Antagonistic Action of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 Toward Spoilage Organisms in Cottage Cheese*

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ABSTRACT

Cells of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 were added to cottage cheese stored at 7°C in different amounts to determine if they would inhibit the growth of *Pseudomonas fluorescens*, also inoculated into the cheese samples. In addition, experiments were conducted in which no spoilage organisms were added to determine the effect of the lactobacilli on the natural background flora in the cottage cheese. For most experiments, as the numbers of lactobacilli increased, the numbers of spoilage organisms were lower than in the control on any given day of storage. In cheese inoculated with *P. fluorescens*, the numbers of spoilage organisms in the control had increased 5 log cycles by d 7, whereas the treatment containing the highest level of *L. delbrueckii* ssp. *lactis* RM2-5 (1.0×10^9 cfu/g) had not, and did not, increase over the course of the 21-d study. In the experiments where no spoilage organisms were added, lactobacilli significantly retarded the growth of gram-negative bacteria in the cheese. However, in these experiments, mold growth on the samples became a limiting factor during extended storage. The results of these experiments indicate that lactobacilli could be effective at helping control gram-negative spoilage bacteria in cottage cheese, thus potentially extending its shelf life.

(**Key words:** *Lactobacillus delbrueckii* ssp. *lactis*, cottage cheese, psychrotrophic spoilage)

Abbreviation key: CVT = crystal violet tetrazolium chloride, LBS = lactobacillus selection, MRS = de Man, Rogosa, Sharpe.

INTRODUCTION

An excellent source of nutrients for the human diet, cottage cheese is also a very appealing source of nutri-

ents for many microorganisms that can cause spoilage of the product. Over the years, a short shelf life (1 to 2 wk) due to microbial growth has been a source of economic loss to processors and has discouraged repeat purchases of cottage cheese by consumers (Stone and Naff, 1967; Rosenberg et al., 1994). Most microorganisms that cause spoilage of cottage cheese are heat sensitive and normally do not survive the cooking process (Bigalke, 1985). Therefore, post-process contamination by psychrotrophic bacteria is a main reason why cottage cheese has the shortest shelf life of any cultured dairy product produced (Weber and Broich, 1986). The growth of these organisms in cottage cheese can cause sliminess, bitter tastes, off-flavors, and color defects, which lead to spoilage of the cheese.

The ability of lactic acid bacteria to preserve food and prevent the growth of undesirable organisms has been studied for several years. Many lactic acid bacteria have the ability to produce substances that are inhibitory at low temperatures to the growth of other bacteria (including pathogens and spoilage organisms). An important factor for these organisms in food systems is their ability to produce inhibitory substances at refrigeration temperatures while not growing (Daly et al., 1972; Gilliland and Speck, 1975; Juffs and Babel, 1975; Martin and Gilliland, 1980). Various researchers have reported the use of *Lactobacillus* spp. as one of the cultures effective at inhibiting the growth of psychrotrophic and pathogenic organisms at refrigeration temperatures. Gilliland and Ewell (1983) reported that *Lactobacillus lactis* was inhibitory to psychrotrophic organisms in raw milk at 5°C. Brashears et al. (1998) showed that *Lactobacillus delbrueckii* ssp. *lactis* was effective at reducing numbers of *Escherichia coli* O157:H7 on raw chicken stored at 5°C. The compound produced by *L. delbrueckii* ssp. *lactis* responsible for this was identified as hydrogen peroxide. Premi and Bottazzi (1972) found that *L. lactis* produced more hydrogen peroxide than other species of lactobacilli. Yap and Gilliland (2000) reported that *L. delbrueckii* ssp. *lactis* RM2-5 produced more hydrogen peroxide than did other strains of *L. delbrueckii* ssp. *lactis* tested. The ability of this organism to produce more hydrogen peroxide at refrigeration temperatures while not growing allows

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for its potential use as a biopreservative in refrigerated foods.

The objective of this study was to determine if cells of *L. delbrueckii* ssp. *lactis* RM2-5 would be effective at inhibiting the growth of spoilage organisms in cottage cheese at 7°C, thus extending the product's shelf life.

MATERIALS AND METHODS

Sources and Maintenance of Cultures

The cultures of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 and *Pseudomonas fluorescens* 1 used in this study were from the stock culture collection of the Food Microbiology Laboratory in the Oklahoma Food and Agricultural Products Research and Technology Center at Oklahoma State University (Stillwater, OK). The culture of lactobacilli was maintained by weekly subculturing using a 1% inoculum in lactobacilli de Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) and incubating for 18 h at 37°C. *Pseudomonas fluorescens* 1 was maintained by subculturing (1% inocula) in tryptic soy broth (Difco Laboratories) and incubating for 18 h at room temperature (23 to 25°C). All cultures were stored at 2 to 5°C. Both cultures were subcultured 3 times immediately before use in the experiments.

Enumeration of Bacteria

The lactobacilli were enumerated using lactobacillus selection (LBS) agar prepared from individual ingredients according to the manufacturer's specifications (BBL, Cockeysville, MD). Plates were prepared using the pour-plate technique with an overlay, placed in plastic bags, flushed with carbon dioxide, sealed, and incubated at 37°C for 48 h.

Crystal violet tetrazolium chloride (CVT) agar, which is selective for gram-negative microorganisms, was used for the enumeration of *P. fluorescens* (Swanson et al., 1992). It was prepared by adding 1 mL of a 0.1% ethanolic solution of crystal violet to 1 L of plate count agar (Difco Laboratories). To better see the colonies in the agar, 1 mL/100 mL of 0.5% aqueous solution of 2,3,5 triphenol tetrazolium chloride was added to the molten agar before plating. The plates were incubated aerobically at 15°C for 5 d.

Populations of lactobacilli in frozen concentrated cell suspension were determined by plating on MRS agar with an overlay of the same medium (MRS broth supplemented with 1.5% agar). Plates were incubated aerobically for 48 h at 37°C.

Sample preparation and dilutions for plating were done using procedures from the Compendium of Methods for the Microbiological Examination of Foods (Swanson et al., 1992). Dilutions were made using ster-

ile peptone diluent containing 0.1% peptone and 0.001% antifoam A (Sigma-Aldrich, St. Louis, MO) in distilled water. Initial dilutions were made using a Dilumat 3 automatic dilution dispenser (AES Laboratoire, Cambray, France). For the initial dilution, approximately 11 g of cottage cheese was weighed into a stomacher bag on the Dilumat 3, which then automatically added enough sterile diluent to create a 1:10 dilution. The sample was then pumped for 60 s in a masticator (RUL Instruments, Barcelona, Spain). The remaining dilutions were done using 99 mL dilution blanks. After the appropriate incubation period, colonies on plates were enumerated using a Quebec Darkfield colony counter (Darkfield-model 3325, Buffalo, NY).

Preparation of Cultures for Treatments

The RM2-5 strain used in the initial experiments was grown in MRS broth for 18 h at 37°C under static conditions, harvested by centrifugation at $5000 \times g$ for 30 min at 2 to 3°C, and resuspended in 15 mL of cold, sterile 10% nonfat milk solids. The resulting concentrated culture was held on ice until used (not more than 30 min).

Cultures of *L. delbrueckii* ssp. *lactis* RM2-5 used in all subsequent experiments were prepared as frozen concentrated suspensions. Thirty milliliters of a freshly prepared culture of lactobacilli were inoculated into 3000 mL of MRS broth in a Bioflo 2000 fermentor connected to a pH 2000 control module (New Brunswick Scientific Co., Edison, NJ) and incubated at 37°C for 6 h. The pH was not permitted to fall below 5.00 by automatic addition of a solution of 20% sodium carbonate in 20% ammonium hydroxide as described by Brashers and Gilliland (1995). After incubation, the cells were harvested by centrifugation at $5000 \times g$ for 30 min at 2 to 3°C and resuspended in enough cold, sterile 10% nonfat milk solids to yield a population of approximately 1.0×10^{10} cfu/g. Sterile glass beads (2 mm diameter) were used to aid in the resuspension of the pellet into the milk. The resulting concentrated suspension was aseptically dispensed in 2-g aliquots into sterile cryogenic vials and frozen in liquid nitrogen at -196°C until used. After 1 d of storage in liquid nitrogen, 1 vial was removed, thawed (by submersion in 1 L of tap water at room temperature for 10 min), and plated on MRS agar to determine the population of the *L. delbrueckii* ssp. *lactis* RM2-5. Before opening each vial, the exterior was sanitized with 70% alcohol. For use in the experiments, the appropriate number of vials needed were thawed then held on ice (less than 1 h) until used.

The *P. fluorescens* was a freshly prepared culture that had been subcultured 3 times in tryptic soy broth before the experiment. On the day of the experiment, the cul-

ture was held on ice until used in the treatments (less than 30 min). The inoculum used in the treatments was made by preparing a 1:1000 dilution of *P. fluorescens* and adding the necessary amount to the cottage cheese cream to yield a population of approximately 1.0×10^2 cfu/g in the cheese.

Preparation of Treatments for Storage

Dry cottage cheese curd was obtained from a commercial dairy processing plant on the day of manufacture and used the same day in experiments. Half-and-half (10.5% fat) was used as the creaming mixture, and was purchased from a local supermarket on the morning each experiment began, and held in the refrigerator until use. Immediately before use, it was repasteurized by transferring it to Pyrex bottles (Fisher Scientific, Pittsburgh, PA), which were placed in boiling water for 30 min, and then rapidly cooled in ice water.

Initial experiment using nonfrozen RM2-5. In the initial experiments, 5 treatments were prepared for storage. The dry curd cottage cheese was mixed and dispensed with a sterile spatula in 300-g portions in each of 5 sterile 1000-mL beakers labeled 1 to 5. To inoculate cream, 3 mL of a 1:1000 dilution of *P. fluorescens* was added to 960 mL of freshly pasteurized half-and-half in a sterile flask, mixed, and then dispensed in 175-g amounts into each of 5 sterile flasks. Sufficient amounts of the cell suspension of *L. delbrueckii* ssp. *lactis* RM2-5 were added to yield populations of 0, 5.0×10^7 , 1.0×10^8 , 5.0×10^8 , and 1.0×10^9 cfu/g in the 5 flasks (treatments 1 to 5, respectively). The contents of flasks 1 to 5 were added to the dry curd in beakers 1 to 5 respectively. The cottage cheese mixture in each beaker was mixed and dispensed, using a sterile spatula, in approximately 100-g portions into 4 appropriately labeled sterile 150-mL beakers (i.e., 4 beakers for each of 5 treatments). All beakers were stored at 7°C. One beaker of each treatment was removed on d 0, 7, 14, and 21 for enumeration of bacteria and for pH measurement. Two replicates were done for this experiment.

Effect of RM2-5 from frozen concentrate. All subsequent experiments were done using the frozen concentrated cell suspensions of *L. delbrueckii* ssp. *lactis* RM2-5. Samples were prepared as described in the previous section except that populations of RM2-5 were 0, 5.0×10^7 , 1.0×10^8 , 2.0×10^8 , and 5.0×10^8 cfu/g.

Effect of RM2-5 on uninoculated cottage cheese. Two separate trials were conducted in which no spoilage organisms were inoculated into the cottage cheese. In the first experiment, dry curd was mixed and 300-g portions were dispensed into each of 5 beakers. Cream (half-and-half) was pasteurized as described previously

and dispensed in 175-g amounts into 5 sterile flasks. The appropriate amounts of frozen concentrated cell suspension of RM2-5 were added to yield populations of 0, 5.0×10^7 , 1.0×10^8 , 2.5×10^8 , and 5.0×10^8 cfu/g in the cheese. Cream was added to appropriately labeled beakers containing dry curd, mixed, and aseptically dispensed in approximately 50-g amounts into each of 5 appropriately labeled, sterile 150-mL beakers. All beakers were stored at 7°C. One beaker of each treatment was removed on d 0, 7, 14, 21, and 28 for enumeration of bacteria and measurement of pH. The experiment was replicated 3 times.

In the second experiment, samples were prepared in the same way as for the first experiment except only 3 treatments (0, 5.0×10^7 , and 1.0×10^8 cfu/g of lactobacilli) were prepared and 8 portions (50 g each) of each treatment were dispensed for storage to permit analysis on d 0, 7, 14, 21, 28, 35, 42, and 49. Three replicates were done.

pH Analysis of Samples

The pH of each sample was measured on each sample day. After removing 11 g for plating, the remaining portion was used to determine pH using an AR 25 dual channel pH/ion meter (Fisher Scientific).

Statistical Analyses

Statistical analysis was conducted for all experiments as a split plot in a randomized block design using PROC MIXED and LSMEANS, where replicates were blocks, treatments served as the main units, and storage time was the subunit. This analysis was conducted using SAS software (SAS Institute, 1985). Least significant differences were used to compare means at the 5% confidence level. Factorial arrangement of the analysis depended on the experiment, because the number of treatments, storage times, and number of replicates varied among experiments.

RESULTS

Initial Experiment Using Nonfrozen RM2-5 in Inoculated Cottage Cheese

Results from an initial experiment in which nonfrozen *L. delbrueckii* ssp. *lactis* RM2-5 were added to cottage cheese inoculated with *P. fluorescens* revealed a significant difference ($P < 0.05$) among treatments over time (Table 1). As the amount of lactobacilli added increased, the amount of spoilage organisms present (as indicated by plate counts on CVT agar) significantly declined during storage ($P < 0.05$). On d 0, there was no significant difference ($P > 0.05$) among the 5 treatments

Table 1. Effect of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 grown statically for 18 h at 37°C on spoilage organisms in cottage cheese inoculated with *Pseudomonas fluorescens*.

Treatment	Target amount of lactobacilli added, ² cfu/g	Count ¹			
		d 0 ³	d 7	d 14	d 21
1	0	2.50 ^a	8.09 ^{xb}	9.47 ^{xb}	9.86 ^{xb}
2	5.0×10^7	2.41 ^a	5.59 ^{xb}	9.05 ^{xc}	9.66 ^{xc}
3	1.0×10^8	2.13 ^a	5.30 ^{xb}	9.05 ^{xc}	9.62 ^{xc}
4	5.0×10^8	1.43 ^a	1.69 ^{ya}	4.36 ^{yb}	6.30 ^{yb}
5	1.0×10^9	<1.00 ^a	<1.00 ^{ya}	<1.00 ^{za}	<1.00 ^{za}

^{a,b,c}Means appearing in the same row with different superscripts are significantly different ($P < 0.05$).

^{x,y,z}Means appearing in the same column with different superscripts are significantly different ($P < 0.05$).

¹Microbial counts expressed as log₁₀ cfu/g; spoilage organisms enumerated on CVT agar incubated at 15°C for 5 d; each value is the mean of 2 replicates.

²Target amount (cfu/g) of lactobacilli added to the cottage cheese. Actual initial lactobacilli counts = 0, 5.8×10^7 , 9.0×10^7 , 4.6×10^8 , and 9.3×10^8 cfu/g for treatments 1 to 5, respectively.

³The count for treatment 1 on d 0 reflects the number of *Pseudomonas fluorescens* inoculated into all samples.

although there was a tendency for lower numbers of spoilage organisms in the samples containing the highest numbers of added lactobacilli. However, by d 7, spoilage organisms in the control (no lactobacilli) had increased more than 5 log cycles, whereas treatments 2 and 3, which contained the 2 smallest amounts of lactobacilli, had only increased 3 log cycles. Treatments 4 and 5, which contained the largest amounts of lactobacilli, did not exhibit any increase in numbers of spoilage bacteria by d 7. By d 14, the first 3 treatments, which contained no or low levels of lactobacilli, had CVT counts of approximately 9.7 log₁₀ cfu/g. Treatments 4 and 5 had significantly lower ($P < 0.05$) CVT counts on d 21 than did treatments 1, 2, and 3. Treatment 4 had a count of 6.3 log₁₀ cfu/g on d 21; treatment 5 had a count of <1.00 log₁₀ cfu/g. The results indicate that, if present in adequate numbers, lactobacilli inhibit growth of *P. fluorescens* in cottage cheese. Lactobacilli enumerated on LBS agar remained moderately constant with only slight decreases over time (data not shown). The pH did not change over time (data not shown).

Effect of RM2-5 from a Frozen Culture in Inoculated Cottage Cheese

In previous experiments in our laboratory, it was observed that *L. delbrueckii* ssp. *lactis* RM2-5 reached maximum growth in 6 h when grown under agitation at 37°C and pH 5. Therefore, we decided to use *L. delbrueckii* ssp. *lactis* RM2-5 grown for 6 h at pH 5 to prepare frozen concentrated cell suspensions, which could then be thawed and added directly to the cottage cheese cream. Freezing and storing the concentrated cell suspensions in liquid nitrogen for up to 3 mo had no detrimental effect on viability of the cells (data not shown).

Cells from the frozen concentrated suspension, however, did not appear to be as effective as the nonfrozen cells (Table 2) in controlling growth of *P. fluorescens* in cheese. However, there were significant differences among treatments over the 21-d storage time. On d 0, there was no significant difference ($P > 0.05$) among treatments, but by d 7 there was a 4 log cycle difference between the control and treatment 5 [which contained the highest number (5.0×10^8 cfu/g) of lactobacilli]. By d 21, although counts of spoilage bacteria were high in all samples, there were significantly fewer ($P < 0.05$) in treatment 5 than in the control. The counts of lactobacilli and pH remained constant over the 21-d storage period (data not shown).

Effect of RM2-5 on Uninoculated Cottage Cheese

To determine the potential for use of *L. delbrueckii* ssp. *lactis* RM2-5 in controlling growth of the natural flora in cottage cheese, experiments were conducted in which no spoilage organisms were added. In the first experiment, the samples were stored for 28 d (Table 3). As the number of cells of lactobacilli increased, there were significant ($P < 0.05$) decreases in the growth of spoilage organisms over the 28-d period. In this trial, no spoilage organisms were detected in treatment 5 (5.0×10^8 cfu/g) until d 14, whereas the control had a count of 4.5 log₁₀ cfu/g by d 7, indicating the effects of RM2-5 on the growth of gram-negative bacteria, which can cause spoilage of cottage cheese. On d 28, there was a 3 to 4 log cycle difference between the control and the 3 treatments with the 3 highest levels of RM2-5. The sporadic growth of molds in the samples was responsible for some of the counts observed on CVT agar. This suggests that the lactobacilli were not effective in controlling growth of molds. In this experiment, the num-

Table 2. Effect of frozen¹ and thawed *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 grown for 6 h with agitation at 37°C in the fermentor on spoilage organisms in cottage cheese inoculated with *Pseudomonas fluorescens*.

Treatment	Target amount of lactobacilli added, ³ cfu/g	Count ²			
		d 0 ⁴	d 7	d 14	d 21
1	0	2.91 ^a	6.25 ^{xb}	9.09 ^{xc}	9.67 ^{xc}
2	5.0 × 10 ⁷	2.77 ^a	3.87 ^{yb}	8.47 ^{xye}	9.46 ^{xc}
3	1.0 × 10 ⁸	2.72 ^a	4.06 ^{yb}	8.10 ^{xye}	9.31 ^{xyd}
4	2.0 × 10 ⁸	2.64 ^a	3.26 ^{yzab}	7.51 ^{yb}	8.99 ^{xye}
5	5.0 × 10 ⁸	2.53 ^a	2.12 ^{za}	5.73 ^{zb}	8.15 ^{ye}

^{a,b,c}Means appearing in the same row with different superscripts are significantly different ($P > 0.05$).

^{x,y,z}Means appearing in the same column with different superscripts are significantly different ($P > 0.05$).

¹Frozen at -196°C.

²Microbial counts expressed as log₁₀ cfu/g; spoilage organisms enumerated on CVT agar incubated at 15°C for 5 d; each value is the mean of 3 replicates.

³Target amount (cfu/g) of lactobacilli added to the cottage cheese. Actual initial lactobacilli counts = 0, 6.2 × 10⁷, 1.1 × 10⁸, 2.0 × 10⁸, and 4.9 × 10⁸ cfu/g for treatments 1 to 5, respectively.

⁴The count for treatment 1 on d 0 reflects the number of *P. fluorescens* inoculated into all samples.

bers of lactobacilli and pH changed very little during storage.

In the second experiment, cheese was stored for 49 d and treatments included 2 levels of lactobacilli (5.0 × 10⁷ and 1.0 × 10⁸ cfu/g) to determine if cells of RM2-5 could control growth of spoilage organisms for a longer time (Table 4). As in the previous trial, mold growth became a factor in some samples. However, as in the other experiments, it was apparent that lactobacilli were effective at slowing the growth of spoilage organisms over an extended period. In treatment 3 (1 × 10⁸/g), after a slight peak of spoilage organisms on d 21, the population remained constant at approximately 2 log₁₀ cfu/g over the rest of the storage period with slight numerical (not significant) decreases. The counts of spoilage bacteria on CVT agar for control and treatment 2 increased significantly ($P < 0.05$) over time. After 49 d, the pH values showed variation among treatments

not seen in previous experiments. On d 0, all treatments had a pH of ~5.00; after 49 d, treatment 1 had a pH value of 5.7, treatment 2 had a value of 5.5, and treatment 3 remained moderately constant at 5.2.

DISCUSSION

A number of researchers have reported the use of lactic acid bacteria to control the growth of spoilage and pathogenic bacteria in a variety of foods. A major benefit of some of these lactic acid bacteria is their ability to produce inhibitory compounds at refrigeration temperatures while not growing themselves. It has been well documented that hydrogen peroxide produced by these lactobacilli at refrigeration temperatures is a main cause for growth inhibition of undesirable organisms in refrigerated foods (Dahiya and Speck, 1968;

Table 3. Effect of frozen¹ and thawed *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 grown for 6 h with agitation at 37°C in the fermentor on cottage cheese that was not inoculated with spoilage organisms.

Treatment	Target amount of lactobacilli added, ³ cfu/g	Count ²				
		d 0	d 7	d 14	d 21	d 28
1	0	<1.00 ^a	4.49 ^{xb}	7.33 ^{xc}	9.67 ^{xc}	9.14 ^{xc}
2	5.0 × 10 ⁷	<1.00 ^a	3.82 ^{xyb}	<i>3.03</i> ^{yab}	5.17 ^{ybc}	7.12 ^{xye}
3	1.0 × 10 ⁸	<1.00 ^a	3.84 ^{xyb}	4.68 ^{yb}	6.19 ^{yb}	<i>4.88</i> ^{yzb}
4	2.5 × 10 ⁸	<1.00 ^a	1.85 ^{yzab}	<i>3.18</i> ^{yabc}	<i>4.93</i> ^{yc}	<i>3.81</i> ^{zbc}
5	5.0 × 10 ⁸	<1.00 ^a	<1.00 ^{za}	2.91 ^{yab}	<i>3.77</i> ^{yb}	4.60 ^{zb}

^{a,b,c}Means appearing in the same row with different superscripts are significantly different ($P < 0.05$).

^{x,y,z}Means appearing in the same column with different superscripts are significantly different ($P < 0.05$).

¹Frozen at -196°C.

²Microbial counts expressed as log₁₀ cfu/g; spoilage organisms enumerated on CVT agar incubated at 15°C for 5 days; each value is the mean of 2 replicates. Means shown in bold indicate that data of 1 replicate was a mold count; means shown in italics indicate that both counts making up the mean were mold counts.

³Target amount (cfu/g) of lactobacilli added to the cottage cheese. Actual initial lactobacilli counts = 0, 5.4 × 10⁷, 1.1 × 10⁸, 3.1 × 10⁸, and 5.6 × 10⁸ cfu/g for treatments 1 to 5, respectively.

Table 4. Effect of frozen¹ and thawed *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 which had been grown for 6 h with agitation at 37°C in the fermentor on cottage cheese that was not inoculated with spoilage organisms.

Treatment	Target amount of lactobacilli added, ³ cfu/g	Count ²							
		d 0	d 7	d 14	d 21	d 28	d 35	d 42	d 49
1	0	<1.00 ^a	2.87 ^{ab}	4.92 ^b	5.54^b	5.82 ^b	4.74^b	5.93 ^b	5.42^b
2	5.0 × 10 ⁷	<1.00 ^a	2.47 ^{ab}	2.35 ^{ab}	4.32^{abc}	6.15 ^c	5.62^{bc}	5.23 ^{bc}	5.63 ^{bc}
3	1.0 × 10 ⁸	<1.00 ^a	<1.00 ^a	2.22 ^a	3.03 ^a	2.93 ^a	2.14^a	2.95 ^a	2.26 ^a

^{a,b,c}Means appearing in the same row with different superscripts are significantly different ($P < 0.05$).

¹Frozen at -196°C.

²Microbial counts expressed as log₁₀ cfu/g; spoilage organisms enumerated on CVT agar incubated at 15°C for 5 d; each value is the mean of 3 replicates. Means shown in bold indicate that data of 1 replicate was a mold count; means shown in italics indicate that both counts making up the mean were mold counts.

³Target amount (cfu/g) of *Lactobacilli* added to the cottage cheese. Actual initial lactobacilli counts = 0, 5.9 × 10⁷, and 8.9 × 10⁷ cfu/g for treatments 1 to 3, respectively.

Price and Lee, 1970; Martin and Gilliland, 1980; Gilliland and Ewell, 1983; Brashears et al., 1998).

The potential for the beneficial effects of adding *L. delbrueckii* ssp. *lactis* RM2-5 to creamed cottage cheese was demonstrated in this study. The results indicate significant ($P < 0.05$) inhibition of growth of spoilage organisms in cottage cheese during storage at 7°C. However, relatively high numbers of the lactobacilli were required. Based on results from earlier studies, strain RM2-5 produces considerable hydrogen peroxide at refrigeration temperatures (Yap and Gilliland, 2000). Because only lactobacilli were added to the cheese, it is probable that hydrogen peroxide produced by RM2-5 was responsible for the inhibition of spoilage organisms over the course of this study. Throughout these experiments, varying amounts of RM2-5 were added in different experiments. The purpose of adding different amounts was to find the least amount of lactobacilli necessary to provide effective results. Weber and Broich (1986) reported that the more beneficial bacteria added the better chance one has in controlling the bacteria that cause spoilage. Because relatively high numbers were required to achieve the greatest degree of inhibition, more research is needed to enhance the antagonistic effect so that fewer numbers of RM2-5 lactobacilli are needed to achieve complete inhibition for a long period.

Use of a combination of antimicrobials might be effective. Several researchers have reported that naturally occurring antimicrobials produced by certain bacteria are effective at controlling undesirable microorganisms in cottage cheese (Weber and Broich, 1986; Tortorello et al., 1991). In a study using naturally occurring substances produced by bacteria and fungi, Tortorello et al. (1991) were able to effectively extend the shelf life of cottage cheese 2 wk beyond its code date. One antimicrobial substance naturally produced by *Propionibacterium shermanii* is Microgard (skim milk fermented by

P. shermanii then dried), a commercially available product that is often used in cottage cheese production (Daeschel, 1989). Weber and Broich (1986) showed the effectiveness of Microgard in controlling gram-negative spoilage organisms in cottage cheese. They also reported fewer instances of mold in cottage cheese that contained Microgard. Another antimicrobial currently added to cottage cheese is potassium sorbate. Much research has been done to show the effectiveness of potassium sorbate in controlling molds and some bacteria in cottage cheese. Because Microgard and potassium sorbate have no effect on the growth of gram-positive bacteria (Daeschel, 1989), it would be possible to develop a combination of Microgard, potassium sorbate, and *L. delbrueckii* ssp. *lactis* RM2-5, which would have the potential to control a variety of spoilage organisms, including gram-negative bacteria and molds, for an extended period.

The results from this study indicated that, when used alone and at an appropriate level, *L. delbrueckii* ssp. *lactis* RM2-5 was very effective at controlling growth of spoilage organisms in refrigerated cottage cheese. Although most treatments eventually showed evidence of spoilage (as evidenced by slime formation or surface mold growth) by the end of the storage period, it was still evident that in the treatments containing RM2-5, it usually took longer for the cottage cheese to exhibit spoilage defects. In most experiments on the final day of storage, the samples containing higher numbers of the lactobacilli had CVT counts several log cycles lower than controls. Results also indicated that pH values for most experiments remained moderately constant over time. These results indicate the effectiveness of using *L. delbrueckii* ssp. *lactis* RM2-5 in creamed cottage cheese to control spoilage organisms and the potential for its use in combination with other antimicrobial products to extend shelf life. Although no sensory evaluations were included in this study, it is not likely that

lactobacilli would cause any adverse flavors in the cheese. In related experiments using this organism, no off-flavors were noted.

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