VIABILITY OF *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS CASEI* IN FERMENTED MILK PRODUCTS DURING REFRIGERATED STORAGE

By

BENTON DOYLE NIGHSWONGER

Bachelor of Science in Dairy Manufacturing

Bachelor of Science in Microbiology

South Dakota State University

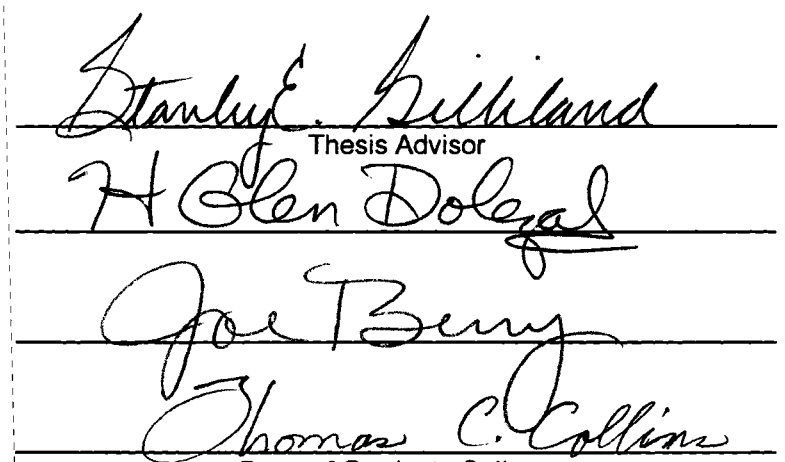
Brookings, South Dakota

1992

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1995

VIABILITY OF *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS CASEI* IN FERMENTED MILK PRODUCTS DURING REFRIGERATED STORAGE

Thesis Approved:



Thesis Advisor

Dean of Graduate College

PREFACE

I would like to thank Dr. Stanley E. Gilliland for his guidance, assistance, and encouragement throughout my graduate study. I also greatly appreciate Dr. H. Glen Dolezal and Dr. J. Berry for serving on the Advisory Committee. I would also like to thank Dr. P. Larry Claypool for his assisitance and guidance with the statistical methods.

Appreciation is expressed to the Mona Division of Campina Melkunie, Woerden, The Netherlands, for partial funding of this research.

I truly appreciate the friendship and help extended to me over the last two years by Freddie Gant, Parinitha Dambekodi, Dong-Ouk Noh and Elba and Gerardo Corzo. Special appreciation goes to Mindy Brashears and Siobhan Reilly. A great portion of the work involved in this study is the result of their help and teamwork and could not have been accomplished without their input.

Finally, I would like to thank my family for all the love, encouragement, understanding, patience and unconditional support in my efforts to further my education.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

INTRODUCTION

In the past few years it has become common practice among some cultured product manufacturers to add special "healthful" bacterial cells to their traditional cultured products to gain the attention of health conscious consumers. Most notably is the addition of "acidophilus and bifidus" cultures to yogurt. Yet, in selling these products the marketing cannot include health claims of these viable "dietary adjunct" bacteria. They will normally only claim that a culture of viable *Lactobacillus acidophilus* and/or *Bifodobacteria* species was added at the time of manufacture.

Some evidence shows that these "dietary adjuncts" decline in numbers before consumers purchase the product. This loss of viability can be rapid, thus very low levels of potentially beneficial bacteria may survive to reach consumers. Most cultured dairy products are consumed within two to three weeks after manufacture yet yogurt may remain in refrigerated storage even longer before consumption. Dietary adjuncts need to remain viable throughout this time period to be of benefit to consumers. The objective of this study was to check the viability of selected *Lactobacillus* species adjuncts in yogurt or cultured buttermilk over a period of twenty-eight days of refrigerated storage.

Chapter II

LITERATURE REVIEW

Early Development of Fermented Milks

According to the International Dairy Federation, fermented milks are products prepared from milk (skimmed or not, concentrated or not) with specific cultures. The microflora remain alive until sale to the consumer and may not contain any pathogenic organisms. It has recommended that the metabolic substances derived from the fermentation should be present in a true fermented milk (71).

There are many forms of fermented milks throughout the world. Manufacture of almost all of them depends on lactic acid bacteria to produce lactic acid and other necessary metabolites for a satisfactory fermented milk.

Fermented milks are popular foods throughout the world. The reasons for their expanse lie far back in history. The time and place of origin of fermented milks for human consumption is not known. There are many accounts of the use of fermented milks throughout Asia and Europe. A Turkish legend describes the first yogurt as coming from an angel (65). Accounts of Buddhists using yogurt as an offering to angels places the first yogurts around present day Turkey (65). These tales were told by word of mouth for ages and the actual dates of these accounts are unknown. Yet by the eighth century, yogurt was a common product in Turkey and called "Yogurut" (65).

The Old Testament offers some of the earliest evidence of use of fermented milk. According to Genesis 8: 8, Abraham offered angles both sweet and soured milk. And Moses reportedly mentioned "soured milk of cows and goats" as stated in Deuteronomy 32: 14. The use of soured milks by ancient Greeks and Romans is mentioned in a biography of Emperor Elagabalum (A.D. 218-222) (65).

Still other reports place the origin of an early yogurt product made from sheep's milk in the Balkans (65). This "art" was then passed on to the Slavs when they took over this area.

Actual proof regarding the origin of fermented milks is certainly lost to history and it is quite possible that there was not one single discovery of fermented milk products but many. Different environmental and cultural aspects could lead to the development of many different fermented products. It is known that ancient man used many versions of sour liquid milks and yogurt throughout the world.

The type of fermentation is very important in producing a product with the appropriate flavor. Likely, ancient man selected fermented milk with the most appealing flavors and would try to duplicate them by "backslopping" an acceptable soured milk into fresh milk for continued production. Yet cultured milks are important for more than just culinary delight, there is evidence that fermented milks provide health benefits. Discussion of this topic began long ago. In the early 1900's, E. Metchnikoff hypothesized that the people of Bulgaria were living extremely long lives because of the fermented milks that they consumed (53).

Characteristics of Cultured Buttermilk

Cultured "buttermilk" originated in the USA. It was an invention of necessity after demand for true buttermilk, a by-product of churning soured or cultured cream, exceeded supply. According to the Encyclopedia of Fermented Fresh Milk Products (49), cultured buttermilk may be better termed "cultured milk" or "cultured lowfat milk" since it is not a by-product of buttermaking as is true buttermilk. True buttermilk is not readily available to the consumer in the US but cultured buttermilk does continue to hold a niche in most supermarkets. Its use in many baked goods has helped keep a demand for this truly fermented milk product (80). It has recently gained more acceptance in Western Europe especially in the Nordic and Baltic states as a healthful drink (54).

Cultured milk is now produced in the Netherlands, Denmark, Germany and some of the Eastern European states (49). It has been most popular in the south and southeastern regions of the US. However, consumption of this product has declined since its peak sales period (approximately 1930-1960).

Cultured buttermilk is usually made from skim or lowfat milk with 9-12 % nonfat solids. The milk is pasteurized at 85°C for 30 min. (or 95°C for 3-5 min.), cooled to 22°C and inoculated with 1% of the appropriate starter culture. Incubation at 22°C for 14-16 hours (with a final pH of approximately 4.6) is followed by stirring and packaging. Cultured buttermilk should be stored at 5°C and distributed to stores within 24 hours for best quality for the consumer (49).

At least one of the species of lactococci is necessary to make a quality cultured buttermilk (69). The function of these bacteria is primarily the production of lactic acid. Lactic acid is not only important for the acidity and texture of the product but also for the development of more subtle flavor components such as diacetyl. The leuconostoc bacteria used in many cultured buttermilks will only metabolize citrate and produce diacetyl after the pH of the product is adequately lowered.

Lactococcus lactis biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* are two bacteria that utilize citrate found at low levels in milk. This results in the production of acetoin, diacetyl and some acetic acid (47,69). *Lactococcus lactis* biovar *diacetylactis* and *Lactococcus lactis* differ only in that *L. lactis* biovar *diacetylactis* metabolizes citrate. This biovariant is reported by Kemper and McKay (42) as possessing a plasmid which allows the citrate metabolism to occur. Care needs to be taken to ensure that strains of this culture do not produce excessive acetaldehyde. Excess acetaldehyde would lead to a "green flavor defect" and this has limited the use of this *Lactococcus* biovariant and helped to promote the production of cultured products with the *Leuconostoc* species instead. (80)

Leuconostoc mesenteroides subsp. *cremoris* grows rather poorly in milk, yet attains a population of 10^8 to 10^9 cfu/ml in milk if grown long enough at 22°C. Leuconostocs are more tolerant of an acidic environment than some of the lactococci (23). *Leuconostoc mesenteroides* subsp. *cremoris* ferments glucose to produce lactic acid, ethanol and CO_2. It utilizes citrate but does not make acetoin. Products from the citrate

metabolism include diacetyl and acetic acid, both of which are very important for the overall flavor characteristics of the final product (69).

Both *L. lactis* biovar *diacetylactis* and *L. mesenteroides* subsp. *cremoris* produce some CO_2 from the metabolism of citrate. The CO_2 production is considered a cause of open texture defects of some cheeses and "floating curd" in the production of cottage cheese. The presence of some CO_2 in cultured buttermilk is considered desirable.

This metabolism is not considered a true fermentation because it is reported that these bacteria do not use citrate as an energy source (23). *Lactococcus lactis* biovar. *diacetylactis* metabolizes citrate as soon as growth begins in milk. Both of the citrate utilizing bacteria can be found in combination in some cultures which are termed "BD cultures"(23). *Leuconostoc mesenteroides* subsp. *cremoris* needs the pH to drop below 5.0 to begin producing diacetyl (71). This pH level is attained in cultured milk by use of the leuconostoc in association with one of the homofermentative lactococci. The end result is the production of appropriate flavor compounds for a high quality cultured milk.

A flavorful "buttermilk" should contain 2-4 ppm of diacetyl for the appropriate "buttery" flavor and quality (49). Care should be taken to ensure that temperatures during incubation do not exceed 24°C because the flavor-producing bacteria may not grow sufficiently. It is proposed by Webb (84) that three things can be done to help ensure the desired levels of diacetyl. First, one may add 0.15% citric acid (or equivalent sodium citrate) to the milk prior to incubation. Secondly, leuconostoc bacteria need to be present in the starter culture to improve the level of this flavor component. Another thing that may improve flavor production is the use of the peroxide-catalase treatment of the milk

(56). The inactivation of any hydrogen peroxide in the milk by catalase would help ensure that this free radical does not interfere with the growth of the starter bacteria.

Antibacterial substances produced by traditional cultured buttermilk bacteria

Leuconostoc ssp. have been shown to produce several substances that may exert antimicrobial actions including: acetic acid, lactic acid, formic acid and potentially some bacteriocin like substances called leucocin or mesentericin (12). According to Oberman (54), acetic acid has bacteriostatic or bactericidal effects against putrefactive microorganisms such as the spore-forming bacilli and coliforms.

Acetic acid is especially important as an inhibitor of many spoilage and pathogenic bacteria (8). Oberman (54) suggests small amounts of ethanol may be produced by the leuconostocs as they do possess an active alcohol dehydrogenase enzyme. Diacetyl also has been implicated by some researchers as being inhibitory (38).

Two review articles by Hoover (38) and by Dodd and Gasson (12) have recently been introduced that cover the scope of bacteriocins. One of the most notable bacteriocins is produced by *L. lactis.* It is an intracellular low weight peptide called nisin which inhibits many Gram positive bacteria such as the *Bacillus* ssp.(12). Another well established bacteriocin called diplococcin was reported by Babel (4) to have been produced by *L. lactis* subsp. *cremoris.* It was found to be inhibitory to *Staphylococcus aureus* and of particular importance is its potential inhibition of *Lactococcus lactis* subsp. *lactis* (4,65). Other bacteriocins of importance are lactostrepins (38), lactococcin (12) and lacticin (12).

The potential effects of these inhibitory substances on *Lactobacillus acidophilus* or *Lactobacillus casei* in buttermilk is not specifically known. One study by Branen et. al. (5) did show that the common buttermilk flavor producing bacteria did produce an inhibitory substance but it appeared that it was most effective against Gram negative bacteria and had no effect on *L. casei*.

While bacteriocins could prove destructive to the *L. acidophilus* or *L. casei* added to buttermilk, other metabolites made by the lactococci and leuconostocs (such as lactic acid, CO_2 and especially the acetic acid) could play more destructive roles. Cultured buttermilk with added *L. acidophilus* and/or *Bifidobacterium* spp. may be an idea slightly ahead of the research in this area. No research has been reported on their survival in cultured buttermilk.

Characteristics of Yogurt

Yogurt is defined by FAO/WHO standards as the coagulated milk product obtained by the lactic acid fermentation, through the action of *Lactobacillus delbreuckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus,* of milk and milk products (86). Traditional yogurt must include these two thermophilic bacteria grown together in the product. Other bacteria can be included as " optional additions" in yogurt but the traditional yogurt must contain at least these two microorganisms (86).

Yogurt can be made from skim, lowfat or whole milk which is usually fortified with two to three percent nonfat dry milk to achieve a total solids percentage of

approximately fifteen to seventeen percent (47). This higher total solids helps to enhance the consistency and flavor of the product (65). Pasteurization (90.6°C for 40-60 seconds, 85°C for 30 minutes or equivalent) is usually well above minimum requirements for fluid milk to help ensure that indigenous thermoduric bacteria are destroyed as well as to free some amino acids for use by starter cultures. The mix is homogenized and allowed to cool to 45°C before inoculation with two to five percent starter unless concentrated starters are used. Incubation proceeds for three to six hours but should be closely monitored for a target pH of no less than 4.4 (0.9 -1.2 % titratable acidity). The yogurt should be cooled to 5-7°C within one hour upon attaining the target pH. Storage temperatures of 3-7°C should permit a shelf life of 30 days (47).

During the fermentation 20 to 30 % of the lactose in the original pre-yogurt mix is hydrolyzed (65). Most of it is utilized for lactic acid production by the starter culture. This still leaves nearly 70% of the lactose intact. Yet the amount of lactic acid produced is enough to cause a precipitation of the casein fraction of milk and create the typical "paste" or "gel" that is recognized as yogurt.

In the manufacture of yogurt, *S. salivarius* subsp. *thermophilus* breaks down (hydrolyzes) the lactose in milk with phospho-β-galactosidase (52). Following phosphorylation, this enzyme hydrolyses phosphorylated lactose (a disaccharide) into its monosaccharide components, glucose and galactose-6-phosphate. The glucose is metabolized through the hexose diphophate pathway to make mostly L (+) lactic acid (23). The remaining galactose-6-phosphate goes through the tagatose phosphate pathway

and makes more L (+) lactic acid. (23) The action of the streptococci on lactose is important for most of the initial lactic acid production in yogurt manufacture.

Lactobacillus delbrueckii subsp. *bulgaricus* hydrolyzes lactose with β-galactosidase to form glucose and galactose (27). The glucose then enters the Embden Meyerhoff pathway and eventually is reduced to lactate (lactic acid) (23). Yet the galactose must be converted to glucose-6-phosphate before it can be metabolized by the EM pathway (23). This is more expensive metabolically than using glucose and some lactobacilli will allow galactose to go unmetabolized. It is therefore released to the surrounding environment which leads to a detectable build up of free galactose in milk during fermentation (65,66). The end result of the fermentation of lactose by *L. delbreuckii.* subsp. *bulgaricus* is D (-) lactic acid (65). The acid production by *L. bulgaricus* is more important later in the fermentation process than the streptococci. The streptococci begin to be inhibited by the lower pH yet the lactobacilli are more tolerant. Therefore the lactobacilli are responsible for the majority of the lactic acid production in the latter stages of the fermentation of milk for yogurt manufacture.

Antibacterial substances produced by the traditional yogurt bacteria

Both the streptococci and lactobacilli produce lactic acid which can be somewhat inhibitory to other bacteria and if accumulated to high levels may become autoinhibitory. Some formic acid may also be produced which could affect the antibacterial potential of yogurt (65)

Hydrogen peroxide, a potent microbial inhibitor, is produced by the lactobacilli (65). *L. bulgaricus* and *L. acidophilus* both can produce hydrogen peroxide (1,16). Yet

it is interesting to note that they are catalase and superoxide dismutase negative (23). This can result in the hydrogen peroxide reaching autoinhibitory levels (16). Luckily streptococci and lactococci are less tolerant of H_2O_2 than the lactobacilli (23). This gives the lactobacilli a slight competitive advantage. Excessive mixing of milk following inoculation can cause incorporation of additional oxygen which can result in even higher levels of H_2O_2 and thus increased inhibition (23).

Rasic and Kurmann (65) reviewed bacteriocin research that has been conducted to find a small protein like substance that may be made by *S. thermophilus*. It is active at low pH values and is destroyed by heating. They suggest that the antimicrobial effects in yogurt can not be solely due to organic acids but must be somewhat the responsibility of hydrogen peroxide and bacteriocins either from *L. bulgaricus* or *S. thermophilus* (65). One last factor which may effect microbial inhibition in yogurt is an ethanol-acetone extract from *S. thermophilus* (63). According to Oberman (54), the antimicrobial effects of (all) fermented milks can be attributed to some kinds of organic acids, antibiotic factors, volatile acids, hydrogen peroxide and to some factors which have not yet been identified (54).

Bulgarican, a bacteriocin produced by *L. bulgaricus* is thermostable and is only active at pH 4.0 (1,65). It is capable of affecting both Gram positive and Gram negative bacteria (38). It is reported to have a rather broad spectrum of inhibitory action, which is not typical of bacteriocins (1,38). Reuterin is another "broad spectrum" bacteriocin linked to some strains of *L. bulgaricus*. It is not a protein but is capable of inhibiting important pathogens such as *Clostridium* species, *Staphylococcus* species, and *Listeria* species.

Some gram negative bacteria that were inhibited included *Pseudomonas fragi* and species of both *Salmonella* and *Shigella* (38).

Growth of traditional cultures

The ratio of rods to cocci in the typical yogurt culture used for the initial inoculum should be in a 1:1 ratio for best results (14). If the ratio is egregiously skewed the products flavor, odor and/or consistency could be poor (14). The following is a listing of the growth stages of these bacteria in mixed culture in milk: The cocci grow rapidly (by the end of the first hour the ratio is 3 or 4 to 1); most of the initial acid is produced from the *S. salivarius* subsp. *thermophilus*. The bacilli's growth rate increases later in the fermentation. When the product is nearing the end of the fermentation process the rods produce the majority of the acid needed to finish the fermentation (14).

The fermentation should not be allowed to proceed too far. If it does, the excessively acidic environment could result in a yogurt with the lactobacilli dominating and potentially affecting the flavor in more ways than just acidity (i.e. proteolysis, texture). Yogurt should be cooled rapidly upon reaching the target pH and kept at refrigeration temperatures (3 to 7°C) until consumption. One should avoid freezing as this will be detrimental to the texture and cultures that one finds appealing in a high quality yogurt.

S. thermophilus can provide stimulatory substances to *L. bulgaricus*. These substance have been found to be formic acid, CO_2 and potentially the lactic acid environment (54,70). *L. bulgaricus* releases free amino acids (especially histadine and glycine) that *S. thermophilus* can use and allow faster growth than without its presence (54,84). Thus both species may grow better together than either alone.

Some other bacteria often encountered in yogurt manufacture are *Lactobacillus helveticus*, *Lactobacillus lactis* and *L. acidophilus*. *Lactobacillus helveticus* and potentially *L. helveticus* subsp. *jugurti* could be heavily involved in some yogurt fermentations (65).

Potential Health or Nutritional Benefits of *L. acidophilus* and *L. casei*.

Fermented milk products do not spoil as quickly as nonfermented milk. If the milk is fermented under controlled conditions, the byproducts of that fermentation can preserve the nutrients from spoilage (17,26,27,50). Fermented milk can also protect the consumer from consuming pathogenic organisms since for the most part pathogens are inhibited by the starter cultures during manufacture of the cultured products (9,20,85). Some bacteria (*L. acidophilus* for example) can be used as starter or as a component of the starter to not only help protect the product from pathogens but to potentially help protect the consumer after consumption of the cultured product. This last factor can be combined with evidence of antitumor activity (3, 31, 67, 76, 79), control of serum cholesterol (10, 24, 36, 62, 82) and improved lactose digestion (22, 33, 43, 45, 51, 55) to support the potential benefits of "dietary adjuncts". These health benefits will be discussed at more length in forthcoming sections.

Definition and requirements of probiotic bacteria

No precise definition is given for the term "dietary adjunct". Yet a dietary adjunct or "probiotic" culture may provide some health benefit by fulfilling one basic goal that

normal starter cultures are unable to meet. The "probiotic" bacteria should be able to establish itself in the intestines. To accomplish this goal, certain requirements of the culture are needed including: 1.) the adjunct must be viable and capable of producing the desired beneficial action(s) upon ingestion; 2.) the adjunct should therefore be able to survive in reasonable numbers after passing through the acidic environment of the stomach; 3.) The bacteria should have the necessary levels of bile tolerance to continue to survive and grow in the intestines; 4.) ideally, it should be a strain originating from the intestines of the host species; 5.) the bacteria should be able to compete and grow in the presence of other normal intestinal flora (20).

According to Gilliland (28) other lactic acid bacteria can be present in the intestinal tract and many of them potentially could supplant efforts to introduce a slow growing adjunct or one with a particular biochemical weakness. Some strains of *L. acidophilus* produce bacteriocins (13,81) or H_2O_2 (9) production which could give them distinct advantages needed to maintain populations and maybe even grow.

Antagonistic Action Towards Pathogens

Lactobacillus acidophilus (20, 60, 61, 73) can be inhibitory against many pathogens. Some pathogens of special interest are *Staphylococcus aureus*, enteropathogenic *Escherichia coli*, *Salmonella typhimurium*, and *Clostridium perfringens*. Hydrogen peroxide production appeared to be one of the main antagonistic compounds responsible for the inhibition of pathogens by *L. acidophilus* (2, 20). In gnotobiotic chicks, *L. acidophilus* significantly reduced mortality for chicks infected with *S. aureus* or *S. typhimurium* (83). Patel et al. (57) reported that four human subjects fed

L. acidophilus or *L. casei* had decreased numbers of fecal coliforms and that the levels of lactobacilli remained elevated and the coliforms reduced for at least 2 weeks after feeding was discontinued.

Lactobacillus casei subsp. *rhamnosus* was found to be inhibitory to *Clostridium* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Vibrio* spp. (77, 78). Mice injected with cell wall fractions of *L. casei* or *L. acidophilus* showed enhanced resistance to *Listeria* infection (72). Perdigon et al. (61) found that consumption of a milk fermented with both *L. casei* and *L. acidophilus* produced a protective effect against *S. typhimurim* in mice. Interestingly, this study found that use of fermented milks with only one of the adjuncts was ineffective. Yet in another study (60) *L. casei,* in pure culture, was found to provide an immunological response and that it could provide protection against *S. typhimurium* infections. *Streptococcus salivarius* subsp. *thermophilus* also caused an immunological effect which was suggested to be protective as well, though this was not as specific as with the *L. casei* treatment.

Growth Stimulation:

In a study conducted in 1952 (68), infants that were partially breast fed exhibited significantly greater weight gain than those that were solely bottle fed. Yet, infants that were only bottle fed but with an addition of *L. acidophilus* to the formula showed significant increase in weight gain compared to those that were solely bottle fed with sterile formula over the first two months. The infants fed *L. acidophilus* were not as heavy as infants that were breast fed.

Benefits for lactose digestion

Fermented milk is an excellent source of nutrition (54). Yet, milk can not be consumed by many adults throughout the world because they lose the ability to digest lactose and therefore exhibit a condition known as lactose maldigestion (also refered to as lactose intolerance or lactose malabsorption). Only select populations (Northern Europeans for example) retain lactase enzymes into adulthood (39,40).

It has been recommended that firm cheeses and yogurt are two forms of fermented milk that may be consumed by "lactose intolerant" individuals (39). The lowering of lactose content in cheese can be mostly attributed to removal of lactose in the whey. The decreased levels of lactose in yogurt (20 to 30 % less lactose than unfermented milk) is likely the result of enzymes from the bacterial cultures (β-galactosidase or phospho-β-galactosidase) that break down lactose in the products (65). Yogurt still contains nearly 70% of the original lactose when consumed, yet lactose malabsorption following consumption of yogurt has been reported as being less severe for lactose maldigestors than following consumption of unfermented milk (22,45,55,65). Traditional yogurt made with *L. bulgaricus* and *S. thermophilus* does not contain bacteria that are expected to survive and grow in the intestine of man (21). However, the traditional yogurt culture contains β-galactosidase which could function in the intestine to improve lactose utilization in lactose maldigestors (22).

In terms of supplementing dairy products with lactobacilli, such as *L. acidophilus,* Kim and Gilliland (43) found that human subjects who were lactose maldigestors fed nonfermented acidophilus milk containing 2.5×10^6 CFU/ml had improved lactose utilization. Some reports (55,58,74) indicate that commercially available nonfermented

acidophilus milk has little or no effect on lactose digestion. These studies depended on the quality of the commercial products and may simply have pointed to the importance of ensuring that viable cultures with adequate β-galactosidase activity be used to cause improvement of lactose utilization in such persons (26,27).

In a review of potential health benefits from lactic acid bacteria, Gilliland (27) suggests that proper strains be used that contain adequate β-galactosidase activity. Two important factors that are considered are growth of the adjunct cultures in a medium which contains lactose so that the β-galactosidase is induced (30). Secondly, this activity should remain stable throughout frozen storage of the culture and the refrigerated storage of subsequent nonfermented acidophilus milk products (27).

Antitumor effects

There is some evidence that dietary adjuncts may aid in preventing some forms of tumors. Goldin and Gorbach (31) reported a decrease in the number of rats which developed chemically induced intestinal cancer for those fed diets containing *L. acidophilus*. Shahani et al. (76) found that consumption of milk fermented with *L. acidophilus* inhibited the formation of Ehrlich ascites tumor cells in lab mice by greater than 30%. Kato et al. (41) reported that mice which had been pre-treated with a Colon 26 tumor mass to illicit a primary immunological response were then better able to suppress the growth of secondary tumors if the mice were fed *L. casei*. There is some evidence that peritoneal macrophage's immune response in mice is enhanced when fed *L. casei* (59). Enzymatic activity of macrophages increased as much as 6-fold for mice fed *L. casei* compared to controls (59). The addition of dietary adjuncts may also effect levels of

carcinogenic compounds in the intestine (75). This may be due in part to the inhibition of bacterial species that produce some toxic or carcinogenic compounds (32).

Potential Benefit of Decreased Serum Cholesterol

There is evidence for and against the possibility that consumption of *L. acidophilus* can help decrease serum cholesterol levels. Gilliland et al. (24) found that pigs fed a high cholesterol diet had significantly decreased levels of serum cholesterol if fed *L. acidophilus* RP32. Danielson et al. (10) reported that pigs fed a yogurt preparation including *S. thermophilus* and *L. acidophilus* and a high cholesterol diet had significantly decreased levels of total serum cholesterol and low density lipoprotein cholesterol compared to control pigs just fed the high cholesterol diet. This appears to be supported in part by the study by Harrison and Peat (34) which showed that serum cholesterol levels in infants decreased as the numbers of *L. acidophilus* cells in the feces increased. In contrast, Pulusani and Roa (62) found that there was no effect of the individual yogurt bacteria or *L. acidophilus* on the cholesterol level in rats.

The mechanism by which the serum cholesterol can be decreased is still unresolved. Gilliland et al. (24) suggest that *L. acidophilus* assimilates cholesterol therefore the cholesterol is removed via the removal of *L. acidophilus* cells in the feces. Gilliland (26) also suggests that lowering of serum cholesterol may be due in part to *L. acidophilus* being able to deconjugate bile acids which would then be less efficient for the absorption of lipids and cholesterol into the blood stream. Free bile acids are less well absorbed from the intestine thus more could be excreted in the feces. The replacement of these bile acids for the bile circulatory system could reduce levels of cholesterol in the

body since cholesterol is a precursor for bile acids. This theory was supported by a recent study (11), that revealed a relationship between reduction of serum cholesterol levels and bile acid deconjugation in the intestines of animals fed cells of *L. acidophilus*.

In contrast, Klavier et al. (44) suggested that this removal of cholesterol from medium is solely due to a bacterial bile salt-deconjugating activity and that there is not any direct action on cholesterol by the cells of *L. acidophilus*.

Viability of *L. acidophilus* and *L. casei* in Milk Based Products During Refrigerated Storage

When products containing *L. acidophilus* were first produced it was found that it is not an extremely competitive bacteria. Many times, more competitive microorganisms would utilize the nutritional components in milk and survive at 37°C (15). The result was a microbial population that had substantially limited the growth of *L. acidophilus* and a product that did not meet the flavor and therapeutic characteristics typically associated with fermented acidophilus milk (15). Pasteurization of milk before inoculation with *L. acidophilus* helped to prevent contaminants from becoming a major concern. Autoclaved, sterilized milk provided an even better solution, however the acidophilus milk made from it did not have a pleasant flavor.

Of equal concern was the shelf-life of these products. Fermented acidophilus milk was considered to contain sufficient viable bacteria for about a week at refrigeration temperatures but the earlier one consumed the product the better (47). In early

acidophilus milk products, it was difficult to quantify the numbers of *L. acidophilus* in the milk. Limitations were imposed by a lack of adequate anaerobic growth chambers, proper growth media and the inability to distinguish *L. acidophilus* colonies from other Gram positive bacilli. According to Foster et al (14).; "If acidophilus therapy is to be of any value, therefore, large numbers of viable cells of a readily implantable strain of the organism must be consumed daily....". Fermented acidophilus milk was recommended for therapeutic treatment of many kinds of gastrointestinal disorders including constipation, nonulcerative colitis and diarrhea (47). According to Kosikowski (47), *L. acidophilus* "succumbs quickly" without sufficient transfer and he recommended that fermented acidophilus milk be "distributed rapidly" to consumers. Yet, Kulp (48) considered *L. acidophilus* stable for more than one week if treated in the proper manner. The practices that are necessary to maintain total counts of *L. acidophilus* in fermented milk are, 1) use a pure culture, 2) avoid development of excessive acidity and 3) store at a temperature below 12 to 16°C (48). *L. acidophilus* is also considered "stable" at 5° C(18). Kulp considered $2x10^8$/ml the minimum required count per ml for fermented acidophilus milk to be useful for intestinal treatments(48). A follow-up study of acidophilus milk reported less viability being maintained over time (46). These experiments by Kopeloff (46) in the 1920's may have seriously lacked the proper techniques to recover all viable *L. acidophilus*.

There are some more recent products which include *L. acidophilus* in a fermented milk product. *Biogarde* is a product made by the fermentation of milk with a *L. acidophilus*, *Bifidobacterium bifidum* and *S. thermophilus* starter (69). It is reported to

contain about 10^7 to 10^8 cells per ml of *L. acidophilus* and 10^6 to 10^7 of *B. bifidum* (69). *Bioghurt* is a yogurt like product produced using *S. thermophilus* and substituting *L. acidophilus* in place of *L. bulgaricus*. According to Roginski (71), both *Biogarde* and *Bioghurt* can retain viable cells of *L. acidophilus* at a level of 10^7 / ml for four weeks at refrigeration temperatures. *Acidophilus Bifidus Yogurt* is a yogurt preparation using the traditional cultures to make the yogurt and then two separately cultivated strains of *L. acidophilus* and *B. bifidum* are added. This results in a product with an initial population of 1 to 3 $x10^7$ /ml of each of these adjunct cultures (49).

Not all products containing *L. acidophilus* need to be fermented. Sweet Acidophilus Milk™ can be defined simply as a non-fermented milk with a concentrated culture of *L. acidophilus* added and chilled to prevent acid development (47). The resulting product tastes like normal milk but it does have *L. acidophilus* in the amount of $5x10^6$/ml (47).

There are a number of factors that effect survival of *L. acidophilus* in milk based products (75). They are as listed below:

1. Fermentation method, substrates and harvesting technique.
2. Microbial preservation technique (cryoprotectant) previous to addition to a product (if a consumer is to ingest the bacteria in the same form as they are "preserved" then this becomes even more important... i.e. dried powders, tablets and capsules ect.).
3. The level of oxygen incorporated into the product (excessive agitation).
4. The storage time before consumption.
5. The acidity of the product during storage.

6. The temperature of storage.
7. The water activity (A*w*) of the product during storage. (esp. for dried preparations).
8. Interactions with other microorganisms in the product (which may include antibacterial substances made by competitive bacteria).
9. Other antimicrobial substances or inhibitory substances in the product.
10. Consumer mishandling of products before ingestion.

According to Gilliland and Speck (18), *L. acidophilus* decreased in numbers during refrigerated storage in yogurt. Three different strains were used, however, none survived well. It may have been that these particular strains were not suited for use as adjunct cultures in yogurt. They all were susceptible to the H_2O_2 produced by the *L. delbrueckii* subsp. *bulgaricus* strain in the yogurt culture (18).

In another study, Gilliland and Speck (19) evaluated several different products that were reported to contain *L. acidophilus*. The products included health food preparations and milk with cells of *L. acidophilus* added. They found that only 3 of 7 products actually contained *L. acidophilus*. One of these three was the milk product. Interestingly, *L. casei* was identified in two of the seven products tested. Their results indicate that either *L. acidophilus* was not really included in the products or that it did not survive during storage.

Brennan et. al. (6) pointed out that freeze drying and vacuum drying adversely effected *L. acidophilus*. Increased sensitivity to NaCl , oxgall and lysozyme was found. Membrane damage is increased and cell surface material may be lost. More than 90% of previously dried cells lost viability after exposure to stresses such as oxgall. β-

galactosidase activity increased for cells that had been dried by either method compared to undried cells.

Rao and Gandhi (64) reported that *L. acidophilus* remained viable in "appreciable numbers" in fermented acidophilus milk for up to 15 days at 5-8°C. However, antibacterial activity (against *E. coli*, *S. aureus* and *B. subtilis*) of these milks decreased with increased storage time.

Gilliland and Lara (25) examined the effect of frozen storage and subsequent refrigerated storage on β-galactosidase activity of *L. acidophilus*. They also monitored the viability of the three strains they used over a period of 4 weeks at 5°C. The cells of *L. acidophilus* from frozen concentrated cultures were added to nonfermented milk for storage at 5°C. They observed variation among strains with respect to survival during the 28 days of refrigerated storage.

The pH at which the culture of *L. acidophilus* are grown prior to being added to nonfermented milk can influence their survival during refrigerated storage (29). Viability in frozen storage (-196°C) was not affected by the pH at which the culture had been grown. Yet, the subsequent viability in refrigerated milk was most significantly effected. Growth of the cells at pH 5.0 was preferable over growth at higher pH levels for attaining maximum viability in nonfermented acidophilus milk during storage at 5°C.

Some attempt to include *L. acidophilus* and *B. bifidum* in contemporary products such as ice cream and soft-serve frozen yogurt has been made. Holcomb, Frank and McGregor (37) placed both of these "probiotic" bacteria into the pre-frozen yogurt mix and sampled before and after freezing. Their results revealed that there was no significant

loss of viability caused by freezing. An interesting side note is that they used MRS agar at specific pH's to select for the two adjunct bacteria. They used pH 5.4 for enumerating the *L. acidophilus* and 6.5 for the bifidobacteria.

The use of ice cream as a carrier of probiotic bacteria was tested by Hekmat and McMahon (35). The fermentation of the mix utilized an inoculum of both *L. acidophilus* and *B. bifidum*. Viability as well as β-galactosidase activity were monitored over a period of 17 weeks at -29°C. Interestingly, they were able to grow their (anaerobic) *Bifidobacterium* in the strawberry flavored ice cream mix without any special atmosphere modifications. Enumeration was performed on reinforced clostridial agar (RCA) incubated in an anaerobic chamber. Actual differential counts for this enumeration were based on colony morphology; *L. acidophilus* colonies were supposed to be small on this agar and *B. bifidum* were large colonies on the same plate. Initial (pre-frozen) values were 5×10^8 CFU / ml for both adjuncts. After freezing the counts were 1.5×10^8 and 2.5×10^8 for *L. acidophilus* and *B. bifidus* respectively. After 17 weeks these researcher reported that they still had viable counts of 4×10^6 CFU / ml *L. acidophilus* and 1.5×10^7 CFU/ml of *B. bifidum*. According to the authors, a 30 % decrease in β-galactosidase activity took place for the product as a whole over 17 weeks. This was considered better retention of activity than with many cultured products and the authors recommended the use of "probiotic" ice cream even for most lactose maldigestors.

Product using *Lactobacillus casei*

Yakult is a drink containing *Lactobacillus casei* var. *Shirota*. This lactic acid containing beverage was developed in1935 by Dr. Minoru Shirota in an attempt to produce a beverage that could help bolster the quantities of lactic acid bacteria in the intestines (77). He assumed that this would help to discourage pathogenic and other "putrefactive" organisms from inhabiting the intestines of consumers and thereby increase their overall health. Dr. Shirota discovered a "lactic acid bacteria growth promoting substance" which he isolated from chlorella (a unicellular green algae)(77). He has since utilized this substance to help culture the "Yakult lactobacilli" at industrial scale.

Lactobacillus casei var. *Shirota* is also considered to be more acid tolerant than *L. acidophilus*. In the report by Dr. Shirota, the *L. casei* var. *Shirota* withstood a pH of 3.1 for 21 days. In the same report *L. acidophilus* died at the same pH after 14 days. Interestingly, *L. acidophilus* survived at a pH of 2.7 for 14 days yet the *L. casei* var. *Shirota* were eliminated in only 12 hours at this pH (77).

According to Dr. Shirota (77) his isolate is able to grow on the carbohydrate rhamnose which may link it to the *L. casei* subsp. *rhamnosus* listed in *Bergey's Manual of Determinative Bacteriology* (7). Many studies have been performed to show that consumption of Yakult can indeed help the treatment of constipation, diarrhea and rehabilitation of patients given full antibiotic treatments (77). This may be linked to some effect Yakult bacteria have on pathogenic bacteria. Some evidence for enhancement of growth for animals consuming Yakult has been shown (77). Interestingly, at least one mouse study compared the growth effects of Yakult with that of yogurt consumption. Yogurt reportedly did not increase growth compared to the control yet the Yakult fed

mice grew nearly twice as large (by weight) (77). Continuous consumption of Yakult may be necessary to receive any of these potential benefits. *L. casei* var. *Shirota* levels decreased from 10 million CFU / ml to below 100,000 CFU / ml in 3 days for adults (77). Dramatic decreases (2 log cycles or more within weeks) do take place after consumption of this product stops.

BIBLIOGRAPHY

1. Abdel-Bar, N., N. A. Harris and R. L. Rill. 1987. Purification and properties of an antimicrobial substance produced by *Lactobacillus bulgaricus*. J. Food Sci. 52: 411-415.

2. Attaie, R., P. J. Whalen, K. M. Shahani and M. A. Amer. 1987. Inhibition of growth of *Staphylococcus aureus* during production of acidophilus yogurt. J. Food Prot. 50 (3): 224-228.

3. Ayebo, A. T. D., K. M. Shahani and R. Dam. 1981. Antitumor component(s) of yogurt: fractionation. J. Dairy Sci. 64: 2318-2323.

4. Babel, F. J. 1977. Antibiosis by lactic culture bacteria. J. Dairy Sci. 60: (5) 815-821.

5. Branen, A. L., and R. P. Genske. 1975. Purification and properties of antimicrobial substances produced by *Streptococcus diacetilactis* and *Leuconostoc citrovorium*. J. Food Sci. 40: 446

6. Brennan, M., B. Wanismal, M. C. Johnson and B. Ray. 1986. Cellular damage in dried *Lactobacillus acidophilus*. J. Food Prot. 49: 47-53.

7. Buchanan, R. E. and N. E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology (8th ed.). The Williams and Wilkins Co., Baltimore, MD.

8. Cogan, T. 1985. The Leuconostocs. pp. 25-40. S. E. Gilliland (ed.) In Bacterial Starter Cultures for Foods. CRC Press, Boca Raton, FL.

9. Dahiya, R. S. and M. L. Speck. 1968. Hydrogen peroxide formation by lactobacilli and its effects on *Staphylococcus aureus*. J. Dairy Sci. 51: 1568-1573.

10. Danielson, A. D., E. R. Peo Jr., K. M. Shahani, A. J. Lewis, P. J. Whalen and M. A. Amer. Anticholesteremic property of *Lactobacillus acidophilus* yogurt fed to mature boars. J. Animal Sci. 67: (4) 966-974.

11. De Rodas, B. Z., 1994. The effect of *Lactobacillus acidophilus* and dietary calcium on serum cholesterol level in swine. Doctoral Dissertation, Oklahoma State University, Stillwater, OK.

12. Dodd, H. M. and M. J. Gasson. 1994. Bacteriocins of lactic acid bacteria. pp. 211-251. Gasson, M. J. and de Vos, W. M (eds). Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional, London.

13. Fernandes, C. F., K. M. Shahani, M. A. Amer. 1987. Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. FEMS-Microbiology-Reviews. 46: 3, 343-356.

14. Foster, E. M., F. E. Nelson, M. L. Speck, R. N. Doetsch and J. C. Olsen. 1957. Dairy Microbiology. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

15. Frost, W. D., T. H. Butterworth and S. M. Farr. 1931. Present status of acidophilus milk. Am. J. Public Health. 21: 862-866.

16. Gilliland, S. E. and M. L. Speck. 1969. Biological response to lactic streptococci and lactobacilli to catalase. Appl. Microbiology. 17: 797.

17. Gilliland, S. E. and M. L. Speck. 1975. Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. J. Food Sci. 40: 903-905.

18. Gilliland, S. E. 1977. Instability of *Lactobacillus acidophilus* in yogurt. J. Dairy Sci. 60 (9): 1394-1398.

19. Gilliland, S. E. and M. L. Speck. 1977. Enumeration and Identity of Lactobacilli in Dietary Products. J. of Food Protection. 40 (11): 760-762.

20. Gilliland. S. E. and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. J. Food Prot. 40: (12) 820-823.

21. Gilliland, S. E. 1979. Beneficial interactions between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. J. Food Protection. 42: 164-167.

22. Gilliland, S. E. and H. S. Kim. 1984. Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. J. Dairy Sci. 67: 1-6.

23. Gilliland, S. E. 1985. The Lactobacilli: Milk Products. pp. 41-55. Gilliland, S. E. (ed.) Bacterial Starter Cultures for Foods. CRC Press, Boca Raton, FL.

24. Gilliland, S. E., C. R. Nelson and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus.* Appl. and Environmental Microbiology. 49 (2): 377-381.

25. Gilliland, S. E. and R. C. Lara. 1988. Influence of storage at freezing and subsequent refrigeration temperatures on beta-galactosidase activity of *Lactobacillus acidophilus.* Applied and Environ. Microbio. 54: (4) 898-902.

26. Gilliland, S. E. 1989. Acidophilus milk products: A review of potential benefits to consumers. J. Dairy Science. 72: 2483-2494.

27. Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiology Reviews. 87: 175-188.

28. Gilliland, S. E. and D. K. Walker. 1990. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. J. Dairy Sci. 73: 905-911.

29. Gilliland, S. E. and C. N. Rich. 1990. Stability during frozen and subsequent refrigerated storage of *Lactobacillus acidophilus* grown at different pH. J. Dairy Sci. 73: 1187-1192.

30. Gilliland, S. E. 1991. Properties of Yogurt. pp.65-80. (ed.) R. K. Robinson. In Therapeutic Properties of Fermented Milks. Elsevier Science Publishers LTD. New York. NY.

31. Goldin, B. R. and S. L Gorbach. 1984. Alterations of the intestinal microflora by diet, oral antibiotics and *Lactobacillus*: Decreased production of free amines from aromatic nitro compounds, azo dyes, and glucuronides. J. Nat. Cancer Institute. 73: 689-695.

32. Goldin, B. R. and S. L. Gorbach. 1984. The effect of milk and *Lactobacillus* feeding on human intestinal bacterial enzyme activity. Am. J. Clin. Nutr. 39: 756-761.

33. Goodenough, E. R. and D. H. Kleyn. 1976. Influence of viable yogurt microflora in the digestion of lactose by the rat. J. Dairy Sci. 59: 601-606.

34. Harrison, V. C. and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nurt. 28: 1351-1355.

35. Hekmat, S. and D. J. McMahon. 1992. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* in ice cream for use as a probiotic food. J. Dairy Sci. 75: (6) 1415-1422.

36. Hepner, G., R. Fried, S. St. Jeor, L. Fusetti and R. Morin. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutrition. 32: 19-24.

37. Holcomb, J. E. and J. F. Frank. 1991. Viability of *Lactobacillus acidophilus* and *Bifidobacteria bifidum* in soft-serve frozen yogurt. Cult. Dairy Prod. J. Aug.: 4-5.

38. Hoover, D. G. 1993. Bacteriocins with Potential for use in Foods. Davidson, P. M. and Branen, A. L. (eds.) Marcel Dekker, Inc., New York, NY.

39. Hourigan, J. A. 1984. Nutritional implications of lactose. Austral. J. Dairy Technol. 39 (3): 114-120.

40. Houts, S. S. 1988. Lactose intolerance. Food Technol. 42 (3): 110-113.

41. Kato, I., K. Endo and T. Yokokura. 1994. Effects of oral administration of Lactobacillus casei on antitumor responses induced by tumor resection in mice. Int. J. Immunopharmac. 16: (1) 29-36.

42. Kempler, G.M. and L. L. McKay. 1981. Biochemistry and genetics of citrate utilization in *Streptococcus lactis* ssp. *diacetylactis*. Journal of Dairy Science. 64. 1561.

43. Kim, H. S. and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. J. Dairy Sci. 66: 959-966.

44. Klaver, F. A. M. and R. Van Der Meer. 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. Applied Envir. Microbio. 59: 1120-1124.

45. Kolars, J. C., M. D. Levitt, M. Aouji and D. A. Savaiano. 1984. Yogurt: an autodigesting source of lactose. New England J. Medicine. 310: 1-3.

46. Kopeloff, N. 1926. Lactobacillus acidophilus. London; Bailliere, Tindall and Cox.

47. Kosikowski, F. W. 1977. Cheese and Fermented Milk Products. 2nd Ed. Ann Arbor, Mich., Edwards Brothers, INC.

48. Kulp, W. L. 1931. Studies on the viability of *Lactobacillus acidophilus* in acidophilus milk. Am. J. Public Health. 21: 873-883.

49. Kurmann, J. A., J. L. Rasic and M. Kroger. 1992. Encyclopedia of Fermented Fresh Milk Products. Von Nostrand Reinhold, NY, NY.

50. Martin, D. R. and S. E. Gilliland. 1980. Inhibition of psychrotrophic bacteria in refrigerated milk by lactobacilli isolated from yogurt. J. Food Protection. 43: 673-678.

51. Martini, M.C., D. E. Smith and D. A. Savaiano. 1987. Lactose digestion from flavored and frozen yogurts, ice milk and ice cream by lactase-deficient persons. Am. J. Clin. Nutrition. 46: 636-640.

52. McKay, L. L., A. Miller, W. E. Sandine, and P. R. Elliker. 1970. Lactose utilization by lactic acid streptococci; enzymatic and genetic analyses. J. Bacteriology. 102: 804.

53. Metchnikoff, E. 1907. The Prolongation of Life. G. P. Putnam's Sons, New York.

54. Oberman, H. 1985. Fermented Milks p. 167-195. in Microbiology of Fermented Foods. Vol. 1. Elsevier Applied Science Publishers. London and New York.

55. Onwulata, C. I., D. R. Roa and P. Vankineni. 1989. Relative efficiency of yogurt, sweet acidophilus milk, hydrolyzed-lactose milk, and a commercial lactase tablet in alleviating lactose maldigestion. Am. J. Clin. Nutr. 49: 1233-1237.

56. Pack, M.Y., Vedamuthu, E.R., Sandine, W.E. and Elliker, P.R. 1968. Hydrogen peroxide-catalase milk treatment for enhancement and establishment of diacetyl in lactic starter cultures. J. Dairy Sci. 51 (4): 511-516.

57. Patel, J. R., J. M. Dave and R. I. Dave. 1992. Effect of feeding milk fermented with strains of lactobacilli on faecal lactobacilli and coliform counts in human test subjects. Indian J. Dairy Sci. 45: 379-382.

58. Payne, D. L., J. D. Welsh, C. V. Manion, A. Tsegaye and L. D. Herd. 1981. Effectiveness of milk products in dietary management of lactose malabsorption. American J. Clin. Nutrition. 34: 2711-2715.

59. Perdigon, G., S. Alverez, M. E. Demacias, R. A. Margni, G. Oliver and A. A. D. De Ruiz Holgado. 1986. Lactobacilli administered orally induce release of enzymes from peritoneal macrophages in mice. Milchwessenschaft. 41: 344-347.

60. Perdigon, G., M. E. N. Demacias, M. E. Roux and A. P. R. Holgado. 1990. The oral administration of lactic acid bacteria increases the mucosal intestinal immunity in response to enteropathogens. J. Food Prot. 53: 404-410.

61. Perdigon, G., M. E. N. Demacias, S. Alverez, G. Oliver and A. A. P. R. Holgado. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Dairy Research. 57: 255-264.

62. Pulusani, S. R. and D. R. Rao. 1983. Whole body, liver and plasma cholesterol levels in rats fed *Streptococcus thermophilus, Lactobacillus bulgaricus* and *Lactobacillus acidophilus* milk. J. Food Sci. 48: 280-281.

63. Rao, D. R., B. M. Reddy, G. R. Sunki and S. R. Pulusani. 1981. Influence of antimicrobial compound(s) extracted from milk fermented by *Streptococcus thermophilus* on keeping quality of meat and milk. J. Food Quality. 4: 247-258.

64. Rao, S. M and D. N. Gandhi. 1987. Studies on the microbial quality of acidophilus milk. Indian J. Dairy Sci. 40: 4, 451-453.

65. Rasic, J. L. and J. A. Kurmann. 1978 Yoghurt: Scientific Grounds, Technology, Manufacture and Preparations. (Fermented Fresh Milk Products Series: Vol. 1.) Technical Dairy Publishing House, Copenhagen, Denmark.

66. Rasic, J. L. 1987. Nutritive value of yogurt. Cult. Dairy Prod. J. 22: 6-9.

67. Reddy, G. V., B. A. Friend, K. M. Shahani and R. E. Farmer. 1983. Antitumor activity of yogurt components. J. Food Protection. 46: 8-11.

68. Robinson,E. L. and W. L. Thompson 1952. Effect on weight gain of the addition of *Lactobacillus acidophilus* to the formula of newborn infants. J. Pediatr. 41: 395-398.

69. Robinson, R. K. (ed.). 1991. Therapeutic Properties of Fermented Milks. Elsevier Applied Science, London, UK.

70. Rodke-Mitchell, L. and W. E. Sandine. 1984. Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*: A review. J. Food Prot. 47 (3): 245-248.

71. Roginski, H. 1988. Fermented milk. Australian Journal of Dairy Technology. 43 (2): 37-46.

72. Sako, K., H. Saito, H. Tomioka and T. Yokokura. 1988. Enhancement of host resistance against *Listeria* infection by *Lactobacillus casei*: Efficacy of cell wall preparation of *Lactobacillus casei*. Microbio. Immunology. 32: 1189-1200.

73. Sandine, W. E., K. S. Muralidhara, P. R. Elliker and D. C. England. 1972. Lactic acid bacteria in food and health: a review with special reference to enteropathogenic *E. coli* as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Milk and Food Tech. 35 (12): 691-702.

74. Savaiano, D. A., A. A. El Ansuar, D. E. Smith and M. D. Levitt. 1984. Lactose malabsorption from yogurt, pasteurized yogurt, sweet acidophilus milk and cultured milk in lactase-deficient individuals. American J. Clin. Nutrit. 40: 1219-1223.

75. Sellars, R. *L.* 1991. *Acidophilus* products. Ch. 5. (p.81-116). Therapeutic Properties of Fermented Milks. Elsevier Applied Science, London, UK.

76. Shahani, K. M., B. A. Friend and P. J. Bailey. 1983. Antitumor activity of fermented colostrum and milk. J. Food Protection. 46: 385-386.

77. Shirota, Minoru. 1971. The summary of reports on Yakult. Yakult Honsha Co., LTD., 1: 1-19. Higashi-Shinbashi, Minato-ku, Tokyo, Japan.

78. Silva, M., N. V. Jacobus, C. Deneke and S. L. Gorbach. 1987. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrobial Agents and Chemotherapy. 31: 1231-1233.

79. Simon, G. L. and Gorbach, S. L. 1984. Intestinal flora in health and disease. Gastroenterology. 86: 174-193.

80. Tamine, A. Y. and R. K. Robinson. 1988. Fermented milks and their future trends. II. Technological Aspects. J. Dairy Research. 55: 281-307.

81. Vincent, J. G., R. C. Veomett and R. F. Riley. 1959. Antibacterial activity associated with *Lactobacillus acidophilus*. J. Bacteriology. 78: 477-484.

82. Walker, D. K. and S. E. Gilliland. 1993. Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *Lactobacillus acidophilus*. J. Dairy Sci. 76: 956-961.

83. Watkins, B. A. and B. F. Miller. 1983. Competitive gut exclusion of avian pathogens by *Lactobacillus acidophilus* in gnotobiotic chicks. Poultry Science. 62: 1772-1779.

84. Webb, B. H. (ed.). 1970. Byproducts from Milk (second ed.). Avi Publishing Company, Inc. Westport, Conn.

85. Wilson, F. L. and F. W. Tanner. 1945. Behavior of pathogenic bacteria in fermented milks. Food Research. 10: 122-134.

86. Winkleman, F. 1986. Legal Aspects of Yogurt. Milk: the Vital Force: Proceedings of the XXII Intern. Dairy Congress. The Hague. D. Reidel Publishing Company, Boston, Mass., USA.

CHAPTER III

VIABILITY OF *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS CASEI* IN FERMENTED MILK PRODUCTS DURING REFRIGERATED STORAGE

Benton D. Nighswonger and Stanley E. Gilliland

Department of Animal Science, Oklahoma State University

Stillwater, Oklahoma 74078

ABSTRACT

Viability of five strains of *Lactobacillus acidophilus* and one strain of *L. casei* added as adjuncts to yogurt and cultured buttermilk during 28 days of refrigerated storage (5-7 C) was investigated. A modification of LBS agar was used for the enumeration of *L. acidophilus* and *L. casei*. This medium allowed the colony formation of the adjunct bacteria while preventing colony formation of the traditional yogurt or buttermilk starter cultures. At each sampling period colonies from the selective agar medium were isolated for characterization and comparison using a commercially available identification kit (API CHL 50). This helped ensure that we were enumerating only the strains of *L. acidophilus* and *L. casei* and that they had not changed during storage. *L. acidophilus* survived better in cultured buttermilk than in yogurt. However, there was variation among the strains of *L. acidophilus* in both cultured products. *L. casei* survived very well in both cultured products. While there was variation in survival among strains of *L. acidophilus* in yogurt, none survived as well as did the one strain of *L. casei*.

INTRODUCTION

In the past, fermented acidophilus milk was considered a potential vehicle by which consumers might receive adequate numbers of cells of *Lactobacillus acidophilus*. Yet this product was considered to contain only sufficient numbers of viable bacteria to permit approximately one week storage at refrigeration temperatures (17). Beyond that, insufficient viable bacteria remained. In early studies involving fermented acidophilus milk, it was difficult to quantify the numbers of *L. acidophilus*. Limitations were imposed by a lack of adequate anaerobic growth chambers, proper growth media and the inability to distinguish colonies of *L. acidophilus* from those of other Gram positive bacilli. According to Kosikowski (17), *L. acidophilus* "succumbs quickly" without regular subculture and he recommended that fermented acidophilus milk be "distributed rapidly" to consumers. Yet, Kulp (18) considered *L. acidophilus* stable for more than one week if treated in the proper manner. Today, fermented acidophilus milk does not have much consumer appeal mostly due to its poor flavor.

Consumption of lactobacilli, such as *L. acidophilus* and/or *L. casei*, has potentials of aiding lactose digestion (16), aiding in the control of serum cholesterol (4,10,12), controlling intestinal infections (9,20,21,24), and exerting antitumor activity (11,15,19,25). For most of these benefits it is likely that adequate numbers of viable cells of *L. acidophilus* and/or *L. casei* need to be consumed. Thus it is important that the lactobacilli remain viable during storage of products containing them.

Cultured or culture containing dairy products supplemented with *L. acidophilus* have gained considerable consumer attention in recent years and for this reason more of these products are now reaching the public than ever before. In the past, few studies have been reported on quantifying the viability of such supplemental cultures in cultured products. Yet, some studies (7,8,14) have indicated that hydrogen peroxide produced by yogurt cultures may be detrimental to viability of added cells of *L. acidophilus*. No research has been reported on the stability of cultures of lactobacillli added to cultured buttermilk as adjuncts, yet this product may also serve as an acceptable vehicle for supplementation with bacteria having potential health benefits.

The objective of this study was to determine the effect of refrigerated storage in cultured buttermilk and yogurt on the viability of five strains of *L. acidophilus* and one strain of *L. casei*.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Two cultures of *Lactobacillus acidophilus* (strains La-5 and MUH-41) and one of *L. casei* were supplied by the Mona Division of Campina Melkunie (Woerden, The Netherlands). Three additional cultures of *Lactobacillus acidophilus* (strains ATCC 43121, L-1 and O-16) were isolated in previous studies in our laboratory (3,10) and are part of our stock culture collection (Department of Animal Sciences, Oklahoma State

University, Stillwater, OK). Before experimental use, all strains were subcultured at least three times in lactobacilli MRS broth (Difco Laboratories, Detroit, MI) using 1% inocula followed by incubation for 18 hours at 37°C. The yogurt cultures (CM-2 and YC-4) and the buttermilk culture (A) were supplied by Campina Melkunie. The yogurt cultures were maintained by using a 2% inocula into sterile 10% reconstituted nonfat dry milk (NDM) with subsequent incubation at 42°C for 5 hours. The buttermilk culture was maintained by using a 2% inocula into sterile 10% reconstituted NDM with subsequent incubation at 22°C for 18 hours. All cultures were stored at 5-7°C between transfers.

Production and Enumeration of *L. acidophilus* and *L. casei*

Lactobacilli MRS broth was used to propagate cells of the supplemental lactobacilli (*L. acidophilus* and *L. casei*). MRS broth was sterilized by autoclaving at 121°C for 15 minutes. A modification of LBS (*Lactobacillus* selection) agar was used for selective enumeration of *L. acidophilus* and *L. casei* in cultured buttermilk and yogurt. The modified LBS agar was prepared from individual ingredients according to the formulation of Baltimore Biological Laboratories (BBL, Cockeysville, MD) except the glucose was deleted and only 9/10th of the water was used. Following heating it was aseptically dispensed in 90 ml volumes into sterile, capped media bottles. Cellobiose, which is sensitive to excessive heating, was dissolved in distilled water to make a 10% solution and subsequently filter sterilized by passage through a sterile Acrodisc® .45μm filter (Gelman Sciences, Ann Arbor, MI) into a sterile, capped media bottle. Previous to

plating, 10 ml of this sterile 10% cellobiose solution was added to 90 ml of modified LBS agar, which had been melted and tempered to 45°C, to serve as a carbohydrate source for the supplemental lactobacilli. This medium was termed "C-LBS" agar. Bile resistant lactobacilli were enumerated using C-LBS agar supplemented with .1% oxgall added before heating ("C-LBSO" agar).

Cell crops of *L. acidophilus* and *L. casei* were grown in 20 ml volumes of MRS broth. For each culture, the broth was inoculated with 1% using a freshly prepared culture of the desired strain of lactobacilli and incubated at 37°C for 18 hrs. They were then centrifuged at 12,000 x g for 10 minutes at 5°C to harvest the cells. The cell pellets were resuspended (by vortexing twice for five and ten seconds, with model 232; Fisher Scientific Co.) in 10ml volumes of sterile 10% reconstituted NDM. These suspensions contained approximately $9x10^8$ cells/ml. They were held in an ice and water mixture until used (within 30 minutes).

For measurement of the total numbers of *L. acidophilus* or *L. casei,* appropriate dilutions were prepared according to the methods described in the *Compendium of Methods for the Microbiological Examination of Foods* (27) using 99-ml dilution blanks containing 1% peptone (Sigma) and 0.01% silicone antifoamer (Sigma Chemical Co., St. Louis, MO.) and plated by the pour plate method with C-LBS agar. Numbers of bile tolerant lactobacilli were measured by plating the appropriate dilutions with C-LBSO agar. The plates were placed, inverted, in plastic bags which were subsequently flushed for 10 seconds with CO_2 and sealed. All plates were incubated at 37°C for 48 hours. The colonies were counted with the aid of a Quebec colony counter.

Two colonies from the highest dilution plated with C-LBS agar containing countable colonies for each strain of *L. acidophilus* or *L. casei* were grown in sterile lactobacilli MRS broth. The identities of the isolates were confirmed to ensure that the organisms being enumerated were the supplemental lactobacilli added to the yogurt or buttermilk.

Confirmation of identity of lactobacilli

Isolates from C-LBS agar were tested for Gram stain reaction and the ability to grow at 15 and 45°C. The API CHL 50 identification system (Biomerieux SA, RCS Lyon B, Marcy-l'Etoile, France) was used to test for the action of the cultures on 49 substrates. The API CHL 50 system was used according to the manufacturer's direction except that mineral oil was not used and the system was incubated anaerobically in a BBL® GasPak system (Becton Dickinson Microbiology Systems, Cockeysville, MD). The identity of the cultures was based on the phenotypic characteristics of the lactobacilli as presented in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (2).

Preparation of Cultured Buttermilk

Approximately 11.5 L of raw cows' milk was obtained from Oklahoma State University's Dairy Cattle Center. The milk was then separated and the appropriate amounts (based on fat content determined by the Babcock Method (23)) of the cream and the skim fractions were combined to yield 0.5% butterfat milk. The 0.5% fat milk was

then homogenized at 1000 psi and pasteurized by heating at 92°C for 6 minutes. The pasteurized milk was then cooled to 21°C and inoculated with 115 ml (1%) of a freshly prepared buttermilk culture (culture A). The inoculated milk was thoroughly mixed and 900ml portions were dispensed into each of eight sterilized capped bottles (approx. 1.2 L capacity bottles). All eight bottles were then tightly sealed and placed in a water bath at 21°C. One of the bottles was used to monitor pH. The bottles were incubated at 21°C until a pH of 4.55 was reached. At this point, the bottles were quickly immersed into an ice-water mixture and allowed to chill. Prior to breaking the curd, the required amounts of suspensions of *L. acidophilus* or *L. casei* were added to appropriately labeled bottles to yield an initial population of approximately 1×10^7 CFU/g. The buttermilk samples containing the 6 supplemental lactobacilli were dispensed into sterile dilution bottles to within a half inch of the top before capping. A bottle of buttermilk without added lactobacilli was dispensed in a similar manner to serve as a control at each sampling period. All bottles were labeled for appropriate sampling day and stored at 5°C. One bottle of each supplemental stain was removed from refrigerated storage on the appropriate day for analyses. A pH determination was performed and numbers of total and bile resistant supplemental lactobacilli were enumerated. The experiment was replicated three times on separate days.

Preparation of Yogurt

Approximately 4 gallons of raw cows' milk was obtained from Oklahoma State University's Dairy Cattle Center. The milk was then separated and the appropriate amounts (based on fat content determined by the Babcock Method (23)) of the cream and the skim fractions were combined to yield 3% butterfat milk. A 20,000 ml portion (approx. 19.37 kg) of the 3% fat milk was then supplemented with 700g of NDM. After thoroughly mixing, the yogurt mix was homogenized at 1000 psi followed by pasteurization at 92°C for 6 minutes. After pasteurization, 6.29 kg of mix was aseptically dispensed into a sterile stainless steel vat. The vat was placed into a water bath and tempered to 45°C. After reaching target temperature, it was inoculated with 130 ml (approximately a 2.07% inoculation) of the desired yogurt culture (CM2 or YC4). The inoculated milk was allowed to incubate at 45°C until a pH 4.9 was reached. The container was then submersed into an ice-water mixture and stirred (with a sterile spoon) gently to help it to cool rapidly and consistently. The chilled yogurt was dispensed in 1000g portions into large sterile beakers. Appropriate amounts of suspensions of cells of the *L. acidophilus* or *L. casei* were added to the appropriately labeled beakers and mixed thoroughly to yield initial populations of approximately 1 x 10^7 CFU/g. Approximately 200g portions of the yogurt containing the supplemental lactobacilli were dispensed into 5 plastic 224g (8 oz) cups. After capping, all cups were labeled for appropriate sampling day and stored at 7°C. One cup for each supplemental culture of lactobacilli was removed

from refrigerated storage on the appropriate day for analyses. A cup of yogurt without added lactobacilli served as a control at each sampling period. The pH of each sample was measured and the numbers of total and bile resistant supplemental lactobacilli were enumerated. This procedure was replicated three times on separate days for each yogurt culture for a total of three replicate trials for both yogurt cultures CM-2 and YC-4.

Statistical Methods

Analysis of variance for each set of data was conducted as a split-split plot in a randomized block design to determine whether significant differences existed (26). Each replication trial was a block, the cultures of supplemental lactobacilli were the main unit treatment, the time of storage was the subunit treatment and the presence or lack of oxgall in the media was a sub-sub unit treatment. Least significant difference analyses were used to compare means for significant differences at the 5% level of confidence. There were no statistical comparisons made between yogurt samples or among the buttermilk samples and the yogurt samples.

RESULTS

The buttermilk culture, yogurt culture CM-2 and yogurt culture YC-4 did not form colonies on C-LBS or C-LBSO agars. All cultures of *L. acidophilus* and *L. casei* formed equal numbers of colonies on both of these media as well as on lactobacilli MRS agar. Plating of the control samples (i.e. those without the supplemental lactobacilli) on C-LBS and C-LBSO agars resulted in no colony formation. Thus, C-LBS and C-LBSO agars were considered suitable for the enumeration of total and bile tolerant numbers of *L. acidophilus* and *L. casei* in cultured buttermilk and yogurt prepared using the indicated starter cultures.

Effect of Storage at 5°C in Cultured Buttermilk:

Total numbers of lactobacilli in cultured buttermilk containing added cells of *L. acidophilus* 43121 declined significantly ($P<.05$) with increased storage time at 5°C (Table 1). There was a slight, although nonsignificant ($P>.05$) increase in numbers from day 0 to day 7. This phenomenon was observed for all strains and may be attributed to the breaking up of clumps or chains of the supplemental lactobacilli. Compared to the initial population, the decline became significant ($P<.05$) on day 21. The numbers of bile resistant *L. acidophilus* 43121 showed a similar behavior during the 28 day storage period. There were significantly lower ($P<.05$) counts on C-LBSO agar than on CLBS agar for strain 43121 on days 14, 21 and 28 (Table 1).

The total numbers of lactobacilli in buttermilk containing added cells of *L. acidophilus* MUH-41 did not decline as rapidly as observed for strain 43121(Table 1). The numbers of viable lactobacilli did not decline significantly ($P>.05$) from the number of viable lactobacilli at day zero. Yet, there was a significant decline ($P<.05$) in numbers of bile tolerant colonies formed on C-LBSO agar. There were significantly lower ($P<.05$) counts on C-LBSO agar for this strain than on C-LBS agar for days 7, 14, 21 and 28 (Table 1).

The total numbers of supplemental lactobacilli in buttermilk containing cells of *L. acidophilus* La-5 during storage exhibited nearly the same pattern of declines as did strain MUH-41. There were, however, significant declines ($P<.05$) for both total numbers and numbers of bile tolerant *L. acidophilus*. There also were significant differences ($P<.05$) between counts on C-LBS and C-LBSO on days 14 and 21 for strain La-5 (Table 1).

Compared to initial counts, the total numbers of lactobacilli and the numbers of bile tolerant lactobacilli in cultured buttermilk containing added cells of *L. acidophilus* L-1, *L. acidophilus* O-16 or *L. casei* did not decline significantly ($P>.05$) from the numbers present on day 0 during 28 days of storage at 5°C (Table 1). There also were no significant differences ($P>.05$) between numbers enumerated on C-LBS agar and on C-LBSO agar for either *L. acidophilus* L-1, *L. acidophilus* O-16 or *L. casei* (Table 1).

The initial pH values (day 0) of cultured buttermilk samples in the three trials were 4.5 to 4.6 and did not change during the 28 days of storage at 5°C (data not shown). This was true for the control samples as well as for those containing added cells of *L. acidophilus* or *L. casei*.

Effect of Storage at 7°C in Yogurt

There were no significant differences (P>.05) between the total numbers and numbers of bile tolerant supplemental lactobacilli for any strain of *L. acidophilus* or for *L. casei* on any day of sampling for yogurt made with culture CM-2 (Table 2). Total and bile tolerant counts for the yogurt containing added cells of *L. acidophilus* 43121 declined significantly on day 28 of storage at 7°C. Total and bile tolerant counts in the yogurt containing added cells of *L. acidophilus* MUH-41 and La-5 declined significantly (P<.05) by day 21 of storage at 7°C. While the total numbers of *L. acidophilus* in the yogurt supplemented with strain O-16 declined significantly (P<.05) by day 21, there was not a significant decline (P>.05) in numbers of bile tolerant *L. acidophilus* O-16 until day 28 (Table 2). Total numbers and numbers of bile tolerant *L. acidophilus* in the yogurt supplemented with cells of *L. acidophilus* L-1 were stable for 28 days, with no significant decline (P>.05). The latter observation also was true for the numbers of *L. casei*.

The initial (day 0) pH values for three batches of yogurt CM-2 were in a range of 4.5 to 5.0. The pH values decreased over time to 4.2 to 4.4 by 28 days of storage at 7°C (data not shown). There were no apparent differences for pH among yogurt CM-2 control samples or the samples with cells of different strains of *L. acidophilus* or *L. casei* after 28 days of storage at 7°C.

Some strains of *L. acidophilus* exhibited significantly higher counts on CLBS-O than on CLBS agar during storage in yogurt made with culture YC-4 (Table 3). For

yogurt made with culture YC-4 and supplemented with cells of *L. acidophilus* 43121 the total numbers and numbers of bile tolerant *L. acidophilus* did not decline significantly (P>.05) during 28 days of storage at 7°C. The total and bile tolerant numbers of *L. acidophilus* in the yogurt containing cells of *L. acidophilus* MUH-41 declined significantly (P<.05) on day 21 of storage at 7°C if compared to day 7 but not if compared to day 0 (Table 3). There were significantly higher numbers enumerated on C-LBS-O agar compared to C-LBS agar for *L. acidophilus* MUH-41 on days 7, 21 and 28 (P<.05).

The total numbers of *L. acidophilus* in yogurt YC-4 containing added cells of *L. acidophilus* O-16 had declined significantly (P<.05) by day 14 and also had an additional significant decline (P<.05) by day 21 and again on day 28. There was a significant decline (P<.05) in numbers of bile tolerant *L. acidophilus* on day 14 and again on day 28. There were significant differences (P<.05) between numbers enumerated on C-LBS agar compared to C-LBSO agar for *L. acidophilus* O-16 on days 21 and 28 (Table 3). As with *L. acidophilus* MUH-41, the counts were higher on C-LBSO than on C-LBS agar.

Total numbers and numbers of bile tolerant *L. acidophilus* in yogurt supplemented with cells of *L. acidophilus* L-1 were stable, with no significant decline (P>.05) until 28 days of storage. The total and bile tolerant numbers of *L. acidophilus* for yogurt made with culture YC-4 and supplemented with cells of *L. acidophilus* La-5 declined significantly (P<.05) by 14 days of storage at 7°C with additional significant declines on days 21 for total numbers and on day 28 for the bile tolerant counts . The total numbers and numbers of bile tolerant *L. casei* for yogurt prepared with culture YC-4 with added

cells of *L. casei* did not decrease significantly (P>.05) during 28 days of storage at 7°C (Table 3).

The initial (day 0) pH values of yogurt YC-4 in three batches were 4.8 to 5.0 and decreased to 4.4 to 4.8 after 28 days of storage at 7°C. There were no apparent differences for pH among the control samples or the samples containing different strains of supplemental *L. acidophilus* or *L. casei* in yogurt YC-4 initially or after storage for 28 days.

Confirmation of Identity of Lactobacilli from Storage Samples

Two colonies were isolated from the highest countable plates from the C-LBS agar at each sampling period for each product and tested for identification using API CHL 50 kits. The isolates all were confirmed to be the *L. acidophilus or L. casei* added to the buttermilk or yogurt samples at the start of the experiments. While, there did appear to be some slight differences in fermentation of some carbohydrates as storage time increased, the overall pattern was always consistent with the *L. acidophilus* or *L. casei* which had been added to the cultured product. None of the bacteria in the cultured buttermilk or yogurt cultures were encountered among these isolates.

DISCUSSION

Viability of *L. acidophilus* during storage at 5°C in cultured buttermilk varied among the strains. Statistical comparisons were not made between strains, yet apparent differences were observed. Based on the higher numbers at day 7, the numbers of *L. acidophilus* 43121 declined by approximately one log cycle (90% decline in viable cells) by the twenty-eighth day of storage at 5°C, whereas the number of viable cells of *L. acidophilus* O-16 declined by approximately 68% (Table 1). The increase of numbers from day 0 to day 7 for all strains of lactobacilli added to cultured buttermilk and most yogurts was likely due to chains or clumps of the supplemental lactobacilli breaking up during mixing and storage. Despite sampling methods being kept consistent between the cultured buttermilk trials and the yogurt trials, there appeared to be larger increases in the buttermilk. Comparison of the values at day 7 with those at day 14 for the buttermilk indicated significant declines for all strains from day 7 to day 14. All strains retained viable populations at 28 days of storage above $1x10^6$ CFU/g in cultured buttermilk stored at 5°C. No previous research on addition of lactobacilli to cultured buttermilk has been reported, yet the antimicrobial effects of diacetyl, acetic acid, lactic acid and potentially some bacteriocins (5) could be responsible for the declines of viability of the organisms in cultured buttermilk.

The data suggest that cultured buttermilk can be a suitable carrier food for supplying consumers with lactobacilli having potential health/nutritional benefits. However, care should be used in selecting strains of lactobacilli to provide maximum

survival during refrigerated storage of the cultured buttermilk. The buttermilk culture also could influence survival of the added lactobacilli. Thus the choice of the starter culture also should be considered.

Gilliland and Speck (7) reported that *L. acidophilus* is capable of remaining viable in an acidic environment such as milk acidified with lactic acid. However, they found that numbers of viable cells of *L. acidophilus* placed in yogurt declined markedly within 7 days based on enumeration of bile tolerant lactobacilli on LBS agar supplemented with oxgall (7). Our results, utilizing different strains of *L. acidophilus* and different yogurt cultures, indicate that some strains are capable of remaining viable in yogurt for up to 28 days of storage at 7°C. This improved storage stability also could be related to the use of different media for the enumeration of *L. acidophilus*.

Hull et al. (14) concluded that *L. acidophilus* could have improved stability during refrigerated storage if added to yogurt at the same time as the traditional yogurt cultures and allowed to grow during the fermentation process. They found that *L. acidophilus*, added after the yogurt manufacture, died rapidly. They reported only 1 percent survival after 4 days of storage at 5°C. Death of cells of *L. acidophilus* was attributed to the effects of hydrogen peroxide produced in the yogurt. They concluded that the hydrogen peroxide did not cause the same results in yogurt prepared with the strains of *L. acidophilus* included in the yogurt fermentation and attributed this increased tolerance to H_2O_2 to an acquired or "induced" mechanism. Theoretically the *L. acidophilus* grown with the yogurt cultures developed a hydrogen peroxide splitting activity that remained "uninduced" in *L. acidophilus* added post-fermentation (14).

These researchers used a medium to enumerate the *L. acidophilus* that consisted of a modified MRS agar that excluded glucose and replaced it with maltose as the only carbohydrate source (14). This allowed them to enumerate *L. acidophilus*, which could utilize maltose, in the presence of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which could not readily utilize maltose (14).

We did not observe as great of declines in numbers of any of the strains of *L. acidophilus* or *L. casei* that were added after the yogurt was fermented and cooled, as was reported by Hull et al. (14). In our study, there were differences among the strains of *L. acidophilus*. However, even the least stable strains (La-5 for example) still retained viable cells capable of growing in the presence of bile salts at levels exceeding 1×10^6 CFU/g in both yogurt CM-2 and YC-4 after twenty-one days of storage at 7°C. Even though not designed for statistical comparison, there appeared to be differences between the two yogurts with respect to influence on survival of *L. acidophilus* during storage at 7°C. This could be due to variations of antimicrobial substances produced in the different yogurt cultures.

Of particular importance for the impact of yogurt on the stability of added *L. acidophilus* or *L. casei* would be the production of hydrogen peroxide (6,7,14) and potentially some bacteriocins (1,5,13,22) by *L. bulgaricus* and/or *S. thermophilus*. The narrow spectrum of activity of most bacteriocins against other members of its own genus could play an important role in survival of lactobacilli added as adjuncts to cultured milk products. The differences observed between the survival of *L. acidophilus* and *L. casei* in

yogurts made with two different cultures suggests the need to carefully select yogurt cultures for such products supplemented with cells of *L. acidophilus* or *L. casei*.

In summary, strains of *L. acidophilus* varied in their ability to remain viable during refrigerated storage in the two fermented milk products in this study. The addition of an appropriate strain of *L. acidophilus* to cultured buttermilk or yogurt after fermentation at a level of approximately $1x10^7$ CFU/g can result in numbers of viable *L. acidophilus* in excess of $1x10^6$ CFU/g after 28 days of storage at 5 and 7°C, respectively. Results of this study focus attention on the necessity of choosing appropriate strains of *L. acidophilus* or *L. casei* as well as the starter culture for manufacture of the cultured product to which probiotic type cultures are to be added. *L. casei* shows promise as another *Lactobacillus* species in addition to *L. acidophilus* for use as a bacterial supplement to fermented products as its refrigerated storage stability in these products was apparently equal to, or greater than that of the strains of *L. acidophilus* tested. More research also is needed to substantiate the potential health benefits consumers may receive by consuming these bacterial species having appropriate metabolic activity and in adequate numbers in products at the time of consumption.

Table 1. Influence of Storage in Cultured Buttermilk (Culture A) at 5°C on Total and Bile Resistant Numbers of Five Strains of *L. acidophilus* and One Strain of *L. casei*

		SUPPLEMENTAL CULTURE OF LACTOBACILLI[1]					
PLATING		*Lactobacillus acidophilus*					
MEDIUM	DAYS AT 5° C	43121	MUH-41	O-16	L-1	La-5	*L. casei*
C-LBS	0	7.38^{ab}	7.30^{ab}	7.89^{b}	6.85^{b}	6.97^{ab}	7.47^{b}
	7	7.55^{a}	7.58^{a}	8.33^{a}	7.48^{a}	7.20^{a}	8.16^{a}
	14	7.09^{b}	7.16^{b}	7.84^{b}	6.91^{b}	6.73^{bc}	7.58^{b}
	21	6.69^{c}	6.99^{b}	7.85^{b}	7.13^{ab}	6.62^{bc}	7.39^{b}
	28	6.36^{c}	7.00^{b}	7.83^{b}	6.79^{b}	6.57^{c}	7.34^{b}
C-LBSO	0	7.34^{ab}	7.17^{ab}	7.96^{b}	6.83^{b}	6.93^{a}	7.49^{b}
	7	7.44^{a}	7.34^{a*}	8.35^{a}	7.41^{a}	7.13^{a}	8.15^{a}
	14	6.74^{b*}	6.76^{c*}	7.79^{b}	6.81^{b}	6.55^{b*}	7.64^{b}
	21	6.52^{b*}	6.84^{bc*}	7.91^{b}	7.00^{b}	6.46^{b*}	7.40^{b}
	28	6.19^{b*}	6.78^{c*}	7.78^{b}	6.66^{b}	6.52^{b}	7.29^{b}

[1]Each value represents the mean of three trials; numbers with the same alphabetic superscripts, within one strain, and one assay did not differ significantly ($P>.05$), all others were different; reported as $\log_{10}$ colony forming units/g (SE between media = .0073) (pooled SE within media = .1010).

*Indicates that the C-LBSO count is significantly less ($P<.05$) than the C-LBS count on the same sampling day for the same strain.

Table 2. Influence of Storage in Yogurt CM-2 at 7°C on Total and Bile Resistant Numbers of Five Strains of *L. acidophilus* and One Strain of *L. casei*

PLATING MEDIUM	DAYS AT 7° C	SUPPLEMENTAL CULTURE OF LACTOBACILLI [1*] *Lactobacillus acidophilus* 43121	MUH-41	O-16	L-1	La-5	*L. casei*
C-LBS	0	7.67^{a}	7.13^{a}	7.87^{a}	7.28^{a}	6.94^{a}	7.29^{b}
	7	7.68^{a}	7.16^{a}	7.81^{a}	7.34^{a}	6.82^{a}	7.67^{a}
	14	7.50^{a}	7.06^{ab}	7.68^{ab}	7.33^{a}	6.64^{a}	7.65^{a}
	21	$7.42^{a\,b}$	6.79^{b}	7.39^{bc}	7.13^{a}	5.72^{b}	7.71^{a}
	28	7.13^{b}	6.83^{ab}	7.29^{c}	7.03^{a}	5.39^{b}	7.62^{ab}
C-LBSO	0	7.69^{a}	7.25^{a}	7.83^{a}	7.30^{ab}	7.05^{a}	7.26^{b}
	7	7.68^{a}	7.27^{a}	7.84^{a}	7.36^{a}	6.92^{a}	7.68^{a}
	14	7.52^{ab}	7.11^{ab}	7.77^{a}	7.38^{a}	6.74^{a}	7.69^{a}
	21	7.38^{ab}	6.71^{b}	7.54^{ab}	7.12^{ab}	6.03^{b}	7.68^{a}
	28	7.23^{b}	6.85^{b}	7.22^{b}	7.02^{b}	5.82^{b}	7.58^{a}

[1]Each value represents the mean of three trials; numbers with the same alphabetic superscripts, within one strain, and one assay did not differ significantly ($P>.05$), all others were different ($P<.05$); reported as $\log_{10}$ colony forming units/g (SE between media = .0889) (pooled SE within media = .0789).

*There were no significant differences ($P>.05$) between numbers enumerated on C-LBSO and C-LBS agars within any strain on the same sampling day.

Table 3. Influence of Storage in Yogurt YC-4 at 7°C on Total and Bile Resistant Numbers of Five Strains of *L. acidophilus* and One Strain of *L. casei*

		SUPPLEMENTAL CULTURE OF LACTOBACILLI[1]					
PLATING		*Lactobacillus acidophilus*					
MEDIUM	DAYS AT 7° C	43121	MUH-41	O-16	L-1	La-5	*L. casei*
C-LBS	0	7.30^{a}	7.15^{ab}	7.39^{a}	7.41^{a}	7.00^{a}	7.30^{a}
	7	7.29^{a}	7.20^{a}	7.12^{ab}	7.22^{ab}	7.08^{a}	7.45^{a}
	14	7.20^{a}	7.04^{ab}	6.75^{bc}	7.23^{ab}	6.38^{b}	7.46^{a}
	21	7.36^{a}[2]	6.76^{bc}	6.59^{c}	7.14^{ab}	5.86^{c}	7.61^{a}
	28	7.30^{a}[2]	6.63^{c}	6.02^{d}	6.87^{b}	5.56^{c}	7.47^{a}
C-LBSO	0	7.30^{a}	7.21^{ab}	7.35^{a}	7.40^{a}	7.02^{a}	7.33^{a}
	7	7.28^{a}	7.48^{a*}	7.20^{ab}	7.21^{ab}	7.13^{a}	7.47^{a}
	14	7.27^{a}	7.11^{ab}	6.92^{b}	7.27^{ab}	6.59^{b}	7.43^{a}
	21	7.35^{a}[2]	7.45^{a*}	7.05^{ab*}	7.23^{ab}	6.22^{b*}	7.55^{a}
	28	7.24^{a}[2]	6.92^{b*}	6.33^{c*}	6.90^{b}	5.66^{c}	7.45^{a}

[1]Each value represents the mean of three trials; numbers with the same alphabetic superscripts, within one strain, and one assay did not differ significantly ($P>.05$), all others were different ($P<.05$); reported as $\log_{10}$ colony forming units/g (SE between media = .0207) (pooled SE within media = .1235).

[2]Laboratory accident; average of two trials.

*Indicates that the C-LBSO count significantly ($P<.05$) differs from the C-LBS count on the same sampling day for the same strain.

REFERENCES

1. Abdel-Bar, N., N. A. Harris, and R. L. Rill. 1987. Purification and properties of an antimicrobial substance produced by *Lactobacillus bulgaricus*. J. Food Sci. 52: 411.

2. Buchanan, R. E., and N. E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology (8th ed.). The Williams and Wilkins Co., Baltimore, MD.

3. Buck, L. M., and S. E. Gilliland. 1994. Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human origin for ability to assimilate cholesterol during growth. J. Dairy Sci. 77: 2925.

4. De Rodas, B. Z. 1994. The effect of *Lactobacillus acidophilus* and dietary calcium on serum cholesterol level in swine. Doctoral Dissertation, Oklahoma State University, Stillwater, OK.

5. Dodd, H. M., and M. J. Gasson. 1994. Bacteriocins of lactic acid bacteria. pp. 211. Gasson, M. J. and de Vos, W. M (eds). Genetics and Biotechnology of Lactic Acid Bacteria. Blackie Academic and Professional, London.

6. Gilliland, S. E., and M. L. Speck. 1969. Biological response to lactic streptococci and lactobacilli to catalase. Appl. Microbiology. 17: 797.

7. Gilliland, S. E. 1977. Instability of *Lactobacillus acidophilus* in yogurt. J. Dairy Sci. 60 (9): 1394.

8. Gilliland, S. E., and M. L. Speck. 1977. Enumeration and Identity of Lactobacilli in Dietary Products. J. of Food Protection. 40 (11): 760.

9. Gilliland. S. E., and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. J. Food Prot. 40: (12) 820.

10. Gilliland, S. E., C. R. Nelson and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. Appl. and Environmental Microbiology. 49 (2): 377.

11. Goldin, B. R., and S. L Gorbach. 1984. Alterations of the intestinal microflora by diet, oral antibiotics and *Lactobacillus*: Decreased production of free amines from aromatic nitro compounds, azo dyes, and glucuronides. J. Nat. Cancer Institute. 73: 689.

12. Harrison, V. C., and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nurt. 28: 1351.

13. Hoover, D. G. 1993. Bacteriocins with Potential for use in Foods. Davidson, P. M. and Branen, A. L. (eds.) Marcel Dekker, Inc., New York, NY.

14. Hull, R. R., A. V. Roberts, and J. J. Mayes. 1984. Survival of *Lactobacillus acidophilus* in yogurt. Australian J. Dairy Techn. 39: (4) 164.

15. Kato, I., K. Endo, and T. Yokokura. 1994. Effects of oral administration of *Lactobacillus casei* on antitumor responces induced by tumor resection in mice. Int. J. Immunopharmac. 16: (1) 29.

16. Kim, H. S., and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. J. Dairy Sci. 66: 959.

17. Kosikowski, F. W. 1977. Cheese and Fermented Milk Products. 2nd Ed. Ann Arbor, Mich., Edwards Brothers, INC.

18. Kulp, W. L. 1931. Studies on the viability of *Lactobacillus acidophilus* in acidophilus milk. Am. J. Public Health. 21: 873.

19. Perdigon, G., S. Alverez, M. E. Demacias, R. A. Margni, G. Oliver, and A. A. D. De Ruiz Holgado. 1986. Lactobacilli administered orally induce release of enzymes from peritoneal macrophages in mice. Milchwessenschaft. 41: 344.

20. Perdigon, G., M. E. N. Demacias, M. E. Roux, and A. P. R. Holgado. 1990. The oral administration of lactic acid bacteria increases the mucosal intestinal immunity in responce to enterpathogens. J. Food Prot. 53: 404.

21. Perdigon, G., M. E. N. Demacias, S. Alverez, G. Oliver, and A. A. P. R. Holgado. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Dairy Research. 57: 255.

22. Rasic, J. L., and J. A. Kurmann. 1978 Yoghurt: Scientific Grounds, Technology, Manufacture and Preparations. (Fermented Fresh Milk Products Series: Vol. 1.) Technical Dairy Publishing House, Copenhagen, Denmark.

23. Richardson, G. H. (ed.), 1985. Standard Methods of the Examination of Dairy Products, 15th ed. American Public Health Association. Port City Press, Inc., Baltimore, MD.

24. Sandine, W. E., K. S. Muralidhara, P. R. Elliker, and D. C. England. 1972. Lactic acid bacteria in food and health: a review with special reference to enteropathogenic *E. coli* as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Milk and Food Tech. 35 (12): 691.

25. Shahani, K. M., B. A. Friend, and P. J. Bailey. 1983. Antitumor activity of fermented colostrum and milk. J. Food Protection. 46: 385.

26. Steel, R. G. D., and J. H. Torrie. 1980. Principles and Procedures of Statistics: a biometrical approach. McGraw-Hill Publishing Co., New York, NY.

27. Vanderzant , C., and D. F. Splittstuesser. (eds.) 1992. Compendium of Methods for the Microbial Examination of Foods. Third Ed.. Washington D.C. American Public Health Association..

APPENDIX 1

INFLUENCE OF REFRIGERATED STORAGE ON SUPPLEMENTAL LACTOBACILLI IN CULTURED BUTTERMILK

Table 4. Influence of storage in cultured buttermilk at 5°C on total and bile resistant numbers of *L. acidophilus* strains 43121 and MUH-41

PLATING MEDIUM	DAYS AT 5°C	LACTOBACILLI ADJUNCT CULTURE[1]					
		TRIAL 1 43121	TRAIL 2 43121	TRAIL 3 43121	TRAIL 1 MUH-41	TRAIL 2 MUH-41	TRIAL 3 MUH-41
C-LBS	0	7.06	7.57	7.51	6.54	7.68	7.68
	7	8.11	7.11	7.41	7.59	7.68	7.46
	14	6.65	7.40	7.20	6.66	7.52	7.30
	21	6.34	6.74	7.00	6.80	7.20	6.96
	28	5.59	6.66	6.81	6.60	7.32	7.08
C-LBSO	0	7.12	7.45	7.45	6.60	7.49	7.43
	7	7.90	7.08	7.34	7.53	7.26	7.23
	14	6.32	6.83	7.08	6.40	6.94	6.92
	21	6.18	6.49	6.89	6.76	7.08	6.68
	28	5.23	6.62	6.71	6.48	7.08	6.78

[1]Values givin as Log_{10} of CFU/g

Table 5. Influence of storage in cultured buttermilk at 5°C on total and bile resistant numbers of *L. acidophilus* strains O-16 and L-1

PLATING MEDIUM	DAYS AT 5°C	LACTOBACILLI ADJUNCT CULTURE[1] TRIAL 1 O-16	TRAIL 2 O-16	TRAIL 3 O-16	TRAIL 1 L-1	TRAIL 2 L-1	TRIAL 3 L-1
C-LBS	0	7.88	7.88	7.92	7.12	6.28	7.15
	7	9.04	8.04	7.90	7.79	7.45	7.20
	14	7.83	7.86	7.83	6.90	6.98	6.86
	21	7.91	7.85	7.79	7.54	7.00	6.85
	28	7.86	7.89	7.75	6.63	7.00	6.75
C-LBSO	0	7.99	7.95	7.95	7.10	6.30	7.08
	7	9.04	8.08	7.92	7.76	7.40	7.08
	14	7.85	7.80	7.72	6.69	7.00	6.75
	21	7.97	7.91	7.86	7.51	6.99	6.52
	28	7.81	7.72	7.82	6.40	7.04	6.54

[1]Values givin as Log_{10} of CFU/g

Table 6. Influence of storage in cultured buttermilk at 5°C on total and bile resistant numbers of *L. acidophilus* strain La-5 and *Lactobacillus casei*

PLATING MEDIUM	DAYS AT 5°C	LACTOBACILLI ADJUNCT CULTURE[1]					
		TRIAL 1 La-5	TRAIL 2 La-5	TRAIL 3 La-5	TRAIL 1 *L. casei*	TRAIL 2 *L. casei*	TRIAL 3 *L. casei*
C-LBS	0	7.18	6.91	6.81	7.41	7.62	7.38
	7	7.78	7.04	6.78	8.91	7.82	7.76
	14	6.88	6.49	6.83	7.56	7.45	7.74
	21	6.81	6.40	6.66	7.49	7.23	7.46
	28	6.69	6.45	6.57	7.38	7.28	7.36
C-LBSO	0	7.04	6.93	6.83	7.45	7.64	7.38
	7	7.76	6.67	6.95	8.88	7.87	7.71
	14	6.78	6.23	6.64	7.62	7.45	7.86
	21	6.76	6.18	6.45	7.43	7.34	7.43
	28	6.68	6.26	6.62	7.38	7.18	7.32

[1]Values givin as Log_{10} of CFU/g

APPENDIX 2

INFLUENCE OF REFRIGERATED STORAGE ON SUPPLEMENTAL LACTOBACILLI IN YOGURT CM-2

Table 7. Influence of storage in yogurt CM-2 at 7°C on total and bile resistant numbers of *L. acidophilus* strains 43121 and MUH-41

PLATING MEDIUM	DAYS AT 7°C	LACTOBACILLI ADJUNCT CULTURE[1] TRIAL 1 43121	TRAIL 2 43121	TRAIL 3 43121	TRAIL 1 MUH-41	TRAIL 2 MUH-41	TRIAL 3 MUH-41
C-LBS	*0*	7.63	7.57	7.82	7.11	6.99	7.28
	7	7.63	7.77	7.64	7.08	7.18	7.23
	14	7.41	7.28	7.81	7.15	7.04	6.99
	21	7.40	7.57	7.29	6.69	6.86	6.82
	28	7.44	6.76	7.20	6.91	6.93	6.64
C-LBSO	*0*	7.61	7.61	7.84	7.20	7.18	7.38
	7	7.61	7.73	7.71	7.36	7.11	7.34
	14	7.46	7.38	7.70	7.28	7.04	7.00
	21	7.30	7.62	7.21	6.75	6.90	6.49
	28	7.55	6.92	7.20	7.25	6.74	6.57

[1]Values givin as Log_{10} of CFU/g

Table 8. Influence of storage in yogurt CM-2 at 7°C on total and bile resistant numbers of *L. acidophilus* strains O-16 and L-1

		LACTOBACILLI ADJUNCT CULTURE [1]					
PLATING MEDIUM	DAYS AT 7°C	TRIAL 1 O-16	TRAIL 2 O-16	TRAIL 3 O-16	TRAIL 1 L-1	TRAIL 2 L-1	TRIAL 3 L-1
C-LBS	0	7.74	7.76	8.11	7.26	7.18	7.41
	7	7.81	7.65	7.97	7.28	7.26	7.48
	14	7.81	7.36	7.86	7.36	7.20	7.43
	21	7.62	7.11	7.42	7.00	7.15	7.25
	28	7.74	7.05	7.07	7.03	6.91	7.16
C-LBSO	0	7.64	7.71	8.15	7.28	7.20	7.41
	7	7.76	7.72	8.04	7.36	7.28	7.45
	14	7.85	7.59	7.86	7.36	7.23	7.54
	21	7.68	7.51	7.42	7.00	7.15	7.23
	28	7.85	6.65	7.16	7.11	6.79	7.24

[1]Values givin as Log_{10} of CFU/g

Table 9. Influence of storage in yogurt CM-2 at 7°C on total and bile resistant numbers of *L. acidophilus* strain La-5 and *Lactobacillus casei*

MEDIUM	DAYS AT 7°C	LACTOBACILLI ADJUNCT CULTURE [1]					
		TRIAL 1 La-5	TRAIL 2 La-5	TRAIL 3 La-5	TRAIL 1 *L. casei*	TRAIL 2 *L. casei*	TRIAL 3 *L. casei*
C-LBS	0	6.82	6.85	7.15	7.23	7.18	7.46
	7	6.68	6.79	6.99	7.56	7.65	7.81
	14	6.65	6.75	6.52	7.62	7.49	7.83
	21	5.90	5.75	5.50	7.63	7.65	7.85
	28	5.75	4.75	5.66	7.72	7.48	7.65
C-LBSO	0	6.93	6.96	7.26	7.18	7.15	7.46
	7	6.81	6.85	7.11	7.63	7.71	7.69
	14	6.58	6.83	6.82	7.63	7.66	7.78
	21	5.99	6.41	5.68	7.57	7.72	7.74
	28	6.03	5.79	5.64	7.72	7.40	7.60

[1]Values givin as Log_{10} of CFU/g

APPENDIX 3

INFLUENCE OF REFRIGERATED STORAGE ON SUPPLEMENTAL LACTOBACILLI IN YOGURT YC-4

Table 10. Influence of storage in yogurt YC-4 at 7°C on total and bile resistant numbers of *L. acidophilus* strains 43121 and MUH-41

PLATING MEDIUM	DAYS AT 7°C	LACTOBACILLI ADJUNCT CULTURE[1] TRIAL 1 43121	TRAIL 2 43121	TRAIL 3 43121	TRAIL 1 MUH-41	TRAIL 2 MUH-41	TRIAL 3 MUH-41
C-LBS	0	7.57	7.22	7.11	7.20	7.37	6.87
	7	7.79	7.08	7.00	7.38	7.22	7.00
	14	7.43	7.08	7.08	6.85	7.28	7.00
	21	7.48	LA	7.23	6.47	7.08	6.74
	28	7.34	LA	7.25	6.61	6.96	6.31
C-LBSO	0	7.58	7.24	7.08	7.30	7.36	6.97
	7	7.71	7.15	7.00	7.30	7.20	7.92
	14	7.49	7.15	7.18	6.86	7.41	7.04
	21	7.51	5.32	7.20	7.90	7.30	7.15
	28	7.33	4.57	7.15	6.70	7.24	6.82

[1]Values givin as Log_{10} of CFU/g

Table 11. Influence of storage in yogurt YC-4 at 7°C on total and bile resistant numbers of *L. acidophilus* strains O-16 and L-1

MEDIUM	DAYS AT 7°C	LACTOBACILLI ADJUNCT CULTURE [1]					
		TRIAL 1 O-16	TRAIL 2 O-16	TRAIL 3 O-16	TRAIL 1 L-1	TRAIL 2 L-1	TRIAL 3 L-1
C-LBS	0	7.87	7.23	7.08	7.28	7.35	7.61
	7	7.81	6.92	6.62	7.08	7.36	7.20
	14	7.66	6.81	5.77	7.04	7.26	7.38
	21	7.59	6.69	5.49	7.20	7.01	7.23
	28	7.02	5.77	5.28	6.99	6.63	7.00
C-LBSO	0	7.81	7.20	7.04	7.23	7.34	7.61
	7	7.77	7.08	6.76	7.15	7.23	7.26
	14	7.72	7.04	6.00	7.06	7.32	7.43
	21	7.70	7.08	6.38	7.16	7.18	7.34
	28	7.12	6.05	5.82	7.04	6.66	6.99

[1]Values givin as Log_{10} of CFU/g

Table 12. Influence of storage in yogurt YC-4 at 7°C on total and bile resistant numbers of *L. acidophilus* strain La-5 and *Lactobacillus casei*

MEDIUM	DAYS AT 7°C	LACTOBACILLI ADJUNCT CULTURE [1]					
		TRIAL 1 La-5	TRAIL 2 La-5	TRAIL 3 La-5	TRAIL 1 *L. casei*	TRAIL 2 *L. casei*	TRIAL 3 *L. casei*
C-LBS	0	7.11	7.19	6.68	7.38	7.35	7.18
	7	7.77	7.26	6.20	7.90	7.30	7.15
	14	5.72	7.04	6.38	7.76	7.40	7.20
	21	4.55	7.07	5.97	7.68	7.62	7.54
	28	4.63	6.34	5.71	7.48	7.54	7.40
C-LBSO	0	7.18	7.23	6.67	7.57	7.32	7.11
	7	7.81	7.28	6.30	7.82	7.40	7.20
	14	6.10	7.23	6.43	7.75	7.38	7.15
	21	5.03	7.18	6.45	7.58	7.67	7.41
	28	4.63	6.33	6.00	7.40	7.54	7.41

[1]Values givin as Log_{10} of CFU/g

APPENDIX 4

ANALYSIS OF PH OF THE CULURED PRODUCTS CONTAINING CELLS OF SUPPLEMENTAL LACTOBACILLI

Table 13. Results of Analysis of pH at Day 0 and Day 28 for Cultured Buttermilk A (BMA), Yogurt CM-2 , and Yogurt YC-4 (YC-4) Containing Cells of Five Strains of *L. acidophilus* and One Strain of *L. casei* for all three trials

		SUPPLEMENTAL CULTURE OF LACTOBACILLI					
PRODUCT/	*DAY*[1]	*Lactobacillus acidophilus*					
TRIAL		43121	MUH-41	O-16	L-1	La-5	*L. casei*
BMA TRIAL 1	*0*	4.5	4.5	4.5	4.5	4.5	4.5
	28	4.5	4.5	4.5	4.5	4.5	4.5
BMA TRAIL 2	*0*	4.5	4.5	4.55	4.5	4.5	4.5
	28	4.5	4.5	4.5	4.5	4.5	4.5
BMA TRIAL 3	*0*	4.5	4.5	4.5	4.5	4.5	4.55
	28	4.5	4.5	4.5	4.5	4.5	4.5
CM-2 TRIAL 1	*0*	4.6	4.6	4.5	4.5	4.5	4.8
	28	4.3	4.3	4.4	4.3	4.3	4.3
CM-2 TRIAL 2	*0*	4.6	4.6	4.6	4.6	4.6	4.6
	28	4.2	4.2	4.2	4.2	4.2	4.3
CM-2 TRIAL 3	*0*	5.0	5.0	5.0	5.0	4.8	5.0
	28	4.4	4.4	4.3	4.4	4.4	4.2
YC-4 TRIAL 1	0	4.8	4.8	4.8	4.8	4.8	4.9
	28	4.5	4.5	4.5	4.5	4.5	4.4
YC-4 TRAIL 2	0	5.0	5.0	5.0	5.0	5.0	5.0
	28	4.7	4.7	4.7	4.7	4.7	4.7
YC-4 TRIAL 3	0	5.0	5.0	5.0	5.0	5.0	5.0
	28	4.8	4.7	4.7	4.8	4.8	4.7

[1]Days of Storage at 5°C for Cultured Buttermilk (BMA), Days of Storage at 7°C for Yogurts CM-2 and YC-4.

APPENDIX 5

EXAMPLES OF ANALYSIS OF VARIANCE TABLES

TABLE 14. ANALYSIS OF VARIANCE - COUNTS OF FIVE *L. ACIDOPHILUS* STRAINS AND ONE *L. CASEI* STRAIN ON CLBS AND CLBSO AGAR DURING STORAGE IN CULTURED BUTTERMILK AT 5°C.

SOURCE	*DF*	*SS*	*MS*	*F VALUE*	*PR > F*
Corrected Total	179	62.2772			
Trial	2	.0768	.0384		
Strain	5	31.1043	6.2209	12.37	.0005
T * S (Error A)	10	5.0309	.5031		
Day	4	11.9086	2.9771	15.29	.0001
S * D	20	3.4282	.1714	.88	.6108
T * S * D (Error B)	48	9.4374	.1947		
MEDIA	1	.3986	.3986	54.81	.0001
S * M	5	.3284	.0657	9.03	.0001
D * M	4	.1034	.0259	3.56	.0114
S * D * M	20	.1144	.0057	.79	.7183
Error (C)	60	.4363	.0073		

TABLE 15. ANALYSIS OF VARIANCE - COUNTS OF FIVE *L. ACIDOPHILUS* STRAINS AND ONE *L. CASEI* STRAIN ON CLBS AND CLBSO AGAR DURING STORAGE IN YOGURT CM-2 AT 7°C.

SOURCE	*DF*	*SS*	*MS*	*F VALUE*	*PR > F*
Corrected Total	179	52.2907			
Trial	2	.5529	.2765		
Strain	5	22.9835	4.5967	10.01	.0012
T * S (Error A)	10	4.5932	.4593		
Day	4	7.1112	1.7778	25.76	.0001
S * D	20	4.7987	.2399	3.48	.0002
T * S * D (Error B)	48	3.3126	.0690		
MEDIA	1	.1711	.1711	1.93	.1704
S * M	5	2.4187	.4837	5.44	.0003
D * M	4	.0408	.0102	.11	.9768
S * D * M	20	.9743	.0487	.55	.9318
Error (C)	60	5.3337	.0889		

TABLE 16. ANALYSIS OF VARIANCE - COUNTS OF FIVE *L. ACIDOPHILUS* STRAINS AND ONE *L. CASEI* STRAIN ON CLBS AND CLBSO AGAR DURING STORAGE IN YOGURT YC-4 AT 7°C.

SOURCE	*DF*	*SS*	*MS*	*F VALUE*	*PR > F*
Corrected Total	175	65.7278			
Trial	2	3.1958	1.5979		
Strain	5	18.8750	3.7750	2.80	.0782
T * S (Error A)	10	13.5036	1.3504		
Day	4	8.4158	2.1040	9.30	.0001
S * D	20	8.3383	0.4169	1.84	.0441
T * S * D (Error B)	46	10.4092	0.2263		
MEDIA	1	.5280	.5280	25.54	.0001
S * M	5	.5046	.1009	4.88	.0009
D * M	4	.3069	.0767	3.71	.0093
S * D * M	20	.4516	.0226	1.09	.3822
Error (C)	58	1.1992	.0207		

VITA

Benton Doyle Nighswonger

Candidate for the Degree of

Master of Science

Thesis: VIABILITY OF *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS CASEI* IN FERMENTED MILK PRODUCTS DURING REFRIGERATED STORAGE

Major Field: Food Science

Biographical:

Personal Data: Born in Orange City, Iowa, November 22, 1969, the son of Dr. David and Connie Nighswonger. Single.

Education: Graduated from West Sioux Community High School in May, 1988. Graduated with honors with a double major in Dairy Manufacturing and Microbiology in December, 1992 from South Dakota State University, Brookings, South Dakota. Completed the requirements for the Master of Science degree in Food Science at Oklahoma State University in May, 1995.

Experience: Raised outside small rural town of Hawarden, Iowa; employed by father's veterinary clinic for temporary summer help as well as farm labor; employed by State Game Lodge, Custer State Park; employed by SDSU Dairy Plant, Department of Dairy Science. Worked summers as a nightime park ranger and daytime park aid at Newton Hills State Park, S.D. Game, Fish and Parks Department, Canton, S. D.; employed by Gold Bond Ice Cream, Inc., Sikeston, MO., as part of S.D.S.U. cooperative education program. Employed by United States Department of Agriculture as an Inspector/Grader in Independance, MO. Employed by Oklahoma State University Department of Animal Science as a graduate research/teaching assistant.

Organizations: Institute of Food Technologists, American Dairy Science Association, Phi Kappa Phi, Alpha Zeta, Gamma Sigma Delta, OSU Food Industry Club, SDSU Dairy Science and Microbiology Clubs