BUTTERMILK MANUFACTURE USING A COMBINATION OF DIRECT ACIDIFICATION AND CITRATE FERMENTATION BY *LEUCONOSTOC CREMORIS*

By

DEREK KEITH WALKER

Bachelor of Science in Agriculture

Oklahoma State University

Stillwater, Oklahoma

1984

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1986

BUTTERMILK MANUFACTURE USING A COMBINATION OF DIRECT ACIDIFICATION AND CITRATE FERMENTATION BY LEUCONOSTOC CREMORIS

Thesis Approved:

Stanley E. Bellland
Thesis Adviser

John J. Guenther

Earl D. M. Fitch Jr.

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Stanley Gilliland for his guidance, assistance, and encouragement throughout this graduate study. Appreciation is also extended to Dr. Earl Mitchell and Dr. Jim Guenther for serving on the Advisory Committee.

Recognition is also extended to Charles Nelson Rich and Jerry Nielsen for their friendship and assistance throughout this program. The author also expresses his thanks to Freddie Gant, Bob Von Gunten, Paul Johnson, and Farm Fresh Inc. for their help during this study.

Finally, the author thanks his wife, Brenita, for her love, patience, understanding, and help during this course of study. Also, the author wishes to thank his daughter, Ashley, and niece, Tara, for their sacrifice during this course of study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The quality of commercial buttermilk is often less than desirable. Even for those dairy processing plants that can produce high quality cultured buttermilk, it is a real challenge to do so on a consistent basis. The manufacture of a high quality cultured buttermilk requires the adequate growth and metabolic action of a mixed species bacterial starter culture. The lactic streptococci (Streptococcus lactis and Streptococcus cremoris) produce lactic acid which gives buttermilk its tart or acid taste. The other species of bacteria normally included is Leuconostoc cremoris which gives buttermilk its main volatile flavor component (diacetyl). The main problem with using the two types of bacteria is that they have different growth characteristics. Often times, the two types of bacteria do not perform together adequately during the incubation period used for buttermilk manufacture and the result is a low quality product. Another choice for a starter culture is S. lactis subsp. diacetilactis which produces both acid and flavor compounds.

One way to avoid inconsistent acid production by the lactic streptococci would be to use chemical acidulants

which could result in more consistency with regard to tartness of the product. Adding a concentrated culture of L. cremoris to the acidified milk followed by incubation at a desired temperature would enable this bacteria to ferment citrate for production of diacetyl and other flavor compounds. Because L. cremoris does not grow at or below pH 4.5 - 5.0, but does produce diacetyl, addition of a concentrated culture of this organism to directly acidified milk should be a feasible process for producing buttermilk of consistent quality. By eliminating the necessity to grow both cultures together, the process would be more easily controlled to yield a more uniform product. The purpose of this study was to check the feasibility of producing buttermilk of consistent quality using direct acidification and citrate fermentation.

CHAPTER II

LITERATURE REVIEW

The Quality of Cultured Buttermilk

Buttermilk is the least popular of the cultured dairy products sold in the United States. In fact, buttermilk consumption has steadily declined since 1954 (Lundstedt and Corbin, 1983). The annual per capita consumption declined from 8.28 pounds in 1954 to 4.20 pounds in 1983, because of the inconsistent quality of the product (Vasavada and White, 1979; Frank, 1983). Vasavada and White (1979) found that 73 percent of cultured buttermilk purchased from retail outlets ranged from fair to good in quality. According to Vedamuthu (1985) many samples of buttermilk purchased from retail outlets ranged from poor to good in quality. Olson (1974) found that 48 percent of 23 commercial buttermilks from various plants throughout the United States were rated poor in quality; the remaining 52 percent rated fair to good in quality.

The major quality problem of cultured buttermilk is the lack of delicate flavor and fine aroma. The most common flavor defects found in cultured buttermilk are the following: coarse, lacks flavor, unclean, and green (Olson, 1974; Lundstedt and Corbin, 1983). The coarse defect is an

excess amount of diacetyl in the product. Lack of flavor results from inadequate volatile flavor compounds, primarily diacetyl. The unclean defect is characterized by a putrid flavor that results from proteolysis by contaminating bacteria (Kosikowski, 1977). The green (acetaldehyde) flavor defect results from excess acetaldehyde in the buttermilk; this defect gives buttermilk a yogurt-like taste.

A good quality buttermilk has the following four characteristics: (1) good body, (2) smooth texture, (3) desired acidity, flavor, and carbonation, and (4) adequate storage stability under normal marketing conditions. To produce a buttermilk with adequate body and texture, the milk must contain 9.0 - 9.5 percent solids not-fat (SNF) (Kosikowski, 1977; Richter, 1977; Vedamuthu, 1977; White, 1978; Vedamuthu, 1985). A titratable acidity (expressed as percent lactic acid) between 0.85 and 0.88 is desirable because maximum diacetyl formation occurs between pH 4.4 and 4.5, with a titratable acidity of 0.85 and 0.88 (Emmons and Tuckey, 1967). Vedamuthu (1977) stated that a desirable diacetyl concentration in cultured buttermilk was 3.0 - 5.0 ppm and that carbonation with CO_2 adds a "fizz" to cultured buttermilk that enhances flavor. Also, acetate is important in the flavor of cultured dairy products (Hempenius et al, 1967). Vedamuthu (1977) claimed that minute amounts of acetic acid and formic acid enhanced the flavor of cultured buttermilk.

There are several factors that affect the quality of cultured buttermilk. According to Lundstedt and Corbin (1983), 60 percent of buttermilk defects are due to the culture and 40 percent are due to the raw product and its processing. Some processing plants use hold-over milk (milk held in the plant over a weekend before processing) to make their cultured products; however, fresh skim milk or lowfat milk free from off odors and flavors make a better quality buttermilk (Richter, 1977; White, 1978; Vedamuthu, 1985). Pasteurizing milk for buttermilk at 185-190^{o}F for 30 minutes increases the water holding properties of the coagulum; thus, it enhances the body of the finished product (Olson, 1974; Vedamuthu, 1985). Also, using milk free from antibiotics is best, because even low levels can inhibit growth and action of starter cultures (Richter, 1977). A supplement of 0.05 - 0.15 percent sodium citrate in the milk will compensate for seasonal variations in citrate concentration.

Diacetyl (the main volatile flavor component of cultured buttermilk) is produced from citrate. When all citrate in the milk is used, diacetyl is rapidly converted to acetoin (flavorless compound). Cogan (1975) found that low levels of citrate in milk resulted in an inadequate production of diacetyl in pure cultures of Leuconostoc cremoris. Incubation at 21-22^{o}C is best for adequate acid and diacetyl production (Frank, 1984; Vedamuthu, 1985).

The lactic streptococci (Streptococcus lactis and Streptococcus cremoris) and Leuconostoc cremoris comprise the starter culture for buttermilk manufacture. If the incubation temperature is too high, an imbalance between the lactic streptococci and L. cremoris can occur that may result in no diacetyl production (Frank, 1984; Vedamuthu, 1985). Excess inoculum of lactic streptococci may result in excess acid production that inhibits growth of L. cremoris and diacetyl production (Lindsay et al., 1965). If the level of inoculum is too low, acid production will be too slow and "wheying-off" (separation of curds and whey) will result (Kosikowski, 1977). If not cooled immediately after incubation, diacetyl is reduced to acetoin by diacetyl reductase (Pack et al., 1968).

Role of "Citric Acid Fermenting" Organisms and Lactic Streptococci in the Manufacture of Cultured Buttermilk

The manufacture of high quality cultured buttermilk requires adequate growth and metabolic action of a mixed species starter culture containing lactic streptocci (S. lactis and S. cremoris) and L. cremoris. Lactic streptococci ferment lactose to produce lactic acid, which gives buttermilk its tart taste. In the presence of a fermentable sugar, cells of L. cremoris use citrate to produce diacetyl, the major volatile flavor component of cultured buttermilk (Vedamuthu, 1982).

Because the lactic streptococci and L. cremoris have different growth requirements, it is difficult to get them to perform together adequately in the manufacture of cultured buttermilk. S. lactis and S. cremoris have an optimum growth temperature of 30°C (Kosikowski, 1977). Pure cultures of L. cremoris have an optimum growth temperature of 24 - 27°C in broth (Cooper and Collins, 1978). Goel and Marth (1968) found that in milk, some strains of L. cremoris grew best 22°C, while others grew best at 30°C. Goel and Marth (1969) also found that some strains of L. cremoris grown in mixed cultures varied in their growth and activity. Pack et al. (1968) stated that L. cremoris grew best in mixed cultures at 30°C; however, higher levels of diacetyl were achieved at 21°C. The generation time of L. cremoris is twice that of the lactic streptococci at 22°C (Cooper and Collins, 1978). This longer generation time can result in inhibition of L. cremoris because of excess acid produced by the lactic streptococci. Instead of using S. lactis or S. cremoris and L. cremoris, S. lactis subspecies diacetilactis can be used to make cultured buttermilk because it produces lactic acid and diacetyl simultaneously. If present in excess numbers, all three species of lactic streptococci will impart a green flavor defect because of excess acetaldehyde production (Lindsay et al., 1965). No literature exists that suggests the appropriate amount of acetaldehyde required in buttermilk flavor. However, Lindsay et al. stated that a 4:1 ratio of diacetyl to

acetaldehyde is desirable for best flavor in products cultured with these bacteria.

Drinan et al. (1976) found that L. cremoris used citrate under neutral and acidic conditions; however, diacetyl was produced only under acidic conditions. Harvey and Collins (1962) reported that as pH dropped below 6.0 citrate uptake increased rapidly because of increased action of citrate permease. This enzyme is responsible for the transport of citrate into the bacterial cells.

Methods for Making Directly Acidified Dairy Products

A major step in the development of cultured dairy products is the lactic acid produced by the starter culture bacteria (Deane and Hamnmond, 1960). Culture activity and incubation temperatures are important to ensure desired acid production; furthermore, strain to strain variation concerning activity makes controlling lactic acid production difficult. Several organic compounds have been tested for possible use as a chemical acidulant to replace the lactic acid produced by the starter culture. Little (1967) used hydrochloric acid and phosphoric acid to acidify skim milk; however, these acids did not produce a smooth coagulum. Campbell (1975) stated that phosphoric acid imparted an undesirable taste to milk. Deane and Hammond (1960) reported that the following chemical acidulants had limited solubility in milk: racemic lactide, succinic anhydride,

diethyl sulfate, sulfuryl chloride, phosphorus oxychloride, phosphorus pentachloride, acetylchloride, and glutaric anhydride. All these compounds yielded an uneven coagulum of the milk. They also found that the use of sulfamic acid, metaphosphoric acid, D-galactano-gamma lactone, and D-glucurono-gamma lactone resulted in little or no production of acid. Acetic anhydride, beta-propiolactone, and glutaric acid changed the properties of the milk coagulum. The two former compounds acylate the amino groups on casein (the primary protein in milk) in addition to producing acid. As a result, the isoelectric point of the milk changes. This type of change could possibly affect the activity of some microbial enzymes. Trop and Kushelevsky (1985) reported that glucono- delta-lactone would also form a complex with free amino groups in milk. Deane and Hammond (1960) and Hempenius and Liska (1964) used glucono-delta-lactone (GDL) to acidify skim milk and produced an acidified product with a smooth coagulum. Trop (1984) compared the following compounds as acidulants for milk: propionic anhydride, lactide, glucono-delta- lactone, ethylbutyrate with its esterase, and tributyrin and its lipase. GDL functioned best. When GDL is dissolved in milk, it hydrolyzed slowly to gluconic acid and forms a stable curd (Mabbit et al., 1955).

While a smooth coagulum is essential in the manufacture of buttermilk, it is not in cottage cheese creaming mixtures. In the latter case the acidified skim is mixed

with cream to make the final creaming mixture. Mather and Babel (1959) developed a method for standardizing the diacetyl content in cottage cheese by using a pure culture of L. cremoris. The culture was grown for 24 hrs. at 21°C in skim milk; then, the pH was adjusted to 4.3 with sterile 15 percent citric acid. The skim milk was then reincubated for 20 - 24 hrs. to allow production of flavor compounds. The cultured skim milk was then mixed with cream and homogenized to make the creaming mixture.

Hempenius and Liska (1964) grew L. cremoris for 24 hrs. at 21°C in skim milk, acidified the skim milk to pH 4.4 with glucono-delta-lactone, and reincubated the skim milk an additional 6 hrs. to allow for adequate diacetyl production. Lundstedt and Fogg (1962) used a method similar to Mather and Babel except they used S. lactis subspecies diacetilactis. The culture was grown for 18 hrs. at 22°C in cottage cheese whey containing 5 percent trisodium citrate and produced a very high level of diacetyl in milk. Gilliland et al. (1970) used concentrated cultures of L. cremoris to inoculate skim milk that was then immediately acidified to pH 4.4 - 4.5 with 15 percent citric acid and incubated for 6 hrs. at 25°C to produce a cottage cheese creaming mixture with desirable aroma.

Lundstedt and Corbin (1983) developed a method using S. lactis subspecies diacetilactis in conjunction with GDL and food grade organic acids to produce a buttermilk with a desirable level of diacetyl within 4 - 5 hours incubation.

However, they pointed out that the cost of the acidulant and the amount of starter required made the process economically infeasible. Also, the use of S. lactis subspecies diacetilactis resulted in a green flavor defect. Gettys and Davidson (1985) used a method of acidification with lactic acid and citrate fermentation by L. cremoris to produce buttermilk. Pasteurized skim milk was inocluated with a mixed starter composed of S. lactis (or S. cremoris) and L. cremoris and incubated at 24°C to pH 5.2, after which it was mixed with pasteurized milk acidified to pH 5.2 with lactic acid. The mixture was then allowed to ferment to a final pH of 4.5, and chilled to 10°C. Taste panel and quantitative analyses showed that the combination buttermilk was not significantly different from traditional buttermilk.

Use of Frozen Concentrated Cultures of Leuconostoc species

The use of frozen concentrated cultures of lactic acid bacteria (bacteria that produce lactic acid) in the dairy industry has eliminated the need to maintain stock cultures by conventional means in dairy processing plants (Gilliland and Speck, 1974; Lawrence et al., 1976). Gilliland (1971) described the basic procedure for making frozen concentrated cultures of L. cremoris. Cells of the starter organism grown in a liquid medium at specified pH and temperature are harvested by centrifugation, resuspended in a small volume of 10 percent non-fat milk solids (NFMS), and stored at

-196°C until used. He also reported that concentrated cultures of L. cremoris made from cells grown under automatic pH control in a broth supplemented with citrate had greater diacetyl producing activity than cells grown in skim milk.

Media for Producing Cell Crops of Leuconostoc cremoris

L. cremoris requires citrate in order to produce diacetyl (Prill and Hammer, 1939). However, excess citrate can be toxic to cells of L. cremoris (Cogan, 1985). Madsen (1955) found that 1.0 percent sodium citrate was toxic to cells of L. cremoris while 0.2 percent sodium citrate had no adverse affect on growth. Gilliland et al. (1970) successfully used 0.5 percent sodium citrate in a growth medium to produce concentrated cultures of L. cremoris that produced desirable levels of diacetyl when subsequently added to acidified milk. Cells grown in broth without sodium citrate did not produce diacetyl when used in a similar manner. Cogan (1985) stated that trace amounts of manganese ions and minute amounts of acetaldehyde stimulate growth of leuconostocs in complex media. Gilliland et al. (1970) showed that growth of cells of L. cremoris in broth at pH 6.0 - 6.5 was best for achieving maximum populations. However, cells grown at pH 5.5 were more active with respect to diacetyl production.

Suspending Menstrum for Preparing Concentrated Starter Culture Bacteria

According to Briggs (1955) the suspending menstrum was the most important factor affecting the survival of bacterial cells during frozen storage. Gibson et al. (1966) found that skim milk was the best suspending menstrum for lactic acid bacteria. He also noted that cryoprotective agents did not significantly increase the survival of cells of lactic streptococci upon freezing. Morichi et al. (1963) found that glutamic acid added to cells of Lactobacillus bulgaricus before freeze drying did not prevent death of some cells. Smittle et al. (1972) found that cryoprotective agents did not increase viability or activity of cells of L. bulgaricus after freezing. Freezing and storage at -196°C did not adversely affect viability or diacetyl producing activity of cells of L. cremoris used for making cottage cheese creaming mixtures (Gilliland et al., 1970).

Storage Temperature for Starter Culture Bacteria

There are differing opinions on the optimum storage temperature for frozen concntrated cultures of lactic streptococci. Storage at -196°C in liquid nitrogen appears to be best with regard to viability and activity (Gibson et al., 1966; Gilliland et al., 1970; Smittle et al., 1972). Cowman and Speck (1963) showed that lactic streptococci had higher enzymatic activity after storage at -196°C than at

-17°C. Peebles et al. (1969) showed that concentrated cultures of lactic streptococci stored at -196°C for up to 231 days remained viable and active.

Evaluation of Diacetyl and Acetoin Production by Cultures of *Leuconostoc cremoris*

Hammer (1935) used the creatine test as a qualitative measure for diacetyl and acetoin in butter cultures. The dairy industry uses the creatine test as a qualitative test for diacetyl in buttermilk and cottage cheese. The method relies on a reaction between creatine and acetoin that in the presence of a strong alkali produces a red color. Subjective evaluation of the intensity of red color determines diacetyl content. Prill and Hammer (1938) discovered a more sensitive method that entailed a reaction between diacetyl and hydroxylamine which combine to form a dimethylglyoxime complex. When this complex reacts with ferrous sulfate, a red color complex forms that can be measured colorimetrically to detemine diacetyl content. Pack et al. (1964) modified the Prill and Hammer method, so that they could analyze a larger number of samples simultaneously within a three hour period. Walsh and Cogan (1974) further modified the Pack et al. method by using steam distillates of buttermilk samples and analyzing them for diacetyl content. The disadvantage of this modification is that the alpha acetolactic acid in buttermilk and cottage

cheese is oxidatively decarboxylated to diacetyl during distillation. As a result, the recovery of diacetyl is too high. However, by raising the pH of the product to 9.0 before distillation, the error in diacetyl content is almost negligible (Veringa et al., 1984). The major disadvantage of the latter three methods is the considerable amount of time required to analyze samples. Hill et al. (1953) used a modification of the Westerfield method for the determination of blood acetoin to determine the amount of diacetyl and acetoin produced in orange juice by contaminating cultures of leuconostocs and lactobacilli. This method relies on the reaction of diacetyl and/or acetoin with a strong alkali solution (contaning 0.3 percent creatine) and alpha napthol. These react to form a red color complex that can be measured colorimetrically. This method is very simple and permits the analyses of a large number of samples in a short time. However, it is not specific for diacetyl, since it also measures acetoin.

CHAPTER III

EXPERIMENTAL PROCEDURES

Source and Maintenance of Cultures

The fourteen cultures of Leuconostoc cremoris used in this study were from the stock culture collection in the Dairy Foods Microbiology Lab at Oklahoma State University. The cultures were routinely propagated in sterile peptonized milk nutrient (PMN) broth supplemented with 0.1% sodium citrate using 1% inocula and incubation at 21°C for 24 hours. The cultures were stored at 2 - 4°C between transfers and subcultured at least three times prior to use.

PMN broth contained 5% PMN (Sheffield Products, Kraft Inc. Norwich, N.Y.), 2% lactose, 2% primatone (Sheffield Products, Kraft Inc. Norwich, N.Y.), 0.1% Tween 80, 0.1% yeast extract, and 0.1% sodium citrate in distilled water. After dissolving, the medium was dispensed in 10 ml quantities into test tubes and autoclaved for 15 minutes at 121°C.

Identification of Cultures

A modification of the procedure of Kempler and McKay (1982) was used to confirm that the cultures were citric acid fermenting organisms. PMN was used as the base medium

for the calcium citrate agar. A modification of the procedure described by Gilliland and Speck (1977) which involved the Minitek system (BBL Microbioligy Systems, Becton Dickinson Co. Cockeysville, MD) was used to test the action of the cultures on various sugars. The modification entailed the preparation and use of additional carbohydrate substrate discs including arbutin, fructose, and ribose. Minitek plates were incubated for 4 - 5 days at 21°C. The following tests were done to identify the cultures: gram stain, catalase test, and the ability to ferment amygdalin, arbinose, arbutin, cellobiose, galactose, mannose, lactose, fractose, mannitol, ribose, melibiose, raffinose, salicin, sucrose, trehalose, and xylose. The ability of the cultures to grow at 37°C was also tested. For each culture, one tube of PMN broth was inoculated using a flame sterilized inoculating loop. Each tube was incubated at 37°C for 24 hours. Visual turbidity was a positive test for growth.

Enumeration of Leuconostocs

Serial dilutions were prepared using 99 ml peptone dilution blanks as described in the Compendium of Methods for the Microbial Examination of Foods (Speck, 1976). Initial dilutions (1:100) were prepared by adding 1 gram of concentrated culture or 1 ml of previously diluted culture to a 99 ml peptone dilution blank.

Total populations of leuconostocs were determined by the pour plate procedure. Plates containing the appropriate

dilutions were poured with molten PMN agar tempered at 45°C. PMN agar was prepared by dissolving 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.) in PMN broth, prior to autoclaving. Duplicate plates for each dilution were prepared in all experiments. After, solidification, the plates were inverted and incubated at 32°C for 48 hours. All colonies visible with a Quebec Colony Counter were counted.

Determination of Amount of Glucono-Delta-Lactone Required to Acidify Milk

Raw milk was separated and standardized to 1% fat, fortified with 0.5% non-fat milk solids (NFMS) and 0.1% sodium citrate, pasteurized at 85°C for 30 minutes, cooled to 7 - 8°C and adjusted, while cold, to pH 5.3 with a mixture of lactic acid and acetic acid (3M lactic acid and 1M acetic acid) (J.T. Baker Chemical Co., Philipsburg, N.J.). Ten ml portions of cold pasteurized milk containing 3, 6, 9, and 12 grams of glucono delta lactone (GDL), (Sigma Chemical Co., St. Louis, MO) were added to separate one liter volumes of the partially acidified milk, mixed, and incubated at 24°C in a water bath until maximum pH reduction was attained. The pH was monitored every half hour.

Storage Stability of Acidified Milk at Refrigeration Temperature

Milk containig 1% fat and supplemented with 0.5% NFMS,

and 0.1% sodium citrate was prepared as described in the previous section; however 500 ml portions of milk were dispensed into quart glass milk bottles and pasteurized at 85°C for 30 minutes. After chilling, 10 ml portions were withdrawn from each bottle and placed in a sterile tube to which the required amount of GDL, based on results from the previous section, was added. The milk in each bottle was adjusted to pH 5.3 with the 3:1 molar solution of lactic acid and acetic acid. The ten ml of cold pasteurized milk containing the GDL was then added back to the appropriate 500 ml sample and mixed. All samples were placed in a 24°C water bath for 5 hours (final pH 4.5). The samples were then chilled to 7 - 8°C; afterwards, the curd was broken by shaking each bottle 5 - 8 times. Samples were stored at 5°C and monitored for depth of whey and pH.

Relative Ability of Cultures to Produce Diacetyl in Whey

Whey was prepared by adjusting 10% NFMS supplemented with 0.1% sodium citrate to pH 4.5 with a 3:1 molar solution of lactic - acetic acid. In some experiments the milk was acidified with a combination of lactic - acetic acid and GDL as described in a previous section. The acidified milk was centrifuged for 20 minutes at 4000 x g and 5°C. The whey was decanted into a sterile flask, and 20 ml of it was then added to each of twelve 20 x 250mm test tubes, and held for 24 hours in an ice-water bath until used.

Cultures of L. cremoris were grown in PMN broth supplemented with 0.1% sodium citrate. Each strain was subcultured three times with incubation at 21°C for 24 hours, prior to use. The 10 ml portions of fresh broth cultures were centrifuged for 20 min. at 12,000 x g and 1°C in a Sorvall Model RC-5 Superspeed Refrigerated Centrifuge (Dupont Co., Newton CT.) The supernatant fluid was discarded, and the cells were resuspended in 10ml of cold sterile 10% NFMS. One gram of each of the culture suspensions was then inoculated into separate 20 x 250mm test tubes containing the acid whey (duplicates for each culture) and incubated for 4 hours at 32°C. Diacetyl content was determined as described by Pack et al. (1964).

Preparation and Freezing of Concentrated Cultures

PMN broth supplemented with 0.1% sodium citrate was prepared as described earlier; however, 5 liter quantities were made. After sterilization the broth was aseptically transfered into a sterile 7l fermentor (New Brunswick Scientific Co., Edison, N.J.). The head of the fermentor jar contained ports for sampling, inoculation, neutralization, and an Ingold pH electrode. A New Brunswick automatic pH controller was used in conjunction with the fermentor, to maintain the culture medium at constant pH during growth of the leuconostocs.

Neutralizer was prepared by dissolving 117g of Na_2CO_3 in 335ml of distilled water and autoclaving for 15 minutes at 121°C. After autoclaving, the mixture was cooled to room temperature and 165 ml of concentrated NH_4OH was added. This provided a solution of 20% Na_2CO_3 in 20% NH_4OH. The neutralizer was placed in the resevoir of the automatic pH controller.

The broth in the fermentor jar was maintained at 24 - 25°C and the automatic pH controller was set to maintain it at pH 6.0 during growth of the culture. The broth was then inoculated with 50 ml of a 24 - hour fresh broth culture of L. cremoris and incubated for 22 - 24 hours at room temperature with moderate agitation. At the end of the incubation period, a 10 ml sample was taken and placed in an ice-water bath. The number of leuconostocs in the sample was determined by plating appropriate dilutions of the sample on PMN agar. The cells were harvested from the remainder of the culture for preparing concentrated cultures.

The cell crop was harvested by centrifuging 20 minutes at 12,000 x g and 1°C. The supernatant fluid was discarded, and the cells were resuspended in twice their weight of cold sterile 10% NFMS. Resuspension was aided by placing sterile glass beads (0.3 cm diameter) into the centrifuge bottle(s) along with the milk and swirling the contents until a uniform resuspension was attained. The resuspended cells were then dispensed in 2 - gram quantities into 2 - ml

polyethylene screw cap cryogenic vials (Dyntoch Laboratories Inc., Alexandria, VA). Cultures were kept cold during the resuspension procedure. The concentrated cultures were then frozen and stored in liquid nitrogen (-196^{o}C).

Evaluation of Concentrated Cultures

Numbers of leuconostocs were determined prior to freezing (Day 0) and after 7 and 14 days storage in liquid nitrogen by plating appropriate dilutions on PMN agar. The vials of frozen concentrated cultures were thawed by submerging them in one liter of distilled water at 25^{o}C for 5 minutes. The exterior of each vial was sanitized by dipping it in 95% ethanol prior to opening. Excess ethanol was wiped from the vials with tissue paper. The ability of the concentrated cultures to produce diacetyl was tested in whey and/or acidified milk as previously described. One gram of thawed concentrated culture was aseptically placed in 99 ml of sterile 10% NFMS and thoroughly mixed. An appropriate amount of this 1:100 dilution was then added to each of two bottles containing 200 ml of whey to achieve a popultion of 5 x 10^{6} cells/ml. For the acidified milk, a sufficient amount of thawed concentrated culture was added directly to each of two bottles containing 100 ml of milk acidified to pH 5.3 to yield a population of 2 x 10^{8} cells/ml. Afterwards, an appropriate amount of GDL was added to each bottle to achieve a final pH of 4.5 - 4.6. All bottles were incubated at 32^{o}C for 14 hours. Samples

were removed at 8, 10, 12, and 14 hours incubation and assayed for diacetyl content via the method described by Pack et al. (1964).

Preparation of Buttermilk

Nineteen liters of milk standardized to contain 1% fat was prepared as described in previous sections with a few modifications. The milk, immediately after being pasteurized, was homogenized at 2000 psi with a single stage homogenizer. After homogenization, it was collected in a sanitized milk can and cooled to 5°C. One liter quantities of milk were aseptically transferred to 1 liter Nalgene beakers. Twenty ml of the cold pasteurized milk was withdrawn, placed in a tube containing 9g of GDL and placed in an ice-water bath. The milk in the beaker was then acidified to pH 5.3 with a 3:1 molar solution of lactic-acetic acid while it was simultaneously inoculated with 4g of concentrated culture of L. cremoris. After reaching pH 5.3, the 20 ml of cold pasteurized milk containing the 9g of GDL was added. Eight hundred ml of the milk was aseptically transferred to 1 quart sterile glass milk bottles, and incubated for 14 hours at 32°C. After incubation, the buttermilk was shaken 5 - 8 times to break the curd, 0.1% salt was added and the shaking repeated. It was then chilled in an ice-water bath. The buttermilk was stored at 5°C and analyzed for diacetyl content on days 0, 7, and 14 as described by Pack et al. (1964).

An additional 100 ml of the milk was aseptically transferred to a sterile milk dilution bottle immediately after the GDL was added and incubated for 14 hours at 32°C. The resulting buttermilk was shaken, cooled, and stored in the same manner as the 800 ml sample. It was also stored at 5°C and monitored for pH and depth of whey on days 0, 7, and 14.

A 10 ml sample of the milk immediately following inoculation was placed in a sterile test tube and held in an ice-water bath. The number of leuconostocs was determined by plating on PMN agar.

Two commercial cultured buttermilk samples were also tested for diacetyl content during storage at 5°C. They were dispensed in 100 ml quantities into sterile milk dilution bottles for storage.

Sensory Evaluation of Buttermilk

The experimental buttermilk made via the direct acidification and citrate fermentation method and two commercial buttermilk samples were evaluated by an experienced taste panel for flavor, body/texture, and overall sensory quality. Each characteristic was scored on a scale of 1 - 9 with 9 being the most desirable. If the flavor or body/texture score was below 9, panelists checked appropriate comments or wrote in any additional ones that best described the defect. Figure I is the score sheet used in the sensory evaluation. During sensory evaluation

presentation of samples was randomized among the panelists to help avoid any bias.

Statistical Analyses

Comparisons of the relative ability of various strains of L. cremoris to produce diacetyl was analyzed using simple analysis of variance. Analysis of variance was also used to determine the effect of GDL in acidified milk on the ability of strains of L. cremoris to produce diacetyl. Sensory Panel scores also were analyzed using analysis of variance. Means were compared using Least Significant Different for Means. All statistical analyses are outlined in Principles and Procedures of Statistics (Steele and Torrie, 1980).

CODE _____

SAMPLE _____

SENSORY EVALUATION OF BUTTERMILK

CHARACTERISTIC	SCORE*
FLAVOR	_____
BODY/TEXTURE	_____
OVERALL	_____

*Score each characteristic on a scale of 1 to 9 with 9 being the most desirable.

COMMENTS: [If flavor or body/texture score is below 9, please check appropriate comments or write in any additional one(s) that best describe the defect.]

FLAVOR		BODY/TEXTURE	
____	Flat	____	Weak
____	Coarse	____	Too Viscous
____	Green	____	Other _____
____	Other _____		_____

Figure I. Score card used by members of sensory panel to evaluate buttermilk.

CHAPTER IV

RESULTS

Confirmation of Identity of Cultures

The results of the tests to confirm the identity of all strains of Leuconostoc cremoris used in this study are given in Table I. The characteristics of each strain matched those of L. cremoris as described in Bergey's Manual of Determinative Bacteriology (Buchanan, 1974).

Selection of Required Amount of Glucono-Delta-Lactone to Acidify Milk

Considerable variation was observed in acid development in milk when different levels of glucono-delta-lactone (GDL) were used to acidify the milk (Figure II). Generally, as the level of GDL increased, the acid production increased. The sample containing 3g GDL/liter did not produce sufficient gluconic acid to reduce pH to a level which favors diacetyl production (pH 5.0) by L. cremoris. In addition, 6g GDL/liter did not reduce the pH to that normally found in commercial buttermilk. The pH began to level off in both samples at about 3.5 hours which indicated little if any further acid production would occur. In the sample containing 12g GDL/liter, the pH of the milk was

TABLE I

BIOLOGICAL CHARACTERISTICS OF CULTURES OF *LEUCONOSTOC CREMORIS*

		STRAIN													
TEST	Bergey's	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Gram Stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellular Morphology	c[a]	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 37°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilize Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of Esculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from litmus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reduce litmus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid From:															
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE I (continued)

		STRAIN													
TEST	Bergey's	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Indicates cocci in pairs and short chains.

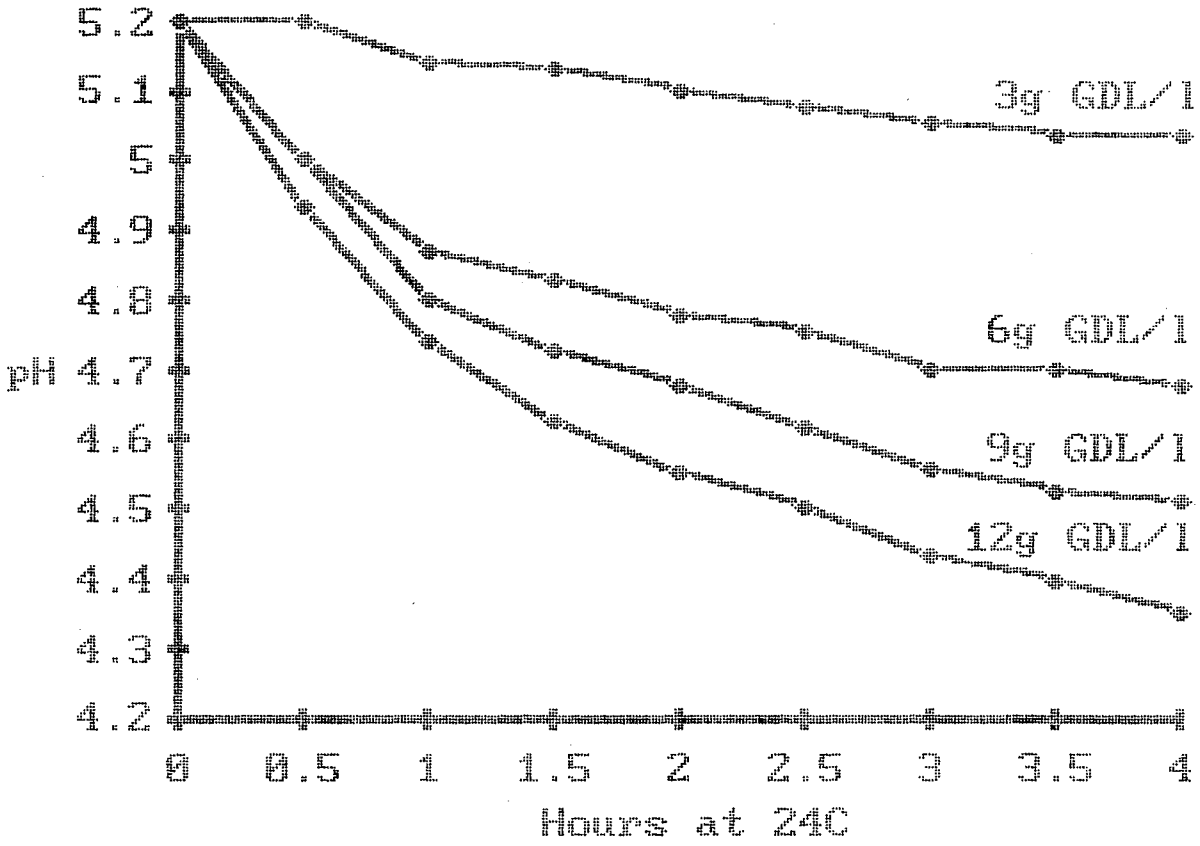

FIGURE II. INFLUENCE OF INCREASING CONCENTRATIONS OF GLUCONO-DELTA-LACTONE ON ACID DEVELOPMENT IN MILK (AVERAGES FROM TWO TRIALS)

reduced below that normally found in commercial buttermilk. When 9g GDL/liter was used to acidify the milk, there was a sufficient amount of acid produced. Also, the pH changed very little throughout extended incubation. The very small difference between pH at 3, 3.5, and 4 hours indicated that additional acid development was unlikely.

Table II shows the effect of storage on the stability of acidified milk. There was very little fluctuation in pH during storage between days 0 and 14 at 5°C. Also, there was no evidence of wheying-off in any of the samples during the two week period of storage at 5°C

Selection of Strains of Leuconostoc cremoris for Maximum Diacetyl Production

Considerable variation was observed in the relative ability of various strains to produce diacetyl in "lactic-acetic acid whey" (Table III). Cells which had been grown statically without pH control in broth were used for these comparisons. Strains J, K, and N produced significantly more ($P < .05$) diacetyl than did the other eleven strains tested (ANOVA in Table XII, appendix).

Concentrated cultures were prepared from strains J, K, and N; however, strain J did not yield a concentrated culture with a population that was adequate to use for buttermilk manufacture; thus, it was deleted from the study. Table IV shows the ability of cells of L. cremoris K and N from frozen concentrated cultures to produce diacetyl in

TABLE II

INFLUENCE OF STORAGE AT 5°C ON pH AND STABILITY OF DIRECTLY ACIDIFIED MILK[A]

Observation	Day	Trial[C]		
		A	B	C
pH	0	4.60	4.60	4.55
	3	4.60	4.60	4.50
	7	4.60	4.60	4.50
	14	4.60	4.60	4.50
Depth of Whey	0	N[B]	N	N
	3	N	N	N
	7	N	N	N
	14	N	N	N

[A] Standardized to 1% fat; fortified with 0.5% NFMS and 0.1% sodium citrate

[B] No whey separation evident

[C] Each value is an average from 3 trials

TABLE III

COMPARISON OF STRAINS OF *LEUCONOSTOC CREMORIS* FOR DIACETYL PRODUCTION IN LACTIC-ACETIC ACID WHEY

Strain	Diacetyl[a] (ug/10^8 cells)
A	0.392[e]
B	0.455[e]
C	1.080[d]
D	0.750[de]
E	0.605[de]
F	1.043[d]
G	0.688[de]
H	0.977[d]
I	0.622[de]
J	4.970[b]
K	4.143[c]
L	0.896[d]
N	4.790[b]

[a] Each value is an average from 3 trials

[bcde] Means with different superscript letters differ significantly ($P < .01$)

milk acidified with GDL during a 14 hour incubation period. Strain N tended to produce more total diacetyl than strain K throughout the incubation, but the difference was not significant ($P > .05$). However, strain N produced significantly more diacetyl than strain K ($P < .01$) when compared on the basis of ppm diacetyl/10^8 cells (ANOVA in Table XIV, appendix).

Compared to the amounts of diacetyl produced in the "lactic-acetic acid whey" by these strains (Table III), considerably less diacetyl was produced in the milk acidified with GDL (Table IV). Apparently, GDL inhibited diacetyl production in the acidified milk, so the level of inoculum in milk acidified with GDL ($2.0 - 2.5 \times 10^8$/ml) was increased by approximately 50-fold over that used in whey (4.0×10^6/ml) for initial screening of all cultures. The confirmation of the inhibitory action of GDL on diacetyl production by L. cremoris is shown in Table V. After 12 hours, L. cremoris N in whey made from milk acidified with lactic-acetic acid had produced significantly more ($P < .005$) diacetyl than it did in whey made from milk acidified with GDL (ANOVA in Table XV, appendix). Plate counts at the end of the 14 hours incubation indicated that the final population of L. cremoris N in whey made from lactic-acetic-acid was six-fold higher than that in whey made from milk acidified with GDL, even though they were equal at 0 hours (Table VI).

TABLE IV

DIACETYL PRODUCTION BY LEUCONOSTOC CREMORIS IN MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE

Strain	Hours Incubation	Diacetyl[a]	
		ppm	ppm/10^8 cells
K[1]	8	2.25[b]	0.87[c]
	10	2.30[b]	0.88[c]
	12	2.55[b]	0.98[c]
	14	3.00[b]	1.15[c]
N[2]	8	2.35[b]	1.18[d]
	10	2.75[b]	1.38[d]
	12	2.90[b]	1.45[d]
	14	3.10[b]	1.55[d]

a Each value is an average from 4 trials

bcd Means in the same column at the same hour incubation with different superscripts differ significantly ($P < .01$)

1 2.6×10^8 cells/ml

2 2.0×10^8 cells/ml

TABLE V

INHIBITORY ACTION OF GLUCONO-DELTA-LACTONE ON DIACETYL PRODUCTION BY LEUCONOSTOC CREMORIS N

Type of Whey	ppm diacetyl/10^8 cells[a]			
	8[b]	10	12	14
Lactic-Acetic Acid	32[c]	72[c]	107[c]	86[c]
Glucono-delta-lactone	4[d]	8[d]	12[d]	16[d]

[a] Each value is an average from 5 trials

[b] Hours incubation in whey

[cd] Means in the same column with different superscripts differ significantly ($P < .005$)

TABLE VI

APPARENT GROWTH OF LEUCONOSTOC CREMORIS IN WHEY PREPARED FROM MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE OR LACTIC-ACETIC ACID

	$\log_{10}$ cfu/ml[a]	
Hours of Incubation	Whey (Lactic-Acetic Acid)	Whey (GDL)
0	6.72	6.67
14	7.53	6.55

[a] Each value is the average $\log_{10}$ cfu/ml from 5 trials; cfu = colony forming units

Subjective and Objective Analysis of Buttermilk

Table VII shows the results of sensory panel evaluation of the experimental buttermilk made using L. cremoris K and N, and two samples of commercial buttermilk. Commercial brand FF had the highest overall mean score (7.3); buttermilk made with L. cremoris N had the highest flavor mean score (7.2), and commercial sample GS had the highest body/texture mean score (8.2). However, there were no significant differences ($P > .05$) among buttermilk samples with respect to mean scores for flavor, body/texture and overall quality. Diacetyl contents of the four samples before and after 7 and 14 days of storage at $5^{o}C$ are shown in Table VIII. Commercial sample GS contained considerably less diacetyl initially than did the other three samples. However, after one week of storage at $5^{o}C$, experimental buttermilk made with strains K and N had lost considerable amounts of diacetyl. After 14 days of storage, the concentration of diacetyl in the experimental buttermilk had decreased even more. Loss of diacetyl in the two commercial samples was minimal compared to that observed in the experimental samples.

TABLE VII

ORGANOLEPTIC EVALUATION OF BUTTERMILK[a]

Sample	Mean Scores[b]		
	Overall	Flavor	Body/Texture
Direct acid + culture K	6.7[c]	6.8[c]	7.2[c]
Direct acid + culture N	7.0	7.2	7.4
Commercial FF	7.3	6.8	7.6
Commercial GS	6.4	6.8	8.2

[a] Direct acid and commercial buttermilk were evaluated the day following manufacture of Direct acid buttermilk

[b] Average scores from 3 trials

[c] There were no significant differences among the mean scores for the 4 samples ($P > .05$)

TABLE VIII

LOSS OF DIACETYL DURING REFRIGERATED STORAGE OF BUTTERMILK AT 5°C

Sample	ppm diacetyl[a]		
	Day 0	Day 7	Day 14
Direct acid + culture K	3.10	1.00	0.05
Direct acid + culture N	3.55	1.40	0.30
Commercial FF	4.65	4.05	3.40
Commercial GS	1.30	1.50	1.20

[a] Each value is an average from 3 trials except for commercial brands FF and GS which are averages from 2 trials

CHAPTER V

DISCUSSION

Cultures most frequently used for the manufacture of cultured buttermilk contain different strains of S. lactis or S. cremoris for lactic acid production and L. cremoris for diacetyl production. These cultures are available in either a lyophilized or frozen concentrated form (Richter, 1977). Inoculation is usually at a level of 1 percent using a milk culture or an amount of concentrated culture containing comparable numbers of bacteria, and incubation is typically for 14 - 16 hours at 21 - 22°C. Improper incubation temperatures may adversely affect diacetyl production in two ways: (1) Rapid acid production by S. lactis or S. cremoris inhibits growth of L. cremoris and subsequently diacetyl production. (2) Insufficient acid production does not adversely affect the growth of L. cremoris; however, because uptake of citrate begins after the acidity reaches pH 6.0, insufficient lactic acid production will result in little if any diacetyl production (Harvey and Collins, 1962). Lundstedt and Corbin (1983) stated that 86.7 percent of the citrate in milk is fermented at acidities below pH 5.2 by L. cremoris.

Goel and Marth (1969) reported that different strains of L. cremoris grown in conjunction with lactic streptococci, at various temperatures, will vary in their growth and activity. In this study all strains of L. cremoris were grown as pure cultures at 21°C for 24 hours prior to inoculating into acid whey and incubating at 32°C. The variation observed with regard to diacetyl production may have been due to the level of manganese in the whey. Cogan (1985) stated that when manganese is added to milk, diacetyl production by pure strains of L. cremoris is increased. He attributed this increase in diacetyl to enhanced growth of the organism and not to increased activity of the enzymes involved in diacetyl synthesis. Also, the variation may be due to the rate of transport of citrate into the cell. Harvey and Collins (1962) and Seitz et al. (1962) showed that citrate enters the cell by means of an inducible permease, and is converted to acetate and oxaloacetate (OAA) by citrate lyase. OAA is then decarboxylated to form pyruvate and CO_2 by OAA decarboxylase. Pyruvate is normally required for cell synthesis; however, in the presence of lactose, excess pyruvate is produced that is toxic to the cell, so the organism reacts by decarboxylating pyruvate to yield a thiamine pyrophosphate complex that reacts with acetyl CoA, in the presence of diacetyl synthase, to produce diacetyl and acetoin (Cogan, 1976). When all the citrate in the medium is used, diacetyl reductase (DR) activity increases

which results in the reduction of diacetyl to acetoin. However, Cogan et al. (1981) reported that at acidities below pH 5.0, DR activity was minimal. Because DR is pH dependent and because this experiment used acid whey (pH 4.5 - 4.6) it is very unlikely that DR played a major role in the variation among cultures with respect to diacetyl production.

Strain M lost the ability to produce diacetyl in whey and was deleted from the study. This loss in ability to produce diacetyl may have been due to loss of plasmid(s). Marshall and Law (1984), and O'Sullivan and Daly (1982) found that in S. lactis subsp. diacetilactis and L. mesenteroides respectively, citrate permease activity was coded by a plasmid.

The level and ratio of organic acids are important in the flavor of buttermilk. Harper (1968) showed that other than lactic acid, acetic acid was the most prevalent organic acid in good quality cultured buttermilk. Also, Bennet et al. (1965) showed that acetic acid significantly enhanced the perception of low concentrations of diacetyl in artificial buttermilk. Based on these reports, glacial acetic acid was used in conjunction with lactic acid to acidify the milk to pH 5.2.

Concentrated starter cultures are used extensively in the production of cultured dairy products. Gilliland et al. (1970) explained that when growing cells of L. cremoris for concentrated cultures it is desirable to attain as high a

population as possible. Because acid inhibits the growth of L. cremoris, growing these cells under conditions where the pH is controlled at a favorable level enhances their growth. Gilliland et al. (1970) showed that there was approximately a two-fold increase in the population of L. cremoris when grown in broth with the growth medium automatically controlled at pH 6.0 when compared to cells grown without pH control. Therefore, in the present study cells were grown at pH 6.0. While L. cremoris J grown at pH 6.0 did not yield a final population that was adequate to use as a concentrated starter culture, sufficient growth was obtained for the other strains.

To form a smooth coagulum, chemical acidulants used to acidify milk should mimmick acid production by starter culture bacteria. Thus, to ensure a smooth coagulum for buttermilk, the formation of acid must be relatively slow and uniform throughout the milk. The use of glucono-delta-lactone (GDL) as an acidulant can achieve this need. The addition of GDL to cold milk does not immediately result in reduced pH; thus, the chemical can be uniformly distributed throughout the milk by mixing while the milk is cold. When the temperature is raised the GDL is hydrolyzed to form gluconic acid. This results in uniform acid development throughout the milk which forms a smooth coagulum. Deane and Hammond (1960) found that by increasing the temperature of incubation from 20°C to 35°C that the time required to acidify milk with GDL was decreased by 75 - 80 percent.

They relied soley on GDL to coagulate the milk to pH 4.6; consequently, the time required to achieve a smooth coagulum was substantial. In this experiment the time required to acidify the milk was greatly reduced because both acid and GDL were used to acidify the milk. The pH of the cold milk was adjusted to a level (5.1 - 5.3) just above the coagulation point; then, 9g GDL/liter was able to acidify the milk to approximately pH 4.5 within 3.5 hours.

Theoretically using a concentrated culture of L. cremoris will enable immediate synthesis of diacetyl without waiting for growth of the organism. The ability of concentrated cultures of L. cremoris to produce diacetyl in whey and milk was adversely influenced by GDL. Gilliland et al. (1970) successfully used concentrated cultures of L. cremoris to produce diacetyl in an acidified creaming mixture for cottage cheese. However, they did not use GDL as an acidulant because the smooth coagulum was not necessary for the product being made. Deane and Hammond (1960) speculated, but, did not confirm that GDL was nonreactive with amino groups in milk protien. Trop (1984) reported that GDL reacted with lysine, tyrosine, arginine, and serine residues of protein. This reaction not only may affect the milk protein but also may alter the enzymes involved in diacetyl synthesis.

The fact that the final population of L. cremoris in whey made from milk acidified with lactic-acetic acid was eight-fold higher than that in whey made from milk acidified

with GDL indicates that GDL also may inhibit the growth of L. cremoris. However, because L. cremoris does not reportedly grow at pH 4.5 - 4.6 (Gilliland et al., 1970), this is only speculation. The significant difference in diacetyl produced by leuconostocs in the two media also indicates that GDL may inhibit diacetyl synthesis by L. cremoris. The mechanism by which GDL may act to decrease diacetyl synthesis is unknown; however, it appears that it may somehow alter the enzymes responsible for synthesis of diacetyl. Marshall and Law (1984) reported that the rate of entry of citrate into the cell is independent from growth for S. lactis subsp. diacetilactis. In contrast, Cogan (1985) stated that diacetyl synthesis by L. cremoris will not begin until an appropriate population is obtained. Whether inhibition of growth and diacetyl synthesis are coupled is unknown; however, because lower populations were always associated with minimal diacetyl production, it appears that the two may be coupled.

Diacetyl content in experimental buttermilk may have been adversely affected by GDL. In order to attain an acceptable concentration of diacetyl in the acidified milk, the level of inoculum was increased 40-fold over that in "lactic-acetic acid whey". However, after one week of storage, the concentration of diacetyl in the product was negligible. Although diacetyl reductase is not normally active at pH 4.5 (Cogan et al., 1981), GDL may act by somehow enabling the enzyme to remain active at acidities

below pH 5.0. These results indicate that GDL may be involved in inhibiting diacetyl synthesis and destruction of diacetyl during storage of experimental buttermilk made using direct acidification and citrate fermentation. In contrast to these results, Lundstedt and Corbin (1983) reported that buttermilk made using S. lactis subsp. diacetilactis and GDL as an acidulant maintained good flavor after 46 days of storage. Perhaps this organism is not adversely affected by GDL. However, they did state that the level of starter culture required, in addition to the amount of acidulant, made the process economically infeasible.

The 3:1 molar solution of lactic acid and acetic acid did not appear to result in severe comments relative to flavor and perception of diacetyl in the experimental buttermilk. Buttermilk made using L. cremoris K or N in conjunction with GDL and the acid mixture was rated good by an experienced taste panel. The most common flavor defects for the experimental buttermilk were coarse and acid. The coarse defect may indicate that the level of acetic acid was excessive and overenhanced the perception of diacetyl. Additional work should be done to determine the optimum level of acetic acid in the product. This should be done using a more detailed sensory evaluation.

The fact that the experimental buttermilk was acceptable when compared to commercially cultured buttermilk shows that this method may be feasible. However, due to the apparent inhibitory affect of GDL on diacetyl synthesis by

L. cremoris, and destruction of diacetyl during storage, more research is needed to identify an alternate acidulant or to modify the process so that the adverse influence of GDL can be minimized. Perhaps S. lactis subsp. diacetilactis would be a more suitable organism for this process.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Experimental buttermilk was made using two different strains of L. cremoris. The purpose of this study was to determine if direct acidification and citrate fermentation by frozen concentrated cultures of L. cremoris could produce buttermilk that would be commercially acceptable.

Studies comparing diacetyl production by various strains of L. cremoris at 32°C in "lactic-acetic acid whey" containing 0.1% sodium citrate indicated that there were significant differences among strains with respect to diacetyl production. The most active strains were selected for preparing frozen concentrated cultures.

Concentrated cultures were prepared from cells of L. cremoris J, K, and N grown in media containing 0.5% sodium citrate and compared for their ability to produce diacetyl in milk acidified with glucono-delta-lactone(GDL). L. cremoris N produced significantly more diacetyl on a "ppm/10^8 cells basis" than did L. cremoris K. However, both strains were adversely affected by the apparent inhibitory action of GDL on diacetyl formation. Thus, much higher levels of inoculum were required than anticipated to produce

sufficient diacetyl in the milk acidified with lactic acid, acetic acid, and GDL.

Confirmation of the inhibitory action of GDL was achieved by inoculating a concentrated culture of L. cremoris N into whey made from milk acidified with lactic-acetic acid, and whey made from milk acidified with GDL and incubating both at 32°C. The culture produced significantly higher levels of diacetyl in the whey made from lactic-acetic acid than it did in whey made from milk acidified with GDL.

The final evaluation of the method was the ability of frozen concentrated cultures of L. cremoris K and N, separately, to produce an acceptable buttermilk from 1% fat milk supplemented with 0.5% non-fat milk solids (NFMS), 0.1% sodium citrate, pasteurized at 85°C, and homogenized at 2000 psi. The buttermilk samples containing L. cremoris K and N were not siginificantly different from commercial buttermilk when analyzed by an experienced taste panel.

This study reveals that buttermilk can be made using direct acidification and citrate fermentation by L. cremoris; however, the negative affect that GDL has on growth of L. cremoris, synthesis of diacetyl, and stability of diacetyl during storage make this an infeasible process. Factors such as the optimum level of GDL to prevent or decrease the inhibitory action of GDL, and the proper level of acetic acid to accentuate diacetyl need to be researched.

BIBLIOGRAPHY

Bennett, G., B.J. Liska, and W.L. Hempenius. 1965. Effect of other flavor components on the perception of diacetyl in fermented dairy products. J. Food Sci. 30: 35-37.

Briggs, M., G. Tull, M. G. Newland, and C.A.E. Briggs. 1955. The preservation of lactobacilli by freeze drying. J. Gen. Microbiol. 12: 503-512.

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

Campbell, J.R., and R.T. Marshall. 1975. The Science of Providing Milk for Man. McGraw-Hill Book Co. N.Y., New York.

Cogan, T.M. 1975. Citrate utilization in milk by Leuconostoc cremoris and Streptococcus diacetylactis. J. Dairy Res. 42: 139-146.

Cogan, T.M. 1976. The utilization of citrate by lactic acid bacteria in milk and cheese. Dairy Industries International reprint from paper delivered at the 4th Marschall Symposium, London. 1975.

Cogan, T.M., M. O'Dowd, and D. Mellerick. 1981. Effects of pH and sugar on acetoin production from citrate by Leuconostoc lactis. J. Appl. and Environ. Microbiol. 41: 1-8.

Cogan, T.M. 1985. "The Leuconostocs" Bacterial Starter Cultures for Foods. Ed. S.E.Gilliland. CRC Press N.Y., New York.

Cooper, R.K., and E.B. Collins. 1978. Influences of temperature on growth of Leuconostoc cremoris. J. Dairy Sci. 61: 1085-1088.

Cowman, R.A., and M.L. Speck. 1963. Activity of lactic streptococci following ultra-low temperature storage. J. Dairy Sci. 46: 609.

Deane, D.D., and E.G. Hammond. 1960. Coagulation of milk for cheese making by ester hydrolysis. J.

Dairy Sci. 42: 1421-1429.

Drinan, D.F., S. Tobin, and T.M. Cogan. 1976. Citric acid metabolism in hetero- and homofermentative lactic acid bacteria. J. Appl. and Environ. Microbiol. 31: 481-486.

Emmons, D.B., and S.L. Tuckey. 1967. Cottage Cheese and other Cultured Dairy Products. Chas Pfizer Co. Inc. N.Y., New York.

Frank, J.F. 1984. Improving the flavor of cultured buttermilk. Cult. Dairy Prod. J. 19(3): 6-9.

Gettys, S.C., and P.M. Davidson. 1985. A comparison of buttermilks made using fermentation, direct acidification, and a combination of both. J. Dairy Sci. 68: 620-627.

Gibson, C.A., C.B. Landerkin, and P.M. Morse. 1966. Effects of additives on the survival of lactic streptococci in frozen storage. Appl. Microbiol. 14: 665-669.

Gilliland, S.E., E.D. Anna, and M.L. Speck. 1970. Concentrated cultures of Leuconostoc citrovorum. Appl. Microbiol. 19: 890-893.

Gilliland, S.E. 1971. Strain balance of multiple strain lactic streptococcus concentrated cultures. J. Dairy Sci. 54: 1129-1133.

Gilliland, S.E., and M.L. Speck. 1974. Frozen concentrated cultures of lactic starter bacteria in review. J. of Milk and Food Tech. (J. Food Protection). 37: 107-111.

Gilliland, S.E., and M.L. Speck. 1977. Use of the minitek system for characterizing Lactobacilli. J. of Appl. and Environ. Microbiol. 33: 1289-1292.

Goel, M.C., and E.H. Marth. 1968. Growth patterns of Leuconostoc citrovorum. J. Dairy Sci. 51: 926.

Goel, M.C., and E.H. Marth. 1969. Growth of Leuconostoc citrovorum in skim milk at 22 and 30°C. J. Dairy Sci. 52: 1207.

Hammer, B.W. 1935. The creatine test for acetylmethylcarbinol plus biacetyl in butter cultures. J. Dairy Sci. 18: 579-581.

Harper, W.J. Cultured products flavor--with emphasis on biological factors. Cult. Dairy Prod. J. 3: 3-8.

Harvey, R.J., and E.B. Collins. 1962. Citrate transport system of Streptococcus lactis susp. diacetilactis. J. Bacteriol. 83: 1005-1009.

Hempenius, W.L., and B.J. Liska. 1964. Preliminary observations on production of natural flavor in chemically acidified cream and skim milk. J. Dairy Sci. 47: 1099-1101.

Hempenius, W.H., B.J. Liska, and R.B. Harrington. 1967. Consumer research on flavor levels in sour cream. J. Dairy Sci. 50: 954.

Hill, E.C, F.W. Wenzell, and A. Baretto. 1954. Colorimetric method for detection of microbilogical spoilage in citrus juices. Food Tech. 8: 168-171.

Kempler, G.M., and L.L. McKay. 1982. Improved medium for detection of citrate-fermenting Streptococcus lactis subsp. diacetylactis. Appl. Environ. Microbiol. 39: 926.

Kosikowski, F. 1977. Cheese and Fermented Milk Foods. Edwards Brothers Inc. Ann Arbor, Michigan.

Lawrence, R.C., T.D. Thomas, and B.E. Terzaghi. 1976. Reviews of the progress of dairy science: Cheese Starters. J. Dairy Res. 43: 141-193.

Lindsay, R.C., E.A. Day, and W.E. Sandine. 1965. Green flavor defect in lactic starter cultures. J. Dairy Sci. 48: 863-869.

Little, L. 1967. Techniques for acidified dairy products. J. Dairy Sci. 60: 434-440.

Lundstedt, E., and W.B. Fogg. 1962. Citrated whey starters II. Gradual formation of flavor and aroma in creamed cottage cheese after the addition of small quantities of citrated cottage cheese whey cultures of Streptococcus diacetilactis. J. Dairy Sci. 45: 1327-1331.

Lundstedt, E., and E.A. Corbin. 1983. Controlled fermentation buttermilk. Cult. Dairy Prod. J. 18(3): 6-8.

Mabbitt, L.A., H.R. Chapman, and N.J. Berriridge. 1955. Experiments in cheese-making without starter. J. Dairy Res. 22: 365-373.

Madsen, F. 1955. Biacetyl production by Leuconostoc citrovorum in simplified media. Dairy Sci. Abstr.

17: 1039.

Marshall V.A., and B.A. Law. 1984. "Physiology and Growth of Dairy Lactic Acid Bacteria". Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk. F.L. Davies and B.A. Law (ed). Elsevier Appl. Sci. Publ. London.

Mather, D.W., and F.J. Babel. 1959. A method for standardizing the diacetyl content of creamed cottage cheese. J. Dairy Sci. 42: 1045.

Morichi, T.R.I., N. Yano, and H. Kembo. 1963. Protective effect of glutamic acid and related compounds on bacterial cells subjected to freeze drying. J. Gen. Appl. Microbiol. 9: 149-161.

Olson, H.C. 1974. Cultured Dairy Products Scoring Clinic. Cult. Dairy Prod. J. 9: 6-7.

O'Sullivan, T. and C. Daly. 1982. Plasmid DNA in Leuconostoc species. Irish J. Food Sci. Tech. 6: 206.

Pack, M.Y., E.R Vedamuthu, W.E. Sandine, P.R. Elliker, and H. Leesment. 1964. Owades and Jakovac method for diacetyl determination in mixed strain starters. J. Dairy Sci. 47: 981-986.

Pack, M.Y., E.R. Vadamuthu, W.E. Sandine, P.R. Elliker, and H. Leesment. 1968. Effect of temperature on growth and diacetyl production by aroma bacteria in single-and mixed-strain lactic cultures. J. Dairy Sci. 51: 339-344.

Peebles, M.M., S.E. Gilliland, and M.L. Speck. 1969. Preparation of concentrated lactic streptococcus starters. Appl. Microbiol. 17: 805-810.

Prill, E.A., and B.W. Hammer. 1938. A colorimetric method for the determination of diacetyl. Iowa St. Coll. J. Sci. 12: 385-395.

Prill, E.A., and B.W. Hammer. 1939. Production of diacetyl from citric acid in butter cultures. J. Dairy Sci. 22: 67-77.

Richter, R.L. 1977. Manufacture of superior quality buttermilks. Cult. Dairy Prod. J. 12(1): 22-28.

Seitz, E.W., W.E. Sandine, P.R. Elliker, and E.A. Day. 1962. Studies on diacetyl biosynthesis by Streptococcus diacetilactis. Can. J. Microbiol. 9: 431-441.

Smittle, R.B., S.E. Gilliland, and M.L. Speck. 1972. Death of Lactobacillus bulgaricus resulting from liquid nitrogen freezing. Appl. Microbiol. 24: 551-554.

Speck, M.L. editor. 1976. Compendium of Methods for the Microbial Examination of Foods. American Public Health Association, Inc. Washington, D.C.

Steele, R.G.D., and J.H. Torrie. 1980. Principles and Procedures of Statistics. 2nd Edition. McGraw Hill Book Co. N.Y., New York.

Trop, M. 1984. Simulation of bacterial fermentation of milk and possible acylation of its proteins by acidogen hydrolysis. J. Dairy Sci. 67: 1381-1389.

Trop, M., and A. Kushelevsky. 1985. The reaction of glucono-delta-lactone with proteins. J. Dairy Sci. 68: 2534-2535.

Vasavada, P.C., and C.H. White. 1979. Our Industry Today: The quality of commercial buttermilk. J. Dairy Sci. 62: 802-806.

Vedamuthu, E.R. 1977. Better Buttermilk. Microlife Technics. Sarasota, Florida.

Vedamuthu, E.R., and A.H. Rose. 1982. "Fermented Foods" Economic Microbiology (7). Acedemic Press. Bath, England.

Vedamuthu. E. 1985. What's wrong with cultured buttermilk today? Dairy and Food Sanitation. 5: 8-13.

Veringa, H.A., E.H. Verburg, and J. Statdhouders. 1985. Determination of diacetyl in dairy products containing alpha aceto lactic acid. Netherlands Milk and Dairy Journal (1984) 38(4): 251-263. (En, n.l. 36 ref), Netherlands. Dairy Sci. Abstracts. 47(7): 496.

Walsh, B., and T.M. Cogan. 1974. Further studies on the estimation of diacetyl by methods of Price and Hammer and Owades and Jakovac. J. Dairy Res. 41: 31-35.

White, C.H. 1978. Manufacturing better buttermilk. Cult. Dairy Prod. J. 13: 16-20.

APPENDIXES

APPENDIX A

INHIBITORY ACTION OF GLUCONO-DELTA-LACTONE ON DIACETYL PRODUCTION BY *LEUCONOSTOC CREMORIS*

TABLE IX

INHIBITORY ACTION OF GLUCONO-DELTA-LACTONE ON DIACETYL PRODUCTION BY LEUCONOSTOC CREMORIS

Hours Incubation at 32°C	Medium	Trial[a]					
		1	2	3	4	5	x[b]
8	LA Whey[c]	40.0	32.7	41.1	20.0	26.5	32.1
	GDL Whey[d]	6.4	6.5	5.3	2.1	2.1	4.4
10	LA Whey	65.5	72.7	71.4	88.0	65.3	72.1
	GDL Whey	6.4	6.5	8.5	8.5	8.3	7.7
12	LA Whey	101.8	123.6	121.4	88.0	102.0	107.4
	GDL Whey	12.8	13.0	17.0	8.5	9.4	12.1
14	LA Whey	87.3	65.5	84.8	88.0	102.0	85.5
	GDL Whey	17.0	17.4	17.0	19.1	9.4	16.0

[a] Each value represents ppm diacetyl from duplicate 20g samples

[b] Each value is an average from 5 trials

[c] Whey made from milk acidified with lactic-acetic acid

[d] Whey made from milk acidified with glucono-delta-lactone

APPENDIX B

EFFECT OF STORAGE AT 5°C ON pH AND DEPTH OF WHEY IN EXPERIMENTAL BUTTERMILK

TABLE X

AFFECT OF STORAGE AT 5°C ON pH AND DEPTH OF WHEY IN EXPERIMENTAL BUTTERMILK

		Trial					
		1		2		3	
Sample	Day	pH	Depth of Whey	pH	Depth of Whey	pH	Depth of Whey
K	0	4.55	N[a]	4.65	N	4.65	N
	7	4.55	N	4.65	N	4.60	N
	14	4.60	N	4.60	N	4.65	N
N	0	4.50	N	4.50	N	4.65	N
	7	4.55	N	4.60	N	4.60	N
	14	4.55	N	4.60	N	4.60	N

[a] Indicates no wheying-off in 100 ml of sample

APPENDIX C

STORAGE STABILITY OF DIACETYL IN EXPERIMENTAL AND COMMERCIAL BUTTERMILKS DURING STORAGE AT $5^{\circ}C$

TABLE XI

STORAGE STABILITY OF DIACETYL IN EXPERIMENTAL AND COMMERCIAL BUTTERMILKS DURING STORAGE AT 5°C

Sample	Days at 5°C	ug Diacetyl/ml[a] Trial 1	Trial 2	Trial 3	x[b]
Direct acid + culture K	0	2.25	3.10	2.60	2.65
	7	0.20	1.00	0.15	0.45
	14	0.05	0.05	0.10	0.07
Direct acid + culture N	0	3.10	3.55	2.63	3.10
	7	1.30	1.40	0.18	0.96
	14	0.28	0.30	0.05	0.21
FF	0	3.70	4.65	0.50	2.95
	7	-	4.05	0.38	2.21
	14	-	3.40	0.15	1.78
GS	0	1.60	1.30	1.03	1.31
	7	-	1.50	0.85	1.18
	14	-	1.20	1.03	1.12

[a] Each value also represents ppm diacetyl from duplicate 20g samples

[b] Each value is an average from 3 trials

APPENDIX D

ANALYSIS OF VARIANCE OF DATA FROM COMPARISON OF STRAINS OF *LEUCONOSTOC CREMORIS* FOR DIACETYL PRODUCTION IN LACTIC-ACETIC ACID WHEY

TABLE XII

ANALYSIS OF VARIANCE OF DATA FROM COMPARISON OF STRAINS OF LEUCONOSTOC CREMORIS FOR DIACETYL PRODUCTION IN LACTIC-ACETIC ACID WHEY

Analysis of Variance					
Source	df	SS	MS	F-Ratio	Critical F (0.5% Level)
Total	38	103.57			
Strain	12	100.63	8.38	79.28	3.42
Rep	2	0.40	0.20	1.90	6.73
Error	24	2.54	0.11		

Significant at $P < .005$; LSD required for differences among means at $P < .01$ and $P < .05$ are .4287 and .3165 respectively

APPENDIX E

ANALYSIS OF VARIANCE OF DATA FROM DIACETYL PRODUCTION BY *LEUCONOSTOC CREMORIS* IN MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE

TABLE XIII

ANALYSIS OF VARIANCE OF DATA FROM DIACETYL PRODUCTION BY *LEUCONOSTOC CREMORIS* IN MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE

Source	Analysis of Variance df	SS	MS	F-Ratio	Critical F (5% Level)
Total	7	1.21			
Strain	1	0.26	0.26	1.88	10.13
Rep	3	0.53	0.17	1.27	9.28
Error	3	0.42	0.14		

APPENDIX F

ANALYSIS OF VARIANCE OF DATA FROM DIACETYL PRODUCTION BY *LEUCONOSTOC CREMORIS* IN MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE II

TABLE XIV

ANALYSIS OF VARIANCE OF DATA FROM DIACETYL PRODUCTION BY LEUCONOSTOC CREMORIS IN MILK ACIDIFIED WITH GLUCONO-DELTA-LACTONE II

Analysis of Variance (ppm/10^8 cells)					
Source	df	SS	MS	F-Ratio	Critical F (5% Level)
Total	7	0.52			
Strain	1	0.43	0.43	16.99	10.13
Rep	3	0.12	0.004	0.16	9.28
Error	3	0.07	0.03		

Significant at $P < .05$; LSD required for differences among means at $P < .01$ and $P < .05$ are .3286 and .1789 respectively

APPENDIX G

ANALYSIS OF VARIANCE OF DATA FROM INHIBITORY ACTION OF GLUCONO-DELTA-LACTONE ON DIACETYL PRODUCTION BY *LEUCONOSTOC CREMORIS*

TABLE XV

ANALYSIS OF VARIANCE OF DATA FROM INHIBITORY ACTION OF GLUCONO-DELTA-LACTONE ON DIACETYL PRODUCTION BY LEUCONOSTOC CREMORIS

Analysis of Variance					
Source	df	SS	MS	F-Ratio	Critical F (0.5% Level)
Total	9	2.36			
Rep	4	0.06	0.02	2.07	31.33
Media	1	2.27	2.27	295.95	23.15
Error	4	0.03	0.01		

APPENDIX H

ANALYSIS OF VARIANCE OF BODY/TEXTURE SCORES FROM SENSORY EVALUATION OF BUTTERMILK

TABLE XVI

ANALYSIS OF VARIANCE OF BODY/TEXTURE SCORES FROM SENSORY EVALUATION OF BUTTERMILK

Analysis of Variance					
Source	df	SS	MS	F-Ratio	Critical F (5% Level)
Total	19	18.8			
Sample	3	2.8	0.93	1.33	3.49
Panelist	4	7.6	1.89	2.68	3.26
Error	12	8.5	0.70		

APPENDIX I

ANALYSIS OF VARIANCE OF FLAVOR SCORES FROM SENSORY EVALUATION OF BUTTERMILK

TABLE XVII

ANALYSIS OF VARIANCE OF FLAVOR SCORES FROM SENSORY EVALUATION OF BUTTERMILK

Analysis of Variance					
Source	df	SS	MS	F-Ratio	Critical F (5% Level)
Total	19	13.80			
Sample	3	0.60	0.20		
Panelist	4	8.80	2.20	0.55	3.49
Error	12	4.40	0.37	5.90	3.26

APPENDIX J

ANALYSIS OF VARIANCE OF OVERALL SCORES FROM SENSORY EVALUATION OF BUTTERMILK

TABLE XVIII

ANALYSIS OF VARIANCE OF OVERALL SCORES FROM SENSORY EVALUATION OF BUTTERMILK

Analysis of Variance					
Source	df	SS	MS	F-Ratio	Critical F (5% Level)
Total	19	10.95			
Sample	3	0.55	0.18	1.00	3.49
Panelist	4	8.20	2.05	11.80	3.26
Error	12	2.20	0.18		

VITA

Derek Keith Walker

Candidate for the Degree of

Master of Science

Thesis: BUTTERMILK MANUFACTURE USING A COMBINATION OF DIRECT ACIDIFICATION AND CITRATE FERMENTATION BY LEUCONOSTOC CREMORIS

Major Field: Food Science

Biographical:

Personal Data: Born in Philadelphia, Pennsylvania, July 31. 1961, the son of Wilmar and Mary E. Walker.

Education: Graduated from Franklin Delano Roosevelt High School, Dallas, Texas in May 1979; received Bachelor of Science degree from Oklahoma State University in Animal Science--Food Industry option in May 1984; completed requirements for Master of Science degree in Food Science at Oklahoma State University in July, 1986.

Professional Experience: Graduate Research Assistant, Oklahoma State University, Department of Animal Science, 1984-1986.

Organizations: American Dairy Science Association.