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Characterizations of Environmental Factors in Conjugated Linoleic Acid Production by Mixed Rumen Bacteria

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Conjugated linoleic acid (CLA) production by rumen bacteria is closely related to biohydrogenation of linoleic acid (LA) and affected by various environmental factors. Ruminal biohydrogenation and isomerization were characterized in view of incubation conditions using a mixed culture of microorganisms obtained from surgically prepared cows. Free-floating bacteria (FFB) produced more CLA than particle-attached bacteria (PAB). Some major factors affecting the ruminal environment such as diet, concentrations of fat substrates, incubation time, pre-incubation, and the presence of glucose in the medium were found to be important determinants for the ruminal production of CLA and in a close relationship with biohydrogenation. The mixed bacterial culture, which was pre-exposed to LA, produced more CLA than an unexposed control in a medium containing 30% rumen fluid. The rate of conversion of fat substrate (LA) to hydrogenated products (*trans*-C18:1, C18:0) was negatively correlated with the initial LA concentration. Overall, the present study showed that CLA accumulation can be increased by modification of diet-induced environmental conditions, which affect changes in ruminal microflora.

KEYWORDS: CLA; rumen bacteria; biohydrogenation; glucose; linoleic acid

INTRODUCTION

The biohydrogenation of polyunsaturated fatty acids (PUFAs) is a characteristic biochemical process carried out by various ruminal microorganisms (*1*). The biochemical characteristics of rumen bacteria involved in the biohydrogenation pathway have been studied extensively for decades. Through the process of consecutive reactions by membrane-associated enzymes, dietary PUFAs, which are inhibitory to bacterial growth, are typically hydrogenated to stearic acid to which rumen bacteria are immune (*2*).

Conjugated linoleic acid (CLA), which refers to a mixture of positional and geometric isomers of linoleic acid (LA; *cis*-9, *cis*-12-octadecadienoic acid), is an intermediate that occurs during the biohydrogenation process. CLA has been well-characterized as a health-promoting factor present in dairy foods (*3*). Animal studies have shown that CLA has anticarcinogenic effects against a variety of cancers (*4*, *5*), antiatherogenic effects (*6*), and antiobesity effects (*7*). On this basis, there have been continuous attempts to increase the CLA concentration of beef and dairy products (*8*, *9*).

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Before the advantages of CLA in human health were recognized, Kepler and Tove (*1*) noted the fatty acid conversion by ruminal bacteria. Ruminal biohydrogenation is composed of sequential, but distinct, enzymatic processes to yield saturated fatty acids: the spontaneous isomerization of LA to CLA, followed by the energy-requiring reduction steps of CLA to VA and to stearic acid. The isomerization process is the CLA-producing step in the pathway of LA biohydrogenation and, thereafter, partially purified isomerase can be obtained from the rumen bacterium, *Butyrivibrio fibrisolvens* A38 (*10*). However, the roles of factors affecting CLA production as a byproduct in ruminal biohydrogenation remain to be clarified. We previously reported the characteristics of *B. fibrisolvens* A38 and *Megasphaera elsdenii* YJ-4 for CLA production, which are influenced by numerous factors (*11*, *12*). The population of these rumen bacteria was altered by the diet fed to cows, which induced rumen environmental changes for their fat metabolism.

In the present study, mixed populations of rumen bacteria from a fistulated cow were used to study factors affecting ruminal CLA formation. The objective was to better understand how CLA is formed in the rumen and how mixed rumen cultures are influenced by various environmental factors in vitro.

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Table 1. Ingredients and Chemical Compositions of Bovine Diets (DM Basis)

	diets (g/kg of DM)	
	HCD[a]	HFD[b]
ingredient composition		
corn grain	630	92
alfalfa silage	135	170
alfalfa hay	94.0	600
soybean meal	12.0	12.0
limestone	10.0	10.0
calcium phosphate	5.0	5.0
sodium bicarbonate	5.0	5.0
calcium sulfate	0.5	0.5
magnesium oxide	35.0	35.0
protein supplement[c]	2.5	2.5
mineral-vitamin supplement[d]	60.0	60.0
chemical composition		
DM (g/kg)	605	440
ADF	124	205
NDF	245	355
CP	175	167
fatty acid composition		
C16:0	6.8	7.6
C16:1	0.04	0.06
C18:0	1.5	2.4
C18:1	12.4	10.5
C18:2	25.0	31.0
C18:3	1.8	3.5
other	0.4	0.6

[a] HCD, high-concentrate diet. [b] HFD, high-forage diet. [c] Commercial supplement containing blood meal, feather meal, and corn and gluten meal (Taylor By-Products, Wyalusing, WA). [d] Mineral−vitamin mix contained (mg/kg mix) Mn, 3500; Zn, 3000; Cu, 750; Fe 20; I, 85; Co15; and Se, 35; retinyl acetate (2,100,000 IU/kg); cholecalciferol (390,000 IU/kg); and α-tocopheryl acetate (7500 IU/kg).

MATERIALS AND METHODS

Animals and Diets. Three nonlactating ruminally fistulated dairy cows (Holstein), weighing 600 ± 50 kg, were fed either a high-forage diet (HFD; hay 78%) or a high-concentrate diet (HCD; grain 63%). The alfalfa hay had approximately 14% crude protein (CP) and 30% neutral detergent fiber (NDF). The same cows were used for both experiments. First, the animals were fed approximately 9 kg/day of HFD (770 g/kg of dry matter (DM) hay) twice a day for 5 weeks. After a 4 week interval, they were switched to the HCD (630 g/kg of DM corn grain) and acclimated for another 5 weeks thereafter. The feeding period comprised 3 weeks for adaptation to the diets and 2 weeks for sampling. The feed compositions of the HCD and HFD diets are shown in **Table 1**. The cows were fitted with ruminal cannulae (83 mm i.d.) by surgical procedures approved by the guidelines of Korea University (Seoul, Korea) for the ethical treatment of laboratory animals. The surgery was performed 8 weeks before the rumen sampling so the cows could recover from the surgery. The DM and crude protein (CP) chemical compositions were analyzed using AOAC (*13*) methods (methods 930.15 and 954.01, respectively). NDF and acid detergen fiber (ADF) were determined using methods described by Goering and Van Soest (*14*).

Mixed Rumen Culture. A mixed rumen culture was obtained from the fistulated cows. The rumen samples were taken 3−4 h after feeding. The ruminal fluid was centrifuged at low speed to remove large feed particles (150*g*, 2 min, 22 °C). The mixed bacterial supernatant fluid was centrifuged again at higher speed (4000*g*, 5 min, 22 °C) to separate particle-attached bacteria (PAB) from free-floating bacteria (FFB). Both bacterial preparations (PAB and FFB) were resuspended in a similar volume of clarified, autoclaved (121 °C, 20 min) basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4000 mg of Na_2CO_3, 600 mg of L-cysteine·HCl, 10 g of trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g of yeast extract, and branched-chain volatile fatty acids (1 mM each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals (*15*). Glucose was prepared as a separate solution and was added after autoclaving. The rumen culture was incubated at 39 °C in 150 × 18 mm tubes (capped with butyl rubber stoppers and aluminum seals) that had been flushed with O_2-free CO_2. LA (95%; Sigma Chemical Co., St. Louis, MO) solution, as a fat substrate (100 mg/mL water with 200 mg of bovine serum albumin, Sigma), was sterile filtered (pore size = 0−22 μm) prior to use for the incubation experiments and analysis (*11*). The LA stock solution was serially diluted in sterile anaerobic water to decrease the concentration and then added to cultures.

Fatty Acid Analyses. All of the chemicals used for GC analysis were of analytical grade and purchased from Sigma. The lipid from samples was extracted with hexane/isopropanol (3:2 vol/vol). The fatty acids were converted to methyl esters as described in our previous study with some modifications (*16*). Briefly, 0.5 mL of toluene and 2 mL of 5% KOH−MeOH were added to the lipid, and the sample was vortex-mixed and heated at 70 °C for 8 min and then cooled in cold water. Two milliliters of 14% $BF_3 \cdot$MeOH was added to the sample, which was then heated at 70 °C for an additional 8 min. The sample was cooled, and then 3 mL of 5% NaCl was added and mixed. Five milliliters of distilled water and 0.5 mL of hexane were added to extract the fatty acid methyl ester. The mixture was vortexed and centrifuged at 5000*g* for 10 min, and then the upper phase was collected and dried with sodium sulfate. The fatty acid methyl esters were separated by a Supelcowax-10 fused silica capillary column (60 m × 0.53 mm, 0.5 μm film thickness; Supelco., Inc., Bellefonte, PA) using a Hewlett-Packard model HP5890 gas chromatograph equipped with a flame ionization detector and a model HP3392 integrator. The conditions were as follows: 2.4 mL/min helium flow; injector temperature, 200 °C; detector temperature, 250 °C. The oven temperature was increased from 40 °C (5 min) to 220 °C at the rate of 20 °C/min and held for 30 min. A sample (1 μL) containing 0.5−5 μg of LA or CLA was injected into the column in the splitless mode. Heptadecanoic acid (C17:0) was used as an internal standard. *cis*-9-, *trans*-11-, and *trans*-10,*cis*-12-CLA isomers (95% purity, Sigma) were used to identify and quantify each CLA isomer. Other fatty acid standards were obtained from Sigma. A standard mixture of fatty acids was used to identify other fatty acids.

Statistical Analysis. All data were in triplicate and expressed as the mean ± standard deviation. Analysis of variance was performed by ANOVA procedures. Duncan's multiple-range test was used to determine the differences between means, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

When the PAB and FFB were incubated with LA (200 mg/L) for 6 h, biohydrogenated products (C18:0 + *trans*-C18:1) accumulated in the culture (**Figure 1**). The PAB of HCD produced more biohydrogenated products compared to the PAB of HFD ($P < 0.05$). In addition, the FFB produced fewer biohydrogenated products compared to the PAB ($p < 0.05$), and the FFB of HFD produced fewer biohydrogenated products than the FFB of HCD ($p < 0.05$). CLA was also detected, and there was an inverse relationship between total CLA isomer (*cis*-9-, *trans*-11-, and *trans*-10,*cis*-12-CLA) content and the amount of biohydrogenated products. The PAB of HCD produced less *cis*-9,*trans*-11-CLA than the PAB of HFD ($p < 0.05$). FFB tended to produce more *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA than PAB in both feeding groups, but there was no significant difference in *trans*-10, *cis*-12-CLA content ($p > 0.05$).

The concentration of the fat substrates has been found to be a major factor that affects the hydrogenation patterns of rumen bacteria (*17*), and high levels of fat supplements increase energy density, significantly affecting ruminal fermentation (*18*). Ruminal microorganisms can tolerate fat levels up to 3−5%, but this can be increased with the presence of feed particles in the rumen, because the bacteria can be protected from the fat substrates by binding to the particles. The feed particles could decrease the inhibitory effect of fatty acids on bacterial growth and biohydrogenation because the fatty acid binding to the particles could decrease the availability of fat substrate. Biohydrogenation was more active and less CLA accumulated in the incubations with

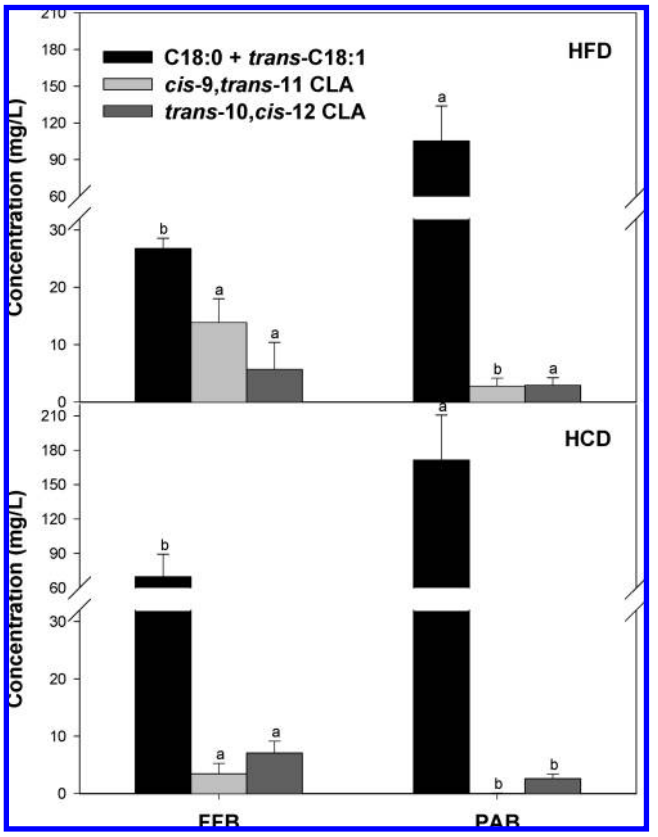

Figure 1. Effect of feeding particle presence on biohydrogenation by rumen bacteria. HFD, high-forage diet; HCD, high-concentrate diet; PAB, particle-attached bacteria; FFB, free-floating bacteria. Bars with different letters are significantly different in each fatty acid ($p < 0.05$).

feed particles than in the incubations without feed particles. Indeed, most of the lipid distributed in the rumen is associated with feed particles (*19*). However, large feed particles were excluded in our studies due to the difficulty in applying the same amount of cells in a controlled environment.

The FFB of HFD were used to examine the effects of different LA concentrations on fatty acid profiles using diluted rumen fluid cultured for 6, 12, and 24 h (**Figure 2**). Incubations with LA at concentrations lower than 40 mg/L showed no CLA accumulation at 6 h of incubation, but at LA concentrations higher than 40 mg/L, CLA accumulation was evident. However, as incubation time increased, CLA declined to a lower degree with higher LA concentrations. The highest level of CLA accumulation was shown with the LA concentration of 320 mg/L, above which the rate of CLA accumulation was significantly reduced. There were only small increases in *trans*-C18:1 at all of the LA concentrations tested until 12 h, but at 24 h of incubation a significant increase was found at concentrations lower than 320 mg/L. There were no significant time-dependent differences in C18:0 concentration at any tested LA concentrations. At LA concentrations lower than 200 mg/L, *trans*-C18:1 concentration was relatively higher than C18:0, indicating that complete biohydrogenation was inhibited by the fat substrate. With an excessive amount of fat substrate, both CLA production and hydrogenation could be reduced due to the inhibition of bacterial growth. These patterns are consistent with some previous results (*11*, *20*).

To determine the effect of pre-exposure to LA on the CLA-producing capacity of mixed populations of rumen bacteria, the cells, transferred from cultures with or without LA, were incubated in the rumen fluid (**Figure 3**). Stationary phase cells grown for 36 h with 1 g/L of glucose were pre-incubated with LA (2.5 g/L)

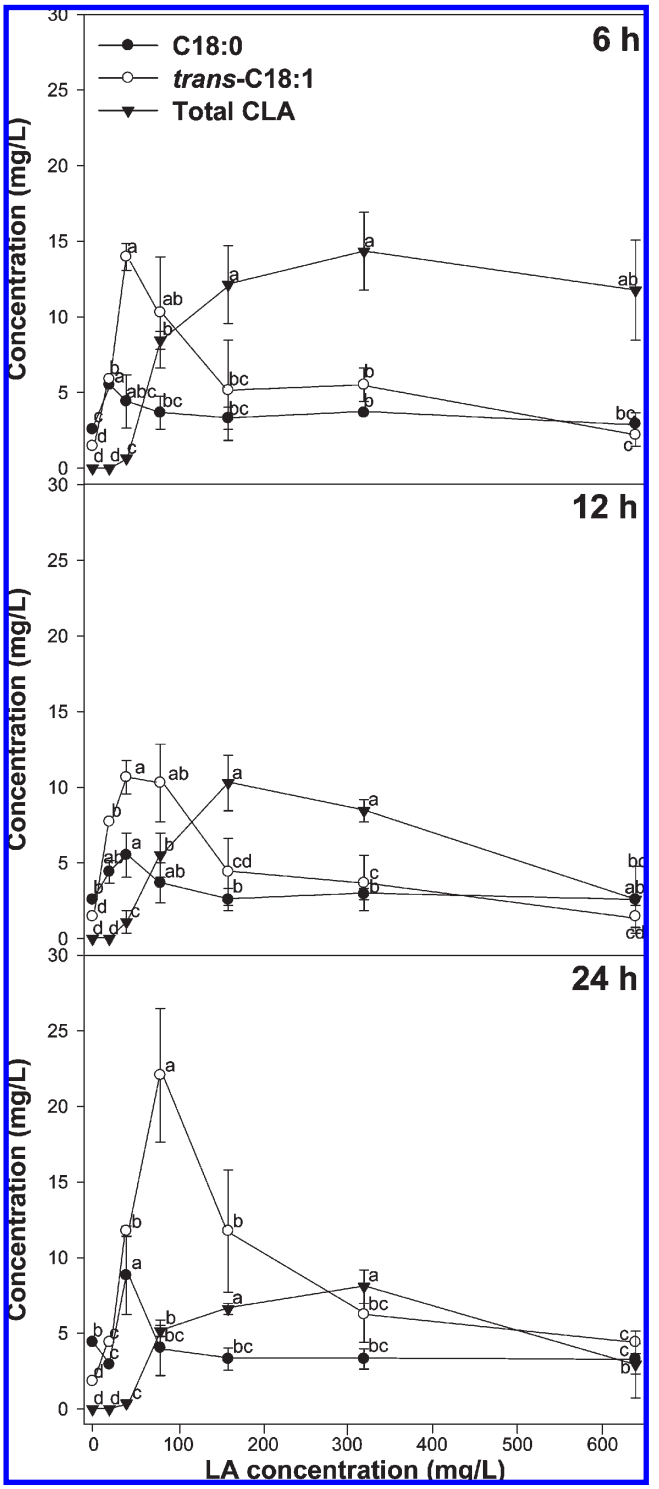

Figure 2. Effects of LA concentrations on biohydrogenation by rumen bacteria. Values having different letters are significantly different in each fatty acid ($p < 0.05$).

and then used as an inoculum. When the cells were incubated with LA (200 mg/L) and glucose (200 mg/L), CLA production was 2.1 mg/L at 1 h of incubation and then increased about 8-fold (17 mg/L) in 6 h. When the cells without pre-incubation were transferred directly to the sterilized rumen fluid with glucose, no CLA production was detected within 1 h, but CLA accumulation was evident at 6 h of incubation (0.8 mg/L), which was about half of the CLA level from the pre-incubated cells. The cells transferred from pre-incubated cells produced more *trans*-C18:1 than the control.

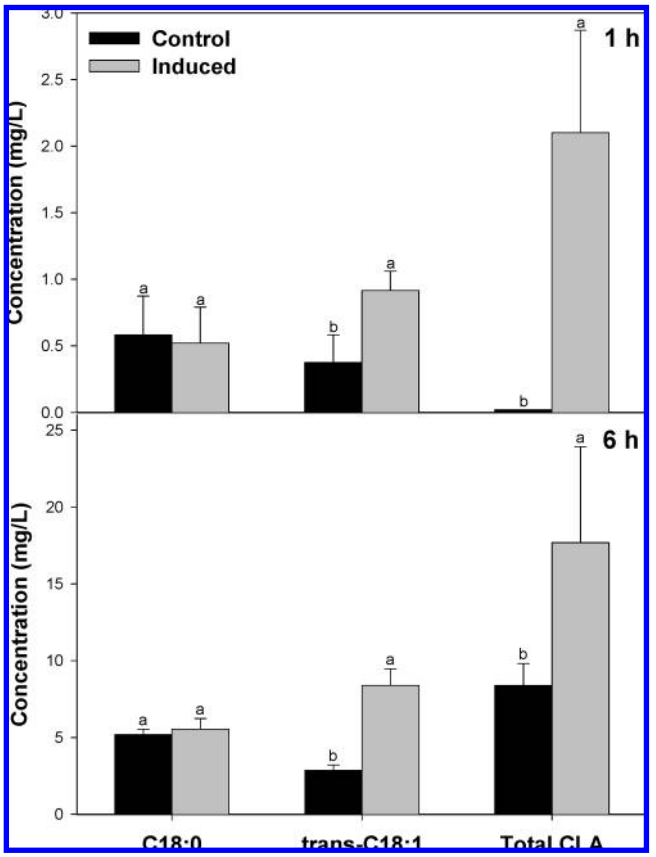

Figure 3. Fatty acid profiles of induced rumen bacterial mixture. Bars with different letters are significantly different in each fatty acid ($p < 0.05$).

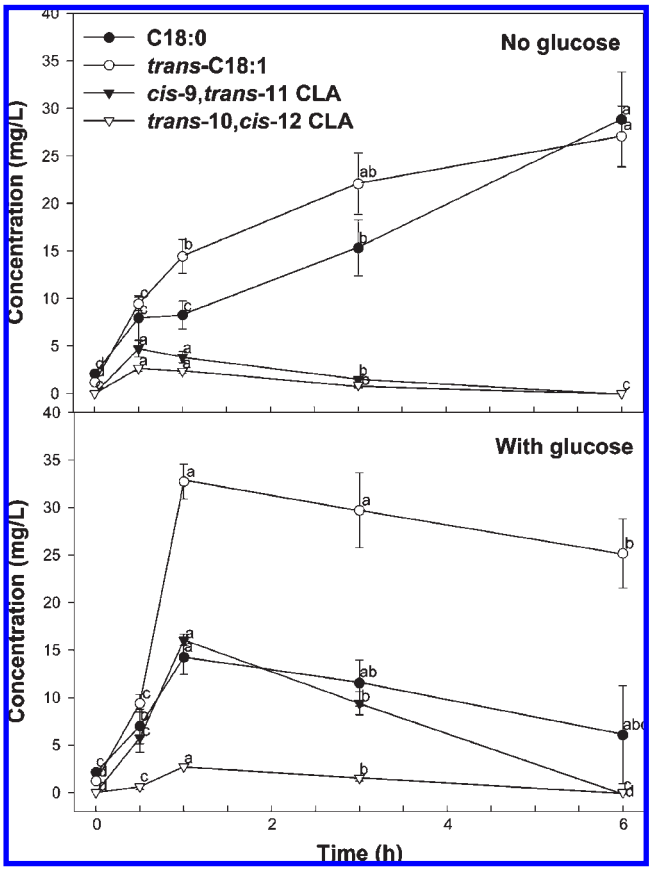

Figure 4. Fatty acid profiles of rumen bacterial mixture with glucose and without glucose supplementation. Values having different letters are significantly different in each fatty acid ($p < 0.05$).

The cells pre-incubated with LA converted LA to biohydrogenated products such as *trans*-C18:1 at a much higher rate than untreated cells, and higher levels of CLA and *trans*-C18:1 were observed over a short incubation time (1 h), indicating that pre-exposure to the substrate increased the level of biohydrogenation. The levels of the final product of biohydrogenation (C18:0) were similar in both conditions, but the ratio of *trans*-C18:1/C18:0 was increased 2.8-fold by substrate induction. Hawke et al. (*21*) noted that the biohydrogenation pathway is completed by two different types of rumen bacteria: bacteria that convert LA → CLA → *trans*-C18:1 (type A) and bacteria that convert CLA → *trans*-C18:1→ C18:0 (type B). The B-type bacteria are likely to be slow in responding to high LA levels as compared to the A-type bacteria, which seem to be more sensitive to LA. LA and CLA were much more toxic than C18:1 or C18:0 to bacterial cells (data not shown). This explains why type A bacteria are more sensitive than type B bacteria to fat substrates. However, feeding high-LA fat sources to cows in the free form failed to increase CLA levels in milk over an extended time (> 3 weeks), indicating that CLA-producing bacteria may not be dominant in the rumen after a long period of adaptation to the fat substrate (*11*).

In the incubation of the control cells without glucose at the LA concentration of 200 mg/L (**Figure 4**), biohydrogenation was less active, and a lower amount of CLA accumulated over a short-term incubation time (1 h). All of the fatty acids except CLA increased throughout the experiments (6 h), and the final C18:0 concentration was about 3 times higher (29 mg/L) than that of the cells incubated with glucose. In the incubation of the cells with glucose, however, all of the biohydrogenation products accumulated at a faster rate, and the concentrations dropped after 1 h of incubation. The concentration of the *cis*-9,*trans*-11-CLA isomer at 1 h of incubation was 3 times higher as compared to the cells without glucose, but the presence of glucose did not affect *trans*-10,*cis*-12-CLA isomer production as significantly as *cis*-9,*trans*-11-CLA isomer production. Both isomers eventually receded to undetectable levels at the end of the incubation time, irrespective of the presence of glucose. The rate of decrease in CLA concentration was slower in the incubation with glucose.

We found an increase in CLA accumulation and biohydrogenation within 1 h, and then CLA declined dramatically thereafter, irrespective of the presence of glucose; higher CLA accumulation, however, was obtained by the addition of glucose. Harfoot et al. (*22*) suggested that the intake of an energy source by cows can be advantageous for reduction steps due to the availability of more reducing equivalents. With a readily utilizable energy source such as glucose, little CLA can accumulate over a long-term incubation due to more active reduction steps. However, bacteria that utilize glucose more efficiently than other bacteria could be selected during incubations with LA, such that they are likely to produce more CLA, especially the *cis*-9,*trans*-11-CLA isomer rather than the *trans*-10,*cis*-12-CLA isomer. This indicates that some *cis*-9,*trans*-11-CLA producers are active glucose utilizers.

In this study, factors affecting biohydrogenation and CLA production were examined under certain artificial conditions to characterize and simulate ruminal CLA production. A high LA concentration (> 100 mg/L) was required to maintain CLA concentration in the rumen during incubation up to 24 h. Pre-incubation with LA was favorable both for the accumulation of CLA and for reduction steps in biohydrogenation. Although the results from the present study do not completely reflect real rumen conditions due to various factors, we could expect that the

accumulation of natural CLA in animal products would be increased by the manipulation of dietary factors such as fat substrate and energy source. In vivo studies under optimized conditions are clearly needed to confirm these results and to better understand the CLA production mechanism.

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