INFLUENCE OF HARVEST TIME ON STABILITY OF CELLS OF *LACTOBACILLUS ACIDOPHILUS* DURING FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

By

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PREFACE

The effect of harvest time on the viability, bile tolerance, ß-galactosidase activity, and cholesterol assimilation of three strains of *Lactobacillus acidophilus* during frozen storage at -196°C and subsequent refrigerated storage in milk at 7°C was evaluated.

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CHAPTER I

INTRODUCTION

Persons who are lactose maldigestors (persons who cannot adequately digest lactose) should not exclude dairy products from their diet because it is a primary source of calcium and other nutrients. One alternative for persons with this condition is nonfermented acidophilus milk. The ß-galactosidase provided by *Lactobacillus acidophilus* can hydrolyze lactose in the intestinal tract and enable the lactose maldigestors to digest it.

Nonfermented acidophilus milk also has the potential to provide health benefits to persons with elevated serum cholesterol levels. Human and animal studies indicate that *L. acidophilus* can play a role in helping control serum cholesterol levels.

Other potential health benefits from consuming *L. acidophilus* are control of certain types of intestinal cancer and control of intestinal infections.

In order for these potential health benefits to be realized, the organism must remain viable and retain its biological activity during production and storage of the milk. Variations in the survival rate and biological activity exist among the different strains of *L. acidophilus*. Research indicates that altering the production procedures such as controlling pH during their growth, can influence the organism's ability to survive during storage.

Research indicates that *L. acidophilus* continues to produce acid during the stationary phase of growth when pH is maintained at 5 to 7. This acid production indicates that the cells continue to utilize lactose as an energy source. The energy could be used to form storage compounds such as capsular material that could protect

the cells during frozen and refrigerated storage. The purpose of this study was to determine the effect of harvest time (i.e. late log phase or stationary phase of growth) on the viability and biological activity of *L. acidophilus* during frozen and subsequent refrigerated storage in nonfermented milk.

CHAPTER II

REVIEW OF LITERATURE

In 1908 the Russian scientist Eli Metchnikoff (47) stated that the consumption of milk fermented by lactobacilli could suppress the growth of putrefactive bacteria in the digestive system and prolong life. Since then extensive research has focused not only on the potential nutritional benefits associated with *Lactobacillus acidophilus*, but also on the production, storage, and development of acidophilus products acceptable to consumers.

Health Benefits Associated with *Lactobacillus acidophilus*

Several potential health benefits have been associated with the consumption of acidophilus products. Among these potential benefits are improved digestion of lactose in persons who are lactose maldigestors, control of intestinal infections, control of serum cholesterol levels, and control of certain types of intestinal cancer.

Benefits to Lactose Maldigestors

Gilliland (32) noted that the terms "lactose intolerance" and "lactose malabsorption" were inappropriate to describe persons unable to digest lactose . He suggested that the term lactose maldigestion be used instead because, according to Dahlqvist et al. (12), the symptoms arise due to a deficiency of lactase in the digestive system. Lactose maldigestion will be the term used to describe this condition throughout this review.

Persons with this condition experience bloating, flatulence, diarrhea and abdominal pain following consumption of milk (40, 50). Lebenthal and Rossi (43) reported that families with lactose maldigestion drink less milk or exclude it from their diet. Philips and Briggs (54) found that milk, in addition to a providing a good source of protein and other nutrients, is the primary source of calcium in the diet. Therefore, in order to prevent any long term nutrient deficiencies, milk should not be eliminated from the diet.

An alternative for lactose maldigestors is reduced-lactose milk which has had lactase added either by the manufacturer or by the consumer. Reasoner (56) observed that these products were effective in alleviating symptoms, but consumers were not likely to accept them because of their sweeter taste and weak body. The unacceptability of this product illustrates the need for a better product.

Gilliland and Kim (26) reported the benefits of *L. acidophilus* to lactose maldigestors. They observed that milk containing either 2.5×10^6 or 2.5×10^8 *L. acidophilus*/ml improved lactose utilization in individuals who were lactose maldigestors. Others have reported that nonfermented acidophilus milk failed to relieve the symptoms of lactose maldigestion (50, 51, 60). Gilliland (32) later reported that these studies gave little information on the cultures used and the procedures used in their production (32). ß-galactosidase is inducible in *L. acidophilus* (49). In the manufacturing of nonfermented acidophilus milk the organism is usually not grown in the milk. Thus if *L. acidophilus* is to provide a source of ß-galactosidase in nonfermented acidophilus milk it is necessary that the cells be grown in a medium containing lactose. To be most effective the cultures should contain a high ß-galactosidase activity and they must be handled so that this activity is maintained.

Studies conducted by Kolars and Levitt (26) and Gilliland and Kim (39) indicated that yogurt containing viable starter bacteria improved lactose utilization in persons who were lactose maldigestors. They suggested that the yogurt cultures which

readily utilized lactose provided the enzyme ß-galactosidase which was responsible for hydrolyzing lactose in the intestinal tract.

Gilliland and Lara (28) observed that ß-galactosidase activity could be significantly reduced in nonfermented acidophilus milk in as early as seven days after storage at 5°C for some strains of *L. acidophilus*. However, some of the strains maintained a high level of activity for 21 days of storage at 5°C. Noh and Gilliland (52) reported wide variation in ß-galactosidase activity among strains of *L. acidophilus*. These observations indicate the importance of selecting a strain with a high ß-galactosidase activity that will be maintained during refrigerated storage.

Antagonistic Action Toward Pathogens

Another potential health benefit associated with *L. acidophilus* is its antagonistic action toward enteric pathogenic bacteria. Studies indicate antagonistic action toward *Staphylococcus aureus, Salmonella typhimurium, Clostridium perfringens*, and *Escherichia coli.*

Gilliland and Speck (23)reported that *L. acidophilus* exerted antagonistic action against *S. aureus, S. typhi, E. coli,* and *C. perfringens* when grown simultaneously with the individual pathogens in broth cultures (23). Watkins and Miller (68) and Watkins et al. (69) tested the effects of *L. acidophilus* on the proliferation of *S. typhi, S. aureus,* and *E. coli* in chicks. The results indicted, for all three pathogens, that mortality was lowest for chicks fed *L. acidophilus* as a "prophylactic" treatment before the pathogen was introduced in comparison with the chicks fed no *L. acidophilus* or fed *L. acidophilus* as a "therapeutic" treatment after the pathogen was introduced. Fuller (17) conducted a similar study in which two sets of chicks were inoculated, one with *E. coli* and one with *E. coli* plus *L. acidophilus*. The numbers of intestinal *E. coli* were significantly lower for chicks fed *L. acidophilus* than in the control group.

In a review paper, Gilliland (29) noted that human studies involving intestinal pathogens are difficult to conduct. Most studies in the past were poorly designed, omitting appropriate controls. It is difficult to determine if all patients were infected with the same pathogen. Results from some of these studies have been conflicting due to such circumstances.

The exact mechanism for the antagonistic effect is not clear but research suggests that an antibiotic-like substance or bacteriocins may be responsible. Vincent et al. (67) observed that an antimicrobial agent was produced by *L. acidophilus* cultures isolated from mice, rats, rabbits, hamsters, and man. They called the substance lactocidin. Similar studies conducted by Hamdam and Mikolajcic (35) and Shahani et al. (62) found antibiotic type substances which they called acidolin and acidophilin, respectively.

Ferreira and Gilliland (15) reported that a bacteriocin was produced by *L. acidophilus* NCFM. Gilliland (32) suggested that such bacteriocins may be important in enhancing the ability of *L. acidophilus* to colonize the intestinal tract of man in the presence of other lactobacilli rather than having an effect on enteric pathogenic bacteria.

Ability to Assimilate Cholesterol

Research indicates *L. acidophilus* has the ability to assimilate cholesterol during growth. Since a decrease in serum cholesterol levels seems to reduce the risk of heart attacks (44) this property has the potential to be very useful for persons with abnormally high serum cholesterol levels.

Harrison and Peat (36) found that infants fed formula supplemented with *L. acidophilus* during the first 6-9 days of life had decreased serum cholesterol levels compared to infants fed sterile formula.

Gruenwald (33) observed that rats consuming skim milk fermented with *L. acidophilus* had lower serum cholesterol levels than rats fed water or plain milk. It was suggested that the culture produced a factor that reduced cholesterol synthesis.

Gilliland et al. (27) reported that in order for *L. acidophilus* to assimilate cholesterol from laboratory media, it must be grown anaerobically in the presence of bile (27). They found much variation among strains isolated from intestines of pigs with respect to the relative amounts of cholesterol assimilated. They conducted a feeding trial in which one strain (RP32) that assimilated cholesterol very actively and one (P47) that assimilated little or no cholesterol were administered to pigs previously fed a high cholesterol diet. The pigs fed strain RP32 had significantly lower serum cholesterol levels than did the pigs fed either strain P47 or no *L. acidophilus*. Similar results were observed in a study using boar hogs in which acidophilus yogurt was being tested (13). The yogurt fed to the hogs was fermented with *Streptococcus thermophilus* and *L. acidophilus* rather than traditional yogurt cultures.

The mechanism for cholesterol uptake in the body has been studied. The GI tract is the primary route for removal of cholesterol from the body. Eyssen (14) studied the blood cholesterol levels and the excretion of bile acids of germ free chicks and rats fed a high cholesterol diet competed to animals having normal intestinal flora. The germ free animals had blood cholesterol levels two times higher and excreted less cholesterol in the feces than did the normal animals. Also, more bile acids were excreted in the feces of the normal animals than in the germ free animals. He concluded that intestinal flora interferes with absorption of cholesterol from the small intestine by deconjugating bile acids thus promoting their excretion and turnover. This in turn causes some cholesterol in the body to be converted to bile acids faster in order to replace the deconjugated ones. Chikai et al. (8) studied germ free rats and rats inoculated with either *Bacteroides vulgatus, Bifidobacterium longum, C. ramosum* or *E.coli* fed a sterile normal diet . The inoculated rats, with the exception of the ones

inoculated with *E. coli*, excreted more total and deconjugated bile acids in their feces than did the germ free rats. They suggested that deconjugated bile acids are excreted more rapidly than conjugated ones by adhesion to microorganisms or dietary fiber. Gilliland and Walker (30) observed that most strains of *L. acidophilus* that assimilated cholesterol also had the ability to deconjugate bile acids but there was no significant correlation between the two activities. However, Gilliland (32) has suggested that the ability of *L. acidophilus* to deconjugate bile acids may be an important factor in helping control serum cholesterol levels.

Antitumor Compounds

Several studies have indicated that *L. acidophilus* may be inhibitory toward some types of cancer. Review articles by Sellars (29) and Gilliland (61) sum up this information.

Sellars (29) reports in his review article that some fecal enzymes produce toxic compounds which are potentially carcinogenic. *L. acidophilus* has been shown to reduce the formation of these carcinogens. He also notes the epidemiological evidence that countries which consume a large amount of acidophilus products have a lower rate of colon cancer.

Gilliland (61) noted other studies that indicate *L. acidophilus* is inhibitory toward some types of cancer. *L. acidophilus* could be antagonistic toward certain bacteria which produce carcinogenic substances. He reviewed studies which reported a reduced amount of proliferation of tumor cells in rats injected with tumor inducing substances when their diets were supplemented with *L. acidophilus*.

The potential health benefits found to be associated with *L. acidophilus* indicate that Metchnikoff's theory was at least partially correct. In order to realize these benefits, cultures of *L. acidophilus* must be selected carefully. The selection process should be based on the desired characteristic for which the culture will be used (32).

Once the appropriate culture is selected it must be propagated and stored under conditions which retain viability and desirable characteristics.

Available Products

Several products containing *L. acidophilus* are available to consumers. These include fermented dairy products, liquid or dried cultures and nonfermented acidophilus milk. However, not all products purchased may contain the indicated organisms (24).

Fermented acidophilus milk has an extremely sour taste produced from the *L. acidophilus* which discourages its consumption (41). It is normally consumed in Scandinavian and Eastern European Countries (61). In the United States a milder, less harsh tasting product would be more acceptable.

Health food stores and pharmacies sell freeze-dried or liquid products claiming to contain *L. acidophilus*. Many times the viability of these cultures are low, and the strains are not selected on the bases of host specificity or biological activity (24).

A commercially available product call "Lactinex" is a dried product containing *L. acidophilus* ATCC 4962 and *L. bulgaricus* ATCC 33404. This product has been evaluated in two separate studies to determine if it would reduce the incidence of travellers diarrhea (55, 9). Results indicated that the product did not prevent or reduce the duration of travellers diarrhea. It may be that the cultures were not selected based on host specificity or ability to inhibit organisms responsible for causing the diarrhea.

A relatively new product is nonfermented acidophilus milk which contains cells of *L. acidophilus* as a dietary adjunct. Cells of the culture are simply added to pasteurized lowfat milk. Thus the product has the appearance and taste of regular lowfat milk. Gilliland (25) reported the desirable characteristics which should be possessed by such a culture for use as a dietary adjunct in milk. These characteristics

included, the organism should be normally found in the human intestinal tract, produce the desired effects in the intestinal tract and remain viable during storage of the product. Nonfermented acidophilus milk is probably the most convenient and organoleptically acceptable acidophilus product available in this country. Again, it is very important that the cultures used to prepare acidophilus milk are of human origin, have a high level of biological activity to produce the desirable action and are propagated and stored under conditions which will maintain the viability and activity.

Development of Frozen Concentrated Cultures

In the past, cultures used to inoculate dairy products were purchased in a liquid form. These liquid cultures were propagated in an appropriate medium and transferred daily until maximum activity was reached. The cultures were then used to produce bulk cultures which could be used to inoculate a large amounts of milk (16). This method was usually successful but had several drawbacks.

Daily transfers and propagation required a great deal of time and media. Also, contamination of the culture was sometimes difficult to avoid using this technique. Furthermore, researchers Simmons and Graham (63) reported that the desirable balance among strains in a mixed strain cultures could be lost after several propagations.

These drawbacks led to the development of a frozen concentrated cultures which would be added directly into milk to be cultured without any daily propagation. Accolas and Auclair (2) described a method for preparing a frozen concentrated culture of lactic streptococci. They propagated cells at a constant pH, harvested them by centrifugation, and resuspended the cells in a solution of glycerol in water. The cultures were stored at -30^{o}C and they maintained viability and acid producing activity for several months. Concentrated cultures can also be made by lyophilization but

research indicates that these cultures are not as viable and active as frozen concentrated cultures (57, 3). Several advances have been made on this technique and will be discussed in the next section of this review.

Preparation of Frozen Concentrated Cultures

Several factors must be considered when preparing a frozen concentrated culture. Among these factors are growth requirements, storage conditions and production procedures. Extensive research has been conducted to determine the conditions which will produce a concentrated culture with the greatest storage stability.

Growth Media

Growth media needed for producing bacterial cells for concentrated cultures of lactobacilli may be very complex. The media not only provides the nutritional requirements for the cells but may also need to provide ingredient which contribute to the storage stability of the concentrated culture.

An important ingredient to provide both nutrients for growth and cells that will survive freezing is Tween 80. Growth of lactobacilli was increased when oleic acid was added to the growth media (5, 35). Tween 80 can serve as a source of oleic acid. Briggs (6) reported a significant increase in growth of lactobacilli when Tween 80 was added to tomato juice and casein digest media. Several years later, frozen cultures becoming more popular, Calcott and Postgate (7) observed that Tween 80 protected *Aerobacter aerogenes* from freezing damage but the mechanism was not reported. Smittle et al. (64) investigated concentrated cultures prepared from cells of *Lactobacillus bulgaricus* propagated with and without Tween 80 and stored in liquid nitrogen. Following freezing in liquid nitrogen, survival of the cells which had been propagated in media containing Tween 80 was significantly higher than the cells grown

without it. Sodium oleate portion of Tween 80 was responsible for the improved survival of the cultures (64). Cells grown in its presence had a higher concentration of a nineteen carbon cyclopropane fatty acid in the lipid portion of the cells compared to cells grown without it. The cellular content of the cyclopropane fatty acid was directly related to the improved survival of the cultures in liquid nitrogen.

Tween 80 is also responsible for morphological changes in lactobacilli. Rogosa and Mitchell (58) observed that when Tween 80 was added to an agar growth medium, smooth shaped colonies were predominantly formed. Without Tween 80, rough shaped colonies predominated. Wright and Klaenhammer (70) reported that when *L. acidophilus* was propagated in MRS broth containing Tween 80, smooth shaped colonies grew on agar plates. Rough shaped colonies formed when the Tween 80 was omitted. The cultures that formed rough colonies were less tolerant of freezing at -20°C than those that formed the smooth shaped colonies.

A similar phenomenon was observed when calcium was added to the growth medium. Wright and Klaenhammer (70) reported a five fold decline in numbers of viable *L. bulgaricus* after storage at -20°C when the cells had been grown in the presence of calcium compared to a 200 fold decrease when the cells were grown without calcium. Abraham et al. (1) also observed that calcium improved the viability of frozen and thawed cells of *L. bulgaricus*. Wright and Klaenhammer (70) reported that the calcium supplementation caused a morphological change in the cells. Cells grown in the presence of calcium were transformed from filamentous to bacilloid rods. The bacilloid rods were more resistant to freezing than the filamentous rods.

In addition to contributing to the storage stability of the culture, the media may need to contain ingredients to induce certain desirable characteristics of the culture. If a culture is prepared for use in a fermented product, acid production is very important. Gibson et al. (18) reported that malic acid was an essential ingredient in the growth medium for lactic streptococci in order to stimulate acid production. In another study,

Gilliland et al. (20) observed that citrate was required in the growth medium of *Leuconostoc citrovorum* in order for concentrated cultures of the organism to produce diacetyl when added to acidified milk.

Although *L. acidophilus* does not produce acid when it is used as a dietary adjunct to produce nonfermented acidophilus milk there is an inducible enzyme very much dependent upon the growth medium. This enzyme is ß-galactosidase which is very important when the culture is being used to improve lactose utilization in persons with lactose maldigestion. Nielson and Gilliland (49) reported ß-galactosidase activity is induced by the presence of either lactose or galactose in the growth medium. Because the cells are not allowed to grow in the nonfermented acidophilus milk, it is important that the growth medium used to propagate the cells to be used in making the concentrated culture contain lactose.

Storage Conditions

The storage conditions used for concentrated cultures are responsible in part for the amount of viability preserved during storage. Research has focused on freezing temperature, freezing rate and the presence of cryoprotective agents in the cell suspension.

In the 1960's liquid nitrogen became readily available and researchers focused on its use to freeze and store concentrated cultures. Lactic cultures frozen at -196°C (liquid nitrogen) survived significantly better than those stored at higher temperatures, 20-23.3°C (10, 11, 18, 66). Tsvetkov and Shishkova (66)also reported that lactic streptococci were more resistant to freezing than lactic acid producing rods. Peebles et al. (53) reported that concentrated cultures of *S. cremoris* could be stored 80 days in liquid nitrogen without significant loss in viability (53). Concentrated cultures of *L. acidophilus* also can be stored at -196°C with no loss of viability or activity (28, 31, 48).

Nonfermented acidophilus milk is stored at 5-7°C, after it is prepared using a concentrated culture. It is held at that temperature for storage and distribution until purchased by the consumer. Therefore, cultures of *L. acidophilus* must maintain viability not only during frozen storage, but also during subsequent refrigerated storage in the milk product.

Gilliland and Lara (28) investigated the effect of frozen and subsequent refrigerated storage on three strains of *L. acidophilus*. All three strains of *L. acidophilus* maintained the same level of viability and ß-galactosidase activity during 28 days of storage at -196°C. On the other hand, after the concentrated cultures were used to prepare nonfermented acidophilus milk, both the viability and enzymatic activity were significantly reduced during 14 days of storage at 5°C. The study also indicated that the frozen storage of the culture -196°C up to 28 days had no effect of the subsequent reduction in viability at storage at 5°C. Gilliland and Rich (31), in a study to evaluate the influence of pH during growth on stability of *L. acidophilus* during frozen and subsequent refrigerated storage reported no significant declines in viability of two strains of *L. acidophilus* during storage at -196°C. The cells which had been propagated at pH 5.0, however were significantly more stable during storage at 5°C in milk than were those which had been grown at pH 5.5, 6.0 or 6.5.

Production Procedures

Several studies have focused on the methods used to propagate cells to be stored as frozen concentrated cultures. The parameters of production procedures include pH control of the media, type of neutralizer used, and amount of growth allowed before cells are harvested.

Peebles et al. (53) indicated that cultures of *Streptococcus cremoris* grown at constant pH of 6.0 reached a population 15 times higher than those grown without pH control. Gilliland and Speck (22) reported that cultures of lactic streptococci grown at

pH 6.0 maintained viability during frozen storage at -17°C better than those grown without pH control.

Several studies have compared the effect different pH have on the cultures. Gilliland et al. (20) and Peebles et al. (53) reported no difference in the maximum population attainable at pH levels of 5.5-7.0. Peebles et al. (53) observed that the culture reached maximum population much sooner when it was grown at pH 5.0, but the maximum population was reached more slowly than that at the other pH levels. Gilliland and Rich (31) compared the viability during frozen and subsequent refrigerated storage of *L. acidophilus* which had been grown at pH 5.0, 5.5, 6.0, 6.5 and 7.0. They observed that cultures grown at pH 5.0 did not exhibit significant decreases in viability during 28 days of storage in liquid nitrogen at -196°C nor during 21 days of subsequent storage at 5°C. While the cultures grown at the other pH levels did not lose viability during 28 days at -196°C they all decreased significantly during subsequent storage in milk for 21 days at 5°C.

The type of neutralizer used to maintain the pH of the media during growth of lactic acid bacteria also can play an important role in the production of cell crops for preparing concentrated cultures. Researchers have compared sodium hydroxide and ammonium hydroxide and their effects on concentrated cultures. Peebles et al. (53) observed that populations of *S. cremoris* grown at pH 6.0 were 1.5 to 2.5 times higher when ammonium hydroxide was the neutralizer for maintaining constant pH than when sodium hydroxide was used as the neutralizer. Gyorgy (34) noted that NH_4^+ was stimulatory to the growth of lactic cultures. Peebles et al. (53) reported that this could possibly be the reason higher populations were reached with ammonium hydroxide than with sodium hydroxide.

Another neutralizer which has been studied is calcium carbonate. Rudnick et al. (59) reported that lactic cultures grown in the presence of calcium carbonate had better viability after frozen storage than those grown without it. Longsworth and MacInnes

(45) reported that carbon dioxide was essential for the growth of *L. acidophilus*. Mitchell and Gilliland (48) utilized this information when they used a neutralizer containing 20% sodium carbonate and 20% ammonium hydroxide which would allow carbon dioxide to be released slowly during growth of the culture at pH 6.

A parameter which has been given very little attention is the amount of growth allowed before the cells are harvested. Stapert and Dokolski (65) observed that viable populations of *L. leichmannii* harvested in the log phase of growth were reduced by two-thirds during frozen storage. Normally, cells are harvested in the late logarithmic/early stationary phase since this is when the maximum population is reached (10, 11, 65, 4). Peebles et al. (53) reported that after the cells of *S. cremoris* entered the stationary phase, acid was still produced. This indicated that lactose was being converted to lactic acid by the cells, but the energy was not being used for cell division. This and other studies indicated that a slimy, viscous material was present in the cell pellets after centrifugation which was not present on pellets of cells harvested in the late log phase (2, 37). It is possible that this is capsular material. Gilliland and Speck (22) reported that as the amount of capsular material in a cell increased, the percentage of suriviors increased. However, others reported either no difference, or less growth from cells of lactic streptococci harvested later in the stationary phase compared with those harvested in the early stationary phase (53, 46, 42, 64).

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CHAPTER III

INFLUENCE OF HARVEST TIME ON STABILITY OF CELLS OF *LACTOBACILLUS ACIDOPHILUS* DURING FROZEN AND SUBSEQUENT REFRIGERATED STOAGE

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ABSTRACT

Concentrated cultures of 3 strains of *Lactobacillus acidophilus* were monitored for numbers of viable and bile tolerant lactobacilli, ß-galactosidase activity, and cholesterol assimilating acitivity after 0, 1, 2, 3, and 4 weeks of storage at -196°C. Nonfermented acidophilus milk prepared following 28 d at -196°C for each culture was monitored in a like manner afer 0, 7, 14, 21, and 28 days of storage at 7°C. Cells for the concentrated cultures were propagated in PMN broth maintained at pH 5 and harvested in both the late logarithmic phase of growth and six hours into the stationary phase. Concentrated cultures were prepared from each age of cells. No decrease in numbers of viable or bile tolerant lactobacilli, ß-galactosidase activity, or cholesterol assimilating activity was observed for any strain during storage at -196°C. Although there were variations in biological activity among the three strains. There were no significant differences between the two harvest times during storage at this temperature. When the cells were suspended in milk and stored at 7°C, the numbers of viable and bile tolerant lactobacilli declined over time as did the ß-galactosidase activity and the amount of cholesterol assimilated. However, the cells from *L. acidophilus* 223 harvested six hours into the stationary phase decreased significantly less in total numbers during storage at 7°C than did the cells harvested in the late logarithmic phase. There were little or no differences in the decline in viability between the two harvest times for the other two strains. All strains showed significant decreases in ß-galactosidase activity and cholesterol assimilation during storage in milk at 7°C with no differences between the two harvest times.

INTRODUCTION

As a dietary adjunct *Lactobacillus acidophilus* can provide potential health benefits to consumers. Persons who are lactose maldigestors digest the lactose in products containing *L. acidophilus* more easily than in products without it (4). This is probably due to the ß-galactosidase produced by *L. acidophilus*. Also, preliminary studies suggest that *L. acidophilus* has the ability to assimilate cholesterol thus decreasing serum cholesterol levels of persons consuming the cells (5, 9, 10). These benefits illustrate the need to provide consumers with a readily available product containing viable and active cells of *L. acidophilus*.

Currently one source of *L. acidophilus* provided to consumers is in the form of nonfermented acidophilus milk. This product is prepared by adding a frozen concentrated culture of the organism to pasteurized lowfat milk and then storing the milk at refrigeration temperature. In order for this product to provide adequate numbers of lactobacilli, the cells must survive frozen and subsequent refrigerated storage.

Previous studies indicate that several factors can influence stability of the cells during storage. Among these factors are the strain of *L. acidophilus* used (3, 6, 8), the media used to produce the cell crops (16), and the pH at which the cells are propagated (8). Research in this laboratory has indicated that after a cell population reaches the log phase, the cells continue to produce acid which indicates they are still using lactose as an energy source. Other research reports suggest that a slimy, viscous material is present on cells of some lactic acid bacteria harvested later in the stationary phase (1, 2). The objective of this study was to determine if harvesting cells of *L. acidophilus* in

the late log phase versus later in the stationary phase of growth affected the viability, bile resistance, ß-galactosidase activity, and cholesterol assimilation of three strains of *L. acidophilus* during frozen and subsequent refrigerated storage.

MATERIALS AND METHODS

Bacterial Growth Media

A peptonized milk nutrient (PMN) medium was used for propagation and enumeration of *Lactobacillus acidophilus*. The broth medium used for propagation of cell crops of *L. acidophilus* contained 5% Peptonized Milk Nutrient (PMN Humco Sheffield), 2% lactose, 2% primatone (Humco Sheffield), .1% yeast extract and .1% polysorbitan monooleate (Tween 80; Sigma Chemical Co., St. Louis, Mo.). A PMN agar medium was prepared by adding 1.5% agar to PMN broth and was used to enumerate the lactobacilli. Bile resistant lactobacilli were enumerated using PMN agar supplemented with .15% oxgall (PMNO agar). All media were sterilized by heating 15 minutes at 121°C.

Source and Maintenance of Cultures

Three strains of *L. acidophilus* from the laboratory stock culture collection were used in this study. The identities of *L. acidophilus* 606, *L. acidophilus* 107, and *L. acidophilus* 223 were confirmed in an earlier study in our laboratory (11).

All three strains were maintained by subculturing in PMN broth. One percent inocula and 18 hours incubation at 37°C were used routinely for subcultures. All cultures were stored at 2°C between transfers. Three subcultures were performed immediately before a culture was used experimentally.

Production of Cell Crops

The procedure for the production of the cell crops is similar to that reported by Gilliland and Rich (8). The cells were propagated at 37°C and pH 5.0 in a New Brunswick 7.5 l fermentor which was equipped with a combination pH electrode connected to an automatic pH control unit (New Brunswick Scientific Co., Inc. Edison NJ) and a stirrer for constant agitation of the medium. The neutralizer for automatic control of pH was 20% sodium carbonate in 20% ammonium hydroxide. The sodium carbonate (30 % aqueous) solution was sterilized by heating 15 min at 121°C prior to mixing with sufficient concentrated ammonium hydroxide to yield 20%.

The sterile fermentor was placed into a water bath maintained at 37°C by an Isotemp Immersion Circulator (Fisher Scientific, Pittsburgh Pa.). Four liters of sterile PMN broth were aseptically added to the fermentor. After the broth temperature reached 37°C, the pH control unit was calibrated and the neutralizer pump (New Brunswick Scientific Co., Inc. Edison, NJ) was adjusted to maintain the pH at 5.0.

The broth was inoculated with 40 ml (1%) of a freshly prepared 18 hour PMN broth culture of the desired strain of *L. acidophilus*. One liter samples were aseptically removed at 16 and 22 hours for *L. acidophilus* 107 and at 18 and 24 hous for *L. acidophilus* 223 and 606. The sample times were selected to coincide with the late log phase of growth and six hours into the stationary phase for each culture. The appropriate sample times for each culture were determined in preliminary experiments for each culture in which populations were determined hourly. The broth culture was placed in an ice-water mixture for 30 minutes to stop growth.

Preparation of Frozen Concentrated Cultures

The preparation of frozen concentrated cultures was similar to the method described by Gilliland and Lara (6). Bacteria cells were harvested from the broth culture by centrifugation for 20 minutes at 4000 x g and 0-1°C. The cells were resuspended in twice their weight of cold (0-1°C) sterile 10% reconstituted nonfat dry milk (NDM) to yield a population of approximately 2 x 10^{10}/g. This concentrated culture was aseptically dispensed in two g aliquots into sterile 2 g cryogenic vials held in an ice-water bath. All vials but one were submerged into liquid nitrogen for storage at -196°C.

The remaining vial was held in an ice-water mixture and analyzed within 2 hours (Day 0). Additional vials were removed from the liquid nitrogen on days 7, 14, 21, and 28 for analysis to permit measurement of the influence of frozen storage on the culture. The cultures were thawed by placing each frozen vial in 35 ml of warm (40°C) tap water for 10 minutes. The analyses included measurement of total numbers of lactobacilli and of numbers of total bile resistant lactobacilli as well as the ability of the cultures to assimilate cholesterol. ß-galactosidase activity also was measured.

Preparation and Storage of Nonfermented Acidophilus Milk

Following 28 days of storage of the concentrated culture in liquid nitrogen, sterile (121°C for 15 minutes), reconstituted 10% NDM was used to make nonfermented acidophilus milk. An appropriate amount (diluted as necessary with peptone ditution blanks) of the thawed concentrated culture was added to the milk to yield a population of approximately 2 x 10^7 lactobacilli per ml in the nonfermented milk. Four bottles, each containing 100 ml of the nonfermented milk were stored at 7°C. A bottle was removed on days 0, 7, 14, 21, and 28 for analyses. (Day 0 was the

day the nonfermented acidophilus milk was prepared). The nonfermented acidophilus milk samples were evaluated for the ability of the culture to assimilate cholesterol and for ß-galactosidase activity. The samples were also evaluated for the total numbers of lactobacilli and for numbers of bile resistant lactobacilli.

Measurement of Total Numbers of Lactobacilli and of Numbers of Bile Resistant Lactobacilli

To measure the total numbers of *L. acidophilus*, appropriate dilutions were prepared according to the methods describe in the *Compendium of Methods for the Microbiological Examination of Foods* (15) and plated by the pour plate method with PMN Agar. Numbers of bile tolerant lactobacilli were measured by plating the appropriate dilutions with PMNO agar. All plates were incubated at 37°C for 48 hours. All colonies visible with the aid of a Quebec colony counter were counted.

Measurement of ß-galactosidase Activity

Appropriate dilutions of the sample to be analyzed were made with cold (1-2°C) phosphate buffer to yield a population of 2×10^6 lactobacilli per ml. The diluted samples were used for the evaluation of enzyme activity. 0rtho-nitrophenyl ß-D-galactopyranoside (ONPG) was used as a substrate to measure the activity of the enzyme. The samples were evaluated for ß-galactosidase activity according to the procedure reported by Gilliland and Lara (6).

Assimilation of Cholesterol

One ml portions of an appropriate dilution of the sample were inoculated individually into tubes containing 10 ml of MRS broth (Difco Laboratories, Detroit MI)

supplemented with 2% sodium thioglycollate, .3% oxgall, and 10% (v/v) cholesterol micelles (containing approximately 1 mg cholesterol/ml). The cholesterol micelles were prepared according to the method of Razin et al. (13). Cultures were incubated at 37°C for 19 hours. Cells were removed by centrifugation for 10 minutes at 12,000 x g. The spent broth was collected and analyzed for cholesterol using the method described by Rudel and Morris (14). Uninoculated control broth also was analyzed for cholesterol by the same method. An earlier study showed that cholesterol removed from the broth during growth was recovered with the cells (5). Thus the difference in cholesterol content of the uninoculated control broth and the spent broth was taken as the amount assimilated by the culture.

Statistical Methods

Analysis of variance for each set of data was conducted as a split plot in a randomized block design to determine whether significant differences existed. Each fermentation was a block, harvest time was the main unit treatment, and days of storage was the subunit treatment. Least significant difference analyses were used to compare means for significant differences at the 5% level of confidence.

RESULTS

Effect of Storage at -196°C

Storage at -196°C had practically no effect on any of the three strains of *L. acidophilus* (Table 1). There were no significant ($P > .05$) declines in total numbers of lactobacilli nor numbers of bile tolerant lactobacilli. The levels of ß-galactosidase activity and amounts of cholesterol assimilated also did not decline for any of the three strains evaluated. There were also no significant ($P > .05$) differences between the two harvest times for any of the three strains for any of the evaluations. There were apparent differences among the the strains in both the ß-galactosidase activity and the amount of cholesterol assimilated. The ß-galactosidase activity of strains 107 and 223 was similar while strain 606 had less activity than the other two. Strain 606 assimilated the most cholesterol. Strains 107 assimilated an intermediate amount while strain 223 assimilated the smallest amount of cholesterol.

Effect of Storage at 7°C

Total and Bile Tolerant Lactobacilli

Total numbers of lactobacilli in nonfermented acidophilus milk prepared using *L. acidophilus* 107 declined significantly ($P < .05$) with increased storage time at 7°C regardless of harvest time (Table 2). There were significantly ($P < .05$) lower numbers of lactobacilli after 7 days of storage at 7°C for both harvest times. Viable numbers of cells continued decreasing significantly through day 21 for cells harvested in the stationary phase of growth and day 28 for both harvest times. There were no

significant differences ($P > .05$) in numbers of lactobacilli between the two harvest times at any day of analysis. The numbers of bile tolerant lactobacilli in the nonfermented acidophilus milk containing *L. acidophilus* 107 declined in a similar manner as observed for total numbers of lactobacilli. There were no apparent differences between the numbers of bile tolerant lactobacilli and total lactobacilli for this strain. There were no significant differences ($P > .05$) in numbers of bile tolerant lactobacilli between the two harvest times on any day of analysis.

The total numbers of lactobacilli in nonfermented acidophilus milk made from cells of *L. acidophilus* 606 did not decline as rapidly as observed for *L. acidophilus* 107 (Table 2). The numbers of viable and bile resistant lactobacilli did not decline significantly ($P < .05$) during the first 21 days of storage for either harvest time. However, the numbers from both harvest times were significantly ($P < .05$) lower than on day 0 after 28 days of storage at 7°C. There were no significant differences ($P > .05$) between the two harvest times or between numbers of viable and bile tolerant lactobacilli at any day of analysis.

The total numbers of lactobacilli for *L. acidophilus* 223 from both harvest times did not decline significantly ($P > .05$) during 28 days of storage at 7°C (Table 2). There were no significant differences ($P > .05$) in the total numbers of *L. acidophilus* 223 between the two harvest times on any day of storage. The numbers of bile tolerant lactobacilli from both harvest times exhibited a pattern similar to those observed for the total numbers of lactobacilli. There was no apparent difference between numbers of viable and bile tolerant lactobacilli for either harvest time.

Although there were no significant declines in total numbers of *L. acidophilus* 223 during the 28 day storage period at 7°C, there was a trend toward lower numbers as storage time increased. Thus, the storage period for this strain was extended for an additional 35 days (total storage time of 63 days) to see how long the cells would remain viable (Figure 1). The trend toward decline of total numbers of cells harvested

in the stationary phase continued throughtout the 63 days of storage. It became significant ($P<.05$) on day 56. The numbers of cells harvested from the late log phase began a dramatic decline following 28 days of storage. The numbers on day 35 were significantly lower ($P<.05$) than on day 0. The decline continued throughout the 63 days of storage.

ß-galactosidase Activity

Cells of *L. acidophilus* 107 had the highest ß-galactosidase activity of the three strains evaluated. The initial ß-galactosidase activity of cells harvested in the stationary phase was significantly ($P<.05$) higher than for those harvested from the late log phase. The activity of cells of *L. acidophilus* 107 from both harvest times decreased with increased storage time (Table 3). The decrease during the first 7 days of storage for cells from the stationary phase was much greater than for those from the late log phase. The decline for the latter did not become significant ($P<.05$) until day 14 of storage while that for the former was significant on day 7. Although the initial level of ß-galactosidase activity was higher for cells from the stationary phase than for those from the late exponential phase, after 7 days of storage there were no differences ($P>.05$) between the 2 harvest times.

The ß-galactosidase activity of *L. acidophilus* 606 declined slower than did that of *L. acidophilus* 107 (Table 3). Declines became significant ($P<.05$) on day 28 for cells from both harvest times. There were no significant differences ($P>0.5$) between the two harvest times on any day during storage.

The decline in ß-galactosidase activity for cells of *L. acidophilus* 223 was similar for both harvest times. The declines became significant ($P<.05$) on day 21 when compared to day 0 for both harvest times (Table 3). There were no significant differences ($P>.05$) between the two harvest times at any day of analysis.

Cholesterol Assimilation

The relative ability to assimilate cholesterol by all strains of *L. acidophilus* stored in milk at 7°C decreased with increased storage time (Table 4). There was apparent variability in both the amount of cholesterol assimilated and in the amounts of decline among the three strains. Compared to day 0, the decline for cells of *L. acidophilus* 107 harvested in the late log phase of growth was significant ($P < .05$) after 14 days of storage at while that for cells harvested in the stationary phase was significant after only 7 days of storage. After 7 days of storage, cells harvested in the stationary phase of growth took up significantly less cholesterol ($P < .05$) than cells harvested in the late log phase. However, there were no there significant differences ($P > .05$) between the two harvest times at any other day of analysis.

The declines in assimilation of cholesterol by *L. acidophilus* 606 were similar to those observed for *L. acidophilus* 107. Cells harvested in both the late log phase and the stationary phase exhibited significant ($P < .05$) declines in assimilation of cholesterol after 14 days of (Table 4). Cells harvested in the stationary phase took up significantly more cholesterol ($P < .05$) after 28 days of storage than those harvested in the late log phase of growth. The amount of cholesterol assimilated by cells of this culture from the stationary phase was greater ($P < .05$) on day 28 than on day 21. This was consistently observed in all three replicate trials for this strain. It did not occur for the other two strains.

The cells of *L. acidophilus* 223 harvested in the late log phase took up significantly less cholesterol ($P < .05$) after 7 days of storage at 7°C than on day 0 (Table 4). The amount assimilated by cells harvested in the stationary phase did not exhibit significant ($P < .05$) declines until after 21 days of storage. There were no significant differences between the two harvest times at any day of analysis.

DISCUSSION

The lack of declines in numbers of viable lactobacilli, ß-galactosidase activity, and ability to assimilate cholesterol during storage at -196°C indicates that all three strains of *L. acidophilus* were stable under these conditions. Storage of frozen concentrated cultures of *L. acidophilus* at -196°C resulted in little loss of viability or activity in previous studies (6, 8). On the other hand, in the present study, there were significant declines in stability of cells of all three strains from both harvest times during storage in milk at 7°C. Previous reports on the stability of *L. acidophilus* support the observation that storing at refrigeration temperature is more damaging than storage at -196°C (9, 11).

Considerable differences in both the decline of viability and activity were observed among the three strains regardless of harvest time during storage at 7°C. While statistical comparisons were not made among the three strains, apparent differences were noted. Gilliland and Rich (8) observed differences in the numbers of viable lactobacilli during storage in milk at 5°C between two strains of *L. acidophilus*. In their study, cells grown at different levels of pH exhibited differences with respect to stability during storage at 5°C. Although there was a trend toward fewer numbers, both strains survived significantly better in milk at 5°C when they had been grown at pH 5. All three strains used in the present study had similar initial populations, but as storage time at 7°C increased the differences in the numbers of viable lactobacilli among the strains become apparent. Storage at 5°C was most damaging to *L. acidophilus* 107 while strains 606 and 223 maintained relatively high numbers.

While there were no significant differences between the two harvest times for any of the three strains, there was a trend toward better survival during the 28 days of

storage at 7°C for all three strains for cells harvested from the stationary phase compared to those harvested from the late exponential phase. However, when the storage time was extended for strain 223, cells harvested in the late log phase declined in total numbers faster than those harvested in the stationary phase. Thus, there is an indication of a benefit to harvesting cells of *L. acidophilus* in the stationary phase to prepare frozen concentrated cultures for use in making nonfermented acidophilus milk. This is especially true if the milk is to be subjected to long storage periods. The improved stability of the cells could be attributed to the formation of cellular capsular material by the lactobacilli during the stationary phase. Gilliland and Speck (11) reported that as the amount of capsular material associated with the cells of lactic streptococci increased, the percentage of the survivors increased. Peebles et al. (12) noted that cells of lactic streptococci growing in a medium maintained at pH 6 continued to produce acid, an indication of energy utilization, well into the stationary phase even though there was no increase in growth. They reported as did Accolas and Auclair (1) that the cell pellet of the streptococci harvested by centrifugation in the late stationary phase was slimy and viscous while cells harvested in the late log phase lacked this characteristic. This was attributed to capsular material which was thought to be responsible for the better survival of the cells harvested in the stationary phase.

L. acidophilus 107 exhibited much greater loss in viability during the 28 days of storage at 7°C than did the other two strains, but the decline in ß-galactosidase activity was more comparable to that of strains 223 and 606. Because it is not necessary that the cells grow to exhibit ß-galactosidase activity, it is possible that even though *L. acidophilus* 107 may have had much reduced viability following storage, the enzyme remained active. Gilliland and Lara (6) evaluated three strains of *L. acidophilus* for viability and ß-galactosidase activity during storage at -196°C and subsequent storage in nonfermented acidophilus milk at 5°C. They grew the cell crops in PMN broth at pH 6 and the cultures survived well during storage in liquid nitrogen. However, two of

the three strains exhibited significant declines in viability after only 7 days of storage at 5°C, and the other strain declined significantly after 14 days. The ß-galactosidase activity of all three strains declined with increased storage time in a manner similar to the declines in viability. In the present study, with the exception of *L. acidophilus* 107, declines in ß-galactosidase activity were similar for cells from both the late log phase and the stationary phase throughout storage. For strain 107, even though the initial ß-galactosidase activity for cells from the stationary phase was significantly greater than that of cells harvested from the late log phase, activity declined rapidly during the first seven days of storage. The ß-galactosidase activity of cells from both harvest times was then similar for the remainder of the storage period. This difference was not observed for strains 223 and 606. Thus, there appears to be no benefit from extended incubation beyond the late log phase to produce cells for concentrated cultures of *L. acidophilus* to ensure maintenance of ß-galactosidase activity during refrigerated storage in nonfermented acidophilus milk.

The amount of cholesterol assimilated by the cells decreased with increased storage time regardless of harvest time. The decline in uptake was probably related to the declines in numbers of viable cells. Although there were no significant declines as storage time at 7°C increased in numbers of viable lactobacilli for two of the strains (223 and 606), there was a trend toward fewer numbers as storage time at 7°C increased. Gilliland et al. (5) reported that in order for cells of *L. acidophilus* to assimilate cholesterol in vitro, they must be growing in the presence of bile under anaerobic conditions. Thus a decrease in numbers of viable cells during storage would result in apparent reduced uptake of cholesterol because a fixed incubation time was used in the assays. Also, even though there are viable cells, as indicated by growth on PMN agar, they may not grow as well in the assay broth containing bile following storage at 7°C. Because of this the reductions in relative ability to assimilate

cholesterol also might have resulted from poorer growth of the lactobacilli in the presence of bile.

There were variations in the initial amount of cholesterol assimilated and the decline in the amount assimilated among the three strains. Other studies reported similar variations among strains thus emphasizing the importance of selecting the appropriate strain to be used as a dietary adjunct (5, 7).

In summary, extending the incubation of cultures of *L. acidophilus* being grown at pH 5 six hours into the stationary phase prior to harvesting cells for preparation of frozen concentrated cultures has no great advantage over harvesting them in the late exponential phase. This is especially true if the concentrated cultures are to be used for making nonfermented acidophilus milk which likely would not need a shelf-life greater than 28 days. Harvesting cells in the stationary phase of growth could increase the survival of *L. acidophilus* added to products subjected to longer storage times such as yogurt and frozen yogurt. However, if longer storage periods are necessary at 7°C, there appears to be an advantage of harvesting cells from the stationary phase over the late log phase. This research illustrates the importance of careful selection of the strain of *L. acidophilus* because there were variations among strains in survival, ß-galactosidase activity and relative ability to assimilate cholesterol following storage at 7°C.

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Table 1. Effect of storage at -196°C on three strains of *Lactobacillus acidophilus*

Assay	Days at -196°C	*L. acidophilus* 107		*L. acidophilus* 606		*L. acidophilus* 223	
		Late Log [1]	Stationary[1]	Late Log	Stationary	Late Log	Stationary
Total Numbers of Lactobacilli[2]	0	10.54	10.45	10.43	10.41	10.77	10.78
	28	10.55	10.45	10.45	10.43	10.82	10.77
Numbers of Bile Tolerant Lactobacilli[2]	0	10.58	10.56	10.42	10.34	10.84	10.79
	28	10.47	10.39	10.29	10.50	10.79	10.67
ß-galactosidase Activity[3]	0	.16	.14	.09	.09	.13	.15
	28	.16	.13	.09	.10	.14	.15
Cholesterol Assimilation[4]	0	46.64	46.95	53.19	51.87	39.51	37.50
	28	48.28	48.12	51.40	49.91	38.46	35.68

[1]Time of harvest of cells for preparation of concentrated culture; Late Log = late log phase of growth, Stationary = stationary phase of growth; Each value represents the mean from three trials.

[2]Reported as Log_{10} colony forming units/ml

[3]Reported as μmoles o-nitro-phenol released/min/ml

[4]Reported as μg cholesterol assimilated/ml in 19 hours

Table 2. Influence of storage in milk at 7°C on total and bile resistant numbers of three strains of *Lactobacillus acidophilus*

Strain	Days at 7 C	PMN[2]		PMNO	
		Late Log[1]	Stationary	Late Log	Stationary
107	0	7.76[a]	7.44[a]	7.73[a]	7.43[a]
	7	6.06[b]	6.44[b]	6.43[b]	6.14[b]
	14	5.91[b]	6.25[b]	5.46[c]	5.62[bc]
	21	5.56[b]	5.49[c]	4.80[d]	5.19[c]
	28	4.91[c]	4.75[d]	4.64[d]	4.45[d]
606	0	7.99[a]	7.88[a]	7.85[a]	7.87[a]
	7	7.73[ab]	7.64[ab]	7.70[ab]	7.70[ab]
	14	7.74[ab]	7.74[ab]	7.71[ab]	7.62[ab]
	21	7.74[ab]	7.76[ab]	7.74[ab]	7.66[ab]
	28	7.54[b]	7.48[b]	7.47[b]	7.29[b]
223	0	7.61[a]	7.76[a]	7.61[a]	7.59[a]
	7	7.53[a]	7.71[a]	7.53[a]	7.65[a]
	14	7.50[a]	7.77[a]	7.29[a]	7.61[a]
	21	7.31[a]	7.47[a]	7.18[a]	7.54[a]
	28	7.48[a]	7.58[a]	7.43[a]	7.43[a]

[1]Time of Harvest of cells for preparation of concentrated culture; Late Log=late log phase of growth: Stationary=stationary phase of growth

[2]Each value represents the mean of three trials; Numbers with different superscripts within one strain, one harvest time, and one assay differ significantly ($P<.05$); Reported as Log_{10} colony forming units/ml.

[3]There were no significant differences ($P>.05$) between the two harvest times at any day of analysis for any of the three strains.

Table 3. Influence of storage of nonfermented acidophilus milk at 7°C on ß-galactosidase activity of three strains of *Lactobacillus acidophilus*

Strain	Days at 7°C	μmoles ONP released/min/ml[1]	
		Late Log[2]	Stationary[2]
107	0[3]	.16[a]	.27[a]
	7	.14[ab]	.13[b]
	14	.12[bc]	.13[b]
	21	.08[d]	.10[b]
	28	.09[cd]	.10[b]
606	0	.12[a]	.10[a]
	7	.10[a]	.08[a]
	14	.11[a]	.09[a]
	21	.10[a]	.08[a]
	28	.07[b]	.07[b]
223	0	.09[a]	.09[ab]
	7	.08[ab]	.10[a]
	14	.08[ab]	.07[bc]
	21	.06[bc]	.05[c]
	28	.05[c]	.05[c]

[1]Each value represents the mean of three trials; Numbers with different superscripts within one strain and one harvest time differ significantly ($P < .05$); ONP= O-nitrophenol

[2]Time of harvest of cells for preparation of concentrated culture; Late Log=late log phase of growth; Stationary=stationary phase of growth

[3]There were no significant differences ($P > .05$) between the two harvest times for any of the three strains except for strain 107 day 0.

Table 4. Influence of storage on nonfermented acidophilus milk at 7°C on cholesterol assimilation by three strains of *Lactobacillus acidophilus*.

Strain	Days at 7°C	Cholesterol assimilated (μg/ml)[1]	
		Late Log[2]	Stationary[2]
107	0	33.73[a]	31.15[a]
	7[3]	28.12[ab]	17.01[b]
	14	21.04[bc]	17.01[b]
	21	14.61[c]	19.17[b]
	28	18.64[c]	19.29[b]
606	0	31.15[a]	32.96[a]
	7	25.19[ab]	28.99[ab]
	14	19.69[bc]	23.56[bc]
	21	15.67[c]	14.67[d]
	28[3]	12.97[c]	22.68[bc]
223	0	48.10[a]	47.64[a]
	7	39.86[b]	45.18[a]
	14	37.76[b]	40.27[a]
	21	22.87[c]	24.48[b]
	28	14.90[d]	11.69[c]

[1]Each value represents the mean of three trials; Numbers with different superscripts within one strain and one harvest time differ significantly ($P<.05$)

[2]Time of harvest of cells for preparation of concentrated culture; Late Log=late log Phase of Growth; Stationary=stationary phase of growth

[3]There were no significant differences ($P>.05$) between the two harvest times at any day of analysis except for strain 107, day 7, and strain 606, day 28.

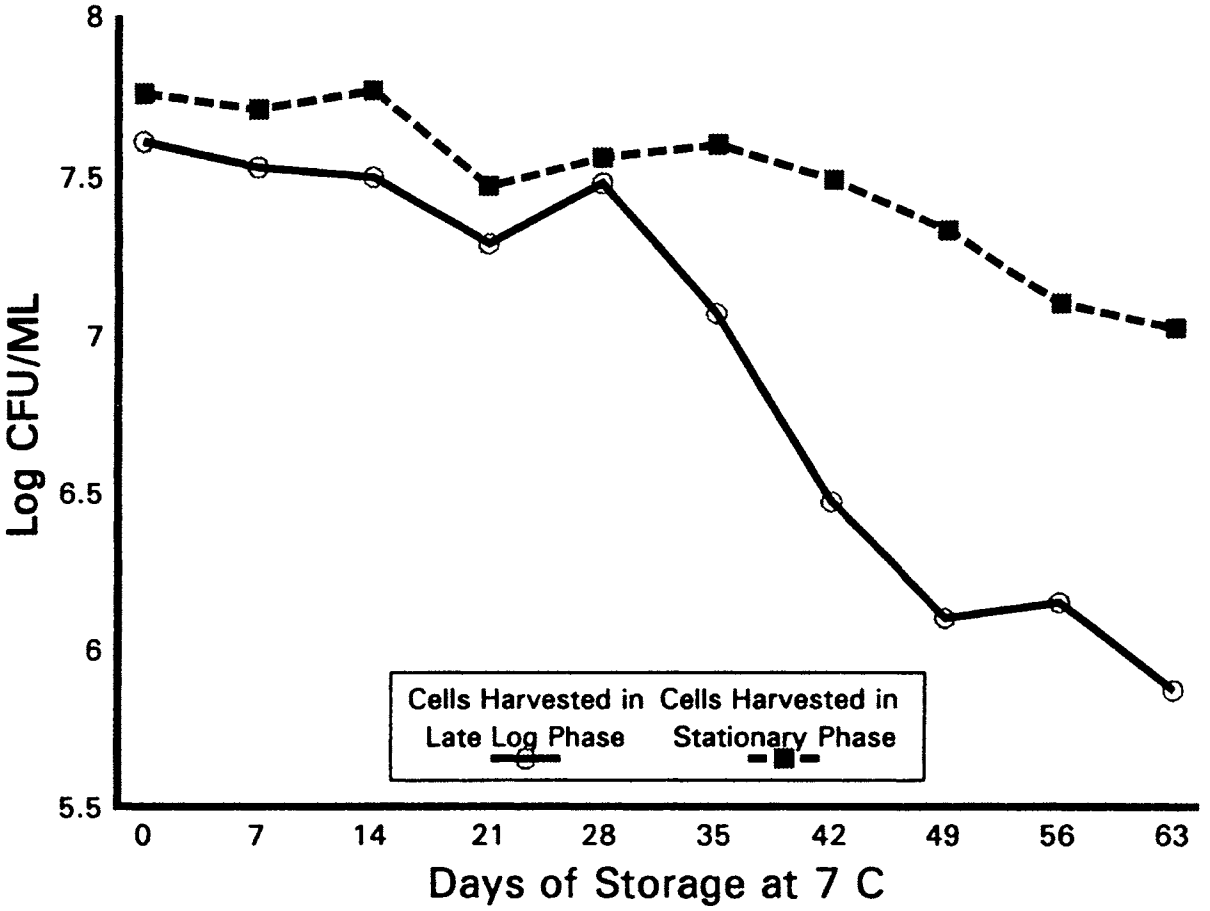

Figure 1. Total numbers of Lactobacillus acidophilus 223 during storage in milk at 7 C

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APPENDIX 1

TOTAL NUMBERS OF LACTOBACILLI

TABLE 5

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMN AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.36	10.41
	7	10.28	10.38
	14	10.28	10.36
	21	10.38	10.38
	28	10.20	10.45
2	0	10.57	10.28
	7	10.54	10.18
	14	10.53	10.23
	21	10.71	10.20
	28	10.72	10.23
3	0	10.69	10.65
	7	10.86	10.82
	14	10.78	10.61
	21	10.79	10.93
	28	10.72	10.69

TABLE 6

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 606 ON PMN AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.68	10.67
	7	10.85	10.66
	14	10.81	10.83
	21	10.79	10.76
	28	10.92	10.83
2	0	10.87	10.97
	7	11.04	10.92
	14	10.83	10.78
	21	10.78	10.78
	28	10.73	10.72
3	0	10.75	10.71
	7	10.66	10.69
	14	10.85	10.86
	21	10.69	10.76
	28	10.81	10.75

TABLE 7

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 223 ON PMN AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.11	10.20
	7	10.04	10.04
	14	10.08	10.00
	21	10.08	10.18
	28	10.15	10.00
2	0	10.54	10.28
	7	10.49	10.45
	14	10.34	10.38
	21	10.63	10.46
	28	10.43	10.43
3	0	10.64	10.76
	7	10.86	10.81
	14	10.66	10.83
	21	10.83	10.81
	28	10.77	10.86

TABLE 8

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMN AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.59	7.28
	7	5.96	5.93
	14	4.93	5.48
	21	4.59	4.78
	28	4.49	4.79
2	0	7.86	7.43
	7	6.15	6.26
	14	5.18	5.23
	21	4.81	5.00
	28	4.54	4.39
3	0	7.92	7.59
	7	7.18	6.23
	14	6.28	6.15
	21	5.00	5.81
	28	4.89	4.18

TABLE 9

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 606 ON PMN AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.84	7.83
	7	7.69	7.56
	14	7.64	7.66
	21	7.59	7.56
	28	7.61	7.49
2	0	8.20	8.08
	7	7.73	7.83
	14	7.71	7.69
	21	7.67	7.79
	28	7.18	6.99
3	0	7.93	7.72
	7	7.76	7.54
	14	7.88	7.86
	21	7.96	7.93
	28	7.83	7.97

TABLE 10

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 223 ON PMN AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.15	7.63
	7	7.04	7.64
	14	7.23	7.68
	21	6.84	7.38
	28	7.27	7.52
	35	6.73	7.65
	42	6.20	7.76
	49	5.84	7.26
	56	6.08	7.08
	63	5.65	6.92
2	0	7.92	7.76
	7	7.88	7.67
	14	7.72	7.73
	21	7.45	7.54
	28	7.48	7.49
	35	7.15	7.51
	42	7.11	7.08
	49	6.34	7.11
	56	6.26	7.11
	63	6.04	7.00
3	0	7.77	7.88
	7	7.66	7.82
	14	7.56	7.89
	21	7.68	7.50
	28	7.69	7.72
	35	7.66	7.64
	42	7.02	7.65
	49	6.62	7.61
	56	6.28	7.11
	63	6.32	7.15

APPENDIX 2

BILE TOLERANT LACTOBACILLI

TABLE 11

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMNO AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.28	10.39
	7	10.28	10.32
	14	10.11	10.23
	21	10.23	10.39
	28	10.15	10.32
2	0	10.69	10.61
	7	10.53	10.11
	14	10.45	10.15
	21	10.69	10.18
	28	10.64	10.26
3	0	10.79	10.69
	7	10.84	10.89
	14	10.77	10.73
	21	10.79	10.74
	28	10.63	10.60

TABLE 12

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 606 ON PMN0 AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.62	10.68
	7	10.91	10.74
	14	10.82	10.77
	21	10.78	10.73
	28	10.89	10.72
2	0	11.04	10.91
	7	10.94	10.88
	14	10.86	10.81
	21	10.69	10.72
	28	10.66	10.69
3	0	10.88	10.79
	7	10.72	10.71
	14	10.83	10.82
	21	10.79	10.79
	28	10.83	10.61

TABLE 13

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 223 ON PMN0 AGAR DURING STORAGE AT -196°C

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	10.04	10.08
	7	10.08	10.04
	14	10.00	10.00
	21	9.96	10.04
	28	10.08	10.34
2	0	10.58	10.26
	7	10.26	10.34
	14	10.30	10.26
	21	10.26	10.39
	28	10.15	10.43
3	0	10.65	10.68
	7	10.67	10.79
	14	10.72	10.84
	21	10.76	10.88
	28	10.64	10.75

TABLE 14

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMNO AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.59	7.28
	7	5.96	5.93
	14	4.93	4.48
	21	4.59	4.78
	28	4.49	4.79
2	0	7.68	7.43
	7	6.15	6.26
	14	5.18	5.23
	21	4.81	5.00
	28	4.54	4.39
3	0	7.92	7.59
	7	7.18	6.23
	14	6.28	6.15
	21	5.00	5.81
	28	4.89	4.18

TABLE 15

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 606 ON PMNO AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.86	7.79
	7	7.57	7.59
	14	7.65	7.54
	21	7.61	7.28
	28	7.53	6.93
2	0	8.04	8.11
	7	7.72	7.88
	14	7.60	7.72
	21	7.63	7.72
	28	7.08	6.96
3	0	7.64	7.71
	7	7.81	7.63
	14	7.89	7.60
	21	7.99	7.97
	28	7.81	8.00

TABLE 16

COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 223 ON PMNO AGAR DURING STORAGE AT 7°C IN MILK

		Log_{10} CFU/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	7.11	7.52
	7	7.11	7.51
	14	7.04	7.53
	21	6.48	7.31
	28	7.15	7.49
	35	5.89	7.83
	42	6.74	7.57
	49	5.63	7.28
	56	5.98	6.86
	63	5.61	6.72
2	0	7.93	7.76
	7	7.85	7.59
	14	7.72	7.48
	21	7.36	7.38
	28	7.49	7.04
	35	7.08	7.46
	42	7.04	7.08
	49	6.32	7.08
	56	6.18	7.04
	63	4.94	6.95
3	0	7.79	7.50
	7	7.62	7.86
	14	7.11	7.82
	21	7.69	7.92
	28	7.66	7.75
	35	7.61	7.68
	42	7.72	7.68
	49	7.57	7.53
	56	7.28	7.04
	63	6.08	7.11

APPENDIX 3

ß-GLACTOSIDASE ACTIVITY

ß-GALACTOSIDASE ACTIVITY OF NONFERMENTED ACIDOPHILUS MILK

REAGENTS:

A. Sodium Phosphate Buffer pH 7 (.05 M)

4.1 g anhydrous Na_2HPO_4
2.9 g NaH_2PO_4 . H_2O

Dissolve and make up to 1 liter in volumetric flask with distilled water.

B. O-nitrophenyl-ß-D-galactopyranoside (ONPG) .012 M

180 mg ONPG
Dissolve and make up to 50 ml in volumeteric flask with 0.5 M phosphate buffer.

C. Lysozyme (50 mg/ml Grade III Sigma) in .05 M phosphate buffer (pH 7)

D. .625 M Sodium Carbonate

6.6 g dissolved and made up to 100 ml in volumetric flask with distilled water.

PROCEDURE:

1. Prepare milk containing 5 x 10^6 cells/ml by adding .1 ml MRS broth culture to 10 ml 10% NDM. Dilute milk 1 : 10 with cold .05 M pH 7 phosphate buffer.

2. Add the following to each of six test tubes in an ice water bath.

   1 ml ONPG
   2 ml diluted cell suspension
   .2 ml lysozyme

3. Add the following to each of three other test tubes in an ice water bath:

   1 ml .5 M phosphate buffer
   2 ml diluted cell suspension
   .2 ml lysozyme

   These are the blanks.

4. Mix all tubes on a vortex mixer and incubate in 37°C water bath.

5. After exactly 15, 25, and 35 minutes remove two sample tubes and one blank. Add 2 ml of cold .625 sodium carbonate to each and vortex to stop reaction.

6. Centrifuge contents of all four tubes 10 minutes at 10000 RPM at room temperature to remove suspended milk solids.

7. Read A_{420} nm of samples tubes against appropriate blank.

9. Determine the amount of o-nitro phenol (ONP) released by comparing the reading to a standard curve.

EQUATION FROM STANDARD CURVE:

$$Y = -.00291071 + .5955357\ X$$

TABLE 17

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 107 DURING STORAGE AT -196°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.18	.09
	7	.21	.15
	14	.15	.12
	21	.16	.11
	28	.16	.12
2	0	.19	.14
	7	.19	.13
	14	.19	.14
	21	.20	.12
	28	.18	.14
3	0	.17	.13
	7	.20	.15
	14	.19	.15
	21	.17	.13
	28	.15	.13

TABLE 18

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 606 DURING STORAGE AT -196°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.17	.18
	7	.17	.17
	14	.14	.17
	21	.14	.17
	28	.17	.15
2	0	.11	.15
	7	.18	.15
	14	.15	.18
	21	.12	.15
	28	.16	.17
3	0	.12	.14
	7	.13	.13
	14	.13	.16
	21	.14	.16
	28	.12	.13

TABLE 19

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 223 DURING STORAGE AT -196°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.07	.05
	7	.07	.07
	14	.07	.06
	21	.08	.07
	28	.07	.07
2	0	.11	.12
	7	.08	.12
	14	.09	.12
	21	.09	.13
	28	.09	.12
3	0	.10	.12
	7	.10	.12
	14	.09	.09
	21	.10	.12
	28	.09	.13

TABLE 20

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 107 DURING STORAGE AT 7°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.21	.24
	7	.16	.12
	14	.12	.14
	21	.08	.11
	28	.06	.11
2	0	.15	.18
	7	.12	.12
	14	.13	.13
	21	.09	.09
	28	.10	.09
3	0	.14	.20
	7	.16	.16
	14	.09	.11
	21	.09	.10
	28	.11	.10

TABLE 21

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 606 DURING STORAGE AT 7°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.13	.12
	7	.09	.09
	14	.10	.09
	21	.11	.09
	28	.08	.08
2	0	.12	.09
	7	.10	.08
	14	.12	.09
	21	.12	.10
	28	.07	.07
3	0	.11	.08
	7	.11	.09
	14	.11	.07
	21	.09	.06
	28	.06	.06

TABLE 22

ß-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* 223 DURING STORAGE AT 7°C

		μmoles ONP Released/min/ml	
Replication	Days of Storage	Late Log	Stationary
1	0	.07	.07
	7	.07	.07
	14	.07	.07
	21	.05	.04
	28	.06	.04
2	0	.09	.07
	7	.08	.09
	14	.06	.05
	21	.05	.04
	28	.04	.04
3	0	.09	.13
	7	.09	.12
	14	.09	.10
	21	.08	.07
	28	.05	.07

APPENDIX 4

CHOLESTEROL ASSIMILATION

CHOLESTEROL ASSIMILATION BY *LACTOBACILLUS ACIDOPHILUS* CULTURES STORED AT -196°C OR 7°C

1. Prepare MRS-THIO-OX broth as follows (the total volume may be adjusted. The amount below is sufficient for three cultures):

Distilled Water	36 ml
MRS Broth	2 g
Sodium Thioglycollate	72 mg
Oxgall	108 mg

   Sterilize in a nonchipped bottle.

2. Prepare cholesterol micelles accoring to Razin (Biochem. Biophys. Acta. 598: 628).

3. After cooling add 4 ml of the micelles to the 36 ml of broth; mix by inverting the bottle six times.

4. Aseptically dispense 10 ml into each of 4 sterile screw cap test tubes. Label one as "uninoculted control" and hold in refrigerator for chelesterol assay. Label the remaining three with the appropriate culture identification.

5. Preparation of cultures for assay (for each culture):
   -Add .1 ml of freshly prepared MRS broth culture to 10 ml sterile 10% NFDM.
   -Mix thoroughly.

6. Innoculate the appropriate tube from step 4 with 1 ml of diluted culture (Step 5).

7. Incubate the innoculated tubes 19 hrs at 37°C.

8. Centifuge contents of each tube 10 min at 10,000 rpm and 1-2°C to remove the cells and precipitated milk.

9. Carefully recover the clear supernatant (spent broth) from each culture.

10. Assay spent broth from each culture and the uninoculated control for cholesterol using the Rudel and Morris method (J. Lipid Res. 14: 364).

Cholesterol Assimilated = (Cholesterol in Uninoculated Broth) - (Cholesterol in Spent Broth)

PREPARATION OF CHOLESTEROL - PHOSPHOLIPID MICELLES[A]

1. Dissolve 9.1 mg cholesterol in approximately 1 ml chloroform in small tube suitable for use with sonicator probe.

2. Add 0.2 ml of L-α-phosphatidyl choline (Lecithin).

3. Evaporate chloroform under a flow of nitrogen gas with mild heat ($<45^{o}C$).

4. Add 10 ml of 0.4 M sucrose.

5. Sonicate at maximum output for 3 15 minutes periods. Suspend sonication vessel in ice-water. Allow 5 minutes between each sonication.

6. Centrifuge 30 minutes at 12000 rpm and $4^{o}C$ in small head to remove metal fragments from sonicator probe.

7. Filter sterilize supernatant fluid through 0.45μ filter and store in refrigerator.

   The solution should contain about 1 mg cholesterol per ml. The molar ratio of lecithin:cholesterol in 1 : 0.9.

One half ml of the solution contains approximately as much cholesterol as does 1 ml of PPLO serum.

[A](1980. Biochem et Biophys. Acta. 598: 628.)

CHOLESTEROL ASSAY[A]

1. Place 0.5 ml sample (spent broth from cell suspension) in appropriately labeled test tubes. Include one as a blank containing 0.5 ml distilled water).

2. Add 3 ml of 95% ethanol.

3. Add 2 ml of 50% potassium hydroxide and mix on vortex mixer.

4. Heat 10 min in a 60°C water bath.

5. Cool and add 5 ml of hexane; mix 30 seconds on vortex mixer.

6. Add 3 ml distilled water, remix and set aside until phases separate.

7. Transfer 2.5 ml hexane layer into a clean dry test tube.

8. Evaporate to dryness under a flow of nitrogen gas under a hood.

9. Add 4 ml of o-phthalaldehyde reagent and mix to dissolve sample.

10. Let set 10 min at room temperature.

11. Carefully add 2 ml concentrated sulfuric acid down inside of tube.

12. Mix immediately on vortex mixer and set aside 10 min at room temperature for color development.

13. Read A_{550} nm to standard curve to determine cholesterol content.

EQUATION FROM STANDARD CURVE:

$$Y = .0101071 + .0057029\ X$$

REAGENTS:

A. o-phthalaldehyde reagent (0.5 mg o-phthalaldehyde/ml glacial acetic acid) Prepare fresh daily.

B. 50% potassium hydroxide

C. Concentrated sulfuric acid

D. Hexane.

[A](J. Lipid Res. 14: 364)

TABLE 23

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 107 DURING STORAGE AT -196°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	49.44	48.58
	7	50.86	41.56
	14	40.50	50.32
	21	41.90	39.98
	28	50.50	51.72
2	0	47.70	49.10
	7	47.88	48.58
	14	42.26	39.98
	21	48.58	37.52
	28	51.72	60.32
3	0	42.78	37.18
	7	38.92	38.92
	14	42.62	44.88
	21	40.60	37.34
	28	42.62	32.32

TABLE 24

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 606 DURING STORAGE AT -196°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	39.98	41.74
	7	38.92	40.86
	14	42.08	41.38
	21	43.66	43.84
	28	42.44	39.46
2	0	34.90	34.72
	7	37.18	37.88
	14	32.26	44.88
	21	40.34	45.94
	28	32.26	34.54
3	0	43.66	36.04
	7	41.04	31.74
	14	39.98	35.42
	21	38.92	37.62
	28	40.68	33.06

TABLE 25

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 223 DURING STORAGE AT -196°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	57.70	52.26
	7	60.50	45.24
	14	55.06	49.1
	21	54.54	48.22
	28	51.56	54.18
2	0	55.06	57.16
	7	53.3	48.38
	14	52.26	52.60
	21	55.76	40.68
	28	52.42	51.02
3	0	46.82	46.20
	7	55.76	59.10
	14	48.58	61.28
	21	54.00	65.24
	28	50.32	44.54

TABLE 26

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 107 DURING STORAGE AT 7°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	32.44	37.53
	7	27.53	20.87
	14	21.92	15.96
	21	6.14	17.54
	28	12.45	14.73
2	0	25.08	32.44
	7	26.48	15.43
	14	18.24	18.24
	21	17.36	14.38
	28	21.04	19.64
3	0	43.66	23.49
	7	30.34	14.73
	14	22.97	16.83
	21	20.34	25.60
	28	22.44	23.49

TABLE 27

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 606 DURING STORAGE AT 7°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	27.00	32.44
	7	25.25	16.83
	14	14.73	15.61
	21	16.66	17.88
	28	11.39	19.28
2	0	40.51	41.56
	7	22.79	41.73
	14	23.32	33.84
	21	17.54	15.43
	28	16.31	33.14
3	0	25.95	24.89
	7	27.53	28.41
	14	21.04	21.22
	21	12.80	10.69
	28	11.22	15.61

TABLE 28

CHOLESTEROL ASSIMILATION OF *LACTOBACILLUS ACIDOPHILUS* 223 DURING STORAGE AT 7°C

		μgrams Cholesterol Assimilated	
Replication	Days of Storage	Late Log	Stationary
1	0	51.20	51.38
	7	45.42	51.73
	14	40.86	38.58
	21	18.58	22.44
	28	13.68	12.98
2	0	45.59	45.94
	7	36.12	35.59
	14	38.75	32.26
	21	22.79	19.81
	28	13.85	9.29
3	0	47.52	45.59
	7	38.05	48.22
	14	33.67	49.97
	21	27.23	31.21
	28	17.18	12.80

APPENDIX 5

EXAMPLES OF ANALYSIS OF VARIANCE TABLES

TABLE 29

ANALYSIS OF VARIANCE - COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMN AGAR DURING STORAGE AT -196°C

SOURCE	DF	SS	MS	F VALUE	Pr > F
Model	13	1.38	.1061	14.18	.0001
Error[b]	16	.12	.0075		
Corrected Total	29	1.49			
Fermentation	2	.9403	.4702	62.82	.0001
Harvest Time	1	.0864	.0864	11.54	.0037
F * H[a]	2	.3188	.1594	21.30	.0001
Day	4	.0321	.0080	1.07	.4023
H * D	4	.0019	.0005	.07	.9912

[a]Error(a) or Main Unit Error Term
[b]Error(b) or Sub Unit Error Term

TABLE 30

ANALYSIS OF VARIANCE - COUNTS OF *LACTOBACILLUS ACIDOPHILUS* 107 ON PMN AGAR DURING STORAGE AT 7°C

SOURCE	DF	SS	MS	F VALUE	Pr > F
Model	13	27.81	2.1391	16.08	.0001
Error[b]	16	2.13	.1331		
Corrected Total	29	29.94			
Fermentation	2	.9032	.4516	3.39	.0591
Harvest Time	1	.0456	.0456	.034	.5663
F * H[a]	2	.2937	.1468	1.10	.3557
Day	4	26.19	6.549	49.21	.0001
H * D	4	.3700	.0925	.70	.6062

[a]Error(a) or Main Unit Error Term
[b]Error(b) or Sub Unit Error Term

VITA 2

Mindy Malynn Brashears

Candidate for the Degree of

Master of Science

Thesis: INFLUENCE OF HARVEST TIME ON STABILITY OF CELLS OF *LACTOBACILLUS ACIDOPHILUS* DURING FROZEN AND SUBSEQUENT REFRIGERATED STORAGE

Major Field: Food Science

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Personal Data: Born in Amarillo, Texas, May 13, 1970, the daughter of Gary and Becky Hardcastle. Married to Todd Brashears on August 12, 1989.

Education: Graduated with an advanced diploma from Wheeler High School, Wheeler, Texas in May 1988. Received Bachelor of Science degree in Food Technology from Texas Tech University, Lubbock, Texas in May 1992. Completed the requirements for the Master of Science degree in Food Science at Oklahoma State University in May 1994.

Experience: Raised on a farm near Wheeler, Texas; employed as a farm laborer during summers; employed by Texas Tech University, Department of Agronomy and Department of Animal Science and Food Technology as a student research assistant; employed by Oklahoma State University Department of Animal Science as a graduate research assistant.

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