2004

Effect of probiotic bacteria on the conjugated linoleic acid (CLA) content and quality of yogurt

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Effect of probiotic bacteria on the conjugated linoleic acid (CLA) content and quality of yogurt

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

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Iowa State University
Ames, Iowa
2004

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ABSTRACT

Conjugated linoleic acid (CLA) is present primarily in dairy products with many positive nutritional benefits. The predominant CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, function as an anticarcinogen and body fat reducer in animal models, respectively. Other health benefits include roles as an immune system modulator, anti-atherosclerosis agent and antidiabetic agent. Linoleic acid isomerase activity in rumen microorganisms and probiotic bacteria contributes to CLA formation. Free linoleic acid is the primary substrate for linoleic acid isomerase and the biohydrogenation pathway of rumen bacteria. There is strong interest to effectively increase the content of CLA in yogurt and other cultured dairy products using probiotic bacteria and unique processing methods.

The objectives of this study were to 1) evaluate the ability of probiotic bacteria to convert linoleic acid (as the free or esterified fatty acid) to CLA in model systems characteristic of yogurt, and 2) develop yogurts with enhanced CLA content and acceptable quality attributes using probiotic bacteria with CLA-producing activity with and without conventional yogurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus,* 1:1).

The 11 probiotic bacteria evaluated were able to produce CLA from linoleic acid in the model system containing hydrolyzed soy oil (1%) emulsified in milk, but not in model systems of unhydrolyzed soy oil (1%) emulsified in milk or 1% fat milk. The propionibacteria demonstrated the greatest increase in CLA content compared to other lactic acid bacteria in model system.

A yogurt processed with yogurt cultures in conjunction with probiotic bacteria resulted in the greatest formation of the CLA and similar acidity, texture and flavor as the control yogurt.

The combination of *Lactobacillus rhamnosus* with yogurt cultures produced a yogurt with the highest content of CLA and acceptable quality characteristics. The addition of yogurt cultures or rennet greatly increased the microbial counts and CLA content of yogurts processed with propionibacteria at 45 °C. The CLA formation, microbial counts, and quality attributes were stable over a 14-day storage time at 4 °C. Further study showed that the inoculation concentration of *L. rhamnosus* and yogurt cultures did not have significant effect on CLA content and texture, but affected acidity and volatile flavor compounds of yogurts.

These findings indicate that probiotic dairy foods with enhanced CLA content and acceptable quality attributes can be effectively developed. The research results would help the food industry produce a more nutritional product for consumers.

GENERAL INTRODUCTION

Conjugated linoleic acid (CLA) refers to a mixture of conjugated positional and geometric isomers of linoleic acid. As a functional component in dairy products, CLA have shown numerous nutritional benefits in *in vitro* and animal studies. The predominant isomer, *cis*-9, *trans*-11 octadecadienoic acid, functions as an anticarcinogen against several types of cancer (Ha and others 1990; Ip and others 1991; Cesano and others 1998). Another important CLA isomer, *trans*-10, *cis*-12 octadecadienoic acid, is more effective in reducing body fat (Park and others 1999) and protecting body weight (Chin and others 1994). Other health benefits of CLA include the use of CLA as an antiatherogenic agent (Nicolosi and others 1997), an antidiabetic agent (Houseknecht and others 1998), and immune system modulator (Hayek and others 1999). These results have exciting implications for improved human health.

Ruminant food products, especially dairy foods, are the major dietary sources of CLA (Chin and others 1992; Shantha and others 1994). Fermented dairy products often contain higher levels of CLA than nonfermented milk (Shantha and others 1995; Jiang and others 1998; Kim and Liu 2002). Several factors, such as the CLA content of the raw milk, starter cultures, aging time and other processing treatments, have been shown to influence the CLA content of cultured dairy foods (Chin and others 1992; Lin and others 1995, 1999a; Shantha and others 1995).

The consumption of CLA-enriched food products is an effective approach to increase human's CLA intake and obtain the potential benefits of CLA (Chamruspollert and Sell 1999). A primary mechanism for the formation of CLA involves the biohydrogenation

pathway through rumen bacteria. The differences in the activity and amounts of linoleic acid isomerase will affect the activity of isomerization, and subsequently the CLA content of foods (Ha and others 1987; Chin and others 1992, 1993).

Currently, much research has focused on the CLA-producing ability of probiotic bacteria in model systems with linoleic acid. The lipid source, a limiting factor during fermentation of dairy products by probiotic bacteria, should be further investigated. Based on our knowledge, there is no published research reporting the CLA formation and quality attributes of dairy products. Since the differences exist between the model system and dairy food production, the CLA formation of probiotic bacteria might be affected in real dairy products.

This study was conducted to develop a yogurt with increased CLA content produced with probiotic bacteria. The long-term goal is to study the mechanism involved in improving human health through the consumption of CLA-enriched dairy products and to further improve the consumer acceptability of dairy foods. The first project was designed to identify strains of probiotic bacteria commonly used in dairy products that were able to produce CLA in milk model systems with different lipid sources (hydrolyzed soy oil, unhydrolyzed soy oil, or milk fat) at 1% fat content. Probiotic bacteria with the high CLA-forming ability were included as adjunct cultures to yogurt cultures in the next experiment. The second project focused on the incorporation of the probiotic bacteria with the conventional yogurt cultures to evaluate the ability of those probiotic bacteria to produce a yogurt with increased CLA content and acceptable quality attributes. Several processing factors including the interaction of probiotic bacteria with conventional yogurt cultures, inoculation level and storage time

following processing were evaluated to determine their effect on the CLA formation and organoleptic characteristics of the yogurt.

Dissertation organization

This dissertation is composed of a general introduction, a literature review, three papers, and a general conclusion. The literature review includes structure, biological activity, and sources of CLA, formation mechanisms of CLA and factors which affect the content of CLA, and analytical methods for CLA determination. The first paper was published in the Journal of the American Oil Chemists' Society in June 2004. The second paper was submitted to the Journal of Food Science. The third paper will also be submitted to the Journal of Food Science. Following the third paper are general conclusions and a list of references cited in the general introduction and literature review.

LITERATURE REVIEW

Structure of CLA

Linoleic acid (LA) is an 18-carbon fatty acid containing double bonds in the *cis*-9 and *cis*-12 configuration. Conjugated linoleic acid (CLA) refers to a mixture of conjugated positional and geometric isomers of LA. The conversion of LA to one isomer of CLA is shown in Figure 1. Ip and others (1994) identified 9 CLA isomers, which differ in the position and geometric configuration of double bonds (Table 1). The main double bonds of CLA exist at positions 9 and 11, or 10 and 12 (Ha and others 1987). The predominant CLA isomer in foods is *cis*-9, *trans*-11 CLA, which accounts for between 73 to 93% of the total CLA in dairy products (Chin and others 1992; Werner and others 1992; Parodi and others 1996). Chin and others (1992) indicated the *cis*-9, *trans*-11 CLA isomer contributed to more than 83% of the total CLA isomers in natural and processed cheeses. In 1992, Werner and others investigated the level of CLA isomers in 3 types of cheese. The results showed that the *cis*-9, *trans*-11 CLA isomers accounted for 82-88% of the total CLA and the *trans*-9, *trans*-11 and *trans*-10, *trans*-12 CLA isomers accounted for 8-11% of the total CLA. *Trans*-10, *cis*-12 CLA is the other major fatty acid form.

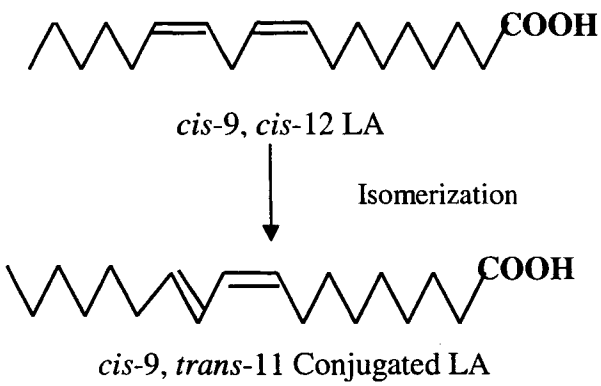

Fig. 1. The conversion of LA to CLA

Table 1. Isomer forms of CLA

Main CLA isomers		
9, 11-isomers[1]	10,12-isomers	11,13-isomers
cis, cis[2]	*cis, cis*	*cis, cis*
cis, trans	*cis, trans*	
trans, cis	*trans, cis*	
trans, trans	*trans, trans*	

[1] The numbers refer to the position of double bonds.
[2] The terms *cis* and *trans* refer to the geometric configuration of the double bonds.

Biological Activity of CLA

As a functional component, conjugated LAs have shown numerous nutritional benefits. *Cis*-9, *trans*-11 CLA was first found to have biological activity because it was incorporated into the phospholipid fraction of cell membranes. This isomer functions as an

anticarcinogen against several types of cancer (Ha and others 1990; Ip and others 1991; Cesano and others 1998). Another bioactive CLA isomer, *trans*-10, *cis*-12 CLA, can act as a body weight protector (Chin and others 1994; Miller and others 1994) and body fat reducer (Park and others 1999). Other health benefits of CLA include roles as an immune system modulator (Hayek and others 1999), a bone metabolism modulator (Watkins 1999), an antiatherogenic agent (Nicolosi and others 1997), and an antidiabetic agent (Houseknecht and others 1998).

The relationship between dietary CLA and arachidonate-derived eicosanoids

Arachidonic acid is the precursor of a group of hormones known as prostaglandins, such as prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and prostaglandin D_2 (PGD_2). The eicosanoids derived from arachidonic acid mediate several processes required for tumorigenesis, including cell proliferation (induction of ornithine decarboxylase and DNA synthesis) and inflammation (local and systemic immune responses) (Belury 1995). PGE_2 suppresses T-lymphocyte proliferation and production of tumor necrosis factor (TNF)-α (Scales and others 1989). PGE_2 has also been reported as a potential agent that stimulates bone formation and resorption (Li and Watkins 1998).

The CLA-induced reduction of phospholipid arachidonate has been suggested to inhibit eicosanoid synthesis and affect tumorigenesis. Since CLA can readily be incorporated into membrane phospholipids, it may compete in elongation and desaturation steps with other polyunsaturated fatty acids (PUFA) that are precursors of arachidonic acid (the precursor of

PGE_2). Thus, eicosanoid biosynthesis is altered due to changes in arachidonic acid synthesis (Abou-El-Ela and others 1989; Leyton and others 1991). Competition between CLA and other PUFAs is likely for the phospholipase, cyclooxygenase, and lipoxygenase enzymes. Dietary CLA modulation of Δ^6-desaturase activity has also been suggested to reduce phospholipid arachidonate so that arachidonate-derived eicosanoids are decreased (Belury and Kempa-Steczko 1997). Since CLA may be elongated and desaturated to 20:4 isomers (Sebedio and others 1997), these isomers can compete with arachidonic acid for cyclooxygenase enzyme and lower PGE_2 in a manner similar to n-3 PUFA. These results explain why CLA is able to reduce basal levels of TNF-like activity (Liu and Belury 1997). Thus, the effects of CLA on arachidonate-derived eicosanoids may significantly affect the development of cancer, immune responses, and bone metabolism.

Carcinogenesis

Conjugated LAs have shown anticarcinogenic effects in many studies. A mutagenesis modulator in fried ground beef was identified by Pariza and others (1983). This substance was suggested to mitigate the harmful effects of low levels of mutagens and carcinogens ingested with daily food consumption (Pariza and Hargrave 1985). In subsequent research, this substance was identified as CLA. The four major CLA isomers were *cis*-9, *trans*-11 CLA, *trans*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, and *trans*-10, *trans*-12 CLA (Ha and others 1987). Belury (1995) showed CLA may regulate carcinogenesis by affecting the different stages of cancer development, such as initiation, promotion, progression, and regression.

Several mechanisms for the anticarcinogenic properties of CLA have been proposed. The antioxidative activity of CLA both *in vitro* and *in vivo* may contribute to its anticarcinogenic effect (Ha and others 1990; Ip and others 1991; Pariza 1991). Free radicals are important in the tumor promotion phase of carcinogenesis (Ha and others 1990; Pariza and Ha 1990; Pariza 1992). CLA functions as a protective agent against the formation of free radicals. It has been shown to be more potent in inhibiting the formation of iron-thiocyanate-induced peroxides (Ha and others 1990) and TBARS (thiobarbituric reactive substances) (Ip and others 1991) than BHT (butylated hydroxytoluene). In female Sprague-Dawley rats using the DMBA (dimethylbenz[a]anthracene)-induced mammary tumor model, although 0.25% CLA in the diet led to maximal antioxidant activity, maximal tumor inhibition was achieved at about 1% CLA (Ip and others 1991). Therefore, the antioxidant effect of CLA may not be the only mechanism in cancer protection.

The regulation of eicosanoid synthesis by CLA also contributes to the anticarcinogenic properties of CLA. Arachidonate-derived eicosanoids can modulate carcinogenesis in the mammary gland (Abou-El-Ela and others 1989), skin (Fischer and others 1989), and colon (Bull and others 1989). PGE_2 is most frequently associated with the enhancement of tumor induction. Dietary CLA (0.5% w/w) was reported to partially displace arachidonate, a precursor of PGE_2, in rat fat pad (Cook and others 1993).

Another proposed mechanism involves the inhibition of enzymes that promote cell proliferation, such as ornithine decarboxylase (ODC) and protein kinase C (Benjamin and others 1990, 1992; Chin and others 1993). As a tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA) can activate ODC and protein kinase C. Increased activity of ODC and protein kinase C results in cell proliferation, which may be associated with tumor promotion

(Parodi 1994). However, CLA inhibits the induction of mouse forestomach ODC and protein kinase C activity induced by TPA (Benjamin and others 1990, 1992).

Conjugated LA also can inhibit the biosynthesis of proteins and nucleotides to suppress cancer cell growth (Shultz and others 1992). When human breast cancer cells were incubated with CLA, significantly less labeled leucine, uridine, and thymidine were incorporated than with control cultures. Similarly, colorectal and melanoma cancer cells incubated with CLA incorporated less labeled leucine (Parodi 1994). The reduction of PGE_2 at the skeletal muscle level has been reported to depress protein degradation (Rodemann and Goldberg 1982). The inhibition effect of CLA on the synthesis of eicosanoid may explain its role on the biosynthesis of proteins and nucleotides.

Skin Cancer

CLA, isolated from fried ground beef, inhibited the initiation of mouse epidermal carcinogenesis induced by DMBA (Ha and others 1987). Compared with control mice, only half as many pappilomas and a lower tumor incidence (20%) were observed when mice were treated with CLA at 7 d, 3 d and 5 min prior to DMBA carcinogen application at doses of 20, 20, and 10 mg, respectively. Cytochrome p450 is necessary for DMBA metabolism *in vitro* to produce its ultimate carcinogenic form. The mechanism for CLA inhibition of the initiation of epidermal tumors by DMBA is partially due to its direct inhibition of microsomal cytochrome p450 enzyme activity. By modifying the carcinogen's metabolism, CLA can inhibit skin carcinogenesis.

CLA also shows an inhibitory effect on tumor promotion. The role of CLA in modulating skin tumor promotion was investigated (Belury 1995). Mice were fed control (no CLA) diets during initiation, and then switched to diets containing various doses of CLA (0-

1.5% w/w) during skin tumor promotion by the phorbol ester TPA. There was an inverse correlation between tumor yield and CLA content in the diet during 22 weeks of TPA treatment. This result suggested that CLA inhibited tumor promotion in a dose-responsive manner. Diets containing 1.0% and 1.5% CLA inhibited tumor yield compared to diets without CLA or 0.5% CLA in skin tumors promoted by 12-0-tetradecanoylphorbol-13-acetate (TPA) in mice (Belury and others 1996). CLA inhibits skin tumor promotion partially through a PGE-dependent mechanism. In addition, the study of multistage carcinogenesis in mouse skin demonstrated that TPA-induced promotion was also associated with increased ODC activity (DiGiovanni 1992). The increased ODC activity resulted in the synthesis of polyamines, which are required to stabilize increased DNA synthesis involved with hyperproliferative cells and tumor promotion (Pegg and others 1986; Clifford and others 1995). Due to the inhibition of ODC activity by CLA, CLA was less able to support hyperproliferation induced by TPA.

Mammary Cancer

CLA has been shown to significantly inhibit mammary carcinogenesis. Ip and others (1991) were the first to report the chemoprotective effect of dietary CLA on mammary carcinogenesis. Dietary CLA at 0.25% or more reduced the formation of TBARS in mammary tissues so that mammary carcinogenesis was inhibited. This result suggested that CLA inhibition of mammary carcinogenesis is partially attributed to the inhibition of oxidative stress. A dose-responsive effect of CLA was observed. Mammary tumor incidence and tumor multiplicity were significantly reduced at a dietary level of 0.5% CLA and inhibited at a dietary level of 1% CLA in female Sprague-Dawley rats using the DMBA-induced (10 mg DMBA in corn oil) mammary tumor model (Ip and others 1994). An inverse

relationship was demonstrated between dietary CLA and oxidation, and between CLA and mammary carcinogenesis. The incorporation of CLA during mammary gland morphogenesis in the rat provided lasting protection against subsequent cancer risk (Ip and others 1994). The proliferative activity of lobuloalveolar units (sites of carcinogenic transformation) was reduced by feeding CLA. By reducing the proliferation of epithelial end bud cells, CLA can render the target cell population less susceptible to carcinogen-induced neoplastic transformation (Ip and others 1994). The effect of dietary fat on anticarcinogenic properties of CLA was further studied. The level (10, 13.3, 16.7 or 20% by weight in the diet) or type of fat (corn oil or lard) in the diet did not affect the cancer inhibitory efficacy of CLA (1%) in the rat (Ip and others 1996).

The role of CLA in inhibiting promotion/progression of mammary carcinogenesis was investigated (Ip and others 1995). Since various doses of dietary CLA administered prior to mammary carcinogen treatment and continued through tumorigenesis inhibit mammary tumor incidence and yield, CLA has also been suggested to modulate the promotion stage of cancer in addition to initiation. Using the methylnitrosourea (MNU)-induced mammary carcinogenesis model, the administration of 1.0% CLA during early post-weaning and puberty was sufficient to reduce subsequent mammary tumorigenesis. Since continuous CLA intake leads to maximal inhibition of tumorigenesis, it is hypothesized that some active metabolites of CLA are necessary to suppress the process of neoplastic promotion / progression (Ip and others 1995). Further study demonstrated that 1% dietary CLA effectively prevented the post-initiation phase (20 weeks) and resulted in 50% reduction in tumor incidence, but had no effect on the initiation period of mammary cancer induced by DMBA in rats (Ip and others 1997). A continuous supply of CLA is necessary for maximal

tumor inhibition in the post-initiation phase of mammary carcinogenesis. In the target organ, an abundant source of CLA plays a critical role in blocking or delaying tumor appearance. Thompson and others (1997) also reported feeding CLA during the time of mammary gland maturation reduced target cell vulnerability to carcinogen-induced transformations.

The growth and mestatic spread of transplantable mammary tumors were studied. Dietary CLA (1.0%) administered 2 weeks prior to inoculation could block both the local growth and systemic spread of human breast cancer to lungs, peripheral blood, and bone marrow (Visonneau and others 1997). However, Wong and others (1997) found that CLA had no significant effect on the growth of established marine mammary tumors.

Colon, Prostate and Lung Cancers

The effects of CLA on tumors were also studied in other sites. The effect of CLA against 2-amino-3-methylimidazo [4, 5-f] quinoline (IQ)-induced colon carcinogenesis was studied (Liew and others 1995). In the colon of the F344 rat, CLA protected against IQ-DNA adducts and colonic aberrant crypt foci. Cesano and others (1998) studied the effect of three different diets (standard, 1% LA or 1% CLA added) on the local growth and mestatic properties of DU-145 human prostatic carcinoma cells in SCID mice. Mice fed the CLA-supplemented diet displayed smaller local tumors and a drastic reduction in lung metastases as compared with standard diet-fed group. However, mice fed LA-supplemented diet resulted in significantly higher local tumor load than the other two groups of mice. Schonberg and Krokan (1995) indicated CLA (control, 10 μg, 20 μg, and 40 μg) showed a dose-dependent reduction in proliferation of the lung adenocarcinoma cell lines. The results showed the CLA-supplemented diet led to smaller local tumors and significantly reduced lung metastasis in mice compared to that for the LA-supplemented diet.

Body fat metabolism

CLA decreases body fat accumulation and increases lean body mass in animals (Pariza and others 1996, 1997). The reason has been assumed that CLA affects the adipocytes (the major site of fat storage) and skeletal muscle cells (the major site of fat oxidation) to play a major role in the body composition changes. The effect of CLA on increased lean body mass is linked to CLA's protection against the catabolic effects of immune stimulation (Cook and others 1993; Miller and others 1994).

One proposed mechanism is that CLA increases the hormone-sensitive lipase activity and enhances lipolysis. CLA also increases the rates of fatty acid oxidation. Dietary CLA supplementation increases carnitine palmitoyltransferase (CPT) activity, which is a rate limiting factor for fatty acid β-oxidation (Pariza and others 1997; Park and others 1997). Mice fed a diet supplemented with CLA (5% corn oil plus 0.5% CLA) enhanced fatty acid β-oxidation in skeletal muscle and fat tissue. CLA also increased hormone-sensitive activity of lipase and free glycerol (22%), and reduced the activity of heparin–releasable lipoprotein lipase (LPL) (-66%), intracellular triacylglycerol concentration (-8%) and glycerol (-15%) compared to controls. The changes in enzyme activity were correlated with the reduction in body fat. In this study, the CLA-fed mice had lower body fat (57% in males, 60% in females) and increased lean body mass (5% in males, 14% in females) compared to controls. Therefore, decreased fat deposition was attributed to increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation in both muscle cells and adipocytes (Park and others 1997).

In the milk fat of dairy cows, a distinct pattern of fatty acids originates from *de novo* fatty acid synthesis, uptake of preformed fatty acids and desaturation of a portion of the long-

chain fatty acids. The addition of the CLA supplement increased the CLA content of milk fat in a dose-dependent manner from 7 mg CLA/g fat at the zero dose to 64 mg CLA/g fat at the high dose of 150 g/d of CLA supplement. However, the milk fat content of lactating cows was greatly reduced when CLA supplement was infused abomasally to by-pass rumen biohydrogenation (Chouinard and others 1999). Specifically, the reduction of milk fat was relatively greater for the short- and medium- chain fatty acids which are partially synthesized *de novo* by the mammary epithelial cells. In addition, the fatty acid ratios, including $C_{14:0}$:$C_{14:1}$, $C_{16:0}$: $C_{16:1}$ and $C_{18:0}$: $C_{18:1}$, were all increased with the CLA supplement. These results might be due to effects of CLA on *de novo* fatty acid synthesis and the desaturation process. More study is necessary to explore whether body fat reduction and muscle mass increase will occur in humans when given CLA.

Further evidence shows CLA-associated body composition changes result from feeding the *trans*-10, *cis*-12 CLA, but not the *cis*-9, *trans*-11 CLA. The *cis*-9, *trans*-11 CLA isomer was not correlated with fat content of milk (Griinari and others 1997; Chouinard and others 1999). However, the *trans*-10, *cis*-12 CLA isomer inhibited the activity or synthesis of key enzymes involved in *de novo* fatty acid synthesis to decrease milk fat synthesis (Baumgard and others 2000). The *trans*-10, *cis*-12 CLA isomer reduced LPL activity in cultured 3T3-L1 adipocytes and enhanced triacylglycerol release from these cells. Both LPL activity and intracellular triglyceride (TG) were reduced in a dose-dependent fashion with addition of *trans*-10, *cis*-12 CLA (Park and others 1999). Thus, the specific CLA isomer, *trans*-10, *cis*-12 CLA, affects fat synthesis in the mammary gland (lactation) and adipose tissue (growth).

Immune function

The immune modulation of CLA may be related to its effect on fat deposition and growth. Immune stimulants stimulate the macrophages to produce cytokines and cause catabolism of muscle cells (Roura and others 1991; Chamblee and others 1992). Lipopolysaccharide (LPS), an endotoxin, stimulates immunity and reduces growth of chicks. However, chicks fed CLA and injected with LPS continued to grow, while those not fed CLA either failed to grow or lost weight following the injection of LPS (Cook and others 1993). CLA decreased arachidonic acid in skeletal muscle so that immune-induced catabolic effects were decreased. Dietary CLA had no adverse effects on immune variables and prevented the catabolic effects on immune stimulation in both chicks and rats (Cook and others 1993; Miller and others 1994). The ability of CLA to prevent endotoxin-induced growth suppression was also studied in mice which were fed fish oil, CLA, or a basal diet. The results showed that anorexia caused by endotoxin injection was effectively prevented by CLA. As a dietary supplement, feeding 0.5% CLA to chicks, rats, and mice decreased body weight loss compared to feeding 0.5% fish oil. Rats and chicks fed CLA had decreased muscle arachidonic acid and reduced muscle degradation compared with the basal-fed controls (Miller and others 1994). Like fish oil, CLA has been suggested to exert an effect on the immune system by altering arachidonic acid formation of cyclooxygenase or lipooxygenase pathway. The change in cell membrane fatty acids alters prostaglandin production and prevents body weight loss (Hellerstein and others 1989).

The effect of CLA on immune regulation is independent of the type of PUFA in the diet. In a study, Sprague-Dawley rats were fed diets containing soybean oil or menhaden oil and safflower oil, with CLA (10 g/kg) or without CLA for 42 days. The effect of CLA on

PGE_2 production in spleen or liver was not affected by the type of PUFA (Turek and others 1998). PUFA and CLA interacted to affect eicosanoid regulation of cytokine production. PUFA, mainly arachidonic acid, are associated with eicosanoid synthesis. CLA possibly interacts with LA and arachidonic acid for incorporation and biological activity in cellular phospholipids (Belury 1995). The interaction between CLA, LA and arachidonic acid influences eicosanoid production and subsequent cellular events. CLA has been reported to change the PUFA composition of phospholipids of cell membranes. So the inhibition of eicosanoid synthesis is caused by CLA-induced reduction of arachidonic acid in phospholipids (Belury 1995; Liu and Belury 1997).

Bone metabolism

A large amount of evidence has indicated that PGE potentially stimulates bone formation (Marks and Miller 1993; Raisz 1993). Bone tissue and cells produce IGF-I (insulin-like growth factor-I) and IGF-II (insulin-like growth factor-II). They are stored in skeletal tissue of vertebrates, such as chickens and humans (Bautista and others 1990; Isgaard 1992; McCarthy and Centrella 1993). Although IGF-I shows lower amounts in bone than IGF-II, IGF-I is under greater regulatory control (McCarthy and others 1991). PGE_2 increased IGF-I transcript and polypeptide contents in rat calvaria cells (McCarthy and others 1991; Schmid and others 1992) and stimulated the expression of mRNA for IGF binding protein-3 (IGFBP-3) to enhance the IGFBP-3 binding affinity to rat calvaria (Schmid and others 1992). The effect of dietary lipids on bone PGE_2 production and bone formation in chicks was studied (Watkins and others 1997). Feeding anhydrous butter oil to chicks produced a moderate level of arachidonic acid and PGE_2 in bone compared with feeding

soybean oil. At a moderate level (10^{-7} M), PGE_2 could stimulate bone formation; but at a higher concentration (10^{-5} or 10^{-6} M), PGE_2 would reduce bone formation. PGE_2 has been recognized as a mediator of bone formation and bone resorption *in vivo* (Marks and Miller 1993). The CLA source in animal fat has been suggested to produce a moderate level of PGE_2.

CLA-derived eicosanoids can regulate the synthesis of arachidonic acid metabolites like PGE_2. So, the effects of PGE_2 on bone formation and resorption may be mediated locally by inducing the biosynthesis of IGF-I. This result explained why animal fats affected bone formation in chicks. Further study on the effects of CLA on tissue fatty acids composition and *ex vivo* PGE_2 formation in rats was conducted (Li and Watkins 1998). Dietary CLA (10 g / kg of the diet) lowered *ex vivo* PGE_2 production in bone organ cultures and has the potential to impact bone formation and resorption. The reduction in PGE_2 by CLA might be due to a competitive inhibition of n-6 fatty acid formation (Sebedio and others 1997). It was also reported that the PUFA and CLA interacted to affect eicosanoid regulation of cytokine production (Turek and others 1998). Therefore, CLA indicates positive effects on bone health by modulating cytokine production.

Antioxidant potential

CLA has been shown to inhibit formation of free radicals *in vivo* and *in vitro*. CLA showed stronger antioxidative activity than α-tocopherol and was as effective as BHT in inhibiting iron thiocyanate–induced peroxide formation (Ha and others 1990). In an *in vivo* rat study (Ip and others 1991), CLA was as effective as vitamin E and BHT in inhibiting the formation of TBARS, a biomarker often used to assess the extent of lipid peroxidation in

biological systems, in the mammary gland. These findings indicated a relationship between dietary CLA, oxidation, and carcinogenesis. The reaction of CLA with peroxyl or hydroxyl radicals, followed by a reaction with oxygen forms an active antioxidant, β-hydroxyacrolein moiety. The resonance enolization of the β-hydroxyacrolein moiety may contribute to its antioxidant activity. The effective chelation of iron by β-hydroxyacrolein derivatives of CLA is another important mechanism to inhibit membrane peroxidation *in vivo* (Ha and others 1990). The antioxidant effect of CLA may contribute to the reduction of atherosclerosis in rabbits (Lee and others 1994) and anticarcinogenic effects (Ha and others 1990; Pariza 1991; Ip and others 1991).

However, some ambiguous conclusions still exist about the antioxidative activity of CLA. The protective effects of CLA (0.25 μM, 0.50 μM, 0.75 μM) on membranes composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) from oxidative modification under conditions of metal ion-dependent or independent oxidation stress were studied (Van den Berg and others 1995). Their results demonstrated that CLA did not act as a free radical scavenger. Since CLA is readily converted to furan fatty acids, the antioxidant activity of CLA may be due to furan fatty acid, which can scavenge free radicals (Yurawecz and others 1995; Okada and others 1996). The antioxidant effect may be due to a metabolite or oxidized form of the fatty acid because CLA was as susceptible to oxidation as LA (Van den Berg and others 1995).

However, CLA and its metabolites have been reported to steadily decrease under oxidative stress and were more susceptible to oxidation than their corresponding methylene–interrupted fatty acids (Banni and others 1998). Decker (1999) reported CLA did not directly inhibit lipid oxidation, but instead altered the fatty acid composition of tissues to increase

oxidative stability. Since the CLA used in the above research was mainly a mixture of isomers, the antioxidant activity of two individual isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, were investigated (Leung and Liu 2000). *Trans*-10, *cis*-12 CLA demonstrated antioxidative activity at all concentrations evaluated (2 mM, 20 mM, and 200 mM). It was a more effective antioxidant than *cis*-9, *trans*-11 CLA and alpha-tocopherol at lower concentrations (2 and 20 mM). The *cis*-9, *trans*-11 CLA only possessed weak antioxidant activity at lower concentrations. At 200 mM, *cis*-9, *trans*-11 CLA functioned as a strong pro-oxidant. However, no pro-oxidant activity was shown for *trans*-10, *cis*-12 CLA. These results suggested that the antioxidative activity of CLA was determined by a balance of antioxidant properties of *trans*-10, *cis*-12 CLA and pro-oxidant properties of *cis*-9, *trans*-11 CLA in different oxidation conditions and concentrations (Leung and Liu 2000).

Atherosclerosis

Some potent antioxidants, such as BHT (Freyschuss and others 2001) and probucol (Kita and others 1987) have shown antiatherogenic effects in rabbits. Since CLA has demonstrated antioxidant activity *in vivo* and *in vitro*, it may act as an antiatherogenic agent in animals.

A study to feed hamsters a hypercholesterolemic diet supplemented with 0.05% CLA was conducted (Nicolosi and others 1993). In CLA-fed hamsters, a significant reduction was observed in plasma total cholesterol (-26%), low density lipoprotein (LDL) cholesterol (-27%), TGs (-28%) and in the formation of fatty lesions on aortic surface compared with hamsters not fed CLA. Similar results were observed by Lee and others (1994) in rabbits. After 12 weeks, the CLA-supplemented diet significantly reduced the LDL cholesterol to

HDL cholesterol ratio, total cholesterol to HDL cholesterol ratio, total and LDL cholesterol, and TGs. The reduced plasma LDL content in the rabbits and hamsters fed CLA would reduce atherosclerosis (Goldstein and Brown 1977). Nicolosi and others (1997) reported that CLA reduced the fatty streak formation in arteries and total serum cholesterol content in hypercholesterolemia hamsters fed CLA (control, 0.06%, 0.11% and 1.1%). Although LA (1.1%) effectively reduced total serum cholesterol and TG levels, CLA (1.1%) was five times more effective in decreasing plaque on arterial walls.

CLA in human body

CLA has been identified in human blood, milk, bile, and duodenal juice (Cawood and others 1983; Fogerty and others 1988). The concentration of *cis*-9, *trans*-11 CLA in human serum and milk, has been shown to be 0.3-0.54% and 0.31-1.25% of total fatty acids, respectively (Fogerty and others 1988). The content of the *cis*-9, *trans*-11 CLA ranges from 2.23 to 5.43 mg / g fat in human milk (McGuire and others 1997).

The majority of CLA in human tissues is due to dietary intake. Fogerty and others (1988) showed that Hare Krishna women who consume greater amounts of butter or ghee produce milk with a higher CLA content (11.2 mg CLA/g fat) than do women consuming a conventional diet (5.8 mg CLA/g fat). Thus, dietary CLA intake can increase the CLA content in human milk. The content of CLA in plasma is also increased by increases of CLA in the diet (Huang and other 1994). The addition of 112 g cheddar cheese (178.5 mg CLA / day) to diets of 9 healthy men daily for 4 weeks enhanced CLA content from 7.1 to 9.6 μmol / L of plasma. The CLA content in plasma or human milk is not significantly altered through increased intake of LA or fish oil (Herbel and others 1998; Jensen and others 1998). Jiang

and others (1999) reported a correlation (r=0.42) between *cis*-9, *trans*-11 CLA in human adipose tissue and milk fat intake. These studies indicated it is necessary to reconsider the positive health properties of milk fat associated with its transfer into CLA.

Endogenous synthesis may also contribute to CLA formation in human tissues. *Butyrivibrio fibrisolvens* has been found in human fecal material (Brown and Moore 1960). Kepler and others (1966) reported this microorganism contains LA isomerase, which converts LA to *cis*-9, *trans*-11 CLA in ruminant animals. So, CLA can be formed through microbial biohydrogenation of dietary LA in the intestine.

Dietary *trans* fatty acids can be converted to CLA to increase CLA content in human plasma. Salminen and others (1998) provided a dairy fat diet to healthy volunteers for the first 5 weeks, then a high *trans* fatty acid diet and a high stearic acid diet for the second 5 weeks. The serum CLA content was significantly increased by 30% for volunteers consuming the *trans* fatty acid diet compared with the dairy fat diet. However, during consumption of the stearic acid diet, the serum CLA decreased to about 50% of that during the dairy fat diet. The increase in serum lipid CLA following increased levels of *trans* fatty acids in the diet was attributed to the desaturation of trans-vaccenic acid (TVA) by Δ^9-desaturase (Corl and others 2001).

Sources of CLA

Major sources of CLA include dairy products and ruminant meats. Animal foods, especially foods from ruminant animals, contain more CLA than vegetable foods. Fats of

lamb, beef, or bovine milk contained ten times higher CLA (0.5 to 1%, CLA / lipid base, w/w) than pork, salmon, and egg yolk (Chin and others 1992). A synthetic CLA mixture consists of 30% *cis*-9, *trans*-11 CLA, 31% *trans*-10, *cis*-12 CLA, 24% *cis*-11, *trans*-13 CLA, and 14% *cis*-8, *trans*-10 CLA (Christie and others 1997). Although CLA could be supplied in a synthetic form, dairy products provided a higher percent (>80%) of *cis*-9, *trans*-11 CLA, the more biologically active isomer (Chin and others 1992; Werner and others 1992).

CLA in ruminant animal products

Dairy products are principal dietary sources of CLA (Table 2). The CLA content in dairy products ranged from 2.9 to 11.3 mg / g fat (Riel and others 1963; Chin and others 1992; Shantha and Decker 1993; Parodi 1996). However, Parodi (1994) found some dairy products from pasture-fed animals may have a CLA content as high as 30 mg / g fat.

The CLA content in dairy products varies considerably. As early as 1963, Riel and others reported that the CLA content of milk often varies widely among herds due to dietary differences. CLA content of the unprocessed raw materials and the final fat content (Ha and others 1989), feed types and dietary regimens (Jiang and others 1996; Stanton and others 1997; Kelly and others 1998; Boylston and others 2002), starter culture and microbial activity (Lin and others 1995; Jiang and others 1998; Kim and Liu 2002; Kishino and others 2002), processing, packaging, and storage (Ha and others 1989; Werner and others 1992; Lin and others 1995; Shantha and others 1995; Lin and others 1998) are among the factors that contribute to variability in the CLA content of dairy products. These factors will be further discussed in Chapter 4.

Ruminant meats are also good dietary sources of CLA. Chin and others (1992) reported that lamb contained the highest amount of CLA (5.6 mg/g fat) in ruminants while veal had the lowest amount (2.7 mg/g fat). Fresh ground beef products ranged from 2.9 mg/g fat to 4.3 mg/g fat (Chin and others 1992). The effects of cooking methods and cooking temperatures on CLA content in beef steaks and ground beef were evaluated by Shantha and others (1994). Raw steaks contained CLA between 3.1 and 8.5 mg/g fat with 57 to 85% *cis*-9, *trans*-11 CLA. They also found freshly cooked rare (60°C) or well-done (80°C) ground beef patties contained 6.6 to 8.2 mg CLA / g fat. No significant differences in CLA occurred among cooking methods or degrees of doneness.

CLA in non-ruminant animal products

Non-ruminant animal products contain less CLA than do ruminant products. In non-ruminant foods, turkey, chicken, pork, and egg yolk contained 2.5, 0.9, 0.6, and 0.6 mg CLA /g fat, respectively (Chin and others 1992). Only the CLA content of turkey is comparable with ruminant foods. Seafood contained 0.3 to 0.6 mg CLA / g fat (Chin and others 1992). The deposition of CLA in the tissues of non-ruminant animals has been attributed to dietary intake or conversion of LA to CLA by bacterial flora (Chin and others 1992). Intestinal microflora in the rat (Chin and others 1992) and other non-ruminants (Chin and others 1994) are able to isomerize LA to CLA. This result demonstrates that rumen bacteria are not the only source for the biosynthesis of CLA (Ip and others 1994).

Table 2. CLA content in dairy products

Dairy products	CLA content (mg / g fat)	Reference
Natural cheese		
Cheddar cheese	3.3	Ha and others 1989
Medium cheddar	4.1	Chin and others 1992
Sharp cheddar	3.6	Chin and others 1992
Sharp Cheddar	4.6	Lin and others 1995
Aged cheddar cheese	5.3	Werner and others 1992
Cottage cheese	4.5	Chin and others 1992
Cottage cheese	4.8	Lin and others 1995
Brick	7.1	Chin and others 1992
Reduced fat Swiss	6.7	Chin and others 1992
Blue	5.7	Chin and others 1992
Mozzarella	4.9	Chin and others 1992
Cream cheese	3.6	Ha and others 1989
Cougar Gold	3.7	Lin and others 1995
Processed Cheese		Lin and others 1995
Cheese whiz	2.1	Ha and others 1989
Cheez whiz™	5.0	Chin and others 1992
Kraft cheese whiz	6.4	Shantha and others 1992
American cheese	5.0	Chin and others 1992
American cheese	4.26	Lin and others 1995
Kraft squeeze cheese	8.9	Shantha and others 1992
Cheese spread	4.02-4.26	Lin and others 1995

Table 2. (Continued)

Dairy products	CLA content (mg / g fat)	Reference
Fluid milk products		
Evaporated milk	3.4-6.4	Lin and others 1995
Whole milk	4.5	Lin and others 1995
2% milk	4.1	Lin and others 1995
Fermented dairy products		
Plain yogurt	4.8	Chin and others 1992
Yogurt	3.8	Lin and others 1995
Butter milk	5.4	Chin and others 1992
Butter milk	4.7	Lin and others 1995
Sour cream	4.6	Chin and others 1992
Sour cream	4.1	Lin and others 1995
Custard style yogurt	4.8	Chin and others 1992
Low fat yogurt	4.4	Chin and others 1992
Frozen yogurt	2.8	Chin and others 1992
Nonfat yogurt	1.7	Chin and others 1992

CLA in plant products

Plant oils are not a major source of CLA in the typical human diet. In plant oils, such as canola, corn, and olive oils, CLA contents range from 0.1 to 0.7 mg CLA / g fat, indicating the low CLA concentration of plant oils (Chin and others 1992). The distribution of individual CLA isomers in plant oils is also different from that in animal fat. Safflower oil contains the highest CLA content of plant oils, but the *cis*-9, *trans*-11 and *trans*-10, *cis*-12

CLA isomers account for 44% and 41% of the total CLA content, respectively. Due to the lack of LA isomerase from ruminal bacteria, LA doesn't specifically form *cis*-9, *trans*-11 CLA. Therefore, the *cis*-9, *trans*-11 CLA isomer is not the predominant CLA isomer form in plant oils. Plant oils contain less *cis*-9, *trans*-11 CLA isomer and more *trans*-10, *cis*-12 CLA isomer than animal fat.

Formation of CLA

The mechanisms of CLA formation

A major way for humans to increase their CLA intake and obtain the potential benefits of CLA is to consume CLA-enriched food products (Chamruspollert and Sell 1999). An understanding of the mechanism of CLA formation and factors, which affect CLA formation is needed to increase the CLA content in foods.

Several mechanisms for the formation of CLA have been proposed. The biohydrogenation pathway involves the isomerization of linoleic and linolenic acids in the rumen to form CLA as shown in Figure 2 (Ha and others 1989; Harfoot and Hazlewood 1997). The *cis*-9, *trans*-11 CLA isomer is identified as an intermediate in the biohydrogenation of LA by a LA isomerase from the bacterium, *Butyrivibrio fibrisolvens*, in the rumen (Kepler and others 1966). The *cis*-9, *trans*-11 CLA undergoes hydrogenation to form TVA. Further hydrogenation forms the end product, stearic acid. The concentration of the intermediate compounds, CLA and TVA, increases with a decrease in the rate of hydrogenation. The differences in the activity and amount of LA isomerase will affect the

activity of isomerization, and subsequently the CLA content of foods (Ha and others 1987; Chin and others 1992, 1993).

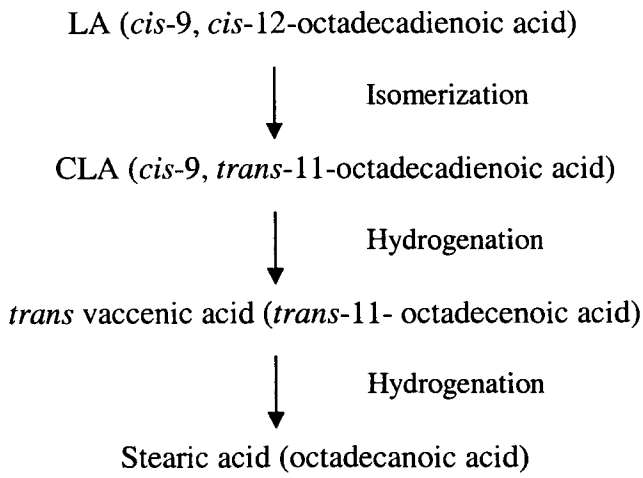

Fig. 2. The biohydrogenation pathway of LA in the rumen (Harfoot and Hazlewood 1997)

Linoleic acid isomerase plays a key role in converting LA to CLA in the biohydrogenation pathway. LA isomerase is specific for free fatty acids with a *cis*-9, *cis*-12 diene configuration (Kepler and Tove 1969; Chin and others 1994; Fellner and others 1997). The ability of enzyme extracts from dairy cultures (*Lactobacillus acidophilus* and *Propionibacterium freudenreichii* subsp. *shermanii*) to isomerize LA into CLA *in vitro* is attributed to the presence of LA isomerase (Lin and others 2002). With *L. acidophilus*, the content of CLA isomers, including *trans*-10, *cis*-12; *cis*-11, *trans*-13; and *cis*-9, *trans*-11 CLA, is greater at pH 5 than at pH 6, 7 and 8. These results suggest that pH 5 is optimal for the activity of LA isomerase. The pH values affect the activity of LA isomerase so that CLA formation is influenced.

Although biohydrogenation of LA has been shown as a major pathway to produce CLA in the rumen, CLA can be endogenously synthesized from ruminally derived *trans*-11 C18:1 by Δ^9-desaturase in the mammary and adipose tissue of lactating cows (Griinari and Bauman 1999; Offer and others 1999; Corl and others 1998). An emulsified mixture of *trans*-11 and *trans*-12 C18:1 fatty acid (25 g / d) was continuously infused into the abomasums of cows. After 3 day infusion, the content of *cis*-9, *trans*-11 CLA increased by 55% compared to the pre-treatment value, but the content of *trans*-11 C18:1 only showed minimal changes (Corl and others 1998). Corl and others (2001) found that infusion of partially hydrogenated vegetable oil, a source of *trans*-11 C18:1, resulted in a 17% increase in *cis*-9, *trans*-11 CLA in the milk fat. However, the content of *cis*-9, *trans*-11 CLA was decreased by 60-65% with the addition of sterculic oil which specifically inhibits Δ^9-desaturase. Since the desaturation of C14:0 by Δ^9-desaturase also forms *cis*-9 C14:1, the formation of *cis*-9 C14:1 was used to estimate Δ^9-desaturase in the presence of sterculic oil. Using this correction factor, endogenous synthesis by Δ^9-desaturase produced 78% of total *cis*-9, *trans*-11 CLA in milk fat and was the major source of *cis*-9, *trans*-11 CLA in milk fat of lactating cows. These results suggest that ruminal formation of *trans*-11 C18:1 is important in developing feeding strategies to produce CLA-enriched milk. A feasible option to increase milk CLA content is to feed supplements containing *trans*-11 C18:1 or dietary management of rumen biohydrogenation to enhance the formation of *trans*-11 C18:1 (Griinari and Bauman 1999).

Another mechanism of CLA formation, proposed by Ha and others (1989), involves a free radical reaction. The LA is first converted to a LA radical, and then the conjugated double bond system is formed with the addition of hydrogen. Heat treatment, atmospheric conditions, and hydrogen donors have been suggested to increase the formation of LA

radicals and facilitate the conversion of LA radicals to CLA (Kosikowski 1982; Ha and others 1989; Shantha and others 1992; Garcia-Lopez and others 1994; Lin and others 1995).

Several methods, such as organic synthesis, microbial fermentation, enzymatic isomerization, or genetic engineering / bioengineering have been used in commercial CLA production (Dunford 2001). As a traditional method, organic synthesis is highly capital intensive and produces isomeric mixture of CLAs. In the alkaline isomerization of LA, LA with *cis*-9, *cis*-12 double bonds is converted to *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. With the extension of time, the *cis*-9, *trans*-11 isomer is further isomerized to the *trans*-8, *cis*-10, and *trans*-10, *cis*-12 isomers, while the *trans*-10, *cis*-12 isomer forms *cis*-9, *trans*-11 and *cis*-11, *trans*-13 isomers (Dunford 2001). Bioengineering can produce CLA as the predominant fatty acid produced in plant oil (Dunford 2001). However, a long time is required to discover the desired genetic traits for developing bioengineered products and consumers may reject genetic modification products. Therefore, a viable alternative is to produce CLA by utilizing microorganisms containing LA isomerase. Biocatalysis by whole microorganisms or purified enzyme can produce CLA isomers with high specificity.

Effects of dietary modification on CLA formation

Individual differences between ruminal animals also contribute to differences in CLA formation. CLA content in milk has been shown to be affected by individual genetic regulation of rumen microflora (Moore and others 1993), individual differences in cow breed (Kelly and others 1998) and individual animal differences to variable diet treatments (Peterson and others 2002). These individual differences affect both rumen biohydrogenation and Δ^9-desaturase activity in the mammary gland through the rumen microflora, rumen pH,

the feeding behavior of the cow, the efficiency of incorporation of CLA into the milk or the conversion of TVA to CLA in the mammary gland.

Several dietary strategies, such as the type of feed or dietary regimen, have been adopted to increase the CLA content of milk fat. The quantity and composition of animal feed affects the microbial population in the rumen. In ruminant tissue and milk, the content and isomer distribution of CLA are modulated through altering the lipid composition related to the formation of CLA or *trans* 18:1, the microbial activity associated with rumen biohydrogenation, and microbial population in the rumen (Vivani 1970).

The feed plays an important role in increasing CLA content in milk fat. The CLA content during the summer is two or three times higher than during the winter (Jahreis and others 1997; Parodi 1999). In the summer, cows fed grass, rich in PUFA, produce a greater content of CLA and TVA in milk than do cows fed maize silage in winter. Pasture feeding produces a higher CLA content in the milk fat than control, which contains hay, corn silage, and concentrates (Jahreis and others 1997; Precht and Molkentin 1997; Kelly and others 1998). There is a linearly positive relationship between the proportion of grazed grass from pasture in the diet of dairy cows and the CLA content of milk (Dhiman and others 1999). In the grazing pasture, linolenic acid is the major dietary unsaturated fatty acids. The biohydrogenation of linolenic acid is suggested to form CLA as an intermediate (Viviani 1970). However, Harfoot and Hazlewood (1988) found linolenic acid biohydrogenation does not produce CLA as an intermediate, but produces *trans*-11 C18:1 as one of final products. The endogenous synthesis of *cis*-9, *trans*-11 CLA occurs from *trans*-11 C18:1 by Δ^9-desaturase (Griinari and others 1997).

The dietary regimen also affects CLA formation. Ecological feeding provides the cows grazing during summer season and silage (clover / alfalfa / grass silage) feeding in other seasons. Ecological feeding produced the highest CLA (0.8%) and TVA (2.67%) concentration in milk fat compared to indoor feeding with silage the whole year and pasture feeding during the summer (Jiang and others 1996). A positive linear correlation has been shown between *cis*-9, *trans*-11 CLA and TVA (r = 0.85) (Jahreis and others 1997). This conclusion is in agreement with results of Jiang and others (1996). This relationship suggests the first two steps in the biohydrogenation pathway (from C18:2 vis *cis*-9, *trans*-11- C18:2 to *trans*-11- C18:1) are not rate-limiting.

Milk CLA content is increased by the addition of dietary oil with a high PUFA content to the diet (Griinari and Bauman 1999; Dhiman and others 2000). Plant oils high in LA, such as sunflower (63% LA), soybean oil (50% LA) and corn oil (50% LA), efficiently increase milk CLA and *trans*-18:1 contents (McGuire and others 1996; Kelly and others 1998; Dhiman and others 2000). These results may be explained because more LA substrate is available for LA isomerase to form CLA. Boylston and Beitz (2002) investigated the effects of feed supplementation on CLA and fatty acid composition of yogurt. The dietary supplementation (5% soy oil and 1% CLA) increased CLA contents of milk by 2.8 fold. Soybean oil supplementation decreased contents of saturated fatty acids and increased *trans*-octadecenoic acids.

Some disadvantages in the dietary supplementation of plant oils have been studied. Free oil, which contains a high content of PUFA, inhibits microbial (particularly cellulolytic) activity in the rumen (Jenkins 1993; Boyaval and others 1995; Jiang and others 1998; Lin and others 1999). As early as 1970, Vivani indicated the microbial population is inhibited by

long-chain fatty acids, in the free or esterified form, in the rumen. The inhibitory effect of fatty acids on the growth of bacteria is influenced by strains, concentration, and availability of fatty acids (Desmazeaud 1996). Some bacterial strains can produce variants that can resist inhibition by fatty acids. In general, intact seeds can effectively decrease the inhibition of fatty acids on bacteria compared to free oil. The higher amounts of plant oils and fish oil decreased ruminal metabolism. The addition of 2% fish oil or 2% soybean oil in the diet decreased milk fat percentages and yield, but increased milk CLA concentrations by 3.2 and 1.9 fold, respectively (Dhiman and others 2000).

An alternative method is to use intact seeds that are processed to provide unsaturated fatty acids for microbial biohydrogenation in the rumen. The partial replacement of corn and soybean meal with soybean oil, linseed oil, raw cracked soybeans, and roasted cracked soybeans showed that the CLA content of cow's milk was increased when free oils, but not full-fat intact seeds, were added to the diet (Dhiman and others 1997). Therefore, the accessibility of lipids to the rumen microorganisms is important to enhance milk CLA content. Although linolenic acid is not a precursor of CLA in the rumen, feeding linseed oil, which is high in linolenic acid, greatly increased the production of rumen TVA, which can be used for CLA synthesis in the mammary gland (Chilliard and others 2000). However, other researchers found the addition of intact feeds to the diets enhanced CLA formation in milk fat. A high-rapeseed-supplemented diet and a low-rapeseed-supplemented diet increased CLA content from 4.8 mg CLA / g fat to 7.9 mg CLA / g fat and 5.2 mg CLA / g fat, respectively (Stanton and others 1997). There was a positive correlation between CLA and *trans*-11 C18:1. The contents of CLA in milk and cheese were increased 1.75 to 2.0 times by the inclusion of full-fat extruded cottonseed (ECS) and full-fat extruded soybeans (ESB),

respectively, compared to the inclusion of soybean meal (Dhiman and others 1999). The ESB and ECS make the oils more readily available for digestion. They also found the milk from the cows fed the ECS diet contained a higher content of C18:0 and a lower unsaturated fatty acid content (C18:1, C18:2 and C18:3) than did cows fed ESB or soybean meal. The reasons may be due to the slightly lower fatty acid content in the ECS diet, different availability of fatty acids in the rumen, or differences in rumen biohydrogenation between the ECS and ESB diets. These results also suggested more complete rumen biohydrogenation for cows fed the ECS diet than for cows fed the ESB diet. Therefore, the milk CLA content can be enhanced by the addition of full fat ESB or ECS to the diets of dairy cows.

The concentration of CLA in milk was greatly increased when cows were fed marine algae (Franklin and others 1999). Abu-Ghazaleh and others (2001) demonstrated that milk protein percentages and the contents of CLA, TVA, and omega-3 fatty acids in milk fat were increased when fish meal (0.5% fish oil from fish meal) completely replaced soybean meal in the diet of lactating cows. When a blend of fish meal and extruded soybeans (0.5% fish oil from fish meal and 2% soybean oil from extruded soybeans) was fed, the content of CLA and TVA was 91-109% greater than the additive effect of fish meal and extruded soybeans (Abu-Ghazaleh and others 2002). Thus, fish oil increased the production of CLA and TVA by stimulating the conversion of linoleic and linolenic acid supplied from other dietary sources, such as extruded soybeans. Feeding lactating dairy cows a blend of 0.5% fish oil from fish meal and 2% soybean oil from extruded soybeans resulted in the greatest increase in CLA content (Abu-Ghazaleh and others 2002).

Effects of CLA as dietary lipid on milk fat content

CLA incorporated into the diet has been shown to reduce the milk fat of cattle (Loor and Herbein 1998; Baumgard and others 1999; Hanson and others 1999). Loor and Herbein (1998) found that CLA concentration in blood plasma at 24 h after first abomasal infusion of 200 g LCLA (1 part LA plus 1 part CLA) to cows was elevated five-fold compared to infusion with 200 g LA. The concentration of CLA in milk increased from 0.5 g / 100 g total fatty acid to 3.3 g / 100 g total fatty acid when time was extended from 0 to 36 h in response to the LCLA infusion. LCLA contained 35 g *cis*-9, *trans*-11 CLA / 100 g total fatty acids and 15 g *trans*-10, *cis*-12 CLA / 100 g total fatty acids. However, from 24 to 72 h, milk fat yield was 34% lower in response to LCLA than LA infusion due to decreased yields of short- and medium-chain fatty acids. Infusion with LCLA resulted in milk with twice the stearic acid content compared to infusion with LA. The oleic acid and arachidonic acid contents in milk declined with the increase of stearic acid content in response to LCLA infusion. These results suggested that CLA may be a potent inhibitor of de novo fatty acid synthesis and desaturation in mammary gland by directly inhibiting the enzymes involved, such as acetyl-CoA carboxylase, fatty acid synthase, Δ^9 stearoyl-CoA desaturase, and Δ^6 desaturase (Loor and Herbein 1998).

Recent studies have shown that *trans*-10, *cis*-12 CLA, but not *cis*-9, *trans*-11 CLA, contributes to a reduction in milk fat. Baumgard and others (1999) reported that 4 day abomasal infusion of *trans*-10, *cis*-12 CLA (10 g / d) resulted in a 44% decrease in milk fat yield, whereas infusing a similar amount of *cis*-9, *trans*-11 CLA had no effect. Further study was conducted to determine the dose effects of CLA on milk fat content. Milk fat yield was decreased by 25, 33, and 50% in response to 5-day abomasal infusion of 3.5, 7.0 and 14.0 g /

day of *trans*-10, *cis*-12 CLA. The fatty acid ratios, such as $C_{14:0}$:$C_{14:1}$, $C_{16:0}$: $C_{16:1}$, $C_{18:0}$: $C_{18:1}$, increased with the infusion of high doses of *trans*-10, *cis*-12 CLA. This result demonstrated that higher doses of *trans*-10, *cis*-12 CLA (7.0 and 14.0 g / day) inhibited Δ^9-desaturase activity so that milk fatty acid composition was changed, but the lower dose *trans*-10, *cis*-12 CLA (3.5 g / day) had no effect (Baumgard and others 2001). Thus, dietary *trans*-10, *cis*-12 CLA inhibited *de novo* fatty acid synthesis and desaturation in the mammary gland. However, the reduced desaturase was not a prerequisite to decrease milk fat yield. For example, a 25% reduction of milk fat was more equally distributed among short- and medium-chain fatty acids (28%), palmitic and palmitoleic acids (35%), and longer-chain fatty acids (36%). Therefore, a substantial reduction in uptake or utilization of preformed fatty acids was also a factor which contributed to the inhibition of milk fat synthesis. T*rans*-10, *cis*-12 CLA has also been found to reduce the expression of fatty acid synthase in cultures of bovine mammary epithelial cells (Loor and Herbein 1998; Matitashvili and Bauman 2000).

Effects of bacteria on CLA formation

The effect of starter culture on CLA concentration has been widely studied in model systems with LA. Lin and others (1999) investigated the CLA-producing ability of six lactic cultures (*Lactobacillus acidophilus, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis,* and *Streptococcus salivarius* subsp. *thermophilus*) in sterilized skim milk with added LA. The results showed LA addition is necessary to produce CLA. CLA was effectively produced at 1000 μg / mL LA by all six bacteria during a 24-h incubation period. Under these conditions, *L.*

acidophilus produced the maximal CLA content (105.5 μg CLA / mL). In the medium with no LA added, CLA concentration only reached 14.5-19.5 μg CLA / mL. Starter cultures did not influence CLA formation without the presence of LA. Ogawa and others (2001) demonstrated that washed cells of *Lactobacillus acidophilus* AKU 1137 under microaerobic conditions could produce CLA from LA. More than 95% of the added LA (5 mg / mL) was converted into CLA after a 4-day incubation with *L. acidophilus* AKU 1137. Linoleic acid was decomposed by washed cells of *L. acidophilus* AKU 1137 into an important hydroxy fatty acid, 10-hydroxy-*cis*-12-octadecenoic acid. The accumulation of hydroxy fatty acid prior to CLA formation and its concomitant decrease with increased formation of CLA suggest that 10-hydroxy-*cis*-12-octadecenoic acid is also an intermediate of CLA production. This result demonstrated that the conversion of LA to CLA is not a one-step isomerization of a nonconjugated diene to a conjugated diene. Kishino and others (2002) found *Lactobacillus plantarum* AKU 1009a could produce high contents of CLA (3.88 mg/mL) in a nutrient medium with 0.06% (w/v) LA after screening 14 genera of lactic acid bacteria. At LA concentration below 0.06%, a lower CLA concentration (0.04 mg/mL) resulted. However, a higher content of LA (0.2%) inhibited the growth of the bacteria and decreased CLA production. Thus, optimal culture conditions are important to enhance CLA concentration. Kim and Liu (2002) reported that *Lactococcus lactis* I-01 showed very high CLA-producing ability compared with the level present in normal milk fat when sunflower oil (SOL; containing 70% LA) was a substrate. The maximal CLA production resulted when *L. lactis* I-01 cells were preincubated with the optimal concentration of SOL (0.1 g / L) in whole milk for 24 hr. The result indicated that esterified LA was an effective substrate for biohydrogenation by *L. lactis* I-01 *in vitro*. Since lactic acid fermentation decreased pH, LA

isomerase was possibly inactivated by lower pH. Potassium phosphate buffer has been found to increase CLA concentration by preventing a pH drop.

However, not all strains of commonly used dairy starter cultures are able to form CLA *in vitro*. Jiang and others (1998) evaluated 19 strains of lactococci, lactobacilli, streptococci, and propionibacteria in *in vitro* systems with free LA. None of the lactic acid bacteria and only *Propionibacterium freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* demonstrated the ability to form CLA.

The content of PUFA may possibly affect the CLA-producing ability of bacteria by inhibiting their growth. The inhibitory effect of free LA on the growth of 19 strains was studied (Jiang and others 1998). Most of the 19 strains of bacteria (lactococci, lactobacilli, streptococci, and propionibacteria) were inhibited upon inclusion of 25 μg / mL LA into the MRS broth. When washed-cell suspensions were incubated anaerobically with more than 350 μM LA or aerobically with 350 μM LA, biohydrogenation and growth of *Butyrivibrio fibrisolvens* A38 were inhibited and the content of *cis*-9, *trans*-11 CLA was significantly increased. When the LA concentration reached 1,800 μM, growth of bacteria was completely inhibited and no CLA formation was detected (Kim and others 2000). Thus, CLA accumulation was due to an inhibition of biohydrogenation. CLA was not a normal end-product of growing cultures. Therefore, if microbial fermentation is used in large-scale production of CLA, the microorganisms' low tolerance to LA and the poor solubility of LA in water are the main concerns. However, Rainio and others (2001) indicated that the growth inhibitory effect of LA could be efficiently reduced when LA was dispersed in a sufficient concentration of polyoxyethylene sorbitan monooleate detergent. At an optimal LA: detergent ratio of 1:15 (w/w) in the whey-based growth medium, *Propionibacterium*

freudenreichii subsp. *shermanii JS* could tolerate at least 1000 μg / mL LA and the conversion of LA to CLA reached 57% - 87%. This result suggests the microbial growth is a key factor to CLA formation in a whey-based system.

The CLA-producing ability of bacteria can also be influenced by additives. Among six evaluated bacteria (*Lactobacillus acidophilus, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis, and S. salivarius* subsp. *thermophilus*), the addition of 60 g / L sweeteners (sucrose, lactose, or fructose) and 10 g / L sodium chloride (NaCl) significantly decreased the *cis*-9, *trans*-11 CLA content compared to the medium with no added sweeteners or NaCl (Lin 2000). Only *L. lactis* subsp. *cremoris* produced a higher CLA content than the control with the addition of fructose. In the presence of sucrose, the CLA formation was strongly inhibited due to the inversion of sucrose into glucose and fructose in an acid medium. The increased solute molecules assisted in lowering the water activity of the product so that growth of bacteria and CLA isomerizing activity of the cultures was lowered (Banwart 1989). Of all 6 lactic acid bacteria evaluated, *L. acidophilus* incubated in skim milk medium containing 60 g / L sweetener and 10 g / L NaCl under aerobic conditions produced the greatest CLA concentration for 24 hr incubation. In the fermented milk products, sweeteners or NaCl can affect the metabolism of starter cultures so that the CLA formation decreases.

Effects of processing and storage on CLA formation

The effect of processing on CLA concentration of dairy products has been widely investigated. CLA concentration has been shown to increase through processing from milk to processed cheese (Ha and others 1989; Shantha and others 1992; Shantha and Decker 1993).

The Cheddar-based processed cheeses have higher CLA concentration than unprocessed cheeses, except for American cheese (Dhiman and others 1999). Processing skim milk into nonfat yogurt and cream into butter increased the CLA concentration 1.2-fold and 1.3-fold, respectively (Shantha and others 1995). However, the CLA content in low-fat yogurt, low-fat and regular ice cream, sour cream, Mozzarella, Gouda and Cheddar cheeses did not show great differences when compared with raw milk. Werner and others (1992) reported that different starter cultures, processing conditions, and aging time showed only negligible effects on the total CLA content of Cheddar cheese but did influence the CLA isomer distribution. Chin and others (1992) also reported that CLA content was comparable in processed and natural cheeses, as well as unprocessed milk.

Heat, presence of oxygen, additives, aging, and packaging have been suggested as important processing factors for CLA formation. Heat treatment and oxygen environment contributed to the enhanced CLA content of processed cheeses. In processed cheese, CLA formation increased at a higher processing temperature (80 °C) (Kanner and others 1987). Increasing the temperature when cheese was processed under nitrogen did not increase CLA content. When cheese was produced under normal atmospheric conditions, CLA content increased with an increase in temperature from 80 to 90 °C. However, heating temperatures of 70 or 85 °C did not show any effect on CLA content (Shantha and others 1992). In this study, lipid peroxides were not detected under either normal atmospheric conditions or nitrogen during processing. Therefore, the LA radicals formed during processing were primarily being converted to CLA instead of lipid peroxides. The atmospheric conditions resulted in a higher CLA content due to the greater formation of oxygen radicals which increased the formation of the LA radical. Under atmospheric conditions, higher

temperatures increased formation of LA and / or oxygen radicals to enhance CLA content. Garcia-Lopez and others (1994) also reported that CLA content increased from 9.5 mg / g of fat in the raw ingredients to 10.7 mg /g of fat in the processed cheese without apparent changes in the isomer distribution. The increase of CLA content was mainly attributed to the heat treatment.

In the free radical oxidation pathway, a LA radical abstracts a proton from a hydrogen donor to produce CLA (Ha and others 1989). Shantha and Decker (1993) found that hydrogen donors (butylated hydroxytoluene, propyl gallate, cysteine, ascorbic acid) could increase the CLA content of processed cheeses. Propyl gallate, the most effective hydrogen donor, increased the total and *cis*-9, *trans*-11 CLA content of processed cheese by 1.59 and 1.42–fold, respectively. As proton donors, cysteine and ascorbic acid gave similar increases in CLA concentration. However, the addition of BHA, tyrosine and lysine produced a lower CLA content than in processed cheese with no additives (Posati and others 1975; Hughes and others 1982). The antioxidant activity of BHA or tyrosine resulted in decomposition, isomerization, or saturation reactions which reduced the CLA content. Ferrous iron also significantly increased CLA concentration in processed cheeses by catalyzing free radical initiation. Compared to processed cheese with no added iron, addition of 500 ppm Fe^{2+} and Fe^{3+} / ascorbate increased total CLA 1.56- and 1.44-fold, respectively (Shantha and Decker 1993).

The effect of the protein component on the CLA concentration of dairy products has attracted much attention in the past couple of decades. Protein additives (sodium caseinate, sweet whey powder, and nonfat dry milk) have been found to increase the CLA content of processed cheeses (Caric and Kalab, 1987; Shantha and Decker 1993). Whey protein

contains relatively high contents of lactalbumin and lactoglobulin that can provide a hydrogen source and facilitate CLA formation (McDermott and others 1987). The most effective dairy additive was sodium caseinate, which increased CLA content 1.65-fold. Nonfat dry milk and whey powder increased CLA concentration 1.57- and 1.56-fold, respectively. Shantha and others (1992) showed CLA concentration in processed cheese was increased by 35% and 19% with a 6% increase of whey protein concentrate (WPC) and its low molecular weight (LMW) fraction, respectively. The CLA concentration was not increased by the high molecular weight (HMW) fraction of WPC. The enzymatic hydrolysis of proteins to LMW fractions also provided better hydrogen donors and facilitated CLA formation (Ha and others 1989; Shantha and Decker 1993). Since LMW in WPC plays a major role in the increase of CLA, whey ultrafiltration permeate can be used to enhance the content of CLA in processed cheese.

Packaging type is another factor that affects CLA content in dairy products. Lin and others (1998) showed that CLA content in canned cheese (3.03 mg / g lipid) was significantly higher than in a vacuum pouch packed cheese (2.70 mg / g lipid) after 6 months of aging. The free radical oxidation mechanism may have contributed to the differences in CLA content. The interior of the canned cheese has more residual air than the pouch-packed cheese so that lipid oxidation, formation of allyl radicals, and protonation of the radicals are enhanced, resulting in increases in CLA formation.

Other factors during dairy processing were also investigated to determine their effect on CLA content. Cheddar-type cheese aged for 3-month produced the highest CLA content (Lin and others 1999). However, when aging time was extended to more than 10 months, the CLA content decreased. Additional aging time resulted in further enzymatic hydrogenation

of CLA to monoenoic and stearic acids (Hughes and others 1982). However, Werner and others (1992) showed aging did not cause changes in the CLA content of Cheddar-type cheeses. Only the CLA isomer distribution was affected by aging. Milling pH is also a factor which affected the CLA concentration of cheese during the aging period (Lin and others 1998). Since lipolytic enzymes showed optimal activity at about pH 6.0 (Kitchen 1985), the milling pH of 5.9 resulted in increases in free fatty acids and produced higher CLA content in cheese than pH 5.5 and 5.7 after 1 month of aging. However, decomposition of CLA isomers occurred with longer aging periods. Therefore, the standard milling pH 5.7 showed maximal CLA value with 3 month of aging.

Storage does not affect the CLA content in dairy products, such as low fat yogurt, regular yogurt, low fat and regular ice cream, sour cream, or cheeses (Shantha and others 1995; Steinhart 1996). Boylston and Beitz (2002) demonstrated that no significant changes of CLA content or fatty acid composition in yogurt were observed after processing and storage for 7 days at 4°C. These results indicated that CLA is a stable component. Therefore, commercial dairy products are possible to provide health benefits to consumers.

Analytical Methods for CLA Determination

Dairy products are the predominant CLA sources. Since milk fat contains up to 400 different fatty acids, which are different in chain length, degree of branching or unsaturation, geometric and positional configuration, there are many challenges in CLA analysis.

Numerous spectroscopic and chromatographic methods have been developed for CLA analysis. Ultraviolet spectroscopy is an early method used to quantitate conjugated acids. CLA absorbs between 230 and 235 nm (Reil 1963; Dormandy and Wickens 1987). However, complex mixtures of fatty acids containing other conjugated fatty acids often interfere with the absorbance of CLA (Shantha and others 1993). This method is also limited because it can not separate all the individual CLA isomers. Therefore, a chromatographic method is needed to get more accurate results about the content of CLA isomers. Although liquid chromatography (LC) has been used in clinical studies to quantitate CLA, most CLA exist as *cis*-9, *trans*-11 CLA isomer in human body fluids and cervical cells (Iverson and others 1984; Green and others 1988). However, LC is not suitable to analyze CLA isomers in food because the CLA isomers are not separated. So gas chromatography (GC) is a common and convenient method for CLA analysis.

GC is routinely used for the analysis of fatty acid due to its rapidity, high resolution and sensitivity. Fatty acids are often converted to fatty acid methyl esters (FAME) to increase volatility and nonpolarity. Lipid extraction is often the first step in CLA analysis. In total lipid extraction, solvent mixtures, such as chloroform / methanol /water, have been successfully used (Folch and others 1957; Blight and Dyer 1959). After extraction of lipid, GC with flame ionization detection has been widely used to analyze FAME. The preparation methods of FAME include acid-catalyzed methylation, base-catalyzed methylation, and basic and acidic reagents methods (AOCS Official Method 1990).

Acid-catalyzed methylation has been extensively used to analyze conjugated dienes in milk and dairy products. The catalysts included boron trifluoride (BF_3) in methanol (Ha and others 1989; Shantha and others 1992; Werner and others 1992; Jiang and others 1996), HCl

(Ha and others 1990; Ip and others 1991), and H_2SO_4 (Wolff 1994; Chardigny and others 1996). Since acid-catalyzed methylation (HCl and H_2SO_4) results in the isomerization of conjugated dienes, BF_3 in methanol (BF_3–MeOH) at room temperature has been recommended as an efficient method to reduce isomerization (Werner and others 1992; Jiang and others 1996). The effects of methylation time (0.5-120 min) and temperature (20-100 °C) on CLA isomerization were studied (Werner and others 1992). The results demonstrated that 14% BF_3–MeOH at room temperature (20 °C) for 30 min effectively methylated the CLA isomers and prevented intraisomerization of CLA isomers. However, the use of BF_3–MeOH for methylation at high temperature (100 °C) for 10, 15 or 20 min resulted in increased intraisomerization of CLA isomers.

Base-catalyzed methylation, such as $NaOCH_3$ or tetramethylguanidine, methylates the TGs but not free fatty acids (Aneja and Muethi 1991). The (trimethylsilyl) diazomethane can partially methylate free fatty acids. However, artifacts (trimethylsilyl CLA esters) and impurities (trimethylsilyl) are generated, which will interfere with the analysis of short-chain fatty acids by GC (Park and others 2001). Our samples contain a high content of free fatty acids and thus, are not suitable for base-catalyzed methylation. Therefore, the acid-catalyzed methylation was selected to analyze CLA.

After methylation, analysis of FAME by GC using long capillary columns is a generally accepted method to separate and identify CLA isomers. The slight differences of columns, temperature program, and concentration have an impact on retention times of FAME. With conventional 25 m Carbowax columns, the methyl ester of *cis*-9, *trans*-11 18:2 will elute in an area of the chromatogram free of potential contaminants. However, this column is unable to separate a wide range of geometrical and positional CLA isomers in

healthy food. Six CLA peaks were first detected in cheese using 60 m Supelcowax-10 fused silica capillary column (Ha and others 1989). Then, the GC resolution of CLA isomers was improved using 100 m CP-Sil88 WCOT fused silica capillary column, and 10 CLA isomers were separated (Kramer and others 1997). The 100 m highly polar cyanosilicone capillary columns have been shown to be the best GC column to analyze the closely related geometric and positional isomers of CLA. Shorter capillary columns (50 and 60 m) are sufficient to separate a mixture of two major CLA isomers (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA). However, they would not resolve the minor CLA isomers in most natural matrices (Kramer and others 2001).

The identification of CLA is often based on comparison of retention times with standards and gas chromatography- mass spectrometry (GC-MS). Some CLA standards for *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are available. GC-MS is also an effective pathway to identify and confirm CLA isomers.

Effect of Lipid Source on Probiotic Bacteria and Conjugated Linoleic Acid Formation in Milk Model Systems

A paper published in the Journal of the American Oil Chemists' Society 81(6):589-595

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Running Title: CLA Formation in Milk Model Systems

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ABSTRACT: The objective of this research was to study the effects of probiotic bacteria, lipid source, and fermentation time on the CLA content of a milk model system. The evaluation of 11 probiotic bacteria showed that they were able to produce CLA from linoleic acid in a model system containing hydrolyzed soy oil (1%) emulsified in milk, but not in model systems of unhydrolyzed soy oil (1%) emulsified in milk or 1% fat milk. *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23 demonstrated the greatest increase in CLA content. *Propionibacterium. freudenreichii* subsp. *shermanii* 51 produced the highest *cis*-9, *trans*-11 CLA content and also produced the greatest increase in *trans*-10, *cis*-12 CLA content as fermentation time was increased from 24 to 48 h. The fermentation of probiotic bacteria for 24 h was often most effective in increasing CLA content. Viable counts of probiotic bacteria increased significantly from 0 to 24 h. These results demonstrated that the content of CLA during the fermentation was primarily dependent on the strain of probiotic bacteria and the lipid sources in the milk model system. This research suggests an efficient approach to produce CLA-enriched cultured dairy products.

KEY WORDS: Conjugated linoleic acid, fermentation time, hydrolyzed soy oil, isomers, probiotic bacteria, cultured dairy products

CLA is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. This new functional component, found in dairy products, has been shown to have numerous nutritional benefits. The predominant isomer, *cis*-9, *trans*-11 CLA, has demonstrated anticarcinogenic activity in animal models (1). *Trans*-10, *cis*-12 CLA, another important isomer, inhibits fat synthesis and deposition in the adipose tissue (2). CLA also functions as an immune system modulator (3), antidiabetic agent (4), and antiatherosclerosis agent (5). These nutritional benefits associated with CLA have contributed to interest in enhancing the CLA content of foods and increasing the daily intake of consumers.

Dairy products are the most important dietary sources of CLA. The CLA content of yogurts, cheeses, and other cultured dairy products ranges from 3.6 to 8.0 mg/g of lipid (6-8). Dahi, an Indian equivalent of yogurt, has a higher CLA content (26.5 mg/g lipid) than raw milk (5.5 mg/g lipid) (9). Variability in the CLA content of cheeses, yogurts, and other commercial dairy products depends on CLA content of raw milk, starter cultures, aging time and other processing treatments (6-8).

A primary mechanism for the formation of CLA is the isomerization of linoleic and linolenic acids through a biohydrogenation process in the rumen. Kepler et al. (10) reported that an isomerase from *Butyrivibrio fibrisolvens* forms *cis*-9, *trans*-11 CLA as an intermediate. Further biohydrogenation reactions result in the formation of vaccenic acid (*trans*-11 octadecenoic acid), elaidic acid (*trans*-9-octadecenoic acid), and stearic acid (octadecanoic acid) (10, 11). A high concentration of linoleic acid or an aerobic condition inhibits the biohydrogenation reaction so that the CLA accumulates (12).

Another CLA production pathway is free radical oxidation of linoleic or linolenic acid during processing. Processing conditions, such as oxygen level, the addition of protein, elevated temperature and aging will affect CLA content. However, yogurts with higher fat content (1%, 2% and 3.25%) showed no significant changes in CLA content during processing (8).

Several studies have focused on the ability of probiotic bacteria to form CLA in model systems. Six lactic cultures (*Lactobacillus acidophilus, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis,* and *Streptococcus salivarius* subsp. *thermophilus*) demonstrated the ability to increase CLA content of model systems of sterilized skim milk and free linoleic acid (13). Kishino et al. (14) showed *Lactobacillus plantarum* produced CLA in a nutrient medium with 0.06% (wt/vol) linoleic acid. Kim and Liu (15) also reported that *Lactococcus lactis* I-01 showed the highest ability among 13 lactic acid bacteria (lactobacilli and lactococci) to produce CLA in model systems with sunflower oil containing 70% esterified linoleic acid as the lipid source. However, of the 19 strains of lactococci, lactobacilli, streptococci, and propionibacteria evaluated, none of the lactic acid bacteria and only *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* demonstrated the ability to form CLA from free linoleic acid in *in vitro* systems (16).

Probiotics are health-promoting bacteria with many potential benefits. These bacteria have been shown to preserve intestinal integrity, mediate the effects of inflammatory bowel diseases, and reduce the risk for colon, liver and breast cancers (17).

The objective of this research was to study the effects of probiotic bacteria, lipid source, and fermentation time on CLA content in a milk model system consisting of 1% fat.

Hydrolyzed and unhydrolyzed soy oil were compared with milk fat to determine the effect of linoleic acid and FFA contents on CLA formation. A yogurt with probiotics and enhanced CLA content would bring the consumers great health benefits. The study would also provide valuable information for the biohydrogenation mechanism of CLA formation.

EXPERIMENTAL PROCEDURES

Treatments. Eleven different strains of probiotic and lactic acid bacteria were selected. *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23 were obtained from the National Collection of Food Bacteria (Reading, England). *Lactobacillus acidophilus* 74-2, *L. casei* 163, *L. plantarum* L2-1, *L. rhamnosus*, *Enterococcus faecium* M74, *Pediococcus acidilactici* and *Bifidobacterium bifidum* 420 were obtained from Danisco Cultor Inc. (Milwaukee, WI). Yogurt culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* 1:1) was obtained from Chr. Hansen's Inc. (Milwaukee, WI). Lactic acid bacteria were grown in Lactobacilli MRS broth (Difco, Detroit, MI) for 15 h at 37°C and the propionibacteria were grown in sodium lactate broth for 20-22 h at 32°C. The sodium lactate broth contained 1% (w/v) tryptic soy broth, 1% (w/v) yeast extract, and 1% (w/v) sodium lactate syrup.

The model milk systems consisted of milk solids with different fat sources: hydrolyzed soy oil, unhydrolyzed soy oil, or milk fat at a 1% fat content. The hydrolyzed soy oil was prepared from soybean oil (Wesson, Conagra Grocery Products Company, Irvine, CA). Soybean oil (20-22 g) was hydrolyzed with ethanolic potassium hydroxide (60 mL of 3 N potassium hydroxide in 3.3% ethanol) at 70°C with stirring for 24 h. The hydrolysis

reaction was neutralized with the addition of 12 N hydrochloric acid (16 mL) and refluxing at 80°C for 3 h. Potassium hydroxide and hydrochloric acid were obtained from Fisher Scientific (Fair Lawn, NJ). The mixture was cooled to 50°C and the upper layer was passed through sodium sulfate to recover hydrolyzed soy oil.

The soy oil (hydrolyzed and unhydrolyzed) was emulsified into a 10% acacia solution at a 1:1 (w/w) ratio to facilitate uniform incorporation of the oil into the milk. Each mixture (with hydrolyzed and unhydrolyzed oil) was homogenized into reconstituted non-fat dry milk (12% solids-not-fat; Hy-Vee Inc., West Des Moines, IA) to obtain a 1% fat content. A 1%-fat milk (Swiss Valley Farm, Co., Davenport, IA) was used for the milk-fat treatment. The model milk systems were autoclaved at 121°C for 15 min, then cooled to room temperature (25°C). The target initial inoculation for all bacteria was 10^7 cfu/mL; actual inoculation levels ranged from 10^6 to 10^7 cfu/mL. Individual containers were prepared for each storage time to avoid disruption of the gel during fermentation. The milk systems were incubated at 37°C for lactic acid bacteria or 32°C for propionibacteria as set gel. The model systems were sampled for lipid, microbiological and acidity analyses at 0, 24 and 48 h.

Lipid analysis. Lipids were extracted from milk model systems using a modified Bligh and Dyer chloroform-methanol extraction method (6). The lipid extracts were hydrolyzed with 1 N NaOH in methanol at 100°C for 15 min and methylated with 14% boron trifluoride in methanol (Alltech Associates Inc., Deerfield, IL) at room temperature for 30 min to prevent intraisomerization of CLA isomers. Heptadecanoic acid ($C_{17:0}$, Sigma Chemical Co., St. Louis, MO) was used as an internal standard. FAME were analyzed on a gas chromatograph equipped with a FID (HP6890, Hewlett Packard Inc., Wilmington, DE) and separated using a CP-Sil 88 column (100 m × 0.25 mm ID; Chrompack, Middelburg,

Netherlands). The sample (1.0 μL) was injected onto the column with a 5:1 split ratio. The temperature of the GC oven was held initially at 30°C for 5 min, increased to 125°C at 10°C/min and held 1 min, increased to 145°C at 2°C/min, increased to 160°C at 1°C/min and held 10 min, and finally increased to 190°C at 2°C/min and held for 10 min. The total run time was 75.5 min. The detector temperature was 225°C. CLA and FAME were identified and quantified by comparison with the retention time and peak areas of CLA standards (NuChek-Prep, Inc., Elysian, MN) and FA standards (Supelco, Inc., Bellefonte, PA). A gas chromatograph-mass spectrometer (Trio 1000, Fisons Instruments, Danvers, MA) with a quadrupole mass analyzer was used to confirm the identity of the FA. The GC conditions were the same as those of the chromatographic analysis. Mass spectrometer conditions were as follows: source electron energy at 70 eV, source electron current at 150 μA, ion source temperature at 220°C, interface temperature at 220°C, source ion repeller at 3.4 V, electron multiplier voltage at 600 V and scan range between 41 and 350 m/z. Mass spectra of the FA compounds were compared to a spectral library (NBS Library) for identification.

Microbiological analysis. The microbial counts were determined by plating serial dilutions of suspensions on MRS agar for lactic acid bacteria and sodium lactate agar for propionibacteria. Buffered peptone water (2%, Difco, Detroit, MI) was sterilized and used for dilution blanks. The plates were incubated under anaerobic conditions at 37°C for 48 h (lactic acid bacteria) or 32°C for 72-96 h (propionibacteria).

pH measurement. The pH of milk model systems was recorded using a digital pH meter (Fisher Scientific, Accumet Model AB15, Pittsburgh, PA). Decreases in pH were expressed as positive values relative to the initial pH.

Statistical analysis. The project was designed as a three-way factorial experiment with probiotic bacteria, lipid source and fermentation time as the main factors. The experiment was replicated two times using different sources of milk and oil. All analyses were conducted in duplicate. Experimental data were analyzed by using ANOVA (mixed linear model procedures) with Duncan's multiple range tests (SAS version 8.2, Cary, NC) with a significance level of 0.05 to determine the main effects and interactions between the main effects.

RESULTS AND DISCUSSION

The 11 different strains of bacteria were selected based on their current applications in dairy products, related literature research demonstrating the effectiveness of probiotic and lactic acid bacteria in CLA formation, and availability of the bacteria from commercial sources. Propionibacteria that have also been reported to enhance the CLA concentration in dairy products were also selected for our study. Conventional yogurt production uses *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*, in a 1:1 ratio, as the starter cultures. The fermentation continues for 4 to 7 hr until a pH of 4.4 to 4.7 is reached. The bacteria evaluated in this study differ in their rate of acid production; thus, fermentation times of 24 and 48 hr were studied to allow the time necessary for the pH to decrease to the pH range of yogurt. Our study focused on a milk model system to identify bacteria with the ability to increase CLA content.

CLA production in milk model systems. Hydrolyzed soy oil provided the best lipid source for all 11 probiotic bacteria to produce CLA (Table 1). This model system, containing FFA, resulted in significantly higher CLA contents than the model systems with esterified fatty

acids. The formation of CLA was accompanied by an 11% decrease in the content of linoleic acid (data not shown). No significant differences in the content of CLA and other FA were noted in the model systems containing the unhydrolyzed soy oil emulsified in nonfat dry milk or milk fat.

Linoleic acid isomerase, which is specific for free linoleic acid (18), catalyzes the formation of CLA from linoleic acid (19). Chin et al. (7) reported that linoleic acid isomerase isolated from *B. fibrisolvens* was able to isomerize linoleic acid of hydrolyzed safflower oil into CLA. Many model systems designed to evaluate the ability of bacteria to form CLA have used free linoleic acid as a lipid source (13, 14, 16). However, with sunflower oil (70% linoleic acid) as the lipid source, esterified linoleic acid was almost as effective as free linoleic acid as a substrate for the formation of CLA by *L. lactis* I-01. The possible reason is *L. lactis* strains showed a high tolerance to sunflower oil and also that biohydrogenation is efficient as a detoxification system for unsaturated long-chain FA (15). Therefore, linoleic acid is the key precursor initiating the biohydrogenation process and promote the formation of CLA.

The limiting factor in determining whether CLA formation will occur during the fermentation of dairy products by lactic acid and probiotic bacteria is the availability of free linoleic acid for the isomerization reaction. Linoleic acid is a relatively minor FA in cow's milk, accounting for approximately 2.4% of the total FA (19). The relatively high content of TAG (97.5%) and low content of FFA (0.027%) in cow's milk (19) suggests that the content of free linoleic acid in milk is inadequate to facilitate the formation of CLA during fermentation unless FFA are produced through the lipase activity of the bacteria. Soy oil contains a high concentration of esterified FFA, which are unable to enter the

biohydrogenation pathway directly. Therefore, only the model system containing hydrolyzed soy oil had a high enough content of FFA to facilitate CLA formation through linoleic acid isomerase.

The 11 lactic acid and probiotic bacteria showed CLA-producing ability only in the model system containing the hydrolyzed soy oil. Bacterial species did have a significant effect on CLA formation. The effect of bacterial species and fermentation time on the formation of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers is presented for the milk model system with hydrolyzed soy oil as the lipid source (Table 2).

Of the lactic acid and probiotic bacteria evaluated in this study, the propionibacteria demonstrated the greatest increase in CLA content. In particular, the total CLA content of the hydrolyzed oil model system fermented with *P. freudenreichii* subsp. *shermanii* 51 was significantly greater than the model system fermented with traditional yogurt cultures (Table 1). *P. freudenreichii* subsp. *shermanii* 51 showed the highest CLA-producing ability, with a *cis*-9, *trans*-11 CLA content of 1.45 mg/g lipid at 24 h. This microorganism also produced significantly higher *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA levels at 48 h fermentation (Table 2). *Propionibacterium freudenreichii* subsp. *shermanii* 56 produced the highest *trans*-10, *cis*-12 CLA content at 24 h fermentation. Other bacteria, such as *P. freudenreichii* subsp. *freudenreichii* 23, *L. rhamnosus* and *P. acidilactici* were not significantly different from the control (yogurt cultures) in their abilities to produce total CLA.

The CLA-forming ability of different bacteria *L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis,* and *Streptococcus salivarius* subsp. *thermophilus* has been evaluated in several model systems. Among them, *L. acidophilus* was most effective in increasing the CLA content in a skim

milk medium containing linoleic acid (13). *Lactobacillus plantarum* AKU 1009a efficiently produced CLA in a medium containing linoleic acid (14). *Lactococcus lactis* I-01 showed high CLA-producing ability in sunflower oil containing esterified linoleic acid (15). Our results were consistent with those reported by Jiang et al. (16), in which *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* were better able to produce CLA from free linoleic acid than were the lactic acid bacteria in *in vitro* systems. In the biohydrogenation pathway for CLA formation, linoleic acid isomerase plays a key role in isomerizing linoleic acid into CLA. Linoleic acid isomerase, which catalyzes CLA formation, has been isolated from *L. delbrueckii* subsp. *bulgaricus* (13), *B. fibrisolvens* (8), *L. acidophilus* and *P. freudenreichii* subsp. *shermanii* (20). Thus, the linoleic acid isomerase activity of bacteria used in the production of cultured dairy products may contribute to differences in CLA formation. Starter cultures, because of the differences in their linoleic acid isomerase activity, have been identified as a factor that affects the CLA content of cultured dairy products.

For most probiotic bacteria, increasing the fermentation time from 24 to 48 h did not increase CLA content. *Propionibacterium. freudenreichii* subsp. *shermanii* 51 produced the most *cis*-9, *trans*-11 CLA (1.45 mg/g lipid) after 24 h fermentation; however, the CLA content remained unchanged as the fermentation time increased to 48 h. Only *L. rhamnosus* and *P. acidilactici* produced more *cis*-9, *trans*-11 CLA with prolonged fermentation. Three microorganisms, *P. freudenreichii* subsp. *shermanii* 51, *L. acidophilus* 74-2 and *L. plantarum* L2-1, produced more *trans*-10, *cis*-12 CLA as fermentation increased from 24 to 48 h. Lin et al. (13) showed prolonging fermentation time from 24 to 48 h did not enhance CLA formation for six lactic acid bacteria in skim milk system containing linoleic acid. Kim and Liu (15) reported

that *L. lactis* I-01 formed more CLA when fermentation time increased from 8 to 12 h. Therefore, the effect of fermentation time on the CLA content was dependent on the species of bacteria and isomer forms of CLA. In general, fermentation time had some impact on CLA formation but was not a key factor in determining the increase in CLA content.

Growth and acid production of probiotic bacteria in the milk model systems. No two-way or three-way interaction effects were found for probiotic bacteria, lipid source, and fermentation time on microbial growth or acid production. The relative growth rates and acid production of the probiotic bacteria were similar for each lipid source and fermentation time.

The growth of bacteria in the model systems with the three different lipid sources increased sharply from the initial count (average 7.05 $\log_{10}$ cfu/mL) during the first 24 h of fermentation. Although the increase in viable counts was slightly lower for the bacteria in the hydrolyzed soy oil model system (1.35 $\log_{10}$ cfu/mL), the increase was not significantly different than for the unhydrolyzed soy oil and milk fat model systems (1.55 – 1.56 $\log_{10}$ cfu/mL). The growth of some lactic acid bacteria (21) and propionibacteria (16) are inhibited by FFA. The inhibitory effect is dependent on the bacterial strains and the levels and availability of FA (22), with some bacteria strains being able to produce variants that can resist inhibition by FFA (23). In model systems, the addition of free linoleic acid (1 to 5 mg/mL) had an antimicrobial effect on the growth of specific probiotic bacteria (13, 16). Propionibacteria (*P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*) that were able to produce higher CLA levels were shown to be more susceptible to inhibition by free linoleic acid than propionibacteria (*P. jensenii* and *P. thoenii*), which produced lower CLA levels (16).

Inhibitory FA must be sufficiently water soluble to reach an effective concentration in the aqueous solution and sufficiently hydrophobic to interact with hydrophobic proteins or lipids on the bacterial cell surface (23). In this study, the hydrolyzed oil, consisting of about 47% free linoleic acid, was emulsified into an acacia solution prior to incorporating into the milk. The use of a hydrolyzed oil and acacia solution to disperse the FFA may contribute to the lack of a significant inhibitory effect of the FFA on microbial growth (24).

The initial 24-h increases in the individual probiotic bacterial counts were significantly different; however, there were no significant increases in microbial counts with an increase in fermentation time from 24 to 48 h (Table 3). *Lactobacillus rhamnosus* showed the highest increase in bacterial growth. The increases in bacterial counts of *E. faecium* M74, *L. casei* 163, *L. acidophilus* 74-2 and *P. freudenreichii* subsp. *shermanii* 23 were not significantly different from that of *L. rhamnosus*. *Bifidobacterium bifidum* 420 showed the lowest change in bacterial counts. Many commercial probiotic yogurts also have been reported to have poor viability, particularly with respect to bifidobacterium strains (25), as was demonstrated in this study. Bifidobacteria are strict anaerobes and require bifidogenic factors for growth (25). Thus, the environment of the model milk systems does not appear to promote the growth of bifidobacteria.

The presence of FFA in the hydrolyzed soy oil model system resulted in a lower initial pH (pH 5.85) than the model systems containing hydrolyzed soy oil (pH 6.37) or milk fat (pH 6.54). Acid production, as indicated by the decrease in pH during fermentation, was significantly affected by the lipid source of the model system. The decrease in pH of the hydrolyzed soy oil model system (0.71 pH units) was significantly less than in the model systems containing unhydrolyzed soy oil (1.17 pH units) or milk fat (1.28 pH units).

However, the final average pH of the model systems with the three different lipid sources was not significantly different. The inhibitory activity of FA was higher at pH 5 than at pH 6 (21). The decreased acid production and reduced growth of the bacteria in the hydrolyzed soy oil model system was attributed to the lower initial pH of the model system and the presence of FFA.

The pH of the milk model systems decreases through the production of lactic acid from lactose during fermentation. Table 4 shows data for the milk model system with hydrolyzed oil as the lipid source. There was a significantly greater decrease in pH when the fermentation time was extended from 24 to 48 h, indicating continued acid production by the bacteria although microbial counts did not change significantly.

Table 4 also showed that for the milk model system with hydrolyzed oil, acid production was greatest for the lactobacillus species and the yogurt culture, resulting in pH ranging from 4.0-4.5. The model system with *L. rhamnosus* resulted in the lowest pH. On the other hand, *B. bifidum* 420 and the propionibacteria produced little acid during the 48-h fermentation period. The genus *Lactobacillus* produces lactic acid as its major fermentation product and is the preferred species to produce lactic acid. *Lactobacillus acidophilus* 74-2 is homofermentative, whereas *L. casei* 163, *L. plantarum* L2-1 and *L. rhamnosus* are facultatively heterofermentative. However, for propionibacteria, lactic acid production in fermentation processes can be an intermediate step in the production of other organic acids, such as propionic acid (26). In the model milk system with these lactic acid bacteria, hexoses are almost exclusively fermented to lactic acid, contributing to a rapid decrease in pH. Thus, differences in the production of acids by probiotic bacteria can contribute to the development of acid and reduction in pH during fermentation.

ACKNOWLEDGEMENTS

Funding for the research was provided in part by the National Dairy Council and the National Aeronautics and Space Administration (NASA) Food Technology Commercial Space Center. This article, of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project Number 3574, was supported by Hatch Act and State of Iowa Funds. The lactic acid and probiotic bacteria were donated by Danisco Cultor Inc. (Milwaukee, WI). The yogurt cultures were provided by Chr. Hansen's Inc. (Milwaukee, WI).

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TABLE 1
The Ability of Probiotic Bacteria to Produce Total CLA[a] (mg CLA/g lipid) in Three 1% Fat Milk Model Systems After 24 h Fermentation[b]

Strain	Lipid sources		
	Hydrolyzed soy oil	Unhydrolyzed soy oil	Milk fat
Propionibacterium freudenreichii subsp. *shermanii* 56	1.71 ± 0.78[bc]	—	+
P. freudenreichii subsp. *shermanii* 51	2.21 ± 0.92[b]	—	+
P. freudenreichii subsp. *freudenreichii* 23	1.32 ± 0.07[cd]	—	—
Lactobacillus acidophilus 74-2	1.06 ± 0.14[cd]	—	—
L. casei 163	1.13 ± 0.21[cd]	—	—
L. plantarum L2-1	1.07 ± 0.28[cd]	+	—
Enterococcus faecium M74	0.63 ± 0.00[d]	—	+
L. rhamnosus	1.30 ± 0.57[cd]	+	—
Pediococcus acidilactici	1.40 ± 0.42[cd]	—	+
Yogurt cultures[c]	1.33 ± 0.14[cd]	—	+
Bifidobacterium bifidum 420	1.04 ± 0.35[cd]	+	—

[a] "+": 0 to 0.2 mg CLA/g lipid, "-": CLA not detected.
[b] Means are duplicate analyses from two replications. Means within columns followed by the same superscript roman letters (b-d) are not significantly different ($P > 0.05$). CLA was not detected for any treatment at 0 h.
[c] *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* (1:1).

TABLE 2
CLA Content (mg CLA/g lipid) of Hydrolyzed Soy Oil Model Systems Fermented with Lactic Acid and Probiotic Bacteria[a]

Bacteria	*cis*-9, *trans*-11 CLA		*trans*-10, *cis*-12 CLA	
	24 h	48 h	24 h	48 h
P. freudenreichii subsp. *shermanii* 56	$0.88 \pm 0.41^{ab,x}$	$0.91 \pm 0.94^{ab,x}$	$0.29 \pm 0.00^{a,x}$	$0.18 \pm 0.11^{ab,x}$
P. freudenreichii subsp. *shermanii* 51	$1.45 \pm 0.69^{a,x}$	$1.40 \pm 0.77^{a,x}$	$0.19 \pm 0.09^{ab,x}$	$0.25 \pm 0.07^{a,x}$
P. freudenreichii subsp. *freudenreichii* 23	$0.66 \pm 0.00^{b,x}$	$0.61 \pm 0.00^{b,x}$	$0.16 \pm 0.07^{ab,x}$	$0.20 \pm 0.14^{ab,x}$
L. acidophilus 74-2	$0.45 \pm 0.14^{b,x}$	$0.45 \pm 0.00^{b,x}$	$0.19 \pm 0.00^{ab,y}$	$0.49 \pm 0.00^{a,x}$
L. casei 163	$0.48 \pm 0.00^{b,x}$	$0.16 \pm 0.05^{b,y}$	$0.22 \pm 0.18^{ab,x}$	$0.16 \pm 0.00^{ab,x}$
L. plantarum L2-1	$0.51 \pm 0.14^{b,x}$	$0.46 \pm 0.21^{b,x}$	$0.16 \pm 0.14^{ab,y}$	$0.36 \pm 0.07^{a,x}$
E. faecium M74	$0.63 \pm 0.00^{b,x}$	$0.73 \pm 0.07^{b,x}$	ND	$0.05 \pm 0.01^{c,x}$
L. rhamnosus	$0.31 \pm 0.22^{b,y}$	$0.67 \pm 0.07^{b,x}$	$0.16 \pm 0.07^{ab,x}$	$0.06 \pm 0.01^{c,x}$
P. acidilactici	$0.30 \pm 0.17^{b,y}$	$0.85 \pm 0.07^{b,x}$	$0.12 \pm 0.08^{b,x}$	$0.06 \pm 0.00^{c,x}$
Yogurt cultures[b]	$0.71 \pm 0.07^{b,x}$	$0.59 \pm 0.07^{b,x}$	$0.19 \pm 0.06^{ab,x}$	$0.15 \pm 0.00^{ab,x}$
B. bifidum 420	$0.46 \pm 0.15^{b,x}$	$0.57 \pm 0.00^{b,x}$	$0.20 \pm 0.14^{ab,x}$	$0.04 \pm 0.02^{c,x}$

[a] Means are duplicate analyses from two replications. Means within columns followed by the same superscript (a-c) roman letters are not significantly different ($P > 0.05$). For each CLA isomer, means within rows followed by the same superscript (x-y) roman letters are not significantly different ($P > 0.05$). CLA was not detected for any treatment at 0 h.
[b] *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* (1:1).

TABLE 3
Growth of Lactic Acid and Probiotic Bacteria in Hydrolyzed Oil Model System After 24 and 48 h Fermentation[a]

Bacteria	Actual viable counts (log_{10} cfu/mL)			Increase in viable counts (log_{10} cfu/mL)	
	0 h	24 h	48 h	24 h	48 h
P. freudenreichii subsp. *shermanii* 56	$7.30 \pm 0.91^{a,x}$	$8.25 \pm 0.88^{abc,x}$	$8.26 \pm 0.86^{abc,x}$	$0.95 \pm 0.03^{abc,x}$	$0.96 \pm 0.05^{cd,x}$
P. freudenreichii subsp. *shermanii* 51	$7.66 \pm 0.18^{a,x}$	$8.36 \pm 0.76^{ab,x}$	$8.75 \pm 0.08^{ab,x}$	$0.70 \pm 0.57^{bc,x}$	$1.09 \pm 0.10^{bcd,x}$
P. freudenreichii subsp. *freudenreichii* 23	$5.37 \pm 1.93^{b,x}$	$6.90 \pm 1.27^{c,x}$	$6.46 \pm 2.06^{c,x}$	$1.53 \pm 0.66^{abc,x}$	$1.09 \pm 0.13^{bcd,x}$
L. acidophilus 74-2	$6.93 \pm 0.00^{ab,z}$	$8.37 \pm 0.20^{ab,y}$	$8.8 \pm 0.11^{ab,x}$	$1.44 \pm 0.20^{abc,x}$	$1.87 \pm 0.11^{abc,x}$
L. casei 163	$7.28 \pm 0.44^{a,y}$	$9.29 \pm 0.02^{a,x}$	$9.59 \pm 0.04^{a,x}$	$2.01 \pm 0.42^{a,x}$	$2.31 \pm 0.40^{a,x}$
L. plantarum L2-1	$7.45 \pm 0.08^{a,y}$	$8.61 \pm 0.13^{ab,x}$	$8.73 \pm 0.08^{ab,x}$	$1.16 \pm 0.06^{abc,x}$	$1.28 \pm 0.16^{bcd,x}$
E. faeciem M74	$7.10 \pm 0.06^{a,y}$	$8.79 \pm 0.06^{ab,x}$	$9.02 \pm 0.42^{ab,x}$	$1.69 \pm 0.00^{abc,x}$	$1.92 \pm 0.35^{abc,x}$
L. rhamnosus	$7.14 \pm 0.36^{a,y}$	$9.02 \pm 0.18^{ab,x}$	$9.15 \pm 0.00^{ab,x}$	$1.88 \pm 0.54^{ab,x}$	$2.01 \pm 0.36^{ab,x}$
P. acidilactici	$7.34 \pm 0.17^{a,y}$	$8.63 \pm 0.04^{ab,x}$	$8.54 \pm 0.16^{ab,x}$	$1.29 \pm 0.21^{abc,x}$	$1.20 \pm 0.01^{bcd,x}$
Yogurt cultures[b]	$6.96 \pm 0.59^{ab,x}$	$7.94 \pm 0.54^{abc,x}$	$8.07 \pm 0.06^{abc,x}$	$0.98 \pm 1.12^{abc,x}$	$1.11 \pm 0.65^{bcd,x}$
B. bifidum 420	$7.03 \pm 0.04^{a,x}$	$7.64 \pm 0.57^{bc,x}$	$7.53 \pm 1.17^{bc,x}$	$0.61 \pm 0.53^{c,x}$	$0.50 \pm 0.77^{d,x}$

[a]Means are duplicate analyses from two replications. Means within columns followed by the same superscript roman letters (a-d) are not significantly different ($P > 0.05$). Means within rows followed by the same superscript roman letters (x-z) are not significantly different ($P > 0.05$).
[b] *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* (1:1).

TABLE 4
Acid Production of Lactic Acid and Probiotic Bacteria in Hydrolyzed Oil Model System After 24 and 48 h Fermentation[a]

Bacteria	Actual pH values			Decrease in pH values	
	0 h	24 h	48 h	24 h	48 h
P. freudenreichii subsp. *shermanii* 56	5.93 ± 0.06^{a,x}	5.51 ± 0.40^{ab,x}	5.36 ± 0.41^{c,x}	0.42 ± 0.34^{ab,x}	0.57 ± 0.35^{de,x}
P. freudenreichii subsp. *shermanii* 51	5.93 ± 0.01^{a,x}	5.52 ± 0.42^{ab,x}	5.16 ± 0.10^{cd,x}	0.41 ± 0.40^{ab,x}	0.77 ± 0.08^{d,x}
P. freudenreichii subsp. *freudenreichii* 23	5.93 ± 0.01^{a,x}	5.92 ± 0.08^{a,x}	5.89 ± 0.01^{a,x}	0.01± 0.00^{b,x}	0.04 ± 0.00^{f,x}
L. acidophilus 74-2	5.77 ± 0.06^{a,x}	5.26 ± 0.21^{ab,x}	4.28 ± 0.18^{f,y}	0.51± 0.15^{ab,y}	1.49 ± 0.11^{ab,x}
L. casei 163	5.77 ± 0.08^{a,x}	4.92 ± 0.06^{b,y}	4.24 ± 0.14^{f,z}	0.85 ± 0.02^{ab,x}	1.53 ± 0.23^{ab,x}
L. plantarum L2-1	5.70 ± 0.04^{a,x}	5.22 ± 0.04^{ab,y}	4.76 ± 0.00^{de,z}	0.48 ± 0.07^{ab,y}	0.94 ± 0.04^{cd,x}
E. faeciem M74	5.89 ± 0.09^{a,x}	5.35 ± 0.09^{ab,y}	5.11 ± 0.15^{cd,y}	0.54 ± 0.18^{ab,x}	0.78 ± 0.24^{d,x}
L. rhamnosus	5.80 ± 0.22^{a,x}	4.80 ± 0.26^{b,y}	3.84 ± 0.15^{g,z}	1.00 ± 0.48^{a,x}	1.96 ± 0.07^{a,x}
P. acidilactici	5.93 ± 0.06^{a,x}	5.57 ± 0.02^{ab,y}	5.45 ± 0.06^{bc,y}	0.36 ± 0.04^{ab,x}	0.48 ± 0.12^{def,x}
Yogurt cultures[b]	5.83 ±0.21^{a,x}	4.92 ± 0.78^{b,x}	4.48 ± 0.27^{ef,x}	0.91 ± 0.99^{ab,x}	1.35 ± 0.48^{bc,x}
B. bifidum 420	5.88 ± 0.04^{a,x}	5.87 ± 0.01^{a,x}	5.81 ± 0.01^{ah,x}	0.01± 0.02^{b,x}	0.07 ± 0.04^{ef,x}

[a]Means are duplicate analyses from two replications. Means within columns followed by the same superscript roman letters (a-g) are not significantly different (P > 0.05). Means within rows followed by the same superscript roman letters (x-z) are not significantly different (P > 0.05).
[b] *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* (1:1).

Production of Conjugated Linoleic Acid in Yogurt Products through Incorporation of Probiotic Bacteria

A paper to be submitted to the Journal of Food Science

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ABSTRACT: The effect of probiotic bacteria and other processing parameters on the formation of CLA in yogurt was determined. Four probiotic bacteria, *Lactobacillus rhamnosus*, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23, were evaluated alone or in conjunction with traditional yogurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). The yogurt used hydrolyzed soy oil as the lipid source and was incubated at 45 °C. *L. rhamnosus*, either alone or in conjunction with yogurt culture, resulted in the highest content of CLA. The yogurt processed with the propionibacteria alone had a lower CLA content than the control yogurt. The growth of propionibacteria was inhibited at 45 °C. The addition of yogurt cultures or rennet greatly increased the microbial counts and CLA content of yogurts processed with propionibacteria at 45 °C. Inoculation concentration of *L. rhamnosus* and yogurt cultures had no significant effect on CLA content of yogurts. The yogurt produced by *L. rhamnosus* in conjunction with yogurt culture with 10^7 CFU/mL total inoculation level resulted in the high content of CLA. In general, the CLA content and microbial counts of yogurts were stable over a 14-day storage time at 4 °C.

Keywords: conjugated linoleic acid, yogurt, probiotic bacteria, propionibacteria, microbial counts

Introduction

Conjugated linoleic acid (CLA) refers to a mixture of conjugated positional and geometric isomers of linoleic acid. The nutritional benefits of CLA have been demonstrated *in vitro* and in animal studies. The predominant CLA isomer, *cis*-9, *trans*-11 octadecadienoic acid, functions as an anticarcinogen in animal models (Ip and others 1994; Corl and others 2001). Another important CLA isomer, *trans*-10, *cis*-12 octadecadienoic acid, can reduce body fat in mice (Park and others 1997). Other health benefits of CLA include roles as an antiatherogenic agent (Nicolosi and others 1997), an antidiabetic agent (Houseknecht and others 1998), immune system modulator (Hayek and others 1999), and body weight protector (Chin and others 1994). These results have exciting implications for improved human health and development of functional food products.

Among all food products, ruminant products, especially dairy products, are the richest sources of CLA (Chin and others 1992; Shantha and others 1994). Fermented dairy products often contain higher levels of CLA than nonfermented milk (Aneja and Murthi 1990; Shantha and others 1995; Jiang and others 1998; Kim and Liu 2002). The CLA content of cultured dairy products is influenced by the CLA content of the raw milk, starter cultures, aging time and other processing and production conditions (Ha and others 1989; Chin and others 1992; Lin and others 1995, 1999a; Shantha and others 1995).

The biohydrogenation pathway has been proposed to be a major mechanism for CLA formation. The *cis*-9, *trans*-11 CLA isomer is identified as an intermediate in the biohydrogenation of linoleic acid by a linoleic acid isomerase from the bacterium, *Butyrivibrio fibrisolvens*, in the rumen (Kepler and others 1966). The *cis*-9, *trans*-11 CLA undergoes hydrogenation to form *trans*-vaccenic acid. Further hydrogenation forms the end

product, stearic acid. Linoleic acid isomerase activity of rumen microorganisms and probiotic bacteria, including *Butyrivibrio fibrisolvens, Lactobacillus acidophilus* and *P. freudenreichii* subsp. *shermanii*, contributes to CLA formation (Kepler and others 1966; Lin and others 2002). Free linoleic acid is the primary substrate for linoleic acid isomerase and the biohydrogenation pathway of rumen bacteria (Kepler and Tove 1969).

Probiotic bacteria, including lactococci, lactobacilli, streptococci and propionibacteria, contributed to the formation of CLA in model systems with linoleic acid as the substrate (Lin and others 1999b; Jiang and others 1998; Kishino and others 2002). Our previous investigation identified several probiotic bacteria that produced increased levels of CLA in a model system containing hydrolyzed soy oil emulsified in non-fat dry milk. These bacteria included *Lactobacillus rhamnosus*, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23 (Xu and others 2004).

The objective of this research was to develop a CLA-enriched yogurt product with probiotic bacteria with the demonstrated ability to form CLA (Xu and others 2004). Yogurt was produced with hydrolyzed soy oil, 4 probiotic bacteria (*Lactobacillus rhamnosus*, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23), and traditional yogurt culture under typical yogurt processing conditions. The effect of incubation temperature, hydrolysis of casein and inoculation concentration on CLA formation was also determined.

Materials and Methods

Bacteria

Lactobacillus rhamnosus, *P. freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51, and *P. freudenreichii* subsp. *freudenreichii* 23 were selected to evaluate CLA formation of yogurt produced with the selected bacteria alone or in conjunction with traditional yogurt cultures (1:1 ratio of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*; YC-180 (YC), Chr. Hansen, Milwaukee, WI). The combination of probiotic bacteria and the yogurt culture was at a 1:1 ratio. *L. rhamnosus* (LB) was obtained from Danisco Cultor Inc. (Milwaukee, WI). *Propionibacterium freudenreichii* subsp. *freudenreichii 23* (PFF-23), *P. freudenreichii* subsp. *shermanii 56* (PFS-56) and *P. freudenreichii* subsp. *shermanii 51* (PFS-51) were obtained from Dr. Bonita Glatz's Collection (Iowa State University, Ames, IA) and stored at – 80 °C. Lactic acid bacteria were activated in Lactobacilli MRS broth (Difco, Detroit, MI) for 15 h at 37 °C and the propionibacteria were activated in sodium lactate broth for 20-22 h at 32 °C. The sodium lactate broth contained 1% (w/v) tryptic soy broth (Becton Dickinson and Company, Cockeysville, MD), 1% (w/v) yeast extract (Becton Dickinson and Company, Sparks, MD), and 1% (w/v) sodium lactate syrup (Fisher Scientific, Fair Lawn, NJ).

Processing of yogurt with hydrolyzed soy oil

Soybean oil (Wesson, Conagra Grocery Products Company, Irvine, CA) was chemically hydrolyzed to increase the content of free fatty acids and emulsified into a 10% acacia solution at a 1:1 (w/w) ratio (Xu and others 2004). The mixture was homogenized into

skim milk (Hy-Vee Inc., West Des Moines, IA) to produce a 1% fat content. The milk solids-not-fat content was adjusted to 12% through the addition of non-fat dry milk (Hy-Vee Inc., West Des Moines, IA). The mixture was heated at 85 °C for 30 min, cooled to 45 °C, and inoculated with probiotic bacteria and/or yogurt cultures and incubated as designated in the following experiments.

Experiment 1: Effect of probiotic bacteria on CLA formation of yogurt

The milk samples were inoculated at 10^7 CFU/mL for single culture (YC, LB, PFS-56, PFS-51 and PFF-23) or 2×10^7 CFU/mL for the combination of YC with probiotic bacteria (YC+LB, YC+PFS-56, YC+PFS-51, and YC+PFF-23) at a 1:1 ratio. The samples were incubated at 45 °C until a pH of 4.4 was reached, and then was transferred to 4 °C. Following transfer, the yogurt was sampled at 1, 7, and 14 days for microbiological and lipid analyses.

Experiment 2: Effect of temperature and protein hydrolysis on growth and CLA-producing ability of propionibacteria

Rennet tablet 0.80 g (Redco Foods Inc., Windsor, CT) was added to 1 L skim milk (Hy-Vee Inc., West Des Moines, IA) containing hydrolyzed soy oil (1% fat) and incubated for 40 min at 25 °C. The milk with added rennet was inoculated with PFS-51 at 10^7 CFU/mL. Milk (without added rennet) was inoculated with YC, PFS-51 at 10^7 CFU/mL and the combination of YC with PFS-51 at 2×10^7 CFU/mL. The samples were incubated at 32 or 45

°C until a pH of 4.4 was reached, and then were transferred to 4 °C. After 1-day storage, the yogurts were sampled for microbiological and lipid analyses.

Experiment 3: Effect of initial concentration of starter culture on CLA formation of yogurt

Yogurt culture, LB, and the mixed cultures of YC and LB, were inoculated at 10^6, 10^7, 10^8 CFU/mL into skim milk (Hy-Vee Inc., West Des Moines, IA) containing hydrolyzed soy oil (1% fat). The combination of *L. rhamnosus* and the yogurt culture was at a 1:1 ratio. The bacteria treatment was expressed as follows: For example, 7YC means yogurt culture with inoculation concentration 10^7 CFU/mL; 8(YC+LB) means the mixture of yogurt culture and *L. rhamnosus* with inoculation concentration about 2×10^8 CFU/mL. The yogurt was incubated at 45 °C until a pH of 4.4 was reached, and then was transferred to 4 °C. Following transfer, the yogurt was sampled at 1, 7, and 14 days for microbiological and lipid analyses.

Lipid analysis

Lipids were extracted from yogurt model systems using a modified Bligh and Dyer chloroform-methanol extraction method (Lin and others 1995). With heptadecanoic acid ($C_{17:0}$, Sigma Chemical Co., St. Louis, MO) as an internal standard, the lipid extracts were hydrolyzed with 1 N NaOH in methanol at 100 °C for 15 min and methylated with 14% boron trifluoride in methanol (Alltech Associates Inc., Deerfield, IL) at room temperature (25 °C) for 30 min to prevent intraisomerization of CLA isomers (Werner and others 1992). The fatty acid methyl esters (FAME) were analyzed on a gas chromatograph equipped with a

flame ionization detector (Model HP6890, Hewlett Packard Inc., Wilmington, DE) and separated using a CP-Sil88 column (100 m × 0.25 mm ID; Chrompack, Middelburg, Netherlands). The column pressure was set at 275.8 KPa with a helium flow rate of 6.2 mL/min. The sample (1.0 μL) was injected onto the column with a 5:1 split ratio. The temperature of the GC oven was held initially at 30 °C for 5 min, increased to 125 °C at 10 °C/min and held 1 min, increased to 145 °C at 2 °C/min, increased to 160 °C at 1 °C/min and held 10 min, and finally increased to 190 °C at 2 °C/min and held for 10 min. The total run time was 75.5 min. The detector temperature was 225 °C. Flow rates of detector gases were air at 400 mL/min, hydrogen at 30 mL/min and nitrogen (make-up gas) at 18 mL/min. CLA and fatty acid methyl esters were identified and quantified by comparison with the retention time and peak area of CLA standards (NuChek-Prep, Inc., Elysian, MN) and fatty acid standards (Supelco, Inc., Bellefonte, PA).

A GC-MS (Trio 1000, Fisons Instruments, Danvers, MA) with a quadrupole mass analyzer was used to confirm the identify of the fatty acid methyl esters. The GC conditions were the same as those of the chromatographic analysis. The mass spectrometer conditions were set as follows: source electron energy at 70 eV, source electron current at 150 μA, ion source temperature at 220 °C, interface temperature at 220 °C, source ion repeller at 3.4 V, electron multiplier voltage at 600 V and scan range between 41 and 350 m/z. Mass spectra of the fatty acid methyl esters were compared to a spectral library (NBS Library) for identification.

Microbiological analysis

The microbial count was determined using Lactobacilli MRS agar (Difco, Sparks, MD, USA) for lactic acid bacteria and sodium lactate agar for propionibacteria. The sodium lactate agar consisted of sodium lactate broth and 2% (w/v) agar. Samples were diluted in buffered peptone water (2%, Difco, Detroit, MI) and plated in duplicate using the surface plating method. The plates were incubated under anaerobic conditions at 37 °C for 48 h (lactic acid bacteria) or 32 °C for 72-96 h (propionibacteria). The mixed cultures of lactic acid bacteria and propionibacteria were plated on the two different agars. The total counts for mixed cultures were calculated by determining the counts of individual bacteria on two agars.

Statistical analysis

Experiment 1 was designed as a 2-way factorial experiment with bacterial culture and storage time as the main factors. Each treatment was replicated three times. Experiment 2 was designed as a 2-way factorial experiment with bacterial treatment and incubation temperature as the main factors. Each treatment was replicated two times. Experiment 3 was designed as a 3-way factorial experiment with inoculation level, bacterial culture and storage time as the main factors. Each treatment was replicated three times. The experimental data were analyzed using analysis of variance (mixed linear model procedures) and Duncan multiple range test (SAS version 8.2, Cary, NC, 2004) with a significance level of 0.05.

Results and Discussion

Effect of probiotic bacteria on CLA formation

The milk was inoculated with either probiotic bacteria alone or probiotic bacteria in conjunction with the yogurt cultures. The microbial counts of the yogurt cultures, *L. rhamnosus*, and the probiotic bacteria with the yogurt cultures ranged from 8.94 to 9.30 $\log_{10}$ CFU/mL following storage at 4 °C for 1 day (Table 1). Total microbial counts decreased slightly with storage, with the counts for these treatments at 14 days ranging from 8.48 to 9.13 $\log_{10}$ CFU/mL. These treatments (yogurt cultures, *L. rhamnosus*, and the probiotic bacteria with the yogurt cultures) had no significant effect on microbial counts after 14 days of storage at 4 °C. For most bacterial treatments, storage time did not have a significant effect on microbial count. Only the combination of *L. rhamnosus* and yogurt culture showed a significant decrease between 1 and 7 days.

The microbial counts of the yogurts processed with the 3 propionibacteria (PFF-23, PFS-51 and PFS-56) were significantly lower than those processed with lactic acid bacteria. Microbial counts in the yogurts processed with the propionibacteria alone decreased to 6.14 to 6.72 $\log_{10}$ CFU/mL after 1 day of storage. Storage time did not have a significant effect on the microbial counts of the bacteria (Table 1). The growth of propionibacteria in the yogurts with propionibacteria and yogurt cultures was significantly higher than in yogurts with only propionibacteria, suggesting that the growth of propionibacteria was enhanced in the presence of the yogurt culture.

Condon and others (2001) indicated that growth of propionibacteria in a whey-based model system was stimulated by lactic acid bacteria, such as *L. helveticus*, *L. bulgaricus* and

S. thermophilus. The extent of the stimulation was dependent on the particular pair of propionibacteria and lactic acid bacteria. The most effective combination was *L. delbrueckii* subsp. *lactis* LL51 and *P. freudenreichii* P23. *L. delbrueckii* subsp. *lactis* LL51 increased the growth rates of *P. freudenreichii* P23 from 1.19 to 2.04 $\log_{10}$ CFU/mL. The metabolic products of the lactic acid bacteria, such as peptides, lactic acid and carbon dioxide, facilitated the growth of propionibacteria (Baer and Ryba 1998; Condon and others 2001). Propionibacteria contain peptidases capable of supplying the cell with all essential amino acids (Vorobjeva 1999). So peptides are an important substrate for the physiological activity of propionibacteria. Lactate is a preferred substrate for the growth of propionibacteria, while carbon dioxide forms a more anaerobic environment to facilitate the growth of propionibacteria.

Previous research demonstrated the ability of probiotic bacteria to produce CLA from linoleic acid only in the model system containing the hydrolyzed soy oil emulsified in non-fat dry milk. Of 11 probiotic bacteria evaluated, 3 propionibacteria (*P. freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23) and *L. rhamnosus* demonstrated the greatest increase in CLA content (Xu and others 2004). These 4 bacteria were selected to evaluate CLA formation of yogurt produced with the selected bacteria alone or in conjunction with traditional yogurt cultures.

In this study, the combination of most probiotic bacteria with the yogurt cultures produced slightly higher contents of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA than yogurt culture alone after 14 days of storage (Table 2). The yogurt processed with the combination of yogurt culture and *L. rhamnosus* showed significantly higher *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA content than the yogurt processed with yogurt culture alone. The yogurt processed with

the propionibacteria (PFF-23, PFS-51 and PFS-56) alone was lower in *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA content than the yogurt processed with the propionibacteria and yogurt culture together. Although the propionibacteria alone were shown to be effective in the formation of CLA in a model system (Xu and others 2004), they were less effective in the formation of CLA in a yogurt system. In the model system, the incubation temperature was optimized for the growth of the propionibacteria (32 °C). However, the fermentation temperature typically used for yogurt processing and used in this study (45 °C) significantly inhibited the growth of propionibacteria. Thus, the combination of yogurt culture and propionibacteria improved microbial counts of propionibacteria and increased CLA formation in the yogurt product compared to propionibacteria alone.

The ability of probiotic bacteria to form CLA has been demonstrated in model systems. *Propionibacterium freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* in *in vitro* systems with 500 or 750 μg/mL free linoleic acid (Jiang and others 1998), *L. acidophilus* in skim milk medium with 1000 μg/mL linoleic acid (Lin and others 1999b), *L. lactis* I-01 in whole milk medium with 100 or 200 μg/mL sunflower oil containing 70% linoleic acid (Kim and Liu 2002), and *L. plantarum* AKU 1009A in a nutrient medium with 0.06% (wt/vol) linoleic acid (Kishino and others 2002), have been shown to significantly increase the CLA content of model systems. Lin (2003) demonstrated the mixed cultures comprising yogurt culture and *Lactobacillus acidophilus* with 0.1% linoleic acid addition significantly improved *cis*-9, *trans*-11 CLA content in non-fat set yogurt. However, few studies have evaluated CLA-forming ability when probiotic bacteria were combined with conventional yogurt cultures.

In general, storage time did not have a significant effect on CLA content of yogurts. For most bacterial cultures including yogurt culture, *L. rhamnosus*, *P. freudenreichii* subsp. *shermanii* 51 and the combination of yogurt culture and probiotic bacteria, the CLA concentration is relatively stable during storage. In previous research, the CLA content of yogurt has been shown to be stable during storage periods of 7 days (Boylston and Beitz 2002) and 6 weeks (Shantha and others 1995). Thus, the CLA-forming ability of the starter cultures rather than storage had an impact on the CLA content of yogurt.

Effect of temperature and casein hydrolysis on propionibacteria

Compared to our previous study (Xu and others 2004), Experiment 1 showed that the microbial growth and CLA formation in yogurt processed with propionibacteria was reduced. A major difference between the two studies was the incubation temperature of the milk samples. The optimal growth temperature for propionibacteria is 32 °C, but the yogurt production is generally conducted at 45 °C. In Experiment 1, the increased microbial growth and CLA formation by the propionibacteria in the presence of the yogurt cultures was attributed to the metabolic activity of the lactic acid bacteria. Experiment 2 was designed to further study the effect of temperature and casein hydrolysis on microbial growth and CLA formation.

The effect of temperature and casein hydrolysis on the microbial growth of propionibacteria was shown in Table 3. At 32 °C, there was no significant difference in the microbial counts of the yogurt culture, PFS-51 with or without rennet addition, and PFS-51 with the yogurt cultures. However, the yogurt produced with PFS-51 showed significantly lower microbial counts than other treatments at 45 °C. Although 45 °C was not the optimal

temperature for the growth of propionibacteria, our results demonstrated that the addition of yogurt cultures or rennet in the milk with PFS-51 greatly increased the microbial counts of the yogurt. The production of peptides by the proteolytic systems of yogurt cultures has been proposed to stimulate the growth of propionibacteria (Piveteau and others 2002). The increased growth of the propionibacteria at 45 °C was attributed to casein hydrolysis by the addition of rennet or yogurt cultures. The hydrolysis of casein through the addition of rennet or proteolytic activity of yogurt cultures increased the microbial counts of propionibacteria in the yogurt incubated at 45 °C.

Table 4 showed the effect of temperature and protein hydrolysis on CLA production. At 32 °C, the yogurt processed by PFS-51 with or without rennet, and the combination of PFS-51 and yogurt cultures showed similar *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA contents. The 3 yogurts processed with PFS-51 had a significantly higher content of *cis*-9, *trans*-11 CLA than yogurt processed with the yogurt cultures. However, the yogurt processed with PFS-51 alone was significantly lower in *cis*-9, *trans*-11 CLA content than the other treatments incubated at 45 °C. Therefore, casein hydrolysis significantly increased CLA formation for PFS-51 under the non-optimal incubation temperature of 45 °C. This is an effective approach for enhancement of CLA content in actual yogurt production. Among the yogurts produced with PFS-51, the addition of yogurt cultures or rennet both greatly increased the CLA content because they both contributed to protein hydrolysis. The casein hydrolysis, either through the metabolic activity of the yogurt cultures or rennet activity, stimulated the growth of the propionibacteria when the incubation temperature is not ideal for propionibacteria. This result also partially explained why propionibacteria in conjunction

with yogurt cultures could overcome the non-optimal temperature effect and produce a significantly higher CLA content than with propionibacteria alone.

Effect of initial concentration of YC and LB on microbial counts and CLA formation of yogurt

The linoleic acid isomerase activity of bacteria contributes to the CLA formation in dairy products. Our study used hydrolyzed soy oil as the lipid source, but only a small percentage (about 11%) of the available linoleic acid was isomerized to CLA. It appears the linoleic acid isomerase rather than the substrate is the limiting factor in the CLA formation. Increasing the initial bacteria level might increase the amount of linoleic acid isomerase available. However, most current studies do not show strict control of the inoculation levels and monitor viability of the starter culture during storage time. Therefore, a further study was conducted to evaluate the effect of initial concentration, composition of starter culture, and storage time on the CLA content and microbial growth of yogurts.

Growth of starter cultures in yogurts

The milk was inoculated with YC, LB, or LB in conjunction with the YC. The microbial cell counts of cultures at the 3 inoculation levels (10^6, 10^7, 10^8 CFU/mL) throughout the 14-day storage period were evaluated (Table 5). The microbial counts increased significantly at 4 °C for 1 day storage with the counts reaching 8.24 to 9.21 $\log_{10}$ CFU/mL. Yogurt culture with initial level 10^8 CFU/mL was an exception. When the inoculation level of yogurt cultures was as high as 10^8 CFU/mL, no significant growth was observed from 0 to 14 d. Although bacteria viability decreased with time, no significant difference in microbial counts was found from 1 d to 14 d at 4 °C. Only 8LB showed a

significant decrease in microbial counts at 14 days. Thus, the yogurt showed a stable level of microbial cells during 14-day cold storage.

The effect of inoculation levels on the growth of bacteria were reported in 3 groups – YC, LB, the combination of YC and LB. For every group, the initial bacteria levels had no significant impact on the microbial counts during 14-day storage at 4 °C. The increased initial concentration of bacteria did not affect the microbial counts of bacteria in yogurts over a 14-day storage time. Only 8YC showed lower microbial counts. When the inoculation level were 10^6 or 10^7 CFU/mL, microbial counts of yogurts didn't show any difference for YC, LB or LB in conjunction with YC. However, the growth of 8YC was significantly lower than 8(YC+LB) at 14 d. In general, initial inoculation level did not affect microbial counts of yogurts.

Since the health benefits of probiotic bacteria depend on microbial cells ingested, it is important to maintain certain levels of microbial bacteria in probiotic foods. The bacterial counts exceeded the suggested minimum count (10^7 CFU/mL) which is needed to affect the gut environment and bring health benefits to people (Vinderola and Reinheimer 1999). Therefore, the determination of microbial numbers of microorganisms in the yogurt product is very necessary. Our results showed all yogurt samples showed high microbial lactic bacteria counts (> 10^8 CFU/mL) at the end of the 14 d storage period.

CLA content of yogurts

Our previous research demonstrated LB, either alone or in conjunction with yogurt culture, resulted in the greatest formation of CLA (Table 2). The CLA formation was further evaluated in the yogurt produced with YC, LB, or the combination of YC and LB with different inoculation levels. In general, the starter culture and inoculation level had no

significant effect on *cis*-9, *trans*-11 CLA content and *trans*-10, *cis*-12 CLA content during storage at 4 °C (Table 6). Of the bacteria treatments evaluated, 6YC and 6(YC+LB) resulted in the highest content of *cis*-9, *trans*-11 CLA at 14-day storage, but not significantly different from other bacterial treatments. Therefore, higher inoculation level did not contribute to a higher CLA content. The partial reason might be attributed to similar microbial counts from 1 to 14 d. Lin (2003) reported inoculation of mixed yogurt culture and *Lactobacillus acidophilus* with 0.1% linoleic acid addition significantly increased *cis*-9, *trans*-11 CLA content compared with individual yogurt culture and *Lactobacillus acidophilus*. The incorporation effect of yogurt culture and another probiotic bacterium in enhancing CLA content is dependent on different bacteria strains.

In this study, no significant difference in *cis*-9, *trans*-11 CLA level was found with the extension of storage time. However, the content of *trans*-10, *cis*-12 CLA increased with storage time at 4 °C (Table 6). Especially for 6LB, 8LB and 7(YC+LB), significant increases were observed between 1 and 14 days of storage. The yogurt produced with 8LB showed 2.3 fold increase of *trans*-10, *cis*-12 CLA content at 14 days. Although no significant difference in *trans*-10, *cis*-12 CLA levels was found at the end of 14 d storage for all bacteria treatments and inoculation levels, the yogurt processed with 6LB or 8LB demonstrated lower CLA formation at 1 and 7 days. Only when the storage time extended, CLA content greatly increased. Therefore, storage time affected the content of *trans*-10, *cis*-12 CLA of yogurts. The differences among bacteria treatments in the CLA-forming ability could be decreased with the extension of storage time.

Conclusions

Probiotc bacteria can be added during processing to enhance the health-promoting potential of yogurt. Yogurt culture in conjunction with probiotic bacteria provided an effective approach to produce similar CLA content as control yogurt. The yogurt processed with the propionibacteria alone resulted in lower CLA content compared to the control yogurt. However, the hydrolysis of casein through either the addition of yogurt cultures or rennet increased CLA formation at incubation temperature not optimal for growth of propionibacteria. Inoculation level did not have a significant effect on microbial growth or CLA formation. Bacterial treatment, incubation temperature, casein hydrolysis, and inoculation level, were shown to affect CLA formation. Further research is needed to evaluate the quality attributes of CLA-enriched yogurt products. The effect of probiotic bacteria on flavor, texture and acidity of yogurts should be studied. These quality attributes must be considered in the development of functional foods with enhanced CLA content.

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ACKNOWLEDGEMENTS

Funding for the research was provided, in part by, the National Dairy Council and NASA Food Technology Commercial Space Center. This article of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project Number 3574, was supported by Hatch Act and State of Iowa Funds. *L. rhamnosus* was donated by Danisco Cultor Inc., Milwaukee, WI. The yogurt cultures were provided by Chr. Hansen, Milwaukee, WI. The statistical consultation of Dr. Philip Dixon is appreciated.

Table 1- Effect of storage time on total microbial counts[a] of yogurts processed with probiotic bacteria

Strain	Microbial counts (log_{10} CFU/mL)[b]		
	1 Day	7 Day	14 Day
YC	8.98a,x	8.98a,x	8.80a,x
YC+LB	9.21a,x	8.63ab,y	8.73a,y
LB	8.94a,x	8.71ab,x	8.64a,x
YC+PFS-51	9.30a,x	8.88a,x	9.13a,x
PFS-51	6.14b,x	6.52c,x	5.75b,x
YC+PFS-56	9.22a,x	8.85a,x	8.48a,x
PFS-56	6.72b,x	7.13bc,x	5.99b,x
YC+PFF-23	9.28a,x	8.83a,x	8.71a,x
PFF-23	6.38b,x	6.68c,x	5.85b,x

[a]Means in the same column followed by the same letter (a-c) are not significantly different ($p > 0.05$) for bacterial treatment effect. Means in the same row followed by the same letter (x-y) are not significantly different ($p > 0.05$) for storage effect. Means are triplicate analyses of three replications.
[b]Inoculation level of every treatment (0 day) was 6.86-7.99 log_{10} CFU/mL.

Table 2- The effect of incorporation of probiotic bacteria with yogurt culture on the *cis-9, trans*-11 and *trans-10, cis*-12 CLA content[a] of yogurts

	CLA content[b] (mg/g lipid)					
	cis-9, *trans*-11 CLA			*trans*-10,*cis*-12 CLA		
Strain	1 Day	7 Day	14 Day	1 Day	7 Day	14 Day
YC	0.45a,x	0.42ab,x	0.57bc,x	0.27ab,x	0.27abc,x	0.38cd,x
YC+LB	0.33a,x	0.39ab,x	0.97a,x	0.33ab,x	0.31ab,x	0.71a,x
LB	0.44a,x	0.44ab,x	0.73ab,x	0.32ab,x	0.34ab,x	0.68ab,x
YC+PFF-23	0.53a,x	0.57a,x	0.65abc,x	0.32ab,x	0.39a,x	0.41abc,x
PFF-23	0.22b,x	0.23b,x	0.39cd,x	0.13b,x	0.11c,x	0.16d,x
YC+PFS-56	0.57a,x	0.51ab,x	0.63abc,x	0.43a,x	0.38a,x	0.50abc,x
PFS-56	0.19b,x	0.21b,x	0.25d,x	0.11b,xy	0.09c,y	0.12d,x
YC+PFS-51	0.42a,x	0.29ab,x	0.60abc,x	0.18b,x	0.31ab,x	0.41abc,x
PFS-51	0.17b,x	0.21b,x	0.24d,x	0.12b,x	0.13bc,x	0.11d,x

[a]Means in the same column followed by the same letters (a-d) are not significantly different ($p > 0.05$) for bacterial treatment effect. Means in the same row followed by the same letters (x-y) are not significantly different ($p > 0.05$) for storage effect. Means are averages of three replications.
[b]CLA was not detected for any treatment at 0 day.

Table 3- Effect of temperature on the microbial counts[a] of yogurts after 1-day storage

	Microbial counts[b] (log_{10}CFU/ml)	
Treatment	**32 °C**	**45 °C**
YC	8.08a,x	8.83a,x
PFS-51	8.83a,x	7.18b,y
PFS-51+YC	9.21a,x	9.23a,x
PFS-51+RE[c]	9.08a,x	8.66a,x

[a]Means in the same column followed by the same letters (a-b) are not significantly different ($p > 0.05$) for treatment effect. Means in the same row followed by the same letters (x-y) are not significantly different ($p > 0.05$) for temperature effect. Means are averages of two replications.
[b]Inoculation level of every treatment (0 day) was 7.61-7.93 log_{10} CFU/ml.
[c]PFS-51+ RE refers to PFS-51 inoculated in the milk with the addition of rennet.

Table 4- Effect of temperature on the CLA content[a] of yogurts after 1-day storage

	CLA content[b] (mg/g lipid)			
	cis-9, *trans*-11 CLA		*trans*-10, *cis*-12 CLA	
Treatment	**32 °C**	**45 °C**	**32 °C**	**45 °C**
YC	0.63b,x	0.72a,x	0.22a,x	0.25a.x
PFS-51	1.95a,x	0.26b,y	0.26a,x	0.18a,x
PFS-51+YC	2.57a,x	0.59a,y	0.26a,x	0.17a,x
PFS-51+RE[c]	1.87a,x	0.73a,y	0.15a,x	0.23a,x

[a]Means in the same column followed by the same letters (a-b) are not significantly different ($p > 0.05$) for treatment effect. For *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, means in the same row followed by the same letters (x-y) are not significantly different ($p > 0.05$) for temperature effect. Means are averages of two replications.
[b]CLA was not detected for any treatment at 0 day.
[c]PFS-51+ RE refes to PFS-51 inoculated in the milk with the addition of rennet.

Table 5- Effect of bacterial cultures and storage time on microbial counts[a] of yogurts

		Microbial counts (log_{10}CFU/mL)			
Strain	**Inoculation level**	**0 Day**	**1 Day**	**7 Day**	**14 Day**
YC	6	6.16c,y	9.17a,x	8.43a,x	8.50ab,x
	7	7.16b,y	8.84a,x	8.81a,x	8.96a,x
	8	8.16a,x	8.24b,x	8.46a,x	8.11b,x
LB	6	6.47c,y	9.20a,x	9.10a,x	8.99a,x
	7	7.47b,y	8.99a,x	9.03a,x	8.78ab,x
	8	8.47a,y	9.10a,x	9.05a,x	8.74ab,y
YC+LB	6	6.66c,z	9.05a,x	8.90a,xy	8.36ab,y
	7	7.66b,z	9.21a,x	8.82a,xy	8.56ab,y
	8	8.66a,y	9.14a,x	8.91a,x	8.97a,x

[a] Means in the same column followed by the same letter (a-c) are not significantly different ($p > 0.05$) for bacterial treatment effect. Means in the same row followed by the same letter (x-z) are not significantly different ($p > 0.05$) for storage effect. Means are triplicate analyses of three replications.

Table 6- Effect of bacterial cultures and storage time on the content of *cis-9, trans*-11 and *trans-10, cis*-12 CLA content[a] of yogurts

		CLA content[b] (mg/g lipid)					
		cis-9, *trans*-11 CLA			*trans*-10, *cis*-12 CLA		
Strain	Inoculation level	1 Day	7 Day	14 Day	1 Day	7 Day	14 Day
YC	6	0.42a,x	0.62a,x	0.58a,x	0.26a,x	0.28ab,x	0.42a,x
	7	0.43a,x	0.39b,x	0.48a,x	0.18ab,x	0.27ab,x	0.39a,x
	8	0.46a,x	0.30b,x	0.36a,x	0.23ab,x	0.24ab,x	0.26a,x
LB	6	0.33a,x	0.27b,x	0.27a,x	0.13b,y	0.15b,y	0.24a,x
	7	0.38a,x	0.42b,x	0.43a,x	0.19ab,x	0.35a,x	0.33a,x
	8	0.54a,x	0.35b,x	0.50a,x	0.12b,y	0.24ab,x y	0.40a,x
YC+LB	6	0.47a,x	0.39b,x	0.52a,x	0.20ab,x	0.33a,x	0.38a,x
	7	0.34a,x	0.38b,x	0.47a,x	0.17ab,y	0.31a,xy	0.45a,x
	8	0.50a,x	0.45ab,x	0.46a,x	0.17ab,x	0.38a,x	0.38a,x

[a] Means in the same column followed by the same letter (a-b) are not significantly different ($p > 0.05$) for bacterial treatment effect. Means in the same row followed by the same letter (x-y) are not significantly different ($p > 0.05$) for each CLA isomer for storage effect. Means are averages of three replications.

[b] CLA was not detected for any treatment at 0 day.

Quality Attributes of Yogurt Products with Probiotic Bacteria

A paper to be submitted to Journal of Food Science

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ABSTRACT: Quality attributes of yogurt with probiotic bacteria incorporated to increase CLA content were evaluated. Four probiotic bacteria, *Lactobacillus rhamnosus*, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23, were evaluated alone or in conjunction with traditional yogurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). Hydrolyzed soy oil was the lipid source in the yogurt. The yogurt processed with probiotic bacteria, *L. rhamnosus* or yogurt cultures in conjunction with probiotic bacteria demonstrated similar acidity, texture and flavor as the control yogurt produced with yogurt cultures. The yogurt processed with the propionibacteria alone showed different texture and flavor attributes than the control yogurt. In general, the quality characteristics of yogurts were stable over a 14-day storage time at 4 °C. The effect of inoculation concentration (10^6, 10^7, and 10^8 CFU/mL) of *L. rhamnosus*, yogurt cultures, and mixed cultures of yogurt cultures and *L. rhamnosus* on quality attributes of yogurt was determined. Inoculation concentration of *L. rhamnosus* and yogurt cultures had no significant effect on texture, but affected acidity and volatile flavor compounds of yogurts. The yogurt produced by *L. rhamnosus* in conjunction with yogurt culture with 10^7 CFU/mL total inoculation level resulted in desirable quality characteristics. This research demonstrated that CLA-enriched yogurt products with *L. rhamnosus* or the combination of probiotic bacteria and yogurt cultures showed acceptable quality attributes.

Keywords: conjugated linoliec acid (CLA), yogurt, flavor, inoculation concentration, texture, acidity

Introduction

Functional foods are considered to elicit benefits to health and well-being or to have disease-preventing properties beyond their inherent nutritional value. Recent growth in the functional foods market stems from the identification of physiologically active components in foods. Functional dairy products with probiotic bacteria and enhanced contents of conjugated linoleic acid (CLA) have attracted much attention.

Probiotics could actively enhance the health of consumers by improving the intestinal microbial balance. The positive effects associated with probiotics include anti-tumor activity, cholesterol reduction, protection against gastroenteritis, improvement of lactose tolerance and stimulation of the immune system through non-pathogenic means (Rastall and others 2000). Lactic acid bacteria (*Lactobacilli, Streptococci, Lactococci, Bifidobacteria*) and propionibacteria constitute promising probiotic bacteria used in dairy food industries.

Conjugated linoleic acid, a mixture of conjugated positional and geometric isomers of linoleic acid, has demonstrated unique biological activities in *in vitro* and animal studies, including an anticarcinogenic agent (Corl and others 2001), body-fat reducer (Park and others 1997), antiatherogenic agent (Nicolosi and others 1997), antidiabetic agent (Houseknech and others 1998), immune system modulator (Hayek and others 1999), and body weight protector (Chin and others 1994).

Several studies have shown that probiotic bacteria can form CLA in model systems. Six lactic acid bacteria (*Lactobacillus acidophilus, L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis,* and *Streptococcus salivarius* subsp. *thermophilus*) (Lin and others 1999), *Lactobacillus*

plantarum (Kishino and others 2002), *Propionibacterium freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* (Jiang and others 1998) have been identified to produce CLA from free linoleic acid. Our research also showed probiotic bacteria, especially *Lactobacillus rhamnosus*, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23, had the ability to produce CLA in the skim milk system containing 1% hydrolyzed soy oil emulsified in non-fat dry milk (Xu and others 2004). Yogurt products processed with *L. rhamnosus*, yogurt cultures, and yogurt cultures in conjunction with probiotic bacteria and hydrolyzed soy oil as the lipid source resulted in increased CLA content. The inoculation concentration of probiotic bacteria had no significant effect on CLA content (Xu and others 2005).

Although consumers are interested in healthful food, quality attributes will directly affect consumer acceptability of a functional yogurt product (Ott and others 2000; Panagiotidis and Tzia 2000). Lactic acid fermentation converts lactose into lactic acid. Lactic acid is responsible for the refreshing tart flavor of yogurt. Plain yogurt has a weak but distinctive and fragile flavor (Ott and others 2000). Consumers in western countries prefer mild, less acidic yogurts. The acid formation also contributes to the viscous, firm and consistent body of the yogurt. A typical yogurt texture is smooth, clotless and without fissures, while whey syneresis is the most undesirable defect (Panagiotidis and Tzia 2000). Starter culture has been shown to affect sensory attributes of yogurt products (Laye and others 1993; Penna and others 1997). As a consumer product, the stability of yogurt products in quality attributes was also important.

The desirable organoleptic properties are important for a successful functional food. Although there are some flavor and texture studies of commercial yogurts, little attention has been paid to the effect of added probiotic bacteria on final yogurt quality characteristics, compared with traditional yogurt cultures. The objective of this research was to evaluate the quality attributes (acidity, texture and flavor) of yogurt with probiotic bacteria added to increase CLA content during the refrigerated storage. The effect of initial concentration of starter culture on these quality attributes of yogurts was also studied.

Materials and Methods

Starter culture

Lactobacillus rhamnosus, *P. freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51, and *P. freudenreichii* subsp. *freudenreichii* 23 were selected to evaluate organoleptic attributes of yogurt produced with the selected bacteria alone or in conjunction with traditional yogurt cultures (1:1 ratio of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*; YC-180 (YC), Chr. Hansen, Milwaukee, WI). The combination of probiotic bacteria and the yogurt culture was at a 1:1 ratio. *L. rhamnosus* (LB) was obtained from Danisco Cultor Inc. (Milwaukee, WI). *Propionibacterium freudenreichii* subsp. *freudenreichii 23* (PFF-23), *P. freudenreichii* subsp. *shermanii 56* (PFS-56) and *P. freudenreichii* subsp. *shermanii 51* (PFS-51) were obtained from Dr. Bonita Glatz's Collection (Iowa State University, Ames, IA) and stored at –80 °C. Lactic acid bacteria were activated in Lactobacilli MRS broth (Difco, Detroit, MI) for 15 h at 37 °C and the propionibacteria were

activated in sodium lactate broth for 20-22 h at 32 °C. The sodium lactate broth contained 1% (w/v) tryptic soy broth (Becton Dickinson and Company, Cockeysville, MD), 1% (w/v) yeast extract (Becton Dickinson and Company, Sparks, MD), and 1% (w/v) sodium lactate syrup (Fisher Scientific, Fair Lawn, NJ).

Processing of yogurt with hydrolyzed soy oil

Soybean oil (Wesson, Conagra Grocery Products Company, Irvine, CA) was chemically hydrolyzed to increase the content of free fatty acids and emulsified into a 10% acacia solution at a 1:1 (w/w) ratio (Xu and others 2004). The mixture was homogenized into skim milk (Hy-Vee Inc., West Des Moines, IA) to produce a 1% fat content. The milk solids-not-fat content was adjusted to 12% through the addition of non-fat dry milk (Hy-Vee Inc., West Des Moines, IA). The mixture was heated at 85 °C for 30 min, cooled to 45 °C, and inoculated with probiotic bacteria and/or yogurt cultures and incubated as designated in the following experiments.

Experiment 1: Effect of probiotic bacteria on quality attributes of CLA-enriched yogurt

The milk samples were inoculated at 10^7 CFU/mL for single culture (YC, LB, PFS-56, PFS-51 and PFF-23) or 2×10^7 CFU/mL for the combination of YC with probiotic bacteria (YC+LB, YC+PFS-56, YC+PFS-51, and YC+PFF-23) at a 1:1 ratio. The samples were incubated at 45 °C until a pH of 4.4 was reached, and then was transferred to 4 °C.

Following transfer, the yogurt was sampled at 1, 7, and 14 days for acidity, texture and volatile flavor analyses.

Experiment 2: Effect of initial concentration of starter culture on quality attributes of CLA-enriched yogurt

Yogurt culture, LB, and the mixed cultures of YC and LB, were inoculated at 10^6, 10^7, and 10^8 CFU/mL. The combination of *L. rhamnosus* and the yogurt culture was at a 1:1 ratio. The bacteria treatment was expressed as follows: For example, 7YC means yogurt culture with inoculation concentration 10^7 CFU/mL; 8(YC+LB) means the mixture of yogurt culture and *L. rhamnosus* with inoculation concentration about 2×10^8 CFU/mL. The yogurt was incubated at 45 °C until a pH of 4.4 was reached, and then was transferred to 4 °C. Following transfer, the yogurt was sampled at 1, 7, and 14 days for acidity, texture and volatile flavor analyses.

Acidity measurement

The pH of yogurt model systems was recorded using a digital pH meter (Fisher Scientific, Accumet Model AB15, Pittsburgh, PA). Titratable acidity was determined by titrating a sample (5 g yogurt + 45 mL distilled water) with 0.1N NaOH to an endpoint of pH 7.0. Titratable acidity was calculated based on lactic acid as the predominant acid and was expressed as grams lactic acid per 100 mL yogurt. Sample temperature was 25 °C for each analysis.

Viscosity and syneresis measurement

Apparent viscosity was determined by using a RVDVII + Brookfield viscometer (Brookfield Engineering Labs Inc., Stoughton, MA) on a 100 mL yogurt sample at room temperature (25 °C). Samples were stirred for 20 sec before measurement. All viscosity values were measured at 10 rpm with spindle #5. Readings were converted to centipoise units (Gassem and Frank 1991). Syneresis (%) was expressed as volume of drained whey per 100 mL yogurt (Rodarte and others 1993).

Volatile Flavor Analysis

Solid-phase microextraction (SPME) technique was used for the isolation and concentration of volatile flavor compounds. A representative yogurt sample (20 g) and 5 mL distilled water was transferred to a 100 mL headspace bottle and sealed with a Teflon septum to prevent volatile loss. Yogurt samples were stirred and held in a 40 °C water bath to increase the concentration of volatile compounds in the sample headspace. The yogurt sample was allowed to equilibrate and absorb onto the SPME fiber (2 cm-50/30 μm divinylbenzene (DVB) / Carboxen / Polydimethylsiloxane (PDMS), Supelco, Inc., Bellefonte, PA) for 45 min. The volatiles were thermally desorbed (220 °C for 3 min) from the SPME fiber via a splitless injection port onto the GC column.

A gas chromatograph equipped with a flame ionization detector (Model HP6890, Hewlett Packard Inc.) and a fused-silica capillary column (SPB-1000, 30 m × 0.25 mm × 0.25 μm film thickness, Supelco, Inc.) was used for separation of flavor compounds. The column pressure was set at 124.0 KPa with a helium flow rate of 1.9 mL/min. The

temperature of the GC oven was held initially at 30 °C for 3 min, increased to 80 °C at 5 °C/min, increased to 95 °C at 4 °C/min, increased to 115 °C at 5 °C/min, and finally increased to 190 °C at 10 °C/min and held for 10 min. The total run time was 38.25 min. The detector temperature was 220 °C. Flow rates of detector gases were air at 400 mL/min, hydrogen at 30 mL/min and nitrogen (make-up gas) at 25 mL/min. Volatile flavor standards were identified using authentic standards (Sigma-Aldrich, Milwaukee, WI; AccuStandard, Inc., New Haven, CT).

Volatile flavor compounds were identified and confirmed with a gas chromatograph-mass spectrometer (Micromass GCT, Waters Corp., Milford, MA). The GC conditions were the same as those of the chromatographic analysis. The mass spectrometer conditions were set as the following: electron ionization positive (EI+) polarity, source electron energy at 70 eV, source electron current at 200 μA, ion source temperature at 180 °C, source ion repeller at 0.8 V, electron multiplier voltage at 2700 V, and scan range between 41 and 400 m/z at a frequency of scanning cycle every 0.75 sec. Mass spectra of the volatile flavor compounds were compared to a spectral library (Wiley Library) and a flavor and fragrance database (Flavor WORKS, Flavometrics, version 2.0, Anaheim Hills, CA) for identification.

Statistical analysis

Experiment 1 was designed as a 2-way factorial experiment with bacterial culture and storage time as the main factors. Each treatment was replicated three times. Experiment 2 was designed as a 3-way factorial experiment with inoculation level, bacterial culture and storage time as the main factors. Each treatment was replicated three times. The experimental

data were analyzed using analysis of variance (mixed linear model procedures) and Duncan multiple range test (SAS version 8.2, Cary, NC, 2004) with a significance level of 0.05.

Principal component analysis (PCA), using varimax orthogonal rotation, was used to examine relationships or groupings of volatile flavor compounds based on bacterial culture effects using the SYSTAT statistical analysis package (version 9.01, SPSS, Inc., Chicago, IL). The biplot of the PCA was completed to show the positions of bacterial cultures in the plane constructed by the first two principal components.

Results and Discussion

Effect of bacteria on quality attributes of CLA-enriched yogurt

In general, interactions between bacteria and storage time did not have a significant effect on the quality attributes of the yogurts. For each starter culture treatment, no significant difference in the quality attributes, such as pH, titratable acidity, viscosity, degree of syneresis and flavor, was observed when the storage time increased from 1 to 14 days at 4 °C. Therefore, data were pooled to focus on the effects of the bacterial culture on the quality attributes of yogurt products.

pH and titratable acidity of yogurts

The yogurts produced with the yogurt cultures, *L. rhamnosus,* or the propionibacteria with the yogurt cultures had pHs in the range of 4.10 to 4.33 (Table 1). These pH values were significantly lower than for the yogurts produced with the propionibacteria (PFF-23, PFS-56, and PFS-51) alone. For propionibacteria, the rate of pH decrease was significantly slower than

for the lactic acid bacteria. The above results are consistent with the growth of the starter cultures (Xu and others 2005). The lower growth rate of propionibacteria resulted in a slower rate of acid production and pH decrease in the yogurts.

The samples with only the yogurt cultures (*L. bulgaricus* and *S. thermophilus*) had the highest titratable acidity (1.79 g lactic acid/100mL yogurt) (Table 1). The yogurts with the probiotic bacteria alone or with probiotic bacteria plus yogurt cultures had lower titratable acidities. The combination of *P. freudenreichii* subsp. *shermanii* 56 and yogurt culture showed the lowest titratable acidity through the whole storage time.

The titratable acidity and pH data did not show parallel effects. In the titratable acidity determination, the acidity is reported based on lactic acid content, which is the predominant organic acid in dairy products. Lactic acid bacteria showed a significantly higher growth rate than propionibacteria (Xu and others 2005). Lactic acid bacteria produce lactic acid as the major product by exclusively fermenting hexose. The genus Lactobacillus is the preferred species to produce lactic acid (Martin 1996). However, for propionibacteria, lactic acid production in fermentation process is only an intermediate step in the production of other organic acids, such as propionic acid and acetic acid. Propionic acid is the main metabolic product of propionibacteria (Vorobjeva 1999). The pK_a values of lactic acid and propionic acid are 3.86 and 4.87, respectively. A lower pK_a indicates a greater dissociation of weak acids so that higher concentration of H^+ is produced. The difference in the pK_a values of the organic acids would contribute to a lower pH in yogurt produced with lactic acid bacteria than in yogurt produced with propionibacteria.

Viscosity and syneresis of yogurts

Yogurt processed with the yogurt cultures, *L. rhamnosus*, or the propionibacteria with the yogurt cultures was significantly more viscous and exhibited less syneresis than yogurt processed with the propionibacteria (PFF-23, PFS-56, and PFS-51) alone (Table 1). Prolonging storage from 1 to 14 days at 4 °C resulted in no significant difference in viscosity and syneresis for all starter culture treatments. Yogurt culture demonstrated the lowest degree of syneresis. Syneresis is an undesirable textural property of yogurt, which is caused by a spontaneous release of water from the gel and accompanied by a reduction in volume (Dannenberg and Kessler 1988). The increase in water-binding capacity of proteins increases curd stability during fermentation and storage (Langton 1991).

The combination of the yogurt cultures with the propionibacteria increased acid production and improved the textural characteristics of the yogurts to result in yogurts with texture similar to that of the control yogurt. The difference in the textural characteristics of the yogurts produced with propionibacteria in comparison to other treatments is attributed in part to the lower rate of acid production by propionibacteria. A comparison of pH and syneresis showed that less syneresis occurred when the pH ranged from 4.10-4.33 (Table 1). The results for viscosity and syneresis of yogurts are consistent with the limited growth and acid production by the propionibacteria.

Volatile flavor compounds in yogurts

Yogurt flavor is mainly generated from fermentation by the starter cultures, the ingredients of yogurt production, and heat processing. Fermentation is a major process to produce lactic acid and volatile compounds. Most of the 34 volatile flavor compounds identified, such as 2,3-butanedione, 2,3-pentanedione, benzaldehyde, acetaldehyde, acetic acid,

ethyl acetate, and 2-heptanone, have been identified in yogurt and milk (Labropoulos and others 1982; Kang and others 1988; Imhof and others 1995; Ott and others 1997). In our research, hydrolyzed soy oil was used as a lipid source to increase CLA content in yogurt products. Therefore, lipid oxidation reactions could produce some compounds, such as *trans*-2-nonenal, 1-octen-3-ol or *trans*-3-octen-2-one, which are not typical yogurt flavors (Ott and others 1997).

Principal component analysis

Principal component analysis (PCA) is one of the most commonly used statistical techniques to study complexities in flavor systems. Principal component analysis identifies patterns of interactions between variables to condense a large set of data into groups of similar characteristics. Principal component analysis grouped the volatile flavor compounds into 4 principal components (PC). PC-1 (14.3%) contained 7 volatile flavor compounds, PC-2 (11.5 %) contained 5 volatile flavor compounds, PC-3 (8.7%) contained 4 volatile flavor compounds and PC-4 (7.5%) contained 18 volatile flavor compounds (Table 2). Volatile flavor compounds were not exclusively grouped into the principal components based on the class of compound, i.e. aldehydes, ketones, alcohols or esters.

The yogurt produced with propionibacteria alone formed a significantly different grouping from that produced with yogurt culture, *L. rhamnosus*, and the probiotic bacteria with the yogurt culture (Figure 1). It is reasonable to assume that yogurt produced with PFS-56, PFS-51 and PFF-23 share certain flavor properties. No significant differences were observed for yogurts processed with the other bacterial treatments. These results demonstrated that the CLA-enriched yogurt products processed with the incorporation of yogurt culture and probiotic bacteria showed similar flavor characteristics as control yogurt produced by yogurt cultures.

However, the yogurt produced with only propionibacteria did not have typical yogurt flavor characteristics.

Effect of bacteria and storage on volatile flavor compounds

The aroma profile of yogurts did not change significantly during refrigerated storage. This result is in agreement with another study (Imhof and Bosset 1994). No interaction between bacterial culture and storage time was detected for all flavor compounds, except for propionic acid. Therefore, the flavor data were pooled to determine the effect of starter culture on volatile organic compounds (Table 2).

Principal component-1 (PC-1) included two major volatiles, 2, 3-butanedione and 2, 3-pentanedione, known to contribute to typical yogurt flavor (Ott and others 1999). The yogurt produced by propionibacteria alone showed higher content of 2, 3-butanedione than the yogurt processed with other cultures. In particular, PFS-51 produced significantly higher amounts of 2, 3-butanedione. Other bacteria resulted in no difference in the production of 2, 3-butanedione compared to the yogurt culture. The yogurt processed by yogurt culture and PFS-51 produced a significantly lower content of 2, 3-butanedione than PFS-51. These results clearly showed the incorporation of probiotic bacteria with yogurt culture effectively improved the yogurt flavor and produced flavor similar to the control yogurt culture.

Another important volatile compound is 2, 3-pentanedione. No significant difference was detected for most bacterial cultures in the production of 2, 3-pentanedione. Only (YC+LB) and LB showed a lower content of 2, 3-pentanedione than YC. During fermentation, 2, 3-butanedione and 2, 3-pentanedione are produced by oxidative decarboxylation of their precursors, 2-acetolactate and 2-acetohydroxybutyrate (Ramos and others 1994). The 2-acetolactate and 2-acetohydroxybutyrate are the metabolic intermediates of the branched chain

amino acids, valine and isoleucine, respectively. The two precursors are unstable under the conditions of heating and oxygen. The formation of 2, 3-butanedione and 2, 3-pentanedione is reduced by 2-acetolactate-dehydrogenase which directly converts their precursors into acetoin (Imhof and others 1995).

Principal component-1 (PC-1) also contained several untypical yogurt flavor compounds, such as dimethyl disulfide and undecanal. In general, the yogurt produced by propionibacteria alone demonstrated significantly higher contents of the flavor compounds that grouped into PC-1 than other bacteria treatments. However, the combination of yogurt culture with propionibacteria effectively reduced the content of these compounds.

In PC-2, acetic acid is a typical yogurt flavor compound. It produces a sour and pungent aroma. Lactic acid bacteria mainly produce lactic acid, while propionibacteria produce propionic acid as an important end-product. Acetic acid is only one of the acids produced by lactic acid bacteria and propionibacteria. Unlike other flavor compounds in PC-2, the content of acetic acid was lower in the yogurt produced by propionibacteria alone than in the yogurt produced by other bacteria. However, the incorporation of propionibacteria and yogurt culture increased the amount of acetic acid. The pH data and microbial counts showed consistent results with the content of acetic acid (Tables 1 and 2, Xu and others 2005). When growth of propionibacteria was slow, acetic acid production was reduced. However, the presence of yogurt culture greatly stimulated the growth of propionibacteria (Xu and others 2005). Thus, the yogurt processed with propionibacteria and yogurt cultures resulted in increased contents of acetic acid.

The yogurt produced by propionibacteria also showed significant differences from that produced with other bacteria in PC-3 and PC-4. In PC-3, hexanal, a product from the oxidation of linoleic acid, contributes to typical yogurt flavor. However, it is presumed that excessive

hexanal production causes strong green, grassy and penetrating aroma and disrupts flavor balance (Imhof and others 1995; McGorrin 2000). In our research, the yogurt produced with propionibacteria, especially PFS-56, resulted in higher concentration of hexanal than with other bacteria. However, the addition of yogurt culture to propionibacteria significantly reduced the production of hexanal. *S. thermophilus* was more effective than *L. bulgaricus* in reducing the hexanal content of fermented peanut milk (Lee and Beuchat 1991). The metabolism of bifidobacteria has been reported to break down hexanal and reduce undesirable flavor of fermented soy milk products (Scalabrini and others 1998). Our study showed similar results in that lactic acid bacteria could effectively reduce the hexanal content of fermented dairy products.

In PC-4, propionic acid is a characteristic flavor compound produced by propionibacteria. In general, the propionibacteria produced a significantly higher content of propionic acid than did the other bacterial treatments. However, the addition of yogurt cultures effectively reduced the content of propionic acid to a similar level as control yogurt. Our results showed the propionic acid content increased with the time extension from 1 d to 7 d. These results were consistent with the growth of propionibacteria (Xu and others 2005).

The aroma profile of yogurt consists of a unique combination of volatile organic compounds. Correct ratios among the different key compounds are essential for a balanced aroma (Ott and others 2000). The volatile compounds contribute to the characteristic note of yogurt but can also cause off-flavors depending on their concentrations compared to other flavor compounds. The reason why the yogurts processed with propionibacteria resulted in untypical flavor was mainly attributed to the different ratio of key flavor compounds compared to the control yogurt. Some desirable fermentation end-products, such as 2,3-butanedione, 2,3-

pentanedione and acetic acid, showed different content in yogurts produced with propionibacteria alone compared to those produced with other bacteria. Overproduction of 2, 3-butanedione by propionibacteria causes a harsh flavor (Lindsay and others 1965). Some off-flavor compounds, such as dimethyl disulfide and undecanal, were present in higher concentration in the yogurt with propionibacteria alone than in the other yogurts. All these differences caused a different ratio of flavor compounds and different flavor characteristics from control yogurt.

The proper acidity is also closely related to yogurt flavor. Desirable pH for typical yogurt flavor ranges from pH 4.0 to 4.4 (Sandine and others 1972). Our study showed similar results. The yogurt produced by yogurt culture, *L. rhamnosus*, and the probiotic bacteria with the yogurt cultures resulted in pH 4.0 to 4.4 and similar flavor characteristics (Table 1; Figure1). However, the yogurt produced by propionibacteria had a higher pH (4.64 to 5.02) and produced different flavor quality compared to control yogurt.

Effect of initial concentration of starter culture on quality attributes of CLA-enriched yogurt

Acid production of yogurts

In this study, storage time did not have significant effect on pH and titratable acidity of yogurts during the 14-day storage time at 4 °C. Thus, data were pooled to determine the effects of inoculation level and bacterial cultures on the acidity of yogurt products. Effect of inoculation level and bacterial cultures on pH and titratable acidity changes in yogurts are presented in Table 3. As lactic acid bacteria, YC, LB, and LB in conjunction with YC rapidly

decreased the pH of the yogurt after 1 day storage ($P<0.05$), followed by much more gradual changes throughout the storage period at 4 °C. The yogurts produced with 8(YC+LB), 6(YC+LB) and 7LB had lower pH values than other starter cultures. The pH of the yogurt processed with 6LB decreased very slowly throughout the storage period as a result of the slower fermentation process. The yogurt processed with 6(YC+LB) showed a faster decrease in pH than 6LB ($p<0.05$). The addition of YC increased the fermentation process through the rapid growth of yogurt cultures. However, the effect of YC on fermentation rate may also be related to the inoculation level.

The pH was considered an effective indicator of starter culture activity as it was directly related to population count. The microbial counts of yogurt gradually decreased with the slight pH drop (Xu and others 2005). The bacteria in yogurt are often exposed to pH 4.3-4.6. Low pH was not suitable for the survivability of bacteria. For example, LB survived best at pH 7 and 8. The number of LB decreased to zero after 48 h at pH 2 (Oliveira and others 2002). Therefore, the increase of acidity would affect the viable counts of bacteria in the yogurts.

The lactic acid bacteria tend to decrease pH and increase titratable acidity by production of lactic acid. The yogurt produced by 8(YC+LB) showed the highest titratable acidity (Table 3). This result was consistent with the pH drop of starter culture. On the other hand, 6LB resulted in the lowest titratable acidity. In this study, the titratable acidity of yogurt appeared to be consistent to the results by Kehagias and Dalles (1984). The titratable acidity reached about 1.0 g lactic acid/100mL yogurt which could be considered as a good quality yogurt. The acid production will affect flavor of yogurt. High acidity (>1.2 g lactic acid/100mL yogurt) would lead to unpleasant acid tastes. Our results indicated that the

titratable acidity of yogurt was close to the optimal acidity (1.0 g lactic acid/100mL yogurt) (Pinthong and others 1980).

Titratable acidity was determined by lactic acid content, which is the predominant organic acid in dairy products. Yogurt cultures and *L. rhamnosus* both belong to the genus Lactobacillus which produces lactic acid as its major product by the fermentation hexose. In this study, titratable acidity is a more precise measure than pH value to describe the acid production of yogurts. For lactic acid bacteria, the pH and titratable acidity showed parallel changes.

Texture properties of yogurt

In this study, storage did not have a significant effect on texture of yogurts. Therefore, data were pooled to evaluate the effects of bacterial treatments on the viscosity and syneresis of yogurt products. In general, bacterial treatments had no significant effect on the viscosity of yogurts at 4 °C (Table 3). The viscosity of yogurt produced with 6LB was the lowest. It is possible that the optimum pH wasn't reached so that gel formation of yogurt was affected. Comparing the viscosity of the yogurt produced with 6LB and 6(YC+LB), the incorporation of LB and YC significantly increased the viscosity from 71,000 to 147,000 centipoise. However, no significant differences were observed for LB and YC+LB when the inoculation level reached 10^7 or 10^8CFU/mL. This result suggests that the combination of YC and LB increases the viscosity of yogurts compared to YC or LB alone at a lower inoculation level.

The degree of syneresis describes the stability of yogurts by measuring the liquid separation. Table 3 showed that bacteria treatments had no significant effect on the degree of syneresis of yogurts ($P>0.05$). The yogurt processed with YC, LB, or LB in conjunction with

YC had a low degree of syneresis at 4 °C. In Table 3, the yogurt processed with 8(YC+LB) with higher acidity showed less syneresis. Oliveria and others (2002) found similar results in lactic beverage for the relationship between acidity and syneresis. In this study, the yogurt after fermentation showed a firm homogeneous curd with little separation of whey.

Volatile flavor compounds in yogurts

Since the storage time did not significantly affect the aroma profile of yogurts, the flavor data were pooled to determine the effect of inoculation level and bacterial culture on volatile compounds (Table 4). Among characteristic yogurt volatile compounds, acetaldehyde, 2, 3-butanedione, and benzaldehyde are important fermentation products (Imhof and others 1995; Ott and others 1997). Yogurt inoculated with LB at 10^6 or 10^7CFU/mL had higher acetaldehyde and 2, 3-butanedione contents than control yogurt (7YC). The overproduction of acetaldehyde in relation to 2, 3-butanedione has been reported to cause harsh flavor (Lindsay and others 1965). Similarly, the yogurt produced with YC demonstrated lower content of benzaldehyde than the yogurt processed with LB. However, the incorporation LB into YC at 10^7CFU/mL effectively decreased the content of benzaldehyde. The yogurt processed with 7(YC+LB) often produced typical volatile compounds, such as acetaldehyde, 2, 3-butanedione and benzaldehyde, which is comparable to the control yogurt.

Some untypical volatile compounds, such as *trans*-2-nonenal, 1-octen-3-ol or undecanal, were identified in the aroma profile of yogurts. Since hydrolyzed soy oil was used to increase CLA content in yogurt products, lipid oxidation reactions contributed to the formation of oxidation products. The results showed that LB in conjunction with YC decreased the content of off-flavor compounds in yogurts compared to LB alone. The content of undecanal

was significantly lower in the yogurt produced with 7(YC+LB) than in yogurt that processed with 7LB and produced similar flavor characteristics as the control yogurt.

Inoculation level of YC, LB or the mixture of YC and LB did not result in significant differences for most volatile flavor compounds. However, some volatile compounds were affected by different bacteria concentration. For the yogurt processed with YC, the content of several fermentation products greatly increased with the increased inoculation level. When the initial bacteria concentration was 10^8CFU/mL, some volatile compounds, such as acetic acid, methanol, 2-propanol, ethyl pentanoate or butanoic acid, were formed in a higher concentration than at other inoculation levels. The increased concentration of bacteria may facilitate the lactic acid fermentation process. However, the yogurt processed with LB alone produced higher contents of volatile compounds, such as acetic acid, acetaldehyde, 2, 3-pentanedione or benzaldehyde, at lower inoculation levels (10^6 or 10^7CFU/mL) than at 10^8CFU/mL. For certain probiotic bacteria, the continuous increase in initial bacteria concentration had no impact on the formation of volatile compounds. Overall, the optimum inoculation level and bacterial cultures had some impact on volatile compounds of yogurts.

Conclusions

The addition of probiotic bacteria to yogurt to increase CLA content must not only improve nutritional value but also result in a product with desirable quality attributes. In our study, the incorporation of probiotic bacteria with yogurt culture and unique processing methods not only increased CLA content but also produced similar quality attributes as

control yogurt. Yogurt processed with yogurt culture, *L. rhamnosus*, and probiotic bacteria in conjunction with yogurt culture showed good storage stability with respect to acidity, texture and flavor attributes. Yogurt culture in conjunction with probiotic bacteria provided an effective approach to produce similar CLA content and quality characteristics as control yogurt. The yogurt processed with the propionibacteria alone showed undesirable quality attributes compared to the control yogurt. Compared to other treatments, the yogurt produced with 7(YC+LB), 6(YC+LB), 6YC and 8LB showed more similar acidity, texture and flavor as control yogurt. Inoculation level of *L. rhamnosus* and yogurt cultures had no significant effect on texture, but affected acidity and volatile flavor compounds of yogurts. This research demonstrated that functional probiotic yogurt products with increased CLA contents have acceptable quality attributes and a potential for greater consumer acceptability.

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ACKNOWLEDGEMENTS

Funding for the research was provided, in part by, the National Dairy Council and NASA Food Technology Commercial Space Center. This article of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project Number 3574, was supported by Hatch Act and State of Iowa Funds. *L. rhamnosus* was donated by Danisco Cultor Inc., Milwaukee, WI. The yogurt cultures were provided by Chr. Hansen, Milwaukee, WI. The statistical consultation of Dr. Philip Dixon is appreciated.

Table 1- Acidity and texture of yogurts processed with probiotic bacteria and/or yogurt cultures[a]

Strain	Titratable acidity (g lactic acid /100ml yogurt)[b]	pH[c]	Viscosity (centipoise)	Degree of syneresis (%)
YC	1.79a	4.23c	225,000a	9b
YC + LB	1.17bc	4.10c	224,000a	12b
LB	1.26bc	4.14c	237,000a	15b
YC + PFS-51	1.02bc	4.16c	234,000a	16b
PFS-51	1.04bc	4.72b	57,300bc	44a
YC + PFS-56	0.83c	4.33c	269,000a	14b
PFS-56	1.35b	5.02a	134,000b	47a
YC + PFF-23	1.04bc	4.32c	223,000a	12b
PFF-23	1.17bc	4.64b	47,400c	51a

[a]Means in the same column followed by the same superscripts (a-c) are not significantly different ($p > 0.05$). Means are triplicate analyses of three replications. Data were pooled across storage times.
[b]Average titratable acidity of all treatments prior to fermentation was 0.31.
[c]Average pH of all treatments prior to fermentation was 5.96.

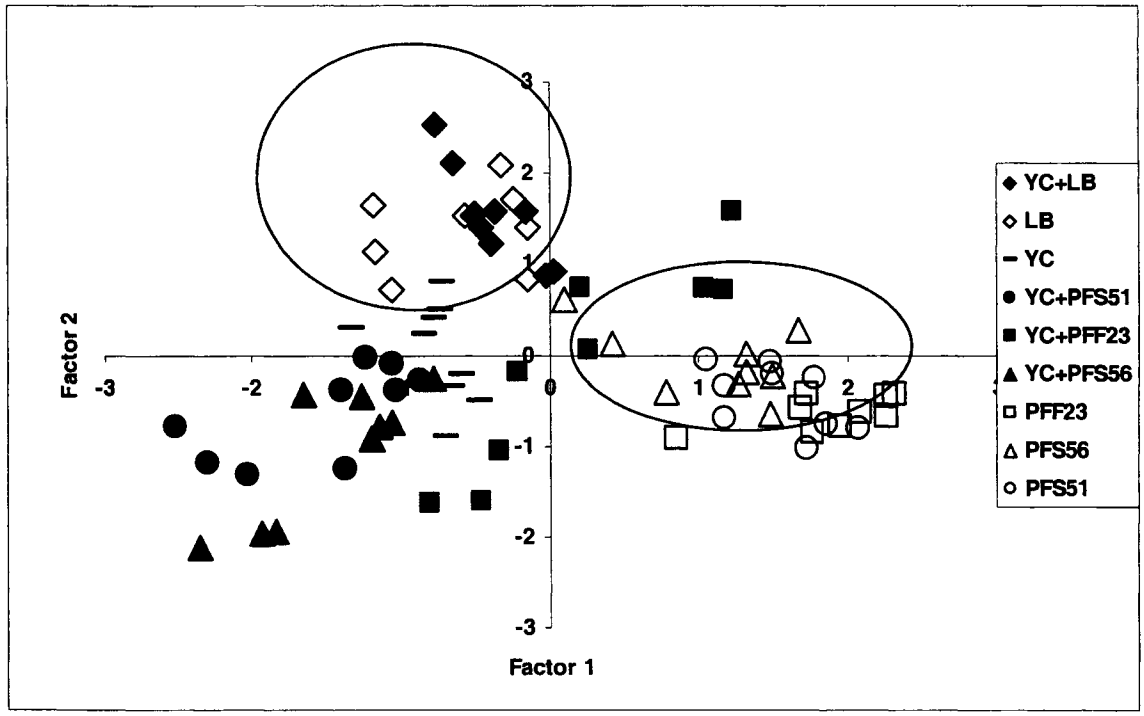

Figure 1 - Plot of the PCA of yogurt showing associations between bacterial cultures and analytical data. Object coordinates representing individual bacterium at 1, 7 and 14 days with three replications.

Table 2 - Effects of bacteria treatments on the volatile flavor compounds of yogurt[a]

Flavor Compounds	Bacterial Cultures								
	YC	YC +LB	YC+ PFS-56	YC+ PFS-51	YC+ PFF-23	LB	PFS-56	PFS-51	PFF-23
COMPONENT 1 (PC-1)									
2-propanol	9.84c	12.45c	9.50c	10.17c	21.57b	12.69c	89.58a	116.67a	94.78a
isobutyl alcohol	35.92c	27.47c	34.39c	28.99c	38.36c	25.98c	63.08ab	61.39ab	72.43a
2,3-pentanedione	80.07ab	52.46c	71.09bc	68.36bc	78.56ab	46.95c	97.95a	81.87ab	104.54a
undecanal	31.61bc	22.24c	16.00c	20.45c	26.97bc	16.38c	45.15ab	41.53ab	52.96a
2,3-butanedione	58.55d	122.40bcd	85.14bcd	73.05cd	93.91bcd	115.98bcd	141.89b	206.26a	128.41bc
ethylbenzene	18.11bcd	12.77cde	27.74a	15.75bcde	19.06abc	23.07ab	9.36de	9.70cde	8.41e
dimethyl disulfide	9.75b	8.46b	8.19b	5.98b	8.53b	10.82b	95.63a	231.71a	227.57a
COMPONENT 2 (PC-2)									
1-hexanol	108.27bcd	68.97de	137.93ab	122.18bc	58.56de	46.08e	144.19ab	186.67a	82.38cde
2-undecanone	20.10a	15.32abc	15.89ab	17.56ab	10.69c	15.17abc	17.49ab	16.59ab	11.28bc
1-butanol	33.32a	14.28b	27.29ab	32.44ab	12.92b	14.24b	21.40ab	13.15b	14.93ab
2-pentylfuran	101.43b	174.67a	71.39b	71.12b	180.68a	128.75ab	117.92ab	130.18ab	100.64b
acetic acid	61.17bc	286.52a	75.71bc	86.66b	76.45bc	266.63a	7.86d	21.08cd	4.60d
COMPONENT 3 (PC-3)									
hexanal	54.59b	35.36bc	27.05c	40.15bc	33.26bc	40.80bc	72.71a	67.96ab	67.74ab
ethyl pentanoate	211.80	180.42	129.98	178.67	192.40	139.56	153.80	185.88	289.67
octanoic acid	89.75abc	65.75d	70.87cd	76.68bcd	95.51ab	86.24abcd	71.91bcd	77.05bcd	109.74a
2-nonanone	34.49b	31.49b	55.29ab	44.92ab	70.55a	42.19b	58.17ab	45.47ab	71.59a

Table 2 - (Continued)

COMPONENT 4 (PC-4)									
methanol	11.03ab	11.94a	7.16bcd	7.56bcd	8.73abcd	10.70ab	10.31abc	5.17d	6.42cd
trans-3-octen-2-one	14.34a	12.38ab	9.00bc	11.13abc	7.42c	12.76ab	11.95ab	9.21bc	10.74abc
1-octen-3-ol	83.84	74.51	64.32	72.80	78.52	96.05	89.53	83.45	80.07
benzaldehyde	49.59bcd	25.34d	105.77a	89.03ab	105.25a	28.03cd	68.57abc	69.17abc	50.18bcd
acetaldehyde	53.54	54.59	39.60	36.74	48.39	62.99	58.74	45.39	44.48
2-heptanone	979.10a	764.40ab	777.4ab	754.70ab	450.70abc	685.10ab	220.30d	482.30abc	301.10bc
ethyl butanoate	65.43	51.65	47.16	67.99	67.64	51.80	42.75	38.08	53.55
1-octanol	19.28c	21.40c	198.28b	336.76a	21.85c	24.52c	42.79c	48.61c	31.21c
trans-2-nonenal	17.42bc	10.56c	27.58a	23.73ab	24.90ab	11.90c	16.37bc	11.95c	19.19abc
nonanal	15.75a	15.76a	11.58a	12.20a	13.79a	16.75a	14.28a	8.91b	13.81a
hexanoic acid	14.00ab	4.04c	7.00bc	3.97c	3.29c	3.51c	4.90c	15.63a	6.03bc
5-nonanone	17.96d	30.30cd	62.54ab	34.51bc	21.14cd	18.93d	42.00bc	72.18a	34.34bc
d-limonene	140.38a	53.20bcd	79.86bc	87.57b	92.91b	39.06cd	30.30d	65.52bcd	80.56bc
butanoic acid	8.71ab	4.37b	10.43ab	5.38b	9.65ab	18.54a	6.00b	8.58ab	7.62ab
ethyl heptanoate	21.10c	27.35bc	27.20bc	68.48ab	27.06bc	92.59a	43.92b	40.92b	21.23c
3-heptanone	10.84c	45.82b	7.38c	6.30c	6.74c	14.16c	121.27a	7.44c	11.07c
1-pentanol	1499.40bc	1544.30bc	1919.40b	1062.30c	2074.50b	2032.60b	2884.00a	3134.30a	1798.70b
ethyl acetate	17.67b	6.93c	2.39cd	4.51cd	42.25a	5.34cd	ND	ND	ND
propionic acid[b]									
1 DAY	11.21c,x	12.91c,x	14.23c,y	14.76c,y	51.54b,y	21.39c,x	76.00ab,y	106.41a,y	97.64a,y
7 DAY	21.20c,x	16.65c,x	60.91b,x	37.96c,x	81.02b,x	36.28c,x	207.21a,x	201.86a,x	228.11a,x
14 DAY	17.68c,x	25.08c,x	30.52c,y	23.74c,xy	50.10b,y	42.05b,x	150.21a,xy	153.17a,xy	180.93a,x

[a]Means in the same row followed by the same superscripts (a-e) are not significantly different ($p > 0.05$). Means are averages of three replications with data for storage time pooled unless interactions between the bacterial culture and storage time were significant ($p < 0.05$).
[b]Means in the same column followed by the same letters (x-y) are not significantly different ($p > 0.05$).

Table 3 - Effect of bacterial cultures and inoculation level on acidity and texture of yogurts[a]

Strain	Inoculation level	Titratable acidity (g lactic acid /100ml yogurt)[b]	pH[c]	Viscosity (centipoise)	Degree of syneresis (%)
YC	6	1.01a	4.47ab	116,000ab	10a
	7	1.09a	4.34bc	192,000a	4a
	8	0.83b	4.55a	153,000ab	6a
LB	6	0.78b	4.65a	71,000b	12a
	7	1.11a	4.26c	135,000ab	6a
	8	0.92a	4.53a	133,000ab	13a
YC+LB	6	1.01a	4.31bc	147,000ab	7a
	7	1.02a	4.36bc	145,000ab	2a
	8	1.17a	4.17c	156,000ab	1a

[a]Means in the same column followed by the same superscripts (a-c) are not significantly different ($p > 0.05$). Means are triplicate analyses of three replications. Data were pooled across storage times.
[b]Average titratable acidity of all treatments prior to fermentation was 0.23.
[c]Average pH of all treatments prior to fermentation was 5.97.

Table 4 - Effects of bacterial cultures on the volatile flavor compounds of yogurt[a]

	Bacterial Cultures								
Flavor Compounds	**6YC**	**7YC**	**8YC**	**6LB**	**7LB**	**8LB**	**6(YC+LB)**	**7(YC+LB)**	**8(YC+LB)**
Characteristic volatile compounds in yogurts									
acetaldehyde	12.46bc	12.72bc	11.39c	17.80ab	20.64a	12.94bc	15.39abc	14.07bc	10.12c
2,3-butanedione	32.90c	32.66c	37.90bc	107.79a	79.11abc	77.53abc	70.45abc	67.13abc	89.34ab
2-propanol	11.95c	15.39bc	69.86a	26.21bc	18.71bc	18.47bc	15.50bc	17.11bc	43.23b
ethyl pentanoate	6.64c	10.79bc	49.37a	20.63b	13.14b	17.42b	11.80bc	16.18b	15.69b
benzaldehyde	9.03c	9.94c	12.41bc	13.96b	19.24a	14.66b	14.30b	14.19b	14.08b
2,3-pentanedione	12.89ab	11.22b	8.50b	14.21ab	16.93a	13.58ab	14.31ab	13.13ab	12.69ab
ethyl acetate	23.99	20.4	23.31	23.57	18.51	17.03	18.89	21.2	14.01
acetic acid	12.55c	14.38bc	19.64ab	19.40ab	12.60c	15.85bc	22.58a	17.82abc	21.84a
butanoic acid	12.59b	13.03b	18.82a	15.30ab	13.36b	13.52b	15.32ab	14.71ab	14.19ab
ethyl butanoate	18.41bc	19.03bc	12.56c	26.09ab	29.72a	20.90bc	17.66bc	18.21bc	19.77bc
2-heptanone	282.50b	267.70b	249.68b	252.26b	216.77b	280.42b	282.44b	273.94b	364.27a
hexanal	12.74	16.06	15.37	19.26	22.43	17.9	17.06	14.03	15.03
1-pentanol	476.20a	149.2c	350.0abc	200.6bc	357.6abc	410.6ab	239.3bc	227.8bc	297.0abc
2-pentylfuran	28.74b	30.56b	54.30b	29.07b	29.94b	62.25b	37.25b	27.55b	140.71a
methanol	9.90b	13.32b	19.98a	14.10b	10.80b	12.08b	11.23b	10.82b	11.10b
2-undecanone	78.93ab	86.56a	86.39a	45.98c	69.74abc	57.97bc	79.11ab	86.87a	58.02bc
2-nonanone	75.98b	100.48a	46.75c	18.91d	24.91d	33.60cd	82.61b	68.10b	78.78b
1-butanol	27.26ab	29.19a	26.65ab	30.19a	24.80ab	29.15a	28.49a	26.97ab	17.52b
ethyl heptanoate	9.69b	9.86b	11.84b	19.96a	13.09b	9.12b	24.24a	12.45b	9.32b
octanoic acid	7.58	7.68	8.78	6.97	6.47	7.13	7.69	8.9	8.2
Untypical volatile compounds									
undecanal	36.77c	63.76ab	67.07ab	51.45b	76.54a	67.34ab	44.65bc	46.61bc	48.95bc
1-octen-3-ol	28.44c	32.62bc	37.17abc	51.45a	45.87abc	41.78abc	49.65ab	41.06abc	38.47abc
trans-2-nonenal	12.94b	15.72ab	39.73a	19.39ab	14.97b	9.15b	15.75ab	13.28b	17.14ab
1-octanol	10.01	11.2	12.37	16.88	15.6	11.31	12.77	11.51	12.8
5-nonanone	15.30bc	21.16ab	21.47ab	29.13a	12.64c	11.36c	23.42ab	13.56c	10.97c

[a]Means in the same row followed by the same superscripts (a-d) are not significantly different ($p > 0.05$). Means are averages of three replications with data for storage time pooled.

GENERAL CONCLUSIONS

Overall, this study demonstrated 1) probiotic bacteria were able to produce CLA from linoleic acid in the model system containing hydrolyzed soy oil (1%) emulsified in non-fat dry milk, but not in model systems of unhydrolyzed soy oil (1%) emulsified in non-fat dry milk or 1% milk fat. Of all evaluated 11 probiotic bacteria, *Propionibacterium freudenreichii* subsp. *shermanii* 56, *P. freudenreichii* subsp. *shermanii* 51 and *P. freudenreichii* subsp. *freudenreichii* 23 demonstrated the greatest increase in CLA content. *P. freudenreichii* subsp. *shermanii* 51 produced the highest *cis*-9, *trans*-11 CLA content. The fermentation of probiotic bacteria for 24 h was often most effective in increasing CLA content and microbial counts. Therefore, the content of CLA during the fermentation was primarily dependent on strains of probiotic bacteria and lipid sources in the milk model system.

2) The incorporation of probiotic bacteria with yogurt culture and unique processing methods increased the CLA content of yogurt. Of the probiotic bacteria evaluated, *Lactobacillus rhamnosus*, either alone or in conjunction with yogurt culture, resulted in the highest content of CLA. However, the yogurt processed with the propionibacteria alone was lower in CLA content than the control yogurt. The addition of yogurt cultures or rennet greatly increased the microbial counts and CLA content of yogurts processed with propionibacteria at 45 °C. Inoculation concentration of *L. rhamnosus* and yogurt cultures had no significant effect on CLA content of yogurts. The yogurt produced by *L. rhamnosus* in conjunction with yogurt culture with 10^7 CFU/mL total inoculation level resulted in the high content of CLA. In general, the CLA content and microbial counts of yogurts were stable over a 14-day storage time at 4 °C.

3) The yogurt processed with yogurt cultures, *L. rhamnosus*, yogurt cultures in conjunction with probiotic bacteria demonstrated similar acidity, texture and flavor as the control yogurt produced with yogurt cultures. The yogurt processed with the propionibacteria alone showed different quality attributes from the control yogurt. In general, the quality characteristics of yogurts were stable over a 14-day storage time at 4 °C. Inoculation concentration of *L. rhamnosus* and yogurt cultures had no significant effect on texture, but affected acidity and volatile flavor compounds of yogurts. CLA-enriched yogurt products with *L. rhamnosus* or the combination of probiotic bacteria and yogurt cultures showed acceptable quality attributes.

Further research in this area should involve sensory evaluation of yogurt under similar conditions as the experiments in this study. Correlations should be made between quality attributes (volatile flavor profiles, acidity and texture) and sensory data. Gas chromatography-olfactometry (GC-O) is a necessary technique to identify desirable and undesirable aromas which have an impact on yogurt flavor. Our research focused on volatile flavor compounds in yogurts using instrumental analysis. However, sensory attributes play a major role in consumer acceptability. GC-O will provide more information in improving yogurt flavor and increasing the popularity in marketplace.

Extended studies with longer storage times are needed to definitely determine the effects of storage time greater than 15 days on CLA content and quality attributes of yogurt. The CLA content of the yogurt throughout the whole shelf-life is important to provide health benefits for consumers.

Finally, the research may also be important to increase the content of free linoleic acid in milk without adding lipids from non-dairy source. Additional processing treatments, such as additives, should be studied to further increase CLA formation in dairy products.

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ACKNOWLEDGEMENTS

I still remember the first day I came to Iowa State University. Time flies so quickly, but leaves many warm and unforgettable memories in my life.

I would like to express sincere appreciation to my major professor, Dr. Terri Boylston, for all of your guidance, support, encouragement and patience. You might not know that your diligence, persistence and deep love for career and life has an invaluable influence on my life.

I would also like to pay my respect and gratitude for my committee members, Dr Earl Hammond, Dr. Bonita Glatz, Dr. Aubrey Mendonca, Dr. Donald Beitz and Dr. Philip Dixon, for all your time, advice and help throughout this project.

I would especially like to thank many fellow students in the food science department for your friendship, enthusiasm and care.

My sincere thankfulness extended to Ms. Lan Zheng. Thank you so much for your help and effort.

My family is the warmest and sweetest place in the world. I have greatest appreciation to my parents for their love, understanding and encouragement throughout my life. I would like to thank my husband, Shihe. Without your love and support, I would not be what I am today.