M]. The collisional dissociation of this adduct yields diagnostic ions characteristic of double bond positions. The double bond geometries of the CLA peaks were determined through relative diagnostic ion intensity as described by Michaud et al. (2003), and each conjugated triene isomer was determined as described by Lawrence and Brenna (2006).

Statistical Analysis

The fatty acid content in the FAME samples was calculated by comparing the fatty acid peak areas on the chromatograms with the internal standard. The result were shown as mg of fatty acid contents within the 10 mL microbial *in vitro* cultures.

In the 1-^{13}C-linolenic acid cultures, the abundance of select ions in all the samples were exhibited by [(M+1)/M]. Enrichment was identified as [(M+1)/M] in the labeled cultures minus [(M+1)/M] in the unlabeled cultures to adjust for the natural occurrence of the ^{13}C isotope. To increase the accuracy of the levels of ^{13}C, the average ratio of the m/z 88 to 87 fraction of the unlabeled cultures was subtracted from the labeled culture instead of using the

molecular weight (Figure 3.1). The abundance of m/z 88 and 87 was higher than the the molecular weight of the FAME sample of the m/z 293 and 292 in linolenic acid. Moreover, using the m/z 88 and 87 fraction made the enrichment calculation easier because these fractions contain three carbons at the end of the carboxyl group, thereby changing the m/z of an individual FAME was not required.

In the U-13C-linolenic acid study, the abundance of samples was shown as [(M+18)/M]. Enrichment was identified as [(M+18)/M] in the labeled cultures minus [(M+18)/M] in the unlabeled cultures.

The enrichment % means ± SEM taken from the analysis of variance (ANOVA) test shown for each fatty acid. The results of the 1-^{13}C-linolenic acid and U-^{13}C-linolenic acid study were combined after a series of two-sample tests because there was minimal difference in the results of the two study sets. For instance, the enrichment of linolenic acid in the 1-^{13}C study was 32.01, 31.55, and 30.65%, and in the U-^{13}C study the enrichment was 35.91, 36.11, and 36.63% at 0, 3, and 24 h incubation, respectively. The difference in the enrichment % of linolenic acid between the two studies was less than 6% for the same incubation time. The results for other fatty acids also showed minimal differences between the two studies. Therefore, the mean of each fatty acid in these two study sets was combined. A Least Significant Difference (LSD) test was conducted to identify the differences between the fatty acid contents and the enrichment of the labeled fatty acids during the incubation time. The fatty acid contents and the enrichments data sets were

subsequently tested for their differences in mean values from zero at a probability of less than 5% (p-value < 0.05) using Student's t-test.

RESULTS

The pH was successfully maintained at 6.5 in continuous cultures (Figure 3.3). The proportions of total and individual VFA concentrations in continuous cultures are shown in Table 3.2. After feeding, the concentrations of acetate and propionate were increased; however, the acetate to propionate ratio was decreased. The butyrate and valerate concentrations increased after the 4 h feeding, whereas the iso-butyrate and iso-valerate did not change before and after feeding.

The fatty acid contents of a 10 mL microbial *in vitro* culture are shown in Figure 3.4. Linolenic acid (*cis*-9 *cis*-12 *cis*-15 C18:3) decreased 97.26% (2.13 mg) over 48 h of incubation. Linoleic (*cis*-9 *cis*-12 C18:2) and oleic (*cis*-9 C18:1) acid also decreased 87.88% (0.87 mg) and 64.29% (0.45 mg), respectively during the same incubation time. Stearic acid (C18:0) was increased by 1.48 mg after 48 h of incubation. The concentration of *trans*-11 C18:1 increased (0.76 mg) over the same amount of time. The C18:2 isomers, including CLA, and C18:3 isomers were not detected at 0 h and were present in very small amounts (<0.05 mg) during 48 h incubation (data are not shown).

The enrichment of linolenic acid was statistically maintained from 0 to 48 h incubation (Table 3.3), being 33.47%, 33.26%, 33.14% and 35.49% at 0, 3, 24 and 48 h incubation, respectively. The stearic acid was significantly

enriched at 24 and 48 h incubation, to 7.19% and 16.25%, respectively. The enrichment of oleic and linoleic acid was not significant during the incubation.

A total of 32 fatty acid peaks were enriched, including four C18:1 isomers, 13 C18:2 isomers, of which eight were CLA, and 15 C18:3 isomers (Table 3.4 and 3.5, and Figure 3.5). The enrichment of all C18:1 isomers, with the exception of *trans*-11 C18:1, was enriched only at 24 and 48 h incubation (Table 3.4). The *trans*-11 C18:1 isomer was enriched by 1.99% at 3 h and then increased to 9.09% at 24 h, 21.12% at 48 h. The enrichment of *cis*-11, and *cis*-12 C18:1 was 4.89 and 1.95%, respectively, at 24 h, and then increased to 8.03% and 5.90%, respectively, at 48 h incubation. An unidentified C18:1 isomer achieved the highest enrichment (26.50%) among the C18:1 isomers. The *trans*-12 C18:1 was not detectable during all of the incubation. The enrichment of C18:2 isomers was not detected at 0 h incubation (Table 3.4). Moreover, all C18:2 isomers, except for the unidentified C18:2 (III) and (IV), were not detected at 3 h. Enrichment of the unidentified (III) C18:2 isomer was 22.34% at 3 h, remained statistically constant from 3 to 24 h, and then increased to 32.03% at 48 h incubation. Enrichment of the unidentified (IV) C18:2 isomer was 5.39% at 3 h and then increased to 26.06% at 48 h incubation. The identified C18:2 (I, II, and V) isomers were enriched only at 24 and 48 h incubation to 24.71, 7.74, and 15.25%, respectively, and 31.60, 17.08, and 16.95%, respectively.

The enrichment of all CLA isomers was also not significantly enriched at 0 h incubation (Figure 3.5). The 7.27% enrichment of *cis*-9 *trans*-11 CLA

was observed at 3 h incubation and then increased from 3 to 48 h (6.27%). The *trans*-10 *cis*-12 CLA was also enriched at 3 h incubation (12.62%) but decreased from 3 to 24 h (1.22%), and then increased from 24 to 48 h (2.17%). The same pattern was observed in *trans*-9 *trans*-11 CLA. The 3.56% enrichment was observed at 3 h incubation but decreased from 3 to 24 h, and then increased to 12.55% at 48 h incubation. The enrichment of *cis*-10 *cis*-12 CLA reached a maximum at 48 h (21.73%) followed by *cis*-9 *cis*-11 (21.11%) and *trans*-8 *trans*-10 CLA (20.14%). During 24 and 48 h incubation, the enrichment of *trans*-9 *cis*-11 and *trans*-11 *trans*-13 CLA increased from 5.46% to 12.59%, and from 0.75% to 9.96%.

C18:3 isomers were not detected or statistically not enriched at 0 h incubation but were significantly enriched at 3, 24, and 48 h incubation, except for the unidentified (IV) and (VII) isomers at 48 h, and unidentified (X) C18:3 isomer at 3 h. The enrichment of *cis*-7 *cis*-9 *cis*-13 C18:3 reached a maximum at 3 h (28.15%) and then decreased thereafter. The pattern observed at the other C18:3 isomers was different during the incubation. However, all other C18:3 isomers were significantly enriched and observed a wide variety of enrichment from 3 to 48 h incubation, from 0.87% to 19.33%.

DISCUSSION

The degree of fatty acid disappearance was highest in linolenic, followed by linoleic, and then oleic acid in this study. Duckett et al. (2002) reported lower levels of biohydrogenation for the linolenic acid (91%) in

cannulated steers with a supplement containing typical corn. In their study, however, more oleic acid (70%) disappeared than in this study. Nam and Garnsworthy (2007a) observed that incubation with mixed ruminal fungi reduced 100% of the linoleic acid within 24 h. Linoleic acid disappearance, during incubation with mixed ruminal bacteria, was not detected after 60 min, a faster rate than that found for incubation with ruminal fungi (Nam and Garnsworthy, 2007b). In the present research, the oleic and linoleic acid biohydrogenation rate was lower than in previous studies. The reason may be related to the injection of a high concentration of linolenic acid, which may inhibit the linoleic and oleic acid biohydrogenation process. Troegeler-Meynadier et al. (2006) reported that the addition of linolenic acid decreased the linoleic acid isomerization rate and efficiency. They suggested that this inhibition may be due to the competitive inhibition of isomerase activation onto the envelope of the ruminal bacteria. Previous research (Kepler et al., 1966; Kepler and Tove, 1967) proposed that the same bacteria and enzyme are related to isomerization of both linolenic and linoleic acid biohydrogenation.

The formation of stearic acid, the final product of biohydrogenation, was inhibited, only producing a small amount during 3 h of incubation. Nam and Garnsworthy (2007a) reported that 100% linoleic acid was converted to stearic acid within 100 min of incubation under a condition of mixed cultures of rumen bacteria. Duckett et al. (2002) also reported that the highest concentration of stearic acid was observed in the duodenum flow of steers. The inhibition or low concentration of stearic acid formation in the research

reported here may be related to the injection of a high concentration of linolenic acid as previously described.

The concentration of *trans*-11 C18:1 was the highest of all the C18:1 isomers. AbuGhazaleth et al. (2005) reported that *trans*-11 was not one of the major *trans*-C18:1 intermediates of oleic acid biohydrogenation. Grinnari and Bauman (1999) suggested that *trans*-11 C18:1 originated from *cis*-9 *trans*-11 CLA (the intermediate of linoleic acid biohydrogenation) and/or *trans*-11 *cis*-15 C18:2 (the intermediate of linolenic acid biohydrogenation). In addition, Ribeiro et al. (2005) suggested that *trans*-11 C18:1 is a major intermediate of linolenic acid biohydrogenation. The accumulation of *trans*-11 C18:1 in cultures is important because this fatty acid is desaturated by Δ^9-desaturase to yield *cis*-9 *trans*-11 CLA in the mammary tissue, which can increase the amount of CLA isomers in meat and milk products (Briinari and Bauman, 1999). In the present study, *trans*-12 C18:1 was not detected. AbuGhazaleh et al. (2005) reported *trans*-12 C18:1 also was not detected during oleic acid biohydrogenation with a treatment of pH 5.5. The absence may be related to the fact that the *trans*-12 C18:1 originates from other sources, such as linoleic acid. Corl et al. (1998) reported that the abomasal infusion of *trans*-12 C18:1 led to an increase in the *cis*-9 *trans*-11 CLA concentration in the milk together with *trans*-11 C18:1.

The enrichment analysis in this study verified that linolenic acid was biohydrogenated, producing several intermediates after the inoculated ^{13}C labeled linolenic acid. The results found here are not in accordance with

currently accepted pathways (Harfoot and Hazlewood, 1988; Griinari and Bauman, 1999) for linolenic acid biohydrogenation. Authoritative pathways suggest that linolenic acid is transformed to *cis*-9 *trans*-11 *cis*-15 C18:3 by isomerase in the rumen, which is subsequently converted to *trans*-11 *cis*-15 C18:2 followed by the hydrogenation of the *trans*-11/*cis*-15 double bond before the formation of stearic acid. Many researchers believe that CLA is not formed through linolenic acid biohydrogenation. However, since 8 CLA and 15 C18:3 isomers were identified in this study, the isomerization and hydrogenation processes appear to be more complex than previously thought.

The enrichment of linolenic acid was maintained during 48 h of incubation. This stable enrichment level was expected during incubation with mixed ruminal microorganisms because ^{13}C labeled linolenic acid is utilized together with unlabeled linolenic acid in the biohydrogenation process with the same efficiency. Moreover, linolenic acid cannot be produced from other fatty acid sources in the ruminal ecosystem.

The enrichment of stearic acid was observed at 24 and 48 h of incubation. The results reported here suggest that stearic acid as the final product of biohydrogenation originated from linolenic acid. In addition, oleic and linoleic acid were not enriched because these two fatty acids cannot originate from linolenic acid by ruminal microbes.

All of the C18:1 isomers were enriched at 24 and 48 h incubation, indicating that they originated from linolenic acid biohydrogenation. Proell et al. (2002) reported that *trans*/*cis*-C18:1 isomers originated from the stable

isotope labeled *trans*-9 C18:1, supporting the possibility that the formation of *trans*/*cis*-C18:1 isomers with a different double bond position can occur during biohydrogenation. Duckett et al. (2002) also reported that several *trans/cis*-C18:1 isomers were observed in the duodenal flow of steers. In this study, the enrichment of unidentified C18:1 was the highest. Accoriding to the other studies, the *cis*-15 C18:1 isomer was the major monoene intermediate of linolenic acid biohydrogenation (Body, 1976; Loor et al., 2002), but we could not determine the double bond position and geometry because of the lack of a standard. However, this finding supports that linolenic acid biohydrogenation is distinct from oleic or linoleic acid biohydrogenation. The *trans*-10 C18:1 isomer was the major *trans* monoene intermediate during oleic acid biohydrogenation (Mosley et al., 2002; AbuGhazaleh et al., 2005), and *trans*-11 C18:1 was the major intermediate derived from linoleic acid (Kepler and Tove, 1967; Loor et al., 2002).

The major C18:2 isomer, except for the identified CLA originating from linolenic acid was unidentified (III), followed by the unidentified C18:2 (III) isomer. Since there is no standard, however, we could not identify these C18:2 intermediates. Loor et al. (2002) proposed that the *trans*-11 *cis*-15 C18:2 is the major C18:2 isomer originating from linolenic acid. A previous study conducted by Wilde and Dawson (1966) contended that this isomer may be the primary one produced from linolenic acid biohydrogenation *in vitro*. Moreover, Hazlewood et al. (1976) reported that the radio isotopes were biophydrogenated to *trans*-11 *cis*-15 C18:2 after the inoculation of ^{14}C-

linolenic acid with a ruminal bacteria. Kepler and Tove (1967) reported that this isomer may be produced from *cis*-9 *trans*-11 *cis*-15 C18:3 by redutase in *Butyrivibrio fibrisolvens*. Based on these results, Destaillats et al. (2005) proposed that *cis*-9 *trans*-13 C18:2 is produced by the reduction of the *cis*-9 *trans*-13 *cis*-15 C18:3 isomer. Moreover, Loor et al. (2005) reported that the *cis*-9 *trans*-13 C18:2 may be endogenously produced by the Δ^9-desaturation of *trans*-13 C18:1, supporting the possibility that *cis*-9 *trans*-13 C18:2 could be formed by both endogenous and ruminal biohydrogenation. The *cis*-9 *trans*-12 C18:2, *trans*-9 *trans*-12 C18:2, *trans*-9 *cis*-12 C18:2 and *cis*-9 *cis*-15 C18:2 isomers were also found in ruminal contents (Loor et al., 2002). The study conducted by Kemp et al. (1975) reported that the *cis/trans, trans/cis and trans/trans* non-conjugated C18:2 isomers are primarly produced from linoleic acid biohydrogenation.

Many researchers believe that ruminal microbes cannot produce CLA from linolenic acid. In contrast, Destaillats et al. (2005) suggested that two CLA isomers, *cis*-9 *trans*-11 CLA and *trans*-13 *cis*-15 CLA, can be produced from linolenic acid biohydrogenation. However, the results of the present study verified that eight CLA isomers originated from linolenic acid. The primary CLA isomers were *cis*-9 *cis*-11, *trans*-8 *trans*-10, and *cis*-10 *cis*-12 CLA. The enrichment of these CLA isomers that were more than 2.5% at 3 h incubation was increased after 3 h in order to exceed 20% enrichment during the incubation.

The formation of *cis*-9 *trans*-11 CLA is considered to be the major

CLA produced from linoleic acid biohydrogenation (Kepler and Tove, 1967). Loor et al. (2002) observed that approximately 88% of the total CLA was *cis*-9 *trans*-11 CLA in the rumen of lactating cows. However, in this study, the enrichment of *cis*-9 *trans*-11 CLA was only 65% compared to 100% of *cis*-10 *cis*-12 CLA. In addition, the enrichment of *trans*-10 *cis*-12 CLA, which is considered the second major CLA, and *trans*-9 *trans*-11 CLA was below 14% for all incubation times. The *cis*-9 *trans*-11, *trans*-10 *cis*-12 and *trans*-9 *trans*-11 CLA isomers are produced as the major CLA of linoleic acid biohydrogenation by ruminal bacteria (Griinari and Bauman, 1999). The study conducted by Kim et al. (2000) reported that *cis*-9 *trans*-11 CLA is produced by the isomerase in *Butyrivibrio fibrisolvens*, and *trans*-10 *cis*-12 CLA is produced by *Propinonibacterium acnes* and P. *freudenreichii* (Jiang et al., 1998) from linoleic acid. Coakley et al. (2006) reported that these three CLA isomers are precursors to one another in the *Bifidobacterium* species. For instance, *cis*-9 *trans*-11 and *trans*-9 *trans*-11 CLA are produced from linoleic acid and *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA are converted to *trans*-9 *trans*-11 CLA.

The remaining CLA isomers (*trans*-9 *cis*-11, *cis*-9 *cis*-11, *trans*-11 *trans*-13, *trans*-8 *trans*-10, and *cis*-10 *cis*-12 CLA) were detected for all incubation times. According to the enrichment calculations, all of them originated from the labeled linolenic acid derived by bacterial isomerases and reductases; however, the biochemical pathways involved are still unknown.

These results indicate that the biohydrogenation pathway of linolenic

acid is different from linoleic acid. In this study, eight CLA were originated, including *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA, from labeled linolenic acid by bacterial isomerases and reductases; however, the biological pathways are still unknown. Loor et al. (2005) found that *cis*-9 *cis*-11, *cis*-11 *trans*-13, and *trans*-11 *trans*-13 CLA accumulated at 2, 1, and 1%, respectively, of the total CLA isomers. Destaillats et al. (2005) suggested that the *trans*-13 *cis*-15 CLA could be produced from the reduction of *cis*-9 *trans*-13 *cis*-15 C18:3.

Enrichment of all of the C18:3 isomers was observed at 3 h, except for the unidentified (X) C18:3 isomer, and they either decreased or increased between 3 and 48 h incubation. This finding supports that the isomerization step occurs within 3 h, yielding several C18:3 isomers from linolenic acid, which are then transformed to other C18:3 or C18:2 isomers. The enrichment of five C18:3 isomers, including *cis*-7 *cis*-9 *cis*-13 C18:3, decreased between 3 and 48 h incubation because other possible sources of unlabeled (^{12}C) polyunsaturated fatty acids were present in the diet. These ^{12}C could transfer to the biohydrogenation intermediates and the ratio of $^{13}C/^{12}C$ might decrease. Kepler and Tove (1967) suggested that the initial intermediate of linolenic acid biohydrogenation is *cis*-9 *trans*-11 *cis*-15 C18:3, and then *trans*-11 *cis*-15 C18:2 is formed by bacterial reductases. They suggest further that the same enzymatic system, Δ^{12}-*cis*, Δ^{11}-*trans*-isomers, of B. *fibrisolvens* contributes to linoleic and linoenic acid biohydrogenation. More recently, Destaillats et al. (2005) suggested that *cis*-9 *trans*-13 *cis*-15 C18:3 was produced from linolenic acid as the precursor of the *cis*-9 *trans*-13 C18:2 isomer. Plourde et al. (2007)

reported that *cis*-9 *trans*-11 *cis*-15 C18:3 was the major C18:3 isomer found in both muscle and milk lipid extracts, while the *cis*-9 *trans*-13 *cis*-15 C18:3 isomer was detected only in muscle lipid extract.

Fully conjugated C18:3 has been of interest to researchers for over a decade as the precursor of CLA or as a distinct biological effect. The most frequently encountered fully conjugated C18:3 is the *cis*-9 *trans*-11 *trans*-13 C18:3 followed by *cis*-8 *trans*-10 *cis*-12, *trans*-8 *trans*-10 *cis*-12, *cis*-9 *trans*-11 *cis*-13 and *trans*-9 *trans*-11 *cis*-13 C18:3 (Hopkins, 1972). In the present study, fully conjugated C18:3 isomers were not identified due to a number of unidentified C18:3 isomers. The 15 C18:3 isomers originated from linolenic acid by bacterial isomerases. The *cis*-7 *cis*-9 *cis*-13 C18:3 isomers were first reported here as the intermediates of linolenic acid.

All of the identified CLA isomers carried a double bond at 8 and 13 with four possible geometries (*cis/cis trans/trans, cis/trans*, and *trans/cis*) verified in linolenic acid via biohydrogenation. As the same enzymatic system would be utilized in linoleic and linolenic acid, isomerization of linolenic acid is initiated at carbon number 11, producing several C18:3 isomers between carbon number 9 and 15 and then several C18:2 isomers including CLA by reductases of different ruminal microbes.

CONCLUSIONS

Several CLA and C18:3 isomers originated from the biohydrogenation of linolenic acid. The current study verified the formation of *cis*-9 *trans*-11

and *trans*-10 *cis*-12 CLA, whereas six additional CLA isomers contained ^{13}C from labeled linolenic acid via biohydrogenation. Moreover, 15 C18:3 isomers including *cis*-7 *cis*-9 *cis*-13 C18:3 isomer were produced from linolenic acid. These results indicate that the biological pathways of linolenic acid in rumen are more complicated than previously described. Additionally, high concentrations of linolenic acid showed an inhibition effect. These results suggest that certain amounts of linolenic acid are required to increase the production of CLA in milk and meat products.

Table 3.1. Ingredient and fatty acid composition of the continuous and batch culture diets.

Culture Tube (mg / 100 mg DM)	Mixed Diet*	1-^{13}C-LNA	U-^{13}C-LNA	Unlabeled LNA
Ingredient				
Alfalfa Pellet	50.00			
Ground corn	24.16			
Soybean meal	10.92			
Soybean hulls	12.13			
Calcium phosphate	1.35			
Trace mineral salt[1]	0.62			
Sodium bicarbonate	0.82			
Fatty acid composition				
C16:0	0.48			
C18:0	0.18			
C18:1	0.55			
C18:2	0.82			
C18:3	0.30	0.90	0.75	0.90 / 0.75[2]
Total	2.73	0.90	0.75	0.90 / 0.75[2]

*Continuous culture diets.
[1]Contained (g/kg): NaCl, 955 to 9.8; Nn, 10.0; Mn, 7.5; Fe, 6.0; Mg, 0.5; Cu, 0.32; I, 0.28, and Co, 0.11.
[2]Contained the same amount of unlabeled linolenic (0.90 or 0.75 mg) + 1-^{13}C-LNA (0.90 mg), or U-^{13}C-LNA (0.75 mg) was added to labeled cultures. Unlabeled cultures contained the sum of the labeled and unlabeled LNA such as 1.80 mg for 1-^{13}C-LNA, and 1.50 mg for U-13C-LNA cultures.

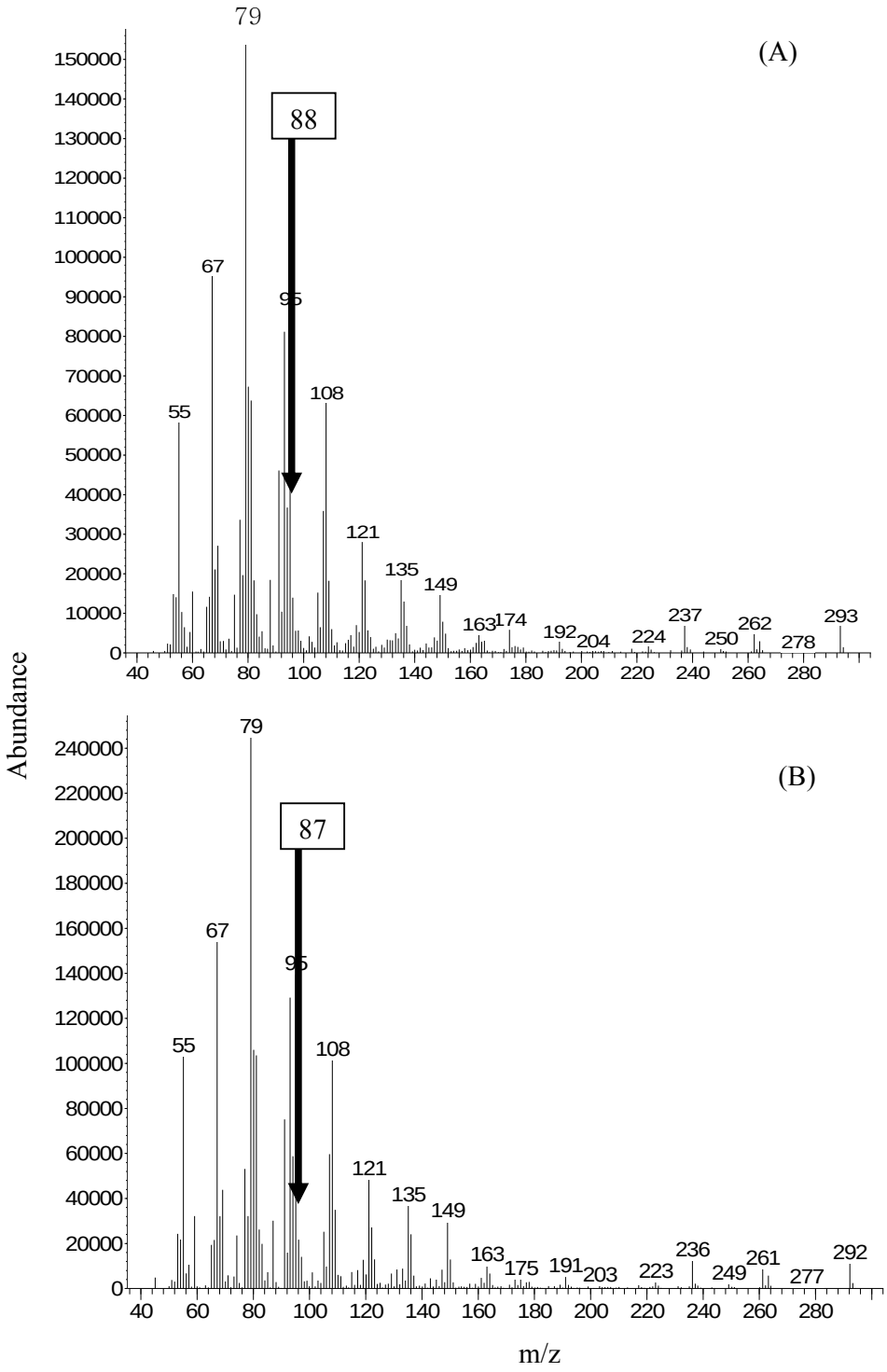

Figure 3.1. Examples of mass spectrum of (A) 1-^{13}C-linolenic acid methyl ester and (B) unlabeled linolenic acid methyl ester.

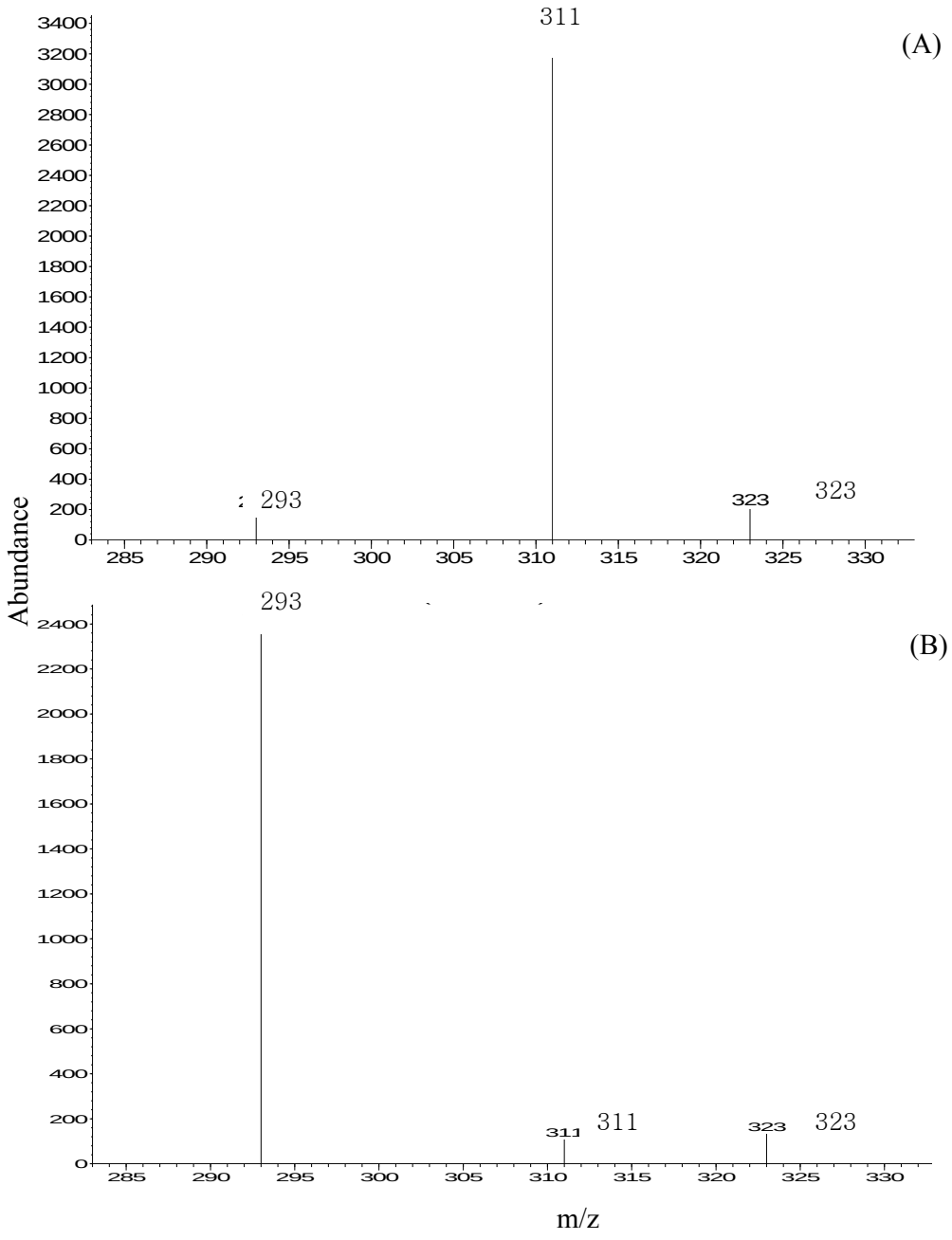

Figure 3.2. Examples of mass spectrum of (A) U-^{13}C-linolenic acid methyl ester and (B) unlabeled linolenic acid methyl ester.

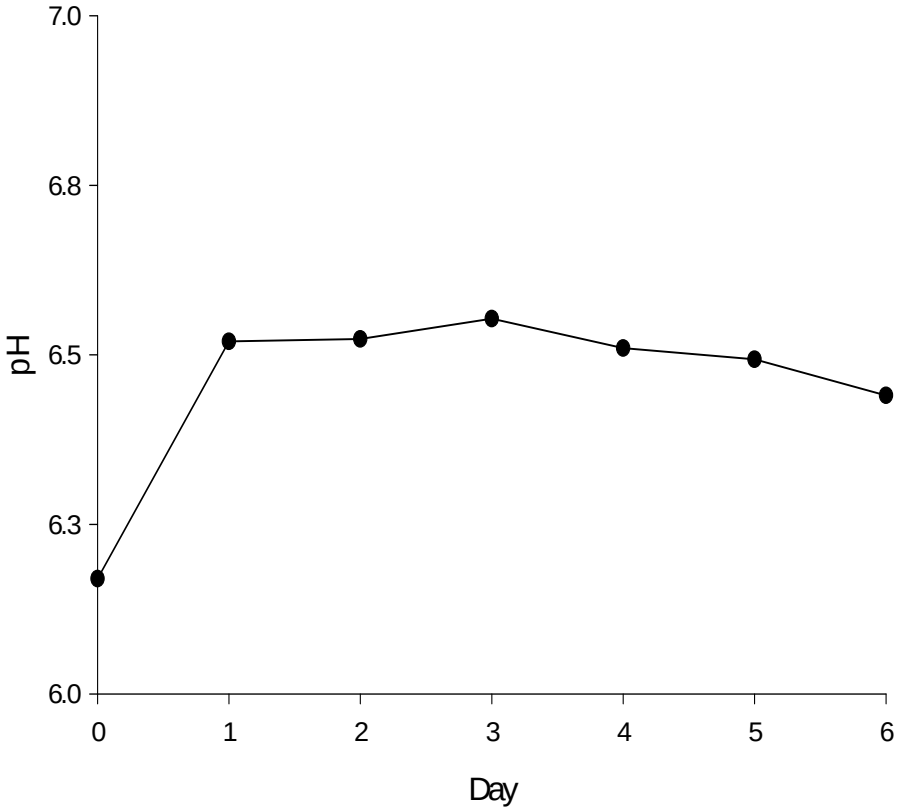

Figure 3.3. pH in continuous culture fermentors for pH 6.5 (n=6). Standard error of the mean was 0.04.

Table 3.2. Proportions of volatile fatty acids in continous culture fermentors on the last day at 0 (before feeding), 2, and 4 h after feeding.

VFA	0 h	2 h	4 h	SEM
Total VFA (mM)	76.46[a]	85.70[b]	103.72[c]	1.54
Individual (mM)				
Acetate	43.21[a]	47.42[b]	55.16[c]	0.96
Propionate	15.56[a]	18.41[b]	23.10[c]	0.43
Butyrate	10.92[a]	13.60[b]	15.55[c]	0.40
Iso-butyrate	0.89	1.05	0.96	0.06
Valerate	3.26[a]	3.91[b]	5.65[c]	0.12
Iso-valerate	1.59	1.40	1.56	0.11
Acetate : Propionate	2.78[a]	2.58[b]	2.39[c]	0.02

Values in a row are the least square means of VFA for each treatment time followed by the pooled SEM (n=4).

[abc]Least square means within a row with different superscript letters are different ($P < 0.05$).

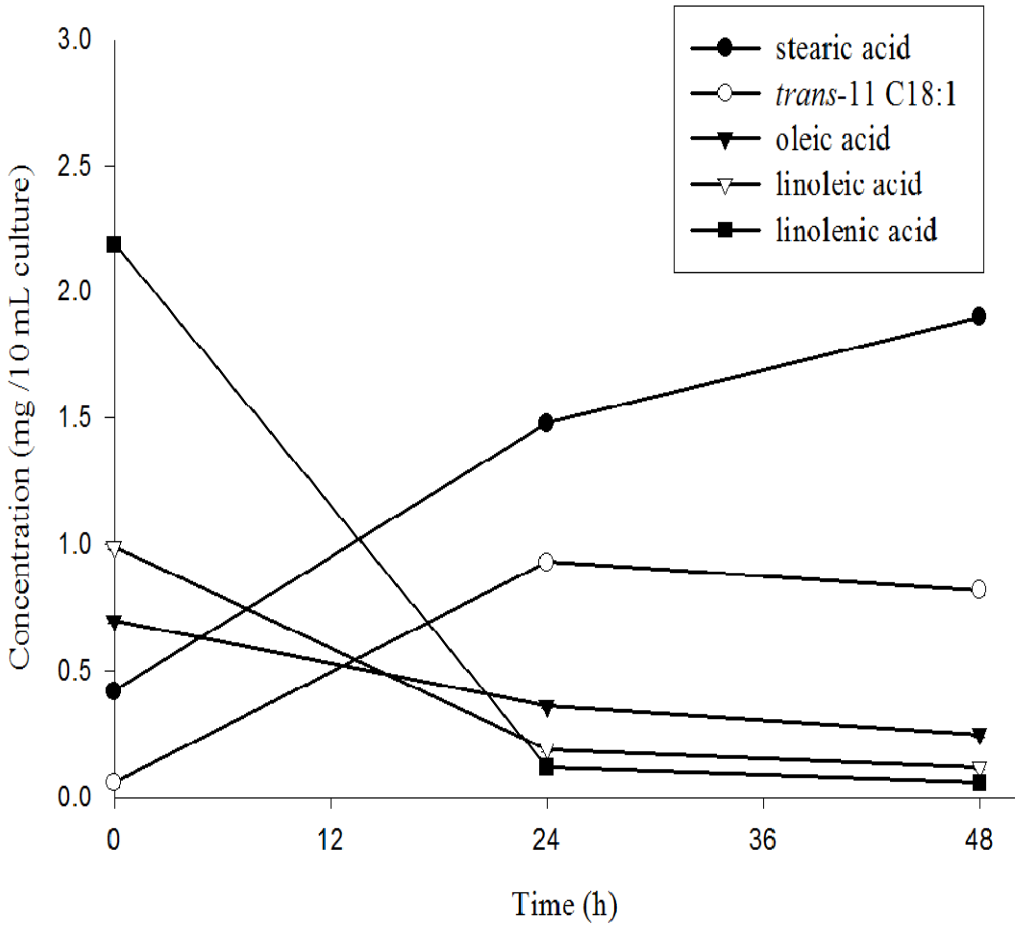

Figure 3.4. The concentration (mg / 10 mL culture) of stearic, oleic, linoleic, linolenic acid, and *trans*-11 C18:1 isomer detected at 0, 24 and 48 h incubation (n=6). Pooled standard errors are shown (<0.02).

Table 3.3. The percentages of enrichment of stearic, oleic, linoleic, and linolenic acid.

	Enrichment, %				
Fatty acid	0h	3h	24h	48h	SEM
Stearic	NS	NS	7.19[a]	16.25[b]	0.10
Oleic	NS	NS	NS	NS	0.12
Linoleic	NS	NS	NS	NS	0.32
Linolenic	33.47	33.26	33.14	35.49	0.34

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=6).

[ab]Least square means within a row with different superscript letters are different ($P < 0.05$).

NS, not significant from zero.

Table 3.4. The percentages of enrichment of C18:1 isomers and C18:2 isomers, except for CLA .

Fatty acid	Enrichment, %				SEM
	0h	3h	24h	48h	
C18:1 isomer					
trans-11	NS	1.99[a]	9.09[b]	21.12[c]	0.15
cis-11	NS	NS	4.89[a]	8.03[b]	0.51
cis-12	NS	NS	1.95[a]	5.90[b]	0.17
Unidentified	ND	ND	25.72	26.50	0.38
C18:2 isomer					
Unidentified (I)	ND	ND	24.71[a]	31.60[b]	0.18
Unidentified (II)	ND	ND	7.74[a]	17.08[b]	0.32
Unidentified (III)	ND	22.34[a]	23.68[a]	32.03[b]	0.39
Unidentified (IV)	ND	5.39[a]	13.04[b]	26.06[c]	0.48
Unidentified (V)	ND	ND	15.25[a]	16.94[b]	0.56

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=6).

[abc]Least square means within a row with different superscript letters are different ($P < 0.05$).

NS, not significant from zero.

ND, not detected.

Table 3.5. The percentages of enrichment of C18:3 isomers .

	Enrichment, %				
Fatty acid	0h	3h	24h	48h	SEM
C18:3 isomer					
C18:3n-3 (I)[1]	ND	11.99	11.84	7.94	0.51
C18:3n-3 (II)[1]	ND	11.37[a]	11.69[b]	16.76[c]	0.43
Cis-7 *cis*-9 *cis*-13	NS	28.15[a]	15.42[b]	5.82[c]	0.52
Partially conjugated (I)	NS	19.33[a]	14.77[b]	2.83[c]	0.40
Partially conjugated (II)	NS	16.87[a]	7.41[b]	6.55[b]	0.70
Unidentified (I)	NS	17.06[a]	11.80[b]	4.77[c]	0.32
Unidentified (II)	NS	9.16[a]	14.42[b]	15.44[c]	0.11
Unidentified (III)	NS	2.71[a]	3.17[a]	7.99[b]	0.40
Unidentified (IV)	NS	1.74[a]	3.98[b]	NS	0.68
Unidentified (V)	NS	2.75[a]	2.29[a]	12.03[b]	0.33
Unidentified (VI)	NS	2.05[a]	2.83[a]	8.64[b]	0.24
Unidentified (VII)	NS	0.97[a]	3.17[b]	NS	0.22
Unidentified (VIII)	NS	2.99[a]	4.36[b]	11.63[c]	0.25
Unidentified (IX)	NS	1.36[a]	0.87[a]	9.17[b]	0.28
Unidentified (X)	NS	NS	2.40[a]	10.72[b]	0.68

Values in a row are the least square means of enrichment percent for each treatment time followed by the pooled SEM (n=6).

[abc]Least square means within a row with different superscript letters are different ($P < 0.05$).

[1]Contained at least one *trans* double bond.

NS, not significant from zero.

ND, not detected.

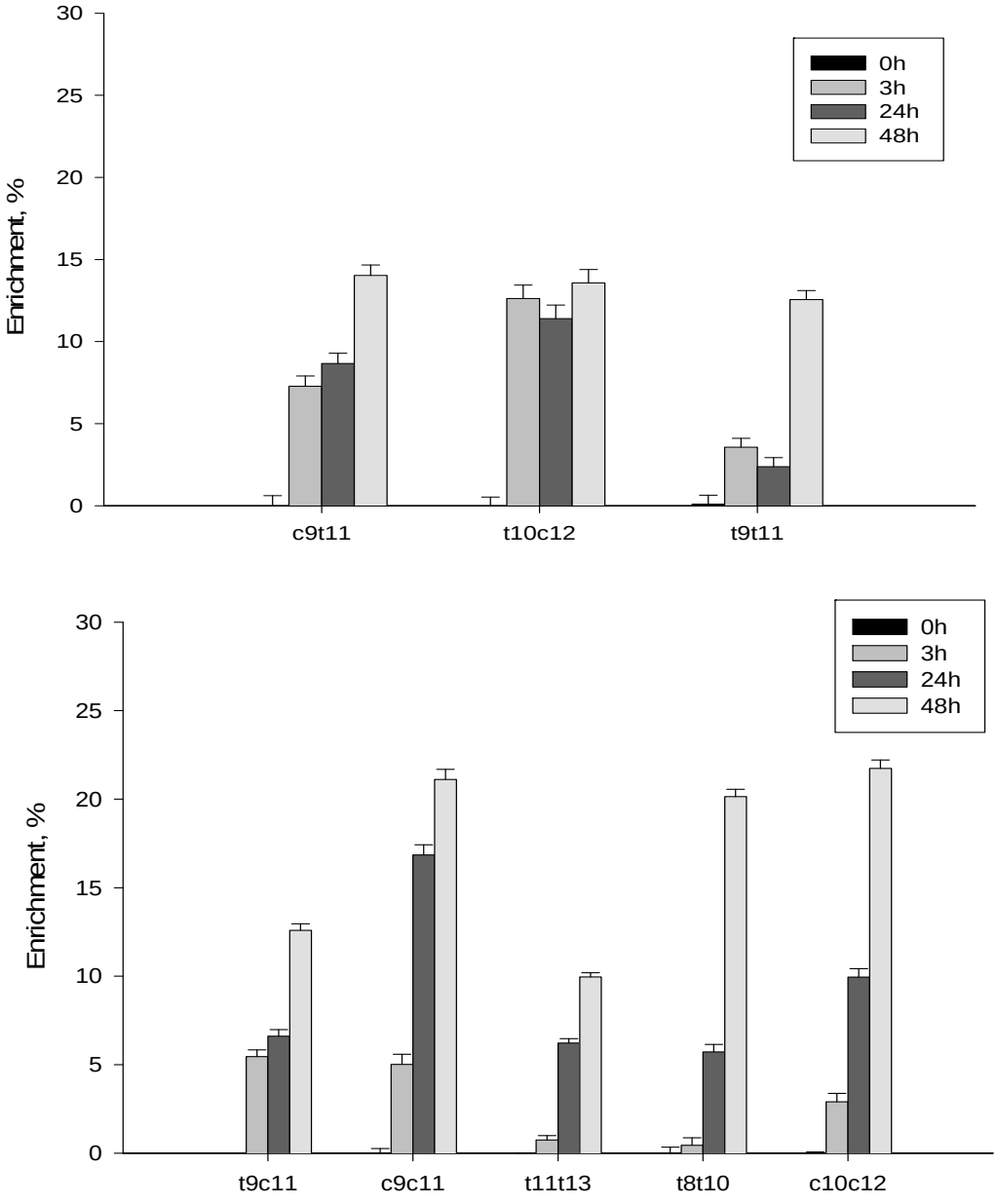

Figure 3.5. The percentages of ^{13}C enrichment of CLA isomers are shown by double bond geometry and position at 0, 3, 24 and 48 h incubation (n=6). Pooled standard errors are shown.

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