Effect of Lipid Source on Probiotic Bacteria and Conjugated Linoleic Acid 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 the CLA content. Viable counts of probiotic bacteria increased significantly from 0 to 24 h. These results demonstrated that the content of CLA during fermentation was primarily dependent on the strain of probiotic bacteria and the lipid source in the milk model system. This research suggests an efficient approach to produce CLA-enriched cultured dairy products.

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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 the CLA content of the 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 temperatures, and aging will affect the CLA content. However, yogurts with a higher fat content (1, 2, and 3.25%) showed no significant changes in the 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, L. 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 that *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 *Propionibacterium 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).

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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 (*L. delbrueckii* subsp. *bulgaricus* and *S. 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% (wt/vol) tryptic soy broth, 1% (wt/vol) yeast extract, and 1% (wt/vol) 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 the hydrolyzed soy oil.

The soy oils (hydrolyzed and unhydrolyzed) were 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 nonfat 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 the 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 the 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 an FID (HP6890; Hewlett-Packard Inc., Wilmington, DE) and separated using a CP-Sil 88 column (100 m × 0.25 mm i.d.; Chrompack, Middelburg, The Netherlands). The sample (1.0 μL) was injected onto the column with a 5:1 split ratio. The temperature of the GC oven was initially held 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 times and peak areas of CLA standards (Nu-Chek-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) was sterilized and used for the dilution blanks. The plates were incubated under anaerobic conditions at 37°C for 48 h (lactic acid bacteria) or at 32°C for 72–96 h (propionibacteria).

pH measurement. The pH of the milk model systems was recorded using a digital pH meter (Accumet Model AB15; Fisher Scientific, 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; SAS 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 h 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 h 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 FA. 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 the 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 that *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 promoting 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

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]

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

[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]

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

[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).

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. s*hermanii* 51 was significantly greater than the model system fermented with traditional yogurt cultures (Table 1). *Propionibacterium 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 the 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 the CLA content. *Propionibacterium freudenreichii* subsp. *shermanii* 51 produced the most *cis*-9,*trans*-11 CLA (1.45 mg/g lipid) after 24 h of 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 that prolonging fermentation time from 24 to 48 h did not enhance CLA formation for six lactic acid bacteria in a skim milk system containing linoleic acid. Kim and Liu (15) reported that *L. lactis* I-01 formed more CLA when the 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 the 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 bacteria in the hydrolyzed soy oil model system (1.35 $\log_{10}$ cfu/mL), the increase was not significantly different 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 bacterial 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 incorporation 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 decreased 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

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

	Actual viable counts ($\log_{10}$ cfu/mL)			Increase in viable counts ($\log_{10}$ cfu/mL)	
Bacteria	0 h	24 h	48 h	24 h	48 h
P. freudenreichii subsp. *shermanii* 56	$7.30 \pm 0.91^{a,x}$	$8.25 \pm 0.88^{a,b,c,x}$	$8.26 \pm 0.86^{a,b,c,x}$	$0.95 \pm 0.03^{a,b,c,x}$	$0.96 \pm 0.05^{c,d,x}$
P. freudenreichii subsp. *shermanii* 51	$7.66 \pm 0.18^{a,x}$	$8.36 \pm 0.76^{a,b,x}$	$8.75 \pm 0.08^{a,b,x}$	$0.70 \pm 0.57^{b,c,x}$	$1.09 \pm 0.10^{b,c,d,x}$
P. freudenreichii subsp. *freudenreichii* 23	$5.37 \pm 1.93^{b,x}$	$6.90 \pm 1.27^{c,x}$	$6.46 \pm 2.06^{c,x}$	$1.53 \pm 0.66^{a,b,c,x}$	$1.09 \pm 0.13^{b,c,d,x}$
L. acidophilus 74-2	$6.93 \pm 0.00^{a,b,z}$	$8.37 \pm 0.20^{a,b,y}$	$8.80 \pm 0.11^{a,b,x}$	$1.44 \pm 0.20^{a,b,c,x}$	$1.87 \pm 0.11^{a,b,c,x}$
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	$7.45 \pm 0.08^{a,y}$	$8.61 \pm 0.13^{a,b,x}$	$8.73 \pm 0.08^{a,b,x}$	$1.16 \pm 0.06^{a,b,c,x}$	$1.28 \pm 0.16^{b,c,d,x}$
E. faeciem M74	$7.10 \pm 0.06^{a,y}$	$8.79 \pm 0.06^{a,b,x}$	$9.02 \pm 0.42^{a,b,x}$	$1.69 \pm 0.00^{a,b,c,x}$	$1.92 \pm 0.35^{a,b,c,x}$
L. rhamnosus	$7.14 \pm 0.36^{a,y}$	$9.02 \pm 0.18^{a,b,x}$	$9.15 \pm 0.00^{a,b,x}$	$1.88 \pm 0.54^{a,b,x}$	$2.01 \pm 0.36^{a,b,x}$
P. acidilactici	$7.34 \pm 0.17^{a,y}$	$8.63 \pm 0.04^{a,b,x}$	$8.54 \pm 0.16^{a,b,x}$	$1.29 \pm 0.21^{a,b,c,x}$	$1.20 \pm 0.01^{b,c,d,x}$
Yogurt cultures[b]	$6.96 \pm 0.59^{a,b,x}$	$7.94 \pm 0.54^{a,b,c,x}$	$8.07 \pm 0.06^{a,b,c,x}$	$0.98 \pm 1.12^{a,b,c,x}$	$1.11 \pm 0.65^{b,c,d,x}$
B. bifidum 420	$7.03 \pm 0.04^{a,x}$	$7.64 \pm 0.57^{b,c,x}$	$7.53 \pm 1.17^{b,c,x}$	$0.61 \pm 0.53^{c,x}$	$0.50 \pm 0.77^{d,x}$

[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 a Hydrolyzed Oil Model System After 24 and 48 h Fermentation[a]

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

[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).

by the bacteria, although microbial counts did not change significantly.

Table 4 also shows 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 to 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.

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REFERENCES

1. Ip, C., S.F. Chin, J.A. Scimeca, and M.W. Pariza, Mammary Cancer Prevention by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 51*:6118–6124 (1991).
2. Chouinard, P.Y., L. Corneau, D.M. Barbano, L.E. Metzger, and D.E. Bauman, Conjugated Linoleic Acids Alter Milk Fatty Acid Composition and Inhibit Milk Fat Secretion in Dairy Cows, *J. Nutr. 129*:1579–1584 (1999).
3. Hayek, M.G., S.N. Han, D. Wu, B.A. Warkins, M. Meydani, J.L. Dorsey, D.E. Smith, and I.N. Meydani, Dietary Conjugated Linoleic Acid Influences the Immune Response of Young and Old C57BL/6NCrIBR Mice, *J. Nutr. 129*:32–38 (1999).
4. Houseknecht, K., J. Helvel, S. Moya-Camarena, C. Portocarrero, L. Peck, K. Nickel, and M. Belury, Dietary Conjugated Linoleic Acid Normalize Impaired Glucose Tolerance in Zucker Diabetic Fatty fa/fa Rat, *Biochem. Biophys. Res. Commun. 244*:678–682 (1998).
5. Nicolosi, R.J., E.J. Rogers, D. Kritchevsky, J.A. Scimeca, and P.J. Huth, Dietary Conjugated Linoleic Acid Reduces Plasma Lipoproteins and Early Aortic Atherosclerosis in Hypercholesterolemic Hamsters, *Artery 22*:266–277 (1997).
6. Lin, H., T.D. Boylston, M.J. Chang, L.O. Luedecke, and T.D. Shultz, Survey of Conjugated Linoleic Acid Contents of Dairy Products, *J. Dairy Sci. 78*:2358–2365 (1995).
7. Chin, S.F., W. Liu, J.M. Storkson, Y.L. Ha, and M.W. Pariza, Dietary Sources of Conjugated Isomers of Linoleic Acid, a Newly Recognized Class of Anticarcinogens, *J. Food Comp. Anal. 5*:185–197 (1992).
8. Shantha, N.C., L.N. Ram, J. O'Leary, C.L. Hicks, and E.A. Decker, Conjugated Linoleic Acid Concentrations in Dairy Products as Affected by Processing and Storage, *J. Food Sci. 60*:695–697 (1995).
9. Aneja, R.P., and T.N. Murthi, Conjugated Linoleic Acid Contents of Indian Curds and Ghee, *Indian J. Dairy Sci. 43*:231–238 (1990).
10. Kepler, C.R., K.P. Hirons, J.J. McNeill, and S.B. Tove, Intermediates and Products of the Biohydrogenation of Linoleic Acid by *Butyrivibrio fibrisolvens, J. Biol. Chem. 241*:1350–1354 (1966).
11. Jenkins, T.C., Lipid Metabolism in the Rumen, *J. Dairy Sci. 76*:3851–3863 (1993).
12. Kim, Y.J., R.H. Liu, D.R. Bond, and J.B. Russell, Effect of Linoleic Acid Concentration on Conjugated Linoleic Acid Production by *Butyrvibrio fibrisolvens* A38, *Appl. Environ. Microbiol. 66*:5226–5230 (2000).
13. Lin, T.Y., C.W. Lin, and C.H. Lee, Conjugated Linoleic Acid Concentration as Affected by Lactic Cultures and Added Linoleic Acid, *Food Chem. 67*:1–5 (1999).
14. Kishino, S., J. Ogawa, Y. Omura, K. Matsumura, and S. Shimizu, Conjugated Linoleic Acid Production from Linoleic Acid by Lactic Acid Bacteria, *J. Am. Oil Chem. Soc. 79*:159–163 (2002).

15. Kim, Y.J., and R.H. Liu, Increase of Conjugated Linoleic Acid Content in Milk by Fermentation with Lactic Acid Bacteria, *J. Food Sci. 67*:1731–1737 (2002).
16. Jiang, J., L. Björck, and R. Fondén, Production of Conjugated Linoleic Acid by Dairy Starter Cultures, *J. Appl. Microbiol. 85*:95–102 (1998).
17. Brassert, D., and E.J. Schiffrin, Pre- and Probiotics, in *Essentials of Functional Foods,* edited by M.K. Schmidt and T.P. Labuza, Aspen Publishers, Gaithersburg, MD, 2000, Chapter 9, pp. 205–216.
18. Kepler, C.R., and S.R. Tove, Linoleate *cis*-9,*trans*-11 Isomerase, in *Methods in Enzymology,* edited by J.M. Lowenstein, Academic Press, New York, 1969, Vol. 14, pp. 105–110.
19. Christie, W.W., G. Dobson, and F.D. Gunstone, Isomers in Commercial Samples of Conjugated Linoleic Acid, *Lipids 32*:1231 (1997).
20. Lin, T.Y., C.W. Lin, and Y.J. Wang, Linoleic Acid Isomerase Activity in Enzyme Extracts from *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* subsp. *shermanii, J. Food Sci. 67*:1502–1505 (2002).
21. Desmazeaud, M.J., Current State of Research on Lactic Acid Bacteria Nutrition, *Lait 63*:267–316 (1983).
22. Desmazeaud, M.J., Growth Inhibitors of Lactic Acid Bacteria, in *Dairy Starter Cultures,* edited by T.M. Cogan and J.P. Accolas, VCH, New York, 1996, Chapter 5, pp. 151–152.
23. Wang, L.L., and E.A. Johnson, Inhibition of *Listeria monocytogenes* by Fatty Acids and Monoglycerides, *Appl. Environ. Microbiol. 58*:624–629 (1992).
24. Rainio, A., M. Vahvaselka, T. Suomalainen, and S. Laakso, Reduction of Linoleic Acid Inhibition in Production of Conjugated Linoleic Acid by *Propionibacterium freudenreichii* ssp. *shermanii, Can. J. Microbiol. 47*:735–740 (2001).
25. Robinson, R.K., *Dairy Microbiology Handbook,* 3rd edn., John Wiley & Sons, New York, 2002, Chapter 9, pp. 457–463.
26. Wood, B.J.B., and W.H. Holzapfel, *The Genera of Lactic Acid Bacteria,* Chapman & Hall, Glasgow, 1995, Chapter 3, pp. 23–30.

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