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Effect of pH on Conjugated Linoleic Acid (CLA) Formation of Linolenic Acid Biohydrogenation by Ruminal Microorganisms

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Conventional beliefs surrounding the linolenic acid (LNA; *cis*-9 *cis*-12 *cis*-15 C18:3) biohydrogenation (BH) pathway propose that it converts to stearic acid (SA) without the formation of conjugated linoleic acid (CLA) as intermediate isomers. However, an advanced study (Lee and Jenkins, 2011) verified that LNA BH yields multiple CLAs. This study utilized the stable isotope tracer to investigate the BH intermediates of ^{13}C-LNA with different pH conditions (5.5 and 6.5). The ^{13}C enrichment was calculated as a ^{13}C/^{12}C ratio of labeled minus unlabeled. After 24 h, eight CLA isomers were significantly enriched on both pH treatment, this result verifies that these CLAs originated from ^{13}C-LNA BH which supports the results of Lee and Jenkins (2011). The enrichment of *cis*-*cis* double bond CLAs (*cis*-9 *cis*-11 and *cis*-10 *cis*-12 CLA) were significantly higher at low pH conditions. Furthermore, the concentration of *cis*-10 *cis*-12 CLA at low pH was four times higher than at high pH conditions after a 3 h incubation. These differences support the LNA BH pathways partial switch under different pH conditions, with a strong influence on the *cis*-*cis* CLA at low pH. Several mono-, di-, and tri-enoic fatty acid isomers were enriched during 24 h of incubation, but the enrichment was decreased or restricted at low pH treatment. Based on these results, it is proposed that low pH conditions may cause a changed or limited capacity of the isomerization and reduction steps in BH.

Keywords: linolenic acid, conjugated linoleic acid, biohydrogenation, pH, enrichment, isomers

Introduction

Natural metabolic conversion of linolenic acid (LNA; *cis*-9 *cis*-12 *cis*-15 C18:3) occurs during the anaerobic rumen ecosystem called biohydrogenation (BH). Conventional beliefs surrounding LNA BH propose that it converts to stearic acid (SA) through the transformation of *cis*-9 *trans*-11 *cis*-15 C18:3 and *trans*-11 *cis*-15 C18:2, followed by hydrogenation of *trans*-11/*cis*-15 double bond. Trienoic, dienoic, and monoenoic *trans* fatty acids are produced as intermediates during LNA BH (Kepler and Tove, 1967; Harfoot and Hazlewood, 1988). This metabolic scheme, confirmed by recent studies, identified several other intermediates produced during the LNA BH such as non-conjugated trienoic isomers (*cis*-9 *trans*-12 *cis*-15, *cis*-9 *trans*-12 *trans*-15, and *trans*-9 *trans*-12 *trans*-15 C18:3) (Loor *et al*., 2004), and partially conjugated trienoic isomer (*cis*-9 *trans*-13 *cis*-15 C18:3) (Destillats *et al*., 2005).

LNA is usually considered to be hydrogenated without the formation of conjugated linoleic acid (CLA) isomers. However, Destillats *et al*. (2005) suggests that two CLA isomers (*cis*-9 *trans*-11 and *trans*-13 *cis*-15 CLA) are produced from LNA BH, but no evidence supporting this route was recorded. Because of several physiological functions reported, determining whether CLA can form from LNA is of prime importance. A recent study by Lee and Jenkins (2011) identified that eight CLAs are produced from LNA BH with a ^{13}C metabolic tracer study.

The main objective of this study is to get a better understanding of how the type and amount of intermediates of LNA BH, especially CLA, are affected by changes in ruminal pH. To complete this objective, the dual-flow continuous culture system was used under two different pH conditions. Culture contents were then transferred to an *in vitro* culture containing a ^{13}C stable isotope.

Materials and Methods

Reagents

Labeled LNA (1-^{13}C-LNA; >99% chemical purity; 99% ^{13}C isotopic purity) was purchased from Medical Isotopes, Inc. (USA). Unlabeled LNA (99% pure) was purchased from Sigma-Aldrich Chemical Company (USA). Technical-grade LNA (including 5.17% palmitic, 3.90% SA, 19.33% OA, 19.59% LA, 49.44% LNA, and 2.69% other fatty acids) was obtained from MP Biomedicals, LLC. (USA).

Experimental design

The experimental design was a triplicate 2×2 Latin squares with two pH ranges (5.5 and 6.5) in six continuous fermentors. Each period consisted of a ten day period for microbial adaptation. On the last day of each period, a portion of the continuous culture contents were taken from each fermentor and inoculated to batch cultures containing 1-^{13}C-LNA. Batch cultures were used rather than continuous cultures because of the extreme cost of the stable isotope. Samples

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Table 1. The ingredient composition of culture diets

Ingredients	g/kg (DM)
Alfalfa pellets	500.0
Ground corn	241.7
Soybean meal	109.2
Soybean hulls	121.4
Calcium phosphate	13.5
Trace mineral salt[a]	6.2
Sodium bicarbonate	8.2

[a] Contained (g/kg): NaCl, 955–9.8; Nn, 10.0; Mn, 7.5; Fe, 6.0; Mg, 0.5; Cu, 0.32; I, 0.28, and Co, 0.11.

were taken from each batch cultures at 0, 3, and 24 h to determine fatty acid content and enrichment in BH intermediates.

Continuous cultures

Ruminal contents were collected from a fistulated Holstein cow two hours after it was given a feed of 50% forage and 50% concentrate in the morning. Within twenty minutes of collection, the large particles of the ruminal contents were removed by filtration through a four-layer of cheesecloth, and then transferred immediately to the laboratory. Six dual-flow continuous culture apparati were used as described by Teather and Sauer (1988), but several parts were modified. Approximately 800 ml of the filtrated ruminal fluid were added to each of the six fermentors. Cultures were maintained for ten days. Thirty grams of feed (Table 1) was inoculated into each fermentor daily in two equal portions at 0800 and 1630 h. A buffer (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 6 N NaOH or 3 N HCl to maintain treatment pH values (5.5 and 6.5). The pH was measured daily at 0800, 1100, and 1630 h using a portable pH meter. The fermentors were continuously infused with CO_2 at a rate of 20 ml/min to maintain anaerobic conditions, and cultures were stirred continuously at 44 rpm. The temperature of fermentors was held at 39°C by a circulating water bath.

In vitro cultures

On day ten of each period (4 h after the morning feeding), mixed culture contents from the continuous fermentors were transferred to *in vitro* batch cultures. Cultures contained 375 mg diet, 7.5 ml of mixed culture contents, 30 ml of *in vitro* media, and 1.5 ml of reducing solution (Goering and Van Soest, 1970). The diet also contained 2.5% technical-grade LNA. Four batch cultures were run for each continuous fermentor in 39°C water baths containing either 14 mg unlabeled LNA or 7 mg unlabeled LNA + 7 mg 1-^{13}C-LNA. Two 5 ml samples were taken from each batch culture flask at 0, 3, and 24 h.

Fatty acid methyl esters (FAME) sample analysis

Culture samples were freeze-dried and converted to methyl esters in sodium methoxide, followed by a methanolic HCl procedure as described by Kramer *et al*. (1997). Contents of FAME samples were analyzed by a Hewlett-Packard 5890 gas chromatograph (GC) with heptadecanoic acid as internal standard. The column on the GC used a 100 m × 0.25 mm Silica Capillary column with 0.2 μm film thickness (Supelco Inc., USA). Helium was used as the carrier gas at 20 cm/sec. The initial temperature for the column was 140°C for 3 min, with a ramp of 3.7°C/min, until is reached and maintaining 220°C holding for 20 min.

The ^{13}C enrichment in individual FAME was determined on an Agilent (USA) 6890N gas chromatograph model 5973 quadrapole mass selective decetor. The 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/sec with a 10:1 split injection. Initial temperature for the column was 140°C, held for five min with a increase of 4°C/min, until 220°C is reached and held for 20 min. The ion chosen for the FAME sample in selected ion mode was the quasimolecular ion; [M]. A [M+1] ion indicated on the

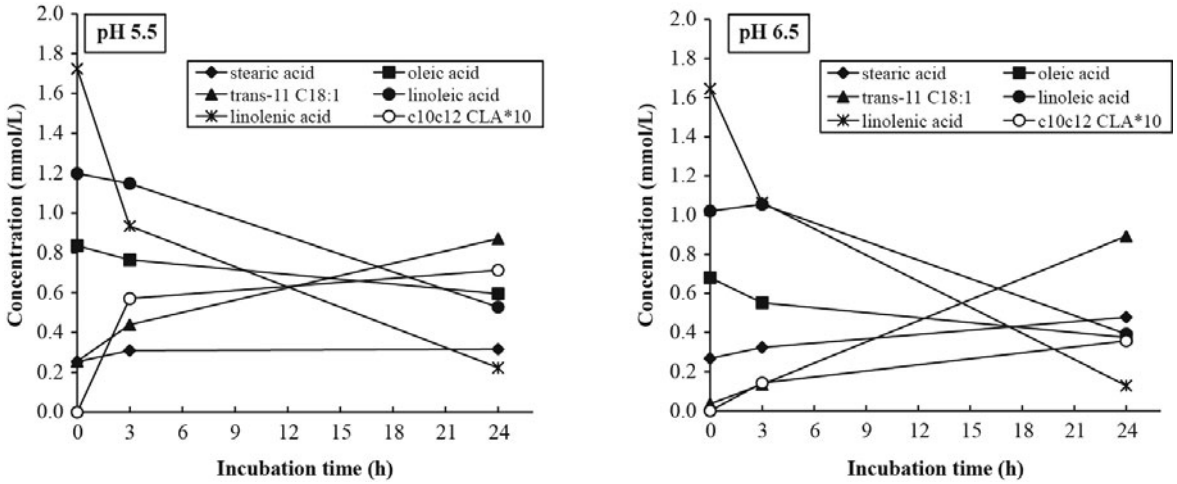

Fig. 1. Fatty acid concentrations in batch cultures with different pH (5.5 and 6.5). Values are least square means, n=6. Pooled SEM was 0.07 for LA and <0.05 for all other fatty acids. The c10c12 CLA was times ten caused of small amounts.

labeled compound.

Initially, FAME peaks were identified based on molecular mass combined with matching retention times of the purchased standard. Further FAME double bond position and geometry were characterized by the GC, followed by covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS), according to detailed methods previously outlined (Michaud *et al*., 2003; Lawrence and Brenna, 2006). FAME samples were separated with a CP-Cil 88 Capillary Column, (100 m × 0.32 mm) with 0.25 μm film thickness. The conditions were programmed of settings of 80°C to 120°C at a rate of 10°C/min and then increased to 220°C at a rate of 2.5°C/min. The total time period was 60 min. FAME eluting into the MS undergo an ion-molecule reaction to form an adduct with [M+54] above that of the parent FAME, [M]. Collisional dissociation of these adduct yield diagnostic ions are characteristic of double bond position. The double bond geometry of CLA peaks were determined by relative diagnostic ion intensity as described by Michaud *et al*. (2003).

Statistical analysis

Data from the continuous cultures was analyzed as a triplicate 2×2 Latin Square with two different pH range treatments by using the general liner model (GLM) procedure of SAS (2003), according to the following model:

$$Y_{ij} = \mu + \rho_i + \gamma_j + \tau_k + e_{ij}$$

Where Y_{ij} is the dependent variable from the i^{th} period and j^{th} fermentor of the Latin Square; μ is the grand mean; ρ_i is the random effect of period i; γ_j is the random effect of fermentor j; τ_k is the effect of the k^{th} treatment; and e_{ij} is the residual error associated with the ij observation.

Long-chain fatty acids were analyzed using the mixed procedure of SAS (2003) including the effects of time and all interactions with time in the model. The pH effects on fatty acids contents and ^{13}C enrichment were determined by analysis of variance (ANOVA) using the GLM procedure of SAS (2003). The fatty acid contents in the FAME samples were calculated by comparing the fatty acid peak areas on the chromatograms with the internal standard.

Abundances of selected ions in all samples were exhibited as [(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. In order to increase the precise level of ^{13}C, the average fractional ratio of m/z 88 to 87 on each of the unlabeled cultures was subtracted from the labeled culture instead of using the whole molecular weight. The abundance of m/z 88 and 87 was measured higher than the whole mass of FAME sample such as m/z 293 and 292 in LNA. Moreover, the m/z 88 and 87 fractional ratios made the enrichment calculation easier because these fractions contained three carbons at the end of carboxyl groups of all FAME isomers, and did not require the consideration of changing m/z by individual FAME. Enrichment % means±SEM taken from ANOVA was shown for each fatty acid. Least Significant Difference (LSD) was measured to identify the main effect and differences of fatty

Table 2. The enrichment of C18:1, non-conjugated C18:2 and C18:3 isomers

Fatty acid	Treatment						SEM	pH Effect (*P* value)
	pH 5.5			pH 6.5				
	0 h	3 h	24 h	0 h	3 h	24 h		
C18:1 isomer								
Trans-11	NS	NS	5.3	NS	7.4	10.1	0.30	NS
Trans-12	ND	5.5	14.2	ND	ND	11.9	1.07	0.10
Unidentified (I)	NS	NS	14.2	NS	NS	20.3	1.35	0.13
Unidentified (II)	NS	NS	13.6	NS	NS	26.79	1.76	0.12
C18:2 isomer (non-conjugated)								
Unidentified (I)	ND	NS	6.46	ND	2.8	11.8	0.87	0.08
Unidentified (II)	ND	ND	24.7	ND	20.8	33.5	0.80	NS
Unidentified (III)	ND	15.3	31.3	ND	9.4	28.6	1.10	NS
Unidentified (IV)	ND	ND	25.5	ND	11.4	25.6	1.49	0.06
C18:2n-7	NS	35.9	37.6	ND	46.5	35.0	1.12	0.07
C18:3 isomer								
Unidentified (I)	NS	3.6	9.9	ND	4.1	18.8	0.77	NS
Unidentified (II)	NS	37.7	19.8	ND	45.9	28.3	2.90	NS
Unidentified (III)	ND	42.6	8.4	ND	46.6	11.8	1.14	NS
Unidentified (IV)	ND	39.7	24.5	ND	32.3	10.0	0.81	NS
Unidentified (V)	ND	34.2	7.0	ND	21.1	9.6	1.25	NS
Unidentified (VI)	ND	21.2	3.7	ND	19.2	5.8	1.04	0.96
Unidentified (VII)	ND	28.2	7.8	ND	34.2	13.1	1.60	NS
Unidentified (VIII)	ND	40.2	16.8	ND	29.0	16.7	1.67	0.09
Unidentified (IX)	ND	12.0	6.9	ND	12.2	4.5	0.74	0.19
Unidentified (X)	ND	13.2	4.5	ND	11.6	6.7	1.23	0.79
Unidentified (XI)	NS	12.7	10.2	NS	8.5	11.3	1.06	0.24

Values in a row are the least square means of fatty acid content for each treatment time followed by the pooled SEM.
NS, non significant from zero (P>0.05).
ND, not detected.

acid contents and enrichments of labeled fatty acids by treatment within the incubation time. Fatty acid content and enrichment data were tested for statistical difference from zero at $P<0.05$ by Student's t-test.

Results

The fatty acid contents in batch cultures are given in Fig. 1. LNA decreased 87.1% (1.50 mmol/L) at a pH of 5.5 and 92.2% (1.52 mmol/L) at a pH of 6.5 over a 24 h incubation period. LA and OA also decreased 55.9% (0.67 mmol/L) and 28.8% (0.24 mmol/L) at pH 5.5, and 61.6% (0.63 mmol/L) and 44.9% (0.31 mmol/L) at pH 6.5. The *cis*-10 *cis*-12 CLA concentration at low pH was four times higher than at the high pH with 3 h incubation. The concentration was only twice as high at 24 h. SA was not significantly increased at the low pH, but comprised 44.1% after 24 h of incubation at the high pH. The concentration of *trans*-11 C18:1 increased (0.62 mmol/L at pH 5.5 and 0.86 mmol/L at pH 6.5) over time.

A total of 28 fatty acids were enriched, including 4 C18:1 isomers and 13 C18:2 isomers (eight of which were CLAs). Eleven C18:3 isomers were also produced (Table 2 and Fig. 2). The enrichment of all C18:1 isomers, with the exception of *trans*-12 C18:1, was enriched but only after 24 h incubation at low pH (Table 2). With a pH of 6.5, all C18:1 isomers, except for *trans*-11 C18:1, were not enriched after only 3 h incubation. The enrichment of non-conjugated C18:2 isomers was either negligible or not detected at 0 h incubation with both pH treatments (Table 2). Except for the unidentified (III)-C18:2 and C18:2n-7, the C18:2 isomers were either not significantly enriched or not detected at 3 h with the low pH. Enrichment of C18:2n-7 isomer was 35.9% at 3 h and then remained statistically constant from 3 to 24 h. With the high pH, all C18:2 isomers were already enriched at 3 h incubation and it kept increasing from 3 to 24 h. All the C18:3 isomers were significantly enriched at 3 and 24 h incubation (Table 2). The enrichment of unidentified (III)-C18:3 reached a maximum at 3 h (42.6% at pH 5.5 and 46.6% at pH 6.5). Other C18:3 isomers were observed to have a wide variety of enrichment, from 3.6% to 40.2% at pH 5.5 and from 4.1% to 45.9% at pH 6.5.

The enrichment of *cis*-10 *cis*-12 CLA reached a maximum at 3 h (35.0% at pH 5.5 and 22.3% at pH 6.5), and decreased significantly from 3 to 24 h at the low pH treatment (26.0%) and remained constant at the high pH (22.5%) (Fig. 2). The 10.6% and 12.9% enrichment of *cis*-9 *trans*-11 CLA was observed at the 3 h incubation with pH 5.5 and 6.5, respectively and then decreased from 3 to 24 h (2.7%, 2.9%, respectively). The *trans*-10 *cis*-12 CLA was also enriched at 3 h incubation but statistically constant from 3 to 24 h of both treatments. The 8.4% and 5.3% enrichment of *trans*-9 *trans*-11 CLA was observed at 3 h incubation. However, enrichment decreased to 4.6% at 24 h of low pH treatment; while it remained statistically constant at the high pH treatment. With the low pH, the enrichment of *cis*-9 *cis*-11 CLA and *trans*-11 *trans*-13 CLA decreased from 19.0% to 14.0% and from 18.3% to 10.6%, respectively. Other CLAs such as *trans*-9 *cis*-11 CLA, and *trans*-8 *trans*-10 CLA were not significantly enriched at 3 h. They increased at 24 h incubation to final values of 3.4%, and 4.8%, respectively. With high pH treatment, the enrichment *trans*-11 *trans*-13 CLA decreased from 20.67% to 12.55%. The enrichment of *trans*-9 *cis*-11 CLA, *trans*-8 *trans*-10 CLA, *cis*-9 *cis*-11 CLA were increased at 24 h incubation to final values of 6.6%, 8.9%, and 10.3%.

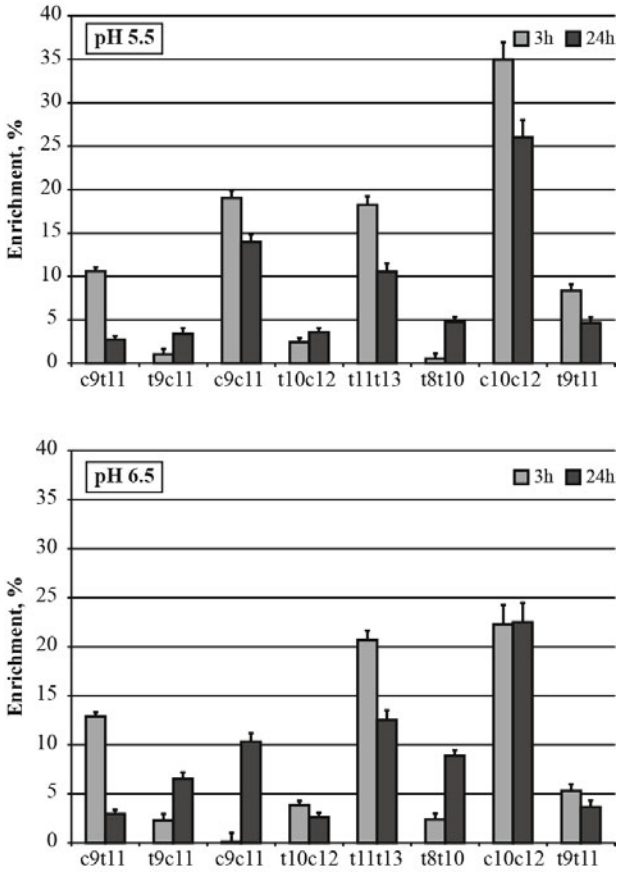

Fig. 2. The percentages of ^{13}C enrichment of CLA isomers are shown by double bond geometry and position at 3 and 24 h incubation. Pooled standard errors are shown.

Discussion

With a high pH treatment, the fatty acid BH rate was higher for LNA, LA, and OA than with low pH treatment after 24 h incubation. Troegeler-Meynadier *et al.* (2003) reported similar results of BH for the LA and LNA by *in vitro* incubations in ruminal contents under two different pH ranges (pH 5.4–6.2 and 6.4–6.9). They found that the rate of BH of these two VFAs treated with a low pH was about one-half that of the high pH buffer treatment. More recently, Troegeler-Meynadier *et al.* (2006) reported that the LA content was significantly decreased for the high pH buffer than with the low pH buffer. Fatty acid disappearance was ranked so that LNA > LA > OA for both pH's in this study. Fuentes *et al.* (2009) reported that the different pH was significantly affected to the microbial biohydrogenation and their growth

rate. The total DNA concentration of *Anaerovibrio lipolytica* and *Butyrivibrio fibrisolvens* vaccinic acid subgroup was significantly reduced by pH 5.6. This indicats that the major ruminant bacteria are very sensitive to the pH conditions because it changes their metabolic processes.

Stearic acid, the final product of BH, was inhibited by low pH treatment and produced only small amounts at high pH treatment during the 24 h incubation. Nam and Garnsworthy (2007) reported that 100% of the LA converted to SA within a 100 min incubation by mixed cultures of rumen bacteria. Fuentes *et al.* (2009) also reported that the highest concentration of SA was observed in the duodenum flow of steers. The inhibition of SA formation in the research reported here may be related to the low pH and the injection of highly concentrated LNA.

The *trans*-11 C18:1 produced the highest concentration of C18:1 isomers at both pH treatments. AbuGhazaleth *et al.* (2005) reported that the *trans*-11 isomer was not the major *trans*-C18:1 intermediate of OA BH. Grinnari and Bauman (1999) suggested that *trans*-11 C18:1 originated from *cis*-9 *trans*-11 CLA (intermediate of LA BH) and/or *trans*-11 *cis*-15 C18:2 (intermediate of LNA BH). Moreover, Ribeiro *et al.* (2005) suggested that the *trans*-11 C18:1 is a major intermediate of LNA BH. The accumulation of *trans*-11 C18:1 in cultures is important because it is desaturated by Δ^9-desaturase to yield *cis*-9 *trans*-11 CLA in mammary tissue, which can increase the amount of CLA isomers in meat and milk products (Griinari and Bauman, 1999).

In our study, several unknown isomers were also enriched, but we did not identify the double bond position and geometry for the purposes of this study. Our main focus was to determine CLA formation under different pH conditions. All C18:1 isomers were enriched at 24 h incubation with both treatments. However, the enrichment resultant was different at the low and high pH treatments. For instance, the production of *trans*-11 C18:1, generally considered the major *trans* fatty acid in the rumen, was inhibited at the low pH treatment while enrichment of *trans*-12 C18:1 was higher than at the pH 6.5. AbuGhazaleh *et al.* (2005) reported that the low concentration of *trans*-11 C18:1 was observed at the low pH levels and *trans*-12 C18:1 showed the opposite result. In our study, the low pH caused an increase of *trans*-12 C18:1. By contrast, AbuGhazaleh *et al.* (2005) reported *trans*-12 C18:1 was not detected at a pH of 5.5 with OA BH. This could be due to *trans*-12 C18:1's origination from LA and LNA, and not from OA.

Many researchers believe that the ruminal microbes cannot produce CLA from LNA. By 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 LNA BH. An advanced study (Lee and Jenkins, 2011) verified that LAN BH yields multiple CLAs. The results of our study also verified that eight CLAs originated from LNA BH in different pH conditions. *Cis*-9 *trans*-11 CLA is considered the major CLA produced from LA BH (Kepler and Tove, 1967). About 88% of total CLA consisted of the *cis*-9 *trans*-11 CLA in the rumen of lactating cows (Loor *et al.*, 2002). In this study, the enrichment of *cis*-9 *trans*-11 CLA was comprised of 30% and 58% at low and high pH treatment respectively, compared to *cis*-10 *cis*-12 CLA isomer. In addition, the enrichment of *trans*-10 *cis*-12 CLA was observed to be the second most produced CLA, and the *trans*-9 *trans*-11 CLA was comprised of below 10% enrichment over the entire incubation time. The *cis*-9 *trans*-11, *trans*-10 *cis*-12, and *trans*-9 *trans*-11 CLA are produced as the major CLAs of LA BH by the ruminal bacteria (Griinari and Bauman, 1999). The study conducted by Kim *et al.* (2000) reported that *cis*-9 *trans*-11 CLA is produced by an isomerase of *Butyvibrio fibrisolvens*, and *trans*-10 *cis*-12 CLA is produced by *Propinonibacterium acnes* and *P. freudenreichii* (Jiang *et al.*, 1998) from LA. Coakley *et al.* (2006) reported that these three CLAs can each be precursors of each other and can be by-products of the *Bifidobacterium* species. For instance, *cis*-9 *trans*-11 and *trans*-9 *trans*-11 CLA are produced from LA and *cis*-9 *trans*-11 and *trans*-10 *cis*-12 CLA are converted to *trans*-9 *trans*-11 CLA. Based on these results, the BH pathway of LNA is different from that of LA. The enrichment of *cis*-9 *cis*-11 and *cis*-10 *cis*-12 CLA were significantly higher at low pH conditions. This supports the idea that the LNA BH pathway is partially switched, with an especially strong influence on *cis-cis* geometric double bond CLA. Furthermore, the concentration of *cis*-10 *cis*-12 CLA at the low pH was four times higher than at the high pH after a 3 h incubation period. However, the exact nature of these biological pathways are still unknown.

All identified CLAs carried a double bond at position 9 through 13 with all four possible geometries (*cis/cis trans/trans*, *cis/trans*, and *trans/cis*) originating from the LNA via BH process. Just as the same enzymatic system would be utilized with LA and LNA. First, isomerization of LNA is initiated at carbon number 11 which produces several C18:3 isomers positioned between carbon number 9 through 15, and then produces several C18:2 isomers, including CLAs, by reductases of different ruminal microbes. Isomerization and reduction of LNA were inhibited by low pH treatments, though not completely, with an especially strong influence on the *cis-cis* geometric CLA.

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