This is to certify that the

thesis entitled

Differential enumeration of viable microorganisms in yogurt products

presented by

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has been accepted towards fulfillment of the requirements for

Master degree in Food Science

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Date April 22, 1983

DATE DUE	DATE DUE	DATE DUE
FEB 0 5 1997		

DIFFERENTIAL ENUMERATION OF VIABLE MICROORGANISMS IN YOGURT PRODUCTS

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1983

ABSTRACT

DIFFERENTIAL ENUMERATION OF VIABLE MICROORGANISMS IN YOGURT PRODUCTS

By

Zain E.M. Saad

The techniques of Lee et al. (1974) and Davis et al. (1971) for differential enumeration of yogurt culture were evaluated and compared. The lactose, glucose, and galactose contents of commercial plain yogurt were determined by high performance liquid chromatography using Aminex HPX-87 cation exchange (calcium form) carbohydrate column.

To evaluate the effect of temperature, time, and pH on the survival rate of yogurt organisms, a series of heat treatments using a tubular heat exchanger (Laboratory Model Spiratherm) was run on a commercial bulk yogurt. A microbial analysis was done to determine the Streptococcus thermophilus to Lactobacillus bulgaricus ratio during storage for 21 days at 4.4^{o}C. Results indicate that Lee's technique is a reliable and easy method for differential enumeration of yogurt starter. Attempts to modify the chemical composition of Lee's agar and the incubation conditions were not fruitful. A decrease in lactose content and an increase in galactose content, while traces of glucose were observed in yogurt stored at 4.4^{o}C for 14 days. The change in carbohydrate content during

storage may be attributed to the growth of yogurt bacteria and residual lactase activity.

In yogurt with pH 4.29, 23.1% of the lactic acid bacteria survived after heat treatment at 65^{o}C, 12 seconds, whereas at pH 3.87, 99.99% of lactic acid bacteria were inactivated under these conditions. These data are not in agreement with other literature. A significant decrease of physical consistency was observed after heat treatment at 70^{o}C, 75^{o}C, and 80^{o}C.

The increase in titratable acidity resulted in shifting the coccus:rod ratio in yogurt stored at 4.4^{o}C for 21 days. The desirable ratio was achieved after 9 days of storage.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude and appreciation to Dr. C.M. Stine for his guidance, encouragement, and help during the course of this study.

Grateful acknowledgment is also expressed to Dr. H.A. Lillevik, Dr. M. Uebersax, and Mr. S. Thompson for their assistance, advice, and effort in reading this manuscript.

Thanks are also expressed to Dr. M.L. Richmond and Mr. J. Partridge for their assistance and advice.

The author feels grateful to his parents and his brother Fathi for their encouragement and moral support.

Very special gratitude is also due to the author's wife Safaa for her devotion, encouragement, patience, and understanding.

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INTRODUCTION

Traditionally, commercial yogurt has been made by incorporating living microorganisms (Streptococcus thermophilus and Lactobacillus bulgaricus) into a milk base with viable bacteria remaining in the final product. Although there have been claims that there is a therapeutic effect from ingestion of yogurt bacteria (which must survive the digestive process and be able to grow in the intestinal tract), clinical research is needed to clarify and substantiate such claims. Yogurt is often consumed with the assumption that there are benefits to be derived from living culture organisms in the product. Many consumers have always expected large numbers of viable bacteria in yogurt. The recently proposed Food and Drug Administration (FDA) definitions and standards for yogurt (Anonymous, 1977) describe yogurt as a product that contains the lactic acid-producing bacteria L. bulgaricus and S. thermophilus. No mention was made of either a method to determine viable organisms or numbers of organisms one might expect to find in fresh yogurt. Rasic and Kurmann (1978) described the methods available to enumerate viable microflora in yogurt. Furthermore they recommended that yogurt should have about $2x10^8$ to $1x10^9$ viable cells per ml of fresh yogurt. The

draft standards of Food & Agriculture Organization (FAO)/ World Health Organization (WHO) for yogurt (Standard No. A-11a, step 7, 1977), indicate that the final product must have "viable and abundant" L. bulgaricus and S. thermophilus present. Many investigators claim that the two species should be present in about equal numbers in the culture in order to obtain optimum consistency, flavor, and odor. Because of the emphasis on the maintenance of a balance between coccus and rod, there is a need for techniques to determine the relative proportions of S. thermophilus and L. bulgaricus. A microscopic examination to determine the ratio of coccus to rod is inadequate because dead cells can not be distinguished from viable ones. Davis et al. (1971) described a double pour plate technique that enabled both organisms to grow in an enriched medium called Living lactic Acid Bacteria (L.A.B.) and then differentiated the cells by colony appearance using a low power binocular microscope. Porubcan and Sellars (1973) described a medium (HYA agar) in which both organisms grow and the differentiation is achieved by adding an appropriate sugar or sugar mixture to the molten agar base before plating. Lee et al. (1974) described another technique for the differential enumeration of yogurt starter bacteria based on acid-producing activity. Shankar and Davies (1977) used the inhibitory effect of β-glycerophosphate to suppress the growth of L. bulgaricus and to selectively isolate and enumerate

S. thermophilus while Johns et al. (1978) used reinforced clostridial agar to allow selective isolation of L. bulgaricus. Kroger (1973) discussed the quality of yogurt in the United States and also reported there was much confusion about legal standards, including microbial content. Robinson and Tamime (1976) reported on the need for standard methods on analysis for this product, including a concern for microbiological examination. Because of confusion regarding the present microbiological standards for fresh plain yogurt, the concern from manufacturers, consumers, and investigators in the field that yogurt should contain viable and abundant bacteria, and the emphasis on maintaining a desirable ratio of coccus to rod, this project was undertaken to evaluate, compare and/or modify procedures of Davis et al. (1971); Lee et al. (1974) for enumeration of yogurt organisms. A series of heat treatments was run on a commercial product to determine the temperature and time relationships in which yogurt organisms can survive, with the goal of also eliminating undesirable contaminants. The ratio of S. thermophilus to L. bulgaricus during storage of commercial products in relation to the rate of acid development was determined. Finally, carbohydrate analyses were made to determine the concentration of sugars during storage of commercial products.

REVIEW OF THE LITERATURE

Historical

Fermented milks came into use many centuries ago, although there is no precise record of the date when they were first made. One legend tells that an angel brought down the pot which contained the first yogurt or leben, while another claims that the ancient Turks, who were Buddhists, used to offer yogurt to the angels and stars who protected them (Krunitz, 1803). The ancient Greeks and Romans were also acquainted with the preparation of soured milks (Walther, 1969). According to various sources yogurt originated in Asia, where the ancient Turks lived as nomads. In the 8th century, the first Turkish name appeared as "yo-gurut" and was changed in the 11th century to its present form. A dried type of yogurt was called "Kurut" and a beverage type "Suvuk yoghurt" (Izmen, 1935). Other data mention Leben of the ancient Arabs or Yoghurt of the Turks, as fermented milks which may become too sour during storage and subsequent drying (Krinitz, 1803). According to other authors, yogurt originated in the Balkans. The inhabitants of Thrace, known for breeding large flocks of sheep, used to

make soured milks called "Prokish" which later became yogurt (Chomacov, 1973).

Originally yogurt was made mainly from sheep, buffalo milk and partly from goat's and cow's milk held or stored in containers of wood or argil. Propagation was carried out by using a small quantity of the previously coagulated milk to seed the next batch of boiled milk. Yogurt was used primarily in the human diet for direct consumption, either as a natural product or fortified with various vegetables and spices, but also for cooking and baking purposes. Yogurt has been prescribed by the ancient physicians of the Near and Middle East for curing disorders of the stomach, intestines and liver and for stimulating the appetite. There are also records of the use of yogurt for the preservation of meat against spoilage during the summer (Kern, 1912). In the early part of the 20th century, Metchnikoff (1907) in his "theory of longevity" noted the beneficial effect of yogurt in the human diet. Although this theory exaggerated the value of yogurt, it significantly influenced the spread of the product to many countries of Europe and promoted extensive studies by subsequent workers. Since 1950, the technology of yogurt and understanding of the factors affecting its organoleptic properties advanced rapidly. The use of pure cultures enabled the manufacture of cultured products and the application of modern equipment has resulted in continuous

processes. Also the use of fruit and flavorings in yogurt has made the product more acceptable to many consumers. More recent research has contributed to a better understanding of some of the beneficial effects of ingestion of such fermented foods and new criteria have been introduced for culture selection.

Technology

The definition of yogurt which was derived from the research of Metchnikoff (1907), who studied the "Yahourth" or fermented milk in Bulgaria and adjoining countries. He ascribed the valuable properties of this food to the presence of a lactic acid organism which he named Lactobacillus (or Bacillus) bulgaricus; this is also called "Massol's bacillus" after one of his collaborators. Traditionally, yogurt may be regarded as milk, heated with or without concentration and soured by an organism producing a large amount of lactic acid and practically nothing else. This definition includes all soured milk, including cheese starter, so that more qualification is required. Special attention has been directed towards flavor aspects and control of growth of Streptococcus thermophilus, which plays an important role in modern yogurt, along with Lactobacillus bulgaricus (Davis, 1971). In recent years, much attention has been focused on the problem of defining yogurt.

Until recently there has been no legal definition for yogurt, but proposals have been made to define it. According to Food and Drug Administration (FDA , 1977), yogurt is described as "the food produced by culturing one or more of the optional dairy ingredients such as cream, milk, partially skimmed milk, or skim milk, with a characterizing bacterial culture that contains the lactic-acid-producing bacteria, Lactobacillus bulgaricus and Streptococcus thermophilus". One or more of the other optional ingredients may also be added such as:

1. Concentrated skim milk, nonfat dry milk, or other milk-derived ingredients to increase the nonfat solids content of the food, provided that the ratio of protein to total nonfat solids of the food and the protein efficiency ratio of all protein present, shall not be decreased as a result of adding such ingredients.
2. Nutritive carbohydrate sweeteners.
3. Characterizing flavoring ingredients.
4. Coloring.
5. Stabilizers.

All ingredients used are safe and suitable. Yogurt contains not less than 3.25 percent milk fat and not less than 8.25 percent milk solids not fat, and has a titratable acidity of not less than 0.5 percent, expressed as lactic acid. All dairy ingredients used shall be pasteurized or

ultra-pasteurized and may be homogenized. Yogurt may be heat-treated, after culturing is completed, to destroy viable microorganisms. Low fat yogurt may contain not less than 0.5 percent nor more than 2.0 percent milk fat and not less than 8.25 percent milk solids not fat, and has a titratable acidity of not less than 0.5 percent, expressed as lactic acid (Federal Register, 1977). Thus, if this definition is accepted, the organisms which can be used as a starter for yogurt production are Streptococcus thermophilus and Lactobacillus bulgaricus. The characteristics of L. bulgaricus and S. thermophilus are presented in Table 1 and Table 2.

Symbiosis

It is well established that Lactobacillus bulgaricus and Streptococcus thermophilus stimulate each other during their associative growth. Acid production was more rapid when mixed cultures of L. bulgaricus and S. thermophilus were inoculated in milk than when single strains were used (Pette & Lolkema, 1950a). Pette & Lolkema also (1950b) further reported that the more rapid acid production by such cultures was attributable to water-soluble, heat stable growth factors for the streptococcus produced by L. bulgaricus. They also indicated that these factors were amino acids and that valine provided the greatest stimulation. Furthermore they indicated that in spring, the streptococci required six

Table 1. Characteristics of *L. bulgaricus*

Characteristics	*L. bulgaricus*
Morphological	- Non-motile, Gram positive, slender rods[a] with rounded ends, often as long forms, cells occur mainly singly or in pairs in young culture. Average size ranges 0.8 to 1.0 by 4 to 6 microns. Granules demonstrated with methylene blue stains (1). - Morphology is markedly affected in the presence of penicillin, aureomycin, subtilin, streptomycin and sanitizers in milk (2).
Colony	- White to light gray, normally rough, 1-3 mm in diameter (1).
Atmosphere	- Anaerobic or facultative (1).
Physiological	- Optimum temperature 40-43°C, minimum 22°C and maximum 52.5°C (some strains grow even at about 60°C), not regarded as thermoduric but certain strains are able to survive heating to 75°C for 20-30 min (1,4). - Growth is inhibited by bile salts and 2% NaCl, some strains may tolerate 2% sodium chloride and exhibit no appreciable growth in the presence of 2 and 4% sodium taurocholate (3). - Many strains are able to grow in the presence of 0.4% phenol. The resistance of phenol may be used as an indication of the intestinal surviving ability of bacterial cultures (4).
Fermentative	- Homogermentative, has the ability to ferment glucose, fructose, galactose and lactose, but fails to utilize sucrose (4).
Biochemical	- Produce up to 1.7% D(-) lactic acid in milk and small quantities of carbonyl compounds, ethanol and volatile acids. The most important carbonyl compounds include acetaldehyde, acetone, butanone-2 and traces of acetoin (5,6).

Table 1. (cont'd).

Characteristics	L. bulgaricus
	- Of the fatty acids the most important are: acetic, propionic, butyric, isovaleric, caproic, caprylic and capric acids - Mildly proteolytic in milk (7). - Hydrogen peroxide produced by the action of NAD (Nicotinamide Adenine Dinucleotide) oxidase (4). - Some strains produce hydrogen sulfide, whereas others are able to transform enzymatically some amino acids to yield ammonia and organic acids (8). - Many strains exhibit an antagonistic effect against a number of saprophytic and pathogenic microorganisms (4). - Weakly lioplytic in milk leading to changes in the fatty acid pattern and free fatty acids (4).

[a]Abnormal rods in yogurt may be due to cooling at low acidities (9). Swelling of rods and the disappearance of streptococci may be caused by some milks heated to 85°C for 5 min. The addition of 10-20% boiled milk to the bulk milk or the use of starter propagation in severely heated milk is recommended to overcome this (10).

Sources

(1) Bergey's manual, Buchman and Gibbons (1974).
(2) Katznelson and Hood (1949).
(3) DeMan (1960).
(4) Rasic and Kurmann (1978).
(5) Goerner et al. (1968).
(6) Bottazzi and Vescovo (1969).
(7) Turcic et al. (1969).
(8) Groux (1973).
(9) Stolk (1956).
(10) Frank (1963).

Table 2. Characteristics of S. thermophilus

Characteristics	S. thermophilus
Morphological	- Non-motile, Gram positive, spherical or ovoid cells[a]) occurring in pairs to long chains. Average size ranges 0.7 to 0.9 micron in diameter (1). - Morphology is markedly affected in the presence of antibiotics, drug preparations and sanitizers in milk (2).
Atmosphere	- Facultatively anaerobic (1).
Physiological	- Optimum temperature 40-45°C, minimum 20°C and maximum 50°C (no growth at 53°C) (1). - Not regarded as thermophilic, since it does not grow at the pasteurization temperature used in the dairy industry (2). - Very sensitive to inhibitory substances, particularly antibiotics such as penicillin and streptomycin (3).
Fermentative	- Homofermentative, has the ability to ferment glucose, fructose, sucrose and lactose, but not maltose (1).
Biochemical	- Produce 0.7-0.8% L(+) lactic acid in milk (some strains produce up to 1.0% lactic acid) and small quantities of volatile acids such as: formic, acetic, propionic, butyric, isovaleric and caproic acids (4,5). Also small amounts of acetaldehyde, acetone, ethanol and butanone-2 (6,7). - Very weak proteolytic in milk (2). - Some strains are antagonistic to a number of microorganisms (2).

[a]Nutritive media and temperature affect the morphology. At 45°C it forms short chains in milk, while at 30°C many strains appear as diplococci. Broth increases the tendency to chain formation and at the higher temperature of 45°C, the chains are longer than at the lower temperature of 30°C. It may form long chains at high acidity of the milk. Cells

Table 2 (cont'd).

of the organism become very irregular (swollen, pointed and often rod-shaped) on solid media as in agar (2).

Sources

(1) Bergey's manual, Buchanan and Gibbons (1974).
(2) Rasic and Kurmann (1978).
(3) Hargrove et al. (1950).
(4) Turcic et al. (1969).
(5) Veringa et al. (1969).
(6) Bottazzi and Vescovo (1969).
(7) Goerner et al. (1972).

amino acids which are: histidine, lysine, leucine, valine aspartic acid and cysteine; while the requirement in autumn increases five amino acids more, which are: glutamic acid, isoleucine, glycine, tyrosine and methionine. Bautista, et al. (1966) conducted a study to clarify further the symbiotic relationship existing between the two bacterial species in yogurt starter cultures and they confirmed the stimulatory effect and further claimed that the higher acid production by a mixed culture was mainly due to the improved growth of the streptococcus resulting from the production of histidine and glycine by the lactobacillus. Galesloot, et al. (1968) showed that it is S. thermophilus which stimulates L. bulgaricus by producing a factor in milk which is equal to or can be replaced by formic acid. This stimulation can only be demonstrated in moderately heated milks and not in sterilized milk which contains formic acid by elevating heating. Davis (1971) stated that S. thermophilus facilitates the growth of L. bulgaricus by removing oxygen which can lead to production of toxic hydrogen peroxide. In turn, L. bulgaricus stimulates the S. thermophilus growth by liberating amino acids and other stimulating substances from the casein. Rasic and Kurmann (1978) stated that when both organisms are mixed, the coagulation time of 2-3 hours at 40-45^{o}C for milk is shorter than in growth of single culture. They stated that the stimulatory effect of amino acids results in shortened

generation time and increases numbers of streptococcal cells. This will result in the streptococci outnumbering the lactobacilli by 3-4:1 after the first hour of incubation. Then the growth of streptococci will slow down as a result of the inhibitory effect of lactic acid and the numbers of lactobacilli will gradually approximate the streptococci. Therefore, it can be concluded that the associated growth of S. thermophilus and L. bulgaricus has mutual symbiotic relationships. L. bulgaricus stimulates the growth of S. thermophilus first by liberating the essential amino acids then the S. thermophilus slow down due to the adverse effect of lactic acid and L. bulgaricus increases through the stimulative action of S. thermophilus.

Yogurt Cultures

Yogurt cultures or starters should be obtained from reliable sources such as a reputable commercial culture producer or from research institutes and should be renewed frequently. Tramer (1973) suggested the following desirable qualities of an ideal yogurt culture:

1. Purity: meaning free from contaminants which can be accomplished via frequent renewal of the culture.
2. Vigorous growth: cultures are more active when grown together prior to their use as inoculum.
3. Should produce the right consistency in the product.
4. Stability: meaning the microbial balance should be

easily maintained.

5. Production of a good flavor without off-flavors.
6. Should have no tendency to induce syneresis.
7. Should be resistant to penicillin and other antibiotics.
8. Should not develop excessive acidity on cold storage.
9. Should have a reasonable tolerance to sugar.
10. Should have easy maintenance.
11. Should be phage resistant.

Yogurt cultures may be supplied in various forms, such as liquid, freeze-dried, concentrated, concentrated and deep-frozen and concentrated freeze-dried. Cultures require great attention and skill in handling due to several facts, namely, that the two bacterial species have different optimal temperatures, that they grow symbiotically and their rate of growth is different. A high-quality yogurt with a pleasant taste depends very much on the ratio of the two bacterial species. There is considerable agreement among various investigators on the desirable ratio of coccus to rod in yogurt starters. Pette and Lolkema (1951a) reported that a coccus:rod ratio of 1:1 is desirable in yogurt after incubation and cooling; furthermore they reported that the proportion of coccus and rod in the finished product is influenced by incubation temperature, acidity of the culture and the rate of inoculum. Stocklin

(1969) recommended that the ratio of the rod to coccus should be maintained at either 1:1 or 1:1.2. Platt (1969) stated that an equal proportion (in terms of inoculation rate, namely 2%) of separate milk cultures of coccus and rod are used for a successful process of yogurt manufacture. Sellars and Babel (1970) recommended a similar ratio for yogurt cultures.

Therapeutic Properties

Traditionally, yogurt should contain viable micro-organisms, namely S. thermophilus and L. bulgaricus in the final product. Some investigators claimed a specific therapeutic value to the presence of living lactic acid bacteria in the yogurt. Chung and Goepfert (1970) and Daly et al. (1971) claimed that lactic acid and other organic acids such as acetic acid, propionic acid and formic acid which produced by lactic acid bacteria have bacteriostatic or bactericidal effect against various acid sensitive organisms, particularly the sporeforming bacteria. The production of antimicrobial substances other than organic acids also was demonstrated and some of them were isolated and identified (Rasic and Kurmann, 1978). An antibiotic called "bulgarican" isolated from L. bulgaricus was active against various gram-positive and gram-negative bacteria (Reddy and Shahani, 1971). Apparently, the

inhibitory effect of Lactobacilli is stronger and more effective than that of the Streptococci (Rasic and Kurmann, 1978). So the number of viable L. bulgaricus in the product at the time of consumption is considered to be of great importance (Davis, 1971). Various investigations dealing with the antagonistic effect of yogurt against various microorganisms, such as Salmonella, Shigella, Staphylococcus, E. coli, Bacillus, Clostridium, Pseudomonas, Brucella, Vebrio, Mycobacterium and others showed different results in vitro. Nichols (1951) stated that the pathogenic enteric organisms such as S. typhosa, S. dysenteriae or V. cholerae died rapidly within 3 hours in a yogurt-like product, which contained 1.0% lactic acid but Makharadze (1961) revealed in his data that V. cholerae suis, var. kunsendorfand and S. enteritidis var. doublin survived for 48 hours in yogurt. Furthermore, Salmonella, Shigella and Vibrio organisms were killed in 5 hours in contact with yogurt, but Seneca et al. (1950) and Ozek (1956) revealed in their investigations that these organisms were killed in 8-24 hours under the same conditions. Yazicioglu and Yilmaz (1966) stated that yogurt inhibits the pathogenic coliform organisms and salmonellae.

Finally, it can be concluded for those who attribute a specific therapeutic value and any healthful benefits from yogurt bacteria that the microorganisms must survive the digestive process and be able to grow in the intestinal

tract in order to produce the antimicrobial agents or other claimed beneficial effects. S. thermophilus would not be able to implant in the intestinal tract because it is not tolerant to very acidic conditions (Humphreys and Plunkett, 1969; Acott and Labuza, 1972). Hawley et al. (1959) stated some factors which are important for a successful implantation of lactobacilli in the human intestinal tract. The most important requirements were:

a. The presence of a sufficient amount of lactose or other fermentable carbohydrates in the product for cell growth, and

b. The presence of a large number of viable bacteria.

Gallagher et al. (1974) recommended that persons with lactose malabsorption be encouraged to consume cultured milk foods as an alternative to regular fluid milk. Kilara and Shahani (1976) stated that the lysing of viable yogurt bacteria in the intestinal tract releases lactase which can hydrolyze lactose. However, in spite of the belief in positive therapeutic value of yogurt, definitive clinical research is still needed to clarify and verify the therapeutic properties, if any, of yogurt.

Differential Enumeration of Yogurt Starter Bacteria

Because of the emphasis on maintaining the desirable 1:1 ratio of coccus to rod for making a high quality yogurt and the concern about the presence ov viable microorganisms in

the final product, research has been conducted to develop a technique and/or media for the differential enumeration of yogurt starter bacteria. Davis et al. (1971) described a method based on growing both organisms in a rich medium (see the Appendix for the composition of the medium) using the double pour plate technique, and differentiating them by colony type under a low power binocular microscope or lens. Porubcan and Sellars (1973) developed an agar medium and the differentiation was accomplished by adding an appropriate sugar or sugar mixture (selected according to the carbohydrate fermentation characteristics of particular strains) to molten agar base before plating. This medium and the one proposed by Davis et al. (1971) suffered the same limitation in that the differentiation is based on a somewhat unreliable criterion; namely, colony morphology. Some investigators (Sellars and Babel, 1970; Mocquot and Hurel, 1970; Johnson, 1945) suggested microscopic examination of yogurt cultures to estimate roughly the ratio of coccus to rod, but this method was considered inadequate because it was difficult to distinguish between dead and live bacteria. Lee et al. (1974) developed an agar medium and the differentiation was achieved according to the carbohydrate fermentation ability of coccus and rod. Generally, S. thermophilus ferments sucrose, whereas L. bulgaricus does not. Accordingly, S. thermophilus will produce sufficient acid to change the color of an acid base indicator (bromcresol purple) and

forms yellow colonies, while L. bulgaricus grows more slowly and produces white colonies (see the Appendix for the composition of the agar medium). There are certain limitations concerning this medium:

a. Incubation conditions are very specific.

b. Some strains of L. bulgaricus can produce yellow colonies.

c. Preponderence of either S. thermophilus or L. bulgaricus in a mixture does not allow distinction between rod and coccus colonies.

Finally, Shankar and Davies (1977) used the inhibitory property of β-glycerophosphate toward L. bulgaricus to selectively isolate and enumerate S. thermophilus from yogurt, while Johns et al. (1978) used reinforced clostridial agar (RCA) at pH 5.5 to suppress the growth of S. thermophilus and thereby allow selective isolation of L. bulgaricus from yogurt cultures.

MATERIALS AND METHODS

Yogurt Manufacture

Culture

Chr. Hansen R-1 concentrated deep-frozen culture cans were obtained and stored at -42.7°C.

Bulk Starter Culture

The concentrated deep-frozen culture was allowed to thaw and was aseptically transferred to the sterilized (at 45.6°C) milk. The inoculated milk (45.6°C) was then held until the desired pH was attained. The bulk starter culture then was added with constant stirring to the yogurt mix at 32.2°C.

Yogurt Mix

Milk obtained from the Michigan Milk Producers Association (MMPA) was pumped into the pasteurizer and the remaining ingredients (see Appendix) were added to the milk as follows:

1. All dry ingredients were mixed with the milk using powder funnel.

2. The water portion of the yogurt mix was added directly to the milk with continuous stirring.

The yogurt mix was then pasteurized at 87.8°C for 40 minutes. At the end of pasteurizing time, the mix was

cooled to 57.2°-62.8°C before homogenizing at 1500 psi, two stages (first stage 1000 psi, second stage 500 psi).

Yogurt Preparation

The yogurt mix was then cooled to 32.2°C and the bulk culture added and mixed well. The yogurt then was incubated 12-14 hours at the same temperature (32.2°C). At the end of incubation period, the pH was measured. Yogurt was aseptically packed into cartons and stored at 4.4°C.

Sampling

Samples were withdrawn from the cartons every 3-4 days for up to 21 days for bacterial count and were diluted to 10^{-7} and 10^{-8} according to Standard Methods for the Examination of Dairy Products. Samples (0.1 mL) of the dilutions were spread evenly on the surface of Living Lactic Acid Bacteria (L.A.B.) agar and Lee's agar with a sterile bent glass-rod. The plates were incubated at 37°C for 48 hours. The pH and titratable acidity of the remaining product was determined at time of sampling. Commercial yogurts (Dannon, Yoplait, and Lunebest) purchased from local markets, were analyzed every 7 days and up to 14 days for pH, titratable acidity, and carbohydrate content. The pH of the heat treated samples was adjusted to pH 4.29, 4.10, 3.95, and 3.87 with NaOH and were set for 24 hours in order to determine the effect of pH on the survival rate.

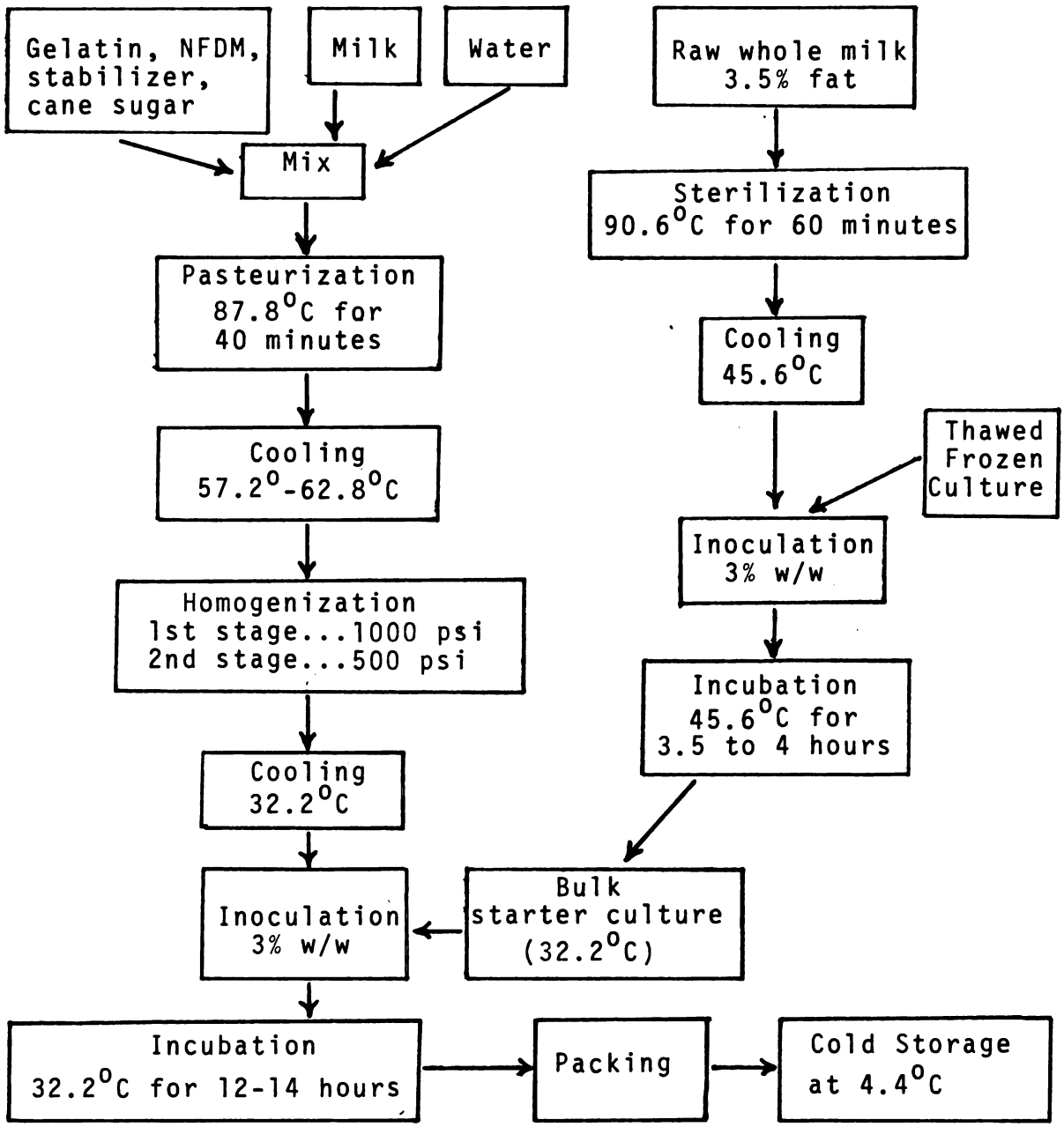

Figure 1. Flow diagram for preparation of yogurt.

Standard Carbohydrate Solutions

Three standard carbohydrate solutions were prepared from analytical grade reagents, and single carbohydrate solutions were used to establish elution times and order. One solution contained 1.0 mg/mL each of lactose, glucose, fructose, galactose, and raffinose; the second solution contained 1.0 mg/mL mixture of lactose, glucose, galactose; the third solution contained 1.0 mg/mL mixture of the above sugars in order to confirm the elution times and order. All solutions were filtered through a Sep-Pak C_{18} cartridge (Water Associates) prior to injection.

Preparation of Carbohydrate Extracts from Yogurt

Samples of plain yogurt obtained from the Michigan State University dairy plant or purchased locally were accurately weighed (5.0 grams) into plastic centrifuge tubes. Absolute ethanol was added to make the final concentration of ethanol 80% (v/v). Slurries were mixed well and allowed to stand for 30 minutes to insure precipitation of proteins. Samples were centrifuged at 3000 rpm for 20 minutes, the supernatant decanted, and the residue washed with about 20 ml of 80% ethanol and centrifuged for 20 minutes at 3000 rpm. The extract plus washing were reduced almost to dryness on a rotary vacuum evaporator. Sample extracts were made to 25.0 mL with water and filtered through a Sep-Pak C_{18} cartridge (Waters Associates).

Chromatography Equipment

The system consisted of a Waters Associates M-45 solvent delivery system, a U6K septumless injector, and a Model R-401 differential refractometer with a programming data module Model 730. The column was a Bio-Rad Aminex carbohydrate HPX-87C column (300x7.8 mm) held at 85°C by a water jacket (Alltech Associates) and a thermo regulator water pump (exacal ex 100). A Bio-Rad Aminex A-25 (40 mmx4.6 mm) Microguard Anion/OH Cartridge was used as a guard column to remove undesirable anions and to prolong analytical column life. The eluent was water purified by ion exchange and vacuum degassing. A Hamilton 25 µl syringe was used to inject 25 µl sample volumes.

Heat Treatment

Samples

Plain yogurt obtained from the MSU dairy plant was heat treated using a tubular heat exchanger (Laboratory Model Spiratherm) at 65°C, 68°C, 70°C, 75°C, and 80°C. Holding time was fixed at 12 seconds. Microbiological analyses were made to determine the survival of culture organisms at different pH.

Laboratory Model Spiratherm

The Lab Model Spiratherm is mounted on a triangular base approximately 27" to a side (Figure 2). It should be

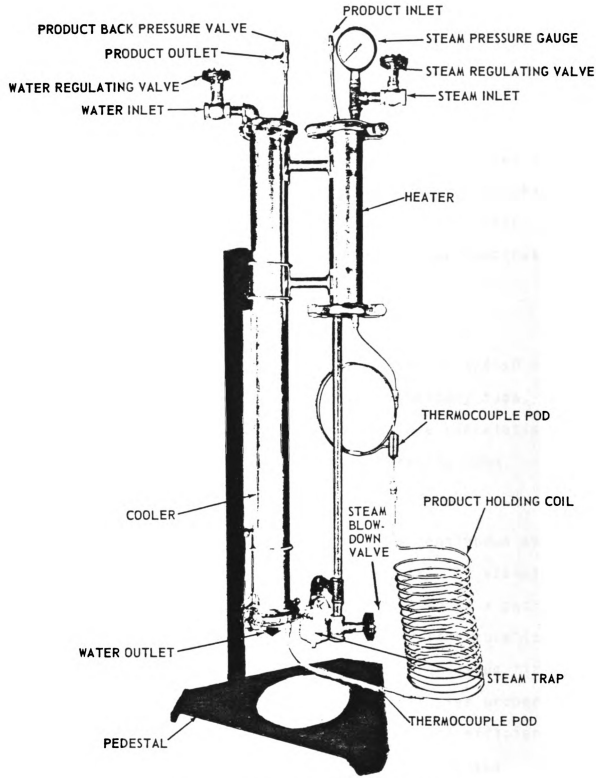

FIGURE 2 – FRONT VIEW OF SPIRATHERM

located on a smooth, level floor close to water and product supply. The unit is connected to a positive displacement pump to transfer product through the heating, holding, and cooling sections.

Temperature Sensors

Two iron constantan (I.C.) in-line thermocouples are provided for sensing product temperature at the discharge of the heater and at the inlet of the cooler. These sensors were connected to a potentiometer for temperature measurement.

Heat Exchanger - Heater

The Spiratherm heater is a spiral coil of 1/4"x0.035" stainless steel tubing mounted in a 3" sanitary tube. The steam cavity or shell is open to allow free circulation of steam around the coiled 1/4" product carrying tube.

Cooler

The Spiratherm cooler is basically a continuous spiral of 1/4" O.D.x0.035" wall tubing mounted in a 4" stainless steel shell and attached to 4" end caps through a packing gland and nut arrangement. Inside of the 1/4" tube is placed a core to displace volume and create a snug fit between the coil and the shell, which will give proper direction and flow to the cooling media. For efficient cooling, product flow should be counterflow to the

heat transfer (cooling) media.

Back Pressure Valve

A back pressure valve is used to prevent flashing of the product at elevated temperatures in the heater, holder and cooling sections. Calculation of flow rate and holding time in a Tubular Heat Exchanger as follow:

Given:

Volume of cylinder (coil) = r^2h

= 9.77 $in.^3$

where: r = radius of coil (0.09 in.)

h = length of coil (384 in.)

A desired holding time of 12 seconds

Volume conversion: 231 $in.^3$/gallon

Calculation:

$$\text{Flow rate} = \frac{\text{Volume of coil}}{\text{Holding time desired}} = \frac{9.77\ in.^3}{12\ sec.}$$

$$= 0.814\ in.^3/sec.$$

$$\text{Flow rate in gallons} = \frac{0.814\ in.^3/sec.}{231\ in.^3/gallon}$$

= 0.0035 gallon/sec.

= 0.035 gal./10 sec.

= 132.5 mL (amount collected in 10 sec. to insure 12 sec. holding time)

RESULTS AND DISCUSSION

Evaluation of Techniques Used for Differential Enumeration of Yogurt Culture

The procedures of Davis et al. (1971) and Lee et al. (1974) were evaluated and compared in order to qualify a method for differential enumeration of yogurt starter bacteria. Using (L.A.B.) medium and a double pour plate technique (Davis et al., 1971), the differentiation was achieved in some cases visually even without using a low power binocular microscope or lens. S. thermophilus appeared as smooth, round colonies of various sizes, while L. bulgaricus gave irregular shaped, rough colonies in the depth of the medium. In other cases a compound light binocular microscope was used for differentiation. Both types of colonies were Gram stained and S. thermophilus appeared as spherical or rounded cells in pairs and long chains while L. bulgaricus appeared as slender rods with rounded ends, mostly as long forms, cells occurred single, pairs, and in long chains. The colonies and cell description agreed with Davis et al. (1971) and Bergey's manual, Buchanan and Gibbons (1974) accordingly. When Lee's agar was used as plating medium and the recommended incubation

conditions were strictly followed, the differentiation between S. thermophilus and L. bulgaricus was very obvious. S. thermophilus appeared as bright, round, and yellow colonies, while L. bulgaricus colonies were diffuse, irregular shaped and white. The differentiation was based on the carbohydrate fermentation patterns of several coccus and rod strains, whereas all S. thermophilus strains fermented lactose and sucrose, on the other hand, most of L. bulgaricus failed to utilize sucrose (Lee et al., 1974). The concentration of lactose was fixed at 0.5% in the formulation of the agar, which was enough to obtain good colony growth of L. bulgaricus without causing the indicator color change from neutral (violet) to acid (yellow) range (Lee et al., 1974). The presence of two utilizable sugars (lactose and sucrose) in the medium and the favorable incubation conditions used resulted in rapid growth of S. thermophilus which produced enough acid to change indicator color to yellow. The uniformly suspended $CaCO_3$ acted as a nondiffusible buffer, localized the acid, producing a narrow intense yellow zone around individual S. thermophilus colonies. A Gram stain was made to confirm the morphological characteristics of both organisms.

Comparison of the Davis and Lee Techniques

The technique of Davis et al. (1971) has a major limitation in that differentiation is based on an unreliable

criterion; namely, colony morphology. It required in most cases using a low power binocular microscope to determine the ratio of S. thermophilus to L. bulgaricus which is time consuming especially when the plate count is high. For comparison of the merits of (L.A.B.) agar to Lee's agar, in terms of efficiency of recovery of the coccus and rod microorganisms (measured as CFU/ml), the same dilutions were inoculated on both media and the incubation conditions (37°C/48 hours) were exactly alike. (L.A.B.) medium proved to have a better efficiency of recovery with a higher count of microorganisms throughout the storage period (21 days). The technique of Lee et al. (1974) allowed easy and reliable differentiation between S. thermophilus and L. bulgaricus throughout the storage period. The most desirable results were obtained when the total number of colonies on the plate did not exceed 100, and the proportions of the coccus and the rod were fairly equal. The technique was based on acid-producing activity and that was accomplished by fixing the lactose concentration in the agar enough for L. bulgaricus to grow but not to change the indicator color to acid (yellow). S. thermophilus will utilize sucrose and lactose and grow rapidly, producing enough acid to change the indicator color to acid (yellow). The limitations of this medium (mentioned previously) could be avoided by:

a. Inoculating the medium to sufficiently higher dilutions to obtain a total plant count

ranging from 50 to 100 CFU/ml which permits distinction between the coccus and rod colonies.

b. Following precisely the incubation conditions ($37^{o}C$/48 hours).

Modification

The observations and the results throughout the experiments favored the use of Lee's agar as a good medium for differential enumeration of yogurt culture and Lee's technique proved to be an easy and reliable. Several trials were made with Lee's agar and the incubation conditions to attempt to modify and improve the technique:

a. Bromcresol purple (BCP) was added to the medium in 0.001%, 0.002%, 0.003%, and 0.004% in order to attain better distinction in colony appearance.

b. Incubation conditions were varied to $37^{o}C$/48 hours; $32^{o}C$/48 hr; $32^{o}C$/72 hours; $45^{o}C$/48 hours; and $45^{o}C$/72 hours.

The results indicated that at 0.002% Bromcresol purple indicator in the formula, the colonies appearance was clear and distinct. For the incubation conditions, $32^{o}C$/48 hours and $45^{o}C$/48 hours did not show good growth and the colonies appearance was not clear, while at $32^{o}C$/72 hours and $45^{o}C$/72 hours, most of L. bulgaricus colonies shifted from white to yellow as the result of prolonged incubation which allowed the lactobacilli to accumulate sufficient acid to shift the indicator color to yellow. The best results

obtained by following the recommended incubation conditions of 37°C/48 hours, and 0.002% Bromcresol purple in the agar.

Analysis of Carbohydrate in Yogurt by High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is becoming widely accepted as an invaluable technique for the analysis of many food components, and it has been suggested as an accurate and a rapid procedure for the analysis of sugars in many food commodities, including fruit juices and dairy products (Conrad and Palmer, 1976; Hurst et al., 1979. Analysis of glucose in ice cream mix was reported by Warthesen and Kramer (1979) using a Waters μ-Bondapak/ carbohydrate column. However they had difficulty in differentiating between glucose and galactose, which were eluted together. Brandao (1980) developed a method in which glucose was oxidized by glucose oxidase and the galactose analyzed by HPLC. By knowing the galactose concentration, the glucose content could be calculated. This method appeared to be time consuming and complicated. Richmond et al. (1981) described a procedure for determining simple carbohydrates (lactose, glucose, galactose) in dairy foods by an Aminex HPX-87 cation exchange (calcium form) carbohydrate column maintained at 80°C and reverse-osmosis, ion-exchanged, degassed water as a solvent. They obtained a good separation between glucose and galactose.

The HPLC Method

When a three component solution containing lactose, glucose, and galactose was injected and the system was set at a flow rate of 0.6 mL/min, the elution order was lactose, glucose, and galactose (Figure 3). This figure shows a good resolution and the base line separation of the three sugars was achieved in 12 min. When samples from yogurt were injected (Figure 4) the carbohyrates isolated included lactose, galactose, and traces of glucose.

Retention times of standard sugars correlated well with actual sample peaks. In addition to sugars, one early eluting peak was present as well as a small late eluting peak (when the elution time was extended to 18 min). This late peak (not shown) had approximately the same retention time as ethanol which could be present because of the extraction procedure. The early eluting peak, which was suspected to be a compound of larger molecular weight such as oligosaccharides was examined to identify it. A standard solution of five sugars containing raffinose, lactose, glucose, galactose, and fructose was injected (Figure 5). The early eluting peak which was present in the samples was eluted before raffinose. This suggests that this peak represent one species of oligosaccharides with molecular weight larger than raffinose, but this possibility was not researched or verified.

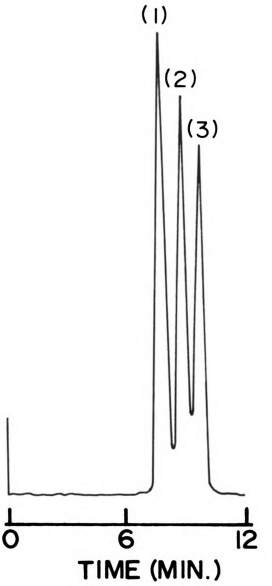

Figure 3. HPLC chromatogram of standard carbohydrate solution; 1) lactose, 2) glucose, 3) galactose. Bio-Rad HPX-87 carbohydrate column (85°C); Aminex A-25 Microguard Anion/OH cartridge; solvent H_2O; flow rate, 0.6 mL/min; injection volume, 25 µL; attenuation, 16x.

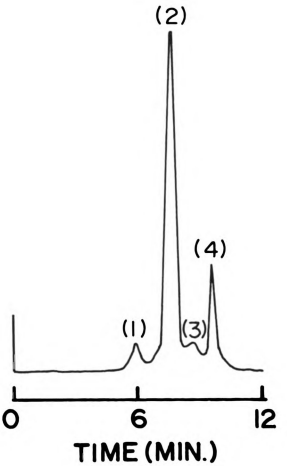

Figure 4. HPLC chromatogram of a yogurt sample; 1) unknown; 2) lactose; 3) glucose; 4) galactose. Bio-Rad HPX-87 carbohydrate column (85°C); Aminex A-25 Micro-guard Anion/OH cartridge; solvent H_2O; flow rate. 0.6 mL/min.; injection volume, 25 mL; attenuation, 16x.

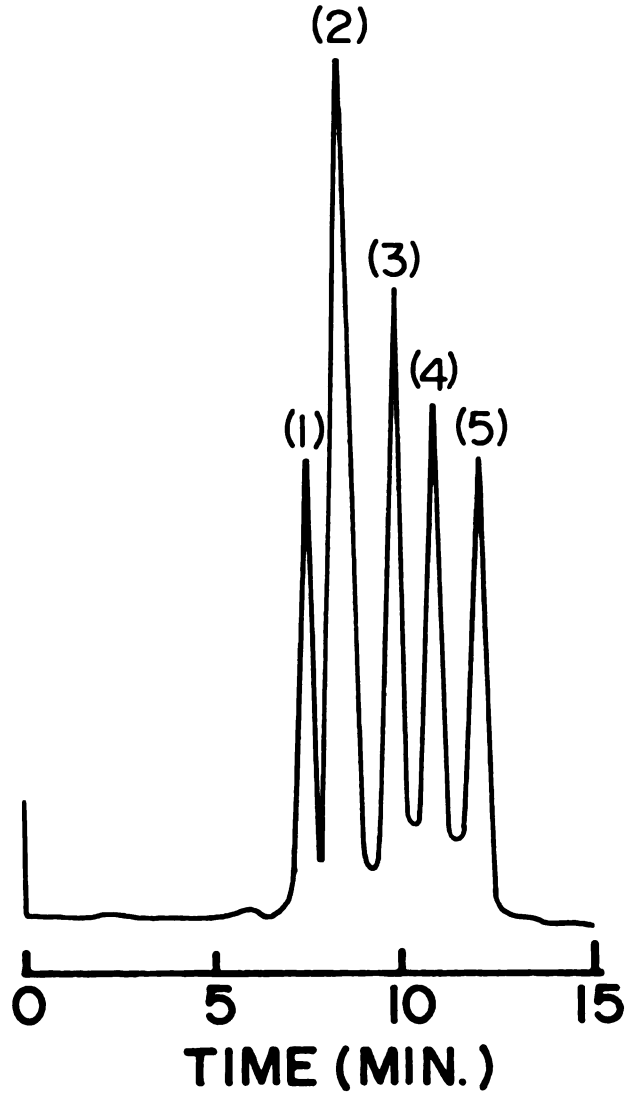

Figure 5. HPLC chromatogram of standard carbohydrate solution; 1) raffinose, 2) lactose, 3) glucose, 4) galactose, 5) fructose. Bio-Rad HPX-87 carbohydrate column (85°C); Aminex A-25 Micro-guard Anion/OH cartridge; solvent H_2O; flow rate, 0.6 mL/min; injection volume, 10 µL; attenuation, 8x.

Changes in the Carbohydrate Content of Yogurt during Storage

According to a United Kingdom Standards Committee (1975), the carbohydrate content of natural yogurt is higher than the content of milk. During processing, fermentation, and storage of yogurt, the carbohydrate contents show a quantitative changes. Brandao (1980) reported a slight decrease of lactose during the pasteurization process and slight increase of monosaccharides (galactose and glucose). Furthermore, a constant decrease of lactose content was observed during fermentation followed by an increase of galactose and glucose. The data in Table 3 shows the quantitative changes in pH, titratable acidity, and carbohydrate content during storage. Lactose content decreased from 6.38% to 3.82% (40.1%) in Dannon yogurt from 7.63% to 4.49% (41.2%) in Yoplait product, and from 6.81% to 4.57% (32.9%) in Lunebest product, while galactose content increased from 0.92% to 2.65%, 0.78% to 2.69%, and from 0.54% to 2.61% in these products, respectively, in 14 days. Trace amounts of glucose were detected during storage. This change may be attributed to the growth of the yogurt bacteria during storage and/or residual lactase activity which will ferment and/or hydrolyze lactose to produce lactic acid, galactose, and glucose. Kilara and Shahani (1974) reported that lactase activity in yogurt increased with time of incubation. They also found that

Table 3. Changes in carbohydrate, pH, and titratable acidity of commercial yogurt during storage for 14 days at 4.4°C.

Samples*	Storage time (days)	Lactose % (w/w)	Galactose % (w/w)	Glucose % (w/w)	pH	% titratable acidity (as lactic acid)
Dannon	1	6.38	0.92	trace	4.20	0.82
	7	5.06	1.78	trace	4.07	1.03
	14	3.82	2.65	trace	3.93	1.29
Yoplait	1	7.63	0.78	trace	4.18	0.83
	7	6.24	0.82	trace	4.06	1.05
	14	4.49	2.69	trace	4.03	1.10
Lunebest	1	6.81	0.54	trace	4.32	0.62
	7	5.29	0.60	trace	4.24	0.75
	14	4.57	2.61	trace	4.22	0.79

*Samples purchased at local market approximately two weeks after date of manufacture.

S. thermophilus contained approximately three times more lactase than did L. bulgaricus. The increase of monosaccharides did not correspond to the observed decrease in lactose content, suggesting that some of the galactose and glucose is been metabolized. There was no significant increase in microbial content during the storage period but there was a shift in the ratio between L. bulgaricus and S. thermophilus. When the titratable acidity increased, the growth of streptococci is slowed down due to the inhibitory effect of lactic acid so that the numbers of lactobacilli gradually increase.

Heat Treatment of Yogurt

Pasteurization after incubation prolongs the shelf life of yogurt by reducing microbial and enzymatic spoilage. Schulz (1966) fermented milk in a closed package at approximately 45°C, sealed, quickly heated it to 60°C and held it at this temperature, then followed with fast cooling. Rasic and Kurmann (1978) stated that organisms such as yeasts and molds are destroyed completely, together with the majority of lactic acid bacteria during pasteurization of the yogurt mix. Furthermore they stated that the bacterial count was reduced by 86.5% at 55°C for 30 min. and 99.5% at 60°C for 5 min. (Source: Pakshy, 1966). Puhan (1979) reported that yeasts, molds, and E. coli were destroyed at 60°C and lactic acid bacteria were

destroyed at 65°C when heating time was fixed at 22 seconds. The data in Table 4 show the survival rate of lactic acid bacteria at holding time of 12 seconds, various temperature and pH. The mix was processed in a pilot plant tubular heat exchanger. Heat-treatment of cultured products is intended to eliminate the contaminants in order to prolong the shelf life and preserve the essential character of yogurt while maintaining viable lactic acid bacteria in the product. In yogurt with pH 4.29, 23.1% of the lactic acid bacteria survived after heat-treatment at 65°C, 12 seconds, whereas at pH 3.87, 99.99% of lactic acid bacteria were destroyed. At pH 4.10, 1.23% of the lactic acid bacteria survived heat-treatment at 68°C, 12 seconds (account for 1.6×10^8 microorganisms/mL). The remaining 0.01 to 0.001% of the lactic acid bacteria practically showed no activity after storage at 4.4°C for one month and no acidity increase was measured. Flueler and Puhan (1972) reported 77.4% survival in yogurt with pH 4.28 at 65°C/22 seconds, whereas the data[a] presented in this thesis showed 23.1% with pH 4.29 at 65°C for 12 seconds, a significant difference. Since a shorter hold time was used, one would presume greater survival. Puhan (1979) did not indicate either the method of heat-treatment or the experimental design which would be essential to

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[a]The experiment was done twice to confirm the data obtained.)

Table 4. Survival of lactic acid bacteria[a] in yogurt after heat treatment at varying temperatures and pH.

Hold time 12 sec. at	Survival (%) at pH			
	4.29	4.10	3.95	3.87
65°C	23.1	6.23	0.92	0.001
68°C	7.46	1.23	0.001	0.001
70°C	0.01	0.01	0.001	0.001
75°C	0.001	0.001	-	-
80°C	0.001	0.001	-	-

[a]Before heat-treatment = 1.3×10^{10} microorganisms/mL.

useful interpretation of this data. The laboratory Spiratherm permitted accurate control of temperature and holding time. Additional trails at other holding times and temperatures would be desirable. A reduced consistency of yogurt was observed after heat-treatment which affected the body and texture of the cultured yogurt. Kroger (1976), Rasic and Kurmann (1978), and Puhan (1979) recommended adding hydrophilic colloids (stabilizers), to increase the milk solids content of the milk (i.e. higher fat and sugar content), and homogenization to improve body of the yogurt. In conclusion, heat-treatment should not aim to convert fresh yogurt to the status of a canned food with extended shelf life. Rather the elimination of contaminating organisms concomitant with survival of significant numbers of L. bulgaricus and S. thermophilus would be desirable if one associates desirable nutritional properties of yogurt to the presence of viable starter organisms.

The Ratio Between S. thermophilus and L. bulgaricus

The mutual symbiotic relationship existing between S. thermophilus and L. bulgaricus resulted in outnumbering streptococci to lactobacilli after the first hour of incubation (Rasic and Kurmann, 1978). During storage for 21 days, the streptococci slowed down due to the effect of lactic acid and the number of lactobacilli increased Figure 6 shows the changes in coccus to rod ratio for

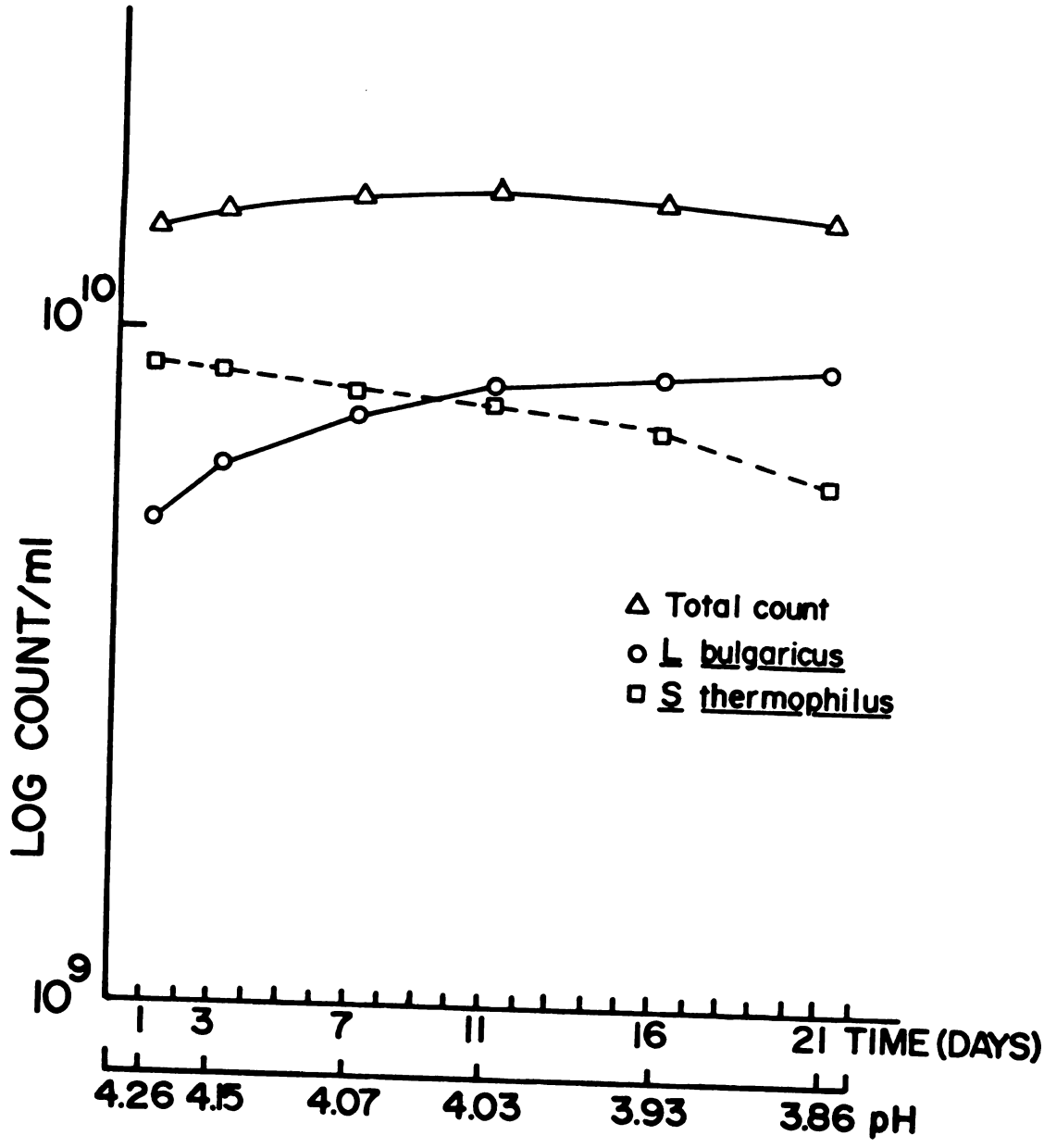

Figure 6. Changes in coccus:rod ratio during storage at 4.4°C.

fresh yogurt stored at 4.4°C. The total number of viable microorganisms increased slightly at the beginning, then a slight decrease was observed. In 21 days, 4.4°C, the pH decreased from 4.24 to 3.86 as a result of increasing the lactic acid, suggesting the presence of lactase activity. Speck (1983) stated that the starter viability and lactase activity decreased during a 20-day period at 1°C, but appreciable levels of lactase activity remained. The increase of titratable acidity from 0.76 to 1.39 had an adverse effect on streptococci and resulted in shifting the coccus:rod ratio from 1.7:1 to 1:1.48 in a 21-day storage period at 4.4°C (Table 5). It can be concluded that the desirable coccus:rod ratio was achieved after 9 days of storage at 4.4°C.

Table 5. The ratio (coccus:rod), ph, and (%) titratable acidity during storage for 21 days at 4.4°C.

Storage time (days)	Lee agar		Total count (countsx10^8)	Ratio (coccus:rod)	pH	% TA
1	yellow white	89 52	141	1.71:1	4.26	0.76
3	yellow white	87 63	150	1.38:1	4.15	0.85
7	yellow white	82 75	157	1.09:1	4.07	1.08
11	yellow white	79 84	163	1:1.06	4.03	1.16
16	yellow white	72 87	159	1:1.21	3.93	1.27
21	yellow white	61 90	151	1:1.48	3.86	1.39

SUMMARY AND CONCLUSION

The techniques of Lee et al. (1974) and Davis et al. (1971) for differential enumeration of yogurt culture were evaluated and compared. The differentiation in Davis's technique was based on colony appearance under a low power binocular microscope. S. thermophilus appeared as smooth, round colonies, while L. bulgaricus showed irregular, rough colonies. Lee's technique was based on the ability of S. thermophilus and L. bulgaricus to ferment carbohydrates. The differentiation was clear and distinct. S. thermophilus appeared as yellow, bright, and rounded shaped colonies, while L. bulgaricus appeared as diffused, irregular shaped, white colonies. The observations throughout the experiments favored Lee's technique as a reliable and easy method for differential enumeration of yogurt starter. An effort was made to modify the chemical composition of Lee's agar and the incubation conditions, but the results were not promising, suggesting that the present composition and incubation conditions are optimal. In yogurt stored at 4.4^{o}C for 14 days, a decrease in lactose content and an increase in galactose content were observed, while traces of glucose were detected. The increase in monosaccharides did not correspond to the decrease in lactose content suggesting

that some of galactose and glucose were metabolized. The change in carbohydrate content during storage may be attributed to the growth of yogurt bacteria and residual lactase activity. The HPLC method used was successful in separating glucose and galactose without sacrifice of near base line resolution. HPLC represent a simple solution to monitoring hydrolysis of lactose to glucose and galactose. Plain yogurt was heat treated in a Laboratory Model Spiratherm tubular heat exchanger. In yogurt with pH 4.29, 23.1% of the lactic acid bacteria survived after heat-treatment at 65^{o}C, 12 seconds, whereas at pH 3.87, 99.99% of lactic acid bacteria were inactivated under these conditions. These data are not in agreement with other literature. The difference may be due to the method of heat-treatment, the experimental design, the culture conditions (i.e. type, age, proportion of the two species, etc). Further studies are recommended to evaluate and/or compare different heat-treatment methods. A slight decrease of physical consistency was observed after heat-treatment at 65^{o}C and 68^{o}C, while a significant decrease in consistency was observed at 70^{o}C, 75^{o}C, and 80^{o}C. In yogurt stored at 4.4^{o}C for 21 days, a slight increase in total number of viable microorganisms was noted up to 11 days, followed by a slight decrease. The increase in titratable acidity resulted in shifting the coccus:rod ratio during a 21-day period. The desirable ratio was achieved after 9 days of storage at 4.4^{o}C.

APPENDICES

APPENDIX I

Composition of Lee's Agar and Plating Technique

Tryptone 1.0%; Yeast extract 1.0%; lactose 0.5%; sucrose 0.5%; $CaCO_3$ 0.3%; K_2HPO_4 0.05%; bromocresol purple (BCP) 0.002%; and agar 1.8%. The pH of the medium was adjusted to 7.0 0.1 before sterilization (121^{o}C for 20 min.). Bromocresol purple was added in the form of 1.0 ml of sterile 0.2% solution (autoclaved at 121^{o}C for 15 min) per 100 ml of sterile agar just before pouring petri plates. Agar was thoroughly mixed to evenly suspend the settled $CaCO_3$ and was then poured into previously chilled, sterile petri plates to obtain a layer 4 to 5 mm thick. Chilling of petri plates (in a refrigerator for 30 min) insure uniform distribution of $CaCO_3$ in the agar layer.

APPENDIX II

Composition of LAB Medium and Plating Technique

Lactose 20 gm; Tryptone 10 gm; Beef extract 10 gm; Yeast extract 10 gm; Tomato juice (filtered) 50 gm; Tween 80 1 gm; Dipotassium phosphate 2 gm; Agar 15 gm; Distilled water 950 ml.

pH was adjusted to 6.6 and autoclaved at 121^{o}C for 15 min. A double pour plate technique was used.

APPENDIX III

Yogurt Formulation

Ingredients	Pounds for 100# batch
Whole milk	42.85
Non-fat dry milk	8.51
Cane sugar	7.00
Stabilizer (stein Hall)	0.50
Gelatin	0.15
Water	40.89
Total	100.00
Yogurt Culture[a] (to be added later)	3.00

[a] Culture

Concentrated deep-frozen culture (Chr. Hansen AY3) cans (stored at -42.7°C) were used.

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