Production of *Trans*-10, *Cis*-12 Conjugated Linoleic Acid by *Megasphaera Elsdenii* YJ-4: Physiological Roles in the Rumen

T. W. Kim[1, a], N. J. Choi[2, a], J. Hwangbo[2], Jih-Tay Hsu[3], Sang S. Lee[4], M. K. Song[5], I. J. Seo[6] and Y. J. Kim*
Department of Food and Biotechnology, Korea University, Chochiwon, Chungnam, 339-700, Korea

ABSTRACT : *Megaspahera elsdenii* YJ-4, which was previously isolated as a producer of *trans*-10*, cis*-12 CLA, was studied for its carbon source on the CLA production. *M. elsdenii* YJ-4, was incubated with glucose and lactose, and cultured in batch and continuous culture systems with linoleic acid at various pHs to investigate CLA production. Batch cultures of the ruminal bacterium, *M. elsdenii* YJ-4, were resistant to stearic acid and linoleic acid, and little growth inhibition was observed even when the fatty acid concentration in the culture was as much as 4 mg ml^{-1}. Stationary phase batch cultures (0.25 mg bacterial protein ml^{-1}) that had been grown on lactate and incubated with linoleic acid (0.20 mg ml^{-1}) produced approximately 12 μg *trans*-10*, cis*-12 CLA mg $protein^{-1}$ and little *cis*-9, *trans*-11 CLA was detected. Some linoleic acid was converted to hydrogenated products (chiefly stearic acid), but these fatty acids were less than 5 μg mg bacterial $protein^{-1}$. Stationary phase batch cultures that had been grown on glucose produced at least 3-fold less *trans*-10*, cis*-12 CLA than ones grown on lactate. Cells from lactate-limited continuous cultures produced less *trans*-10*, cis*-12 CLA than those from batch culture, but only if the pH was greater than 6.4. When the pH of the lactate-limited continuous cultures was lower than 6.4, *trans*-10*, cis*-12 CLA and hydrogenated products declined. Cells from glucose-limited continuous cultures produced less *trans*-10*, cis*-12 CLA and hydrogenated products than the cells that had been limited by lactate, but pH had little impact on this production. These results support the idea that *M. elsdenii* YJ-4 could be one of the major producers of *trans*-10, *cis*-12 CLA which causes cows to produce milk with a low fat content. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 10 : 1425-1429)***

Key Words : Biohydrogenation, Conjugated Linoleic Acid, Fatty acid, *Megasphaera elsdenii*, Rumen

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for different positional and geometric isomers of octadecanoic acid with double bonds in a conjugate configuration. In recent years, research has shown that CLA can inhibit chemically-induced tumors, prevent atherosclerosis and improve the protein to fat ratio of experimental animals (Ha et al., 1987; Lee et al., 1994a, b; Dougan et al., 1997; Choi et al., 2002). CLA is produced in ruminants consuming a diet rich in polyunsaturated fatty acids. The presence of CLA in dairy product is mainly due to isomerization of linoleic acid and linolenic acid during the biohydrogenation by microorganisms in the rumen via a characteristic process called biohydrogenation which is influenced by various dietary factors (Kim, 2003; Wang and Song 2003; Wang et al., 2003). Kepler and Tove (1967) showed that the first step involved in isomerization that created a conjugated fatty acid. It was shown that *B. fibrisolvens* A38 accumulated *cis*-9, *trans*-11 CLA when the substrate fat concentration was high enough to inhibit growth and CLA reduction to further saturated fatty acids (Kim et al., 2000). *B. fibrisolvens* is very sensitive to unsaturated fatty acids (Henderson, 1973), and previous work indicated that *B. fibrisolvens* A38 did not produce *cis*-9, *trans*-11 CLA from linoleic acid until its concentration was high enough to inhibit the growth (Kim et al., 2000). These results are consistent with idea that *cis*-9, *trans*-11 CLA was an intermediate in the conversion of unsaturated fatty acids to less toxic hydrogenated products.

In the 1960's, Davis et al. (1964) noted that dietary oil supplements accentuated the milk fat depression of cattle fed low fiber rations, but abomasal infusions of the same oil did not. These results suggested that milk fat depression was associated with changes in ruminal metabolism rather than a direct effect of the oils on the mammary gland *per se*. These authors also noted that oil supplements increased the amount of *trans*-C18:1 and other isomers of unsaturated fatty acids in the rumen. They concluded that these "unacceptable substrates" might be "rejected" by the mammary gland.

More recently, Gaynor et al. (1994) examined the effect of *trans*- versus *cis*-C18:1 on the milk fat yield of dairy cattle. Neither fatty acids inhibited stearoyl-CoA desaturase or reduced milk fat synthesis. However, Baumgard et al. (2001) demonstrated that abomasal infusion of *trans*-10,

* Corresponding Author: Y. J. Kim. Tel: +82-41-860-1435, Fax: +82-41-865-0220, E-mail: yk46@korea.ac.kr
[1] Department of Animal Science, Cornell University, Ithaca, New York 14853, USA.
[2] National Livestock Research Institute, RDA, Suwon, Kyongki, 441-706, Korea.
[3] Department of Microbiology, Cornell University, Ithaca, New York 14853, USA.
[4] Genebiotech Co., Ltd., Gongju, Chungnam, 314-831, Korea.
[5] Department of Animal Science, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea.
[6] Wootech, Inc.,Youngin, Korea.
[a] First two authors equally contributed to this work.
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cis-12 CLA caused a significant decrease in milk fat yield. These latter results indicated that *trans*-10*, cis*-12 CLA could cause a depression of milk fat, but until recently bacteria responsible for this production had not been identified (Dewhurst et al., 2001; Kim et al., 2002). Fibrolytic rumen bacteria such as *B. fibrisolvens* A38 are typified by high sensitivity to low pH caused by high concentrate diet which selects for starch fermenting ruminal bacteria that produce lactate, but much of this lactate is converted to acetate and propionate by other rumen bacteria, *Megasphaera elsdenii* (Counotte et al., 1981). We hypothesized that lactate utilizing rumen bacteria produce *trans*-10*, cis*-12 CLA and the production could be increased when ruminal fluid was enriched with lactate.

Previous work showed that stationary cultures of *M. elsdenii* YJ-4 that had been grown on lactate produced *trans*-10, *cis*-12 CLA and smaller amounts of *cis*-9, *trans*-11, but the effects of other culture conditions were not examined. The following experiments described the effects of substrate and pH on the CLA production of *M. elsdenii* YJ-4.

MATERIALS AND METHODS

Organism and culture conditions

The isolation, characterization and 16S rDNA sequence of *M. elsdenii* YJ-4 was previously described (Kim et al., 2002). Batch cultures were grown anaerobically at 39°C in a basal medium that contained (per liter), 290 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4$, 64 mg $CaCl_2$, 0.5 g yeast extract, 1.0 g Trypticase, 0.6 g cysteine hydrochloride and 1 mM each volatile fatty acids (isovalerate, isobutyrate, 2-methylbutyrate, and valerate). The basal medium was dispensed anaerobically into tubes (18×150 mm) that were sealed with butyl rubber stoppers and capped with aluminum seals. *M. elsdenii* YJ-4 was also grown with glucose (2 mg/ml) or lactate (4 mg/ml) in continuous culture under O_2-free CO_2 (170 ml culture vessel, 39°C). Yeast extract concentration in the medium of continuous culture was increased to 2.5 g/L to ensure better cell growth. The pH of the continuous culture was decreased by adding concentrated hydrochloride to the medium reservoir. Samples were not taken until at least 4 culture vessel volumes of medium had passed through the continuous culture vessel (98% turnover). Growth was monitored by measuring optical density (600 nm, 1 cm path length, Gilford Spectrophotometer, Oberlin**,** OH) and the relationship between optical density and cell protein was 200 μg protein ml^{-1} optical $density^{-1}$.

Fatty acid analyses

The linoleic acid (100 mg in 10 ml water with 200 mg bovine serum albumin ml^{-1}; Sigma Chemical Co. St. Louis, MO) was filter-sterilized (pore size 0.22 μm), and was added to stationary phase pure cultures to achieve a final concentration of 200 μg linoleic acid ml^{-1}. The bovine serum albumin ensured that the linoleic acid remained in culture media (Kim et al., 2000). Bacterial cell suspensions (10 ml) taken from batch or continuous cultures were incubated with 200 μg linoleic acid ml^{-1} (39°C, 30 min) and immediately extracted with a mixture of organic solvents three times (4 ml, 1 part hexane, 3 parts isopropanol, 1 part acetone, 1 min using a vortex mixer). The suspensions were centrifuged (1,000×g, 3 min, 20°C), the solvent layer (top) was flushed with nitrogen until dry. The fatty acids were dissolved in toluene (1 ml) and methylated as previously described by Kim and Liu (1999). Fatty acid methyl esters were separated by a Supelcowax-10 fused silica capillary column (60 m×0.32 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 model HP3392 integrator. The conditions were: 2.4 ml min^{-1} helium flow; injector 200°C; detector 250°C; the column chamber temperature was initially 40°C (5 min), the column temperature was increased to 220°C at 20°C/min and held for 30 min. Sample (1 μl) containing 0.5-5 μg of linoleic acid or CLA was injected into the column in a splitless mode. Heptadecanoic acid (C17:0) was used as an internal standard and *cis*-9*, trans*-11 octadecadienoic acid and *trans*-10, *cis*-12 octadecadienoic acid were used as a CLA standard (>98% purity Matreya Inc. Pleasant Gap, PA). The recovery of CLA was 83% and C17:0 was 80%. A known standard mixture of fatty acids (Sigma) was used to identify other fatty acids. This protocol was able to separate eight isomers of linoleic acid, but it could not differentiate *cis, trans* versus *trans, cis* configurations in the same position.

Statistical analyses

Data were presented as mean±standard error for at least three replications as a mean values and standard errors of the means. Statistical analyses were conducted using SigmaStat program (Version 8.0; Jandel Corp. San Rafael, CA). Differences among treatments were determined using the Student *t* test ($p<0.05$).

RESULTS AND DISCUSSION

M. elsdenii YJ-4 batch cultures that were grown with either lactate or glucose as a carbon source were relatively resistant to linoleic acid, which serves as substrate for CLA production, and stearic acid, the final product of biohydrogenation. A decrease in growth was not observed until the concentration was 4 mg ml^{-1} (approximately 14

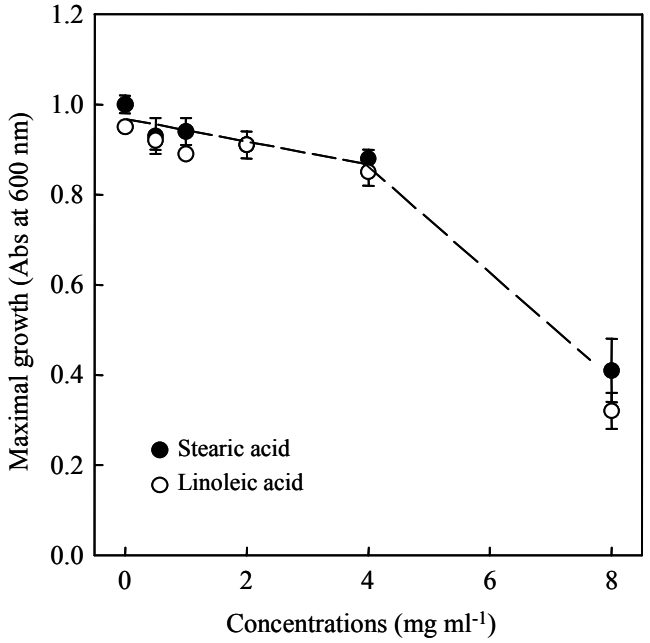

Figure 1. Inhibition of the cell growth of *M. elsdenii* YJ-4 by fatty acids. *M. elsdenii* YJ-4 cells were incubated for 24 h with glucose (20 mM) and stearic acid (●) or linoleic acid (○) at concentrations from 0 to 8 mg ml^{-1} and maximal growth was measured at 600 nm during the incubation.

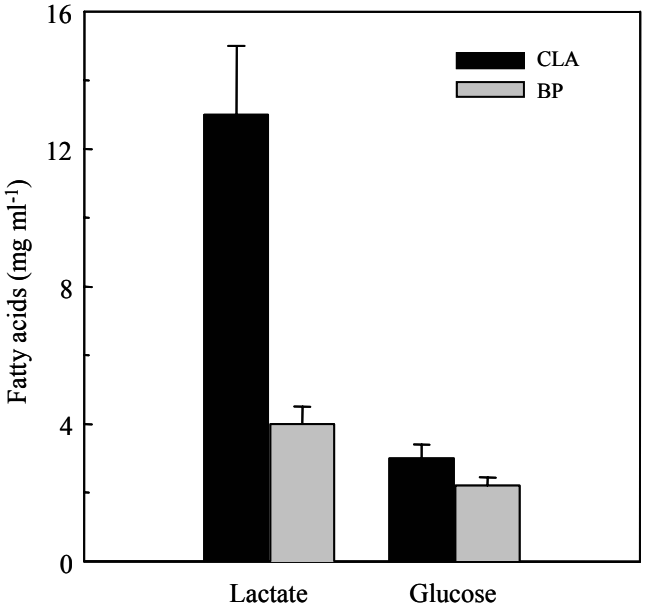

Figure 2. The production of *trans*-10*, cis*-12 CLA (dark bars) by stationary phase *M. elsdenii* YJ-4 cells that were grown in batch culture with lactate (40 mM) or glucose (20 mM) at pH 6.7. The open bars show biohydrogenated products (BP: C18:0 and *trans*-C18:1).

mM) (Figure 1). This indicated that *M. elsdenii* YJ-4 was highly resistant to long chain fatty acids. Indeed, *M. elsdenii* YJ-4 grew rapidly even if the concentration of linoleic acid was 7-fold higher than the amount needed to completely inhibit the growth of *B. fibrisolvens* A38 (Kim et al., 2000). Based on these results, it is unlikely that the biohydrogenation reaction of *M. elsdenii* is a detoxification reaction *per se*, but the reductases of biohydrogenation would serve as sink for reducing equivalents.

M. elsdenii strains are closely related to low G+C Gram positive-bacteria (Stackebrandt et al., 1985), but this bacterium has an outer membrane and is resistant to some potentially toxic compounds (e.g. ionophore and monensin) (Callaway et al., 1999). It has been suggested that unsaturated fatty acids exhibit the inhibitory effect on the growth of various bacteria (Thompson et al., 1994; Kim et al., 2000). Moreover, reducing equivalent disposal can be a problem for anaerobic bacteria in the rumen where oxygen is not available as a terminal electron acceptor. Bacteria with membrane bound hydrogenases transfer reducing equivalents to methanogenic species via interspecies hydrogen transfer. However, some anaerobes must use alternative schemes of oxidation (e.g., lactate and alcohol dehydrogenase), and these schemes ultimately decrease the availability of ATP (Wolin et al., 1997).

Stationary phase *M. elsdenii* YJ-4 batch cultures (0.25 mg bacterial protein ml^{-1}) that had been grown on lactate and were incubated with linoleic acid (0.20 mg ml^{-1}) produced approximately 12 μg *trans*-10*, cis*-12 CLA mg $protein^{-1}$ (Figure 1), and little *cis*-9, *trans*-11 CLA was detected (<2 μg mg $protein^{-1}$) (data not shown). Some linoleic acid was converted to hydrogenated products (chiefly stearic acid), but these acids were less than 5 μg mg bacterial $protein^{-1}$. Stationary phase batch cultures (0.21 mg bacterial protein ml^{-1}) that had been grown on glucose produced at least 3-fold less *trans*-10, *cis*-12 CLA than those that had been grown on lactate (Figure 2; $p<0.05$). The glucose-grown cells tended to produce less hydrogenated products than those grown on lactate, but this trend was not statistically significant ($p>0.05$).

M. elsdenii is capable of producing hydrogen (Hungate, 1966), but lactate is catabolized via a pathway that employs acrylyl-CoA reductase (dehydrase) (Baldwin et al., 1965). Brockman and Wood (1975) provided evidence that this latter reaction was linked to an electron-transferring flavoprotein, but the involvement of this system in biohydrogenation is not known. Glucose carbon is also converted to propionate, but in this case additional reducing equivalents are generated from the Embden Meyerhoff Parnas scheme.

The *M. elsdenii* YJ-4 batch cultures had an initial pH of approximately 6.7, and neither fermentation caused a significant (>0.1 unit) change in final pH (data not shown). Because the batch cultures were buffered by bicarbonate, the pH was difficult to adjust and regulate, but the pH of continuous cultures (dilution rate 0.1 h^{-1}) could be gradually decreased by the addition of concentrated HCl to the

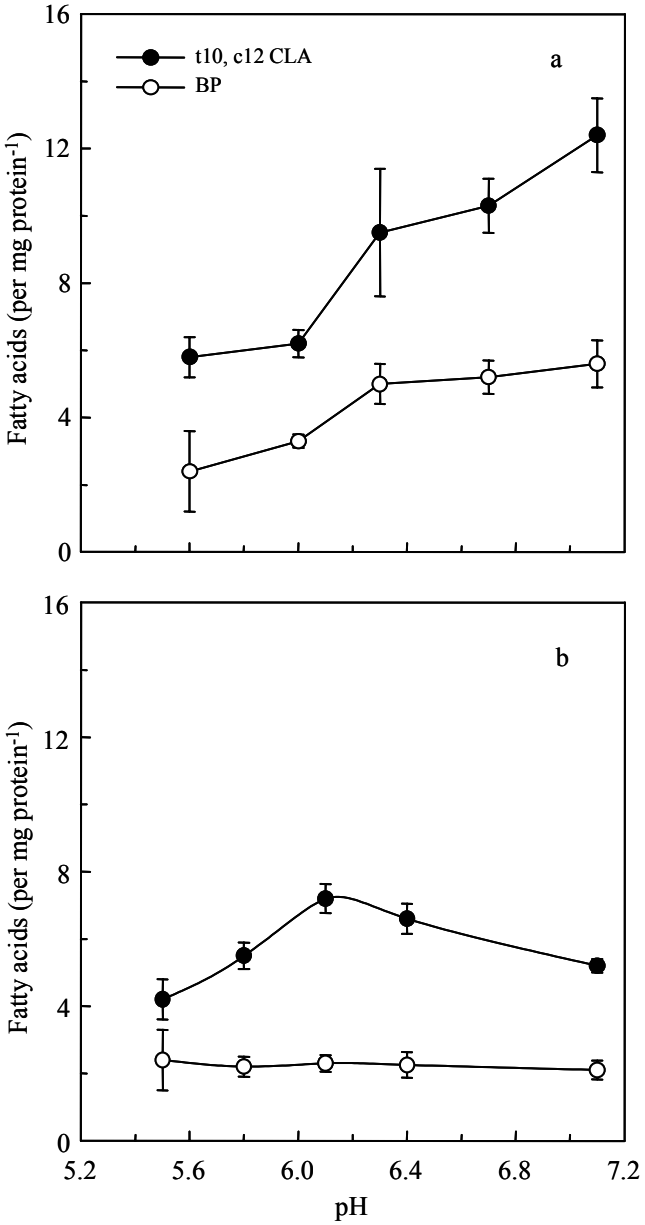

Figure 3. The production of *trans*-10*, cis*-12 CLA (●) and hydrogenated products (HP: C18:0 and *trans*-C18:1) (○) by stationary phase *M. elsdenii* YJ-4 cells that had been grown in continuous culture with lactate (40 mM). pH was decreased by the addition of HCl to the medium reservoir. The production of *trans*-10*, cis*-12 CLA and biohydrogenated products (BP: C18:0 and *trans*-C18:1) by stationary phase *M. elsdenii* YJ-4 cells that had been grown in continuous culture with glucose (20 mM) is shown in part b.

medium reservoir. The continuous cultures reached a steady state after approximately 98% of the original culture medium had been diluted from the culture vessel.

Bacterial cells from glucose-limited continuous cultures produced less *trans*-10*, cis*-12 CLA (Figure 3b) than those from batch culture (Figure 2) when the pH was greater than 6.3 ($p>0.05$). When the pH of the lactate-limited continuous cultures was less than 6.3, the activity of the cells to produce *trans*-10*, cis*-12 CLA declined by half at pH 5.6. The ability of the cells to produce hydrogenated products also decreased ($p<0.05$), and the correlation coefficient was high ($r^2 = 0.86$). Cells from glucose-limited continuous cultures produced less *trans*-10*, cis*-12 CLA than the cells that had been limited by lactate (Figure 3a) ($p<0.05$), and pH had less of an impact compared to that of lactate-limited continuous cultures. The ability of cells from glucose-limited continuous cultures to produce *trans*-10*, cis*-12 CLA was greatest if the pH was 6.1, but the difference between either pH 6.8 and 6.1 or 5.4 and 6.1 was less than 1-fold. Continuous culture pH did not influence the ability of the glucose-limited cells to produce hydrogenated products (Figure 3b) ($p>0.05$).

Fermented silages can contain as much as 10% lactate, and lactate can also be an end-product of ruminal starch and sugar fermentation. Because lactate is a relatively strong acid, ruminal lactate production can cause a significant decrease in ruminal pH. *M. elsdenii* counteracts this decrease by converting lactate to volatile fatty acids that have a significantly higher pK_a value (3.9 versus ≥4.8), and several groups have attempted to use ruminal inoculations with *M. elsdenii* as a tool for increasing ruminal pH. The impact of pH on ruminal fatty acid metabolism has not been examined in a systematic fashion, but it has long been recognized that milk fat depression is generally correlated with an increased concentration of starch in the ration and a reduction in pH (Davis et al., 1964). Based on these earlier results, we had originally thought that acidic pH might enhance the ability of *M. elsdenii* YJ-4 to produce *trans*-10*, cis*-12 CLA, but an opposite effect was observed. When *M. elsdenii* YJ-4 was grown in continuous culture at pH values less than 6.4, the specific rate of CLA production decreased almost 100%.

The success of *M. elsdenii* in the rumen has been linked with tolerance in low pH conditions and its ability to use lactate (Counotte et al., 1981; Miwa et al., 1997), and *M. elsdenii* YJ-4 batch cultures that had been grown on lactate had more than a two-fold greater ability to produce *trans*-10*, cis*-12 CLA than those grown on glucose. The use of batch cultures to model ruminal fermentation is confounded by the fact that substrate concentrations and growth rates are higher *in vitro* than *in vivo*. The rumen tends to operate as a natural continuous culture device (Hungate, 1966), and our results indicated that lactate-limited continuous cultures of *M. elsdenii* YJ-4 also produced more *trans*-10*, cis*-12 CLA than those limited by glucose. The observation that grain feeding promotes the growth of *M. elsdenii in vivo* (Counotte et al., 1981) is consistent with our results that this species survive acidic rumen environment and thus could play some roles in milk fat depression.

We were not able to separate all the isomers of linoleic acid, especially *cis, trans* versus *trans, cis* configurations in

the same position in this study. Further work will be clearly needed to study the effect of each isomer production mechanism as well as to define the role of *M. elsdenii* in ruminal CLA production *in vivo*.

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REFERENCES

Baldwin, R. L., W. A. Wood and R. S. Emery. 1965. Lactate metabolism by *Peptostreptococcus elsdenii*: Evidence for lactyl coenzyme A dehydrase. Biochim. Biophy. Acta. 97:202-213.

Baumgard, L. H., J. K. Sangster and D. E. Bauman. 2001. Milk fat synthesis in dairy cows is progressively reduced by increasing supplemental amounts of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). J. Nutr. 131:1764-1769.

Brockman, H. L. and W. A. Wood. 1975. Acrylyl-coenzyme A. J. Bacteriol. 123:1447-1453.

Callaway, T. R., K. A. Adams and J. B. Russell. 1999. The ability of 'low G+C gram-positive' ruminal bacteria to resist monensin and counteract potassium depletion. Curr. Microbiol. 39:225-230.

Choi, B., S. Kang, Y. Ha, G. Park and R. G. Ackman. 2002. Conjugated linoleic acid as a supplemental nutrient for common carp (*Cyprinus carpio*). Food Sci. Biotechnol. 11:457-461.

Counotte, G. H. M., R. A. Prins, R. H. A. M. Janssen and M. J. A. deBie. 1981. The role of *Megasphaera elsdenii* in the fermentation of DL-(2-13C)-lactate in the rumen of dairy cattle. Appl. Environ. Microbiol. 42:649-655.

Davis, C. L., R. E. Brown and D. C. Beitz. 1964. Effect of feeding high-grain restricted-roughage rations with and without bicarbonates on the fat content of milk produced and proportions of volatile fatty acids. J. Dairy Sci. 47:1217-1226.

Dewhurst, R. J., D. Wadhwa, L. P. Borgida and W. J. Fisher. 2001. Rumen acid production from dairy feeds. 1. Effects on Feed intake and milk production of dairy cows offered grass or corn silages. J. Dairy Sci. 84:2721-2729.

Dougan, M. E. R., J. L. Aalhus, A. L. Schaefer and J. K. G. Kramer. 1997. The effect of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs. Can. J. Anim. Sci. 77:723-725.

Gaynor, P. J., R. A. Erdman, B. B. Teter, J. Sampugna, J., A. V. Capuco, D. R. Waldo and M. Hamsosh. 1994. Milk fat yields and composition during abomasal infusion of *Cis* or *Trans* octadecenoates in Holstein cows. J. Dairy Sci. 77:157-165.

Ha, Y. L., N. K. Grimm and M. W. Pariza. 1987. Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis 8:1881-1888.

Henderson, C. 1973. The effects of fatty acids on pure cultures of rumen bacteria. J. Agric. Sci. 81:107-112.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York.

Kepler, C. R. and S. B. Tove. 1967. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem. 242:5686-5692.

Kim, Y. J. and R. H. Liu. 1999. Selective increase in conjugated linoleic acid in milk fat by crystallization. J. Food Sci. 64:792-795.

Kim, Y. J., R. H. Liu, D. Bond and J. B. Russell. 2000. The effect of linoleic acid concentration on the conjugated linoleic acid (CLA) production of *Butyrivibrio fibrisolvens* A38. Appl. Environ. Microbiol. 66:5226-5230.

Kim, Y. J., R. H. Liu, J. L. Rychlik and J. B. Russell. 2002. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12 isomer of conjugated linoleic acid (CLA) J. Appl. Microbiol. 92:976-982.

Kim, Y. J. 2003. Partial inhibition of biohydrogenation of linoleic acid can increase the conjugated linoleic acid production of *Butyrivibrio fibrisolvens* A38. J. Agric. Food Chem. 51:4258-4262.

Lee, H. S., E. J. Kim, J. S. Cho, H. J. Cho, H. J. Cho, S. K. Lee, K. C. Jung and J. H. Y. Park. 1994a. Conjugated Linoleic Acid Decreases Phosphorylated Akt Levels in Mat-LyLu (MLL) Rat Prostate Cancer Cells. Food Sci. Biotechnol. 13:353-357.

Lee, K. N., D. Kritchevsky and M. W. Pariza. 1994b. Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis. 108:19-25.

Miwa, T., H. Esaki, J. Umemori and T. Hino. 1997. Activity of H(+)-ATPase in ruminal bacteria with special reference to acid tolerance. Appl. Environ. Microbiol. 63:2155-2158.

Stackebrandt, E., H. Pohla, R. Kroppenstedt, H. Hippe and C. R. Woese. 1985. 16S rRNA analysis of *Sporomusa*, *Selenomonas*, and *Megasphaera*: on the phylogenetic origin of Gram-positive eubacteria. Arch. Microbiol. 143:270-276.

Thompson, L., A. Cockayne and R. C. Spiller. 1994. Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori*: a possible explanation of the effect of diet on peptic ulceration. Gut. 35:1557-1561.

Wang, J. H. and M. K. Song. 2003. pH affects the *in vitro* formation of *cis*-9, *trans*-11 CLA and *trans*-11 octadecenoic acid by ruminal bacteria when incubated with oilseeds. Asian-Aust. J Anim. Sci. 16:1743–1748.

Wang, J. H., S. H. Choi and M. K. Song. 2003. Effects of concentrate to roughage ratio on the formation of *trans*-11-$C_{18:1}$ and *cis*-9, *trans*-11-$C_{18:2}$ in rumen fluid and plasma of sheep when fed high oleic or high linoleic acid oil. Asian-Aust. J. Anim. Sci. 16:1604-1609.

Wolin, M. J., T. L. Miller and C. S. Stewart. 1997. The Rumen Microbial Ecosystem (Ed. P. N. Hobson and C. S. Stewart). Blackie Academic & Professional, London, UK. pp. 467-491.