EFFECT OF FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE ON ASSIMILATION OF CHOLESTEROL BY *LACTOBACILLUS ACIDOPHILUS*

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CHAPTER I

INTRODUCTION

Coronary heart disease is a major cause of death in the United States (10). Elevated levels of serum cholesterol (hypercholesterolemia) have been linked to coronary heart disease (2, 3, 4, 5, 7, 8, 9). Therefore, hypercholesterolemic individuals are advised to decrease their dietary intake cholesterol and saturated fatty acids to control their serum cholesterol levels. Foods frequently excluded from the diet are animal products such as meat and milk.

Cells of some strains of Lactobacillus acidophilus are capable of assimilating cholesterol from a broth medium under conditions similar to those in the intestine (2). Pigs on a high cholesterol diet exhibited no significant increases in serum cholesterol levels when they consumed these cells in a nonfermented acidophilus milk product along with a high cholesterol diet. Consumption of a similar non-fermented acidophilus milk could possibly aid in controlling serum cholesterol levels in hypercholesterolemic individuals. Cholesterol is absorbed into the lymphatic system from the intes-

tine. Ingested cells of L. acidophilus could assimilate cholesterol in the intestine and be excreted thus making cholesterol unavailable for absorption into the blood.

The stability of B-galactosidase activity in cells of L. acidophilus during frozen and refrigerated storage has been documented (1). The stability of the ability to assimilate cholesterol by this organism following frozen and refrigerated storage is also of interest. Consumption of a nonfermented acidophilus milk containing strains of L. acidophilus capable of assimilating cholesterol may be beneficial to hypercholesterolemic individuals and to the dairy industry. To provide the most benefit, the ability of the culture to assimilate cholesterol should be maintained throughout production and storage of such a milk product.

The purpose of this study was to evaluate the stability of strains of L. acidophilus, capable of assimilating cholesterol, under the conditions of frozen (-196°C) and subsequent refrigerated (5°C) storage that would be used in the production of a nonfermented acidophilus milk.

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CHAPTER II

REVIEW OF LITERATURE

Cholesterol and Heart Disease

Cholesterol is required for the formation of steroid hormones and bile salts and as a component of all membranes in the body (12). It is synthesized in the body, primarily in the liver and small intestine, or may be at least partially supplied by the diet.

Coronary heart disease is a major cause of death in the United States (43). A popular "diet heart hypothesis" has evolved from the many reports linking a high cholesterol, high fat, animal product diet to the development of coronary heart disease. A positive correlation between serum cholesterol levels, particularly total and low density lipoprotein levels, and coronary heart disease has been established (22). However, the contribution of environmental and genetic factors to atherosclerosis and heart disease cannot be overlooked (13).

Hypocholesterolemic Effect of Milk and Dairy Products

Milk and dairy products are frequently implicated as being undesirable in the relationship of diet to blood cholesterol levels and coronary heart disease. Because one cup of whole milk contains about 34 mg of cholesterol (12), people with elevated blood cholesterol are advised to consume skim milk and low fat milk products. In a study involving teenage males as test subjects, Rossouw et al. (35) found serum cholesterol levels dropped in those subjects consuming skim milk but rose in subjects who consumed yogurt or full cream (whole) milk. These changes were correlated with changes in dietary fat and cholesterol intake. Massey (28) also found no hypocholesterolemic effect for whole milk or yogurt in a study involving college age men and women. Some research suggests, however, the presence of a factor(s) in milk that can lower serum cholesterol levels in rats, pigs, chickens, and humans. The substances identified as possible factors in milk are orotic acid (1, 2, 21, 34), hydroxymethyl glutarate (3, 19, 23), calcium (40), and lactose (15).

Efforts to identify the factor(s) in milk and dairy products responsible for hypocholesterolemic effects began after reports by Mann and co-workers on the low incidence of hypercholesterolemia and heart disease in the Maasai (25, 26, 27). They noted that Maasai warriors were very physi-

cally fit, and that they consumed a diet composed almost exclusively of meat and milk. These warriors did show extensive atherosclerosis and lesions in the arteries, but they did not suffer from a high incidence of coronary heart disease.

Mann and Spoerry (26) later reported on a study in which they fed more than 8 liters of milk per day to Maasai warriors. The milk was cultured with a wild strain of Lactobacillus and was supplemented either with 10 mg of Tween 20 per gram of fat in the milk, or with a like amount of a placebo (pure olive oil). They theorized that consumption of surfactants, such as Tween 20, would cause increased absorption of dietary cholesterol. Thus, they expected to see a greater increase in serum cholesterol levels in the Tween 20 group than in the placebo group due to enhanced absorption of cholesterol. However, serum cholesterol levels decreased in both groups over a three week period. The possibility of the presence of a factor in milk responsible for this hypocholesterolemic effect was suggested. This factor was later described by Mann (24) as a nonprotein, dialysable, heat and acid stable, polar molecule.

Orotic acid has been suggested as a factor in milk involved in the hypocholesterolemic effect of dairy products. Orotic acid is a pyrimidine intermediate in nucleic acid synthesis (34) which inhibits cholesterol biosynthesis at the acetyl CoA synthetase step (2, 34). This substance is

present in bovine milk at a level of 75 mg per milliliter (21). Bernstein et al. (1) demonstrated a significant inhibition of cholesterol biosynthesis in rat liver homogenates by milk, cultured buttermilk, and orotic acid. The inhibition was slightly less for orotic acid than for the dairy products, suggesting other mechanisms may be involved.

The other proposed hypocholesterolemic factor in milk which has been studied is hydroxymethyl glutarate. Bogulslawski and Wrobel (3) noted an increase in sterol biosynthesis from acetate in rats following weaning. They determined that rat milk contains an inhibitor of hydroxymethyl glutaryl CoA reductase. Therefore, the consumption of this milk could be expected to decrease sterol synthesis from acetate in young rats. They also demonstrated the presence of a similar factor in cow's milk. Mann (23) conducted a trial in which human subjects consumed fermented whole or skim milk yogurt, or milk, and radioacetate in water. Blood samples were taken periodically and analyzed for serum cholesterol and for incorporation of radioacetate into the cholesterol. A significant decrease in serum cholesterol level and in cholesterol biosynthesis was seen in subjects consuming yogurt but not in those consuming milk. The factor in yogurt responsible for this effect was postulated to be hydroxymethyl glutarate which inhibits the regulatory enzyme hydroxymethyl glutaryl CoA reductase in cholesterol synthesis. This hypothesis was further studied by Kritchevsky et al. (19) in male wistar rats. Rats fed

whole milk, skim milk, or water were evaluated for serum cholesterol levels and enzyme activities. Serum cholesterol level and hydroxymethyl glutaryl CoA reductase activity was decreased in rats fed whole or skim milk as compared to rats fed water.

Rao et al. (33) and Pulusani and Rao (32) noted decreases in plasma cholesterol in rats fed milk fermented by Streptococcus thermophilus, L. bulgaricus or L. acidophilus. These decreases were thought to be associated with some metabolite produced during the bacterial fermentation of the milk.

Hypocholesterolemic effects have been demonstrated for other milk components. Consumption of lactose decreased serum cholesterol levels in human subjects (15). Calcium was hypocholesterolemic for rabbits but the effect was less than that observed for milk and yogurt (40). However, the calcium supplemented diet contained only 23 mg of calcium compared to 45.5 mg of calcium in the milk and yogurt diets.

Several studies have been done with humans in attempts to identify the milk factor. In a study in which human volunteers consumed whole or skim milk, Howard and Marks (17) noted a greater decrease in serum cholesterol levels in humans consuming skim milk than for those consuming whole milk. Both groups did show a decrease in serum cholesterol levels. Hepner et al. (16) noted a significant hypocholesterolemic effect for both pasteurized and non-pasteurized yogurt fed to humans.

Use of Lactobacillus acidophilus as a Dietary Adjunct to Lower Serum Cholesterol Levels

Several recent studies have dealt specifically with the hypocholesterolemic effect of L. acidophilus. Mott et al. (30) found that germ free pigs monocontaminated with L. acidophilus and then allowed to develop a normal microflora, showed a decrease in serum cholesterol when compared to pigs that remained monocontaminated with L. acidophilus. They hypothesized that alterations of the gastrointestinal tract as well as the intestinal bacteria could have been responsible for the decrease in serum cholesterol.

In a subsequent study by Tortuero et al. (42), chickens were fed a diet containing cholesterol with or without a supplement of L. acidophilus. Serum cholesterol levels were significantly lower in those chickens receiving the supplement than in control chickens indicating that L. acidophilus had an effect on serum cholesterol.

Seventy-five full term infants were divided into three groups and fed either milk formula, milk formula plus sodium bicarbonate, or milk formula supplemented with L. acidophilus in a study by Harrison and Peat (14). By day eight of the study, those babies receiving formula containing sodium bicarbonate or the culture of L. acidophilus had significantly lower serum cholesterol

levels which were correlated with significantly higher numbers of fecal lactobacilli. Blood cholesterol levels of the babies fed only the milk formula increased during the study. Grunewald (11) reported rats fed milk fermented by L. acidophilus had significantly lower serum cholesterol levels than rats fed either noncultured milk or water.

Thompson et al. (41) evaluated the effect of feeding fermented and unfermented milk on serum cholesterol levels in 68 human volunteers. The subjects, who all had normal levels of serum cholesterol, consumed one liter of skim milk, whole milk, 2% milk, "Sweet Acidophilus Milk," buttermilk, or yogurt for 3 weeks. They found no significant decreases in serum cholesterol levels for subjects consuming any of these products at these levels.

Several strains of L. acidophilus were found to be capable of assimilating cholesterol from a broth medium, supplemented with oxgall, during growth under anaerobic conditions (7). This discovery was followed by a feeding trial in which 5 week old pigs, divided into three treatment groups, were fed a control diet, the control diet supplemented with L. acidophilus RP32, or the control diet supplemented with L. acidophilus P47. Although the pigs in all groups exhibited increases in serum cholesterol, the increase in those pigs receiving L. acidophilus RP32 was not significant compared to increases in the control pigs or in those pigs fed L. acidophilus P47, a strain not capable of assimilating cholesterol. This study indicated that a

selected strain of L. acidophilus could assimilate cholesterol in the intestine and thus prevent its absorption into the blood.

Frozen Concentrated Cultures of Lactobacilli

Frozen concentrated starter cultures consist of starter bacteria grown in a broth medium under closely controlled conditions, concentrated into a smaller volume of liquid, and frozen. To be useful, the bacteria must retain all their enzymatic and biological activity while frozen and be able to express this activity when used to make a cultured or culture containing dairy product (8). Such concentrated cultures may be used to prepare bulk cultures for large scale milk fermentations, for direct inoculation of a product vat, or for direct inoculation into pasteurized low fat milk to prepare a nonfermented acidophilus milk product.

Possible health and/or nutritional benefits derived from consuming acidophilus milk led to research to develop a more palatable product. Fermented acidophilus milk possessed undesirable flavors that discouraged consumption (39). Duggan et al. (4) noted that dietary preparations should contain large numbers of viable lactobacilli capable of establishing themselves in the intestines. They prepared concentrated cultures of L. acidophilus and evaluated their survival at different freezing temperatures. They suggested such cultures should be used by storing the concentrated

culture frozen in 5 or 10 ml quantities and adding them directly to a pint or quart of pasteurized milk just prior to consumption. However, the product could be stored at refrigeration temperatures for one week with no change in milk pH and no loss in viable organisms.

Research conducted with lactic streptococci indicated enhanced survival during frozen storage when protective agents such as glycerol were added to the growth medium and when late log or early stationary phase cells grown at pH 6.0 to pH 7.0 were harvested to prepare frozen concentrated cultures (5, 20). Smittle et al. (36) applied these techniques to frozen concentrated cultures of L. bulgaricus and found cultures grown in a medium supplemented with 0.1% Tween 80 (polyoxyethylenesorbitan monooleate) possessed enhanced resistance to freezing. Smittle et al. (37) later determined the sodium oleate present in Tween 80 was responsible for the improved stability of cultures of L. bulgaricus to freezing. This enhanced stability was due to increased production of a C19 cyclopropane fatty acid in the membrane of the cells when grown in a medium containing oleic acid.

In 1975 Speck described a nonfermented milk product containing a population of several million L. acidophilus per milliliter (38). This milk could be maintained at refrigeration temperatures for 2-3 weeks with no change in flavor. Subjects who consumed this milk experienced an increased fecal population of lactobacilli (9).

The stability of L. acidophilus in frozen concentrated cultures during frozen storage for 28 days at -20°C was improved when the culture had been grown in a media supplemented with calcium (44). Rough and smooth variants of L. acidophilus RL8K differed in bile resistance and stability during frozen storage. The presence of these variable morphologies indicates the need for research on effects of growth media and conditions on stability of L. acidophilus strains prepared for use as dietary adjuncts (18).

Mitchell and Gilliland (29) investigated different levels of sweet whey in a pepsinized whey based medium for growing cells of L. acidophilus for frozen concentrated cultures. They evaluated cultures of L. acidophilus after 28 days frozen (-196°C) and 21 days subsequent refrigerated (5°C) storage. They found a concentration of 2.5% sweet whey imparted optimum stability of the culture to freezing and frozen storage.

Stability of Lactobacillus acidophilus During Frozen and Refrigerated Storage

An important consideration in the selection of strains of L. acidophilus for use in the manufacture of nonfermented acidophilus milk is the stability of enzymatic activity and culture viability during frozen and refrigerated storage. Ideally the selected strain should remain viable and active

in the final product without altering the intrinsic properties of the milk. Myers in 1931 (31) prepared an unfermented acidophilus milk by adding cells of L. acidophilus which had not been frozen to pasteurized whole milk. He noted that when the product was held at 2-5°C for one week the lactobacilli remained viable and capable of implantation in the human intestinal tract. The culture did not sour the milk during this storage period.

Duggan (4) and Speck (38) reported populations of L. acidophilus used to prepare nonfermented acidophilus milk were stable at refrigeration temperatures for 1-3 weeks. Young (45) evaluated several commercial brands of "Sweet Acidophilus Milk" and found decreases in populations of lactobacilli, increases in populations of contaminants, and off flavors in the product by day 18 of refrigerated storage. Acidity of the product was not affected. Milk for this study was collected directly from the processing plant, and was 0-3 days old when brought to the laboratory.

Mitchell and Gilliland (29) evaluated L. acidophilus NCFM for stability during refrigerated storage. Concentrated culture which had been stored at -196°C was thawed and added to 10% NFMS and refrigerated (5°C). Samples were evaluated on day 1, 7, 14, and 21 for numbers of total and bile resistant lactobacilli. They found significant decreases in both total and bile resistant lactobacilli over the 21 day storage period.

Gilliland and Lara (6) evaluated viability and B-galactosidase activity of three strains of L. acidophilus during frozen (-196°C) and subsequent refrigerated (5°C) storage. Each strain evaluated possessed a different initial level of enzyme activity and frozen storage for 28 days had little effect on this activity. During refrigerated storage there was a significant decrease in B-galactosidase activity for two of three strains of L. acidophilus tested. The greatest effect was for strain NCFM. The variability among strains, both for retention of viability and for enzymatic activity during storage, accentuates the need for careful evaluation of cultures to be used in the manufacture of nonfermented acidophilus milk to be used for improving lactose utilization or for other nutritional benefits.

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CHAPTER III

Effects of Frozen and Refrigerated Storage on Assimilation of Cholesterol by *Lactobacillus acidophilus*

Abstract

Two strains of *Lactobacillus acidophilus* were evaluated for viability, bile tolerance, and ability to assimilate cholesterol after frozen (-196°C) storage in liquid nitrogen and subsequent refrigerated (5°C) storage in non-fermented acidophilus milk. Frozen storage had little or no adverse effect on cholesterol assimilation, bile tolerance, or viability of either strain. There was no significant interaction between frozen and refrigerated storage for either strain. However, the two strains reacted quite differently to the stress of refrigerated storage in milk. Refrigerated storage had a significant effect on numbers of total and bile resistant lactobacilli for both strains. The decrease in numbers of viable lactobacilli was much greater for *L. acidophilus* NCFM-L than for *L. acidophilus* RP32. Strain RP32 exhibited no significant decrease in either bile tolerance or ability to assimilate cholesterol during refrigerated storage. However, bile tolerance and ability to

assimilate cholesterol decreased significantly for strain NCFM-L during the 21 day storage at 5°C.

Introduction

Results from several studies suggest that consuming certain cultured dairy products can help reduce serum cholesterol levels. Mann and Spoerry (11) found decreased serum cholesterol levels in Maasai warriors fed large quantities of milk fermented by a wild strain of Lactobacillus. Mott noted that pigs monocontaminated with L. acidophilus and then allowed to develop a normal microflora showed significant decreases in serum cholesterol levels compared to pigs that remained monocontaminated (13). They did not, however, indicate that L. acidophilus was involved. Tortuero et al. (16) fed chickens a diet supplemented with L. acidophilus and Grunewald (9) fed rats milk fermented by L. acidophilus. Both researchers noted significant decreases in serum cholesterol levels for their subjects compared to control animals which did not receive the L. acidophilus. In a study with 75 infants, Harrison and Peat (10) noted a decrease in serum cholesterol levels in infants receiving formula supplemented with L. acidophilus. The decrease was correlated with an increase in number of fecal lactobacilli.

The presence of a factor produced or enhanced by the lactobacilli in cultured dairy products has been suggested as being responsible for lowering serum cholesterol (9, 11).

However, results reported by Gilliland et al. (5) suggest that L. acidophilus itself may take up cholesterol during growth in the intestine and make it unavailable for absorption into the bloodstream. They found some strains of L. acidophilus were capable of assimilating cholesterol from a broth medium under conditions similar to those in the gastrointestinal tract. When fed to pigs in a nonfermented acidophilus milk, along with a high cholesterol diet, a selected strain inhibited significant increases in serum cholesterol levels.

Cells of L. acidophilus remained viable during storage at -196°C in liquid nitrogen (4, 12). When thawed and added to cold milk and stored at 5°C the numbers of viable lactobacilli decreased after 10 to 14 days of storage (12). However, all strains of L. acidophilus do not exhibit the same degree of susceptibility to the stress of refrigerated storage (4).

The objective of this study was to determine the stability of the ability of L. acidophilus to assimilate cholesterol during frozen (-196°C) and subsequent refrigerated (5°C) storage. These are conditions similar to those used in the commercial production and storage of nonfermented acidophilus milk.

Materials and Methods

Source and Maintenance of Cultures

Lactobacillus acidophilus strains RP32 (ATCC 43121) and NCFM-L were from the laboratory stock culture collection. Cultures were maintained by weekly subculture using 1% inocula and 18 h incubation at 37°C in Peptonized Milk Nutrient Broth (PMN)> PMN broth contained 5% PMN (Humko Sheffield), 2% lactose, 2% Primatone (Humko Sheffield), 0.1% yeast extract, and 0.1% polyoxyethylenesorbitan monooleate (Tween 80; Sigma Chemical Co.). The medium was sterilized by heating for 15 minutes at 121°C. Cultures were stored at 4°C between transfers. Subcultures were made at least 3 times just prior to experimental use.

Culture Identity

The identity of the cultures was confirmed using the Minitek system as described by Gilliland and speck (7). Identity characterization included tests for hydrolysis of esculin, deamination of arginine, and fermentation of amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Cultures were also tested for the ability to grow at 15°C and 45°C and for Gram stain reaction. Identity was based on characteristics for *L. acidophilus* presented in the

eighth edition of Bergey's Manual of Determinative Bacteriology (1).

Preparation and Storage of Concentrated Cultures

Cell crops of L. acidophilus were grown in a 7 liter fermentor (New Brunswick Scientific Co., Edison, NY) under constant agitation, pH, and temperature control. The fermentor jar, equipped with an autoclavable combination pH electrode and containing 3 liters of distilled water, was sterilized by heating in an autoclave at 121°C for 45 min. The water was aseptically removed just prior to the aseptic addition of the sterile growth medium.

Four liters of sterile PMN broth was aseptically added to the sterile fermentor jar and allowed to equilibrate to 37°C. The broth was adjusted to pH 5.0 with sterile 10% lactic acid, filter sterilized using a 30 ml syringe and a sterile Acrodisc filter, 0.45 mm pore size (Gelman Sciences, Ann Arbor, MI), before inoculation. Broth temperature was controlled by immersing the fermentor in a water bath maintained at 37°C.

The PMN broth was inoculated with 40 ml (1%) of a freshly prepared PMN broth culture (1% inoculum and incubation at 37°C for 18 h) of the desired strain of L. acidophilus. During growth, the culture in the fermentor was maintained at pH 5.0 with a neutralizer composed of 20% Na_2CO_3 in 20% NH_4OH (6). The neutralizer was added automat-

ically as needed by a peristaltic pump controlled by an automatic pH controller (New Brunswick Scientific Co., Edison, NY).

One liter of culture was removed after 12 h of incubation into a sterile one liter flask and held in an ice water bath for one hour to chill. (Preliminary experiments showed both cultures reached maximum populations at 12 h.) Cells were harvested by centrifuging at 4080 x g for 20 min at 0-1°C in a Sorvall RC5 Superspeed Refrigerated Centrifuge (Dupont Co., Newtown, CT). The supernatant was discarded and the cell pellets were resuspended in two times their weight of cold sterile reconstituted 10% nonfat milk solids (NFMS) with the aid of sterile glass beads (0.3 cm diameter). The resulting concentrated culture was then dispensed in 2 g quantities into sterile cryogenic vials (Dynatech Laboratories Inc., Chantilly, VA), which were then frozen and stored at -196°C in liquid nitrogen.

Preparation and Storage of Nonfermented Acidophilus Milk

Milk for refrigerated storage was prepared by dividing 500 ml reconstituted 10% NFMS into one 99 ml and four 90 ml portions in glass dilution bottles. Bottles containing the milk were heated at 100°C for 30 min and cooled at 5°C prior to use.

On day 0 and after 1, 14, and 28 days of frozen (-196°C) storage, one vial of concentrated culture was re-

moved from liquid nitrogen. It was thawed by submersion in 500 ml of tap water at 22°C for five min. The exterior of the vial was sanitized with ethanol and air dried prior to being opened.

To achieve the desired populations of lactobacilli in the nonfermented acidophilus milk, 1-g of thawed concentrated culture was placed into the 99 ml portion of cold milk. This 1:100 dilution was mixed by inverting six times and a 10 ml portion was aseptically transferred into each of the four bottles containing 90 ml of cold milk. The contents of each bottle were mixed by inverting six times prior to storage at 5°C. The nonfermented milk was stored at 5°C for 0, 7, 14, and 21 days.

Numbers of viable L. acidophilus were determined in the concentrated culture following 0, 1, 14, and 28 days of frozen storage and in the nonfermented acidophilus milk during refrigerated storage on days 0, 7, 14, and 21. Ability to assimilate cholesterol was also evaluated after each frozen and each refrigerated storage period.

Measurement of Culture Viability During Frozen and Subsequent Refrigerated Storage

Enumeration of viable lactobacilli was done using the pour plate technique. Serial dilutions were made utilizing 99 ml sterile peptone dilution blanks containing 0.1% Bacto-Peptone (Difco) and 0.01% Antifoam Emulsion A (Sigma

Chemical Co., St. Louis, MO) according to procedures described in the Compendium of Methods for the Microbiological Examination of Foods (2). Initial 1:100 dilutions were prepared by adding 1 ml broth culture, 1 g concentrated culture, or 1 ml of nonfermented acidophilus milk to a 99 ml sterile peptone dilution blank. The diluted samples were plated in duplicate with molten (45°C) PMN agar (PMN broth plus 1.5% Bacto agar) and PMNO agar (PMN agar plus 0.1% oxgall) to determine numbers of viable and bile reistant lactobacilli respectively. Plates were incubated at 37°C for 48 h. Colonies were counted with the aid of a Quebec Colony Counter.

Evaluation of Ability to Assimilate Cholesterol

After each period of frozen and refrigerated storage, cultures were evaluated for ability to assimilate cholesterol from a broth medium. Sterile, freshly prepared lactobacilli MRS broth (Difco) supplemented with 0.2% sodium thioglycollate (BBL), and 0.3% oxgall (Difco) was aseptically dispensed in 20 ml quantities in test tubes for each culture sample to be assayed. Two ml sterile PPLO (pleuro pneumonia like organism) serum (Difco) was aseptically added to each tube to complete the assay broth (MRS-OX-THIO-PPLO). Tubes were mixed by inverting six times and a 5 ml quantity was removed from each, placed in a sterile test tube, and labeled as the uninoculated control for each sample. The

remaining broth (17 ml) was inoculated with 0.17 ml (1%) of the appropriate sample of nonfermented acidophilus milk, mixed by inverting 6 times, and dispensed in 5 ml quantities into 3 additional test tubes. All tubes were held in an ice water bath during the inoculation process and then placed in a 37°C water bath for incubation. One tube of each culture was removed following the desired period(s) of incubation and held in an ice water bath for further analyses.

An indication of the amount of growth of the cultures in MRS-OX-THIO-PPLO broth at each time was obtained turbidimetrically using a spectronic 21 colorimeter. Cultures were diluted 1:5 with lactobacilli MRS broth prior to turbidimetric measurement. The absorbance at 620nm was determined and recorded for each sample.

The remainder of the undiluted broth culture was placed into a glass centrifuge tube and centrifuged at 36,700 x g for 15 min at 0-1°C in the Sorvall Superspeed Refrigerated Centrifuge. Spent broth was collected for each sample and stored at 4°C in a sterile test tube until analyzed for cholesterol content by the method of Rudel and Morris (14).

Initial evaluation of cholesterol assimilation data indicated that maximum amounts of cholesterol were assimilated during a 12 h incubation period for strain RP32 while a 14 h incubation period was necessary for strain NCFM-L. Therefore, only the values for these assay incubation time

periods were included in the results for each culture during storage experiments.

Statistical Analysis

This study involved three trials of each of the two strains of L. acidophilus. Statistical analyses were performed on the data from plate counts obtained on PMN and PMNO agar media, growth (A_{620nm}) in assay broth (MRS-OX-THIO-PPLO), and amount of cholesterol assimilated, using the Statistical Analysis System (SAS) for microcomputers (15). Analysis of variance for a split plot design and the least significant difference test to compare means was performed.

Results

Confirmation of Culture Identity

The two cultures used in this study were confirmed to be L. acidophilus. The fermentation patterns, growth characteristics, and Gram stain reactions of each culture closely matched the description of L. acidophilus presented in the 8th edition of Bergey's Manual of Determinative Bacteriology (1) (Table I). Both strains were composed of Gram positive, catalase negative rods that grew at 45°C but not at 15°C. Both strains hydrolyzed esculin and fermented cellobiose, galactose, glucose, lactose, maltose, mannose, raffinose, salicin, sucrose, and trehalose. Strain NCFM-L fermented amygdalin, strain RP32 did not. Neither strain

TABLE I

IDENTITY CHARACTERISTICS OF STRAINS OF *L. ACIDOPHILUS* USED IN STORAGE TRIALS

Test	Strain		
	RP32	NCFM-L	BERGEY'S[a]
Gram Stain	+	+	+
Cellular Morphology	rods	rods	rods
Catalase	-	-	-
Growth at 15°C	-	-	-
Growth at 45°C	+	+	+
NH_3 from Arginine	-	-	-
Hydrolysis of Esculin	+	+	+
Acid from			
Amygdalin	-	+	+
Arabinose	-	-	-
Cellobiose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	-	-	-
Mannose	+	+	+
Melezitose	-	-	-
Melibiose	-	-	±
Raffinose	+	+	±
Rhamnose	-	-	-
Salicin	+	+	+
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	-	-	-

[a] Characteristics of *L. acidophilus* as described in *Bergey's Manual of Determinative Bacteriology* 8th edition (Buchanan and Gibbons, 1974).

fermented arabinose, mannitol, melezitose, melibiose, rhamnose, sorbitol, or xylose. Strain RP32 was isolated from porcine intestine (4), strain NCFM-L was isolated from a culture of L. acidophilus originating from human intestinal contents (3).

Frozen Storage

Freezing and subsequent frozen storage at -196°C in liquid nitrogen had little or no effect on the viability of either strain of L. acidophilus (Table II). Although a significant decrease ($P<0.05$) in numbers of colony forming units on PMN agar was observed for strain RP32 following 1 day of storage the decrease was slight and probably not of practical importance. The populations on days 14 and 28 were not significantly different from those on day 0 for this culture. No significant differences ($P>0.05$) in PMNO agar counts among storage days were observed for L. acidophilus RP32. No significant differences among storage days for either media were observed for L. acidophilus NCFM-L during frozen storage.

The influence of frozen storage on the ability to assimilate cholesterol by the two strains was based on evaluation of nonfermented milk samples prepared immediately following thawing of the concentrated culture, prior to storage at 5°C. L. acidophilus RP32 assimilated more cholesterol from the broth medium in 12 h than strain NCFM-L assimilated in 14 h. No significant effect ($P>0.05$) on

TABLE II

VIABILITY OF CONCENTRATED CULTURES OF L. ACIDOPHILUS DURING STORAGE AT -196°C

Days at -196°C	Log_{10} CFU/g[a] of concentrated culture			
	L. acidophilus RP3		L. acidophilus NCFM-L	
	PMN[b]	PMNO[b]	PMN[b]	PMNO[b]
0	10.90[c]	10.86[c]	10.34[c]	10.35[c]
1	10.75[d]	10.74[c]	10.33[c]	10.29[c]
14	10.82[c]	10.80[c]	10.35[c]	10.29[c]
28	10.78[c]	10.80[c]	10.39[c]	10.33[c]

[a] Each value is the mean log_{10} CFU/g from 3 trials.

[b] PMN = pepsinized milk nutrient agar; PMNO = PMN + 0.1% oxgall.

[c] Values in the same column followed by different superscript letters differ significantly ($P<0.05$).

ability of either culture to assimilate cholesterol was observed over the 28 day frozen (-196°C) storage period (Table III). The data is presented as the amount (ug) of cholesterol assimilated during the tube assay.

Freezing and storage at -196°C also had no significant effect on the apparent bile resistance of either strain as measured by the ability of the cultures to grow in the MRS-OX-THIO-PPLO broth used in the cholesterol assay (Table IV). Thus, both strains are stable to frozen storage at -196°C for 28 days.

Refrigerated Storage

Statistical evaluation of the data showed no significant interaction ($P>0.05$) between frozen and refrigerated storage for any of the four parameters (i.e. ability to assimilate cholesterol, bile resistance, plate counts on PMN and PMNO agar) measured for either strain. However, the two strains did respond quite differently to refrigerated storage in nonfermented acidophilus milk.

Since there was no significant interaction between frozen and refrigerated storage for either strain, the data for refrigerated storage has been presented as the overall means from each frozen storage period for each strain. Initial populations in the nonfermented milks were similar for the two strains on both PMN and PMNO agar media. Refrigerated storage (5°C) had a significant effect ($P<0.05$) on the

TABLE III

STABILITY OF ABILITY OF L. ACIDOPHILUS TO ASSIMILATE CHOLESTEROL DURING STORAGE AT -196°C

Days at 196°C	Cholesterol assimilated[a] (µg/ml)	
	L. acidophilus RP32[b]	L. acidophilus NCFM-L[b]
0	88.03	66.96
1	87.10	59.98
14	90.93	65.27
28	88.80	61.86

[a] Each value is the mean µg assimilated from three trials; no significant differences among days of storage for either culture.

[b] Data for RP32 is from a 12 h incubation, data for NCFM-L is from a 14 h incubation.

TABLE IV

GROWTH OF L. ACIDOPHILUS IN ASSAY BROTH[a] AFTER STORAGE AT -196°C

Days at -196°C	A_{620nm}[b]	
	L. acidophilus RP32[c]	L. acidophilus NCFM-L[c]
0	.156	.177
1	.168	.166
14	.156	.134
28	.148	.121

[a] MRS-OX-THIO-PPLO broth.

[b] Each value is the mean A_{620nm} from 3 trials.

[c] Data for RP32 is from a 12 h incubations, data for NCFM-L is from a 14 h incubation; no significant differences among days of storage for either culture.

viability of both strains as measured on both PMN and PMNO agar (Table V). However, the reduction in numbers of viable lactobacilli was much greater for strain NCFM-L than for strain RP32.

The significant reductions in viable counts for strain NCFM-L were accompanied by significant decreases ($P<0.05$) in the ability of the culture to grow in the MRS-OX-THIO-PPLO assay broth over the 21 days of refrigerated storage in milk (Table VI). No significant differences were observed between day 0 and day 7 or between day 14 and day 21. However, a significant decrease ($P<0.05$) was observed between day 7 and day 14 of storage in the nonfermented acidophilus milk. Refrigerated storage in the milk had no effect on the ability of strain RP32 to grow in the assay medium.

During refrigerated storage, the stability of the ability of both strains to assimilate cholesterol followed the same pattern as their ability to grow in the assay medium (MRS-OX-THIO-PPLO broth) (Table VII). There was no significant difference in the ability of strain NCFM-L to assimilate cholesterol between day 0 and day 7 or between day 14 and day 21. However, a significant decrease ($P<0.05$) in ability to assimilate cholesterol occurred between day 7 and day 14. No significant decreases ($P>0.05$) in ability of strain RP32 to assimilate cholesterol were observed over the 21 day storage period.

TABLE V

VIABILITY OF L. ACIDOPHILUS IN NONFERMENTED ACIDOPHILUS MILK DURING STORAGE AT 5°C

Days at 5°C	Log_{10} CFU/ml[a]			
	L. acidophilus RP32		L. acidophilus NCFM-L	
	PMN[b]	PMNO[b]	PMN[b]	PMNO[b]
0	7.82[c]	7.81[c]	7.38[c]	7.35[c]
7	7.75[c,d]	7.76[c,d]	7.18[c]	7.09[c]
14	7.69[d,e]	7.70[d]	6.62[d]	6.36[d]
21	7.62[e]	7.59[e]	5.93[e]	5.65[e]

[a] Each value is the mean log_{10} CFU/ml averaged over all 4 frozen storage days from 3 trials.

[b] PMN = pepsinized milk nutrient agar; PMNO = PMN + 0.1% oxgall.

[c,d,e] Values in the same column followed by different superscript letters differ significantly ($P<0.05$).

TABLE VI

GROWTH OF L. ACIDOPHILUS IN ASSAY BROTH[a] AFTER STORAGE AT 5°C

Days at 5°C	A_{620nm}[b]	
	L. acidophilus RP32[c]	L. acidophilus NCFM-L[c]
0	.166[d]	.218[d]
7	.157[d]	.208[d]
14	.162[d]	.090[e]
21	.141[d]	.083[e]

[a] MRS-OX-THIO-PPLO broth.

[b] Each value is the mean A_{620nm} averaged over all 4 frozen storage days from 3 trials.

[c] Data for RP32 is from a 12 h incubation, data for NCFM-L is from a 14 h incubation.

[d,e] Values in the same column followed by different superscript letters differ significantly ($P<0.05$).

TABLE VII

STABILITY OF ABILITY OF L. ACIDOPHILUS TO ASSIMILATE CHOLESTEROL DURING STORAGE OF NONFERMENTED ACIDOPHILUS MILK AT 5°C

Days at 5°C	Cholesterol Assimilated (µg/ml)	
	L. acidophilus RP32	L. acidophilus NCFM-L
0	90.98[c]	83.34[c]
7	87.88[c]	92.87[c]
14	89.57[c]	46.33[d]
21	85.99[c]	31.52[d]

[a] Each value is the mean µg cholesterol assimilated averaged over all 4 frozen storage days from 3 trials.

[b] Data for RP32 is from a 12 h incubation, data for NCFM-L is from a 14 h incubation.

[c,d] Values in the same column followed by different superscript letters differ significantly ($P<0.05$).

Discussion

The two strains of L. acidophilus, RP32 and NCFM-L, used in this study were selected for their ability to assimilate cholesterol from a broth medium supplemented with bile under reduced conditions. The two strains were stored as concentrated cultures for 0, 1, 14, and 28 days at -196°C in liquid nitrogen with subsequent storage in nonfermented acidophilus milk for 0, 7, 14, and 21 days. In an earlier study, L. acidophilus RP32 inhibited significant increases in serum cholesterol levels in pigs on a high cholesterol diet (5). In that study, nonfermented acidophilus milk was prepared fresh each day. Thus, the stability of the culture and maintenance of its ability to assimilate cholesterol during refrigerated storage of the milk was not indicated.

The potential exists for a selected strain of L. acidophilus, consumed in a nonfermented acidophilus milk, to have a beneficial influence on serum cholesterol levels in humans. To be commercially feasible, cells of L. acidophilus in such a product would have to remain viable and able to assimilate cholesterol after refrigerated storage in milk prior to consumption. A strain of L. acidophilus used in such a product should remain viable and highly active for two to three weeks to permit adequate time for processing, distribution, and consumption of the milk.

Both strains evaluated in this study remained viable during storage at -196°C in liquid nitrogen. There were no significant decreases ($P>0.05$) in bile resistance or ability to assimilate cholesterol over the 28 day frozen storage period for either strain. Thus, these two strains have the potential for use as dietary adjuncts if used immediately following thawing and addition of the culture to make nonfermented acidophilus milk.

However, the two strains behaved quite differently during refrigerated storage in nonfermented acidophilus milk. While significant decreases in both total and bile resistant cells were observed for both strains, the decreases were much more pronounced for strain NCFM-L than for strain RP32. Declining populations of lactobacilli in a nonfermented acidophilus milk are undesirable as the decreased number of organisms ingested would lessen the chance of the organism to establish and function in the intestinal tract.

L. acidophilus NCFM-L also exhibited significant decreases in bile tolerance and ability to assimilate cholesterol during refrigerated storage. It may be assumed that decreased activity of the culture during refrigerated storage in nonfermented acidophilus milk would result in decreased activity in vivo. Maintenance of a high level of activity throughout the storage period would be desirable. Even though there was a significant reduction in CFU/ml, no

significant decreases in bile tolerance or ability to assimilate cholesterol were observed for strain RP32 during storage at 5°C.

The data provide evidence of a link between the ability of L. acidophilus to grow in a medium supplemented with bile and the ability to assimilate cholesterol. A concentration of 0.1% oxgall was necessary in an earlier study for cholesterol uptake by L. acidophilus (5). The broth medium used in the present study was supplemented with bile and had a low oxidation-reduction potential, two conditions that would occur in the intestinal tract. The ability of a strain to grow under these conditions is an important factor when screening cultures for use as dietary adjuncts.

The variation between strains with respect to stability during refrigerated storage accentuates the need for careful evaluation and selection of strains of L. acidophilus for use as dietary adjuncts. If such cultures are to be beneficial in controlling serum cholesterol, the selected strain must be able to assimilate cholesterol under conditions found in the intestinal tract. Thus, the strain must maintain the ability to assimilate cholesterol, bile resistance, and viability during production and storage of the product to which it is added as a dietary adjunct. In the present study, L. acidophilus NCFM-L was much more sensitive to the stress of storage at refrigeration temperatures than was L. acidophilus RP32. Thus, strain RP32

appears to be the best candidate for use as a dietary adjunct to aid in the control of serum cholesterol. However, strain RP32 is of porcine intestinal origin and since L. acidophilus exhibits host species specificity (8) it would not be a candidate strain for use in human diets. Further research should be conducted with strains of human intestinal origin to find a strain more suitable for use as a dietary adjunct, to be used in efforts to aid in control of serum cholesterol levels.

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CHAPTER IV

Summary and Conclusions

One of the current concerns in nutrition research is the effect of dietary manipulation on the lowering of serum cholesterol. Elevated serum cholesterol levels have been linked to a high incidence of coronary heart disease. Consuming cells of Lactobacillus acidophilus capable of assimilating cholesterol may be a means of helping lower serum cholesterol levels in hypercholesterolemic individuals. The purpose of this study was to evaluate the stability of strains of L. acidophilus under the conditions of frozen and refrigerated storage used in the commercial manufacture of nonfermented acidophilus milk.

The two strains used in this study were confirmed to be L. acidophilus. Initial evaluation of cholesterol uptake by the two strains during growth in a broth medium indicated a 12 hour incubation time for strain RP32 and a 14 hour incubation time for strain NCFM-L allowed for maximum uptake of cholesterol.

Frozen storage (-196°C) of the concentrated cultures for 28 days had little or no effect on the bile resistance or viability of either strain. Also, no significant decrease in the ability to assimilate

cholesterol was observed during frozen storage for either culture.

Numbers of total and bile resistant lactobacilli decreased significantly during 3 weeks of refrigerated (5°C) storage in nonfermented acidophilus milk for both strains. However, the decreases were much less for strain RP32 than for strain NCFM-L. L. acidophilus RP32 remained stable with respect to bile resistance (based on growth in the assay broth containing 0.3% oxgall) and ability to assimilate cholesterol during refrigerated storage. Strain NCFM-L exhibited significant decreases in both bile resistance and the ability to assimilate cholesterol during refrigerated storage.

L. acidophilus RP32 exhibited much better viability and activity after frozen and subsequent refrigerated storage than did L. acidophilus NCFM-L. Thus, strain RP32 might be considered a better choice for use as a dietary adjunct for humans. However, since RP32 is of porcine intestinal origin and cells of L. acidophilus are host species specific, it would probably not be useful as a dietary adjunct for humans. In this study, strain NCFM-L, of human origin, did not retain sufficient viability, bile resistance, or ability to assimilate cholesterol during storage at refrigeration temperatures to be used commercially. Further research should be conducted with other strains of L. acidophilus of human intestinal origin to identify a

strain suitable for production of a nonfermented acidophilus milk for consumption by hypercholesterolemic individuals.

APPENDIXES

APPENDIX A

TABLES OF MEANS

TABLE VIII

CHOLESTEROL ASSIMILATION[a] BY L. ACIDOPHILUS RP32 AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Refrigeration (days)				Great[c] Means
	0	7	14	21	
0	81.3[b]	86.3	101.6	83.0	88.1
1	94.3	81.9	87.0	83.3	86.6
14	104.5	89.2	86.6	83.4	90.9
28	83.9	94.1	84.2	93.3	88.9
Great[d] Means	91.0	87.9	89.9	85.8	88.6[e]

[a] Expressed as μg cholesterol removed from the assay medium.

[b] Each value is the average from three trials.

[c] Each value is the average from 12 observations across refrigeration.

[d] Each value is the average from 12 observations across freezing.

[e] Overall mean.

TABLE IX

GROWTH[b] OF L. ACIDOPHILUS RP32[a] IN ASSAY BROTH[b] AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Refrigeration (days)				Great[d] Means
	0	7	14	21	
0	.129[c]	.137	.195	.161	.156
1	.178	.175	.180	.125	.165
14	.197	.162	.134	.133	.157
28	.160	.156	.139	.138	.148
Great[e] Means	.166	.158	.162	.139	.157[f]

[a] Expressed as A_{620nm}.

[b] MRS-OX-THIO-PPLO broth.

[c] Each value is the average from three trials.

[d] Each value is the average from 12 observations across refrigeration.

[e] Each value is the average from 12 observations across freezing.

[f] Overall mean.

TABLE X

VIABILITY OF L. ACIDOPHILUS RP32 BASED ON COUNTS ON PMN AND PMNO AGARS AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Media	Refrigeration (days) 0	7	14	21	Great[b] Means
0	PMN	7.83[a]	7.81	7.69	7.64	7.74
	PMNO	7.78	7.79	7.69	7.59	7.71
1	PMN	7.78	7.69	7.63	7.51	7.65
	PMNO	7.77	7.70	7.66	7.50	7.66
14	PMN	7.81	7.74	7.74	7.73	7.76
	PMNO	7.83	7.78	7.70	7.67	7.75
28	PMN	7.84	7.75	7.71	7.57	7.72
	PMNO	7.84	7.77	7.70	7.56	7.72
Great[c] Means	PMN	7.82	7.75	7.69	7.61	7.72[d]
	PMNO	7.81	7.76	7.69	7.58	7.71[d]

[a] Each value is the average $\log_{10}$ CFU/ml from three trials.

[b] Each value is the average from 12 observations across refrigeration.

[c] Each value is the average from 12 observations across freezing.

[d] Overall mean.

TABLE XI

CHOLESTEROL ASSIMILATION BY L. ACIDOPHILUS NCFM-L[a] AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Refrigeration (days)				Great[c] Means
	0	7	14	21	
0	86.8[b]	103.3	43.2	34.6	67.0
1	65.5	92.3	52.0	30.1	60.0
14	90.6	83.9	59.0	27.6	65.3
28	90.5	92.0	31.1	24.2	59.5
Great[d] Means	83.4	92.9	46.3	29.1	62.9[e]

[a] Expressed as μg cholesterol removed from the assay medium.

[b] Each value is the average from three trials.

[c] Each value is the average from 12 observations across refrigeration.

[d] Each value is the average from 12 observations across freezing.

[e] Overall mean.

TABLE XII

GROWTH[a] OF L. ACIDOPHILUS NCFM-L IN ASSAY BROTH[b] AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Refrigeration (days)				Great[d] Means
	0	7	14	21	
0	.280[c]	.269	.087	.072	.177
1	.261	.180	.143	.082	.167
14	.190	.200	.069	.075	.133
28	.139	.181	.061	.102	.121
Great[e] Means	.218	.298	.090	.083	.150[f]

[a] Expressed as A_{620nm}.

[b] MRS-OX-THIO-PPLO broth.

[c] Each value is the average from three trials.

[d] Each value is the average from 12 observations across refrigeration.

[e] Each value is the average from 12 observations across freezing.

[f] Overall mean.

TABLE XIII

VIABILITY OF L. ACIDOPHILUS NCFM-L BASED ON COUNTS ON PMN AND PMNO AGARS AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Freezing (days)	Media	Refrigeration (days)				Great[b] Means
		0	7	14	21	
0	PMN	7.36[a]	7.28	6.49	5.37	6.63
	PMNO	7.30	7.13	6.33	4.97	6.43
1	PMN	7.39	7.21	6.68	6.54	6.96
	PMNO	7.36	7.15	6.08	6.35	6.74
14	PMN	7.35	7.34	6.64	6.23	6.89
	PMNO	7.35	7.25	6.52	6.02	6.79
28	PMN	7.41	6.89	6.69	5.58	6.64
	PMNO	7.36	6.80	6.50	5.27	6.48
Great[c] Means	PMN	7.38	7.18	6.63	5.93	6.78[d]
	PMNO	7.34	7.08	6.36	5.65	6.61[d]

[a] Each value is the average $\log_{10}$ CFU/ml from three trials.

[b] Each value is the average from 12 observations across refrigeration.

[c] Each value is the average from 12 observations across freezing.

[d] Overall mean.

APPENDIX B

ANALYSIS OF VARIANCE TABLES

TABLE XIV

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE THE INFLUENCE OF FREEZING AND SUBSEQUENT REFRIGERATION ON ASSIMILATION OF CHOLESTEROL BY L. ACIDOPHILUS RP32

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	46	18051.33		
Among Freezing				
Trial	2	4795.71	5.04	0.0519
Freezing (F)	3	65.83	0.05	0.9856
Error (a)	6	2852.58		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	166.87	0.16	0.9252
F x R	9	1931.21	0.60	0.7843
Error (b)	23	8239.13		

TABLE XV

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE GROWTH OF L. ACIDOPHILUS RP32 IN MRS-OX-THIO-PPLO BROTH AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	46	0.095		
Among Freezing				
Trial	2	0.033	12.03	0.0079
Freezing (F)	3	0.003	0.75	0.5619
Error (a)	6	0.008		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.003	0.63	0.6053
F x R	9	0.016	1.32	0.2815
Error (b)	23	0.031		

TABLE XVI

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY OF L. ACIDOPHILUS RP32 BASED ON COUNTS ON PMN AGAR AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	46	0.939		
Among Freezing				
Trial	2	0.351	66.33	0.0001
Freezing (F)	3	0.049	6.13	0.0294
Error (a)	6	0.016		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.230	7.10	0.0015
F x R	9	0.045	0.47	0.8819
Error (b)	23	0.248		

TABLE XVII

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY OF L. ACIDOPHILUS RP32 BASED ON COUNTS ON PMNO AGAR AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	46	1.132		
Among Freezing				
Trial	2	0.485	18.49	0.0027
Freezing (F)	3	0.035	0.90	0.4937
Error (a)	6	0.079		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.296	10.45	0.0002
F x R	9	0.020	0.47	0.9851
Error (b)	23	0.217		

TABLE XVIII

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE THE INFLUENCE OF FREEZING AND SUBSEQUENT REFRIGERATION ON ASSIMILATION OF CHOLESTEROL BY L. ACIDOPHILUS NCFM-L

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	47	89780.86		
Among Freezing				
Trial	2	21003.03	5.49	0.0441
Freezing (F)	3	362.41	0.06	0.9774
Error (a)	6	11469.75		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	03893.95	10.68	0.0001
F x R	9	2910.81	0.34	0.9540
Error (b)	24	23140.91		

TABLE XIX

ANALYSIS OF VARIANCE ON DATA FROM TRIALS TO EVALUATE GROWTH OF L. ACIDOPHILUS NCFM-L IN MRS-OX-THIO-PPLO BROTH AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	47	0.470		
Among Freezing				
Trial	2	0.076	4.05	0.0770
Freezing (F)	3	0.025	0.89	0.4969
Error (a)	6	0.056		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	0.192	19.76	0.0001
F x R	9	0.043	1.46	0.2193
Error (b)	24	0.078		

TABLE XX

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY OF L. ACIDOPHILUS NCFM-L BASED ON COUNTS ON PMN AGAR AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	45	31.85		
Among Freezing				
Trial	2	5.23	10.07	0.0121
Freezing (F)	3	1.54	1.98	0.2186
Error (a)	6	1.56		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	13.74	13.48	0.0001
F x R	9	2.30	0.75	0.6583
Error (b)	22	7.47		

TABLE XXI

ANALYSIS OF VARIANCE ON DATA FROM TRIALS EVALUATING THE VIABILITY OF L. ACIDOPHILUS NCFM-L BASED ON COUNTS ON PMNO AGAR AFTER FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Source of Variation	Degrees of Freedom	Sum of Squares	F	P>F
Corrected Total	45	43.81		
Among Freezing				
Trial	2	8.50	15.52	0.0043
Freezing (F)	3	1.80	2.19	0.1899
Error (a)	6	1.64		
Among Refrigeration Within Freezing				
Refrigeration (R)	3	18.94	15.01	0.0001
F x R	9	3.67	0.97	0.4890
Error (b)	22	9.25		

VITA

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Master of Science

Thesis: EFFECT OF FROZEN (-196°C) AND SUBSEQUENT REFRIGERATED (5°C) STORAGE ON ASSIMILATION OF CHOLESTEROL BY LACTOBACILLUS ACIDOPHILUS

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