JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Conjugated Linoleic Acid Content and Organoleptic Attributes of Fermented Milk Products Produced with Probiotic Bacteria

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The effect of probiotic bacteria on the formation of conjugated linoleic acid (CLA), microbial growth, and organoleptic attributes (acidity, texture, and flavor) of fermented milk products 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 individually or in coculture with traditional yogurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*). The lipid source was hydrolyzed soy oil. *L. rhamnosus*, in coculture with yogurt culture, resulted in the highest content of CLA. Growth and CLA formation of propionibacteria were enhanced in the presence of yogurt cultures. Texture and flavor attributes of fermented milks produced with propionibacteria were significantly different than the fermented milks processed with yogurt cultures. The fermented milks processed with probiotic bacteria in coculture with yogurt cultures demonstrated similar acidity, texture, and flavor as the fermented milk produced with yogurt cultures.

KEYWORDS: Conjugated linoleic acid; fermented milk products; probiotic bacteria; acidity, flavor; texture

INTRODUCTION

Functional foods elicit benefits to health and well-being or 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 actively enhance the health of consumers by improving the intestinal microbial balance. Additional health benefits include antitumor activity, cholesterol reduction, protection against gastroenteritis, improvement of lactose tolerance, and stimulation of the immune system through nonpathogenic means (*1*). Lactic acid bacteria (*Lactobacilli*, *Streptococci*, *Lactococci*, and *Bifidobacteria*) and propionibacteria constitute promising probiotic bacteria used in dairy food industries.

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 (*2*, *3*). Another important CLA isomer, *trans*-10,*cis*-12-octadecadienoic acid, can reduce body fat in mice (*4*). Other health benefits of CLA include roles as an antiatherogenic agent (*5*), an antidiabetic agent (*6*), immune system modulator (*7*), and body weight protector (*8*). These results have exciting implications for improved human health and development of functional dairy products.

The biohydrogenation pathway has been proposed as a major mechanism for CLA formation. The *cis*-9,*trans*-11-CLA isomer is an intermediate in the biohydrogenation of linoleic acid by the bacterium, *Butyrivibrio fibrisolvens*, in the rumen (*9*). Linoleic acid isomerase activity of rumen microorganisms, as well as probiotic bacteria, including *B. fibrisolvens*, *Lactobacillus acidophilus*, and *Propionibacterium freudenreichii* subsp. *shermanii*, contribute to CLA formation (*9*, *10*).

The effect of starter culture on CLA concentration has been widely studied in model systems with linoleic acid. Lin and others (*11*) demonstrated the ability of lactic acid cultures to produce CLA in sterilized skim milk with added linoleic acid. *L. acidophilus* produced the maximum CLA content (105.5 μg CLA/mL). However, Jiang and others (*12*) found that none of the lactic acid bacteria and only *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* demonstrated the ability to form CLA. The highest level of CLA formed in the media with linoleic acid was 265 μg/mL. Our previous investigation identified several probiotic bacteria that were able to produce increased levels of CLA in a model system containing hydrolyzed soy oil emulsified in nonfat dry milk. Hydrolyzed soy oil was used to increase the content of free linoleic acid. These bacteria included *Lactobacillus rhamnosus* (LB), *P. freudenreichii* subsp. *shermanii* 56 (PFS-56), *P.*

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10.1021/jf051030u CCC: $30.25
Published on Web 10/15/2005

freudenreichii subsp. *shermanii* 51 (PFS-51), and *P. freudenreichii* subsp. *freudenreichii* 23 (PFF-23) (*13*).

Consumer acceptability of a functional dairy product is dependent on the organoleptic attributes (*14, 15*). A majority of the research on the effect of added probiotic bacteria to improve nutritional value has focused on the viability of the microorganisms, rather than on the flavor and texture. Further study is needed to investigate the incorporation of probiotic bacteria with traditional dairy cultures to improve organoleptic attributes of dairy products.

The objective of this research was to incorporate probiotic bacteria with the ability to form CLA as identified in our previous research (*13*) and unique processing techniques to develop a functional fermented milk product. CLA content, viability of the probiotic bacteria, and organoleptic attributes (acidity, texture, and flavor) of the fermented milks were determined.

MATERIALS AND METHODS

Bacteria. LB, PFS-56, PFS-51, and PFF-23 were selected to evaluate both CLA formation and organoleptic attributes of products produced with the selected bacteria alone or in coculture with traditional yogurt cultures [1:1 ratio of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus 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. LB was obtained from Danisco Cultor Inc. (Milwaukee, WI). The propionibacteria strains were obtained from Dr. Bonita Glatz's Collection (Iowa State University) 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 Fermented Milk Product with Hydrolyzed Soy Oil. Soybean oil (Wesson, Conagra Grocery Products Company, Irvine, CA) was chemically hydrolyzed to increase the content of free fatty acids. The hydrolyzed soy oil was emulsified into a solution of 10% gum acacia (Fisher Scientific, Pittsburgh, PA) at a 1:1 (w/w) ratio (*13*) to increase the dispersability of the hydrolyzed oil into the milk system and to form a stable emulsion. The mixture was homogenized into skim milk (Hy-Vee Inc., West Des Moines, IA) to produce a fat content of 1% with 5000 μg/mL free linoleic acid. The milk solids-not-fat content was adjusted to 12% (w/w) through the addition of nonfat dry milk (Hy-Vee Inc.). 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 and Organoleptic Attributes of Fermented Milk Products. 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 probiotic bacteria (YC + LB, YC + PFS-56, YC + PFS-51, and YC + PFF-23) cocultured with YC at a 1:1 ratio. The samples were incubated at 45 °C until a pH of 4.4 (samples with YC and/or LB) or pH 5.0 (samples with propionibacteria) was reached and then were transferred to 4 °C. All fermented milk products were incubated at the optimal incubation temperature for yogurt cultures (45 °C). Because of the low rate of acid production by the propionibacteria, samples containing only propionibacteria were unable to reach a pH of 4.4. The fermented milks were sampled at days 1, 7, and 14 following transfer to 4 °C for lipid, microbiological, acidity, texture, and volatile flavor 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 of skim milk (Hy-Vee Inc.) 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 or PFS-51 at 10^7 CFU/mL and PFS-51 cocultured with YC 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 of storage, the products were sampled for microbiological and lipid analyses.

Lipid Analysis. Lipids were extracted from fermented milk products using a modified Bligh and Dyer chloroform−methanol extraction method (*16*). Heptadecanoic acid ($C_{17:0}$, Sigma Chemical Co., St. Louis, MO) was added to the lipid extracts 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 (*17*). The fatty acid methyl esters (FAMEs) were analyzed on a gas chromatograph (GC) 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 i.d.; 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 (makeup gas) at 18 mL/min. CLA and FAMEs were identified and quantified by comparison with the retention times and peak areas of CLA (NuChek-Prep, Inc., Elysian, MN) and FAME standards (Supelco, Inc., Bellefonte, PA).

A GC-MS (Trio 1000, Fisons Instruments, Danvers, MA) with a quadrupole mass analyzer was used to confirm the identity of the FAMEs. The GC conditions were the same as those of the chromatographic analysis. The MS 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*. The mass spectra of the FAMEs were compared to a spectral library (NBS Library) for identification.

Microbiological Analysis. The microbial count was determined using Lactobacilli MRS agar (Difco, Sparks, MD) for lactic acid bacteria and sodium lactate agar for propionibacteria. The sodium lactate agar consisted of sodium lactate broth [1% (w/v) tryptic soy broth, 1% (w/v) yeast extract, and 1% (w/v) sodium lactate syrup] and 2% (w/v) agar (Difco Laboratories). The samples produced with mixed cultures of lactic acid bacteria and propionibacteria were plated on the two different agars. The total counts for mixed cultures were determined by identifying the counts of individual bacteria on two agars. Samples were diluted in buffered peptone water (2%, Difco) 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).

Acidity Measurement. The pH of fermented milk products was recorded using a digital pH meter (Accumet model AB15; Fisher Scientific). The titratable acidity was determined by titrating a sample (5 g of sample + 45 mL of distilled water) with 0.1 N NaOH to an end point of pH 7.0. The titratable acidity was calculated based on lactic acid as the predominant acid and was expressed as g lactic acid/100 mL product. The sample temperature was 25 °C for each analysis.

Viscosity and Syneresis Measurement. The apparent viscosity was determined by using a RVDVII + Brookfield viscometer (Brookfield Engineering Labs Inc., Stoughton, MA) in a 100 mL fermented milk product at room temperature (25 °C). Samples were stirred for 20 s before measurement. All viscosity values were measured at 10 rpm with spindle #5 (*18*). Readings were converted to centipoise units. Syneresis (%) was expressed as volume of drained whey per 100 mL sample (*19*).

Volatile Flavor Analysis. A solid phase microextraction (SPME) technique was used for the isolation and concentration of volatile flavor compounds. A representative sample (20 g) and 5 mL of distilled water were transferred to a 100 mL headspace bottle and sealed with a Teflon septum to prevent volatile loss. The samples were stirred and held in

a 40 °C water bath to increase the concentration of volatile compounds in the sample headspace. The sample was allowed to equilibrate and absorb onto the SPME fiber [2 cm 50/30 μm divinylbenzene/carboxen/poly(dimethylsiloxane); Supelco, Inc.] 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 GC 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 as for the GC analysis of FAMEs. 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 GC-MS (Micromass GCT; Waters Corp., Milford, MA). The GC conditions were the same as those of the chromatographic analysis. The MS 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 s. Mass spectra of the volatile flavor compounds were compared to a spectral library (Wiley Library) and a flavor and fragrance database (Flavor WORKS, version 2.0; Flavometrics, Anaheim Hills, CA) for identification.

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

Principal component analysis (PCA) was used to examine relationships or groupings of the volatile flavor compounds based on treatment effects (bacterial treatment and storage time) for experiment 1. A correlation matrix was used for the extraction of the principal components (PCs), with Varimax orthogonal rotation. A minimum eigenvalue of 1.0 was used in the PCA. A biplot analysis was conducted to identify relationships of individual treatment based on the weightings of each objective variable from the first two PCs of PCA and the data obtained for each treatment (SYSTAT, ver. 9.01; SPSS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

Effect of Probiotic Bacteria on CLA Formation. Previous research demonstrated the ability of probiotic bacteria to produce CLA from linoleic acid in the model systems containing hydrolyzed soy oil emulsified in nonfat dry milk. Of the 11 probiotic bacteria evaluated, three propionibacteria (PFS-56, PFS-51, and PFF-23) and LB demonstrated the greatest increase in CLA content (*13*). These four bacteria were selected to evaluate CLA formation in fermented milk products produced with the selected bacteria alone or in coculture with traditional yogurt cultures.

The presence of lactic acid bacteria, either as LB or through the addition of yogurt cultures, had a significant effect on the total microbial counts of the products. The microbial counts of the products processed with yogurt cultures, LB, and the probiotic bacteria with the yogurt cultures ranged from 8.94 to 9.30 log CFU/g following storage at 4 °C for 1 day (**Table 1**). The microbial counts of the products processed with the three propionibacteria (PFF-23, PFS-51, and PFS-56) were significantly lower than those processed with lactic acid bacteria. Microbial counts in the products processed with the propionibacteria alone decreased to 6.14 to 6.72 log CFU/g after 1 day of storage. Total microbial growth in the fermented milk products containing propionibacteria cocultured with the yogurt cultures was significantly higher than in products with only propionibacteria but was not significantly different from the fermented milk products with the yogurt cultures.

Table 1. Effect of Bacterial Treatment and Storage Time on Total Microbial Counts[a] of Fermented Milk Products Processed with Probiotic Bacteria

bacterial treatment	microbial counts (log CFU/g)[b]		
	1 day	7 day	14 day
YC	8.98 a,x	8.98 a,x	8.80 a,x
YC + LB	9.21 a,x	8.63 ab,y	8.73 a,y
LB	8.94 a,x	8.71 ab,x	8.64 a,x
YC + PFS-51	9.30 a,x	8.88 a,x	9.13 a,x
PFS-51	6.14 b,x	6.52 c,x	5.75 b,x
YC + PFS-56	9.22 a,x	8.85 a,x	8.48 a,x
PFS-56	6.72 b,x	7.13 bc,x	5.99 b,x
YC + PFF-23	9.28 a,x	8.83 a,x	8.71 a,x
PFF-23	6.38 b,x	6.68 c,x	5.85 b,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 or y) are not significantly different ($p > 0.05$) for storage effect. Means are averages of three replications. [b] Inoculation level of each treatment (0 day) was 6.86–7.99 $\log_{10}$ CFU/mL.

Storage time only had a minor effect on the microbial counts of the products (**Table 1**). Following 14 days of storage, total microbial counts for treatments with lactic acid bacteria cultures ranged from 8.48 to 9.13 log CFU/g. Microbial counts in products processed with only propionibacteria ranged from 5.75 to 5.99 log CFU/g after 14 days of storage. Only the LB cocultured with the yogurt cultures showed a significant decrease between 1 and 7 days. Thus, refrigerated storage of the fermented milk products did not adversely affect the viability of the lactic acid and probiotic bacteria.

Condon and others (*20*) 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 specific pair of propionibacteria and lactic acid bacteria. The most effective combination, *L. delbrueckii* subsp. *lactis* LL51 and *P. freudenreichii* P23, increased the growth rates of *P. freudenreichii* P23 from 1.19 to 2.04 log CFU/mL. The metabolic products of the lactic acid bacteria, including peptides, lactic acid, and carbon dioxide, facilitated the growth of propionibacteria (*20*, *21*). Peptides are an important substrate for the physiological activity of propionibacteria. The peptidases within the propionibacteria will hydrolyze peptides to supply the cells with all essential amino acids (*22*). Lactate is a preferred substrate for the growth of propionibacteria, while carbon dioxide forms a more anaerobic environment to facilitate the growth of propionibacteria.

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 fermented milk product processed with LB in coculture with yogurt cultures had a significantly higher *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA content than the product processed with yogurt cultures alone.

The ability of probiotic bacteria, including *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*

Table 2. Effect of Bacterial Treatment and Storage Time on the CLA Contents[a] of Fermented Milk Products

bacterial treatment	CLA content[b] (mg/g lipid)					
	cis-9,*trans*-11 CLA			*trans*-10,*cis*-12 CLA		
	1 day	7 day	14 day	1 day	7 day	14 day
YC	0.45 a,x	0.42 ab,x	0.57 bc,x	0.27 ab,x	0.27 abc,x	0.38 cd,x
YC + LB	0.33 a,y	0.39 ab,y	0.97 a,x	0.33 ab,y	0.31 ab,y	0.71 a,x
LB	0.44 a,y	0.44 ab,y	0.73 ab,x	0.32 ab,y	0.34 ab,y	0.68 ab,x
YC + PFF-23	0.53 a,x	0.57 a,x	0.65 abc,x	0.32 ab,x	0.39 a,x	0.41 abc,x
PFF-23	0.22 b,x	0.23 b,x	0.39 cd,x	0.13 b,x	0.11 c,x	0.16 d,x
YC + PFS-56	0.57 a,x	0.51 ab,x	0.63 abc,x	0.43 a,x	0.38 a,x	0.50 abc,x
PFS-56	0.19 b,x	0.21 b,x	0.25 d,x	0.11 b,xy	0.09 c,y	0.12 d,x
YC + PFS-51	0.42 a,x	0.29 ab,x	0.60 abc,x	0.18 b,x	0.31 ab,x	0.41 abc,x
PFS-51	0.17 b,x	0.21 b,x	0.24 d,x	0.12 b,x	0.13 bc,x	0.11 d,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 or y) are not significantly different ($p > 0.05$) for storage effect for each CLA isomer. Means are averages of three replications. [b] CLA was not detected in any treatment at 0 day.

(*12*), *L. acidophilus* (*11*), *L. lactis* I-01 (*23*), and *L. plantarum* AKU 1009A (*24*), to form CLA has been demonstrated in model systems with free linoleic acid as the lipid source. Lin (*25*) demonstrated the mixed cultures comprising yogurt culture and *L. acidophilus* with 0.1% linoleic acid addition significantly improved *cis*-9,*trans*-11-CLA content in nonfat set yogurt. However, few studies have evaluated CLA-forming ability when probiotic bacteria were cocultured with traditional yogurt cultures.

In general, storage time did not have a significant effect on the CLA content of fermented milk products. For the combination of yogurt cultures and propionibacteria, the CLA content was relatively stable during refrigerated storage. However, the fermented milk products produced with LB, either alone or in coculture with yogurt cultures, had a significantly higher CLA content at 14 days. In previous research, the CLA content of yogurt has been shown to be stable during storage periods of 7 days (*26*) and 6 weeks (*27*). Thus, the CLA-forming ability of the bacterial treatments rather than storage has an impact on the CLA content of fermented milk products.

Organoleptic Attributes of Fermented Milk Products. Interactions between bacterial treatment and storage time did not have a significant effect on the organoleptic attributes of the fermented milk products. For each starter culture treatment, no significant difference in the organoleptic 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 organoleptic attributes of fermented milk products.

The fermented milk products produced with the yogurt cultures, LB, or the propionibacteria with the yogurt cultures had pH values in the range of 4.10−4.33 (**Table 3**). These pH values were significantly lower than for the products produced with the propionibacteria (PFF-23, PFS-56, and PFS-51) alone. The slower rate of acid production and pH decrease in the fermented products containing only propionibacteria in comparison to the fermented products with lactic acid bacteria are consistent with the differences in the growth rates (**Table 1**) of these cultures.

The samples with only the yogurt cultures (*L. bulgaricus* and *S. thermophilus*) had the highest titratable acidity (1.79 g lactic acid/100 mL yogurt) (**Table 3**). The products with the probiotic bacteria alone or with probiotic bacteria plus yogurt cultures had lower titratable acidities. The combination of PFS-56 and yogurt culture showed the lowest titratable acidity throughout the storage period.

Table 3. Acidity and Texture of Fermented Milk Products Processed with Probiotic Bacteria

bacterial treatment	pH[c]	titratable acidity (g lactic acid/100 g)[b]	viscosity (centipoise)	degree of syneresis (%)
YC	4.23 c	1.79 a	225000 a	9 b
YC + LB	4.10 c	1.17 bc	224000 a	12 b
LB	4.14 c	1.26 bc	237000 a	15 b
YC + PFS-51	4.16 c	1.02 bc	234000 a	16 b
PFS-51	4.72 b	1.04 bc	57300 bc	44 a
YC + PFS-56	4.33 c	0.83 c	269000 a	14 b
PFS-56	5.02 a	1.35 b	134000 b	47 a
YC + PFF-23	4.32 c	1.04 bc	223000 a	12 b
PFF-23	4.64 b	1.17 bc	47400 c	51 a

[a] Means in the same column followed by the same superscripts (a–c) are not significantly different ($p > 0.05$) for bacterial treatment. 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 g lactic acid/100 g. [c] Average pH of all treatments prior to fermentation was 5.96.

The titratable acidity and pH data did not show parallel changes. In the titratable acidity determination, the acidity is reported only 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 (**Table 1**). Lactic acid bacteria produce lactic acid as the major product by exclusively fermenting hexoses. The genus *Lactobacillus* is the preferred species to produce lactic acid (*28*). However, for propionibacteria, lactic acid production in the 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 (*22*). The pK_a values of lactic acid and propionic acid are 3.86 and 4.87, respectively. At the pH of the fermented milk products, a greater proportion of lactic acid would be dissociated than the propionic acid, thus contributing to the lower pH in fermented milks with lactic acid bacteria than in fermented milks with propionibacteria.

The fermented milk products processed with the yogurt cultures, LB, or the propionibacteria in coculture with yogurt cultures were significantly more viscous than product processed with the propionibacteria (PFF-23, PFS-56, and PFS-51) alone (**Table 3**). Prolonging storage from 1 to 14 days at 4 °C resulted in no significant difference in viscosity for all treatments.

The products processed with the yogurt cultures, LB, or the propionibacteria with the yogurt cultures had significantly less syneresis than products processed with the propionibacteria alone (**Table 3**). Yogurt culture demonstrated the lowest degree

Table 4. Effects of Bacterial Treatments on the Content of Volatile Flavor Compounds (GC Peak Areas) of Fermented Milk Products[a]

	bacterial cultures								
flavor compounds	YC	YC + LB	YC + PFS-56	YC + PFS-51	YC + PFF-23	LB	PFS-56	PFS-51	PFF-23
				PC-1					
2-propanol	9.84 c	12.45 c	9.50 c	10.17 c	21.57 b	12.69 c	89.58 a	116.67 a	94.78 a
isobutyl alcohol	35.92 c	27.47 c	34.39 c	28.99 c	38.36 c	25.98 c	63.08 ab	61.39 ab	72.43 a
2,3-pentanedione	80.07 ab	52.46 c	71.09 bc	68.36 bc	78.56 ab	46.95 c	97.95 a	81.87 ab	104.54 a
undecanal	31.61 bc	22.24 c	16.00 c	20.45 c	26.97 bc	16.38 c	45.15 ab	41.53 ab	52.96 a
2,3-butanedione	58.55 d	122.40 bcd	85.14 bcd	73.05 cd	93.91 bcd	115.98 bcd	141.89 b	206.26 a	128.41 bc
ethylbenzene	18.11 bcd	12.77 cde	27.74 a	15.75 bcde	19.06 abc	23.07 ab	9.36 de	9.70 cde	8.41 e
dimethyl disulfide	9.75 b	8.46 b	8.19 b	5.98 b	8.53 b	10.82 b	95.63 a	231.71 a	227.57 a
				PC-2					
1-hexanol	108.27 bcd	68.97 de	137.93 ab	122.18 bc	58.56 de	46.08 e	144.19 ab	186.67 a	82.38 cde
2-undecanone	20.10 a	15.32 abc	15.89 ab	17.56 ab	10.69 c	15.17 abc	17.49 ab	16.59 ab	11.28 bc
1-butanol	33.32 a	14.28 b	27.29 ab	32.44 ab	12.92 b	14.24 b	21.40 ab	13.15 b	14.93 ab
2-pentylfuran	101.43 b	174.67 a	71.39 b	71.12 b	180.68 a	128.75 ab	117.92 ab	130.18 ab	100.64 b
acetic acid	61.17 bc	286.52 a	75.71 bc	86.66 b	76.45 bc	266.63 a	7.86 d	21.08 cd	4.60 d
				PC-3					
hexanal	54.59 b	35.36 bc	27.05 c	40.15 bc	33.26 bc	40.80 bc	72.71 a	67.96 ab	67.74 ab
ethyl pentanoate	211.80	180.42	129.98	178.67	192.40	139.56	153.80	185.88	289.67
octanoic acid	89.75 abc	65.75 d	70.87 cd	76.68 bcd	95.51 ab	86.24 abcd	71.91 bcd	77.05 bcd	109.74 a
2-nonanone	34.49 b	31.49 b	55.29 ab	44.92 ab	70.55 a	42.19 b	58.17 ab	45.47 ab	71.59 a
				PC-4					
methanol	11.03 ab	11.94 a	7.16 bcd	7.56 bcd	8.73 abcd	10.70 ab	10.31 abc	5.17 d	6.42 cd
trans-3-octen-2-one	14.34 a	12.38 ab	9.00 bc	11.13 abc	7.42 c	12.76 ab	11.95 ab	9.21 bc	10.74 abc
1-octen-3-ol	83.84	74.51	64.32	72.80	78.52	96.05	89.53	83.45	80.07
benzaldehyde	49.59 bcd	25.34 d	105.77 a	89.03 ab	105.25 a	28.03 cd	68.57 abc	69.17 abc	50.18 bcd
acetaldehyde	53.54	54.59	39.60	36.74	48.39	62.99	58.74	45.39	44.48
2-heptanone	979.10 a	764.40 ab	777.4 ab	754.70 ab	450.70 abc	685.10 ab	220.30 d	482.30 abc	301.10 bc
ethyl butanoate	65.43	51.65	47.16	67.99	67.64	51.80	42.75	38.08	53.55
1-octanol	19.28 c	21.40 c	198.28 b	336.76 a	21.85 c	24.52 c	42.79 c	48.61 c	31.21 c
trans-2-nonenal	17.42 bc	10.56 c	27.58 a	23.73 ab	24.90 ab	11.90 c	16.37 bc	11.95 c	19.19 abc
nonanal	15.75 a	15.76 a	11.58 a	12.20 a	13.79 a	16.75 a	14.28 a	8.91 b	13.81 a
hexanoic acid	14.00 ab	4.04 c	7.00 bc	3.97 c	3.29 c	3.51 c	4.90 c	15.63 a	6.03 bc
5-nonanone	17.96 d	30.30 cd	62.54 ab	34.51 bc	21.14 cd	18.93 d	42.00 bc	72.18 a	34.34 bc
d-limonene	140.38 a	53.20 bcd	79.86 bc	87.57 b	92.91 b	39.06 cd	30.30 d	65.52 bcd	80.56 bc
butanoic acid	8.71 ab	4.37 b	10.43 ab	5.38 b	9.65 ab	18.54 a	6.00 b	8.58 ab	7.62 ab
ethyl heptanoate	21.10 c	27.35 bc	27.20 bc	68.48 ab	27.06 bc	92.59 a	43.92 b	40.92 b	21.23 c
3-heptanone	10.84 c	45.82 b	7.38 c	6.30 c	6.74 c	14.16 c	121.27 a	7.44 c	11.07 c
1-pentanol	1499.40 bc	1544.30 bc	1919.40 b	1062.30 c	2074.50 b	2032.60 b	2884.00 a	3134.30 a	1798.70 b
ethyl acetate	17.67 b	6.93 c	2.39 cd	4.51 cd	42.25 a	5.34 cd	ND	ND	ND
				propionic acid[b]					
1 day	11.21 c,x	12.91 c,x	14.23 c,y	14.76 c,y	51.54 b,y	21.39 c,x	76.00 ab,y	106.41 a,y	97.64 a,y
7 day	21.20 c,x	16.65 c,x	60.91 b,x	37.96 c,x	81.02 b,x	36.28 c,x	207.21 a,x	201.86 a,x	228.11 a,x
14 day	17.68 c,x	25.08 c,x	30.52 c,y	23.74 c,xy	50.10 b,y	42.05 b,x	150.21 a,xy	153.17 a,xy	180.93 a,x

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

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 (*29*). The increase in water-binding capacity of proteins increases curd stability during fermentation and storage (*30*). At the isoelectric point of casein (pH 4.6), a stable network forms, which has less susceptibility to syneresis.

The increased acid production in the fermented milks of propionibacteria cocultured with the yogurt cultures improved the textural characteristics to result in final products with texture similar to that of the control yogurt. The difference in the textural characteristics of the products 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 4.1 to 4.33 (**Table 3**). The results for viscosity and syneresis of fermented milk products are consistent with the limited growth and acid production by the propionibacteria.

Fermentation is a major process to produce lactic acid and volatile flavor compounds in fermented milk. Most of the 34 compounds identified in this study, such as 2,3-butanedione, 2,3-pentanedione, benzaldehyde, acetaldehyde, acetic acid, ethyl acetate, and 2-heptanone, have been identified in yogurt and fermented milk products (*31*−*34*). In our research, hydrolyzed soy oil was used as a lipid source to increase CLA content in the fermented milk 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 flavors in fermented milk product (*34*).

PCA is one of the most commonly used statistical techniques to study complexities in flavor systems. PCA condenses a large set of data into groups of similar characteristics. In this study, the volatile flavor compounds were grouped into four PCs, which accounted for 42% of the total variability in the data set (**Table 4**). PC-1 (14.3%) contained seven volatile flavor compounds, PC-2 (11.5%) contained five volatile flavor compounds, PC-3 (8.7%) contained four volatile flavor compounds,

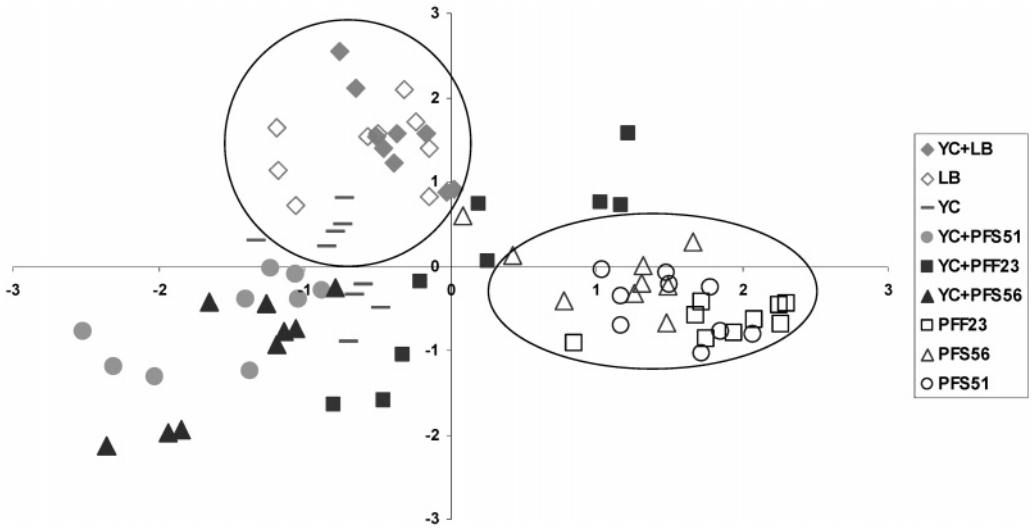

Figure 1. Results of the PCA and biplot analysis showing the relationships between the bacterial treatments for the first two PCs. Object coordinates signify pooled responses for variables (volatile compounds) using the content of volatile compounds at 1, 7, and 14 days with three replications.

and PC-4 (7.5%) contained 18 volatile flavor compounds. Volatile flavor compounds were not exclusively grouped into the PCs based on the class of compound, namely, aldehydes, ketones, alcohols, and esters.

The fermented milk product produced with propionibacteria alone formed a significantly different group from that produced with yogurt cultures, LB, and the probiotic bacteria with the yogurt culture (**Figure 1**). The fermented milk products produced with PFS-56, PFS-51, and PFF-23 shared similar flavor attributes. No significant differences were observed for products produced with the other bacterial treatments. These results demonstrated that the products processed with the probiotic bacteria cocultured with the yogurt culture showed similar flavor characteristics as control yogurt produced by yogurt cultures. However, the product produced with only propionibacteria did not have typical yogurt flavor.

The aroma profile of yogurts did not change significantly during refrigerated storage. These results agreed with research conducted by Imhof and Bosset (*35*). 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 bacterial culture treatments on volatile flavor compounds (**Table 4**).

PC-1 included two major volatiles, 2,3-butanedione and 2,3-pentanedione, known to contribute to typical yogurt flavor (*36*). However, the proper amount of each of these compounds is important to deliver characteristic yogurt flavor. The product produced by propionibacteria alone showed a higher content of 2,3-butanedione than the product processed with other cultures. In particular, PFS-51 produced significantly higher amounts of 2,3-butanedione. Other bacterial treatments resulted in no difference in the production of 2,3-butanedione as compared to the yogurt culture. The fermented milk product 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 fermented milk product flavor and produced volatile flavor compounds similar to that of the control fermented milk.

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 (*37*). 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 (*33*).

PC-1 also contained several untypical yogurt flavor compounds, such as dimethyl disulfide and undecanal. In general, the product 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 fermented flavor compound. It produces a sour and pungent aroma. Lactic acid bacteria produce mainly 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. Propionibacteria often produce more acetic acid than lactic acid bacteria. Unlike other flavor compounds in PC-2, the content of acetic acid was lower in the product produced by propionibacteria alone than in the product 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**, **3**, and **4**). When growth of propionibacteria was slow, acetic acid production was reduced. However, the presence of yogurt culture greatly stimulated the growth of propionibacteria (**Table 1**). Thus, the fermented milk product processed by propionibacteria and yogurt cultures demonstrated an increased content of acetic acid.

The product produced by propionibacteria still showed significant differences from that produced with other bacteria for volatile compounds associated with 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 (*33*, *38*). In our research, the fermented milk products produced with propionibacteria, especially PFS-56, resulted in higher contents

of hexanal than with other culture treatments. However, the addition of yogurt cultures to propionibacteria resulted in significantly lower contents of hexanal. In related studies, the metabolic activities of *S. thermophilus* in fermented peanut milk (*39*) and bifidobacteria in fermented soy milk (*40*) degraded hexanal to reduce the undesirable flavor of these fermented products. Our study showed similar results that lactic acid bacteria could effectively reduce the hexanal content in fermented dairy products.

In PC-4, propionic acid, a characteristic flavor compound produced by propionibacteria, was present at significantly higher contents in the fermented milks cultured with the propionibacteria. For these treatments, the content of propionic acid increased from day 1 to day 7 during storage. These results were consistent with the growth of propionibacteria (**Table 1**).

The aroma profile of yogurt consists of a unique combination of volatile flavor compounds. Correct ratios among the different key compounds are essential for a balanced aroma (*14*). The volatile compounds contribute to the characteristic notes of fermented milk products but can also cause off-flavors depending on their concentrations as compared to other flavor compounds. The atypical flavor of the fermented milks processed with propionibacteria was attributed to the imbalance in the key flavor compounds as compared to the control fermented milk. Some desirable fermentation end products, such as 2,3-butanedione, 2,3-pentanedione, and acetic acid, showed different contents in the products produced with propionibacteria alone as compared to those produced with other bacteria. Overproduction of 2,3-butanedione by propionibacteria causes a harsh flavor (*41*). Some off-flavor compounds, such as dimethyl disulfide and undecanal, were present in higher concentration in the products with propionibacteria alone than in the other products. All of these differences caused a different ratio of flavor compounds and different flavor characteristics from control products.

The proper acidity is also closely related to yogurt flavor. A desirable pH for typical yogurt flavor ranges from pH 4.0 to pH 4.4 (*42*). Our study showed similar results. The fermented milk product produced by yogurt culture, LB, and the probiotic bacteria with the yogurt cultures had a pH ranging from 4.0 to 4.4 and similar flavor characteristics (**Table 3** and **Figure 1**). However, the products produced by propionibacteria had a higher pH (4.64−5.02) and different flavor characteristics than the control fermented milk.

Effect of Temperature and Casein Hydrolysis on Microbial Growth and CLA Formation of Fermented Milk Products. The incubation temperature (45 °C) for the fermented milk products in experiment 1 was higher than the optimal growth temperature for propionibacteria (32 °C) and the incubation temperature (32 °C) for the milk model systems in which propionibacteria were shown to be effective for CLA formation (*13*). In experiment 1, the microbial growth and CLA formation by the propionibacteria in the presence of the yogurt cultures were 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 is shown in **Table 5**. At 32 °C, there was no significant difference in the total microbial counts of the treatments with yogurt culture, PFS-51 with or without rennet addition, and PFS-51 with yogurt culture. However, at 45 °C, the samples produced with PFS-51 alone showed significantly lower microbial counts than the other treatments. 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 significantly increased the microbial counts of the products. Comparing PFS-51 and PFS-51 + RE, it is obvious that rennet played an important role in increasing microbial counts. Although YC and PFS-51 + YC had no great difference, it suggested that proteolytic activity of yogurt cultures showed a similar effect as rennet for enhanced microbial growth of propionibacteria. The production of peptides by the proteolytic systems of yogurt cultures has been proposed to stimulate the growth of propionibacteria (*43*). Therefore, casein hydrolysis and the release of peptides, either through the metabolic activity of the yogurt cultures or rennet activity, contributed to the increased microbial counts of propionibacteria in the products when the incubation temperature was not ideal for growth of propionibacteria.

Table 5. Effect of Casein Hydrolysis and Incubation Temperature on Total Microbial Counts[a] of Fermented Milk Products after 1 Day of Storage

	microbial counts[b] (log CFU/g)	
treatment	32 °C	45 °C
YC	8.08 a,x	8.83 a,x
PFS-51	8.83 a,x	7.18 b,y
PFS-51 + YC	9.21 a,x	9.23 a,x
PFS-51 + RE[c]	9.08 a,x	8.66 a,x

[a] Means in the same column followed by the same letters (a or b) are not significantly different ($p > 0.05$) for treatment effect. Means in the same row followed by the same letters (x or 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 CFU/mL. [c] PFS-51 + RE refers to PFS-51 inoculated in the milk with rennet.

Table 6. Effect of Casein Hydrolysis and Incubation Temperature on the CLA Contents[a] of Fermented Milk Products after 1 Day of 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	0.63 b,x	0.72 a,x	0.22 a,x	0.25 a,x
PFS-51	1.95 a,x	0.26 b,y	0.26 a,x	0.18 a,x
PFS-51 + YC	2.57 a,x	0.59 a,y	0.26 a,x	0.17 a,x
PFS-51 + RE[c]	1.87 a,x	0.73 a,y	0.15 a,x	0.23 a,x

[a] Means in the same column followed by the same letters (a or b) are not significantly different ($p > 0.05$) for treatment effect. For each CLA isomer, means in the same row followed by the same letters (x or 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 refers to PFS-51 inoculated in the milk with rennet.

The effect of temperature and casein hydrolysis on CLA production (**Table 6**) reflected the effects of these treatments on microbial growth. At 32 °C, the products processed with PFS-51 with or without rennet and the combination of PFS-51 and yogurt cultures had significantly higher contents of *cis*-9,*trans*-11-CLA than the treatment with only yogurt cultures. However, when incubated at 45 °C, the products processed with PFS-51 alone were significantly lower in *cis*-9,*trans*-11-CLA content than the other treatments. Therefore, casein hydrolysis significantly increased CLA formation for PFS-51 under the nonoptimal incubation temperature of 45 °C.

In conclusion, probiotic bacteria and CLA both contribute to enhance the health-promoting potential of functional dairy products. The incorporation of probiotic bacteria with yogurt

culture not only increased CLA content but also produced organoleptic attributes that were comparable to the control fermented milk. CLA formation by propionibacteria was inhibited at the optimal temperature for growth of yogurt cultures. However, enhanced proteolysis, through the addition of rennet or yogurt cultures, improved the viability of the propionibacteria and increased CLA formation in nonideal temperatures. With the growing interest in the development on foods with enhanced nutritional value, it is critical to also determine the effect of these treatments on the organoleptic attributes. For these products to be successful and gain wide consumer acceptability, the flavor, textures, and overall sensory attributes must be comparable to the traditional products.

ACKNOWLEDGMENT

LB was donated by Danisco Cultor Inc. The yogurt cultures were provided by Chr. Hansen.

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Received for review May 4, 2005. Revised manuscript received August 16, 2005. Accepted September 6, 2005. 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 3574, was supported by Hatch Act and State of Iowa Funds.

JF051030U