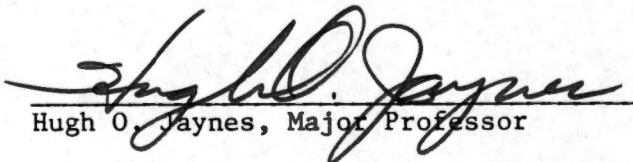
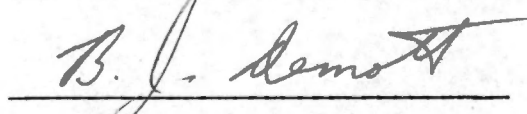
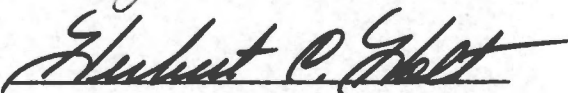


To the Graduate Council:

I am submitting herewith a thesis written by Velagaleti L. Charya entitled "Characterization of a Suitable Strain of *Lactobacillus acidophilus* and Development of Flavored Acidophilus Milks." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.


Hugh O. Jaynes, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Vice Chancellor
Graduate Studies and Research

CHARACTERIZATION OF A SUITABLE STRAIN OF
LACTOBACILLUS ACIDOPHILUS AND
DEVELOPMENT OF FLAVORED
ACIDOPHILUS MILKS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Velagaleti L. Charya

June 1979

1389648

DEDICATION

This thesis is dedicated to my parents, my brother Raghu, and my sister Sreemathi.



ACKNOWLEDGEMENTS

I wish to express sincere appreciation to Dr. W. W. Overcast and Dr. H. O. Jaynes, major professors, for their invaluable guidance and help throughout the graduate program and research presented in this thesis. Sincere appreciation is also extended to Dr. B. J. Demott and Mr. H. C. Holt for their help and constructive criticism while serving on the thesis committee.

Appreciation and thanks are also extended to the faculty, staff, and students of the Department of Food Technology and Science, especially to Mrs. Ola Sanders for her advice and assistance with the sensory evaluation of the products. Also thanks are duly extended to Paula Nelson, Kitty Ninnsteil, Don Maxedon, and Cleva Claiborne for their encouragement and laboratory assistance, and to Cathy Au for her friendship and help. Special thanks are due to Cynthia Meeks who kindly typed the rough draft of this thesis, and for her suggestions and help during the writing.

For their constant support and love, I wish to express gratitude to my parents, all family members including my wife, and especially to my brother, Dr. V. Raghava Charya for his kind support, enthusiasm, and advice during my entire education in the United States.

ABSTRACT

The objective of the study was to characterize four strains of Lactobacillus acidophilus and to select a suitable strain for preparation of five artificially flavored acidophilus milk beverages.

DeMan, Rogosa, Sharpe (MRS) medium was used in studying the characteristics of four strains. Strain 75 was confirmed as L. acidophilus. This strain, which came from raw milk, exhibited the ability to grow in the presence of bile salts, which indicates that it would be able to grow in the intestinal tract. Characteristics of four strains were determined together and compared to strains of L. acidophilus described in the literature.

L. acidophilus 75, selected by a panel for producing a smooth body and texture in acidophilus milk, was used in preparing strawberry, orange, lemon-lime, cherry, and raspberry flavored acidophilus milk beverages. A consumer panel of twenty-three members evaluated the samples. Results indicated that strawberry flavored acidophilus milk was liked best, orange flavor second best, whereas the raspberry flavor was least preferred. The study showed that acceptable flavored acidophilus milk beverages could be prepared with several different flavorings to suit the preferences of consumers.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	3
History	3
Characterization of <u>Lactobacillus acidophilus</u>	4
Importance of <u>Lactobacillus acidophilus</u> in	
Maintaining a Balanced Intestinal Microflora	5
Intestinal Microflora	5
Implantation of <u>Lactobacillus acidophilus</u>	7
Mechanism of <u>Lactobacillus acidophilus</u> Action	14
Acidophilus Milk Products	15
III. MATERIALS AND METHODS	19
Source of Cultures	19
Maintenance of Cultures	19
Characterization of Cultures	20
Colony Morphology	20
Cellular Morphology	20
Catalase Test	20
Motility Test	20
Physiological Tests	21
Preparation of Mother Cultures	24
Preparation of Acidophilus Milk	25
Flavoring of Acidophilus Milk	25
Sensory Evaluation	26
Statistical Analyses	27
IV. RESULTS AND DISCUSSION	28
V. SUMMARY	42
LITERATURE CITED	44
VITA	51

LIST OF TABLES

TABLE	PAGE
I. Colony and Cellular Morphology of <u>L. acidophilus</u> Strains . .	29
II. Physiological Characteristics of Four Strains of <u>L. acidophilus</u>	30
III. Characteristics of <u>L. acidophilus</u> : Data Taken from other Researchers	32
IV. Percent Acidity of the Samples of Acidophilus Milk for Body and Texture Evaluation	35
V. Analysis of Variance of Sensory Responses among Five Flavored Acidophilus Milk Beverages	38
VI. Sensory Scores of Five Flavored Acidophilus Milk Beverages	39

CHAPTER I

INTRODUCTION

Certain species of lactobacilli appear to be important in helping maintain a proper balance among microorganisms in the intestinal tract. Lactobacillus acidophilus is the primary species implicated as being involved in this manner. Consumption of large numbers of viable L. acidophilus cells has been used to reestablish normal intestinal flora following oral antibiotic therapy and to treat patients infected with intestinal pathogens. Therapy involving the consumption of viable L. acidophilus cells has been beneficial in treating certain gastrointestinal disorders (51, 56).

For ages, fermented milks containing the cultures of strains and/or species of lactobacilli have been consumed for their desirable attributes such as their therapeutic benefits. Acidophilus milk containing L. acidophilus is a fermented milk which has some definitely desirable attributes such as mentioned earlier. Elie Metchnikoff reasoned that consumption of these fermented milks would prevent the onset of premature old age (10, 36).

Acidophilus milk has not been acceptable to the American public until sweet acidophilus milk appeared on the market in 1975 (56, 57). This new sweet acidophilus milk has gained public acceptance in some areas where it is marketed. However, in other areas it has not been successful.

Fruit flavored cultured milks are available in some European countries. Well known is the fact that the per capita consumption of

cultured products is higher in European countries than in the United States. In certain European countries the flavored cultured milk beverages are common. However, flavored cultured milks are relatively unknown to the American public. In the U.S., the readily expanding yogurt sales is good evidence that a flavored cultured dairy product may be merchandised successfully (26, 43).

The work reported herein deals with the characterization of four different strains of Lactobacillus acidophilus, selection of a suitable strain, and the development of flavored acidophilus milk beverages.

CHAPTER II

REVIEW OF LITERATURE

I. HISTORY

For centuries, fermented milks such as yogurt, koumis, and kefir have been consumed for their therapeutic benefits. In the early days of bacteriology, the use of fermented milks was investigated and attempts were made to establish a scientific basis for their action. Microbiologists isolated and studied the organisms in the fermented milks and tried to determine which ones were important and what changes they caused. These early workers, especially von Freudenreich, contributed much information on the lactic acid bacteria and their role in the conversion of milk into fermented products (10).

Much of the interest in fermented milks has stemmed from the ideas and writings of Elie Metchnikoff (10, 36) who worked in France during the early part of the twentieth century. He observed that peoples in the Balkan countries lived to an exceptionally old age and attributed their longevity to their consumption of large quantities of fermented milk. He reasoned that consumption of considerable amounts of fermented milk would change the intestinal flora, preventing autointoxication and resulting old age. Since Metchnikoff's dramatic theories, a tremendous amount of research into the characterization of Lactobacillus acidophilus and the importance of this organism in maintaining a balanced intestinal microflora has been done.

II. CHARACTERIZATION OF LACTOBACILLUS ACIDOPHILUS

The concept of L. acidophilus stems from the descriptions of Moro (38), a German microbiologist, who described organisms of intestinal origin forming irregular and fuzzy colonies. He referred to these organisms as Bacillus acidophilus. In 1920, Holland (28) transferred Moro's species from the genus Bacillus to the genus Lactobacillus. However, literature (23) has indicated that a new name or combination was never proposed and thus it could not be considered validly published by Bergey et al., (1923) who gave the name as L. acidophilus (Moro) without citing the author of the combination. This is further substantiated by the fact that the second (1925), third (1930), fourth (1934), fifth (1939), sixth (1948), and seventh (1957) editions of Bergey's manual all cited Holland as the author of L. acidophilus. In 1970, Hansen and Møcquot (23) proposed the name Lactobacillus acidophilus (Moro) comb. nov. (Basionym: Bacillus acidophilus Moro 1900). They also noted that nothing in Moro's meager description of B. acidophilus contained anything which would disagree with the more complex one given for L. acidophilus. Their description for L. acidophilus (Moro) comb. nov. is as follows:

Gram-positive, non-sporulating rod. The colonies on trypticose-glucose-agar are rough, showing fuzzy outlines.

Forms DL-lactic acid, is homofermentative, usually curdles milk, but at times very slowly, and usually forms between 1.0-1.9% lactic acid.

Nutritional requirements are complex; needed are amino acids and growth factors: pantothenic acid, riboflavin, and niacin. Thiamine not needed.

Catalase negative.

Does not grow below 16C and frequently above 20C only; upper limit varies with the strain, some have a limit about 45C, others may grow at 48C.

Ferments: Fructose, glucose, mannose, galactose, sucrose, trehalose, maltose, cellobiose, and lactose.

Does not ferment: Rhamnose, sorbose, melezitose, starch, glycerol, sorbitol, mannitol, inositol.

Does not form gas from glucose.

Splits esculin, does not form ammonia from arginine.

Habitat: intestinal tract of mammals and fowls.

The description furnished here corresponds very closely to the ones offered by Rogosa et al. (49), Rogosa and Sharpe (48), Wheater (68), and Sharpe and Mattick (54). Bergey's Manual (47) eighth edition (1974) gives the name as Lactobacillus acidophilus (Moro) Hansen and Mocquot. American Type Culture Collection (ATCC) 4356 is designated as the neotype strain.

III. IMPORTANCE OF LACTOBACILLUS ACIDOPHILUS

IN MAINTAINING A BALANCED

INTESTINAL MICROFLORA

A. Intestinal Microflora

Concepts of the intestinal microflora have evolved from Metchnikoff's dramatic theories on microbes and longevity. Gorbach et al. (18) have shown that the small intestine contains lactobacilli, streptococci, staphylococci, and fungi whereas the flora of the colon is composed primarily of bacteroides, anaerobic lactobacilli and coliforms. Moore et al. (39) confirmed this.

Lactobacillus acidophilus is predominant among the intestinal forms of lactobacilli (29). Moore et al. (40) have shown that L. acidophilus is a natural inhabitant of the intestinal tract. Contents from the stomach and seven areas of the intestinal tract were cultured and predominant bacteria from each area identified. Contents from the

stomach and small intestine were cultured aerobically as well as anaerobically; the anaerobic cultural counts exceeded the aerobic cultural counts by one to three orders of magnitude. Culture work was initiated within 4-5 hours after death. From a 17-year-old male, the cultural counts per gram dry weight and predominant kinds were: stomach, 7.5×10^7 , four kinds including Lactobacillus fermentum (78%) and L. acidophilus (16%); duodenum, 3.6×10^7 , four kinds including L. acidophilus (78%) and L. fermentum (19%); jejunum, 1×10^7 , L. acidophilus (30%) and ten other species of anaerobes; ileum, 6×10^8 , L. acidophilus (56%).

Lerche and Reuter (33) also reported that L. acidophilus is abundant in the upper intestine. The numbers of lactobacilli found were of the same order of magnitude as the numbers of Escherichia coli. Eight types of lactobacilli were found, singly or in combination, rarely more than three types together, but with no regularity in composition. L. acidophilus was abundant in the upper intestine but the numbers tended to decrease with further passage through the intestinal tract.

In eubiotic or normal intestinal tracts, cultivable flora consist mainly of anaerobic lactobacilli (more than 50%), the second highest group being putrefactive bacteria, mainly Bacteroides species (20). Coli-aerogenes bacteria, enterococci, and other organisms are in a minority (usually less than 1%). The composition of this flora remains very stable, extreme diets producing only minor changes or none at all. The criteria of dysbiotic or disturbed microflora are: a reduction or elimination of anaerobic lactobacilli and an increase

stomach and small intestine were cultured aerobically as well as anaerobically; the anaerobic cultural counts exceeded the aerobic cultural counts by one to three orders of magnitude. Culture work was initiated within 4-5 hours after death. From a 17-year-old male, the cultural counts per gram dry weight and predominant kinds were: stomach, 7.5×10^7 , four kinds including Lactobacillus fermentum (78%) and L. acidophilus (16%); duodenum, 3.6×10^7 , four kinds including L. acidophilus (78%) and L. fermentum (19%); jejunum, 1×10^7 , L. acidophilus (30%) and ten other species of anaerobes; ileum, 6×10^8 , L. acidophilus (56%).

Lerche and Reuter (33) also reported that L. acidophilus is abundant in the upper intestine. The numbers of lactobacilli found were of the same order of magnitude as the numbers of Escherichia coli. Eight types of lactobacilli were found, singly or in combination, rarely more than three types together, but with no regularity in composition. L. acidophilus was abundant in the upper intestine but the numbers tended to decrease with further passage through the intestinal tract.

In eubiotic or normal intestinal tracts, cultivable flora consist mainly of anaerobic lactobacilli (more than 50%), the second highest group being putrefactive bacteria, mainly Bacteroides species (20). Coli-aerogenes bacteria, enterococci, and other organisms are in a minority (usually less than 1%). The composition of this flora remains very stable, extreme diets producing only minor changes or none at all. The criteria of dysbiotic or disturbed microflora are: a reduction or elimination of anaerobic lactobacilli and an increase of

of other organisms, mainly coli-aerogenes bacteria, enterococci, Proteus species, clostridia, and others. Dysbiotic conditions are frequently found in sick, convalescent or poorly thriving infants, in adults during therapy with tetracyclines and other antibiotics, anacidity of gastric juice, disturbance of peristalsis, operations on the upper gastro-intestinal tract, diseases of the liver or kidney, effects of radiation, and impairment of gastro-intestinal function (20, 21).

The delicate balance between the organisms constituting the normal flora, which is maintained by symbiosis and competition, can be upset in many ways.

Antibiotics and other forms of treatment can cause disturbances of the normal microflora of the intestinal tract. These disturbances vary in severity from mild diarrhea to fatal superinfection by resistant organisms. In the absence of competition and of inhibitory substances such as lactic acid, hydrogen peroxide, and other antimetabolites produced by the normal flora, antibiotic-resistant pathogens can flourish; the risks of this happening grow greater as resistant strains become more prevalent (11, 17, 51). When such intestinal disorders occur, coli-aerogenes bacteria and the enterococci predominate; anaerobic lactobacilli are either no longer present or are outnumbered by these pathogens.

B. Implantation of Lactobacillus Acidophilus

For many years, studies on the implantation of lactobacilli in the intestinal tract have indicated preponderantly beneficial results

when Lactobacillus acidophilus was consumed. While antibiotic therapy and other forms of treatment often lower the lactobacillus population in the intestinal tract with consequent intestinal discomfort, replenishing the intestinal tract with L. acidophilus results in an accelerated return to normalcy in the intestinal microflora and subsequent body comfort (56).

Literature indicated that it is essential to use an intestinal strain of L. acidophilus for successful implantation. For most satisfactory results, 10^8 - 10^9 viable cells must be ingested. A further important factor is that the organisms should be able to grow in the presence of bile salts (25, 57, 58).

Stark et al. (59) reported that by administering L. acidophilus therapy, they could successfully treat 29 out of 43 constipated persons experiencing intestinal difficulties, and a majority of the non-constipated persons reported themselves in a much better physical condition while they were drinking milk containing L. acidophilus.

Rafsky and Rafsky (44) have shown that oral administration of a viable human intestinal strain of L. acidophilus in the form of a tablet concentrate, called Viacil, was successful in treating 39 patients suffering from gastro-intestinal distress and dysfunction associated with irritable colon and diverticulitis. Clinical improvement consisting of relief of pain, gas, constipation, and diarrhea was observed in 32 of the 39 cases within one to three weeks. The therapy was effective in longstanding cases which had resisted other measures, including psychiatric. Daily dosage consisted of 100 billion viable L. acidophilus cells in three tablets.

Bacid, a capsule form of a dried viable culture of L. acidophilus, was used in the treatment of 59 cases with diarrhea of various etiologies, and cases with constipation, abnormal fermentation, and food poisoning. In 22 cases with antibiotic diarrhea, 20 excellent and 2 good therapeutic results were obtained. In 19 cases of epidemic diarrhea, excellent results were obtained in 17 chronic patients and fair results in 2 cases (4). Therapeutic failures occurred in one case of chronic pancreatitis with diarrhea, and in one case of colostomy with diarrhea.

Gordon et al. (19) have shown that L. acidophilus therapy can check the increase of potentially pathogenic staphylococci during treatment with antibiotics. Sixty-six patients receiving the oral antibiotic tetracycline were divided into two groups, and one was given a preparation, "Enpac," containing 10^7 - 10^8 viable cells of L. acidophilus. Fecal specimens from all cases were examined bacteriologically at intervals before, during, and after treatment. It was found that staphylococci continued to increase in the patients taking the antibiotic alone, but in those taking "Enpac," there was a pronounced drop in numbers following the initial rise. Simultaneously, in the group receiving "Enpac," lactobacilli were rapidly established in the intestine and continued to multiply. L. acidophilus used in this preparation was a human intestinal strain.

Read et al. (45) indicated that routine treatment of chronic hepatic encephalopathy is successfully accomplished in many patients by restriction of dietary protein and by the continuous use of oral antibiotics. This type of treatment, though often producing clinical

improvement, has some disadvantages such as the cost of antibiotics, toxic effects due to antibiotics, and disturbance of natural flora. Abnormalities in ammonia metabolism are associated with the hepatic encephalopathy. Although the role of ammonia in this syndrome is not clearly defined, it seems certain that ammonia and other toxic products produced by the common gram-negative rods play an important role in causing this syndrome. Medical colectomy has been reported successful in the management of hepatic encephalopathy (35, 45). Medical colectomy involves the colonization of the colon with non-ammonia producing bacteria.

Macbeth et al. (35) reported that medical colectomy using L. acidophilus was successful in treating two patients with hepatic encephalopathy. L. acidophilus was administered to these patients in the form of acidophilus milk containing 2.9×10^{12} organisms per liter, Dosage was one to two liters per day.

Enpac is a convenient way of attempting "medical colectomy" in hepatic encephalopathy and an effective therapeutic addition to treatment of this condition, according to Read et al. (45). Administration of Enpac containing 1×10^7 cells of L. acidophilus per gram along with lactose produced an improvement in five out of seven patients suffering from chronic hepatic encephalopathy. The precise way in which lactobacilli are able to improve this syndrome is uncertain. It has been theorized that lactobacilli interfere with the growth of colonic coliforms which are subsequently reduced in number, with a resultant fall in the production of ammonia and other protein breakdown products in the gut.

Robinson and Thompson (46) have shown that infants partially nursed, even for 2 or 3 days, showed significantly greater weight gains during the first month than infants completely bottle-fed. They also reported that bottle-fed infants on formulas supplemented with L. acidophilus showed greater gains in weight than control subjects.

Lactobacillus acidophilus therapy in man and domestic animals is more widely practiced in other countries, especially Europe, than in this country.

Tomic-Karovic and Fanjek (62) demonstrated the effectiveness of L. acidophilus milk in destroying pathogenic Escherichia coli, in vitro and in vivo. The in vivo effect was tested on twenty infants suffering from diarrhea caused by pathogenic E. coli. In all cases, the E. coli which was resistant to a number of antibiotics disappeared from the stools of the infants within one to five days after starting the therapy with acidophilus milk, and the infants made a rapid recovery.

Comparable results were obtained by Fedotov et al. (9). Colonization of the digestive tract of babies with L. acidophilus bacteria was achieved through applying acidophilus milk to the mother's nipples.

Aritaki and Ishikawa (3) reported that feeding L. acidophilus fermented milk to infants suffering from infantile diarrhea resulted in improvement of the condition. An increase of L. acidophilus and decrease of gram-negative bacilli in the intestinal flora of bottle-fed infants after feeding acidophilus milk was also reported.

Tomic-Karovic et al. (63) demonstrated the effectiveness of acidophilus milk in the therapy of acute and chronic nutritional disturbances. Two groups of forty infants suffering from this condition

were given a vegetable diet plus acidophilus milk, and a vegetable diet plus antibiotics respectively. The acidophilus milk group averaged a shorter period in the hospital and showed better average weight gains than the antibiotics group.

The therapeutic value of a commercial L. acidophilus preparation (Zyma), in the treatment of different intestinal conditions, including enteritis, dyspepsia, and X-ray damage in young persons, was demonstrated by Lachner and Bieler and Low (31, 34).

Tomic-Karovic (61) has shown that patients with various infections caused by Salmonella cholerae, Staphylococcus aureus, Streptococcus haemolyticus, E. coli, and Shigella species, as well as two children suffering from osteomyelitis, were cured or considerably improved by treatment with a half liter of acidophilus milk daily.

Cattan and Milstein (5) have reported on the effectiveness of L. acidophilus milk therapy following antibiotic treatment of certain intestinal disorders.

A preparation of dried acidophilus milk is used in Russia for feeding and therapeutic purposes (37). Whole or separated milk is pasteurized at 90-92C for twenty minutes, evaporated to 40-43% solids, homogenized, inoculated with 6% acidophilus culture, and incubated at 40-42C to desired acidity. It is then dried with air not exceeding 135C. The product contained 26% fat, 28% protein, 34% lactose, and 1.8% lactic acid. When packed in hermetically sealed containers, the product remained biologically active for more than two years.

Aco-yogurt, a type of yogurt with special therapeutic value, was developed in Switzerland. It contains, in addition to the normal yogurt

flora, L. acidophilus. Siegenthaler et al. (55) successfully treated children suffering from para-typhoid and coli-infections by feeding Aco-yogurt. L. acidophilus was readily implanted in the intestines, and a reduction of pathogenic bacteria in the intestines followed. This product was digestible and palatable to both sick and healthy children. An intestinal strain of L. acidophilus made resistant to antibiotics was used in this product.

In Czechoslovakia, concentrates of pure cultures of L. acidophilus, prepared by freeze-drying, sometimes with the addition of B vitamins and other lactic acid bacteria, are used for therapeutic purposes. They are taken either as such or used at a rate of 0.1% for the preparation of cultured milks in hospitals and clinics (60).

// In addition to the therapeutic benefits of L. acidophilus in treating gastro-intestinal disorders, there is evidence that ingestion of L. acidophilus also helps the skin maintain a smooth complexion. Gillespie et al. (12) studied the effect of the ingestion of large numbers of L. acidophilus cells on the lesions of acne vulgaris and reported that approximately 81% of the test subjects improved and maintained improvement of this condition. Approximately 70 billion viable cells were administered twice daily, five days per week in the form of sweetened and flavored acidophilus milk.

Similarly, feeding of L. acidophilus has been reported to be effective in treating domestic animals suffering from various gastro-intestinal disorders (42, 51, 66).

In the previously cited references, it has been clearly indicated that microbial imbalance in the intestinal tract is the underlying cause

in each disorder mentioned above; L. acidophilus therapy can be successfully used to treat gastro-intestinal disorders caused by microbial imbalance and to maintain a proper microbial balance in the intestinal tract.

C. Mechanism of Lactobacillus Acidophilus Action

L. acidophilus exerts antagonistic action toward the growth of a variety of enteric pathogens. It has been reported to produce antibiotic substances (22, 52, 67). These have been called by various names including lactocidin, acidolin, and acidophilin. Strains of L. acidophilus vary in their ability to produce these substances, and cultural conditions influence the amount produced within the strains. In vitro inhibitory activity has been reported against Salmonellae, Shigellae, Staphylococci, Proteus, Klebsiella, pseudomonads, enteropathogenic E. coli, bacilli, and Vibrio organisms. Gilliland and Speck (13) have confirmed these findings and showed that the antibacterial action produced by L. acidophilus is probably due to a combination of factors including acid, hydrogen peroxide, and inhibitory substances including the antibiotic substances mentioned above. In vivo studies with human medical and intestinal surgery patients are needed to determine if these antibiotic substances benefit intestinal health.

Studies (64, 65) have shown that L. acidophilus exerted an inhibitory effect on Mycobacterium tuberculosis. Their investigations have revealed that both acidophilus milk and whey in vivo had an inhibitory effect on M. tuberculosis.

Literature (50) has indicated that bile acids produced in the liver aid in digestion. Deconjugated bile acids are more inhibitory

for bacteria than the conjugated acids and deconjugation is one of the main microbial reactions which alter bile acids. The small intestine contains little free bile acids but significant quantities appear in the large bowel.

Gilliland and Speck (14) have tested human fecal lactobacilli including L. acidophilus for deconjugation of bile substances. L. acidophilus isolates that were tested all deconjugated taurocholate, a bile substance. Thus L. acidophilus can liberate free bile acids in the intestinal tract and thereby could exert an influence on the balance of bacteria present in the intestinal tract.

D. Acidophilus Milk Products

In spite of the beneficial roles ascribed to the ingestion of L. acidophilus, little has been done to include this bacterium as a dietary component, particularly in the U.S.A. (56).

For many years L. acidophilus was available to the consumer in the form of cultured acidophilus milk. This product, however, had an unpleasant taste and was not accepted well except for an urgent need to correct an intestinal malady (51, 56).

Acidophilus milk is prepared by steaming pasteurized skim or low-fat milk for one hour, cooling steamed milk to 37C, and inoculating with 1-2% active culture of L. acidophilus followed by incubation at 37C until desired acidity is reached (10). The acidic flavor of this product renders it unpalatable.

Studies (30) on the viability of L. acidophilus in acidophilus milk indicated that when stored at 5C, acidophilus milk, with an acidity

not more than 0.65%, will maintain satisfactory viability of the culture. It was also emphasized that milk must be of high quality and foreign bacteria remain in minimum number to produce acidophilus milk of high quality.

To overcome the unsavory quality of regular acidophilus milk, other types of products were developed.

There are a number of products containing L. acidophilus that are dispensed as pharmaceuticals. These often are dried preparations. Also, there are a number of products available in health food stores, but viable lactobacilli in these products vary considerably and the cost is relatively high (51, 56).

In 1931, Myers (41) described an unfermented acidophilus milk which had the taste of fresh whole milk. The product had high numbers of viable L. acidophilus suspended in it. The culture was grown in sterile medium, cells were harvested, and added to pasteurized milk to obtain a population comparable to that in regular acidophilus milk. He also conducted feeding experiments on human subjects and showed that L. acidophilus in this unfermented milk were implantable in the intestinal tract, as measured by the appearance of the L. acidophilus in fecal samples. However, there is no record that this milk was ever used on a commercial scale.

In succeeding years, there were major developments in knowledge on how to preserve bacterial cells, particularly by the use of drying, freeze-drying, and storage in the frozen state. In 1959, Duggan et al. (8) reported on a frozen concentrate of L. acidophilus for preparation of a palatable acidophilus milk. In this study, procedures were

described wherein L. acidophilus was grown, harvested, and stored as frozen concentrates of these cells. Such concentrates maintained satisfactory viability of the cells. Frozen concentrates containing the daily requirement of viable cells were prepared and stored in five to ten millileter quantities. These researchers extended the concept of Myers (41) and indicated that the distribution of frozen concentrates to consumers would provide a means for them to obtain L. acidophilus for therapeutic use in palatable form. They recommended that the concentrates in five to ten millileter quantities be thawed and added to 0.47 or 0.95 liters of whole milk before consumption. However, neither of the two foregoing acidophilus products is known to have received any widespread marketability.

At North Carolina State University, researchers have developed procedures whereby frozen concentrated cultures of L. acidophilus could be produced in a commercial starter culture plant and distributed to milk processing plants for the preparation of unfermented acidophilus milk. They have developed a new "Sweet Acidophilus" low-fat milk which is the only product containing the beneficial L. acidophilus as a dietary component available to the American public. Sweet Acidophilus milk is prepared in a manner to contain four to six million viable cells per milliliter. A human intestinal strain of L. acidophilus is used in this preparation. This product is palatable and tastes just like regular pasteurized cold milk. Consumption of this milk also resulted in the implantation of acidophilus organisms in the intestinal tract (1, 2, 16, 43, 57).

Since its introduction into the market in 1975, Sweet Acidophilus milk has gained favorable acceptance in some areas where it is marketed through vigorous advertisement. However, in other areas it has not been successful. It appears that some dairies have taken this product off the shelves since they did not have enough market for the product.

Fruit flavored cultured milks are available in some European countries. Well known is the fact that the per capita consumption of cultured products is higher in European countries than in the U.S. In certain European countries, the flavored cultured milk beverages are common. However, flavored cultured milks are relatively unknown to the American public (26, 51, 56). In the U.S., the rapidly expanding yogurt sales is good evidence that a flavored cultured dairy product may successfully be merchandised.

Researchers (26, 27) at Michigan State University have successfully developed flavored cultured dairy products and have shown that flavoring of cultured dairy products, such as buttermilk, whipped cultured cream, and cultured milk dessert provide increased sales potential for marketing cultured milk products.

Lack of market for the Sweet Acidophilus milk in some areas offers an important challenge for developing a flavored acidophilus milk beverage which in turn could add to the variety of the present beneficial acidophilus preparations.

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF CULTURES

Lactobacillus acidophilus 4356 and L. acidophilus 4357 strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD. These two strains were of human origin.

L. acidophilus K and L. acidophilus 75 strains isolated from Sweet Acidophilus milk and raw milk respectively by Dr. Woodrow Overcast, Department of Food Technology and Science at the University of Tennessee, Knoxville, were from the culture collection in the Food Technology and Science Department of the University of Tennessee, Knoxville.

II. MAINTENANCE OF CULTURES

All four strains were inoculated into deMan, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) and incubated at $36 \pm 1^\circ\text{C}$ for 48 hours. Growth from MRS broth tubes (with screw caps) was transferred to MRS agar (MRS broth plus 1.5% agar) slants; colonies from MRS agar slants after 48 hours of incubation were picked into MRS broth tubes. After at least three successive transfers through MRS broth and MRS agar culture tubes, characterization experiments were made using the isolated organisms. Active cultures of all strains were routinely propagated and maintained in MRS broth (7). All cultures were stored at 4°C .

III. CHARACTERIZATION OF CULTURES

A. Colony Morphology

One millileter of MRS broth cultures of each strain was used to make dilutions with phosphate buffered water in dilution blanks. Samples from these dilutions were plated by the pour plate method with MRS agar, an overlay of MRS agar poured into the plates and incubated at $36 \pm 1^\circ\text{C}$ for 48-72 hours (15). Colony morphology was studied using a Spenser dissecting microscope.

B. Cellular Morphology

Cellular morphology was determined by examining, with a microscope, gram-stained films of the cultures. Colonies were picked from MRS agar slants after 36 hours of incubation and gram-stained employing the Burke modification (24).

C. Catalase Test

A few drops of 30% hydrogen peroxide were transferred to a spot plate and vigorous growth of all strains from MRS agar slants was added with an inoculating loop. Cultures were considered catalase negative if no visible gassing was observed (24).

D. Motility Test

Hanging-drop preparations of 24 hour MRS agar cultures were examined for motility using the high-dry objective (24).

E. Physiological Tests

Growth Temperatures

The ability of four strains to grow at 15C and 45C was determined by using accurately controlled (± 1 C) water baths (E.H. Sargent and Co., Chicago). One to two loopfuls of inoculum from MRS broth cultures were added to MRS broth tubes and observed for growth at both temperatures over a period of seven days incubation. All strains were tested in duplicate (6).

Production of Gas from Glucose

Gibson's medium with manganese sulfate (MnSO_4 —0.4% w/v) was used to determine the ability of cultures to produce gas from glucose. This medium, consisting of yeast extract (0.3125 grams), D-glucose (6.25 grams), MnSO_4 (1.25 ml of 0.4% w/v solution), skim milk (100 ml), and nutrient agar (25 ml), was dispensed into screw-capped test tubes and sterilized in a steamer for 30 minutes on each of the three successive days. These were cooled to 37C, inoculated with 0.5 ml of MRS broth cultures of all strains and allowed to solidify. An overlay of four ml of non-nutrient agar was added to each tube, and allowed to solidify. After addition of three ml of sterile water, tubes in duplicate for each strain along with the control tubes were incubated at 36 ± 1 C for seven days. Gas production was noted by cracks or disturbance of the agar (24).

Ammonia from Arginine

Ability of cultures to produce ammonia (NH_3) from arginine was determined by using MRS arginine broth: 0.3% w/v of L-arginine

monohydrochloride was added to MRS broth and sterilized by autoclaving for 15 minutes at 121°C. One ml of inoculum of all strains from MRS broth cultures was added to MRS arginine broth. Nessler's solution was used to test for the presence of ammonia after seven days of incubation at $36 \pm 1^\circ\text{C}$ (24).

Acidity in Milk

Seven milliliters of sterile skim milk in screw-capped test tubes was inoculated by loop with 48 hour growth of all strains from MRS agar slants and incubated at $36 \pm 1^\circ\text{C}$ for two weeks. All strains were tested in duplicate for percent acidity. The contents of the tubes were washed into Erlenmeyer flasks with the aid of a stirring rod and titrated with N/10 sodium hydroxide (NaOH), with phenolphthalein as the indicator. Percent acidity produced by the cultures was calculated using the formula:

$$\text{Lactic Acid (\%)} = \frac{(\text{ml NaOH}) (\text{N NaOH}) (\text{meq. wt. lactic acid}) (100)}{\text{ml sample}}$$

and acidity was reported as percent lactic acid produced by the strains (49, 24).

Carbohydrate Fermentations

MRS fermentation medium (7) was used to study the carbohydrate fermentations of the cultures. This medium was prepared by omitting the glucose and meat extract from the usual MRS broth. MRS fermentation medium was prepared as follows:

Peptone	10 g
Yeast extract	5 g
Tween-80	1 ml
Ammonium citrate	2 g
Dipotassium phosphate	2 g

Sodium acetate	5 g
Magnesium sulfate	200 mg
Manganese sulfate	50 mg.

All the ingredients were added to one liter of distilled water, stirred and steamed for 30 minutes. Bromcresol purple indicator was added to this medium at 0.1% (v/v), dispensed into screw-capped test tubes and fermentation vials with cotton plugs, and sterilized by autoclaving at 121C for 15 minutes. A 10% solution (w/v) was made of each of the following sugars: mannitol, melizitose, sorbitol, inositol, cellobiose, salicin, sucrose, raffinose, melibiose, lactose, trehalose, and dextrin. These sugars were sterilized by autoclaving at 121C for 15 minutes. Filter sterilization, using Nalgene Filter Units (Nalge Sybron Corporation, Rochester, NY), was applied to the 10% (w/v) solutions of each of the following sugars (49): galactose, maltose, rhamnose, sorbose, arabinose, xylose, and fructose. Sterility of the filter-sterilized sugar solutions was checked by pouring the plates with standard plate count agar. These plates in duplicate were incubated at 32C for 48 hours. No colonies or growth on the plates indicated sterility of the sugar solutions. All the sugar solutions were added to the fermentation medium to give a final concentration of 0.2% (w/v) of each sugar (7, 68). Aseptic precautions were followed in every case throughout the work. All the tubes with fermentation medium plus sugar substrates were inoculated with one to two drops of inoculum of each culture (42 hour growth) from MRS broth tubes. Sterile Pasteur pipettes were used to dispense the inoculum into the tubes. All the tests in duplicate along with the controls were incubated 36 ± 1 C for seven days. Not only was the color noted, but

pH in each case was also determined using a pH meter (Fisher Accumet Model 230A).

Bile Salt Tolerance

MRS broth was used as the basal medium to check for the tolerance of the bile salt, sodium taurocholate, by the cultures (53). Two and four percent (w/v) of sodium taurocholate was added to MRS broth, dispensed into screw-capped test tubes and sterilized in an autoclave at 121C for 15 minutes. These tubes were inoculated with two to three drops of active MRS broth cultures using sterile Pasteur pipettes, and incubated at 36 ± 1 C for seven days. Tolerance was recorded by the presence or absence of turbidity, indicating growth.

Sodium Chloride (NaCl) Tolerance

Tests for NaCl tolerance were carried out in exactly the same manner as those for bile salt tolerance, except that two and four percent NaCl (w/v) was substituted for sodium taurocholate.

IV. PREPARATION OF MOTHER CULTURES

Skim milk in required quantities was obtained from the University of Tennessee Creamery, dispensed in 100 ml quantities into screw-capped bottles, and sterilized by autoclaving at 121C for 12 minutes. The skim milk was then cooled to 95F and inoculated with 2% inoculum of each strain from MRS broth culture. Inoculated skim milk was incubated at 36 ± 1 C for 48 hours. Mother cultures after incubation were stored in refrigerated water at 4C.

V. PREPARATION OF ACIDOPHILUS MILK

Low-fat milk, prepared by mixing equal parts of pasteurized skim milk and whole milk in Erlenmyer flasks, was used to prepare acidophilus milk. After steaming for one hour, low-fat milk was cooled to 95F, inoculated with 5% mother cultures of each strain and incubated at $36 \pm 1^{\circ}\text{C}$ until a nominal acidity of 0.9% was obtained. Control flasks for each sample were also incubated the same way and were used to determine the percent acidity produced in each acidophilus milk. A ten milliliter sample from each control flask was taken into an Erlenmyer flask with the help of sterile pipettes and titrated with N/10 NaOH, with phenolphthalein as the indicator. The percent acidity produced in each sample was calculated and expressed as percent lactic acid. After the optimum acidity was obtained, samples were removed to the cooler, and stored in refrigerated water.

VI. FLAVORING OF ACIDOPHILUS MILK

Acidophilus milk for flavoring was prepared in 500 ml quantities using 5% inocula of the four mother cultures. A panel of four expert members and four members with previous dairy products evaluation experience evaluated the body and texture of acidophilus milk prepared with the cultures of L. acidophilus 4356, 4357, -K, and -75 strains. L. acidophilus 75 was selected as the most suitable strain for preparing acidophilus milk with good body and texture.

Flavored acidophilus milk beverages were prepared with five different synthetic flavorings (Warner-Jenkins). The flavorings were: natural cloudy lemon-lime flavoring, WONF (with other natural flavors);

natural and artificial wild strawberry flavoring; natural and artificial raspberry flavoring; natural and artificial wild cherry flavoring; and natural fresh orange juice flavoring, WONF.

After reaching a 0.9% acidity, acidophilus milk in 500 ml quantities was transferred into blender jars (Waring Blender with Variac Speed Control); flavorings, sugar and salt were added at appropriate proportions, and the contents were blended at low speed for four minutes. Blending at low speed for four minutes resulted in a satisfactory pourability of the beverage. The flavorings, sugar and salt were added to the acidophilus milk in the following proportions:

Sugar	10.0% w/v
Salt	0.2% w/v
Flavorings:	
Strawberry	0.2% v/v
Raspberry	0.2% v/v
Cherry	0.04% v/v
Orange juice	0.2% v/v
Lemon-lime	0.2% v/v

Immediately after blending, samples with each flavoring were transferred to the cooler and stored in refrigerated water. No colorants were added.

VII. SENSORY EVALUATION

Sensory evaluation of the flavored acidophilus milk beverages was done by a consumer panel of 23 members. Panelists evaluated the samples under red lighting using Hedonic Scale score sheets in the Sensory Evaluation Laboratory of the Food Technology and Science Department at the University of Tennessee. The panelists evaluated the samples both in the morning and afternoon. Panelists were served cold (40-45F) samples of all five flavored acidophilus milk beverages. Numerical

values of one to nine were assigned to the "like extremely" to "dislike extremely" points of the Hedonic scale respectively (32).

VIII. STATISTICAL ANALYSES

Numerical values of each point that the panelists scored for five flavored products in three replications were used as the data for statistical analyses. Analysis of variance (ANOVA) was used to determine the significance of the flavors, replications and the panelists, and Duncan's Multiple Range Test was used to determine the significance of differences among the flavors, replications and panelists (32).

CHAPTER IV

RESULTS AND DISCUSSION

Results from the characterization studies on four strains of Lactobacillus acidophilus, ATCC 4356, ATCC 4357, K, and 75, are presented in Tables I and II. Results presented in Table I conform to the typical colony and cellular morphology of L. acidophilus described in Bergey's (47). All the cultures were facultatively anaerobic, non-motile, and catalase negative.

As can be seen from Table II, the cultures neither produced gas from glucose, nor ammonia from arginine indicating the typical homogermentative characteristic of L. acidophilus. All the cultures grew at 45C but not at 15C. These characteristics are typical of L. acidophilus. Except for L. acidophilus 75, percent acidity in milk produced by all the cultures agrees well with the normal range of a 0.3-1.9% acidity of authentic L. acidophilus cultures (47). Ability of strain 75 to produce a higher amount of acidity than other cultures could only be accounted for as its characteristic.

Ability of the cultures to ferment various carbohydrates is shown in Table II. Nineteen carbohydrates commonly employed by researchers (47, 48, 49, 54, and 68) in the characterization of L. acidophilus were used at a final concentration of 0.2% (w/v) in studying the fermentation reactions; generally, clear-cut results were obtained at such reduced carbohydrate concentrations. Esculin, glucose, inulin, dulcitol, mannose, and amygdalin substrates were not tested; DNA base composition

TABLE I
COLONY AND CELLULAR MORPHOLOGY OF L.
ACIDOPHILUS STRAINS

	STRAIN			
	4356	4357	K	75
Colony ¹ morphology	Colonies rough, irregular, with twisted or fuzzy filamentous projections	Colonies irregular, smooth to slightly rough, with fuzzy filamentous projections; colonies smaller than in 4356	Colonies rough, irregular, with fuzzy filamentous projections	Colonies irregular, with fuzzy filamentous projections
Cellular ² morphology	Gram + rods with rounded ends; mostly in singles and pairs; some in short and long chains, 0.7-0.8 by 3.2-5.6 um	Gram + rods with rounded ends; majority in pairs and short chains, 0.7-0.8 by 3.2-6.4 um	Gram + rods with rounded ends; majority in pairs and chains that look like slender filaments, 0.7-0.8 by 4.0-8.8 um	Gram + rods with rounded ends; in singles, pairs, and short chains, 0.7-0.8 by 3.2-5.6 um

1 = MRS agar pour plates after 48 hour incubation at 35C.

2 = Growth from MRS agar slants after 36 hour incubation at 35C.

TABLE II
PHYSIOLOGICAL CHARACTERISTICS OF FOUR
STRAINS OF L. ACIDOPHILUS

Characteristic	Strain ¹			
	4356	4357	-K	-75
Growth at 15C	-	-	-	-
Growth at 45C	+	+	+	+
Gas from Glucose	-	-	-	-
NH ₃ from arginine	-	-	-	-
Acidity in milk (%)	1.863	1.156	1.369	2.179
Fermentation of:				
arabinose	-(6.37)	-(6.35)	-(6.35)	-(6.35)
rhamnose	-(6.20)	-(6.20)	-(6.25)	-(6.30)
xylose	-(6.35)	-(6.35)	-(6.35)	-(6.35)
fructose	+(5.35)	+(5.35)	+(5.38)	+(5.40)
galactose	V	V	V	V
lactose	+(5.40)	+(5.15)	+(5.00)	+(5.10)
sucrose	+(5.00)	+(5.00)	+(4.98)	+(4.95)
maltose	+(5.35)	+(5.30)	+(5.26)	+(5.59)
trehalose	+(5.12)	+(5.12)	+(5.05)	+(5.08)
melibiose	-(6.25)	-(6.30)	-(6.30)	-(6.28)
cellobiose	+(5.62)	+(5.20)	+(5.37)	+(5.00)
raffinose	-(6.35)	+(5.50)	+(5.20)	-(6.30)
melizitose	-(6.32)	-(6.32)	-(6.25)	-(6.30)
dextrin	-(6.20)	-(6.17)	-(6.05)	-(6.20)
mannitol	-(6.40)	-(6.40)	-(6.40)	-(6.40)
sorbitol	-(6.37)	-(6.30)	-(6.25)	-(6.30)
inositol	-(6.30)	-(6.30)	-(6.30)	-(6.30)
salicin	+(5.25)	+(5.15)	+(5.25)	+(5.30)
esculin				
sorbose	-(6.20)	-(6.10)	-(6.17)	-(6.10)
Tolerance of:				
2% NaCl	+	+	+	+
4% NaCl	-	-	-	-
2% Bile*	+	+	+	+
4% Bile	+	+	+	+

1 "+" = positive results; "-" = negative results;
"V" = variable results; "()" = pH after growth in medium.

* Sodium taurocholate.

of the strains was not determined. Arabinose, rhamnose, xylose, lactose, fructose, sucrose, maltose, trehalose, cellobiose, melizitose, mannitol, sorbitol, salicin, and sorbose carbohydrates gave clear-cut results: all the strains fermented fructose, lactose, sucrose, maltose, trehalose, cellobiose, salicin, but not arabinose, rhamnose, xylose, melizitose, mannitol, sorbitol, and sorbose. These characteristics are consistent with those (Table III) of L. acidophilus as described by other investigators (47, 48, 49, 54, and 68). However, results obtained in the fermentation of galactose, melibiose, raffinose, dextrin, and inositol by the strains were different from those of other researchers presented in Table III. Fermentation of galactose by all four strains gave variable results, whereas Table III shows that galactose was always fermented by L. acidophilus, even when used at a concentration of 0.2% (w/v) in the basal medium (68). None of the cultures in this study fermented melibiose whereas the literature (Table III) has shown that strains of L. acidophilus are generally negative to weakly positive in fermenting this carbohydrate. L. acidophilus 4357 and K strains fermented raffinose, and 4356 and 75 strains did not ferment this substrate. This characteristic is in agreement with the literature (Table III) which indicates that strains of L. acidophilus vary in their ability to ferment this carbohydrate. None of the cultures fermented either dextrin or inositol; however, literature indicates in Table III that dextrin may or may not be fermented and inositol may very rarely be fermented by strains of L. acidophilus.

All strains grew in 2% and 4% bile salt, and 2% NaCl but not in 4% NaCl, whereas Wheater (68) has shown that L. acidophilus does not

TABLE III

CHARACTERISTICS OF *L. ACIDOPHILUS*: DATA TAKEN
FROM OTHER RESEARCHERS

Characteristic	Researcher				
	Rogosa ₁ et al.	Rogosa & 2 Sharpe	Wheater ³	Sharpe & 4 Mattick	Bergey's ₅ Manual
Growth at 15C	-	-		-	-
Growth at 45C	+	+	+(86%)	+	+
Growth at 48C			-		
Gas from glucose	-	-			-
NH ₃ from arginine	-	-			-
Acidity in milk (%)	+s 0.3-1.8	0.3-1.0			0.3-1.9
Fermentation of:					
arabinose	-	-	-		-
rhamnose	-	-	-	-	-
xylose	-	-	-		-
fructose	+		+		+
galactose	+	+	+		+
lactose	+	+	+		+
sucrose	+	+	+		+
maltose	+	+	+	+	+
trehalose	+	+	+(89%)	+	+
melibiose	- or +s	+	+(72%)		+
cellobiose	+	+	+(62%)	+	d
raffinose	+s or -	+	+(62%)		d
melizitose	-	-	-	-	-
dextrin		w	+(83%)	+	(-)

TABLE III (continued)

Characteristic	Researcher				
	Rogosa ₁ et al.	Rogosa & ₂ Sharpe	Wheater ³	Sharpe & ₄ Mattick	Bergey's ₅ Manual
mannitol	-	-	-	-	-
sorbitol	-	-	-	-	-
inositol	-	-	-	-	(-)
salicin	+	+	+	+	+
esculin	-	+	-	+	+
sorbose	-	-	-	-	-
Tolerance of:					
2% NaCl			+	+	
2% bile			+(83%)	+	

¹Rogosa et al. (49). +s = fermented or coagulated slowly.

²Rogosa and Sharpe (48). + = great majority of strains negative, sometimes weak or late positive; w = may be weakly fermented.

³Wheater (68). () = percentage of strains giving the specified reaction.

⁴Sharpe and Mattick (54). + = usually positive, but may sometimes be negative.

⁵Bergey's Manual (47). d = some strains positive (about 89-11% positive); (-) = very rarely fermented.

tolerate 4% bile salt. However, the bile salt and NaCl tolerance tests are neither mentioned in Bergey's Manual (47), nor in the authentic description (mentioned earlier in Chapter II, page 5) of L. acidophilus given by Hansen and Mocquot (23).

Speck (57) indicated that L. acidophilus from human feces does possess the ability to grow in the presence of bile salts, whereas those that are not from human feces do not possess this cultural characteristic. However, from the present study it is evident that L. acidophilus 75, which came from raw milk, does possess the ability to grow in the presence of bile salts. Characteristics of this strain conform to the characteristics of three authentic strains used in this study as well as the strains of L. acidophilus from various sources as characterized by other researchers. Because of its identity as L. acidophilus and its ability to grow in the presence of bile salts, it is reasonable to assume that it should be included in the dietary preparations for implantation in the intestinal tract.

In the characterization of strains of L. acidophilus, although a variety of media and techniques were employed, researchers (Table III) obtained generally consistent results, except for some variations which are now known to occur among the strains of L. acidophilus from not only the different habitats but also from identical sources (15). Nevertheless, the media they have used in the characterization have been reported to be deficient in certain nutrients needed to permit a good growth of certain strains of L. acidophilus (7) and thus may give variable results. MRS broth, MRS agar, and MRS basal fermentation media have now been widely employed in the characterization of L. acidophilus.

However, the characteristics of ATCC 4356, ATCC 4357 and other strains were not readily available. These strains along with L. acidophilus K, which came from a commercial sweet acidophilus milk product, were characterized using MRS medium which is an adequate and convenient medium for the characterization of Lactobacillus acidophilus.

Characteristics of the strains from different sources presented in this study might be of help to those interested in isolation and characterization of the strains.

Samples of acidophilus milk prepared with the mother cultures of four strains characterized in this study were presented to an expert panel for body and texture evaluation. Eight panelists evaluated the samples three times, each time samples having a slight variation in acidity produced by the cultures. Percent acidity of the samples evaluated for body and texture is shown in Table IV.

TABLE IV
PERCENT ACIDITY OF THE SAMPLES OF ACIDOPHILUS
MILK FOR BODY AND TEXTURE EVALUATION

Replication	% acidity of acidophilus milk with the cultures of:			
	4356	4357	K	75
1	0.666	0.252	0.990	0.792
2	0.702	0.252	1.278	0.720
3	0.945	0.375	0.975	0.975

In each evaluation, panelists selected strain 75 as producing a smooth body and texture. Strain 4357 always produced a low amount of acidity, had a weak body, and was rated poor in body and texture. Panelists, during evaluations, used buttermilk as the control for body

and texture. Panelists selected strain 75 as the most suitable for preparing flavored acidophilus milk beverage from its body and texture characteristic.

Methods and formulas of Hedrick and King (27) constituted the basis for the present study on flavored acidophilus milk beverages. They worked with cultured buttermilk. When necessary, methods and formulas used in preparing flavored acidophilus milk beverages were modified from those of Hedrick and King.

For preliminary trials, strawberry and raspberry flavored acidophilus milk beverages were prepared as described in Materials and Methods, and these samples were evaluated in the laboratory by staff and students. Strawberry flavored samples were liked very much and raspberry was rated good, but not liked as much as the strawberry sample. The proportions of flavoring, sugar and salt were considered satisfactory for preparing flavored acidophilus beverages. Blending at low speed for four minutes proved satisfactory for obtaining a medium viscosity. In this preliminary trial, the percent acidity of the samples was 1.008. Extensive preliminary storage tests were not conducted on the samples. However, a 400 ml quantity of the strawberry flavored sample was prepared and stored at 4C for one week. The sample kept well without any wheying-off or objectionable odors and the flavor was retained well in the sample. It was very acceptable during the entire storage period.

Lemon-lime, strawberry, raspberry, cherry, and orange juice flavored acidophilus milk beverages (culture 75) were prepared and a consumer panel of twenty-three members evaluated the samples separately.

using the Hedonic scale score sheets as described in Chapter III. The consumer panel evaluated the five flavored beverages in three replications. The percent acidity of the samples that the panelists received during three replications was 0.972, 1.134 and 0.945 respectively.

Standardization of amount of inoculum used, and incubation time and temperature did not significantly help in controlling the acidity produced by the cultures. This might have been, in part, due to the variation in the composition and/or quality of different batches of milk used to prepare the mother cultures and the acidophilus milk.

After panelists evaluated the five flavored samples, data was analyzed by analysis of variance. Results of the ANOVA are presented in Table V.

As indicated in Table V, there was a significant difference among the flavors at the 1% level of probability. Replications being non-significant indicated that the samples presented to the panelists in each replication did not differ significantly. This also indicated that the variation in the percent acidity of the samples in three replications was not a significant factor in flavor. Flavor-replication interaction was also non-significant. There was a significant difference among the panelists in scoring the samples, and the interaction between the flavors and panelists was also significant.

Since there was a significant difference among the flavors, Duncan's Multiple Range Test was conducted (Table VI) to determine the flavors that were different from each other. As indicated in Table VI, strawberry flavor was significantly better than orange, cherry,

TABLE V
ANALYSIS OF VARIANCE OF SENSORY
RESPONSES AMONG FIVE FLAVORED
ACIDOPHILUS MILK BEVERAGES

Source	d f	SS	MS	F
Flavors (F)	4	75.032	18.760	22.89**
Replications (R)	2	1.305	0.652	0.80 ^{ns}
F x R	8	6.377	0.797	0.97 ^{ns}
Panelists	22	638.389	29.012	35.42**
F x P	88	253.902	2.810	3.43**
Error	220	180.319	0.819	

** = significant at 0.01 level.

ns = non-significant.

TABLE VI
 SENSORY SCORES OF FIVE FLAVORED
 ACIDOPHILUS MILK BEVERAGES

Flavors	Mean ¹	
Raspberry	3.812+	a
Lemon-lime	3.580	a b
Cherry	3.536	a b
Orange	3.304	b
Strawberry	2.463	c

* = at 0.05 level of probability

¹ = means with the same letters
 are not significantly different

+ = Hedonic Scale for Sensory Evaluation

- 1 = Like extremely
- 2 = Like very much
- 3 = Like moderately
- 4 = Like slightly
- 5 = Neither like nor dislike
- 6 = Dislike slightly
- 7 = Dislike moderately
- 8 = Dislike very much
- 9 = Dislike extremely

lemon-lime, and raspberry flavors. Mean scores for five flavors, as presented in Table VI, also indicated that strawberry flavored acidophilus milk was liked very much to moderately, whereas orange, cherry, and lemon-lime flavored beverages were liked slightly to moderately. Strawberry flavored milk was liked the best of all the flavors. Approximately 43.5% of the panelists liked the strawberry flavored milk "extremely well" to "very much"; 43.5% of the panelists liked this flavored beverage "very much" to "moderately"; about 14% of the people liked this beverage "slightly" or below. Approximately 80% of the panelists indicated that this beverage had a pleasing texture and mouthfeel; excellent flavor and body; good and refreshing taste; fine flavor and aroma; smooth, light and good; whereas 20% of the panelists indicated that it had a slightly strong flavor; slight after-taste; slightly chalky. As indicated in Table VI, orange flavored beverage was ranked second by the panel. Cherry and lemon-lime flavors were ranked third and fourth among the flavors, and raspberry was the least preferred.

From this study, it is evident that strawberry flavored acidophilus milk beverage could easily be prepared and marketed. Through consumption of this product, the beneficial L. acidophilus can be ingested to maintain a normal microflora of the intestine and good health. Not only strawberry flavor but also several other flavoring possibilities could be tried. For calorie conscious people, artificial sweetener could be substituted for sugar. However, for optimum acceptance, the exact amounts of flavorings and sweeteners need to be established for the specific culture used and the consumer preference of

the areas served. Selection of a good culture that has a smooth body and texture cannot be overemphasized.

CHAPTER V

SUMMARY

The purpose of this investigation was two-fold: to characterize four strains of L. acidophilus from different origins, using MRS medium, and to select a suitable strain to develop flavored acidophilus milk beverages.

A strain of L. acidophilus isolated from raw milk, along with three authentic strains of L. acidophilus, was characterized using MRS broth, MRS agar, and MRS fermentation medium. The purpose of this characterization was to confirm the identity of strain 75 as L. acidophilus by comparing its characteristics with those of three authentic strains used in this study as well as the strains of L. acidophilus from various sources as characterized by other researchers. Characteristics of all four strains were presented together for the convenience of other researchers interested in characterization and comparison of strains of L. acidophilus. Data of other researchers was also included. It was also found that strain 75 possessed the ability to grow in the presence of bile salts (sodium taurocholate) which indicates that this strain would be implantable in the intestinal tract. MRS medium was found to be an adequate and convenient medium for characterization studies on L. acidophilus.

An expert panel selected strain 75 as producing a smooth body and texture for preparation of flavored acidophilus milk beverages. Using the strain 75, five artificially flavored acidophilus milk

beverages were prepared. A consumer panel evaluated the samples using Hedonic scale score sheets in three replications. Results of the statistical analyses indicated that strawberry flavored acidophilus milk was liked best, while orange flavor was liked second best. This study indicated that flavored acidophilus milk beverages can add to the variety of the presently available beneficial acidophilus preparations. With further investigations, this product could be refined and introduced into the commercial market.



LITERATURE CITED

LITERATURE CITED

1. Anon. 1976. Many dairies are selling new sweet acidophilus milk. Amer. Dairy Review 38(7):30.
2. Anon. 1976. Acidophilus milk introduced regionally. Dairy and Ice Cream Field 159(5):57.
3. Aritaki, S., and Ishikawa, S. 1962. Application of Lactobacillus acidophilus fermented milk in the pediatric field. Acta. Pediat. Jap. 66:811-815. Cited in Dairy Sci. Abst. 26:745.
4. Beck, C., and Necheles, H. 1961. Beneficial effects of administration of Lactobacillus acidophilus in diarrhea and other intestinal disorders. Amer. J. Gastroenterol. 35:522.
5. Cattani, R., and Milstein, J. 1960. The use of lactic preparations in therapy of intestinal disorder. Pr. Med. 68(25):951-953. Cited in Dairy Sci. Abst. 25:1698.
6. Davis, G.H.G. 1955. The classification of lactobacilli from the human mouth. J. Gen. Microbiol. 13:481.
7. DeMan, J.C., Rogosa, M., and Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130.
8. Duggan, D.E., Anderson, A.W., and Elliker, P.R. 1959. A frozen concentrate of Lactobacillus acidophilus for preparation of a palatable acidophilus milk. Food Technol. 13:465.
9. Fedotov, P.V., Zotova, A.B., and Buwich, P.E. 1966. Colonization of digestive tract of infants with acidophilus bacteria. Pediatriya, Mosk. 45:26-30. Cited in Dairy Sci. Abst. 29:225.
10. Foster, E.M., Nelson, F.E., Speck, M.L., Doetsch, R.N., and Olson, Jr., J.C. 1957. Dairy Microbiology. Prentice-Hall, Inc., Englewood Cliffs, NJ.
11. Garrod, L.P. 1955. Present position of the chemotherapy of bacterial infections. Brit. Med. J. 1955(II):756.
12. Gillespie, H.P., Potter, H.W., Lambelet, I.D., Schnable, L.D., and Poshko, E.P. 1963. Control of the lesions of acne vulgaris by the ingestion of viable Lactobacillus acidophilus cells. Bacteriol. Proc. 1963, p. 163.
13. Gilliland, S.E., and Speck, M.L. 1977. Antagonistic action of Lactobacillus acidophilus toward intestinal and foodborne pathogens in associative cultures. J. Food Protection 40:820.

14. Gilliland, S.E., and Speck, M.L. 1977. Deconjugation of bile acids by intestinal lactobacilli. *Appl. Environ. Microbiol.* 33:15.
15. Gilliland, S.E., Speck, M.L., and Morgan, C.G. 1975. Detection of Lactobacillus acidophilus in feces of humans, pigs, and chickens. *Appl. Microbiol.* 30:541.
16. Gilliland, S.E., Speck, M.L., Nauyok, Jr., G.E., and Geisbrecht, F.G. 1978. Influence of consuming non-fermented milk containing Lactobacillus acidophilus on fecal flora of healthy males. *J. Dairy Sci.* 61:1.
17. Gorbach, S.L. 1971. Intestinal microflora. *Gastroenterology* 60:1110.
18. Gorbach, S.L., Plaut, A.G., Nahas, L., and Weinstein, L. 1967. Studies of intestinal microflora. II. Microorganisms of the small intestine and their relation to oral and fecal flora. *Gastroenterology* 53:856.
19. Gordon, D., MacRae, J., and Wheeler, D.M. 1957. A lactobacillus preparation for use with antibiotics. *Lancet* 272:899.
20. Haenel, H. 1961. Some rules in the ecology of the intestinal microflora of man. *J. Appl. Bacteriol.* 24:242.
21. Haenel, H. 1970. Human normal and abnormal gastrointestinal flora. *Amer. J. Clin. Nutr.* 23:1433.
22. Hamdan, I.Y., and Mikolajcik, E.M. 1974. Acidolin: An antibiotic produced by Lactobacillus acidophilus. *J. Antibiotics* 27:631.
23. Hansen, P.A., and Møcquot, G. 1970. Lactobacillus acidophilus (MORO) comb. nov. *Internl. J. Syst. Bacteriol.* 20:325.
24. Harrigan, W.F., and McCance, M.E. 1976. Laboratory Methods in Food and Dairy Microbiology. Academic Press Inc., New York, NY.
25. Hawley, H.B., Shepherd, P.A., and Wheeler, D.M. 1959. Factors affecting the implantation of lactobacilli in the intestine. *J. Appl. Bacteriol.* 22:360.
26. Hedrick, T.I. 1970. Cultured buttermilk with added flavoring materials. *Cult. Dairy Prod. J.* 5(2):4.
27. Hedrick, T.I., and King, C. 1969. Flavored cultured dairy products. *Amer. Dairy Review* 31(6):14.
28. Holland, D.F. 1920. Generic index of the commoner forms of bacteria. *J. Bacteriol.* 5:215.

29. King, J.W., and Rettger, L.F. 1942. Gram-positive non-sporulating anaerobic rod-shaped bacteria of the intestinal tract. J. Bacteriol. 44:301.
30. Kulp, W.L. 1931. Studies on the viability of Lactobacillus acidophilus in "Acidophilus Milk." Amer. J. Pub. Health 21:873.
31. Lachner, O., and Bieler, A. 1961. Treatment of non-specific intestinal diseases with acidophilus preparation. Wien. Med. Wschr. 111(20):361. Cited in Dairy Sci. Abst. 25:153.
32. Larmond, E. 1970. Methods for Sensory Evaluation of Food. Bull. 1284, Canada Dept. of Agric., Ottawa, Canada.
33. Lerche, M., and Reuter, G. 1962. The presence of gram-positive rods of the genus Lactobacillus beijerinck in the intestine of adults. Zbl. Bakt. I. Orig. 185:446. Cited in Dairy Sci. Abst. 27:77.
34. Low, J. 1959. Treatment of intestinal disturbances in children with acidophilus zyma. Munch. Med. Wschr. 101:762-764. Cited in Dairy Sci. Abst. 22:1024.
35. MacBeth, W.A.A.G., Pass, E.H., and McDermott, Jr., W.V. 1965. Treatment of hepatic encephalopathy by alteration of intestinal flora with Lactobacillus acidophilus. Lancet 1:399.
36. Metchnikoff, E. 1908. The Prolongation of Life. 1st ed. G.P. Putnam's Sons, New York, NY.
37. Mironenko, A. 1958. Dried acidophilus milk for feeding and therapeutic purposes. Mol. Prom. 19:18-20. Cited in Dairy Sci. Abst. 21:321.
38. Moro, E. 1900a. Ueber die nach Gram farbbaren Bacillen des Sauglingsstuhles. Wienerklin. Wochenschr. 13:114-115 and 1900 b. Ueber den Bacillus acidophilus n. spec. Ein Beitrag zur Kenntniss der normalen Darmbakterien des Sauglings. Jahrb. Kinderheilk, Ser. 3, 52:38-55. In Hansen, P.A., and Mocquot, G. 1970. Lactobacillus acidophilus (MORO) comb. nov. Internl. J. Syst. Bacteriol. 20:325.
39. Moore, W.E.C., Cato, E.P., and Holdeman, L.V. 1969. Anaerobic bacteria of the gastrointestinal flora and their occurrence in clinical infections. J. Infect. Dis. 119:641.
40. Moore, W.E.C., Ryser, M., and Holdeman, L.V. 1975. Predominant kinds of bacteria in gastrointestinal contents. Abst. of the Annual Meeting of ASM, p. 61.

41. Myers, R.P. 1931. Transformation of the intestinal flora through feeding of unfermented acidophilus milk. *Amer. J. Pub. Health* 21:687.
42. Nedyalkov, S., Stoichev, S., and Iotov, I. 1967. Production and use of Lactobacillus acidophilus preparation. *Vet. Sбив. Sof.* 64:13-14. Cited in *Dairy Sci. Abst.* 30:2108.
43. Nielsen, V.H. 1976. Sweet acidophilus milk gains wide public acceptance. *Amer. Dairy Review* 38(10):40.
44. Rafsky, H.A., and Rafsky, J.C. 1955. Clinical and bacteriological studies of a new L. acidophilus concentrate in functional gastrointestinal disturbances. *Amer. J. Gastroenterol.* 24:87.
45. Read, A.E., McCarthy, C.F., Heaton, K.W., and Laidlaw, J. 1966. Lactobacillus acidophilus (Enpac) in treatment of hepatic encephalopathy. *Brit. Med. J.* 1:1267.
46. Robinson, E.L., and Thompson, W.L. 1952. Effect on weight gain of the addition of Lactobacillus acidophilus to the formula of newborn infants. *J. Pediatrics* 41:395.
47. Rogosa, M. 1974. Lactobacillus acidophilus (MORO) Hansen and Mocquot 1970, 326. In *Bergey's Manual of Determinative Bacteriology*. R.E. Buchanan and N.E. Gibbons, eds. 8th ed. The Williams & Wilkins Co., Baltimore, MD.
48. Rogosa, M., and Sharpe, M.E. 1959. An approach to the classification of the lactobacilli. *J. Appl. Bacteriol.* 22:329.
49. Rogosa, M., Wiseman, R.F., Mitchell, J.A., and Disraely, M.N. 1953. Species differentiation of oral lactobacilli from man including descriptions of Lactobacillus salivarius nov. spec. and Lactobacillus cellobiosus nov. spec. *J. Bacteriol.* 65:681.
50. Sandine, W.E. 1978. Roles of Lactobacillus in the intestinal tract. *J. Food Protection* 42:259.
51. Sandine, W.E., Muralidhara, K.S., Elliker, P.R., and England, D.C. 1972. Lactic acid bacteria in food and health: a review with special reference to enteropathogenic Escherichia coli as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. *J. Milk Food Technol.* 35:691.
52. Shahani, K.M., Vakil, J.R., and Kilara, A. 1977. Natural antibiotic activity of Lactobacillus acidophilus and bulgaricus. *Cult. Dairy Prod. J.* 12(2):8.
53. Sharpe, M.E. 1962. Taxonomy of the lactobacilli. *Dairy Sci. Abst.* 24:109.

54. Sharpe, M.E., and Mattick, A.T.R. 1957. The identification of Lactobacillus acidophilus. Milchwissenschaft 12:348.
55. Siegenthaler, E., Hawley, H.B., Schmid, F., and Berger, B. 1960. Acyoghurt. Schweiz. Milchztg. 86:537-544. Cited in Dairy Sci. Abst. 22:2470.
56. Speck, M.L. 1976. Interactions among lactobacilli and man. J. Dairy Sci. 59:338.
57. Speck, M.L. 1978. The development of sweet acidophilus milk. Dairy and Ice Cream Field 161(3):70A.
58. Speck, M.L. 1978. Enumeration of viable Lactobacillus acidophilus organisms in dairy products. J. Food Protection 41:135.
59. Stark, C.N., Gordon, R., Mauer, J.C., Curtis, L.R., and Schubert, J.H. 1934. Studies on acidophilus milk. Amer. J. Pub. Health 24:470.
60. Teply, M. 1961. Pure dairy cultures for therapeutic purposes. Ciste metekarske kultury lecebne Vyzive. Vyziva Lidu. 16:(6): 106-107. Cited in Dairy Sci. Abst. 23:3495.
61. Tomic-Karovic, K. 1963. Therapeutic effect of acidophilus milk—A ten year experience. Rad. Immunot. Zavoda. 1:45-48. Cited in Dairy Sci. Abst. 26:2666.
62. Tomic-Karovic, K., and Fanjek, J.J. 1962. Acidophilus milk in therapy of infantile diarrhea caused by pathogenic Escherichia coli. Ann. Pediat. 199:625-634. Cited in Dairy Sc. Abst. 25:1697.
63. Tomic-Karovic, K., Fischer-Sartorius, F., and Bijelic, A. 1961. Lactobacillus acidophilus in the therapy of diarrhea in infants. Neve. Ost. Z. Kinderheilk. 6(1):1-7. Cited in Dairy Sci. Abst. 24:1356.
64. Tomic-Karovic, K., Sudic, D., and Marjanovic, C. 1964. The effect of acidophilus whey in vitro and in vivo on M. tuberculosis. Cons. Acads. RSF Yougosl. 9(6):163-165. Cited in Dairy Sci. Abst. 27:2581.
65. Tomic-Karovic, K., Sudic, D., and Tomljenovic, C. 1959. Antibiotic action of L. acidophilus upon Mycobacterium tuberculosis.
66. Tortuero, F. 1973. Influence of the implantation of Lactobacillus acidophilus in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. Poultry Sci. 52:197.

67. Vincent, J.G., Veomett, R.C., and Riley, R.F. 1959. Antibacterial activity associated with Lactobacillus acidophilus. J. Bacteriol. 78:477.
68. Wheeler, D.M. 1955. The characteristics of Lactobacillus acidophilus and Lactobacillus bulgaricus. J. Gen. Microbiol. 12:123.

VITA

Velagaleti Lakshmana Charya was born on February 24, 1951, to Mr. and Mrs. Velagaleti Narasimha Acharya, in Mahabubabad, A.P., India. In 1967 he graduated from the Zilla Parishad Multi-Purpose High School, Mahabubabad, India. He began his undergraduate work at the Osmania University in 1968, and in 1970 graduated with a Bachelor of Science degree in Botany, Zoology, and Chemistry. For one year, 1972-1973, he served as a teacher in a primary school, Sri Vivekananda Vidyalaya, at his native place. In March 1973 he came to the United States and attended Valdosta State College, Valdosta, Georgia, where he graduated with a Bachelor of Science degree in Biology in December 1975. In March 1976 he began a graduate program in the Department of Food Technology and Science at the University of Tennessee, Knoxville. Requirements for the Master of Science degree were completed in June 1979. He is married to the former Kidambi Vijaya Lakshmi.