DEVELOPMENT OF A WHEY BASED MEDIUM FOR PREPARING CONCENTRATED CULTURES OF *LACTOBACILLUS ACIDOPHILUS* TO BE USED AS DIETARY ADJUNCTS

By

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CHAPTER I

INTRODUCTION

Currently there is much interest in the use of Lactobacillus acidophilus as a dietary adjunct. Cells of L. acidophilus, usually added to refrigerated low-fat milk in the form of a concentrated culture, reportedly can help maintain intestinal health and improve intestinal function. The availability of non-fermented milk containing cells of L. acidophilus has been made possible by the commercial development of frozen concentrated cultures. One of the most important considerations in preparing a frozen concentrated culture is the growth medium used to grow the cells. For L. acidophilus the growth medium should permit maximum growth of the bacterial cells and produce cells that will retain viability and their desirable characteristics during frozen storage of the resulting concentrated cultures. These characteristics should also be maintained during refrigerated storage of the milk containing these cells.

This thesis study was undertaken to develop a whey based medium for growing cell crops of L. acidophilus to be used in the preparation of frozen concentrated cultures.

In these experiments, three different levels of dried sweet whey solids (2.5%, 5.0%, or 7.5%) were used in the preparation of pepsinized whey media for growing cells of L.

acidophilus NCFM. Concentrated cultures were prepared using the cells and frozen at -196 C in liquid nitrogen. The storage stability of these concentrated cultures during storage at -196 C and their subsequent survival in milk at 5 C were evaluated.

CHAPTER II

LITERATURE REVIEW

Bases for Use of *Lactobacillus acidophilus* as a Dietary Adjunct

Soured milks such as yogurt and kefir were used as therapeutic products in Europe and Asia long before the existence of bacteria was known (Hawley et al., 1959). Metchnikoff (1908) believed that life could be prolonged by the ingestion of soured milk containing cells of *Lactobacillus bulgaricus*. He felt that these lactic acid bacteria could reduce the toxic effects of undesirable bacteria in the human intestine. Cheplin and Rettger (1920, 1921), however, were unsuccessful in their attempts to implant *L. bulgaricus* in the intestine, but found that *L. acidophilus* would survive implantation. Later researchers also reported the successful implantation of *L. acidophilus* in the human intestine (Kopeloff, 1926; Myers, 1931; Morey, 1953; Gilliland, et al., 1978).

Metchnikoff's early writings led to the development of acidophilus milk. This early acidophilus milk was a fermented product (Kopeloff, 1926) and very unpalatable due to its extreme cooked flavor and high acidity (Prouty and Bendixin, 1932). Speck (1975a) said the early product was

primarily used for medicinal purposes and the cells of L. acidophilus rapidly lost their viability during refrigerated storage. Speck (1980) further reported that the early acidophilus milk also did not always contain intestinal strains of L. acidophilus.

Prouty and Bendixin (1932) and Kopeloff (1934) found that the viability of L. acidophilus in fermented milk was higher when stored at room temperature than at refrigerated temperatures. Black (1931) and Black and Harris (1931), however, reported that less reduction in numbers of L. acidophilus occurred at 0-9 C than at 37 C. He attributed the reduced viability of L. acidophilus at room temperature to its high acidity. Kulp (1931) showed that commercial acidophilus milk could be stored at 5 C for 2 days to 1 week without serious damage to the product.

The benefits of consuming acidophilus milk have been widely researched for many years. Acidophilus milk has been suggested to treat a variety of intestinal disorders such as constipation and diarrhea (Rettger, 1929; Stark et al., 1934; Frost et al., 1931; Morey, 1953). Others have shown the benefits of using intestinal lactobacilli to replace bacterial flora destroyed after the prolonged oral use of antibiotics (Morey, 1953; Gillespie et al., 1956; Speck, 1975b). Vincent et al. (1959) and Hosono (1977) reported that L. acidophilus produces an antibiotic-like substance that has anti-bacterial properties.

Goldin and Gorbach (1980) conducted a study involving

rats to determine the effect of dietary supplements of L. acidophilus on chemically induced cancer. They found that these supplements delayed the developement of colon cancer in the test animals exposed to the carcinogen. Goldin et al. (1980) also did a study on the effect of supplements of L. acidophilus on humans and suggested that the metabolic activities of the fecal bacteria could be favorably altered by these supplements.

Acidophilus milk has also been suggested as a source of the enzyme B-galactosidase to aid lactose-intolerant patients (Sandine, 1979). Gilliland and Kim (1981) fed non-fermented milk containing viable cells of L. acidophilus to humans who were "lactose malabsorbers." Lactose malabsorbers can not normally hydrolyze lactose in the small intestine due to the absence of lactase. In these people the lactose passes into the large intestine where it is fermented. Hydrogen is produced in the fermentation and is expelled in the breath of the person. The concentration of hydrogen in the breath is an indication of the severity of lactose malabsorption. The test subjects exhibited significantly lowered levels of hydrogen in their breath after consuming milk containing cells of L. acidophilus as compared to that after consuming milk without L. acidophilus. Thus the organism appears to be beneficial for people who cannot utilize lactose.

Successful acidophilus therapy depends upon the ingestion of large numbers of viable cells of L. acidophilus

(Kopeloff, 1934; Hawley et al., 1959). These strains of L. acidophilus should be readily implantable in the human intestine (Frost et al., 1931; Myers, 1931; Gillespie et al., 1956). The culture of L. acidophilus must also maintain its viability and bile resistance during preparation of the milk (Gilleland and Speck, 1977), and it must be viable and bile resistant at the time of consumption (Gilliland, 1979).

Other Products as Sources of Lactobacillus Acidophilus

Products other than milk were suggested as carriers of L acidophilus, but the cells did not maintain viability during storage (Speck, 1980). Prouty and Bendixin (1932) added L. acidophilus to a frozen sherbet mix, and Gilliland and Speck (1977a) stored cells of L. acidophilus in yogurt. The viability of the cells of L. acidophilus was not satisfactorily maintained in either product.

Through the years many inferior products have appeared on the market. Rettger (1929) found that few so-called acidophilus products on the market contained living cells of L. acidophilus, and a survey by Gillespie et al. (1956) produced similar results. In a more recent survey of commercial acidophilus products, Gilliland and Speck (1977b) also reported that few products sold in "health food stores" as sources of L. acidophilus tested in their study contained viable cells of the organism.

Unfermented Acidophilus Milk

The low number of viable L. acidophilus in fermented milk and its unpleasant flavor led to the development of an unfermented acidophilus milk. Myers (1931) added large numbers of viable cells of L. acidophilus which had been grown in broth to milk and found that they would implant in the human intestine. Duggan et al. (1959) prepared concentrated cultures of L. acidophilus to be added to pasteurized milk just prior to consumption. In 1975 a low fat non-fermented milk product containing a human intestinal strai of L. acidophilus was made available commercially (Speck 1980). The product is prepared by adding a concentrated culture of L. acidophilus to pasteurized low fat milk. This milk contained several million viable and bile resistant cells of L. acidophilus per ml., and the flavor was the same as ordinary low-fat milk. Gilliland et al. (1978) evaluated the qualities of such an unfermented low-fat milk to which a commercially available concentrated culture of L. acidophilus was added following pasteurization. The flavor of the milk was unaltered by the culture, and consumption of the milk by a group of human volunteers resulted in significantly increased numbers of facultative lactobacilli in their feces.

Frozen Concentrated Cultures

The availability of frozen concentrated cultures of lactic bacteria that can be added directly to the milk has

eliminated the need for daily transfer of starter cultures (Lawrence, 1976). Gilliland and Speck (1974) described the general procedure for producing frozen concentrated cultures. The starter culture bacteria are grown in a sterile liquid medium under carefully controlled conditions, concentrated, and placed in frozen storage at the desired temperature. They further indicated that the growth medium used to grow the cell crop can affect the subsequent survival of the cells after freezing and thawing. The development of technology for commercially producing frozen concentrated cultures has made it feasible to provide the consumer with a pleasant tasting nonfermented milk containing cells of L. acidophilus (Gilliland et al., 1978).

Media for Producing Cell Crops of Lactobacilli

There have been many growth media suggested for the propagation of lactobacilli. Duggan et al. (1959) used a whey based medium in the preparation of concentrated cultures of L. acidophilus. The medium contained 7.0% "deionized" whey powder, 2.0% powdered casein digest, 0.1% monobasic potassium phosphate, and 0.5% sodium acetate. The pH of the mixture was adjusted to 7.0 prior to sterilization. The sterilized broth was translucent with no precipitate. High populations of L. acidophilus were attained during growth, and the cells were easily harvested by centrifugation. One disadvantage of the medium would be the high cost of some of the ingredients.

Other media used to cultivate lactobacilli were APT broth (Evans and Niven, 1951), lactobacilli MRS broth (de Man et al., 1960), and a calcium supplemented MRS broth (Wright and Klaenhammer, 1981). Smittle et al. (1972) grew cells of L. bulgaricus in a broth containing tryptone, yeast extract, lactose, and Tween 80. The major disadvantage of these media would be their high cost. This cost makes them ecoomically impractible to be used in the commercial production of cells of L. acidophilus for preparing concentrated cultures. Lawrence (1976) also noted the limited buffering capacities of most semi-synthetic media.

Smith (1943) and Jakubowska et al. (1978) reported that yeast extract was an essential ingredient in the growth medium. Braz and Allen (1939) and Speck et al. (1958) indicated that yeast extract in the growth media stimulated the growth of lactobacilli. Speck suggested that the yeast extract provided preformed peptides required by the lactobacilli for most rapid growth.

Certain ingredients in the growth media may affect the stability of cells of L. acidophilus during frozen storage. Smittle et al. (1972, 1974) observed that Tween 80 in the growth medium could increase the survival of cells of L. bulgaricus during freezing and storage in liquid nitrogen. Goldberg and Eschar (1977) later showed that Tween 80 in the growth medium could also improve the survival of cells of L. acidophilus during storage at -196 C.

Maintaining the pH of the medium at a favorable level

during the growth of starter bacteria has been shown to increase the numbers of cells produced. Rogers and Whittier (1928) attained higher bacterial populations when the acid produced by the culture was neutralized than when the acid was not neutralized. When growing cultures of L. acidophilus, Longsworth and MacInnes (1936) found that automatic pH control resulted in a four-fold increase in the cell crop.

Suspending Menstrum for Cells

Another factor that can affect a culture's survival during freezing, storage, and thawing is the menstrum in which the bacterial cells are suspended prior to freezing. Briggs (1955) felt that the most important factor affecting survival rate during frozen storage was the suspending medium. The recommended suspending medium for lactic cultures is skim milk (Gibson et al., 1966; Keogh, 1970). They found that the addition of cryoprotective agents to skim milk was unnecessary when cells of lactic streptococci were frozen and stored in liquid nitrogen.

Many researchers have tried to improve the survival of lactobacilli during frozen storage by adding cryoprotective agents to the suspending menstrum. Morichi et al. (1963) found that the addition of glutamic acid did not adequately protect cells of L. bulgaricus during freeze drying. Malkk et al. (1970) suspended cells of L. acidophilus in a medium consisting of 9 parts non-fat milk and 1 part glycerol.

They adjusted the pH to 6.8 prior to freezing at -30 C. More cells curvived in the milk-glycerol medium than in non-fat milk alone.

Concentrated cultures of L. casei were frozen in skim milk at -70 C and stored at -30 C (Jabukowski et al., 1978). Only a slight decrease in viability was observed after 12 weeks of storage. Smittle et al. (1972) used monosodium glutamate, glycerol, and Tween 80 as cryoprotective agents when freezing concentrated cultures of L. bulgaricus in liquid nitrogen. None of the additives offered any significant improvement in survival of cells of L. bulgaricus during frozen storage over skim milk alone.

Storage Temperature

There are varied opinions on the optimum temperature for frozen storage of concentrated cultures of lactic acid bacteria. In a review article, Lawrence (1976) indicated that the rate of freezing and temperature of storage were very important in the survival of frozen concentrated starter cultures. Storage in liquid nitrogen (-196 C) results in the greatest survival of viable cells or biological activity (Gibson et al., 1966; Gilliland et al., 1970; Johannsen, 1972; Keogh, 1970; Smittle et al., 1972). Cowman and Speck (1963) reported that storage at 196 C resulted in the highest enzymatic activity for lactic streptococci after frozen storage, and they later also showed that viability, acid production, and proteinase activity were much higher for

cells stored in liquid nitrogen at 196 C than in a freezer at 20 C (Cowman and Speck, 1965).

Evaluation of Concentrated Cultures

Several media have been used to determine the numbers of lactobacilli in concentrated cultures of L. acidophilus. Rogosa et al. (1951) developed a selective medium (Lactobacillus Selective Agar, Baltimore Biological Laboratories, Cockeysville, MD) to enumerate lactobacilli and de Man et al. (1960) formulated a non-selective medium (Lactobacilli MRS broth, Difco Laboratores, Detroit, Mich.) which has been modified by incorporating agar for enumerating lactobacilli.

In order for a culture to be useful as a dietary adjunct to benefit the intestinal flora, it must be able to survive and grow in the human intestine. Thus it has been assumed that if cultures of L. acidophilus are to be used in such a manner, the cells must be able to grow in the presence of bile (i.e., be bile resistant). Bile acids can determine the types of bacteria that will grow in the intestinal tract of man (Gilliland and Speck, 1977b).

Gilliland and Speck (1977b) also reported that non-intestinal lactobacilli will not grow on Lactobacillus Selective Agar (LBS) containing 0.15% oxgall (LBSO-.15). Strains of lactobacilli from the intestine, however, did grow on LBSO-.15. Since intestinal strains of lactobacilli will grow well on both LBS and LBSO agars, LBS agar containing oxgall can be used to enumerate the numbers of

bile-resistant lactobacilli. Cultures of lactobacilli that will not maintain their bile resistance during storage presumably would be less suitable than bile resistant ones for use in acidophilus products. Wright and Klaenhammer (1981) used MRS agar plus 0.15% oxgall to quantitatively evaluate the numbers of bile resistant cells of L. acidophilus after storage in liquid nitrogen.

CHAPTER III

EXPERIMENTAL PROCEDURE

Source and Maintenance of Culture

Lactobacillus acidophilus NCFM (of human origin) was obtained from the stock culture collection in the Dairy Food Microbiology Lab at Oklahoma State University. The strain was propagated in sterile 10% non-fat milk solids (NFMS) using a 1.0% inoculum and incubation at 37 C for 18 hours. The culture was stored at 5 C between transfers.

Identification of Culture

The procedure described by Martin (1979) was used to confirm the identity of the culture. The following tests were done: Gram stain, catalase test, the ability to deaminate arginine, to hydrolyze esculin, and to ferment amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. The ability of the culture to grow at 15 C and 45 C was also tested. Two tubes of MRS broth were inoculated using a flame sterilized inoculating loop. One tube was incubated at 15 C for 1 week and the other tube was incubated at 45 C for 24 hr. Visual turbidity was a positive test for growth.

Determination of Populations of Lactobacilli

Serial dilutions were prepared with 99-ml. dilution blanks composed of 0.1% peptone (Difco Laboratories, Detroit, Mich.) and 0.001% Antifoam A Emulsion (Sigma Chemical Company, St. Louis, MO) in distilled water. The dilution blanks were sterilized by heating 15 minutes at 121 C. Initial dilutions (1:100) for evaluation of concentrated cultures were prepared by aseptically adding 1 g of the thawed concentrated culture to a sterile 99-ml. dilution blank. Broth cultures were measured volumetrically for preparing the initial dilutions. All subsequent dilutions in this study were made according to the procedures described in the Compendium of Methods for the Microbiological Examination of Foods (Speck, 1976).

Total populations of lactobacilli were determined by a pour plate procedure. Plates containing the appropriate dilutions were poured with melted MRS agar at 45 C. MRS agar was prepared by dissolving 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.) in MRS broth prior to autoclaving. Numbers of bile resistant organisms were enumerated using a pour plate procedure with MRSO agar prior to sterilization. MRSO agar was prepared by adding 0.1% oxgall (Baltimore Biological Laboratories, Cockeysville, MD) to MRS agar. Duplicate plates for each dilution were prepared with each medium in all experiments. After solidification, the plates were inverted and incubated at 37 C for 48 hours.

All colonies visible with the aid of a Quebec Colony Counter were counted.

Preparation of Whey Base Media

Dried sweet whey (Associated Milk Producers Incorporated, Tulsa, OK) was reconstituted at the desired concentration (2.5%, 5.0% or 7.5%) in 4 liters of distilled water containing 0.1% Tween 80 (Sigma Chemical Company, St. Louis, MO). The pH was adjusted to 3.0 with 5N phosphoric acid, and pepsin (Sigma Chemical Company, St. Louis, MO) was added to the mixture in the amount of 1 g/4,000 ml. This mixture was incubated in a 37 C water bath for 30 minutes. Afterward the digested whey was adjusted to pH 7.0 with 5 N ammonium hydroxide. One tenth percent yeast extract (Baltimore Biological Laboratories, Cockeysville, MD) and 1% thiotone (Baltimore Biological Laboratories, Cockeysville, MD) were added to the mixture. After dissolving the added ingredients the medium was equally divided into two 4-liter erlenmeyer flasks. It was then autoclaved for 15 minutes at 121 C. Ingredients for the three whey media are listed in Table 1.

TABLE I

COMPOSITION OF PEPSINIZED WHEY BASED MEDIA

Medium	Ingredients (percent)				
	Dried Whey Solids	Tween 80	Pepsin	Yeast Extract	Thiotone
1	2.5	0.1	0.025	0.1	1.0
2	5.0	0.1	0.025	0.1	1.0
3	7.5	0.1	0.025	0.1	1.0

Preparation of Neutralizer

Sixty g of Na_2CO_3 was dissolved in 200 ml. of distilled water and autoclaved for 15 minutes at 121 C. After autoclaving, the mixture was cooled to room temperature, and 100 ml. of concentrated NH_4OH was added. This provided a solution of 20% Na_2CO_3 in 20% NH_4OH. The neutralizer was placed in the reservoir of the automatic pH controller (New Brunswick Scientific Co., Edison, N.J.)

Growth in Whey Media

Cell crops of L. acidophilus were grown in a 6-liter fermentor jar equipped with a conbination pH electrode (New Brunswick Scientific Co., Edison N.J.). The fermentor was used in conjunction with a New Brunswick Automatic pH controller. The head of the fermentor contained ports for sampling, inoculation, neutralization, and an electrode. A self-contained stirrer provided agitation, and the jar was equipped with baffles to reduce foaming. Three liters of distilled water were added to the jar, and the entire assembly was autoclaved for 45 minutes at 121 C. The water was discarded prior to using the fermentor.

Four liters of sterile pepsinized whey was aseptically added to the empty sterile fermentor jar. The temperature of the whey was adjusted to 37 C and the automatic pH controller was set to maintain the growth medium at pH 6.0. The whey medium was then inoculated with an 18-hour milk culture (37C) of L. acidophilus NCFM at a level of 1% and

incubated for 24 hours at 37 C with moderate agitation. The medium was maintained at pH 6.0 during growth of the culture with a neutralizer consisting of 20% Na_2CO_3 in 20% NH_4OH.

Ten ml. samples of the culture were aseptically removed from the fermentor using sterile 10-ml. pipettes and placed in an ice-water bath. These samples were taken at two hour intervals beginning at 10 hours of incubation and continuing to 24 hours. The numbers of lactobacilli in the samples were determined by plating the samples on MRS and MRSO agars.

Preparation and Freezing of Concentrated Cultures

The cell crop was harvested when the culture of L. acidophilus reached the late exponential phase of growth in the whey medium at pH 6.0. This time was based on growth curves determined in twelve preliminary trials in which the culture of L. acidophilus was grown in the three whey media at pH 6.0. Maximum population of L. acidophilus NCFM under these conditions was reached after 14 to 18 hours of incubation at 37 C.

Eight hundred ml. of the culture was aseptically removed from the fermentor into a sterile flask. The cells were harvested by centrifugation at 4,080 x g for 10 minutes at 0 C in a Sorvall model 5C-5 Superspeed Refrigerated Centrifuge (DuPont Company, Newton, CT). The supernatant fluid was discarded and the cells were resuspended in twice their weight of cold sterile 10% NFMS. Resuspension was

aided by adding sterile glass beads (0.3 cm diameter) with the milk, and the contents were swirled until a uniform suspension was attained. The resuspended cells were then aseptically dispensed in 2-gram quantities into sterile 2-ml. polytheylene screw cap freezing vials (Dynatech Laboratories, Inc., Alexandria, VA). Care was taken to keep the culture cold during these operations. The concentrated cultures were frozen and stored in liquid nitrogen (-196 C).

Evaluation of Concentrated Cultures

Numbers of lactobacilli were determined prior to freezing (Day 0) and after 1, 14 and 28 days of storage in liquid nitrogen by plating on MRS and MRSO agar. The vials of frozen concentrated cultures were thawed by submerging them in one liter of tap water at 30 C for 5 minutes. The exteriors of the vials were sanitized by dipping them in ethanol prior to opening. Excess ethanol was wiped from the vials with a Kim Mark Wiper (Kimberly-Clark Corporation, Neenah, WI).

Subsequent Survival of Cells from Concentrated Cultures in Refrigerated Milk

The milk was prepared by equally dividing 400 ml. of 10% reconstituted NFMS into four milk dilution bottles and heating at 100 C for 30 minutes. The bottles of heated milk were then cooled at 5 C.

One gram of the thawed concentrated culture was aseptically placed in 9 ml. of sterile 10% NFMS. Two tenths of this 1:10 dilution was then added to each of the four bottles containing the cold 10% NFMS to achieve a population of about $2x10^6$/ml. The bottles were stored at 5 C. Bottles were removed for microbiological examination on Days 1, 7, 14 and 21. The total numbers of lactobacilli and bile resistant lactobacilli were determined.

Sampling Scheme

The sampling scheme for monitoring growth of the organism in the whey based media and for evaluating the concentrated cultures is outlined in Figure 1. Six trials were conducted using the media containing 2.5% and 5.0% whey solids. There were 4 trials using the medium containing 7.5% whey solids.

Statistical Analyses

The effect of different levels of whey solids in the growth media on the populations of L. acidophilus at pH 6.0 and the bile resistance of the cells was studied using a split-split plot design. Split-split plot designs were also used to determine the effect of different levels of whey solids in the growth media on the bile resistance and storage stability of cells of L. acidophilus during frozen and refrigerated storage.

Statistical analyses were done to compare the media containing 2.5% and 5.0% whey solids. The methods for these analyses are outlined in Statistical Methods (Snedecor and Cochran, 1967).

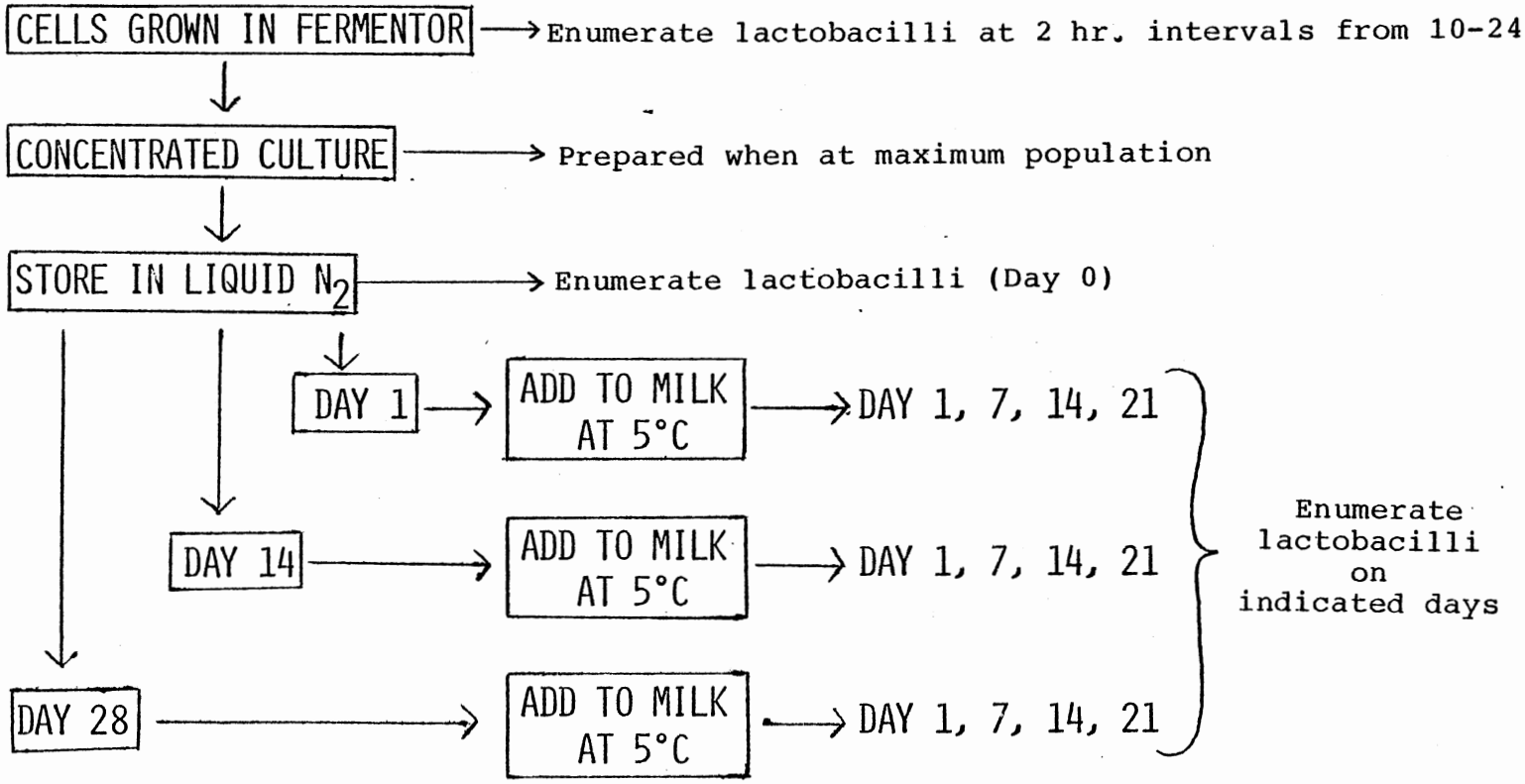

Figure 1. Flow Chart for Evaluating Stability of Lactobacillus acidophilus in Frozen and Refrigerated Storage

CHAPTER IV

RESULTS

Confirmation of Identity of Culture

The results of tests to confirm the identity of Lactobacillus acidophilus NCFM used in this project are presented in Table II. The characteristics of the culture matched those of L. acidophilus as described in Bergey's Manual of Determinative Bacteriology, 8th Edition (Buchanan, 1974).

Growth of Lactobacillus in Whey Media at pH 6.0

Considerable variation was observed in the growth of the culture of L. acidophilus in the media containing different levels of whey solids. Table III shows the growth of L. acidophilus in the whey media at pH 6.0. The values are presented as $\log_{10}$ count/ml. Each value for the media containing 2.5% and 5.0% whey solids represents the average count obtained in 6 trials. For the medium containing 7.5% whey solids, each value represents the average from 4 trials. The $\log_{10}$ of the highest population based on counts on MRS agar was 9.0 and was attained in the medium containing 2.5% whey solids. The second highest numbers of lactobacilli ($\log_{10}$ = 8.81) was observed in the medium containing 5.0%

TABLE II

BIOLOGICAL CHARACTERISTICS OF CULTURE OF *L. ACIDOPHILUS*

Test	*Lactobacillus acidophilus* NCFM	*acidophilus* Bergey's[a]
Gram stain	+	+
Cellular Morphology	rods	rods
Catalase	-	-
Growth at 15° C	-	-
Growth at 45° C	+	±
NH_3 from Arginine	-	-
Acid from:		
Amygdalin	+	+
Arabinose	-	-
Cellobiose	+	+
Galactose	+	+
Glucose	+	+
Inositol	-	-
Lactose	+	+
Maltose	+	+
Mannitol	-	-
Mannose	+	+
Melezitose	-	-
Melebiose	+	±
Raffinose	+	±
Rhamnose	-	-
Salicin	+	+
Sorbitol	-	-
Sucrose	+	+
Trehalose	+	+
Xylose	-	-

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

TABLE III

GROWTH OF LACTOBACILLUS ACIDOPHILUS NCFM IN PEPSINIZED WHEY MEDIA AT PH 6.0 AND 37 C

Hours of Incubation	2.5% Whey Solids[a]		5.0% Whey Solids[a]		7.5% Whey Solids[b]	
	MRS	MRSO	MRS	MRSO	MRS	MRSO
10	8.92	8.67	8.63	8.34	7.96	7.32
12	8.93	8.85	8.51	8.34	8.26	7.30
14	8.95	8.88	8.63	8.54	7.89	7.29
16	9.00	8.82	8.76	8.64	7.84	7.27
18	8.96	8.76	8.81	8.62	7.62	6.67
20	9.00	8.83	8.81	8.62	7.68	6.48
22	9.01	8.81	8.80	8.54	7.68	6.38
24	9.00	8.82	8.77	8.51	7.32	6.51

[a]Each value is the average $\log_{10}$ count/ml from 6 trials

[b]Each value is the average $\log_{10}$ count/ml from 4 trials

whey solids. Growth of L. acidophilus in the 2.5% and 5.0% media reached maximum numbers at 16 to 18 hours and then remained constant in numbers for the remainder of the incubation time. The growth of L. acidophilus in the medium containing 7.5% whey solids, however, reached its maximum ($\log_{10}$ = 8.26) at approximately 12 hours and then slowly decreased throughout the remainder of the incubation period.

As the level of whey solids was increased in the media, the maximum populations attained during growth at pH 6.0 were decreased. This relationship is illustrated graphically in Figure 2. The data in this figure were taken from MRS agar counts in Table III. The figure more clearly shows the stability of the populations attained in the 2.5% and 5.0% pepsinized whey solids media compared to the instability of the population in the 7.5% medium. The maximum numbers of L. acidophilus attained during growth in the 7.5% medium was much lower than the maximum numbers attained in the other two media.

It was also observed that lower counts were obtained on MRSO agar than on MRS agar for cultures grown in each level of pepsinized whey. The difference in counts on these two media increased as the level of whey solids was increased (Table III). When the culture was grown in the 7.5% medium the MRSO counts were much lower than the MRS counts.

Due to the poor performance of the culture in the medium containing 7.5% whey solids further experiments with this medium were not done.

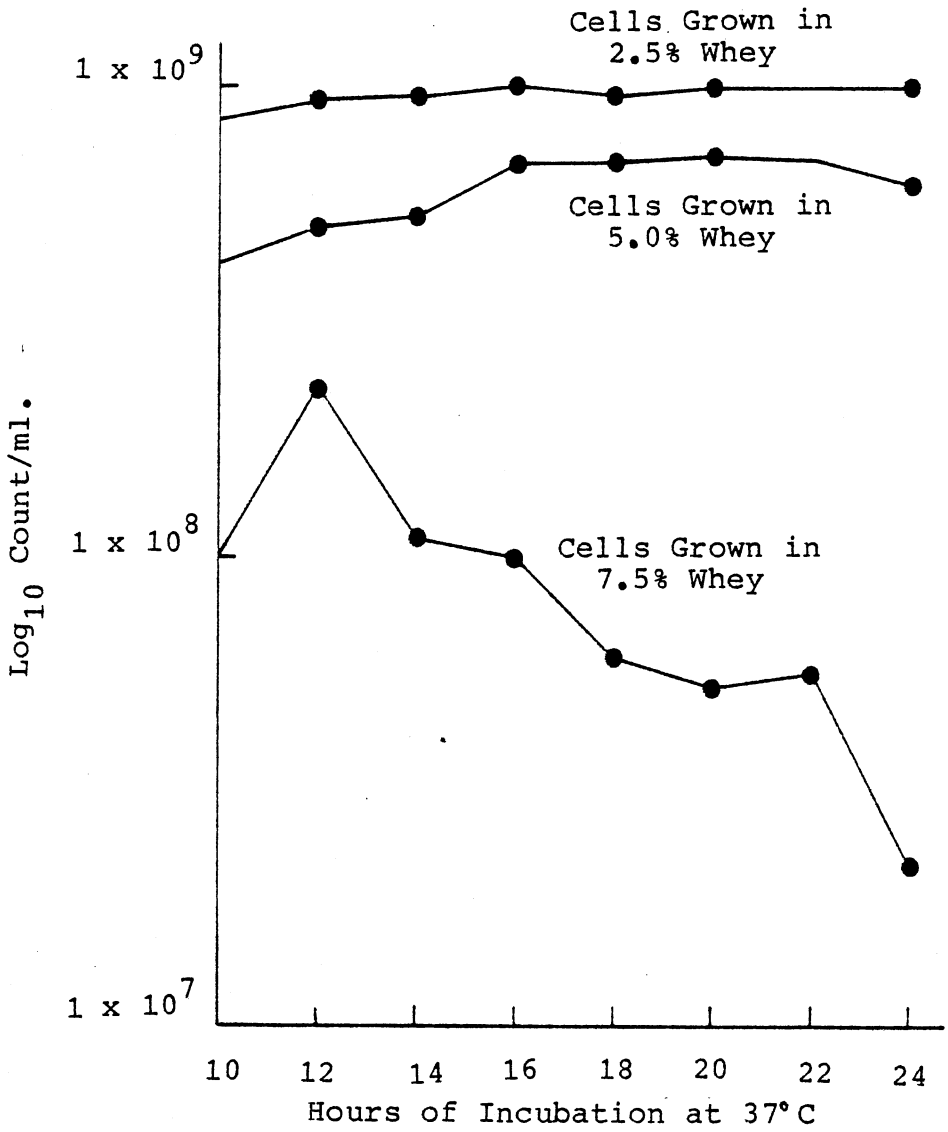

Figure 2. Growth of Lactobacillus acidophilus NCFM at pH 6.0 in Media Containing 2.5%, 5.0%, and 7.5% Ppsinized Whey Solids

Statistical analyses were conducted only for the data collected when the culture was grown in the 2.5% and 5.0% pepsinized whey media. A split-split plot design was used to compare the performance of the culture when grown in the media containing the two levels of whey solids (Table IV). There was a significant difference ($P < .05$) between the maximum populations of L. acidophilus obtained in the 2.5% and 5.0% pepsinized whey media. The maximum populations achieved in the 2.5% whey medium were thus greater than that in the 5.0% whey medium. Also, the MRS counts were higher ($P < .005$) than the counts on MRSO agar for the culture grown in both 2.5% and 5.0% pepsinized whey media.

Survival of Concentrated Cultures During Storage in Liquid Nitrogen

Concentrated cultures were prepared when the growth of L. acidophilus in the whey media was estimated to be in the late exponential phase and then stored in liquid nitrogen. The survival of the concentrated cultures of L. acidophilus NCFM during storage in liquid nitrogen is shown in Table V. The values are presented as $\log_{10}$ count/g and each value represents an average from 6 trials. For the concentrated cultures prepared from cells grown in the medium containing 2.5% pepsinized whey solids counts on both the MRS and MRSO agar remained fairly constant during 28 days of storage in liquid nitrogen. However, the MRS and MRSO counts for the concentrated cultures prepared from cells grown in the 5.0%

TABLE IV

ANALYSIS OF VARIANCE OF DATA FROM GROWTH OF *LACTOBACILLUS ACIDOPHILUS* NCFM IN WHEY MEDIA AT PH 6.0

Source of Variation	Analysis of Variance df	ss	MS	F value	OSL
Total	95				
Among Batches	11	5.06	0.46	1.48	>.25
Whey Level, A[a]	1	1.94	1.94	6.26	<.05
Error (a)	10	3.12	0.31		
Sub-units	36				
Time, B[b]	3	0.38	0.13	2.86	<.25
AXB	3	0.27	0.09	1.29	>.25
Error (b)	30	1.96	0.07		
Sub-sub units					
Medium, C[c]	1	0.69	0.69	46.0	<.005
AxC	1	0.01	0.01	0.67	>.25
BxC	3	0.04	0.013	0.87	>.25
AxBxC	3	0.02	0.007	0.47	>.25
Error (c)	40	0.60	0.015		

[a] Pepsinized whey media containing 2.5% and 5.0% whey solids

[b] 12, 16, 20 and 24 hr. of incubation at 37 C

[c] Samples plated on MRS and MRSO agars

TABLE V

SURVIVAL OF LACTOBACILLUS ACIDOPHILUS NCFM IN CONCENTRATED CULTURES DURING STORAGE IN LIQUID NITROGEN[a]

Days in Liquid Nitrogen	2.5% Whey Solids		5.0% Whey Solids	
	MRS	MRSO	MRS	MRSO
0	10.31	10.26	9.73	9.64
1	10.27	10.13	9.66	9.51
14	10.28	10.24	9.59	9.33
28	10.27	10.21	9.61	9.26

[a] Each value is the average $\log_{10}$ count/g from 6 trials

pepsinized whey medium showed a reduction after 1 day of storage in liquid nitrogen. The MRS count dropped from 9.81 to 9.67 and the MRSO count dropped from 9.65 to 9.53. The MRS counts then remained about the same from Day 1 to Day 28, but the MRSO count ($\log_{10}$ = 9.36) was further reduced after 28 days of storage in liquid nitrogen.

A split-split plot design was used to compare the storage stability of the concentrated cultures prepared from cells grown in the two levels of whey solids (Table VI). The numbers of lactobacilli in the concentrated cultures prepared from cells grown in the 2.5% pepsinized whey medium were much greater ($P<.005$) than for the concentrated cultures prepared from cells grown in the 5.0% pepsinized whey medium. The concentrated cultures prepared from the 2.5%

TABLE VI

ANALYSIS OF VARIANCE OF DATA FROM TRIALS EVALUATING THE STORAGE STABILITY OF *LACTOBACILLUS ACIDOPHILUS* IN CONCENTRATED CULTURES STORED IN LIQUID NITROGEN

Source of Variation	Analysis of Variance df	SS	MS	F Value	OSL
Total	95				
Among Batches	11	12.97	1.18	9.83	$<.005$
Whey Level, A[a]	1	11.74	11.74	97.83	$<.005$
Error (a)	10	1.23	0.12		
Sub-units	36				
Time, B[b]	3	0.35	0.12	4.00	$<.05$
AxB	3	0.26	0.09	3.00	$<.05$
Error (b)	30	0.89	0.03		
Sub-sub units	48				
Medium, C[c]	1	0.54	0.54	18.00	$<.005$
AxC	3	0.14	0.14	4.64	$<.05$
BxC	3	0.04	0.01	0.33	$>.25$
AxBxC	3	0.08	0.03	1.00	$>.25$
Error (c)	40	1.05	0.03		

[a] Pepsinized whey media containing 2.5% and 5.0% whey solids

[b] 0, 1, 14, and 28 days of storage in liquid nitrogen

[c] Samples plated on MRS and MRSO agars

whey medium were also more stable ($P<.05$) during 28 days of storage in liquid nitrogen. Counts obtained on MRSO agar were significantly lower ($P<.005$) than on MRS agar for cultures grown in both levels of pepsinized whey. There was significant interaction ($P<.05$) between levels of whey and counts obtained on MRS and MRSO agars. The difference in counts on MRSO and MRS agar was greater for the culture grown in 5.0% pepsinized whey medium. There was also a significant interaction ($P<.05$) between levels of whey and days of storage in liquid nitrogen. The cells grown in the 2.5% pepsinized whey medium were more stable than those grown in the 5.0% medium.

The percentage survivors during storage in liquid nitrogen were calculated from the counts obtained on MRS agar using the following equation:

$$\text{Percent Survivors} = \frac{B_x}{A_x} \times 100$$

A_x = The average count/ml on Day 0

B_x = The average count/ml on Day 1, 14, or 28 of frozen storage

The results ($\log_{10}$ of average percent survivors from 6 trials) are presented graphically in Figure 3. The greatest difference in percent survivors between cultures that had been grown in the 2 media was observed after 14 and 28 days of frozen storage. After 28 days of storage in liquid nitrogen the percent survivors for concentrated cultures prepared from cells grown in the 2.5% pepsinized whey medium was 90% compared to 66% for the concentrated cultures from

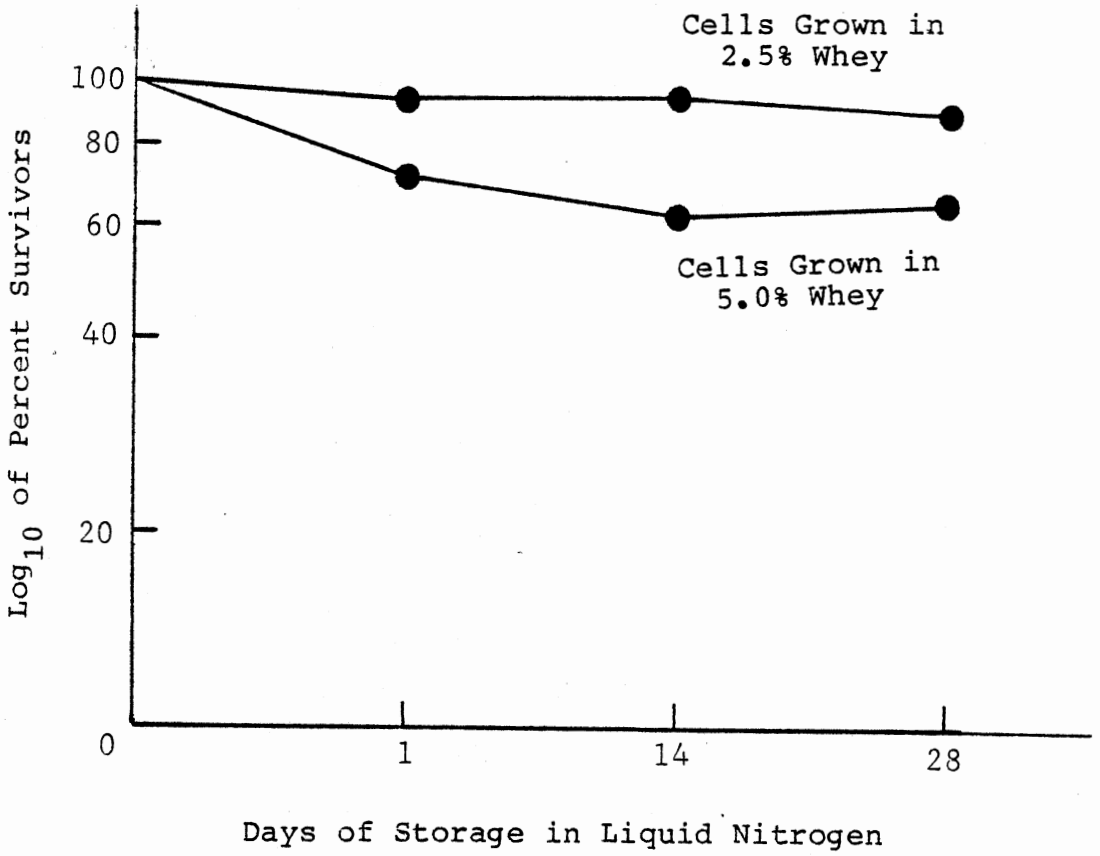

Figure 3. Survival in Liquid Nitrogen of a Concentrated Cultures of Lactobacillus acidophilus NCFM Prepared from Cells Grown in Media Containing 2.5% and 5.0% Whey Solids

the 5.0% pepsinized whey medium.

Stability in Refrigerated Milk of Cells of *Lactobacillus acidophilus* from Frozen Concentrated Cultures

The effect of storage in refrigerated milk on the stability of cells of *L. acidophilus* NCFM which were grown in the medium containing 2.5% pepsinized whey solids is shown in Figure 4. The values are presented as $\log_{10}$ count/ml. and each value is an average from 6 trials. The concentrated cultures used to prepare the milk had been stored in liquid nitrogen for 28 days. Counts obtained on MRS agar and MRSO agar dropped steadily during storage in milk at 5 C. The MRSO counts obtained on MRSO agar showed the sharpest decrease from Day 7 to Day 14 while the MRS agar counts showed the sharpest decrease from Day 14 to Day 21. The greatest difference between MRS and MRSO agar counts was observed after 14 and 21 days of refrigerated storage. The $\log_{10}$ count/ml on MRS agar after 14 days of storage was 5.77. This was significantly higher ($p<.005$) than the $\log_{10}$ of MRSO agar count/ml. of 4.87 after 14 days of refrigerated storage (Table VII). Similar results were observed after 21 days of storage in refrigerated milk.

The refrigerated storage stability in milk of cells of *L. acidophilus* grown in the 5.0% whey medium is presented in Figure 5. Once again the values are $\log_{10}$ count/ml. and an average from 6 trials. The milk was prepared using con-

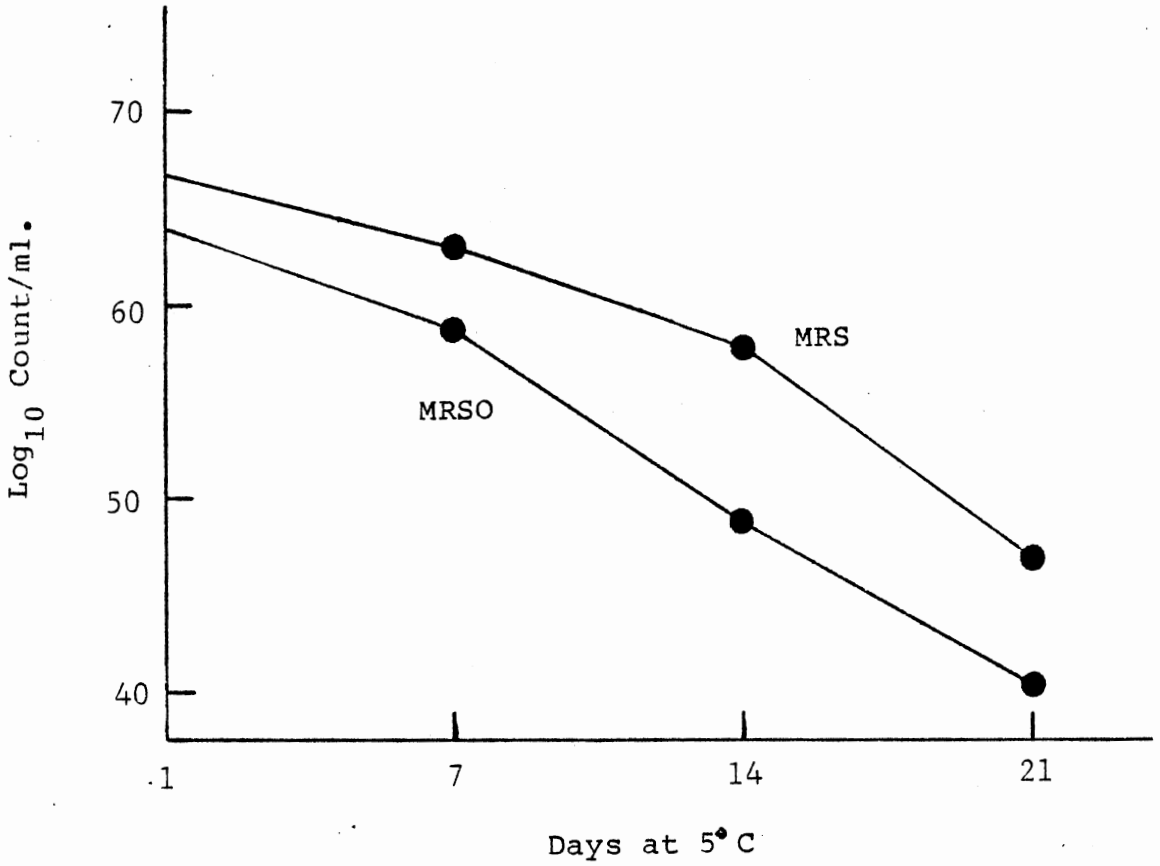

Figure 4. Stability in Refrigerated Milk of Cells of Lactobacillus acidophilus NCFM Grown in Medium Containing 2.5% Pepsinized Whey Solids

TABLE VII

ANALYSIS OF VARIANCE OF DATA FROM TRIALS EVALUATING THE STABILITY IN REFRIGERATED MILK OF *LACTOBACILLUS ACIDOPHILUS* NCFM FROM CONCENTRATED CULTURES WHICH HAD BEEN STORED 28 DAYS IN LIQUID NITROGEN

Source of Variation	df	SS	MS	F value	OSL
Total	95				
Among Batches	11	46.60	4.24	1.85	.25
Whey Level, A[a]	1	23.70	23.70	10.35	.01
Error (a)	10	22.90	2.29		
Sub-units	36				
Time, B[b]	3	70.46	23.49	293.63	.005
AxB	3	0.29	0.10	1.25	.25
Error (b)	30	2.31	0.08		
Sub-sub units	48				
Medium, C[c]	1	17.37	17.37	34.06	.005
AxC	1	2.01	2.01	3.94	.10
BxC	3	1.92	0.64	1.25	.25
AxBxC	3	0.83	0.28	0.55	.25
Error (c)	40	20.51	0.51		

[a] Pepsinized whey media containing 2.5% and 5.0% whey solids

[b] 1, 7, 14, and 21 day of storage in milk at 5 C

[c] Samples plated on MRS and MRSO agars

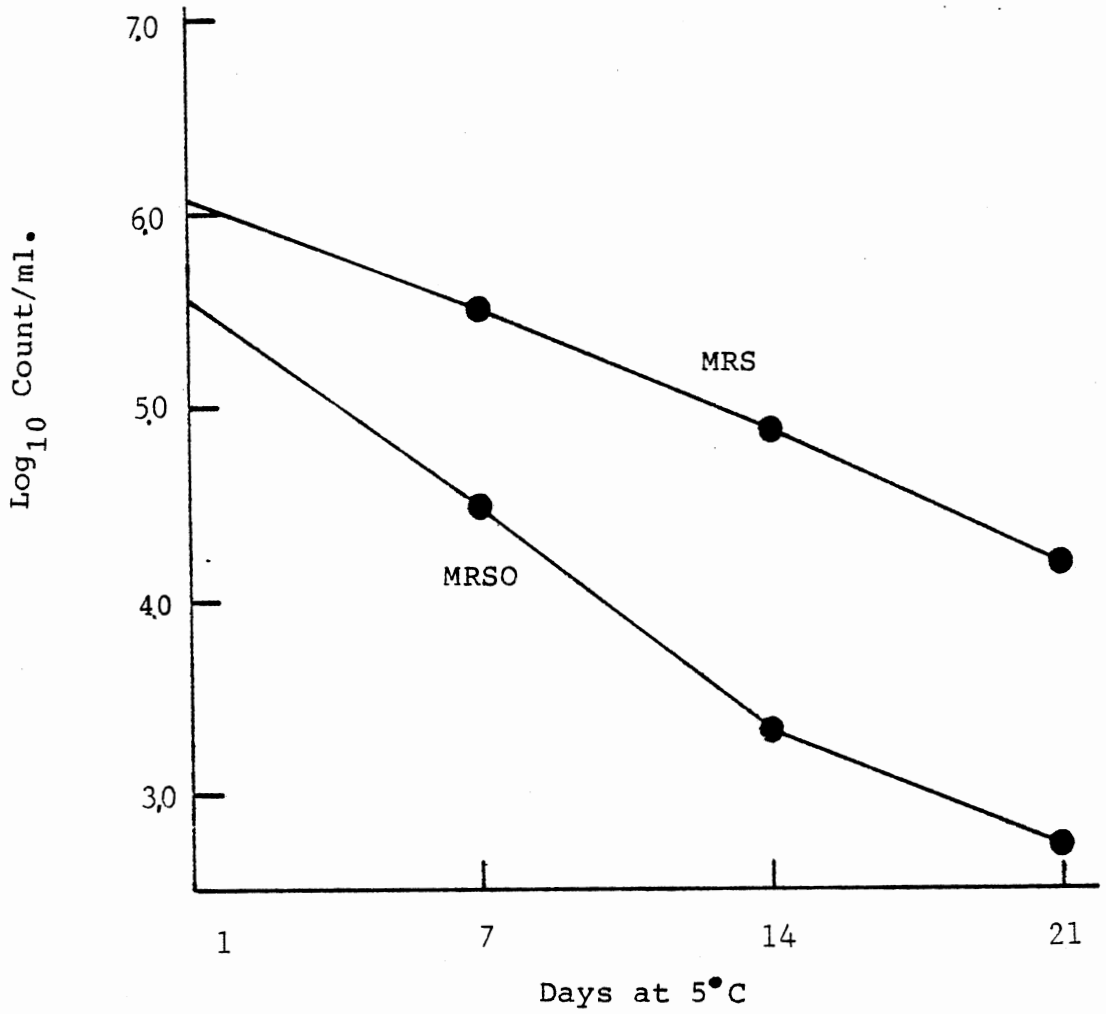

Figure 5. Stability in Refrigerated Mild of Cells of Lactobacillus acidophilus NCFM Grown in Medium Containing 5.0% Pepsinized Whey Solids

centrated cultures which had been stored 28 days in liquid nitrogen. Both the MRS and MRSO agar counts dropped during storage in milk at 5 C, but the MRSO agar counts dropped much more rapidly than the MRS agar counts. There was a significant difference ($P<.005$) between counts obtained on MRS and MRSO agars. As in the 2.5% whey medium, the greatest difference between MRS and MRSO counts was seen after 14 and 21 days of refrigerated storage. The MRS agar count at 14 days of storage was 4.88 as compared to a $\log_{10}$ count of 3.32 on MRSO agar.

Once again a split-split plot design was used to compare the two media. There was a significant decrease ($P<.005$) in the numbers of lactobacilli during storage in refrigerated milk. Also, the MRS and MRSO agar counts for cells grown in the 2.5% pepsinized whey medium during storage at 5 C were significantly higher ($P<.01$) than for cells grown in the 5.0% medium.

Differences in storage stability for cells grown in the two media is illustrated graphically in Figure 6. The values are presented as $\log_{10}$ of percent survivors. The percentages of survivors during storage in refrigerated milk were calculated from the counts obtained on MRS agar as previously indicated. There was little difference in percent survivors for the culture grown in the two media at 7 and 14 days of storage at 5 C, but at 21 days there was a large difference.

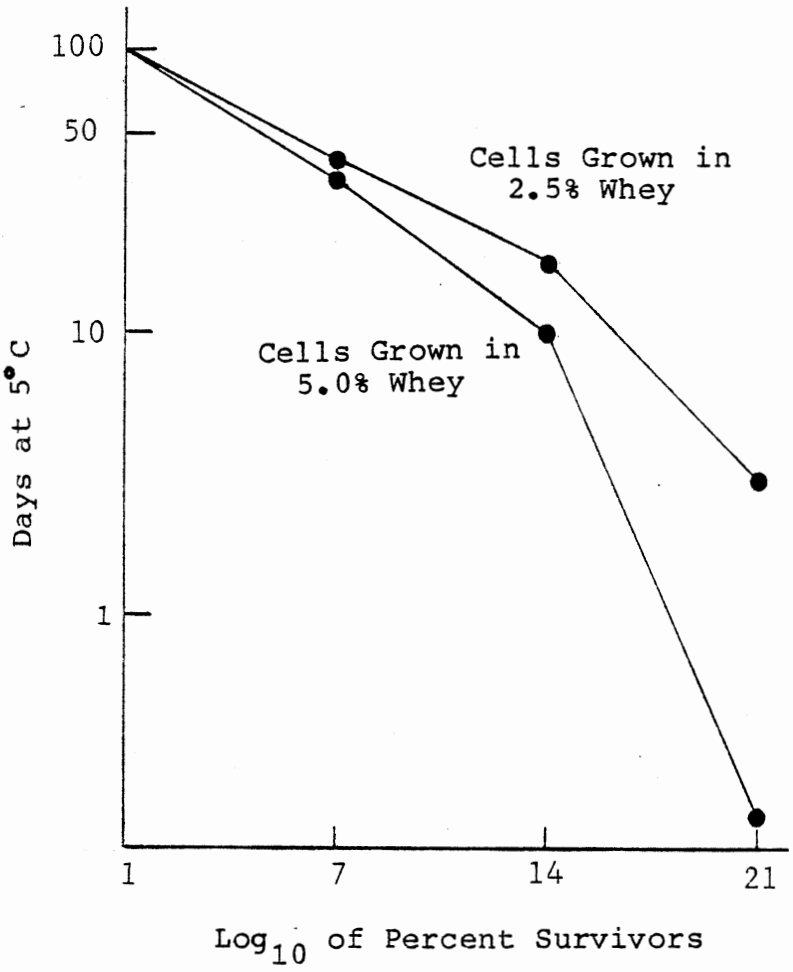

Figure 6. Survival in Refrigerated Milk of Cells of Lactobacillus acidophilus NCFM in Media Containing 2.5% and 5.0% Pepsinized Whey Solids

CHAPTER V

DISCUSSION

Cell crops of L. acidophilus to be used in the preparation of concentrated cultures are usually harvested from the growth medium by centrifugation. To allow efficient harvesting of the cells of L. acidophilus the ingredients of the growth medium should be in solution. Since the proteins in milk are not in solution, it is difficult to efficiently harvest bacteria from it. Duggan et al. (1959) adjusted the pH of the whey medium to pH 7.0 and sterilized the medium at 121.1 C for 45 minutes. After autoclaving this 7.0% whey suspension was translucent and allowed easy harvesting of the cells by centrifugation. Duggan did not utilize pH control during growth of L. acidophilus and he allowed the pH to drop to 4.3 which is below the isoelectric point of whey proteins. However, the pH of the medium was adjusted to 6.5 just prior to centrifugation to reduce the amount of whey proteins in the cell pellet. The pepsinized whey media used in the present study were adjusted to a pH of 7.0 prior to autoclaving at 121 C for 15 minutes. This resulted in clear media with minimum amounts of precipitation. When the pH was adjusted to 5.5, 6.0 or 6.5 prior to heating heavy precipitation resulted after heating. Heating times longer than 15 minutes also resulted in heavy precipitation. This

precipitated material was probably precipitated whey proteins or calcium salts. The volume of medium sterilized also played a major role in determining the amount of precipitation after sterilization. Smaller volumes (250 ml.) resulted in heavy precipitation. This indicates that the rate of heating may affect the amount of precipitation in the whey medium during autoclaving since the smaller volumes would heat much faster in the autoclave.

The maximum populations of L. acidophilus NCFM attained during growth at pH 6.0 and 37 C was influenced by the concentration of whey solids in the pepsinized whey media. As the level of whey solids was increased the maximum populations of L. acidophilus attained during growth were decreased. Also, the bile resistance of the culture decreased as the level of pepsinized whey solids was increased in that lower counts were obtained on MRSO agar than on MRS agar. The lower populations of L. acidophilus and the decreased bile resistance of the culture in the 7.5% pepsinized whey medium could be due to inhibitory metabolic products produced from the higher concentration of whey components. Increasing concentrations of whey components might also have created an "imbalance" amoung nutrients in the medium. For instance, the whey media with higher levels of pepsinzed whey solids might require different levels of yeast extract or thiotone to attain maximum growth of lactobacilli. Williams et al. (1947) and Kitay and Snell (1950) found that lactobacilli require oleic acid for optimum growth. While the requirement

for oleic acid can be provided by the addition of Tween 80, different levels may have been necessary to support maximum growth of the culture in the media containing higher concentrations of whey components.

Lloyd and Pont (1973) grew lactic streptococci in a trypsin digested cheese whey containing autolyzed yeast cells. They found that maintaining the pH with NH_4OH plus $Ca(OH)_2$ increased the yield of cells 50-100% as compared to the yield using only NH_4OH. Peebles et al. (1969) found that populations of Streptococcus cremoris, when NH_4OH was used as the neutralizer, were twice that when NaOH was the neutralizer. Gilliland et al. (1970), however, observed that maximum populations of Leuconostoc citrovorum were equal at pH 6.0, 6.5, or 7.0 when either NaOH or NH_4OH was used as the neutralizer. The neutralizer used in this project was 20% Na_2CO_3 in 20% NH_4OH. This neutralizer would allow the slow release of CO_2 during growth of the culture of L. acidophilus at pH 6.0 in the fermentor. Longsworth and MacInnes (1936) reported that CO_2 was essential for the growth of L. acidophilus. It is possible that a different neutralizer may have been more effective in the media containing higher concentrations of whey. While there is no imformation available as to the optimum level of various minerals and/or salts on the growth of L. acidophilus, an alteration in the ratio of the various salts caused by increasing the whey solids could have influenced the culture.

Many researchers have studied the effect of the physiological age of lactic cultures on their subsequent survival during frozen storage. The culture of L. acidophilus NCFM did not enter a death phase during the 24 hour observation period when grown in the media containing 2.5% and 5.0% pepsinized whey solids. It reached maximum populations at approximately 16 hours of incubation at 37 C, and this is when the cells were harvested by centrifugation, concentrated, and frozen and stored in liquid nitrogen. Smittle et al. (1972) found that the freezing stability of L. bulgaricus in liquid nitrogen was not influenced by the physiological age of the culture. Lamprech and Foster (1963), however, reported that the physiological age of cells of Streptococcus lactis at which they were harvested influenced their storage stability. Younger cells harvested at 10 to 15 hours of incubation were hardier and survived storage at -20 C better than did older cells. Johannsen (1972) found that cells of L. leichamannii younger that 8 hours were more susceptible to damage by freezing at -196 C than older ones. Data from the present study cannot be used to show the effect of physiological age of the cells on survival of L. acidophilus during freezing and subsequent refrigerated storage in milk. However, this is a factor which should be considered in future studies.

In comparing the survival of concentrated cultures of L. acidophilus during 28 days of storage in liquid nitrogen the medium containing 2.5% pepsinized whey solids produced

more stable cells than the 5.0% whey medium. The lower stability of the concentrated cultures in liquid nitrogen prepared from cells grown in the 5.0% whey medium could be due to some damage to the cells during growth at 37 C. Damage or injury to the cells is also indicated by the larger difference between MRS and MRSO agar counts for the culture grown in the 5.0% pepsinized whey medium than for the cells grown in the 2.5% pepsinized whey medium. The lower counts on MRSO agar indicate reduced resistance of the culture to bile salts.

Many factors can affect the survival of L. acidophilus in concentrated cultures during frozen storage. Bauman et al. (1966) felt that fast freezing of lactic cultures at -196 C resulted in significantly greater survival and activity after storage than slow freezing at -20 C. He suggested that there is little opportunity for ice crystal growth at -196 C and biochemical activity within the cell is at a virtual standstill. Smittle et al. (1972) found that cells of L. bulgaricus grown in a medium containing Tween 80 survived storage in liquid nitrogen better than cells grown in the medium without Tween 80. He suggested that Tween 80 could play a role in developing cell membrances whose integrity was maintained during storage in liquid nitrogen. Smittle et al. (1974) later reported that sodium oleate was the active portion of Tween 80 responsible for producing cells of L. bulgaricus stable to storage in liquid nitrogen. They indicated that the increased protection during frozen

storage was due to a favorable balance of cyclopropane and saturated fatty acids. Goldberg and Eschar (1977) also reported that the addition of Tween 80 to the growth medium could cause changes in the fatty acid composition in the lipid fractions of lactic acid bacteria. They concluded that the damage from freezing was correlated to specific alterations in the cellular fatty acids.

Wright and Klaenhammer (1981) found that cells of L. acidophilus NCFM grown in calcium supplemented MRS broth were more resistant to frozen storage in liquid nitrogen than cells of L. acidophilus grown in unsupplemented MRS broth. They suggested that calcium may aid in transforming the cells of L. acidophilus to a physiological state less susceptible in injury from frozen storage.

In order for a concentrated culture of L. acidophilus NCFM to be useful as a dietary adjunct, it must maintain viability and bile resistance during extended storage in liquid nitrogen. Straka and Stokes (1959) said that the percentage of injured bacteria varied with the time and temperature of frozen storage. They felt that cold injury to bacteria was manifested by an increase in their nutritional requirements. Speck (1978) reported that the bile resistance of a culture might be lowered when the organism has been subjected to certain stresses such as frozen storage. The bile resistance of concentrated cultures of L. acidophilus NCFM prepared from cells grown in the 5.0% pepsinized whey medium was lower after 28 days of storage in

liquid nitrogen than concentrated cultures prepared from cells grown in the medium containing 2.5% pepsinized whey solids.

Since a concentrated culture must maintain its viability and bile reisitance during long periods of storage in liquid nitrogen, the milk containing added cells of L. acidophilus in this study was prepared from concentrated cultures of L. acidophilus NCFM stored 28 days in liquid nitrogen. The cells of L. acidophilus grown in the 2.5% pepsinized whey medium rapily lost bile resistance after 14 days of storage in pasteurized low-fat milk at 5 C. The cells of L. acidophilus grown in the medium containing 5.0% pepsinized whey solids, however, rapidly lost bile resistance after only 7 days of refrigerated storage in milk. This loss of bile resistance was indicated by the large difference in the counts obtained of MRS and MRSO agar. The viability of cells of L. acidophilus grown in both concentrations of pepsinized whey solids also steadily decreased during 21 days of refrigerated storage as indicated by the reduction in counts obtained on MRS agar.

Myers (1931) found that cells of L. acidophilus maintained their viability in unfermented milk when stored at 2 to 5 C for 4 to 5 days. Myers grew the cells of L. acidophilus in a sterile medium, harvested them by centrifugation, and resuspended them in pasteurized milk. He attributed the low death rate to the low acid content of the product.

Young and Nelson (1978) did a survey of several brands of commercial acidophilus milk and evaluated them for microbial and chemical changes during 23 to 24 days of storage at 4 C. The percentages of survivors of lactobacilli during extended storage of the commercial products at 4 C were similar to the percentages of survivors of cells of L. acidophilus NCFM during storage in milk at 5 C grown in the medium containing 2.5% pepsinized whey solids. They reported that the count of viable L. acidophilus declined markedly during 23 days of refrigerated storage. They further stated that the final level of viable cells of L. acidophilus in the milk was influenced by the initial level of inoculation and the strain of L. acidophilus used.

Different strains of L. acidophilus may differ in their nutrient requirements during growth and their ability to survive storage in liquid nitrogen. They may also differ in their bile resistance and in their ability to survive refrigerated storage in pasteurized low-fat milk. Thus when selecting a strain of L. acidophilus to be used as a dietary adjunct, it is important to consider the organism's nutrient requirements, bile resistance, and ability to survive frozen and refrigerated storage.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Pepsinized whey based media were prepared using three different concentrations of dried sweet whey solids. The purpose of this study was to develop a growth medium that would permit the production of high populations of cells of L. acidophilus NCFM that would be stable during storage in liquid nitrogen and subsequent storage in refrigerated pasteurized milk.

Studies comparing the growth of L. acidophilus NCFM in the media containing different concentrations of pepsinized whey solids (2.5%, 5.0%, or 7.5%) indicated that the maximum populations attained during growth at pH 6.0 significantly decreased as the level of whey solids was increased. The bile resistance of the resulting cultures also decreased as the level of whey solids was increased.

Concentrated cultures were prepared from cells of L. acidophilus NCFM grown in media containing 2.5% and 5.0% pepsinized whey solids and compared for their ability to maintain viability and bile resistance during storage in liquid nitrogen. The numbers of lactobacilli in the concentrated cultures prepared from cells of L. acidophilus grown in the 2.5% pepsinized whey based medium were much

greater than for cells grown in the 5.0% medium. Stability of the cultures during storage in liquid nitrogen also significantly decreased as the concentration of whey solids in the growth medium was increased.

The final evaluation of the growth media was their ability to produce cells of L. acidophilus NCFM capable of maintaining their stability during storage in pasteurized low-fat milk at 5 C after 28 days of storage in liquid nitrogen. The viability and bile resistance of cells of L. acidophilus grown in the 2.5% pepsinized whey based medium during storage at 5 C were significantly higher than for cells of L. acidophilus grown in the 2.5% pepsinized whey based medium showed a significant reduction in viability and bile resistance during 21 days of storage in pasteurized milk at 5 C.

This study emphasizes the effect of medium composition on the growth of L. acidophilus NCFM at pH 6.0 and the subsequent ability of the organism to maintain viability and bile resistance during frozen and refrigerated storage. Based on the data obtained in this study, the growth medium containing 2.5% pepsinized whey solids would be the most desirable for producing cells of L. acidophilus NCFM to be used in the preparation of frozen concentrated cultures. However, the survival of the culture during storage in refrigerated milk needs to be improved. Factors other than the concentration of whey solids in the medium such as pH during growth, neutralizer, for maintaining constant pH, and growth temperature need to be researched.

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APPENDIXES

TABLE VIII

GROWTH OF LACTOBACILLUS ACIDOPHILUS NCFM IN MEDIUM CONTAINING 2.5% PEPSINIZED WHEN SOLIDS AT PH 6.0 AND 37 C

Hours of Incubation	Medium	Trials[a]						$\bar{x}$[b]
		1	2	3	4	5	6	
12	MRS	9.04	9.11	8.53	9.04	9.04	8.81	8.93
	MRSO	8.97	9.00	8.52	8.93	9.93	9.93	8.85
16	MRS	9.04	9.11	8.94	9.11	8.92	8.90	9.00
	MRSO	8.72	9.08	8.94	8.83	8.78	8.64	8.82
20	MRS	9.08	9.04	8.91	9.15	8.93	8.93	9.01
	MRSO	9.04	9.00	8.94	9.00	8.70	8.28	8.83
24	MRS	9.04	9.08	9.00	9.11	8.91	8.83	9.00
	MRSO	8.92	8.97	9.00	9.04	8.77	8.20	8.82

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

TABLE IX

GROWTH OF LACTOBACILLUS ACIDOPHILUS NCFM IN MEDIUM CONTAINING 5.0% PEPSINIZED WHEY SOLIDS AT PH 6.0 AND 37 C

Hours of Incubation	Medium	Trials[a]						x^b
		1	2	3	4	5	6	
12	MRS	8.58	8.08	8.83	8.88	7.69	9.00	8.51
	MRSO	8.08	8.18	8.74	8.67	7.58	8.79	8.34
16	MRS	8.89	8.20	8.85	9.00	8.52	9.00	8.76
	MRSO	8.79	8.30	8.86	8.74	8.38	8.78	8.64
20	MRS	8.92	8.46	8.66	8.89	8.79	9.11	8.81
	MRSO	8.75	8.52	8.36	8.72	8.52	8.87	8.62
24	MRS	8.81	8.57	8.63	8.88	8.77	8.89	8.77
	MRSO	8.56	8.58	8.30	8.66	8.20	8.76	8.51

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

TABLE X

STABILITY IN LIQUID NITROGEN OF CELLS OF LACTOBACILLUS ACIDOPHILUS NCFM GROWN IN MEDIUM CONTAINING 2.5% PEPSINIZED WHEY SOLIDS

Days in Liquid N_2	Medium	Trials[a]						x^b
		1	2	3	4	5	6	
0	MRS	10.38	10.40	10.28	10.42	10.34	10.04	10.31
	MRSO	10.36	10.45	10.26	10.20	10.23	10.04	10.26
1	MRS	10.26	10.30	10.20	10.49	10.34	10.04	10.27
	MRSO	10.26	10.36	10.20	10.30	10.00	9.64	10.13
14	MRS	10.31	10.41	10.26	10.36	10.30	10.04	10.28
	MRSO	10.36	10.42	10.30	10.40	10.04	9.93	10.24
28	MRS	10.32	10.36	10.23	10.38	10.30	10.04	10.27
	MRSO	10.28	10.42	10.04	10.42	10.26	9.83	10.21

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

TABLE XI

STABILITY IN LIQUID NITROGEN OF CELLS OF LACTOBACILLUS ACIDOPHILUS NCFM GROWN IN MEDIUM CONTAINING 5.0% PEPSINIZED WHEY SOLIDS

Days in Liquid N_2	Medium	Trials[a]						x^b
		1	2	3	4	5	6	
0	MRS	9.82	9.46	9.72	9.72	9.86	10.08	9.78
	MRSO	9.59	9.49	9.72	9.66	9.60	9.76	9.64
1	MRS	9.57	9.46	9.73	9.73	9.73	9.71	9.66
	MRSO	9.26	9.41	9.54	9.59	9.70	9.58	9.51
14	MRS	9.81	9.40	9.49	9.59	9.65	9.62	9.59
	MRSO	9.51	9.38	9.43	9.49	9.51	8.65	9.33
28	MRS	9.59	9.49	9.71	9.51	9.54	9.83	9.61
	MRSO	9.45	9.40	9.66	9.43	8.90	8.70	9.26

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

TABLE XII

STABILITY IN REFRIGERATED MILK OF CELLS OF LACTOBACILLUS ACIDOPHILUS NCFM GROWN IN MEDIUM CONTAINING 2.5% PEPSINIZED WHEY SOLIDS

Days at 5 C	Medium	Trials[a]						x^b
		1	2	3	4	5	6	
1	MRS	6.73	7.15	6.51	6.70	6.59	6.32	6.67
	MRSO	5.65	7.04	6.53	6.69	6.46	6.04	6.40
7	MRS	6.08	6.49	6.45	6.59	6.28	5.93	6.30
	MRSO	5.28	6.00	6.11	6.51	6.20	5.15	5.88
14	MRS	6.30	5.92	6.20	6.08	5.51	4.62	5.77
	MRSO	5.08	4.90	5.90	5.90	4.48	2.95	4.87
21	MRS	4.92	4.90	5.85	5.15	4.64	2.70	4.69
	MRSO	4.41	4.26	5.71	4.04	3.94	1.90	4.04

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

TABLE XIII

STABILITY IN REFRIGERATED MILK OF CELLS OF LACTOBACILLUS ACIDOPHILUS NCFM GROWN IN MEDIUM CONTAINING 5.0% PEPSINIZED WHEY SOLIDS

Days at 5 C	Medium	Trials[a]						$\bar{x}$[b]
		1	2	3	4	5	6	
1	MRS	5.85	5.83	6.08	5.85	6.15	6.63	6.07
	MRSO	5.52	5.78	6.11	4.23	5.95	4.72	5.56
7	MRS	4.84	5.28	5.80	5.11	5.76	6.20	5.50
	MRSO	3.52	5.08	5.75	3.15	4.57	4.72	4.47
14	MRS	5.11	4.08	5.20	3.97	5.53	5.36	4.88
	MRSO	4.43	3.00	2.68	1.95	4.41	3.46	3.32
21	MRS	3.51	3.91	4.00	4.89	4.94	3.82	4.18
	MRSO	2.91	2.76	3.36	1.30	3.66	2.30	2.72

[a]Each value is the $\log_{10}$ count/ml.

[b]Average $\log_{10}$ count/ml. from 6 trials.

VITA

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