INHIBITION OF PSYCHROTROPHIC BACTERIA IN MILK BY LACTOBACILLI FROM YOGURT

By

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INHIBITION OF PSYCHROTROPHIC BACTERIA IN MILK

BY LACTOBACILLI FROM YOGURT

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CHAPTER I

INTRODUCTION

The trend toward centralized milk processing operations indicates that raw milk must be held for several days at refrigeration temperatures before processing. Psychrotrophic bacteria can grow readily at refrigeration temperature and seriously limit the keeping quality of the raw milk. Even though the majority of psychrotrophic bacteria are destroyed by pasteurization, some of the enzymes they produce while growing in the raw milk will survive (Speck and Adams, 1976). These heat resistant enzymes can cause lipolysis and proteolysis in heat processed milk and in some products made from it. Since conventional pasteurization and ultra-high temperature (UHT) pasteurization do not destroy these enzymes, other effective methods need to be found to prevent the growth of psychrotrophic microorganisms in milk, thus decreasing the presence of the enzymes.

Concentrated suspensions of starter culture bacteria have been shown to suppress the growth at refrigeration temperatures of spoilage bacteria in ground beef and of psychrotrophic bacteria inoculated into autoclaved milk. Hydrogen peroxide was identified as the antagonistic agent produced by the lactobacilli included in the starter cultures tested.

The objective of this investigation was to isolate lactobacilli from yogurt which when added to refrigerated milk would control the growth of psychrotrophic bacteria.

CHAPTER II

LITERATURE REVIEW

Raw milk to be used for pasteurized milk products is commonly held for several days at refrigeration temperature before processing. Psychrotrophic microorganisms grow readily at these commercial refrigeration temperatures and can cause undesirable changes in the quality of dairy products. Bjorck (1978) noted that psychrotrophic bacteria in raw milk did not generally grow during the first 48 hrs of refrigerated storage, but they multiplied rapidly beyond this time period.

The bacterial quality of raw milk influences the quality of the pasteurized product. Elliott et al. (1974) reviewed the effect of poor sanitary conditions in the production area on the bacteriological quality of the bulk-tank raw milk. Under unsanitary milking conditions, rancid flavor developed within 2-3 days of milking. This off-flavor could not be removed by heat treatment. Patel and Blankenagel (1972) studied the relationship of bacterial counts in raw milk and the flavor of pasteurized milk during storage. When psychrotrophic bacteria reached levels of 10^6-10^7/ml before pasteurization, off-flavors and bitterness developed even though the pasteurized product had a low microbial count and was not contaminated after pasteurization.

Growth of psychrotrophic microorganisms in raw milk can influence the flavor and yield of cheeses made from the milk. When milk was inoculated with psychrotrophic bacteria before separation and pasteurization, there were reduced yields of cottage cheese and a greater formation of abnormal curd particles (Mohomed and Bassette, 1979). High psychrotrophic counts in raw milk resulted in marked flavor defects and a high concentration of free fatty acids in Gruyere cheeses (Thomas et al., 1971). In cheddar cheese produced from raw milk with a high bacterial-count ($>10^7$/ml), there was a higher concentration of free fatty acids than in control cheese made from milk with a low microbial count (Law et al., 1976; Ohren et al., 1969). In addition, rancidity developed in the cheese even though the psychrotrophic bacteria were destroyed by pasteurization. Yates and Elliott (1977) indicated that milk inoculated with a culture of a proteolytic psychrotrophic bacterium resulted in reduced yields of cheese.

Psychrotrophs are easily inactivated by heat during pasteurization. However, during growth in raw milk, some produce heat stable proteases (Speck and Adams, 1976) and lipases (Law et al., 1976). These enzymes survive heat processing and can cause undesirable proteolysis and lipolysis in the finished product. Adams et al. (1975) noted that 70-90% of samples from two dairy plants and one dairy farm contained psychrotrophs which produced heat resistant proteases. Pseudomonas species was identified as the organisms producing the heat stable proteases (Adams et al., 1975) and lipases (Law et al., 1976).

The successful storage of ultra high temperature (UHT) sterilized milk has been hindered by heat stable proteases produced in raw milk by Pseudomonas species. From studies on storage stability, proteases

attacked milk caseins, causing gelation (Adams et al., 1975; Law et al., 1977) and bitterness (Adams et al., 1975; Mayerhofer et al., 1973). The gelation of the UHT milk during storage was dependent on the psychrotrophic growth before heat treatment (Law et al., 1977).

The heat resistance of these enzymes to pasteurization temperatures and UHT heat processing has been well documented. Mayherhofer et al. (1973) isolated a heat-stable extracellular protease from Pseudomonas flourescens. The test organism had a D value (time at a specific temperature to kill 90% of cells in a culture) of 2.6 minutes at 62.8°C. However, the proteolytic enzyme was only 90% inactivated after 14 hrs at 62.8°C. For UHT treatments, Speck and Busta (1968) reported that 4 sec at 149°C was needed for a 12D reduction in the spores of Bacillus stearothermophilus 1518 or Putrefactive Anaerobe 3679. Adams et al. (1975) stated that a heat resistant protease produced by a psychrotrophic bacteria isolated from raw milk was hundreds of times more heat resistant than the bacterial spores used by Speck and Busta. They reported that less than 10% of the protease was destroyed by a heat treatment of 149°C for 4 sec.

Methods have been studied for reducing the level of heat resistant enzymes in milk. Barach et al. (1976) found that heat-stable proteases were less resistant at sub-pasteurization temperature. A more feasible method for reducing the amount of heat resistant enzymes in milk would be to prevent growth and metabolism by psychrotrophic microorganisms in the milk. Lactic acid bacteria have been shown to suppress the growth of many food-borne pathogens and spoilage microorganisms. There have been numerous studies on the antagonism(s) of lactic acid bacteria toward Staphylococcus aureus (Dahiya and Speck, 1968; Daly et al., 1972),

Salmonella species (Daly et al., 1972; Gilliland and Speck, 1972) and Pseudomonas species (Daly et al., 1972; Gilliland and Speck, 1972; Gilliland and Speck, 1975; Price and Lee, 1970). Most of these interaction studies have been done using associative cultures of the starter culture bacteria with undesirable microorganisms at temperatures favorable for the growth of both types of organism.

Recent research has focused on using lactic acid bacteria to prevent the growth of psychrotrophs at refrigeration temperature. Reddy et al. (1970) showed that mixtures of Streptococcus lactis and Leuconostoc citrovorum suppressed growth of spoilage bacteria in ground beef during storage at 7°C. Gilliland and Speck (1975) reported the inhibition of psychrotrophic bacteria by lactobacilli and pediococci at 5 and 7°C in milk, ground beef and crab meat. These starter culture bacteria did not grow at refrigeration temperature. They observed that Lactobacillus bulgaricus had a marked antagonism toward Pseudomonas fragi in autoclaved milk at 7°C. The intensity of the antagonism increased as the numbers of lactobacilli were increased. The lactic acid bacteria were more effective in controlling growth of psychrotrophic bacteria when the initial counts in ground beef were low. In the presence of catalase, there was less inhibition of psychrotroph MC-60N by L. bulgaricus, indicating that the lactobacilli produced hydrogen peroxide as the antagonistic agent. Since the lactobacilli and pediococci used in these studies did not grow at refrigeration temperatures, these antagonisms occurred with very little change in the acidity of the food.

Juffs and Babel (1975) used commercial mixed species lactic cultures (mixtures of lactic streptococci and Leuconostoc cremoris) to

restrict growth of psychrotrophs in sterile milk and raw milk stored at 3.5 and 7°C. The lactic culture exhibiting the greatest inhibition of psychrotrophs at both temperatures produced more hydrogen peroxide and less acid than the other cultures. This culture produced a higher percentage inhibition of the psychrotrophs in raw milk when the initial population of psychrotrophs was lower. They theorized that at a higher initial count, the psychrotrophs overcame the inhibition by starter cultures at a faster rate.

The lactoperoxidase/thiocyanate system (LP system) which is present in raw milk, was initially shown to inhibit some groups of streptococci (Reiter, 1978). Recently it was shown to inhibit some catalase-positive gram-negative organisms. The LP system depends on all three components for bactericidal activity (Bjorck, 1978). Bovine milk contains about 30 ug/ml of lactoperoxidase, variable amounts of thiocyanate and no hydrogen peroxide. Bjorck and Rosen (1976) used a two-enzyme system consisting of B-galactosidase and glucose oxidase to generate hydrogen peroxide from their action on lactose. In another study, glucose and glucose oxidase supplied enough hydrogen peroxide to activate the LP system and inhibit Escherichia coli and Pseudomonas species (Bjorck et al., 1975). A more pronounced inhibition was observed in raw milk with the addition of 0.25 mM thiocyanate and 0.25 mM hydrogen peroxide (Bjorck, 1978). The number of viable psychrotrophic bacteria declined sharply during the first 2 days and then remained constant until day 5. Lactoperoxidase catalyzed the oxidation of thiocyanate by hydrogen peroxide to form a compound which inhibited growth of psychrotrophic bacteria.

Partial bacteriostasis (increase in lag period) appears to be the mechanism by which hydrogen peroxide inhibits the growth of psychrotrophic bacteria (Juffs and Babel, 1975; Price and Lee, 1970). The lag period for Pseudomonas species at 30°C in 1% peptone broth increased 1 to 7 hrs when 2 to 8 ug of hydrogen peroxide per ml was added with an indefinite lag period when 25 to 40 ug per ml was added (Price and Lee, 1970).

CHAPTER III

EXPERIMENTAL PROCEDURE

Isolation and Maintenance of Cultures of Lactobacilli

Samples of different commercial brands of plain yogurt were purchased from local supermarkets and health food stores. The yogurt samples were inoculated (1%) into 10 ml of sterile (autoclaved 15 min at 121°C) 10% non-fat milk solids (NFMS) and incubated at 37°C for 24 hrs. The resulting cultures were subcultured once more in a similar fashion.

The milk cultures were then streaked onto a "milk-peroxidase" agar medium designed for differentiating colonies that produce hydrogen perioxide from those that do not. The agar medium was prepared as follows. Five ml of 10% NFMS (steamed for 30 min) containing 0.5% thiotone (Baltimore Biological Laboratories, Cockeyville, Maryland) was tempered to 45°C and added to 5 ml of sterile, melted 3% Bacto agar (Difco, Detroit, Michigan) at 45°C. One-tenth ml of peroxidase (0.2 mg/ml) (Sigma Chemical Company, St. Louis, Missouri) and 0.1 ml of O-tolidine (20 mg/ml) (Sigma Chemical Company, St. Louis, Missouri) were then aseptically added to the mixture. The medium was mixed by inverting the container six times and poured into a sterile petri dish. One-tenth ml of an appropriate dilution of the test culture was spread onto

the surface of the solidified "milk-peroxidase" agar with a sterile glass hockey stick. The plate was inverted and incubated 24 hrs at 37°C. At least three colony types (when present) were selected from each yogurt culture; those with no color zones surrounding them, and those with intermediate and large brown zones. It was assumed that zone size was related to the amounts of hydrogen peroxide produced by the colonies. The colony morphology and zone size (mm) were recorded. Cells from the selected colonies were inoculated into 10 ml of sterile lactobacilli MRS broth (Difco, Detroit, Michigan) with the aid of a sterile inoculating needle and incubated at 37°C until growth was visibly evident (turbidity). The broth cultures were restreaked on the "milk-peroxidase" agar to ensure purity of culture. If a mixed culture was indicated, a colony having morphology and zone size like that of the original isolate was isolated and transferred into lactobacilli MRS broth.

The isolates were transferred from lactobacilli MRS broth with a sterile needle into screw capped tubes containing 10 ml of sterile MRS agar (the MRS agar was prepared by adding 1.5% Bacto agar to lactobacilli MRS broth, dispensing in 10 ml volumes and heating at 121°C for 15 minutes). The stab cultures were incubated at 37°C for 18 hr and stored at refrigeration temperature (6°C). The isolates were subcultured into fresh stabs of MRS agar at monthly intervals for the duration of the study.

Isolation and Maintenance of Psychrotrophic Bacterial Culture

Fresh raw milk was obtained from the Oklahoma State Dairy Farm.

After incubation at 5°C for 6 days, the milk was plated on Plate Count Agar (PCA) (Difco, Detroit, Michigan). The plates were incubated at 21°C for 5 days. Cells from the predominating type of colony were inoculated into Trypticase Soy Broth (TSB) (Baltimore Biological Laboratories, Cockeyville, Maryland) with the aid of a sterile inoculating needle and incubated at 21°C until growth was evident (~24 hrs). This was to insure that the isolate represented the predominating psychrotrophic bacteria that had grown in raw milk at 5°C. To make certain the isolate retained its ability to grow well in milk, the culture was subcultured (1% inoculum) in sterile 10% NFMS and incubated for 19-20 hrs at 21°C.

The culture was maintained by weekly transfers in sterile 10% NFMS using a 1% inoculum and incubation at 21°C for 19-20 hrs. For the interaction studies in autoclaved milk, the psychrotroph was subcultured twice on successive days in sterile 10% NFMS for 19 hrs at 21°C just prior to use.

Enumeration of Bacteria

Enumeration of Facultative Lactobacilli

Decimal dilutions were made in 99 ml dilution blanks containing 0.1% peptone (Difco, Detroit, Michigan) and 0.001% Anti-foam A Emulsion (Sigma Chemical Company, St. Louis, Missouri) in distilled water. The dilution blanks were autoclaved at 121°C for 15 minutes prior to use. The necessary dilutions were made according to the procedures outlined in Standard Methods for the Examination of Dairy Products (Marth, 1978).

Duplicate plates containing the required dilutions were poured with melted lactobacilli MRS agar tempered at 45°C. After solidification, the plates were inverted and incubated at 37°C for 48 hrs. All colonies visible with the aid of a Quebec Colony Counter were counted.

Enumeration of Non-Lactobacilli

Dilutions were made as described in the previous section. Duplicate plates containing the required dilution were poured with Plate Count Agar (PCA) and incubated at 21°C for 5 days. The lactobacilli used in this study did not form colonies on this medium at 21°C. All colonies visible with the aid of a Quebec Colony Counter were enumerated.

Identification of Facultative Lactobacilli

Each isolate was streaked onto a plate of MRS agar and incubated anaerobically in a Gas Pak anaerobic system (Baltimore Biological Laboratories, Cockeyville, Maryland) for 24 hrs at 37°C. After incubation, the colonial morphology of each culture was observed as an indication of whether or not the culture was pure. A Gram stain was prepared by the Burke method (Burke, 1922). A portion of the growth was collected with a sterile polyester-fiber swab (Falcon, Oxnard, CA) and transferred into 2.5 ml of sterile basal broth to an optical density equivalent to a McFarland 5 standard. Composition of the basal broth and its use were described by Gilliland and Speck (1977). The remaining growth on the petri plate was flooded with 3% hydrogen peroxide for the catalase test. A positive test for the presence of catalase was indicated by effervescence from the colonies.

The Minitek system (Baltimore Biological Laboratories, Cockeyville,

Maryland) was used as described by Gilliland and Speck (1977) to determine the ability of the organisms to hydrolyze or ferment certain substrates. The basal broth containing cells of the culture was used to inoculate the substrates. The following biochemical tests were done for each culture: the deamination of arginine, the hydrolysis of esculin and the fermentation of amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhammose, salicin, sorbitol, sucrose, trehalose, and xylose. A tube of MRS broth (10 ml) was also inoculated using a sterile loop. The tube was incubated at 15°C for 1 week and a visual turbidity was a positive test for growth.

Preparation and Stability of Frozen Concentrated Cultures of Lactobacilli

Growth Curves

Each isolate was subcultured twice in 10 ml of MRS broth at 37°C for 18 hrs to prepare fresh cultures. A 250 ml Erlenmeyer flask containing 110 ml of MRS broth was inoculated with 1.1 ml of the freshly prepared culture. After mixing, ten ml volumes were aseptically dispensed into each of 10 sterile test tubes and incubated in a 37°C water bath. When growth became evident (visual observance of turbidity), turbidimetric measurements were made with a Klett-Summerson colorimeter (Filter No. 42, 400-465 mm) and recorded at hourly intervals until there was no additional increases in turbidity. A partial growth curve (optical density versus incubation time) was plotted for each culture to determine when the cultures had reached the late exponential phase of growth.

Preparation of Frozen Concentrates

Each isolate was inoculated (1%) into 400 ml of MRS broth and incubated at 37°C until the culture reached the late exponential phase of growth. The cells were harvested by centrifugation at 16,000 x g for 20 minutes at 5°C in a Sorvall model RC-5 Superspeed Refrigerated Centrifuge (DuPont Company, Newtown, Connecticut). The supernatant fluid was discarded and the cells were resuspended in 40 ml of cold sterile 10% NFMS. Resuspension was facilitated by adding approximately 25 sterile glass beads (0.3 cm diameter) along with the milk to the centrifuge bottles and swirling the contents. This helped to assure a uniform suspension of cells. Two ml quantities of the resulting concentrated culture were dispensed into sterile 2-ml plastic freezing vials (Cooke Pro-vial, Dynatech Laboratories Inc., Alexandria, Virginia) and submerged in liquid nitrogen (-196°C) for storage.

Storage Stability of Frozen Concentrates

The numbers of lactobacilli in the concentrated cultures were determined by plating on MRS agar before freezing and after 24 hrs of storage in liquid nitrogen (-196°C). Storage stability was determined by plating the concentrates again after several months of storage.

To thaw the culture concentrates, the vials were placed in approximately a 500 ml water bath (35°C) for a few minutes and immediately transferred to an ice bath until needed. The exterior of the vial was dried with a Kimwipe (Kimberly-Clark) prior to opening.

Hydrogen Peroxide Production

The isolates of lactobacilli were tested for peroxide production

in 10% NFMS at 5°C (Gilliland and Speck, 1975). In preliminary studies, each isolate was subcultured twice in MRS broth (1% inoculum and incubation for 18 hrs at 37°C). The cells from 15 ml of broth culture were removed by centrifugation at 12,000 x g for 10 minutes at 2°C. With the aid of sterile glass beads, each pellet was resuspended in 10 ml of cold 10% NFMS which had been heated at 100°C for 30 minutes and held under refrigeration prior to use. The mixture was transferred to a 50 ml Erlenmeyer flask containing an additional 25 ml of cold steamed 10% NFMS. The samples were incubated at 5°C on a platform shaker (76 strokes per minute). After 22 hrs, the amount of residual peroxide in the milk was measured using the peroxidase o-dianisidine method described by Gilliland (1969). The numbers of lactobacilli were enumerated on MRS agar. In reporting the amount of peroxide in milk, optical density was used instead of ug/ml because it is possible to measure only the residual peroxide and not the total amount produced by the culture (Gilliland, 1969). The optical density values were used to compare cultures with respect to peroxide production.

In testing for peroxide production by cells from the frozen cultures, the required amount of each thawed concentrate was added to 25 ml volumes of cold 10% NFMS contained in 50 ml Erlenmeyer flasks to yield a population of 1×10^8/ml. The 10% NFMS had been heated at 100°C and held in the refrigerator prior to use. The same procedure was followed for incubation and peroxide determination as described in the previous paragraph. Preliminary experiments showed that the numbers of lactobacilli in the milk did not change during the incubation period. Therefore, the numbers of facultative lactobacilli were enumerated and reported only at 0 hr.

Effect of Numbers of Lactobacilli on the Growth of a Psychrotroph in Refrigerated Milk

The effect of increasing populations of lactobacilli on growth of psychrotrophs was determined in autoclaved 10% NFMS inoculated with a pure culture of a psychrotrophic bacteria and in raw milk. The raw milk was obtained from the University dairy farm and placed in an ice bath until needed (usually 2 hr). The reconstituted 10% NFMS was prepared on the day of the experiment and dispensed into dilution bottles (100 ml per bottle). After autoclaving for 10 minutes at 121°C, the milk was cooled in an ice bath. A freshly prepared culture of the psychrotroph was used to inoculate the milk so the initial population was 1×10^3/ml. This was done by aseptically adding 1.2 ml from a 1:1000 dilution of the psychrotroph culture to 100 ml of cold sterile 10% NFMS. The inoculated milk was kept in an ice bath during the remaining steps in preparing the experiment.

The desired populations of lactobacilli were prepared from frozen concentrated cultures. Concentrated cultures of L. bulgaricus D3 and L. bulgaricus E2 were diluted to a population of 2×10^8/ml in cold sterile 10% NFMS. The following volumes of the diluted cultures were measured into sterile dilution bottles contained in an ice bath: 0 ml (control), 0.5 ml, 0.75 ml, 1.0 ml, 2.5 ml, 5.0 ml, 7.5 ml, and 10.0 ml. The volume in each bottle was adjusted to 10 ml with cold sterile 10% NFMS. Either 10 ml of the autoclaved milk containing the psychrotroph or 10 ml of raw milk were added to the dilution bottles to achieve a final volume of 20 ml with the following populations of lactobacilli: 0 (control), 5.0×10^6/ml, 7.5×10^6/ml, 1.0×10^7/ml, 2.5×10^7/ml, 5.0×10^7/ml, 7.5×10^7/ml, and 1.0×10^8/ml. The contents of each

bottle were mixed by inverting six times. From each test sample, a five ml aliquot was aseptically transferred to a sterile test tube for determining day 0 bacterial counts (facultative lactobacilli and non-lactobacilli). The bottles were stored in an upright position in a 5.5°C water bath for 6 days. The samples were mixed and the numbers of non-lactobacilli determined. Increases in numbers of non-lactobacilli from day 0 to day 6 was presumably due to growth of psychrotrophic microorganisms in the milk. The pH of each sample was measured with a Beckman Zeromatic pH Meter to the nearest .01 pH unit on the initial and final day of the experiment to determine if the lactobacilli affected the acidity.

Effect of Different Cultures of Lactobacilli on the Growth of the Psychrotrophic Culture in Autoclaved Milk

A comparison was made of the inhibition of the psychrotrophic culture by seven cultures of lactobacilli. Based on the results obtained from the effect of numbers of L. bulgaricus E2 in autoclaved milk, seven cultures of lactobacilli were comapred at a population of 2.5×10^7/ml. Each concentrated culture was diluted to a population of 5×10^7/ml in cold sterile 10% NFMS. Ten ml volumes of the milk containing the lactobacilli were mixed with 10 ml portions of cold 10% NFMS containing the psychrotroph (2×10^3/ml) in sterile dilution bottles. Once these test samples were prepared, the same procedure for monitoring bacterial numbers was followed as discussed in the previous section.

For comparing the inhibition by cultures of lactobacilli toward the psychrotroph culture, the following formula was used:

$$\% \text{ inhibition} = \left[\frac{A_x - B_x}{A_x} \right] 100$$

A_x = number of non-lactobacilli in control on day x.

B_x = number of non-lactobacilli in sample containing added lactobacilli on day x.

Statistical Analyses

Duncan's new multiple range test was used to compare the means of the percent inhibition produced by cultures of lactobacilli. It was also used to compare the effect of different numbers of lactobacilli on the growth of the psychrotroph. The methods for these analyses are described in Principles and Procedures of Statistics (Steele and Torre, 1960).

CHAPTER IV

RESULTS

Hydrogen Peroxide Production by Isolates From Yogurt

An agar medium was designed to differentiate colonies of bacteria that produced hydrogen peroxide from those that did not. Isolates from yogurt cultures were selected on the basis of the size of the colored zone surrounding the colony. Each isolate was then tested for hydrogen peroxide production in refrigerated 10% NFMS. The numbers of lactobacilli were determined initially on MRS agar. The data in Table I indicates a lack of correlation between the zone size produced by the colonies on the "milk-peroxidase" agar and peroxide production by the isolate in 10% NFMS at 5°C. In most cases, the relative amount of peroxide produced by the cultures in refrigerated milk could not be predicted by the size of the zone observed for colonies of the culture on the "milk-peroxidase" agar. Examples of this can be seen by comparing results from several of the cultures. Isolate E1 with a small (< 1.0 mm) zone produced slightly more peroxide than isolate E3 with a much larger (2.0 mm) zone. It also produced much more peroxide in refrigerated milk than did isolate B2 which exhibited the largest (3.5 mm) zone on the agar medium. Isolates C3 and C4 with no zone on the "milk-peroxidase" agar did not produce detectable peroxide in

TABLE I

PEROXIDE FORMATION ON "MILK-PEROXIDASE" AGAR AT 37°C AND IN MILK AT 5°C BY ISOLATES FROM YOGURT

Culture	Zone Size[a] (mm)	Test for Peroxide Production in Refrigerated Milk[b]	
		Lactobacilli/ml at 0 hr	$A_{400\ nm}$
A1[c]	1.3	1.5×10^8	.33
A2	1.3	1.5×10^8	.38
B1	1.6	4.0×10^8	.16
B2	3.5	4.3×10^8	.17
C1	<1.0	6.8×10^6	.12
C2	2.4	2.4×10^6	.41
C3	0	1.3×10^6	0
C4	0	3.1×10^7	0
D1	<1.0	1.4×10^8	.33
D2	1.2	3.5×10^8	.34
D3	1.2	3.5×10^8	.34
E1	<1.0	1.6×10^8	.42
E2	1.0	6.9×10^7	.40
E3	2.0	1.0×10^8	.40

[a]Diameter of brown zone surrounding colony on "Milk-Peroxidase" agar.

[b]Measured after 22 hrs incubation at 5°C; the higher the absorbance reading, the more peroxide is present.

[c]Each letter designates a culture of lactobacilli isolated from a different yogurt sample.

refrigerated milk. Since the initial numbers of lactobacilli varied, it was difficult to make comparisons in the peroxide production of the isolates.

Identity of Cultures of Lactobacilli

Those cultures which showed differences in their ability to produce hydrogen peroxide in milk were selected for identification. Cultures D2 and E3 which produced amounts of peroxide similar to the isolate already selected from that yogurt sample were not included for identification. Cultures (B1, B2, C3 and C4) which produced little or no peroxide in refrigerated milk and cultures (C1 and C2) which did not appear to grow well in MRS broth were also excluded. Two additional laboratory cultures (F1 and F2) were included for identification since they produced relatively high amounts of peroxide in milk at 5.5°C.

To confirm the identity of the selected cultures of lactobacilli, a series of tests were conducted using the Minitek system. The characteristics of isolates A1 and A2 matched those of Lactobacillus acidophilus as described in Bergey's Manual of Determinative Bacteriology 8th Edition (Buchanan, 1974) with the exception that A2 fermented mannitol (Table II). The remaining isolates (D1, D3, E1, E2, F1 and F2) were identified as Lactobacillus bulgaricus. They, however, did ferment mannose and did not ferment galactose.

Characteristics of Psychrotrophic Bacterial Culture

Psychrotroph RM was a gram-negative rod shaped bacterium isolated from raw milk.

TABLE II

IDENTIFY CHARACTERISTICS OF CULTURES OF LACTOBACILLI

Test	*Lactobacillus acidophilus*			*Lactobacillus bulgaricus*						
	A1	A2	Bergey's[a]	D1	D3	E1	E2	F1	F2	Bergey's[a]
Gram stain	+	+	+	+	+	+	+	+	+	+
Cellular Morphology	rods	rods	rods	rods	rods	rods	rods	rods	rods	rods
Catalase	-	-	-	-	-	-	-	-	-	-
Growth at 15°C	-	-	-	-	-	-	-	-	-	-
NH_3 from Arginine	-	-	-	-	-	-	-	-	-	-
Hydrolysis of Esculin	+	+	+	-	-	-	-	-	-	-
Acid from:										
Amygdalin	+	+	+	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	-	-	-	-	-	-	-
Galactose	+	+	+	-	-	-	-	-	-	+
Glucose	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	-	-	-	-	-	-	-
Mannitol	-	+	-	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	-

TABLE II (Continued)

Test	*Lactobacillus acidophilus*			*Lactobacillus bulgaricus*						
	A1	A2	Bergey's[a]	D1	D3	E1	E2	F1	F2	Bergey's[a]
Melezitose	-	-	-	-	-	-	-	-	-	-
Melebiose	+	+	±	-	-	-	-	-	-	-
Raffinose	+	+	±	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	+	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	-	-	-	-	-	-	-
Trehalose	+	+	+	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-

[a]Characteristics of *L. acidophilus* and *L. bulgaricus* as indicated in *Bergey's Mannual of Determinative Bacteriology* 8th Edition (Buchanan, 1974).

Frozen Concentrated Cultures

Frozen Concentrated cultures of lactobacilli were prepared from those cultures selected for identification. The stability of eight cultures of lactobacilli to storage in liquid nitrogen is shown in Table III. Viability was determined with the plate count method before freezing and after storage for 24 hrs and 8 months. Most of the damage to those cultures (L. acidophilus A1 and L. bulgaricus E1) with lower survival after 8 months apparently occurred during the freezing since the counts did not decrease much from day 1 to 8 months. L. acidophilus A2 appeared to survive the freezing step but the count decreased during the 8 months storage so that the level of survival was 54%. All other cultures appeared to survive at -196°C storage very well.

After storage for 1 month at -196°C, the cultures were tested for hydrogen peroxide production in refrigerated milk. Results in Table IV indicate that cells from frozen concentrated cultures were able to produce hydrogen peroxide in 10% NFMS at 5°C. All cultures were compared in a single experiment. The relative amount of peroxide produced by the cultures varied; culture A1 produced hardly any detectable peroxide, cultures E1 and E2 produced intermediate amounts of peroxide and cultures D1, D3 and F2 produced higher levels of peroxide. All cultures were compared at approximately 1×10^8 lactobacilli per ml. The ability of cultures to produce hydrogen peroxide could be more easily compared since the numbers of lactobacilli were fairly constant.

TABLE III

STABILITY OF FROZEN CONCENTRATED CULTURES OF LACTOBACILLI TO STORAGE IN LIQUID NITROGEN (-196°C)

Culture	Lactobacilli/ml			% Surviving[b]
	0 Day[a]	1 Day	8 Months	
L. acidophilus A1	1.4×10^{10}	6.0×10^{9}	5.4×10^{9}	39
L. acidophilus A2	2.6×10^{10}	2.7×10^{10}	1.4×10^{10}	54
L. bulgaricus D1	2.1×10^{10}	1.8×10^{9}	1.9×10^{9}	90
L. bulgaricus D3	7.0×10^{9}	7.2×10^{9}	6.6×10^{9}	94
L. bulgaricus E1	1.2×10^{10}	7.7×10^{9}	7.6×10^{9}	63
L. bulgaricus E2	4.2×10^{9}	3.6×10^{9}	3.6×10^{9}	86
L. bulgaricus F1	4.4×10^{9}	4.4×10^{9}	4.5×10^{9}	100
L. bulgaricus F2	1.7×10^{9}	1.6×10^{9}	1.9×10^{9}	100

[a]Lactobacilli count/ml prior to freezing in liquid nitrogen.

[b]After storage for 8 months.

TABLE IV

HYDROGEN PEROXIDE PRODUCTION IN STEAMED 10% NFMS BY CELLS FROM CONCENTRATED CULTURES OF LACTOBACILLI[a]

Yogurt Isolates		Lactobacilli Count at 0 hr	H_2O_2[b] $A_{400\ nm}$
L. acidophilus	A1	1.1×10^8	.06
L. acidophilus	A2	1.0×10^8	.13
L. bulgaricus	D1	9.0×10^7	.33
L. bulgaricus	D3	9.0×10^7	.33
L. bulgaricus	E1	1.2×10^8	.24
L. bulgaricus	E2	8.8×10^7	.24
L. bulgaricus	F1	7.9×10^7	.15
L. bulgaricus	F2	1.1×10^8	.47

[a]Stored 1 month in liquid nitrogen.

[b]After 22 hrs incubation at 5°C with continuous agitation; the higher the absorbance reading, the more peroxide is present.

Effect of Numbers of Lactobacilli on the Growth of a Psychrotrophic Culture in Refrigerated Milk

To determine a reasonable population for comparing different cultures of lactobacilli for the ability to inhibit a psychrotrophic culture in refrigerated milk, increasing numbers of L. bulgaricus E2 were added to autoclaved 10% NFMS containing 2×10^3 psychrotroph RM per ml (Figure 1). L. bulgaricus E2 was selected since it produced a relatively high amount of peroxide in refrigerated milk. The data from three trials are presented graphically in Figure 1 as the $\log_{10}$ of the numbers of non-lactobacilli per ml after 6 days of storage at 5.5°C plotted against the numbers of lactobacilli per ml. The growth of the psychrotroph did not appear to be affected by a population of 1×10^7 L. bulgaricus E2 per ml (increase noted in Trial 1) but at 2.5×10^7 per ml, there was marked antagonism toward the psychrotrophic culture. This sharp decline in the numbers of psychrotrophs continued at higher populations of lactobacilli.

Duncan's new multiple range test was used to test for significant differences among the means of the non-lactobacillus counts in the milk containing the various numbers of added lactobacilli. A summary table of results obtained from the three trials is shown in Table IX of the Appendix. The test for significant differences among means of these results appears in Table V. The sources of variation were the replicates and the treatments. Each difference was declared significant if it exceeded the corresponding calculated least significant range. The first significant ($P < .05$) difference from the control count occurred at 2.5×10^7 lactobacilli per ml. There was also significant

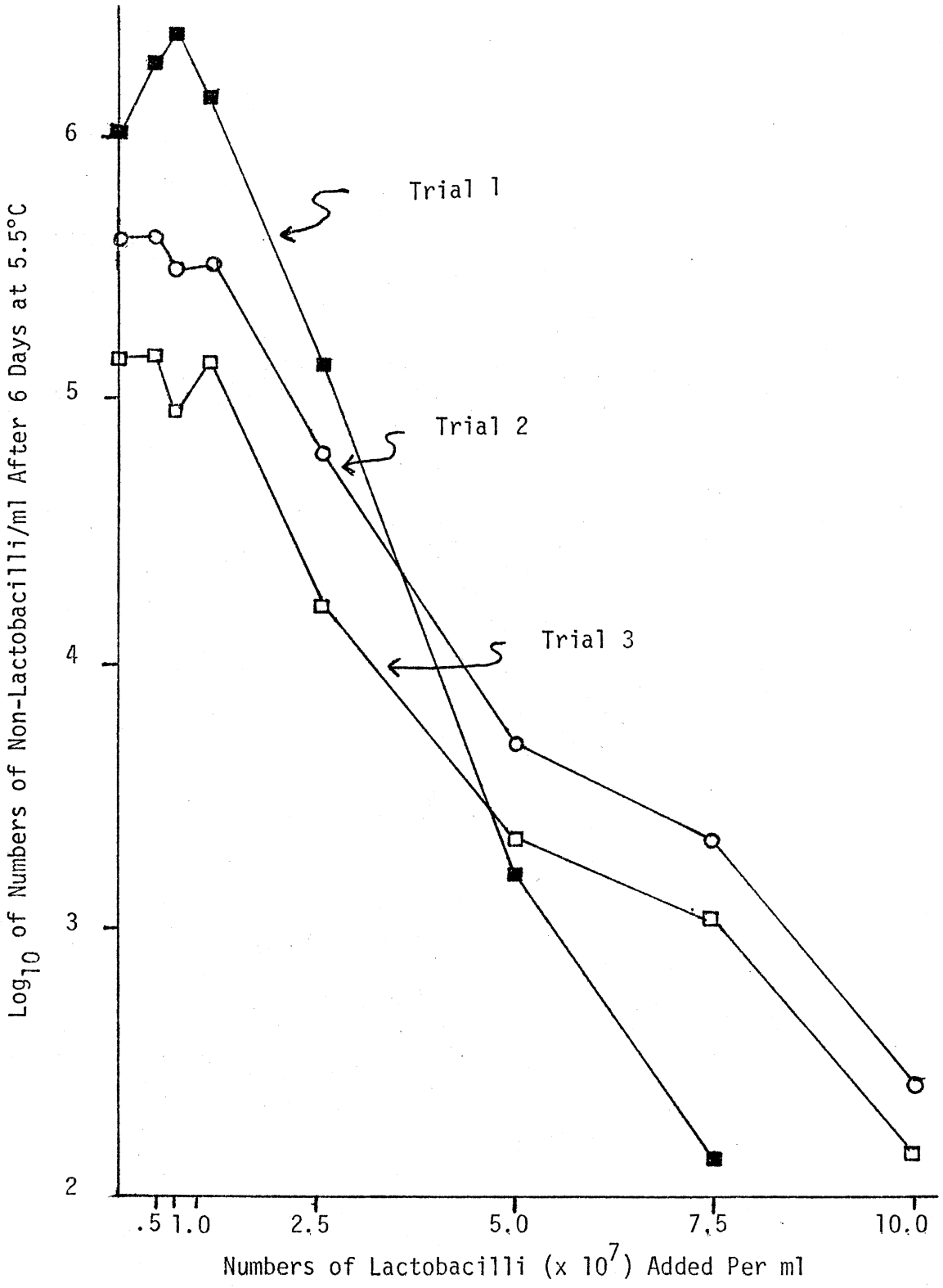

Figure 1. Effect of Numbers of Lactobacillus bulgaricus E2 on the Growth of Psychrotrophic Culture in Autoclaved 10% NFMS

TABLE V

TEST FOR SIGNIFICANT DIFFERENCES AMONG AUTOCLAVED MILK SAMPLES CONTAINING INCREASING NUMBERS OF LACTOBACILLUS BULGARICUS E2 WITH RESPECT TO INHIBITION OF GROWTH OF PSYCHROTROPHIC BACTERIA

Source	df	SS	MS
total	23	49.16	
replicates	2	1.04	
treatment	7	44.97	
error	14	3.56	.22

Means Compared[a]	Differences Between Means	LSR[b]	Means Compared	Differences Between Means	LSR
A-B	5.69-5.58 = .11	.90	C-F	5.62-3.41 = 2.21*	.91
A-C	5.62-5.58 = .04	.87	C-G	5.62-2.84 = 2.78*	.92
A-D	5.61-5.58 = .03	.83	C-H	5.62-2.06 = 3.56*	.93
A-E	5.58-4.73 = .85*	.83			
A-F	5.58-3.41 = 2.17*	.87	D-E	5.61-4.73 = .88*	.87
A-G	5.58-2.84 = 2.74*	.90	D-F	5.61-3.41 = 2.20*	.90
A-H	5.58-2.06 = 3.52*	.91	D-G	5.61-2.84 = 2.77*	.91
			D-H	5.61-2.06 = 3.55*	.92
B-C	5.69-5.62 = .07	.83			
B-D	5.69-5.61 = .08	.87	E-F	4.73-3.41 = 1.32*	.83
B-E	5.69-4.73 = .96*	.91	E-G	4.73-2.84 = 1.89*	.87
B-F	5.69-3.41 = 2.28*	.92	E-H	4.73-2.06 = 2.67*	.90
B-G	5.69-2.84 = 2.85*	.93			
B-H	5.69-2.06 = 3.63*	.94	F-G	3.41-2.84 = .57	.83
			F-H	3.41-2.06 = 1.35*	.87
C-D	5.62-5.61 = .01	.83			
C-E	5.62-4.73 = .89	.90	G-H	2.84-2.06 = .78	.83

[a] A = control; B = 5.0×10^6 lactobacilli/ml; C = 7.5×10^6 lactobacilli/ml; D = 1.0×10^7 lactobacilli/ml; E = 2.5×10^7 lactobacilli/ml; F = 5.0×10^7 lactobacilli/ml; G = 7.5×10^7 lactobacilli/ml; H = 1.0×10^8 lactobacilli/ ml.

[b] least significant ranges at .05 significance level

*These differences marked with an asterisk are significant at $P<.05$.

differences in the means of the psychrotrophs between 2.5×10^7 and 5.0×10^7 per ml and between 5.0×10^7 and 1.0×10^8 per ml.

Comparison of Intensity of Inhibition Produced by Cultures of Lactobacilli

A comparison was made of the inhibition by seven cultures of lactobacilli (2.5×10^7/ml) against psychrotroph RM in autoclaved 10% NFMS. The results are tabulated in Table VI. Since the numbers of lactobacilli added to the milk was the same for each culture, the inhibition by these cultures could be compared. The percentage inhibition for each culture was calculated relative to the control count for that trial. There was considerable variation in percentage inhibition among cultures in the three trials. A comparison of the average percentage inhibition indicates that L. bulgaricus D1 was the most active inhibitor toward psychrotroph RM; L. bulgaricus E2 was an intermediate inhibitor and L. acidophilus A1 was one of the least active inhibitors.

Duncan's new multiple range test was used to test for significant differences among the means for percent inhibition by cultures of lactobacilli (Table VII). The statistical analyses shows that the three most active cultures - L. bulgaricus D1, L. bulgaricus D3 and L. acidophilus A2 were significantly ($P < .05$) more inhibitory toward psychrotroph RM than was L. acidophilus A1 and L. bulgaricus F1. L. bulgaricus E2 and L. bulgaricus F2 which appeared to be intermediate among the cultures with respect to inhibition were not significantly different from the other five cultures.

TABLE VI

INHIBITION OF GROWTH OF PSYCHROTROPH RM IN AUTOCLAVED 10% NFMS BY CELLS OF DIFFERENT CULTURES OF LACTOBACILLI[a]

Culture	% Inhibition			
	Trial 1	Trial 2	Trial 3	Avg.
L. acidophilus A1	-13	55	38	27
L. acidophilus A2	66	92	74	77
L. bulgaricus D1	48	99	99	82
L. bulgaricus D3	62	91	75	76
L. bulgaricus E2	30	83	40	51
L. bulgaricus F1	-48	77	49	26
L. bulgaricus F2	48	83	32	54

[a] 2.5×10^7 lactobacilli added/ml for each culture; initial population of psychrotroph - 1×10^3/ml.

TABLE VII

TEST FOR SIGNIFICANT DIFFERENCES AMONG THE MEANS FOR PERCENT INHIBITION PRODUCED BY CULTURES OF LACTOBACILLI

Source	df	SS	MS
total	20	26525.24	
replicates	2	10737.81	
treatment	6	9957.24	
error	12	5830.19	485.85

Means Compared[a]	Differences Between Means	LSR[b]	Means Compared	Differences Between Means	LSR
A1-A2	77-27 = 50*	42.8	D1-D3	82-76 = 6	41.1
A1-D1	82-27 = 55*	43.3	D1-E2	82-51 = 31	42.8
A1-D3	76-27 = 49*	42.4	D1-F1	82-26 = 56*	43.5
A1-E2	51-27 = 24	39.2	D1-F2	82-54 - 28	42.4
A1-F1	27-26 = 1	39.2			
A1-F2	54-27 = 27	41.1	D3-E2	76-51 = 25	41.1
			D3-F1	76-26 = 50*	42.8
A2-D1	82-77 = 5	39.2	D3-F2	76-54 = 22	39.2
A2-D3	77-76 = 1	39.2			
A2-E2	77-51 = 26	42.4	E2-F1	51-26 = 25	41.1
A2-F1	77-26 = 51*	43.3	E2-F2	54-51 = 3	39.2
A2-F2	77-54 = 23	41.1			
			F1-F2	54-26 = 28	42.4

[a]Cultures of lactobacilli being compared.

[b]Least significant ranges at .05 significance level.

*Those differences marked with an asterisk are significant at $P<.05$.

Effect of Numbers of L. bulgaricus D3 on the Growth of Psychrotrophs in Milk

Based on comparisons among cultures of lactobacilli, the effect of numbers of L. bulgaricus D3 (an active inhibitor) was determined. Concurrent interaction experiments were set up in raw milk and in autoclaved 10% NFMS containing 1×10^3 psychrotroph RM per ml (the results in Figure 2 represent the average of three trials). The average initial non-lactobacillus counts in raw milk were 8×10^3 per ml. Fewer numbers of the psychrotroph were detected in autoclaved milk containing 2.5×10^7 L. bulgaricus D3 per ml after 6 days of storage than in samples containing fewer lactobacilli. The growth of non-lactobacilli in raw milk was apparently not affected by increasing numbers of L. bulgaricus D3. Slightly greater numbers of non-lactobacilli were noted for samples containing 7.5×10^7 and 1.0×10^8 lactobacilli per ml.

Significant differences among the means of the non-lactobacillus counts in the milk containing the various numbers of added lactobacilli were determined by the Duncan's multiple range test. A summary table of results obtained on the effect of increasing numbers of L. bulgaricus D3 toward psychrotroph RM in autoclaved milk appears in Table X of the Appendix. The test for significant differences among means of these results is shown in Table VIII. There was a significant ($P < .05$) difference between the numbers of non-lactobacilli detected after 6 days storage in the control and in the sample containing 2.5×10^7 lactobacilli per ml. Significant differences in the counts also occurred between samples containing 2.5×10^7 and 5.0×10^7 lactobacilli

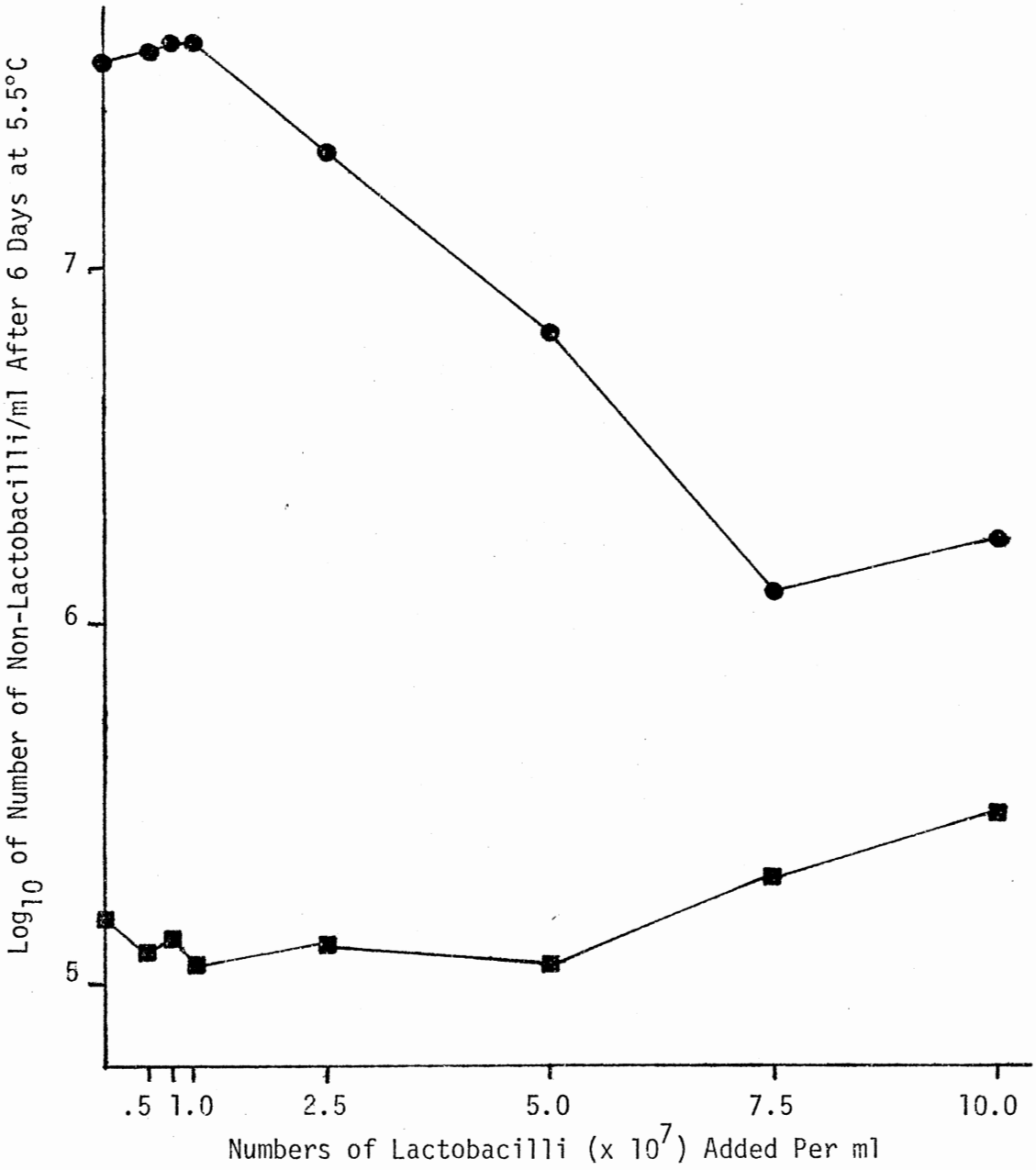

Figure 2. Effect of Numbers of Lactobacillus bulgaricus D3 on the Growth of Psychrotrophic Culture in Autoclaved 10% NFMS and Raw Milk (each point represents the average count from three trials) Autoclaved 10% NFMS ● , Raw Milk ■

TABLE VIII

TEST FOR SIGNIFICANT DIFFERENCES AMONG AUTOCLAVED MILK SAMPLES CONTAINING INCREASING NUMBERS OF LACTOBACILLUS BULGARICUS D3 WITH RESPECT TO INHIBITION OF PSYCHROTROPHIC BACTERIA

Source	df	SS	MS
total	23	62.14	
replicates	2	54.45	
treatment	7	7.22	
error	14	.47	.033

Means Compared[a]	Differences Between Means	LSR[b]	Means Compared	Differences Between Means	LSR
A-B	6.12-6.12 = 0	.32	C-F	6.09-5.13 = .96*	.34
A-C	6.12-6.09 = .03	.34	C-G	6.09-4.84 = 1.25*	.35
A-D	6.12-6.03 = .09	.34	C-H	6.09-4.80 = 1.29*	.36
A-E	6.12-5.56 = .56*	.35			
A-F	6.12-5.13 = .99*	.36	D-E	6.03-5.56 = .47*	.32
A-G	6.12-4.84 = 1.28*	.36	D-F	6.03-5.13 = .90*	.34
A-H	6.12-4.80 = 1.32*	.36	D-G	6.03-4.84 = 1.19*	.34
			D-H	6.03-4.80 = 1.23*	.35
B-C	6.12-6.09 = .03	.32			
B-D	6.12-6.03 = .09	.34	E-F	5.56-5.13 = .43*	.32
B-E	6.12-5.56 = .56*	.34	E-G	5.56-4.84 = .72*	.34
B-F	6.12-5.13 = .99*	.35	E-H	5.56-4.80 = .76*	.34
B-G	6.12-4.84 = 1.28*	.36			
B-H	6.12-4.80 = 1.32*	.36	F-G	5.13-4.84 = .29	.32
			F-H	5.13-4.80 = .33	.34
C-D	6.09-6.03 = .06	.32			
C-E	6.09-5.56 = .53*	.34	G-H	4.84-4.80 = .04	.32

[a] A = control; B = 5.0×10^6 lactobacilli/ml; C = 7.5×10^6 lactobacilli/ml; D = 1.0×10^7 lactobacilli/ml; E = 2.5×10^7 lactobacilli/ml; F = 5.0×10^7 lactobacilli/ml; G = 7.5×10^7 lactobacilli/ml; H = 1.0×10^8 lactobacilli/ml.

[b] Least significant ranges at .05 significance level.

* These differences marked with asterisk are significant at $P<.05$.

per ml. The data from the raw milk was not analyzed statistically since no apparent decreases in psychrotroph counts were observed.

Hydrogen Peroxide Production by Lactobacilli During Extended Storage

Four cultures were selected on the basis of their ability to inhibit the psychrotrophic culture and tested for peroxide production after various times of incubation at 5.5°C (Figure 3). The milk was inoculated with approximately 1 x 10^8 lactobacilli per ml. Peroxide production by the cultures reached a peak after 12 to 24 hrs incubation and then slowly dissipated during the remaining incubation time. The most active culture (L. bulgaricus D1) in the interaction experiments produced substantially more peroxide than a less active culture (L. acidophilus A1). The detectable peroxide produced by L. bulgaricus E2 (intermediate culture) decreased more rapidly after 48 hrs than L. bulgaricus D1. However, L. acidophilus A2 was a very active inhibitor in the interaction experiments but produced much lower levels of peroxide than L. bulgaricus D1.

Effect of Lactobacilli on pH of Milk During Refrigerated Storage

The pH of the autoclaved 10% NFMS containing all levels of lactobacilli was 6.10 to 6.60 initially and was 6.00 to 6.55 after 6 days storage at 5.5°C. The pH of the raw milk containing all levels of lactobacilli was 6.50 to 6.68 initially and was 6.40 to 6.70 after 6 days storage at 5.5°C.

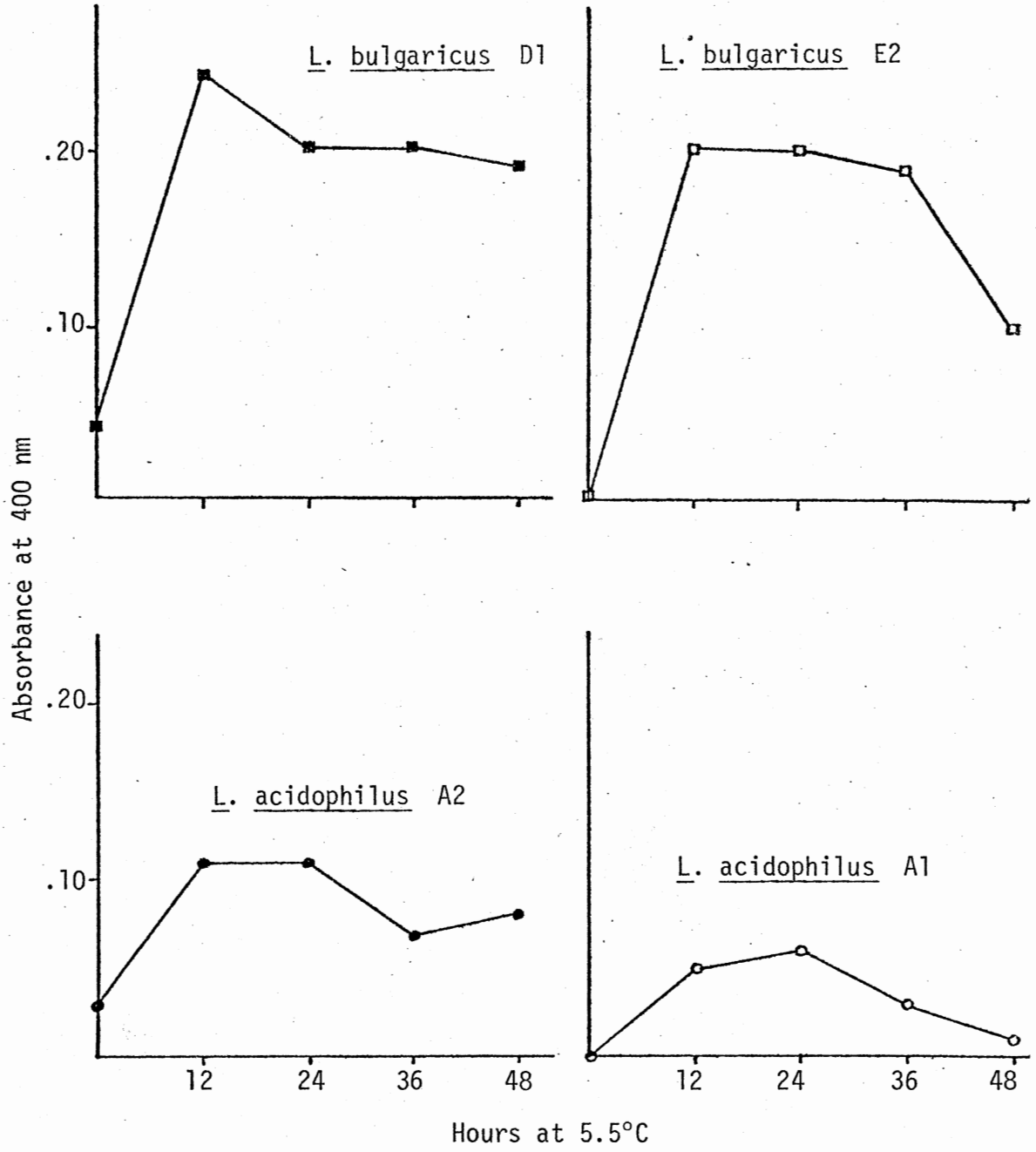

Figure 3. Hydrogen Peroxide Production by Selected Cultures of Lactobacilli in Refrigerated Milk

CHAPTER V

DISCUSSION

Most studies on the antagonistic action of lactic acid bacteria toward food borne pathogens and spoilage microorganisms have involved measuring the inhibitory actions produced during growth of the starter culture bacteria (Dahiya and Speck, 1968; Daly et al., 1972; Gilliland and Speck, 1972; Price and Lee, 1970). However, some reports have also indicated there is inhibition toward undesirable microorganisms during storage of cultured foods at refrigeration temperatures (Goel et al., 1971; Goepfert and Chung, 1970). The growth of psychrotrophic bacteria in raw milk during refrigerated storage not only limits the keeping quality of the raw milk but also can affect the quality of heat processed milk and some products made from it. Consequently, recent research has emphasized controlling the growth of psychrotrophic bacteria in raw milk. Lactobacilli and pediococci have been shown to suppress the growth at refrigeration temperatures of spoilage bacteria in ground beef and of a psychrotrophic culture inoculated into autoclaved milk (Gilliland and Speck, 1975). The three cultures of lactobacilli used in their study did not grow at refrigeration temperature, thus very little change in the acidity of the food was observed. When catalase was added to the milk the inhibition toward the psychrotroph culture in milk was relieved. This suggested that hydrogen peroxide was the antagonistic agent.

Since hydrogen peroxide appears to be the major inhibitory agent produced by the lactobacilli during refrigerated storage, an attempt was made in this study to select cultures most active with regard to hydrogen peroxide production based on the zone size formed by them on "milk-peroxidase" agar. The apparent lack of correlation between zone size on the agar medium at 37°C and peroxide production in refrigerated milk could be due to the difference in the incubation temperatures. If the cultures of lactobacilli had been able to grow and form colonies on the peroxidase agar medium at 5°C, the zone size may have predicted the amount of peroxide the cultures would produce in refrigerated milk. The zone size at 37°C may be an accurate indication of the peroxide the cultures would produce in milk at that temperature. The peroxidase agar did appear useful in this study in that if no zones were formed around the colonies, the culture did not produce detectable peroxide in refrigerated milk. However, it was not useful for ranking cultures with respect to the relative amounts of peroxide they could produce in refrigerated milk.

Experiments were done in milk which was not agitated during refrigerated storage. The small volume of sample (15 ml) in the dilution bottles (40 x 40 mm inside dimensions) provided a relative high surface to volume ratio. Under these conditions, the lactobacilli inhibited the growth of the psychrotrophic culture in autoclaved milk. However, the intensity of the antagonistic action may have been greater had the surface to volume ratio been greater or if the culture had been aerated during refrigerated storage. An aerated culture of lactic streptococci produced much higher levels of hydrogen peroxide than a static culture (Gilliland and Speck, 1969). Presumably the production of peroxide is

dependent on the presence of oxygen. Thus, if hydrogen peroxide is the inhibitory agent, greater inhibitory action might be expected at higher concentrations of oxygen. The first statistically significant inhibition of psychrotrophic bacteria in autoclaved milk by two cultures of L. bulgaricus occurred at a population of 2.5×10^7 lactobacilli per ml. Numbers of lactobacilli greater than this (5.0×10^7 to 1.0×10^8/ml) caused greater inhibition of the psychrotrophic culture. However, rather than having to add the higher numbers it may be better to increase the amount of oxygen available to the culture in milk to increase the amount of antagonism.

Using 2.5×10^7 lactobacilli per ml, cultures of lactobacilli were effectively compared for percentage inhibition against the psychrotrophic culture in autoclaved milk. A comparison of the inhibitory action of seven cultures indicated a wide variation; three were very active, two were intermediate and two were only slightly inhibitory. These results show that careful selection of cultures of lactobacilli for use in controlling the growth of psychrotrophic bacteria in refrigerated milk is very important.

Based on comparisons of the inhibition toward the psychrotrophic culture, four cultures of lactobacilli were further tested for peroxide production in refrigerated milk. The most active inhibitor (L. bulgaricus D1) in the interaction experiments produced considerably more peroxide during a 48 hr period in refrigerated milk than an intermediate inhibitor (L. bulgaricus E2) and one of the least active cultures (L. acidophilus A1). However, one of the three most active inhibitors (L. acidophilus A2) produced lower levels of peroxide than an intermediate inhibitor (L. bulgaricus E2). Apparently, other

inhibitory substances in addition to hydrogen peroxide were responsible for the inhibitory action produced by L. acidophilus A2. Sandine (1979) reported that L. acidophilus can produce antibiotic-like substances such as acidophilin, acidolin or lactocidin. Gilliland and Speck (1975) stated that other factors were responsible for the inhibition of psychrotrophic bacteria in refrigerated ground beef by Pediococcus cerevisiae, since no detectable hydrogen peroxide was produced by P. cerevisiae.

In preliminary experiments in raw milk, equal volumes of autoclaved 10% NFMS and raw milk were mixed to achieve desired populations of lactobacilli. Increasing numbers of lactobacilli from 5.0×10^6 to 1.0×10^8 per ml had no effect on the growth of psychrotrophic bacteria at 5.5°C. Juffs and Babel (1975) observed low levels of inhibition of psychrotrophic bacteria when a commercial lactic culture (lactic streptococci and Leuconostoc cremoris) was added to raw milk. However, no hydrogen peroxide was detected in these samples.

Lactoperoxidase is naturally present in raw milk (Bjorck, 1978). Thus, this enzyme could destroy any hydrogen peroxide produced by the lactobacilli in the raw milk samples. However, raw milk contains a lactoperoxidase-thiocyanate system (LP system) which can inhibit the growth of psychrotrophic bacteria (Bjorck, 1978). The LP system is activated only in the presence of all three components - lactoperoxidase, thiocyanate and hydrogen peroxide. Since hydrogen peroxide is the only component normally missing, it could be added exogenously (Bjorck, 1978), generated enzymatically (Bjorck et al., 1975; Bjorck and Rosen, 1976), or produced by lactobacilli (Reiter, 1978). Since hydrogen peroxide is very labile, active cultures of lactobacilli could be used to

continuously generate substantial levels of peroxide for several days at refrigeration temperatures.

In recent years, frozen concentrated starter cultures have been used in the manufacture of cultured foods. The increased availability of highly concentrated cell suspensions makes it possible to consider adding the cultures to raw milk for controlling the growth of psychrotrophic bacteria. In the present study, the concentrated cultures survived quite well in liquid nitrogen. However, the effect of freezing on the inhibitory system still needs to be determined.

An important consideration in future studies is the selection of lactobacilli capable of producing high levels of peroxide. Premi and Bottazzi (1972) reported that L. lactis produced much higher levels of peroxide than L. bulgaricus in a phosphate buffer solution with added glucose after 6 days storage at 5°C. Similar results were obtained by Gilliland and Speck (1975) in autoclaved 10% NFMS at 5°C. Thus, strains of L. lactis which are capable of producing high amounts of hydrogen peroxide need to be selected for future use.

Temperature not only affects growth of psychrotrophic bacteria but also may play an important role in the amount of peroxide produced. More peroxide was detected in a commercial lactic culture at 7°C than at 3.5°C (Juffs and Babel, 1975). While at the higher temperatures the psychrotrophic bacteria would be expected to grow more rapidly, the increased production of hydrogen peroxide may increase antagonistic action. The end result might be greater inhibition of the psychrotroph. In future studies, optimum conditions need to be found for maximum inhibition of the growth of psychrotrophic bacteria in raw milk.

CHAPTER VI

SUMMARY AND CONCLUSIONS

An attempt was made to utilize a "milk-peroxidase" agar medium to isolate cultures of lactobacilli that would produce peroxide in milk at refrigeration temperatures. Hopefully the size of the zone surrounding the colonies on "milk-peroxidase" agar would permit isolation of the most active peroxide producing colonies. Even though no correlation was established between the size of the zone produced during growth at 37°C and peroxide production in refrigerated milk, the test system predicted whether or not a culture would produce peroxide in milk.

Interaction experiments were conducted in which cells of lactobacilli from frozen concentrated cultures were added to autoclaved milk which had been inoculated with a psychrotrophic culture. The psychrotrophic culture had been previously isolated from refrigerated raw milk. The cultures of lactobacilli were from commercial yogurt. Results from preliminary experiments indicated that the lactobacilli significantly inhibited the growth of the psychrotrophic bacteria in autoclaved milk when 2.5×10^7 lactobacilli per ml were added. No inhibition was noted when fewer lactobacilli were added. The intensity of the antagonism increased as the number of lactobacilli added was increased (5.0×10^7 to 1.0×10^8/ml).

Comparisons of the percentage inhibition by cultures of lactobacilli

toward a psychrotrophic culture in autoclaved milk at 5.5°C were done at 2.5×10^7 lactobacilli per ml. Wide variations in inhibition were observed among the cultures. The three most active inhibitors were significantly different from the two least active inhibitors.

Some cultures were then compared for their ability to produce hydrogen peroxide in milk during storage at 5.5°C. L. bulgaricus D1 (the most active inhibitor) produced slightly higher amounts of peroxide during 48 hrs than L. bulgaricus E2 (intermediate inhibitor) and substantially more peroxide than L. acidophilus A1 (one of the least active inhibitors). L. acidophilus A2 produced less peroxide than L. bulgaricus E2. However, in the interaction experiments it was more effective in inhibiting the growth of the psychrotroph than was L. bulgaricus E2. Thus it appears that factors in addition to peroxide must have been responsible for the antagonistic action produced by L. acidophilus A2.

In "preliminary" experiments, a culture of L. bulgaricus did not inhibit the growth of psychrotrophic bacteria in raw milk. In future studies, cultures capable of producing high levels of peroxide need to be isolated. L. lactis reportedly produces more peroxide than L. bulgaricus and L. acidophilus (Premi and Bottazzi, 1972). Additional investigations are needed to determine the effect of temperature on the inhibitory action by lactobacilli. A few degrees difference in refrigeration storage temperature could have considerable effect on peroxide production. These studies should emphasize the conditions required to maximize the inhibition by lactobacilli toward the growth of psychrotrophic bacteria in raw milk.

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APPENDIX

TABLE IX

GROWTH OF PSYCHROTROPHIC CULTURE IN AUTOCLAVED 10% NFMS CONTAINING INCREASING NUMBERS OF LACTOBACILLUS BULGARICUS E2

Sample Number	Lactobacilli Count/ml	Non-Lactobacilli Count/ml[a]			
		Trial 1	Trial 2	Trial 3	Avg.
A	control	6.0	5.59	5.15	5.58
B	5.0×10^6	6.28	5.62	5.18	5.69
C	7.5×10^6	6.38	5.51	4.96	5.62
D	1.0×10^7	6.15	5.33	5.15	5.61
E	2.5×10^7	5.15	4.83	4.23	4.73
F	5.0×10^7	3.20	3.70	3.34	3.41
G	7.5×10^7	2.15	3.34	3.04	2.84
H	1.0×10^8	1.60	2.42	2.15	2.06

[a]Data presented as $\log_{10}$ of number of non-lactobacilli/ml after the milk had been stored 6 days at 5.5°C.

TABLE X

GROWTH OF PSYCHROTROPHIC CULTURE IN AUTOCLAVED 10% NFMS CONTAINING INCREASING NUMBERS OF LACTOBACILLUS BULGARICUS D3

Sample Number	Lactobacilli Count/ml	Non-Lactobacilli Count/ml[a]			
		Trial 1	Trial 2	Trial 3	Avg.
A	control	5.82	4.51	8.04	6.12
B	5.0×10^6	5.58	4.70	8.08	6.12
C	7.5×10^6	5.72	4.48	8.08	6.09
D	1.0×10^7	5.68	4.30	8.11	6.03
E	2.5×10^7	4.88	4.00	7.80	5.56
F	5.0×10^7	4.82	3.30	7.28	5.13
G	7.5×10^7	4.72	3.20	6.59	4.84
H	1.0×10^8	4.53	3.15	6.73	4.80

[a]Data presented as $\log_{10}$ of numbers of non-lactobacilli/ml after the milk had been stored 6 days at 5.5°C.

VITA[2]

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Master of Science

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