THE INHIBITION OF PSYCHROTROPHIC MICROORGANISMS IN REFRIGERATED RAW MILK BY *LACTOBACILLUS LACTIS* WITH AND WITHOUT POTASSIUM SORBATE

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CHAPTER I

INTRODUCTION

Today large centralized milk processing plants receive their milk from many different sources. Some of the milk may be held several days before processing. This milk is refrigerated while it is stored but growth of psychrotrophic bacteria during storage can reduce the quality of the milk greatly. While these psychrotrophic bacteria are easily killed by pasteurization, some of them produce heat stable lipase and proteases that can adversely affect the quality of products made from the milk after it has been heat processed (Law et al., 1976; White and Marshall, 1972).

Lactic acid bacteria have been shown to inhibit the growth of food-borne pathogens and spoilage microorganisms in various food at refrigeration temperatures (Daly et al., 1972; Gilliland and Speck, 1972). The inhibitory agent has been identified as hydrogen peroxide (Dahiya and Speck, 1968). Premi and Bottazzi (1972) reported that Lactobacillus lactis produced more H_2O_2 than L. helveticus, L. jugurti, and L. bulgaricus.

Sorbic acid has been used in many foods as a preservative (Luck, 1976). Escherichia coli, Pseudomonas aeruginosa, and Saccharomyces cerevisiae are examples of microorganisms which have been shown to be inhibited by sorbate (York and Vaugh, 1964). In addition, sorbate has been shown to be more inhibitory to catalase positive bacteria than

to catalase negative lactobacilli (Emard and Vaugh, 1952).

The objectives of this study were to determine if the growth of psychrotrophic microorganisms in refrigerated raw milk could be retarded by adding cells of L. lactis. An additional objective was to determine if the addition of potassium sorbate along with cells of L. lactis would inhance any inhibition caused by the lactobacilli.

CHAPTER II

LITERATURE REVIEW

Psychrotrophic bacteria grow well at refrigeration temperatures such as 7°C even though their optimun growth temperature may be between 20 and 30°C. Spoilage of raw milk by these organisms is usually evident when their population reaches 1×10^6 to 1×10^7 per ml (Langveld et al. 1980). However, lower populations can reduce the quality of products prepared from the milk.

Psychrotrophic bateria can, during their growth in raw milk, produce heat stable lipases and proteases that will survive pasteurization or sterilization even though the bacteria are easily killed by the heat process (Adams et al., 1974). These heat stable enzymes can cause defects or spoilage of products manufactured from the milk (White and Marshall, 1972). Heat processed milk, during storage, develop bitter flavor, coagulation, or clearing due to the presence of heat stable proteases which degrade casein and whey proteins (White and Marshall, 1972). Cheddar cheese has been reported to have developed flavor defects such as bitterness when raw milk was inoculated with Pseudomonas fluorescens P26 prior to the heat treatment (79°C for 19 sec). To obtain these results the cheese was stored at 7°C for four weeks and evaluated at weekly intervals by a taste panel (White and Marshall, 1972).

Cheddar cheese may develop rancidity during storage due to the presence of a heat-stable lipase which is produced by P. fragi or

P. fluorescens prior in the raw milk prior to pasteurization (Law et al., 1976). Extracellular heat stable lipases produced by Pseudomonas and Alcaligenes species in refrigerated raw milk can cause rancidity and lipolysis in butter, cheese, and cream (Pinheiro, et al., 1965). Barach, et al. (1975) reported that gelatinization and the development of bitter flavor during storage of commercially sterile milk resulted from the action of heat resistance proteases produced by psychrotrophs in the raw milk.

An agent that could be added to raw milk to inhibit the growth of psychrotrophs and associated production of heat stable enzymes during storage at refrigeration temperatures would be helpful in preventing reduction of the quality of milk and its products. Lactic acid bacteria have been reported to have an antagonistic effect towards psychrotrophs in refrigerated raw milk (Juffs and Babel, 1975; Collins and Aramaki, 1980). Pseudomonas species (Price and Lee, 1970; Bjorck et al., 1975; Daly et al., 1971; Collins and Aramki, 1980; Juffs and Babel, 1975), Staphylococcus aureus (Dahiya and Speck, 1968; Daly et al., 1972; Gilliland and Speck, 1972) and Salmonella species (Gilliland and Speck 1972) can be inhibited in various foods by the growth and action of lactic acid bacteria.

Ground beef was inoculated with a lactic culture consisting of Streptococcus lactis and Leuconostoc citrovorum which inhibited Gram negative bacteria during storage of the meat at 7°C (Reddy et al., 1970).

Mather and Babel (1959) used a special cremaing mixture consisting of 1.5 parts of cream containing 20 percent fat and 1.0 part of acidified culture of S. citrovorus (Leuconostoc citrovorum), to inhibit psychrotropic bacteria in cottage cheese during refrigerated storage. P. fragi

and P. putrefaciens inoculated into the finished cheese were inhibited from producing slime on the cheese by the creaming mixture.

Hydrogen peroxide has been identified as one of the main inhibitory agents produced by some of the lactic acid bacteria.

Lactobacillus lactis was reported to be inhibitory towards an exterotoxigenic strain of S. aureus (Dahiya and Speck, 1968). Cell-free fitrates of spent glucose-phosphate media from cultures of L. lactis accumulated hydrogen peroxide during refrigerated storage and were found to be inhibitory. Catalase nullified the inhibitory effect of the filtrates. A level of 6 ug/ml of H_2O_2 was bacteriostatic while levels of 25-35 ug/ml were bactericidal to staphylococci at 35°C in the cell-free fitrates. Wheater et al. (1952) reported that L. lactis produced hydrogen peroxide (formerly thought to be "lactobacillin," an antibiotic) which at 4 ug/ml inhibited S. aureus. Inhibition decreased after catalase was added to the medium. Gilliland and Speck (1969) concluded that lactic streptococci produced enough hydrogen peroxide during growth in milk to be autoinhibitory. Martin and Gilliland (1980) found that strains of L. bulgaricus causing the greatest amounts of inhibition of a Gram negative psychrotroph isolated from raw milk also produced the greater amounts of hydrogen peroxide at 5.5°C.

L. bulgaricus NCSI was shown to be very antagonistic towards a Gram negative psychrotrophic bacterial culture (isolated from raw milk) in 10 percent non fat milk solids (NFMS) at 5°C. Catalase decreased the antagonism towards the psychrotroph when added to the 10 percent NFMS (Gilliland and Speck, 1975).

Premi and Bottazzi (1972) reported that L. lactis produced far more hydrogen peroxide than L. helveticus, L. jugurti, and L. bulgaricus in

antoclaved milk at 5°C.

A lactic culture was inoculated into raw milk (0.5 percent) inhibited the growth of psychrotrophs present by 50 percent at 3.5 and 7°C (Juffs and Babel, 1975). Catalase decreased the inhibition of the psychrotrophs. The lower the initial population of psychrotrophs the greater the inhibitory effect of the lactic culture. Higher populations of psychrotrophs overcame the inhibitory effect of the lactic culture.

The mechanism of inhibition of bacteria by hydrogen peroxide is associated with an increase in membrane fragility as the concentrations of hydrogen peroxide is increased. In Escherichia coli, hydrogen peroxide was reported to inhibit the uptake and metabolization of ^{14}C-labeled glutamic acid, ^{3}H-labeled uracil, glucose, and galactose. Incubation of cells of E. coli, injured by exposure to hydrogen peroxide, in bouillon at 37°C while shaking returned the cells to normal (Hideo and Fujimoto, 1975).

The lactoperoxidase/thiocyanate/hydrogen peroxide (LP/SCN/H_2O_2) system is indirectly inhibitory to psychrotrophs. The oxidation product(s) of the thiocyanate formed by the action of hydrogen peroxide (catalyzed by lactoperoxidase) inhibited P. fluorescens and E. coli (Bjorck, et al., 1975).

Lactoperoxidase and low levels of thiocyanate occur naturally in bovine milk. The hydrogen peroxide may come from bacteria such as lactic acid bacteria that produce it as a metabolite. Bjorck et al. (1979) reported that the LP/SCN/H_2O_2 system may be activated by increasing thiocyanate to 15 ppm and adding 7.5 ppm at H_2O_2 to raw milk. The amount of inhibition, described as a bacteriostatic effect, decreased as the temperatures increased. For example, the bacteriostatic

effect lasted 24-26 hr, 15-16 hr, 11-12 hr, and 7-8 hr at 15, 20, 25, and 30°C respectively. Rosen and Bjorck (1975) found that the addition of 0.5 percent glucose and 2 units of glucose oxidase to raw milk lowered the population of psychrotrophs from 1×10^4 to 1×10^2 per ml in a few hours and the population remained constant for ten days at 4°C, while the population in the untreated milk increased to 1×10^7/ml in four days. Glucose oxidase in the presence of oxygen produce the hydrogen peroxide. Thus this enzyme system could produce the hydrogen peroxide needed to activate the LP/SCN/H_2O_2.

Sorbic acid and its salts have been used in many foods to prevent the growth of molds, yeasts, and various bacteria. Sorbic acid was reported to be inhibitory towards E. coli, P. aeruginosa, and Saccharomyces cerevisiae in concentrations of 15 to 100 mg per 100 ml of medium (York and Vaugh, 1964).

Example of foods in which sorbate has been used to inhibit microbial growth include the following: cheese, meat, fish, fermented vegetables, pickled vegetables, dried fruits, edible fat emulsions, drinks, fruit juices, bakery goods, and confectionery products (Lück, 1976).

Emard and Vaugh (1952) concluded that sorbic acid was more inhibitory to catalase positive bacteria than to catalase negative bacteria such as lactobacilli and clostridia. The possible cause of this inhibition may be the effect sorbate has upon enzyme systems of the microorganisms. Melnick, et al. (1954) examined the effect of sorbate upon molds and found that at a "high" initial concentration sorbate could inhibit the dehydrogenase system in molds. If the mold population is too high, the sorbate would be metabolized by the molds. Whitaker

(1959) found that sorbic acid inhibited sulfhydryl-containing enzymes, such as ficin and alcohol dehydrogenase. Endogenous catalase in cell-free extracts of Aspergillus niger was undetectable when the medium had 0.1 percent sorbate present (Troller, 1964).

The inhibitory effect of sorbic acid against catalase positive cultures was found to be most effective at pH 5.0 - 5.5 (Emard and Vaugh, 1952). The upper limit of pH for which sorbate was found to be effective was approximately 6.5.

The sensitivity of catalase positive microorganisms to sorbate and the possible inhibition of catalase by sorbate coupled with the resistance of lactobacilli to sorbate suggests that a combination of lactobacilli and sorbate might be useful to control the growth psychrotrophs in raw milk. This is further supported by the fact that most psychrotrophic microorganisms are catalase positive. There has been no work published on the effect of a combined treatment of lactobacilli and sorbate on psychrotrophs in raw milk during refrigerated storage.

CHAPTER III

MATERIALS AND METHODS

Sources and Maintenance of Cultures

The cultures of Lactobacillus lactis used in this study were obtained from various laboratories. L. lactis Farr, F, 39A2, and 12315 were obtained from Dr. W. E. Sandine of Oregon State University. L. lactis 8000 and B were obtained from Dr. K. M. Shahani of the University of Nebraska. Dr. L. L. McKay of the University of Minnesota provided the L. lactis 39A1. L. lactis 403 E-15 was from the dairy-food microbiology culture collection at Oklahoma State University.

These cultures were maintained by subculturing (1 percent inocula) weekly into sterile (121°C for 15 minutes) 10 percent non fat milk solids (NFMS). The inoculated milk was incubated at 37°C for 24 hours. These cultures were stored in a refrigerator between subcultures. Before use in experiments, the cultures were subcultured twice in 10 ml of sterile lactobacilli MRS broth (Difco, Detroit, Michigan) using 1 percent inocula and 18 hours incubation at 37°C.

Confirmation of Identity of Cultures

Each culture was subcultured twice in lactobacilli MRS broth before streaking onto duplicate plates of lactobacilli MRS agar (lactobacilli MRS broth with 1.5 percent agar added). The streak plates were

incubated at 37°C for 24 hours in a Gas-pak anaerobic system (Baltimore Biological Laboratories, Cockeyveille, Maryland). If more than 1 colony type appeared, the predominating colony type(s) were selected from the streak plates and inoculated into MRS broth (incubated 24 hr at 37°C). Each isolate was streaked from the MRS broth culture onto the surface of two plates containing MRS agar and incubated 24 hours at 37°C in a Gas-pak anaerobic system to confirm purity (i.e. the presence of one colony type).

If the culture appeared to be pure, Gram stains (Burke, 1922) were performed on slides prepared from colonies from one of the streak plates prior the flooding it with 3 percent hydrogen peroxide from the catalase test. A positive catalase test was indicated when effervesence occurred around the colonies. Cells from the remaining streak plates were collected and used to determine fermentation patterns using the Minitek (Baltimore Biological Laboratories, Cockeyville, Maryland) system as described by Gilliland and Speck (1977). The substrate reactions included in the tests for fermentation were: amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Other biochemical tests included the ability to deaminate arginine and hydrolyze esculin. In addition cells from each isolate were inoculated into sterile MRS broth contained in screw capped test tubes and incubated at 15°C for five days to determine if the culture would grow at that temperature.

Identity was confirmed by comparing the characteristics observed for each isolate to those reported for L. lactis in the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and

Gibbon, 1974).

Preparation of Frozen Concentrated Cultures of Lactobacillus lactis

A flask containing 600 ml of sterile lactobacilli MRS broth was inoculated with 6 ml of a fresh MRS broth culture of L. lactis. The inoculated broth was incubated in a 37°C water bath until the culture reached the late exponential - early stationary phase of growth (as determined from growth curves for each strain). At the end of the incubation period, the flask was placed in an ice water bath to stop further growth. The contents of the flask were aseptically dispensed into three sterile 250 ml centrifuge bottles and then centrifuged for 20 minutes at 4080 x g (0-2°C) in a Sorvall model RC-5 superspeed refrigerated centrifuge (Dupont Company, Newton, Connecticut). The supernatant fluid (spent broth) was discarded and the bottles were placed in an ice water bath. The pellets were resuspended in 10 ml of sterile 10 percent NFMS with the aid of 20-25 sterile glass beads (0.3 cm diameter). The contents of the three bottles were combined in one bottle and mixed by swirling to insure a uniform suspension. The resulting concentrated culture was aseptically dispersed in 2 gram portions into sterile plastic freezing vials (Cooke pro-vial, Dyatech Laboratories Inc., Alexandria, Virginia). The vials were frozen and stored in liquid nitrogen (-196°C). The concentrated cultures were evaluated for numbers of lactobacilli before and after freezing in order to determine the amount necessary to provide the desired population in the milk storage experiments.

The Enumeration of Microorganisms

Microorganisms were enumerated using a pour plate technique. Appropriate decimal dilutions were prepared using 99 ml dilution blanks containing 0.1 percent peptone (Difco, Detroit, Michigan) and 0.001 percent antifoam A Emulsion (Sigma Chemical Company, St. Louis, Missouri) in distilled water. The dilutions were prepared and placed in appropriately labeled petri plates in accordance with procedures described in Standard Methods for the Examination of Dairy Products (Marth, 1978).

To enumerate lactobacilli, petri plates containing the appropriate dilutions were poured with molten, tempered (45°C) lactobacilli MRS agar. Duplicate plates were prepared for each dilution. The plates were incubated at 37°C for 48 hours. Colonies were counted with the aid of a Quebec Colony Counter. This procedure was used to enumerate lactobacilli in concentrated cultures and in the raw milk immediately after the addition cells from the concentrated culture. The latter was possible since the numbers of cells added greatly exceeded the number of other bacteria in the raw milk.

Non-lactobacilli in raw milk were enumerated by pouring plates, containing the appropriate dilutions, with Plate Count Agar (PCA) (Difco, Detroit, Michigan). The plates were incubated at 21°C for five days. All colonies visible with the aid of the Quebec Colony Counter were counted. The lactobacilli used in this study did not form colonies on PCA at 21°C.

The Interaction Experiments

Raw milk was obtained on the day produced from the bulk storage

tanks at the Oklahoma State University Dairy Cattle Center. The raw milk was aseptically placed in a sterile flask and held in an ice water bath during transport to the laboratory and until used in experiments (never longer than two hours). It was thoroughly mixed and dispensed in 100 ml volumes into sterile cotton stoppered 250 ml Erlenmeyer flasks. The concentrated cultures of L. lactis to be used were thawed by submerging the vials in 1 liter of tap water at 24°C for five minutes. The thawed cultures were added immediately to the raw milk to obtain a population of 1×10^8 lactobacilli per ml for each culture in separate flasks. The contents of the flasks were mixed by swirling 12 times in opposite directions. One flask of raw milk was used as a control. From each of the flasks a 10 ml sample was drawn and placed into sterile screw cap test tubes in an ice water bath for determining initial populations of the bacteria.

The flasks containing the samples were placed into a Gyrotory Water Bath Shaker (Model G76; New Brunswick Scientific Company, Inc., Edison, New Jersey). The temperature had been previously set at the desired level (5 or 7°C) and the agitation was set at 146 rpm. The low temperature control was facilitated by a Frigid Flow Bath Circulator (Model RF-10; New Brunswick Scientific Company, Inc., Edison, New Jersey) connected to the Gyrotory Water Bath Shaker. Additional samples were taken from each flask daily for six days for analyses.

The samples taken initially were analyzed for numbers of lactobacilli and non-lactobacilli. All subsequent samples were analyzed for numbers of non-lactobacilli. Increases in numbers of these bacteria were due to growth of psychrotrophic bacteria in the milk. The pH of all the samples was also determined.

Influence of Sorbate on Inhibitory Action of Lactobacilli

Potassium sorbate (Monsanto Company, St. Louis, Missouri) and cells from concentrated cultures of L. lactis were added to raw milk to determine their combined effect on growth of psychrotrophic bacteria at 5°C. Thawed concentrated cultures of selected strains of L. lactis were added to raw milk to yield a population of 1×10^8 per ml. The strains used were the two which appeared to be most inhibitory in previous experiments. Three flasks of raw milk were prepared for each strain. A set of three flasks of raw milk without lactobacilli was also prepared. To each set of three flasks with and without lactobacilli, a 10 percent solution of potassium sorbate was added to yield 0, 0.1 percent and 0.2 percent concentrations. The 10 percent solution of potassium sorbate had been sterilized by passage through a sterile 0.45 u membrane filter. The samples were stored at 5°C in the Gyrotary Shaker water bath as in previous experiments.

Samples (10 ml) were taken from each flask initially and on the third and fourth days of the trials. Analyses of the samples were done as indicated in the previous experiments.

Comparison of H_2O_2 production by Cultures of L. lactis in Fluid Milk

Comparison of H_2O_2 production by all strains of L. lactis was done in reconstituted 10 percent NFMS at 5°C. The milk was heated 30 minutes at 100°C prior to use. Thawed concentrated cultures of L. lactis were inoculated into 100 ml of the cold 10 percent NFMS contained in 250 ml

Erlenmeyer flasks to yield a population of 1 x 10^8 lactobacilli per ml. The samples were incubated statically at 5°C for 22 hours. The peroxidase O-dianisidine method as reported by Gilliland (1969) was used to determine the relative amounts of residual hydrogen peroxide present in the samples at the end of the incubation. The samples were plated using MRS agar to determine the numbers of lactobacilli prior to the 22 hours of incubation.

Milk - Peroxidase Agar Assay for Comparing H_2O_2 Production by Cultures of Lactobacilli

Raw milk - peroxidase agar plates were prepared and allowed to solidify in the refrigerator (5°C). Each plate was prepared by pouring from a test tube of tempered media (45°C) containing 10 ml of sterile 3 percent agar and 10 ml of raw milk. The test tube also contained 0.1 ml of horseradish peroxidase solution (2 mg in 10 ml distilled water, Sigma, St. Louis, Missouri) and 0.2 ml of 2-2' azino-di-3 ethyl benzthiazoline sulphonic acid (ABTS) (27.4 mg ABTS in 10 ml of distilled water). Both the peroxidase and ABTS solutions had been sterilized by passage through sterile 0.45 u membrane filters.

Autoclaved and dried filter paper discs (1.3 cm diameter) were placed on the milk peroxidase agar plates. These discs were then inoculated with .1 ml of 10 percent NFMS containing 1 x 10^8 lactobacilli per ml. The control disc was inoculated with .1 ml of 10 percent NFMS without lactobacilli. The milk-peroxidase agar plates were incubated 18 hours at 21°C. The reaction of hydrogen peroxide. peroxidase, and ABTS formed a purple color against the white background of the milk agar. The diameter of each purple zone was measured in centimeters from the

bottoms of the plates after the incubation period.

Statistical Analyses

The effects of the various strains of lactobacilli on growth of psychrotrophs in raw milk at 5 or at 7°C were compared using Duncan's new multiple range test. Differences in mean population of psychrotrophs in raw milk due to the various treatments in this study were compared using this method. The statistical analyses used are described in Principles and Procedures of Statistics (Steele and Torre, 1960).

CHAPTER IV

RESULTS

Confirmation of Identity of Cultures

Of the cultures designated as L. lactis obtained from various sources only those confirmed to be L. lactis were used in experiments in this study. All cultures displayed characteristics matching those for L. lactis as described in Bergey's Mannual for Determinative Bacteriology (Buchanan, 1974) with the following exceptions. None fermented glactose. Strains Farr and 12315 did not ferment mannose. Strain 12315 fermented melezitose. Strains B, Farr, 403 E-15, 39A1, and 39A2 did not ferment salicin. Thus none of the strains represented in Table I differed from the characteristics for L. lactis by greater than 2-3 characteristics out of a total of 24 characteristics tested.

Effect of Cells of L. lactis on Growth of Psychrotrophs at 7°C

Results from four trials in which the antagonistic action of six strains of L. lactis toward the growth of psychrotrophs in raw milk at 7°C are summarized in Table II. The data are presented as the $\log_{10}$ of counts of non-lactobacilli per ml obtained after the milk samples had been stored three days at 7°C. Three days of storage was selected for comparing treatment effects for the 4 trials because the population

TABLE I

CONFIRMATION OF IDENTIFICATION OF CULTURES OF L. LACTIS

Test	Strain						
	B	Farr	12315	403E-15	39A1	39A2	Bergey's[a]
Gram Stain	+	+	+	+	+	+	+
Cellular Morphology	rod	rod	rod	rod	rod	rod	rod
Catalase	-	-	-	-	-	-	-
Growth at 15°C	-	-	-	-	-	-	-
NH_3 from Arginine	-	-	-	-	-	-	-
Hydrolysis of Esculin	-	-	-	-	-	-	-
Acid From:							
Amygdalin	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-
Galatose	-	-	-	-	-	-	+
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	-	+	+	+	+
Mannitol	-	-	-	-	-	-	-
Mannose	+	-	-	+	+	+	+
Melezitose	-	-	+	-	-	-	-
Melebiose	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Salicin	-	-	+	-	-	-	+
Sorbitol	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-

[a]Characteristics of L. lactis as reported in the 8th Edition of Bergey's Mannual of Determinative Bacteriology (Buchanan and Gibbon, 1974).

TABLE II

NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH AND WITHOUT ADDED CELLS OF *LACTOBACILLUS LACTIS* AFTER THREE DAYS OF STORAGE AT 7°C

Strain of L. lactis[b]	Non-Lactobacilli Count/ml[1]				
	Trial 1	Trial 2	Trial 3	Trial 4	Avg
B	4.43	5.77	4.97	3.28	4.61
Farr	4.00	5.76	5.71	3.74	4.80
12315	4.40	5.30	4.97	3.62	4.57
403E-15	4.18	4.95	5.18	3.08	4.35
39A1	4.46	5.20	5.65	2.78	4.52
39A2	4.63	5.28	4.97	3.61	4.62
Control	4.63	5.74	6.04	3.90	5.07

[a] Counts recorded as $\log_{10}$ of number of non-lactobacilli/ml on Day three of storage.

[b] *L. lactis* counts were approximately 1×10^8/ml.

in none of the samples had reached the stationary phase of growth. Furthermore the populations in all control samples had increased at least one log cycle from the populations on day 0. When compared to the average count for the control samples, L. lactis 403 E-15 was the most inhibitory followed in order by strains 39A1, 12315, B, 39A2, and Farr. The differences in the amount of inhibition produced by strains 39A1, 12315, B, and 39A2 appeared to be minimal. Strain 403 E-15 appeared to be most consistent among the strains of L. lactis with regard to inhibiting the psychrotrophs.

Duncan's new multiple range test was used to compare differences among means for the counts in the four trials. The comparisons are summarized in Table III. When compared to the control, only L. lactis 403 E-15 and 39A1 were significantly inhibitory (403 E-15 at $P < 0.01$ and 39A1 at $P < 0.05$). The means for the other strains were not significantly different.

To determine if the cells of L. lactis altered the acidity of the milk during storage, pH measurements were made initially and after storage. The results (Table IV) indicated little or no change in acidity of the milk samples stored at 5°C for 5-7 days.

Effect of Cells of L. lactis on Growth of Psychrotrophs at 5°C

Results from six trials in which the antagonistic action of six strains of L. lactis toward the growth of psychrotrophs in raw milk was measured at 5°C are summarized in Table V. The data are presented as $\log_{10}$ of counts of non-lactobacilli per ml after the milk samples had been stored four days at 5°C. The storage period of four days was

TABLE III

TEST FOR SIGNIFICANT DIFFERENCES AMONG THE MEAN NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH AND WITHOUT ADDED CELLS OF LACTOBACILLUS LACTIS AFTER THREE DAYS STORAGE AT 7°C

Source	df	SS	MS
Total	27	21.73	
Replicate	3	18.68	6.24
Treatment	6	1.26	.21
Error	18	1.79	.099

Means Compared	Differences Between Means	LSR .05	LSR .01	Means Compared	Differences Between Means	LSR .05	LSR .01
Control-403E-15	.72[ab]	.53	.72	39A2-403E-15	.27	.51	.70
Control-39A1	.55[a]	.52	.71	39A2-39A1	.10	.50	.69
Control-12315	.50	.51	.70	39A2-12315	.05	.49	.67
Control-B	.46	.50	.69	39A2-B	.01	.47	.64
Control-39A2	.45	.49	.67				
Control-Farr	.27	.47	.64	B-403E-15	.26	.50	.69
				B-39A1	.09	.49	.67
Farr-403E-15	.45	.52	.71	B-12315	.04	.47	.64
Farr-39A1	.28	.51	.70				
Farr-12315	.23	.50	.69	12315-403E-15	.22	.49	.67
Farr-B	.19	.49	.67	12315-39A1	.02	.47	.64
Farr-39A2	.18	.47	.64				
				39A1-403E-15	.17	.47	.64

[a]Significant at $P < 0.05$.
[b]Significant at $P < 0.01$.

TABLE IV

RAW MILK pH ON THE INITIAL AND THE FINAL DAY ON TRIALS AT 7°C

Culture	Trial 1		Trial 2		Trial 3	
	Day 0	Day 3	Day 0	Day 3	Day 0	Day 6
B	6.50	6.50	6.50	6.50	6.50	6.50
Farr	6.40	6.50	6.50	6.50	6.50	6.55
12315	6.55	6.55	6.55	6.50	6.50	6.50
403E-15	6.50	6.50	6.50	6.50	6.50	6.55
39A1	6.60	6.50	6.50	6.55	6.50	6.40
39A2	6.50	6.45	6.50	6.50	6.55	6.50
Control	6.50	6.50	6.50	6.50	6.50	6.50

TABLE V

NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH AND WITHOUT ADDED CELLS OF *LACTOBACILLUS LACTIS* AFTER FOUR DAYS OF STORAGE AT 5°C

Strains of[b] *L. lactis*	Non-lactobacilli/ml[a] Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Avg
B	4.00	3.34	5.85	5.40	4.20	6.76	4.93
Farr	4.04	3.56	5.79	5.72	4.59	6.69	5.07
12315	3.96	3.77	5.92	5.23	4.53	6.70	5.02
403E-15	4.08	3.20	5.69	5.76	4.67	6.68	5.01
39A1	4.15	3.15	5.77	3.89	4.41	6.57	4.66
39A2	4.18	3.57	5.79	5.79	4.54	6.91	5.13
Control	4.20	3.61	7.23	5.40	4.58	7.38	5.40

[a] Counts recorded as $\log_{10}$ of numbers of non-lactobacilli/ml on Day four of storage.

[b] *L. lactis* counts were approximately 1×10^8/ml.

chosen because none of the populations had reached the stationary phase of growth, also the populations in all the con trol samples had increased at least one log cycle from day 0. When compared to the average count for the control samples, L. lactis 39A1 was the most inhibitory followed in order by strains B, 403 E-15, 12315, Farr, and 39A2. Strains 39A1 and B were greatly superior to the others in amount of inhibition. While 403 E-15, 12315, Farr, and 39A2 exhibited minimal differences in inhibition of psychrotrophs.

Duncan's new multiple range test was used to compare differences among means for the counts in these trials. The comparisons are summarized in Table VI. When compared to the control only L. lactis 39A1 and B were significantly inhibitory ($P < 0.05$). L. lactis 39A1 was also significant at $P < 0.01$ when compared to the control. The means for the other four strains were not significantly different from the control or any of the other strains.

The pH of the raw milk samples in these trials did not change appreciably during the storage period (Table VII). Thus the cells of L. lactis had little or no affect on acidity of raw milk stored at 5°C.

Combined Effect of L. lactis and Potassium Sorbate on Growth of Psychrotrophs at 5°C

The results from the six trials in which the antagonistic action of two strains of L. lactis in conjuncetion with potassium sorbate toward the growth of psychrotrophs in raw milk at 5°C are summarized in Table VIII. The data represented as the $\log_{10}$ count of non-lactobacilli per ml after the milk samples had been stored four days at 5°C. The storage period of four days was selected for comparing treatment effects for the

TABLE VI

TEST FOR SIGNIFICANT DIFFERENCES AMONG THE MEAN NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH AND WITHOUT ADDED CELLS OF LACTOBACILLUS LACTIS AFTER FOUR DAYS STORAGE AT 5°C

Source	df	SS	MS
Total	41	60.33	
Replicates	5	54.96	10.99
Treatments	6	1.79	.30
Error	30	3.58	.12

Means Compared	Differences Between Means	LSR .05	LSR .01	Means Compared	Differences Between Means	LSR .05	LSR .01
Control-39A1	.74[ab]	.46	.61	Farr-39A1	.41	.45	.59
Control-B	.47[a]	.46	.61	Farr-B	.14	.44	.59
Control-403E-15	.39	.45	.59	Farr-403E-15	.06	.43	.57
Control-12315	.38	.44	.59	Farr-12315	.05	.41	.55
Control-Farr	.33	.43	.57				
Control-39A2	.27	.41	.55	12315-39A1	.36	.44	.59
				12315-B	.09	.43	.57
39A2-39A1	.47[a]	.46	.61	12315-403E-15	.01	.41	.55
39A2-B	.20	.45	.59				
39A2-403E-15	.12	.44	.59	403E-15-39A1	.35	.43	.57
39A2-12315	.11	.43	.57	403E-15-B	.08	.41	.55
39A2-Farr	.06	.41	.55				
				B-39A1	.29	.41	.55

[a]Significant at P < .05.
[b]Significant at P < .01.

TABLE VII

RAW MILK pH ON INITIAL AND FINAL DAY OF TRIALS AT 5°C

L. lactis Culture	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5		Trial 6	
	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6
B	6.5	6.5	6.5	6.5	6.5	6.45	6.45	6.5	6.45	6.55	6.55	6.6
Farr	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.45	6.5	6.7	6.6	6.6
12315	6.5	6.45	6.5	6.5	6.5	6.6	6.5	6.5	6.5	6.6	6.55	6.6
403E-15	6.5	6.5	6.45	6.5	6.5	6.5	6.5	6.5	6.5	6.55	6.6	6.6
39A1	6.45	6.5	6.5	6.5	6.45	6.5	6.55	6.45	6.5	6.5	6.6	6.65
39A2	6.5	6.5	6.5	6.5	6.45	6.5	6.55	6.5	6.5	6.6	6.55	6.6
Control	6.5	6.5	6.5	6.45	6.5	6.4	6.55	6.45	6.45	6.5	6.6	6.6

TABLE VIII

NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH ADDED CELLS OF LACTOBACILLUS LACTIS AND/OR SORBATE AFTER FOUR DAYS OF STORAGE AT 5°C

Treatment		Non-lactobacilli/ml[a]				
Culture[b]	Sorbate	Trial 1	Trial 2	Trial 3	Trial 4	Avg
	0[c]	3.54	3.63	4.20	4.57	3.99
L. lactis 39A1	0.1	3.26	3.62	3.91	4.54	3.83
	0.2	2.70	2.59	3.91	4.59	3.45
	0	3.65	3.70	4.11	4.71	4.04
L. lactis 39A2	0.1	3.20	3.20	4.00	4.59	3.75
	0.2	3.26	3.00	3.98	4.60	3.71
	0	3.85	3.96	4.26	4.88	4.24
Control	0.1	3.52	3.54	4.26	4.75	4.02
	0.2	3.52	3.49	4.26	4.90	4.04

[a]Counts recorded as $\log_{10}$ of numbers of non-lactobacilli on day four of storage.

[b]L. lactis counts were approximately 1 x 10^8/ml.

[c]Percent potassium sorbate.

four trials because the population in none of the samples had reached the stationary phase of growth. Furthermore the population in all control samples had increased at least one log cycle. When compared to the mean count for the control samples, strains 39A1 and 39A2 with 0.2 percent sorbate were much more inhibitory than either strain alone. Sorbate alone had minimal inhibitory effects on the growth of psychrotrophs.

Differences among mean populations obtained from the samples in these trials were compared using Duncan's New Multiple range test (Table IX). When compared to the control, both strains in conjunction with potassium sorbate (0.1 percent and 0.2 percent) were significantly inhibotory ($P < 0.01$). The most antagonistic treatment was the combination of L. lactis 39A1 and 0.2 percent sorbate. This treatment was significantly superior to all other treatments with the exception of L. lactis 39A2 with 0.2 percent sorbate.

Neither the cells of L. lactis nor potassium sorbate had much effect on the acidity of the milk samples during storage (Table X).

Measurement of Residual Hydrogen Peroxide

The hydrogen peroxide produced in milk at $5^{o}C$ by the strains of L. lactis was determined enzymaticly (Table XI). The color developed from the reaction of peroxide with peroxidase and a chromagen was measured as absorbance at 400 nm. The absorbance values increased as the relative amounts of H_2O_2 increased. Thus the higher absorbance values indicate greater amounts of peroxide produced by the cultures. L. lactis 39A1 produced the most H_2O_2. It was followed in order by strains Farr, 403 E-15, 39A2, 12315, and B.

TABLE IX

TEST FOR SIGNIFICANT DIFFERENCES AMONG THE MEAN NUMBERS OF NON-LACTOBACILLI IN RAW MILK WITH AND WITHOUT ADDED CELLS OF L. LACTIS AND SORBATE AFTER FOUR DAYS STORAGE AT 5°C

Source	df	SS	MS
Total	35	12.92	
Replicates	3	10.32	3.44
Treatments	8	1.78	.22
Error	24	.82	.034

Means[a] Compared	Differences Between Means	LSR .05	LSR .01
Control(0)-39A1(.2)	.79[bc]	.31	.42
Control(0)-39A2(.2)	.53[bc]	.31	.42
Control(0)-39A2(.1)	.49[bc]	.31	.41
Control(0)-39A1(.1)	.41[bc]	.31	.41
Control(0)-39A1(0)	.25	.30	.40
Control(0)-Control(.1)	.22	.29	.39
Control(0)-Control(.2)	.20	.29	.39
Control(0)-39A2(0)	.20	.27	.37
39A2(0)-39A1(.2)	.59[bc]	.31	.42
39A2(0)-39A2(.2)	.33[b]	.31	.41
39A2(0)-39A2(.1)	.29	.31	.41
39A2(0)-39A1(.1)	.21	.30	.40
39A2(0)-39A1(0)	.05	.30	.39
39A2(0)-Control(.1)	.02	.29	.39
39A2(0)-Control(.2)	.00	.27	.37
Control(.2)-39A1(.2)	.59[bc]	.31	.41
Control(.1)-39A1(.2)	.57[bc]	.31	.41
Control(.1)-39A2(.2)	.31[b]	.30	.40
Control(.1)-39A2(.1)	.27	.30	.39
Control(.1)-39A1(.1)	.19	.29	.39
Control(.1)-39A1(0)	.03	.27	.37
39A1(0)-39A1(.2)	.54[bc]	.30	.40
39A1(0)-39A2(.2)	.28	.30	.39
39A1(0)-39A2(.1)	.24	.29	.39
39A1(0)-39A1(.1)	.16	.27	.37
39A1(.1)-39A1(.2)	.38[b]	.30	.39
39A1(.1)-39A2(.2)	.12	.29	.39
39A1(.1)-39A2(.1)	.08	.27	.37
39A2(.1)-39A1(.2)	.30[b]	.29	.39
39A2(.1)-39A1(.2)	.04	.27	.37

TABLE IX (Continued)

Means Compared	Differences Between Means	LSR .05	LSR .01	Means Compared	Differences Between Means	LSR .05	LSR .01
Control(.2)-39A2(.2)	.33[b]	.31	.41	39A2(.2)-39A1(.2)	.26	.27	.37
Control(.2)-39A2(.1)	.29	.30	.40				
Control(.2)-39A1(.1)	.21	.30	.39				
Control(.2)-39A1(0)	.05	.29	.39				
Control(.2)-Control(.1)	.02	.27	.37				

[a]Numbers in parenthesis indicate percent potassium sorbate.
[b]Significant at P < .05.
[c]Significant at P < .01.

TABLE X

RAW MILK pH ON INITIAL AND FINAL DAY OF TRIALS WITH L. LACTIS AND/OR SORBATE AT 5°C

Treatment		Trial 1		Trial 2		Trial 3		Trial 4	
Culture	Sorbate	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5
	0[a]	6.70	6.70	6.70	6.60	6.60	6.60	6.55	6.50
L. lactis 39A1	0.1	6.70	6.70	6.70	6.60	6.60	6.60	6.55	6.50
	0.2	6.70	6.70	6.70	6.60	6.60	6.60	6.50	6.50
	0	6.70	6.70	6.65	6.50	6.60	6.55	6.50	6.50
L. lactis 39A2	0.1	6.70	6.70	6.70	6.50	5.50	6.50	6.55	6.50
	0.2	6.70	6.70	6.70	6.60	6.60	6.55	6.50	6.50
	0	6.70	6.65	6.65	6.65	6.60	6.55	6.55	6.50
Control	0.1	6.70	6.70	6.70	6.65	6.60	6.55	6.50	6.50
	0.2	6.70	6.70	6.70	6.60	6.60	6.55	6.55	6.50

[a]Percent potassium sorbate.

TABLE XI

COLORMETERIC DETERMINATION OF PEROXIDE PRODUCED BY *LACTOBACILLUS LACTIS* IN 10 PERCENT NFMS AT 5°C[a]

Culture	400 nm[b]
L. lactis B	.10 (.15-.06)
L. lactis Farr	.13 (.21-.08)
L. lactis 12315	.11 (.16-.08)
L. lactis 403E-15	.12 (.18-.08)
L. lactis 39A1	.24 (.37-.12)
L. lactis 39A2	.12 (.20-.06)

[a]Determined after incubation for 22 hours at 5°C; 1 x 10^8 lactobacilli/ml for each strain.

[b]Each value represents average of six trials; numbers in parenthesis are ranges.

In other experiments a disc assay on milk-peroxidase agar was used to compare the relative amounts of peroxide produced by the six strains of L. lactis used in this study. In these experiments each culture was tested with and without added potassium sorbate to determine if sorbate influenced the production of peroxide. The results from six trials are summarized in Table XII. L. lactis 39A1 appeared to produce the largest zones, thus the greatest amount of hydrogen peroxide among the six strains. It is followed in order by strains Farr, 39A2, 403 E-15, B, and 12315. Sorbate was observed to have minimal affect upon the zone sizes on milk-peroxidase agar when combined with L. lactis in the inocula.

TABLE XII

MEASUREMENT OF HYDROGEN PEROXIDE PRODUCED BY L. LACTIS WITH AND WITHOUT POTASSIUM SORBATE ON RAW MILK-PEROXIDASE AGAR

Culture[b]	Zone Size (cm)[a]		
	0% Sorbate	.1% Sorbate	.2% Sorbate
L. lactis B	1.1 (1.55-.8)[c]	1.1 (1.55-.7)	1.2 (1.45-.8)
L. lactis Farr	1.3 (1.4-.8)	1.2 (1.5-.9)	1.2 (1.6-.8)
L. lactis 12315	1.0 (1.4-.7)	1.1 (1.6-.8)	1.0 (1.4-.6)
L. lactis 403E-15	1.1 (1.4-1.0)	1.2 (1.4-1.0)	1.1 (1.35-1.0)
L. lactis 39A1	1.6 (2.2-1.2)	1.6 (2.3-1.1)	1.6 (2.25-1.0)
L. lactis 39A2	1.2 (1.5-.6)	1.2 (1.6-.9)	1.2 (1.6-.8)

[a]Diameter of purple zone measured after 18 hours incubation at 21°C.

[b]Population for each strain was 1×10^8/ml.

[c]Each value represents an average from six trials; numbers in parenthesis are ranges.

CHAPTER V

DISCUSSION

The growth of psychrotrophic microorganisms in refrigerated foods has been of great concern to food processors for years. Various lactic acid bacterial cultures have been shown to be antagonistic toward psychrotrophs in various foods (Price and Lee, 1970; Bjorck et al., 1975; Daly et al., 1972; Juffs and Babel, 1975; Martin and Gilliland, 1980; Gilliland and Speck, 1975). The inhibition of psychrotrophic bacteria in raw milk would be of great value in preventing deterioration of raw milk and products prepared from it. Martin and Gilliland (1980) observed that cells of L. bulgaricus had an antagonistic effect in autoclaved milk at refrigerated temperatures toward a Gram negative psychrotrophic bacteria isolated from raw milk. However, the cultures of L. bulgaricus included did not inhibit growth of psychrotrophs in raw milk at 5.5°C.

The major inhibitory agent produced by the lactobacilli during refrigeration has been identified by Price and Lee (1970) as hydrogen peroxide. Premi and Bottazzi (1972) reported that L. lactis produced more hydrogen peroxide than did L. bulgaricus. Thus L. lactis might be more likely to have an inhibitory effect on psychrotrophs in raw milk during refrigerated storage than would L. bulgaricus.

The differences in the intensity of the inhibition of psychrotrophs in raw milk by L. lactis in the present study may have been due to the

variations in the microflora present in the different lots of raw milk. There was no attempt to control types of psychrotrophs other than to include those organisms naturally present in the raw milk. These microorganisms might vary in susceptibility to the inhibitory action of the lactobacilli. The variation in inhibition of psychrotrophs may also have been due to the variations in the amounts of hydrogen peroxide produced by the lactobacilli.

The refrigerated storage time and the initial population of non-lactobacilli present in the raw milk may have influenced the antagonism. If the initial population was too great and increased too rapidly, the high numbers may have overcome the inhibitory action of the lactobacilli. Juffs and Babel (1975) reported that this occurred for lactic starter cultures being used in a similar manner. A slight variation in storage temperature can have considerable influence on the growth of psychrotrophs in raw milk. Storage of the raw milk samples at 7°C resulted in faster growth of the psychrotrophs and less inhibition than was observed at 5°C. The greater inhibition of psychrotrophs in raw milk at 5°C than at 7°C may have been due to the lower temperature's ability to retard the growth of the psychrotrophs and enhance the effect of hydrogen peroxide upon them. The higher temperature may have allowed the psychrotrophs to overcome the inhibitory effect of hydrogen peroxide by a faster growth rate.

The temperature (5 or 7°C) at which the interaction trials were conducted had variable effects on the relative intensity of antagonism produced by individual cultures of L. lactis. L. lactis 39A1 was most inhibitory of the strains tested at 5°C while L. lactis 39A1 also was found to be significantly inhibitory at 7°C, L. lactis 403 E-15

expressed a greater antagonistic action at this temperature.

The most inhibitory strains of L. lactis produced greater amounts of hydrogen peroxide than did less inhibitory strains. This confirms the importance of hydrogen peroxide production and its relationship to inhibition of psychrotrophs. L. lactis 39A1 appeared to be far superior to the other five strains on the basis of peroxide production as measured by both methods.

Emard and Vaugh (1952) concluded that sorbate had an inhibitory effect toward catalase positive bacteria. It had little effect upon the catalase negative lactobacilli. Sorbate was reported to inhibit enzyme systems in microorganisms, such as dehydrogenases in molds (Melnick et al., 1954) and sulfhydrl-containing enzymes such as ficin and alcohol dehydrogenases (Whitaker, 1959). Catalase activity was eliminated from a cell-free extract of Aspergillus niger by sorbate (Troller, 1964). If the catalase in raw milk were inhibited by the addition of sorbate, the hydrogen peroxide produced by L. lactis should be more inhibitory toward psychrotrophs in the milk. Thus the use of sorbate in conjunction with L. lactis should significantly increase the inhibition of psychrotrophs in raw milk. The combined treatments of either L. lactis 39A1 or 39A2 with sorbate (0.1 percent or 0.2 percent) were significantly more inhibitory to the growth of psychrotrophs in refrigerated raw milk than were either strain of L. lactis or sorbate alone. The sorbate did not appear to influence the hydrogen peroxide production as measured by a disc assay. The mechanism where by the combined treatments of L. lactis and sorbate was more effective in inhibiting psychrotrophs is not known. It may have been due to an additive effect.

The hydrogen peroxide production by the lactobacilli appears to be

the most important means where by they inhibit psychrotrophs in refrigerated raw milk. Additional research is needed to find means of improving the hydrogen peroxide production by L. lactis to increase the antagonism towards psychrotrophs in raw milk. This may be achieved by selection of strains of L. lactis that have greater activity with regard to hydrogen peroxide production. The aeration of the raw milk during refrigerated storage may enhance hydrogen peroxide production. Gilliland and Speck (1969) reported that aeration increased the amount of peroxide produced by certain lactic acid bacteria. The growth conditions for producing cells for frozen concentrated cultures of L. lactis may be altered to increase hydrogen peroxide production. It is possible that the addition of catalase and introduction of air into the growth medium for producing cells of L. lactis may result in the cells having improved ability to produce more hydrogen peroxide.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Interaction experiments were conducted to determine the effect of strains of L. lactis on psychrotrophs in refrigerated raw milk during storage at 5 and 7°C. In addition, two strains of L. lactis (39A1 and 39A2) in conjunction with sorbate were added to raw milk to determine their combined effect upon the psychrotrophs. There was inhibition of the growth of psychrotrophs in raw milk by cells of L. lactis at both 5 and 7°C. Little or no change in acidity of the milk occurred during the refrigerated storage of the samples with and without added cells of lactobacilli. The addition of sorbate enhanced the antagonism of L. lactis toward the psychrotrophs at 5°C. Measurements of hydrogen peroxide production revealed that the cultures of L. lactis most antagonistic toward psychrotrophs were also the ones producing the greatest amounts of hydrogen peroxide. The combination of sorbate with a strain of L. lactis had no apparent effect on the amount of peroxide produced.

The addition of cells of L. lactis to refrigerated raw milk may be of great importance in the future. However, to be a reasonable means of inhibiting the growth of psychrotrophs, better strains of L. lactis with respect to peroxide production are needed. Means of enhancing the peroxide production or improving its effectiveness might also make this approach to controlling the growth of psychrotrophs more reasonable. The number of cells of L. lactis per ml used in the present study would

probably be too expensive to be feasible for routine use.

If peroxide production by cells of lactobacilli can be improved it would permit the use of fewer cells. This might make their use less expensive.

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VITA

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Master of Science

Thesis: THE INHIBITION OF PSYCHROTROPHIC MICROORGANISMS IN REFRIGERATED RAW MILK BY LACTOBACILLUS LACTIS WITH AND WITHOUT POTASSIUM SORBATE

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