ISOLATION, PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF YOGHURT STARTER BACTERIA

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İZMİR

It is dedicated to my mother.

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ABSTRACT

ISOLATION, PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF YOGHURT STARTER BACTERIA

Specific bacteria cultures, known as starters, are used for manufacturing of fermented milk products. Yoghurt is made from milk by the protocooperative action of well known starters, namely *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. They lead to coagulation of milk by lactic acid fermentation and other products give the characteristic properties, such as acidity, aroma, and consistency. Traditional method, using part of a previous batch to inoculate a new batch, have been used for centuries. Such cultures lead to variable performance, however industrial production needs consistency. The method of choice is the use of bacterial strains with known physiological, biochemical and genotypic characters.

The isolation and identification of natural starters is a need not only for the dairy industry, which still import starters abroad, but also for the preservation of natural lactic acid bacteria diversity of Anatolia. With this perspective, the aim of our study was the isolation of starters from artisanal yoghurts, and their biochemical and molecular characterization.

At the end of the study, 66 cocci and 71 bacilli were isolated. According to biochemical identification, all the bacilli isolates were found to be *L. delbrueckii* ssp. *bulgaricus,* however cocci isolates showed highly variable sugar fermentation results and only 7 of them were characterized as *S. thermophilus*. The molecular characterization was based on the amplification and restriction fragment length polymorphism (RFLP) of 16S ribosomal DNA (rDNA) and internal transcribed spacer (ITS) region. Species-specific PCR amplification and 16S sequencing were also used for justification. All of the isolates were well identified with the help of molecular techniques.

ÖZ

YOĞURT KÜLTÜRLERİNİN İZOLASYONU, FENOTİPİK VE GENOTİPİK KARAKTERİZASYONU

Fermente süt ürünlerinin üretiminde starter olarak bilinen saf kültürler kullanılır. Yoğurt *L. delbrueckii* ssp. *bulgaricus* ve *S. thermophilus* bakterilerinin sütteki simbiyotik etkileşimleri sonucunda oluşur. Bakteriler laktozdan laktik asit üreterek sütün pıhtılaşmasını sağlarlar. Ürettikleri diğer ürünler de yoğurdun aroması, asitliği ve yapısına katkıda bulunur. Geleneksel olarak bir önceki yoğurtla yeni yoğurdu aşılama metodu yüzyıllardır kullanılmaktadır. Ancak değişken son ürünlerle sonuçlanan bu metod standart ürün almak zorunda olan endüstrinin ihtiyacına yanıt vermemektedir. Endüstri fizyolojik, biyokimyasal ve genetik olarak tanımlanmış kültür kullanımını tercih etmektedir.

Bu nedenle Anadolu'daki doğal yoğurt örneklerinden yoğurt kültürlerinin izolasyonu ve tanımlanması, halen starter kültürlerini yurt dışından alan Türk Süt ve Süt Ürünleri endüstrisi için önem taşımaktadır. Ayrıca Anadolu'daki laktik asit bakteri çeşitliliğinin koruma altına alınmasına da katkıda bulunacaktır. Bu bakış açısıyla, çalışmamızın amacını geleneksel yöntemle yapılmış olan doğal yoğurt örneklerinden starter kültürlerinin izolasyonu, biyokimyasal ve moleküler tanımlaması oluşturmaktadır.

Çalışmanın sonunda toplam 66 kok ve 71 basil izolatı elde edilmiştir. Biyokimyasal tanımlama sonuçlarına göre bütün basil izolatları *L. delbrueckii ssp. bulgaricus* olarak belirlenmiştir. Kok izolatları oldukça değişken şeker fermantasyon profilleri göstermiş olup, ancak 7 tanesi tam olarak S. thermophilus olarak tanımlanmıştır. Moleküler karakterizasyon, ribosomal RNA genlerinin 16S-ITS (Internal Transcribed Spacer) bölümünün amplifikasyonu ve RFLP'lerinin (Restriction Fragment Length Polymorphism) karşılaştırılmasına dayanmaktadır. Moleküler karakterizasyonda türe özgü PCR (Polimeraz Zincir Reaksiyonu) ve 16S dizileme analizleri doğrulama yapılmıştır. Moleküler metodlarla bütün izolatlar başarılı bir şekilde tanımlanabilmiştir.

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ssp.	: Subspecies
NFMS	: Non-fat milk solids
µm	: Micrometer
min	: Minute
g	:Gram
mg	:Miligram
kg	:Kilogram
L	: Liter
µl	:Microliter
h	: Hour
rpm	: Round per minute
LAB	:Lactic Acid Bacteria
S.	:*Streptococcus*
L.	: Lactobacillus
EPS	: Exopolysaccharide
cfu	: Colony forming unit
DVS	: Direct Vat Set
IU	: International Units
Mb	: Megabase
DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
rDNA	: Ribosomal DNA
rRNA	: Ribosomal RNA
dNTP	:Deoxynucleotide triphosphate
kbp	: Kilo base pair
PCR	: Polymerase Chain Reaction
RFLP	: Restriction Fragment Length Polymorphism
ARDRA	: Amplified Ribosomal Restriction Analysis
ATCC	: American Type Culture Collection
RAPD	: Randomly Amplified Polymorphic DNA
PFGE	: Pulsed Field Gel Electrophoresis
MRS	: de Man, Rogosa and Sharpe Medium

EDTA	: Ethylene Diamide Tetra Acetic Acid
ITS	: Internal Transcribed Spacer
TE	: Tris-EDTA
CTAB	: Cethyl Trimethyl Ammonium Bromide
TAE	: Tris Acetate EDTA
TBE	: Tris Borate EDTA
UPGMA	: Un-weighed pair group method with arithmetic averages
NCBI	: National Centre for Biotechnology Infirmation
SDS	: Sodium Dodecyl Sulfate
BSA	: Bovin Serum Albumin
UV	: Ultra Violet

CHAPTER 1

INTRODUCTION

1.1. The History of Yoghurt

For thousands of years yoghurt has been produced throughout the Middle East. Although no records are available regarding the origin, yoghurt is most likely evolved from the nomadic people living in the Middle East part of the world.

The production of milk in the Middle East was seasonal, being restricted to a few months of the year. The main reason for this limited availability of milk was the lack of intensive animal production. Farming was in the hands of nomadic people who moved from one area to another. Hence they were in wilderness away from cities where they could sell their animal produce. Another factor was the climate in the Middle East. At as high as 40°C, milk sours immediately under primitive conditions. The animals were hand-milked, no cooling of milk was possible, and contamination was unavoidable. Under these conditions transportation and keeping of milk for a long time was not possible. However, the nomadic people devised a fermentation process, which as a result led them to keep milk for long times. It was heating the milk over an open fire (Tamime and Robinson 1985).

Heating milk resulted in;

- Concentration of milk
- Modification of casein in milk
- Selection of thermophilic lactic acid bacteria resistant to high temperatures
- Destruction of pathogenic microorganisms
- Fermentation at slightly higher temperatures during cooling, and enrichment of thermophilic lactic acid bacteria

Soured milk with thermophilic lactic acid bacteria became the preservation method of milk, and other communities learnt of this technique. As a result the product “yoghurt”, coming from the Turkish name “Yogurt”, has been widely accepted (Tamime and Robinson 1985).

Today different kinds of products are emerging according to the market demands although the very basic properties of yoghurt remain the same. *Streptococcus thermophilus and Lactobacillus delbrueckii* ssp. *bulgaricus* grow synergistically in milk, increase the acidity by screeting lactic acid, and coagulate the milk proteins while producing the specific yoghurt aroma compounds.

1.2. Manufacture of Yoghurt

The basic steps in the manufacture of yoghurt:

1. Filtration of milk for the removal of debris.
2. Checking the presence of antibiotics that may affect the activity of the starter bacteria.
3. Standardization of milk for making good quality yoghurt

Firstly, the milk fat in yoghurt can vary from 0.5 to 4.5 % according to the market demand. Hence milk fat can be separated or fortified to the desired value for final product.

Secondly, non-fat milk solids (NFMS) is raised to the desired value. The NFMS value in milk differs from 8.5 to 9%. NFMS comprises 4.5% lactose, 3.3% protein (2.6% casein and 0.7% whey protein) and 0.7% mineral salts. The NFMS is vital for good quality product. Protein and mineral content contributes to gel structure and lactose is the energy source for bacteria. However the NFMS value in liquid milk is not high enough for strong gel structure and it should be raised to 13-18% value by adding skimmed milk powder or evaporating under vacuum.

4. Homogenization of milk

Homogenization is the size reduction of fat globules to 1-2µm. It prevents the segregation of fat during production and improves the incorporation of other ingredients such as skimmed milk powder. After the homogenization, the membrane of the fat globule is destroyed and lipase enzymes attacks to the destroyed globules. Hence milk should be heat treated immediately after homogenization to prevent lypolysis.

5. Heat Treatment

The processed milk is pasteurized at 90-95°C for 5-10 min by passing it through plate heat exchanger or at 80-85°C for 30 min in process vessel. Heat treatment destroys all the pathogenic bacteria, and inactivates enzymes such as lipase. It also denatures

whey proteins, β-lactoglobulin and α-lactalbumin. This phenomenon is very important for gel strength. Because a complex is formed between κ-casein and denatured β-lactoglobulin, which increases the hydrophilic nature of the casein. The complex between κ-casein and denatured β-lactoglobulin decreases also the synerysis and improves the stability of coagulum. Optimum temperature value is 85°C, since heat treatment at higher temperatures decreases the hydrophilicity of casein micelles.

6. Inoculation and Incubation

After the heat treatment, the milk is cooled to 43°C and inoculated with the starter culture. The bacilli to cocci ratio in yoghurt starter culture is 1:1 (Wouters 2002, Sokolinska and Pikul 2004). The inoculation amount can vary between 0.5-5 % but the optimum value is 2 %. The starter cells are mixed with milk by stirring. Then milk is dispensed into containers and incubated at 42-43°C for 3-4h. At the end of the incubation time, pH decreases to 4.5.

7. Cooling and Storage

The first alternative to end incubation is cooling the product as quickly as possible at an acidity of 1.2-1.4% (pH 4.3). Otherwise it will lead to overacidification, shrinkage of the protein gel and whey synerysis occurs on the surface. The second alternative for cooling is a controlled two stage cooling. In the first stage the temperature is dropped to about 37°C at the pH of about 4.6. The pH decrease is controlled with slow acidification and then it is further cooled to ≤10°C at a pH of 4.3. During cold storage at 4°C, viscosity of yoghurt increases for 1-2 days. Hydration leads to much firmer gels and casein miscelles continue to stabilize.

1.3. Types of Yoghurt

Yoghurts in the market can be classified according to their chemical composition, manufacturing method, flavoring and the type of postincubation process (Shah 2003). According to chemical composition, they are classified as full-fat, reduced-fat or low-fat yoghurt.

Table 1.1. Types of Yoghurt

	Full-fat yoghurt	**Reduced-fat yoghurt**	**Low-fat yoghurt**
Fat %	≥ 3	0.5-2	≤ 0.5
Non-fat % milk solids	≥ 8.25	≥ 8.25	≥ 8.25
Titratable acidity %	≥ 0.9	≥ 0.9	≥ 0.9
pH	≤ 4.5	≤ 4.5	≤ 4.5

- According to the method of production they can be grouped as set-type and stirred-type. Set-yogurt is fermented in a retail container, however for stirred yoghurt fermentation occurs in tanks and the gel is broken before cooling and packaging by stirring. For example "Ayran" is the stirred yoghurt of low viscosity.
- In terms of flavoring, yoghurt can be classified into subgroups. Plain yoghurt is the known traditional type. Fruit yoghurts are obtained by the addition of fruit particles, and, lastly, flavored yoghurt is manufactured by the addition of sweetening and coloring compounds.
- Finaly the postincubation process includes concentration, pasteurization, freezing, and drying of the yoghurt after fermentation.

In recent years an additional group "according to the type of culture used" should also be included to this classification after the use of probiotics. A wide variety of probiotic cultures are available across the world. Probiotic yoghurts are produced by the incorporation of other lactic acid bacteria, helpful for our digestive or immune system to the starter cultures of *Streptococcus thermophilus and Lactobacillus delbrueckii* ssp. *bulgaricus.* The most common probiotic species are *Lb. acidophilus, Lb. casei, Lactobacillus GG, Lb. plantarum, Lb. reuteri, B. bifidum, B. longum, B. reve*.

1.4. Yoghurt Starter Cultures

The microflora of traditional yoghurt from the Middle East has been examined, and it was found that Gram-positive rods and cocci were predominant. Although the

nomenclature has changed over the years, it is now agreed that the bacteria essential for the production of yoghurt are *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp *bulgaricus* (Robinson 2002). These two species of bacteria are very well known starter microorganisms of yoghurt fermentation. They grow synergistically in milk, increase the acidity by screeting lactic acid, and coagulate the milk proteins while producing the specific yoghurt aroma compounds. In some countries, there is a legal requirement for *Lactobacillus delbrueckii* ssp *bulgaricus* to be included in the starter culture of any dairy product labeled as 'yoghurt', because its typical flavour of yoghurt depends on the presence of *Lactobacillus delbrueckii* ssp *bulgaricus.* However in other countries, such as Australia, other thermophilic lactic acid bacteria, such as *Lactobacillus helveticus* and *Lactobacillus jugurti* are also permitted. According to the food legislations of some countries, the bacteria also should be abundant and viable during consumption.

In literature, starter cultures have been defined as preparations of one or more strains of one or more microbiological species (Wigley 1999). Dairy starters are the most crucial component in the manufacture of high-quality fermented milks. The cultures are harmless, food-grade, active microorganisms which impart desirable and predictable flavour and texture to milk product. Depending on their concentration, they are inoculated directly or subcultured before use.

Historically, a starter culture is simply a sample of fermented food. These types of starters are called artisanal or undefined cultures. They contain historically tested blends of starter culture organisms. Often the actual identities of the organisms present in a mixed culture are not known, and the individual species may not have been characterized microbiologically or biochemically. The proportion of different organisms in a mixed culture may not be constant from one product to another. Thus the main disadvantage of artisanal cultures is that they may yield products of inconsistent quality. In addition, fermentation rates may vary from day to day, affecting production schedules. In large production facilities where precise schedules are essential and consistent product quality is expected, artisanal starters can not be used. Instead defined cultures have become predominant (Durso and Hutkins 2003).

Defined cultures contain physiologically, biochemically and genetically characterized strains, which are used individually or as blends. Most of the defined strains have been isolated from wild or artisanal cultures (Hebert et al. 2000). They are characterized and screened for the desirable traits. Hence they give consistent quality,

and flexibility to modify the production according to demands, e.g. high productivity, quality and safety.

1.4.1. *Streptococcus thermophilus*

Streptococcus thermophilus is the only starter dairy streptococci in Streptococcus genus. According to DNA-DNA homology and membrane fatty acid profile studies, researchers have thought that *S. thermophilus* should be reclassified as *S. salivarius* ssp. *thermophilus.* However *S. thermophilus* and *S. salivarius* are not found in the same ecological niche and these possess large number of physiological differences. In 1987, *S. thermophilus* has been restored to species level again as a result of more sophisticated homology studies, and huge phenotypic differences (Zirnstein and Hutkins 1999).

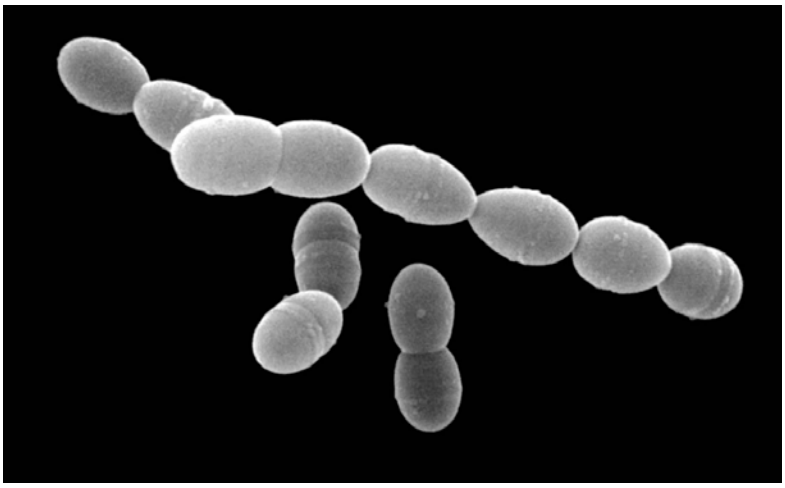

Figure1.1. Electron microscopic image of *S. thermophilus*
(Source: Durso and Hotkins 2003)

General characteristics;

- Gram positive, non-motile coccus
- Spherical/ovoid cells of 0.7-0.9µm diameter
- Occurs in pairs or in long chains of 10-20 cells
- Homofermentative,L(+) lactic acid as the major end product
- Facultative anaerobe

- Catalase negative
- Lacks cytochromes
- Optimum growth temperature is between 40-45°C, a minimum at 20-25°C and maximum at 50-52°C.
- Thermotolerant, survives at 60°C for 30 min
- Weak or no growth at 2 % NaCl
- Does not utilize arginine
- Lacks group specific antigen
- G-C mol ratio is 37-40%
- Above 10g of lactic acid/kg of yoghurt, the growth reaches exp8-9 and, the metabolism of bacterium ceases. The final pH in broth culture is 4-4.5.

The loss or gain of alleles for metabolic functions is common. Lactose, glucose, fructose and sucrose are fermented by *S. thermophilus* (Pearce and Flint 2002, Gobbetti and Corsetti 1999, Tamime and Robinson 1985). According to some other literature, however, lactose, glucose, fructose and mannose are fermented but sucrose, galactose and maltose are strain specific (Robinson 2002).

S. thermophilus prefers the disaccharides lactose and sucrose, and its growth on the constituent monosaccharides, glucose, fructose and galactose is slower than the disaccharides. Some researchers have suggested that the transport systems required to accumulate monosaccharides might be absent or has low activity. Now, it appears that it is dependent on the availability of the necessary transport system. It has been also observed that when cells are grown on excess lactose, galactose accumulates in the medium. Even those strains, which ferment free galactose, do not ferment in the presence of glucose (Hutkins and Ponne 1991). This phenomena has been explained with the findings that the enzymes of the Leloir pathway for galactose metabolism are present in *S. thermophilus,* however their activities are very low and the activity of the first enzyme galactokinase is undetectable under normal growth conditions (Grossiord et al. 1998). Recently it has been shown that the genes coding for these enzymes are present but their transcription is repressed (Zirnstein and Hutkins 1999).

S. thermophilus has limited proteolytic activity and requires free amino acids for its growth. These are glutamic acid, histidine, cysteine, methionine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine. However free aminoacids naturally found in milk are not sufficient. The free amino acids are supplemented during heat treatment

in milk or the absorption of short-chain peptides released by the breakdown of milk proteins by *Lactobacillus delbrueckii* ssp. *bulgaricus* (Pearce and Flint 1999).

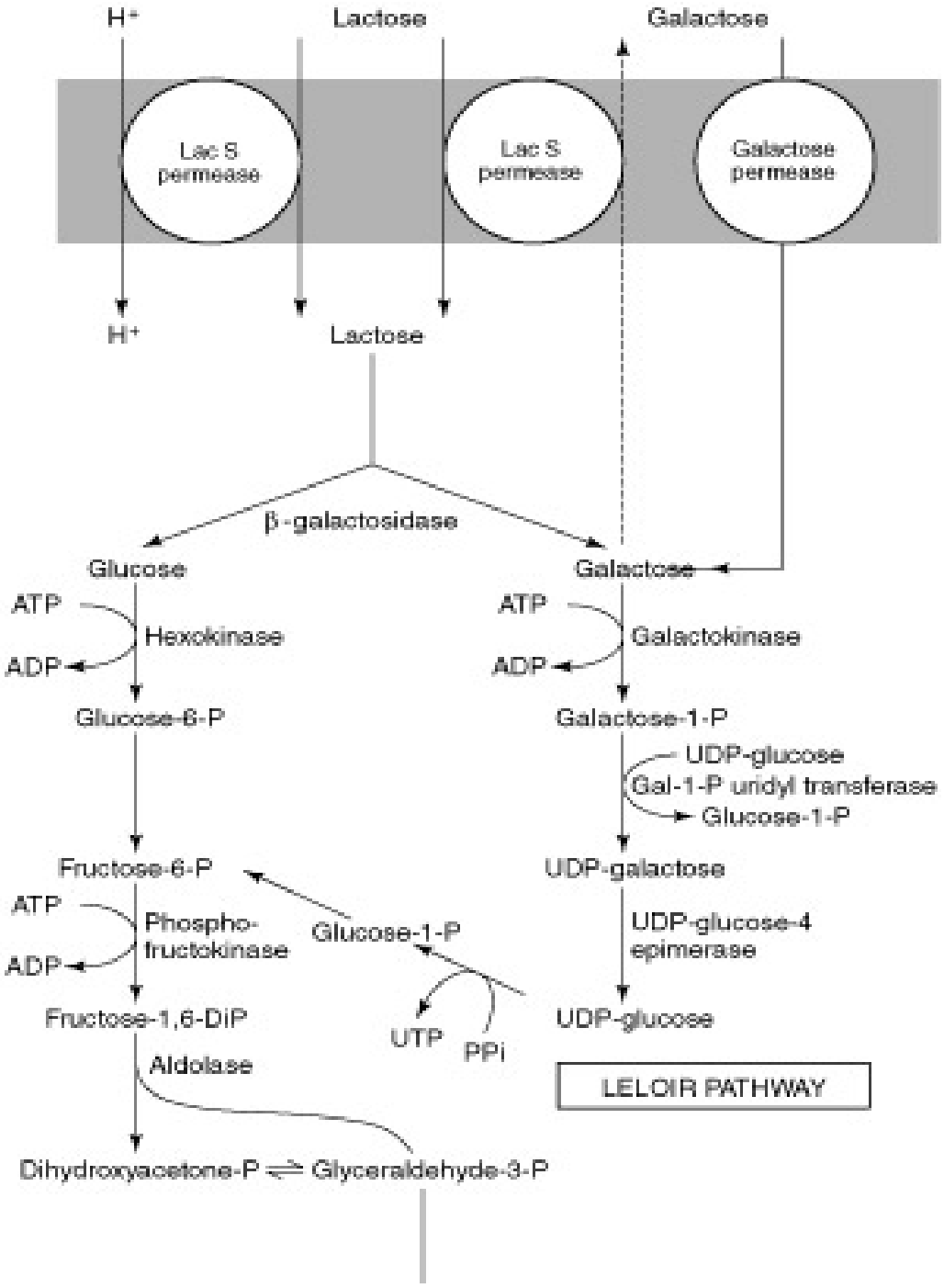

Figure 1.2. Pathways for lactose and galactose transport and their metabolism by *S.thermophilus*, (Source: Zirnstein and Hutkins 1999)

1.4.2 *Lactobacillus delbrueckii* ssp. *bulgaricus*

Lactobacillus delbrueckii ssp. *bulgaricus* is a member of *Lb. delbrueckii* group. This group previously consisted of four separate species with similar phenotypes. These were *Lb. delbrueckii, Lb. leichmanii, Lb. lactis* and *Lb. bulgaricus*. However the DNA homology above 80% led them to be reclassified as single specie with three subspecies. *Lb. delbrueckii* ssp. *lactis* includes the previous species *Lb. lactis* and *Lb. leichmanii*. The remaining two subspecies are *Lb. delbrueckii* ssp. *delbrueckii* and *Lb. delbrueckii* ssp. *bulgaricus* (Limsowtin et al. 2002).

- Gram positive, non-motile rods
- Rod shaped with rounded ends, 0.5-0.8 by 2-9μm.
- Single or short chains of 3-4 cells. Long chains can also be observed at late stationary phase and they are often arranged in palisades.
- Contains metachromic granules. These internal granulations are observed by Gram or methylene blue staining especially for old cells.
- Homofermentative, D(-) lactic acid is the major end product
- Facultative anaerobe and very sensitive to O_2 exposure
- Catalase negative
- Lacks cytochromes
- Optimum growth temperature is 45°C; minimum and maximum growth temperatures are 22°C and 50-55°C, respectively.
- Ferments lactose, fructose, glucose
- Does not utilize arginine
- G-C mol ratio of 49-51%
- Above 10g of lactic acid/kg of yoghurt, the growth reaches exp 8-9 and the metabolism of bacterium ceases. The final pH in broth culture is 4-4.5.

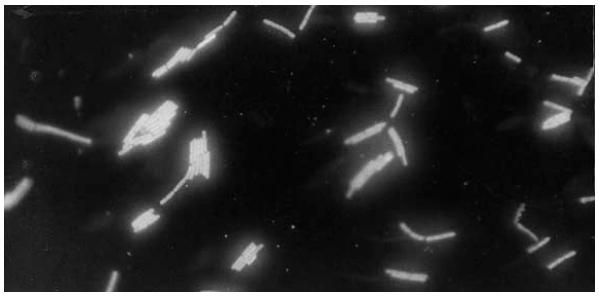

Figure 1.3. Acrydine orange staining of *Lactobacillus delbrueckii* ssp*. bulgaricus* at early stationary phase of growth (Source: Teixeira 1999)

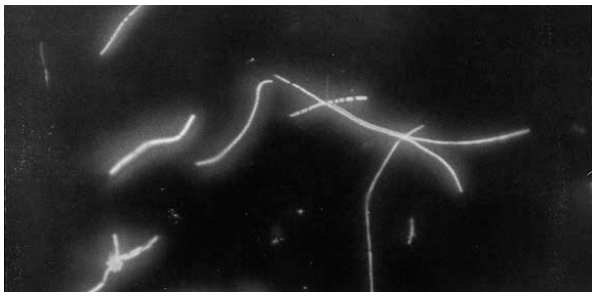

Figure 1.4. Acrydine orange staining of *Lactobacillus delbrueckii* ssp*. bulgaricus* at late stationary phase of growth (Source: Teixeira 1999)

Lactobacillus delbrueckii ssp*. bulgaricus* ferment lactose and possesses β-galactosidase enzyme rather than phospho-β-galactosidase. The chromosomally encoded nucleotide sequence for β-galactosidase has been determined. *Lb. delbrueckii* ssp. *bulgaricus* is galactose negative. As in *S. thermophilus,* lactose, is transported into the cell with the expulsion of galactose via an antiport system (Teixeira 1999).

Proteolytic systems of lactobacilli are composed of proteinases and peptidases with different cellular locations. The proteinases of *Lb. delbrueckii* ssp. *bulgaricus* are mainly found on the cell wall and they are regulated by temperature and growth phase. Its major source of amino acids during growth is casein. The high proteolytic activity of *bulgaricus* is important in yoghurt production because the peptides which are released by *bulgaricus*, stimulates the growth of *S. thermophilus* as well. Although its proteinase activity is high, its peptidase activity is limited and it relies on the metabolism of peptides by *S. thermophilus* for a source of free amino acids (Robinson 2002).

1.4.3 The Associative Growth of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*

For thousands of years these two bacteria have been used to ferment milk to yoghurt together. There are important reasons for this synergistic relationship. It is based on the metabolic compatibility between the two species. Studies have shown that combined culture of starter bacteria produce much higher acidity than the isolated strains. While the combined culture produces an acidity of >10g/L within 4 h, the values in the isolated strain of *S. thermophilus* is 4g/L and 2g/L for *Lb. delbrueckii* ssp. *bulgaricus* (Robinson 2002).

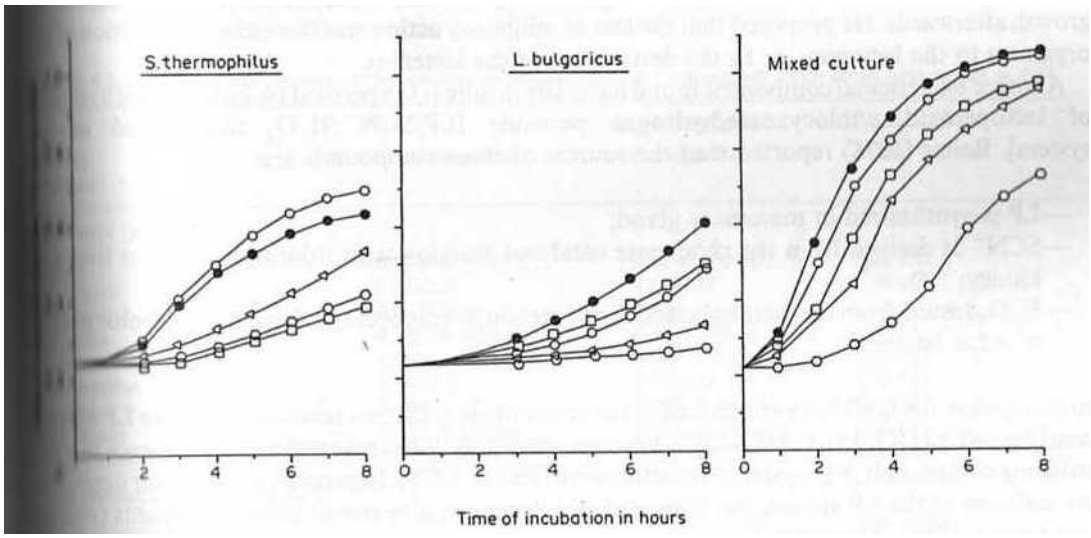

Figure 1.5. The Associative growth of Yoghurt Starter Bacteria
(Source: Tamime and Robinson 1985)

S. thermophilus grows more rapidly than *L. delbrueckii* ssp. *bulgaricus*, at least initially and produces lactic acid. *S. thermophilus* also releases CO_2 from the breakdown of urea and formic acid. It uses up the oxygen in the medium, which let the oxidation-reduction potential be more favorable for bacilli. The increased acidity (around pH of 5,4), CO_2, and formic acid stimulates the growth of bacilli, which is more acid-tolerant than the *S. thermophilus,* and produces large amount of lactic acid. However the growth of *S. thermophilus* is dependent on the metabolic function of bacilli also. *Lb. delbrueckii* ssp. *bulgaricus* has high proteolytic activity. It screets extracellular proteinases that

hydrolyze casein and other milk proteins and produces the essential amino acids especially valine for *S. thermophilus,* which has poor proteolytic activity.

An incubation temperature of 42°C is the optimum temperature of the symbiotic growth. The optimum growth temperature of *S. thermophilus* is 37°C, while the optimum growth temperature of *Lb. delbrueckii* ssp. *bulgaricus* is 45°C. When the incubation temperature is increased above 42°C, the growth of lactobacilli will be favored, while the temperatures below 42°C lead to the increased growth of streptococci. Temperature shift results in a deviation in the ratio of cocci to bacilli. For the optimum yoghurt flavour, the ratio should be 1:1 (Shah 2003).

1.5. The Role of Starter Culture on Yoghurt

The crucial point in yoghurt fermentation is the precipitation of casein miscelles as a result of the increased acidity. Casein miscelles are destabilized at pH 4.5 and the complete precipitation occurs at pH 4.6, which is the isoelectric point of casein. Hence the main role of starter in the process is to produce lactic acid homofermentatively from lactose and lowering the pH below 4.5 in 4 h.

Main characteristic and distinct flavour of yoghurt comes from the other metabolites produced by the protocooperative action of lactobacilli and streptococci. For example, acetaldehyde at levels up to 40mg/kg is the major contributor to the flavour. The major pathway for the production of aldehyde is the conversion of threonine to glycine and acetaldehyde by threonine aldolase. The proteolytic activity of *Lb. delbrueckii* ssp. *bulgaricus* is important for acetaldehyde production, because threonine mainly results from the activity of the peptidases of lactobacilli. However, the conversion of threonine to acetaldehyde could be a function of both species. However, studies have showed that the activity of the threonine aldolase produced by *S. thermophilus* decreases drastically when the incubation temperature is above 30°C, while the enzyme from lactobacilli is not affected (Robinson 2003). Therefore it appears that the source of acetaldehyde is lactobacilli. However, Teixeira has stated that the acetaldehyde is derived from threonine via the threonine aldolase of *S. thermophilus* (Teixeira 1999). Another study has also showed almost linear relationship between the level of acetaldehyde produced during milk fermentation and the threonine aldolase activity measured in different *S. thermophilus* strains (Chaves, A.C.S.D.et al. 1999).

This clearly shows the importance of this enzyme in flavour formation in yoghurt. The other contributors to the flavour with starter origin include diacetyl, acetone, acetoin, organic acids such as acetic, lactic acid, fatty acids and free amino acids but their effects have not been well understood yet.

Another important characteristic of yoghurt, for consumer appeal, is the consistency or viscosity of product, which gives a good mouth feel. The milk solids non fat content of milk or stabilizers contribute to viscosity of the product but exopolysaccharides (EPS) produced by starter bacteria gain special importance especially for the countries where the use of stabilizers are forbidden. Both *S. thermophilus* and *L. bulgaricus* can produce extracellular polysaccharides (EPS). The EPS increases the viscosity by binding free water and preventing the gel fraction and whey synerysis. The EPS producing strains are known as ropy or slime producers. The concentration and composition of EPS are dependent on many factors such as, temperature, pH, vitamins, growth phase, carbohydrate source and the strain (Crow and Curry 2002). EPS are mainly composed of glucose, galactose and rhamnose units. In practice, the amount of EPS does not necessarily correlate with the viscosity. This may be because of the conformation changes and interactions with casein at low pH, or because of the glycohydolases that hydrolyze polymer (Pearce and Flint 2002). The ropyness is very unstable. Unstability is not due to the loss of a plasmid, since many polysaccharide producing strains do not contain plasmids. This is in contrast to the plasmid-carried ropy character of mesophilic lactic acid bacteria confirmed by several groups (Zirnstein and Hutkins 1999).

The starter culture also improves nutritional value and digestibility of yoghurt as probiotic. Although it is evident that *S. thermophilus* do not colonize the intestine, consumption of viable cells can enhance lactose digestion of lactose intolerant people. Such individuals have also been shown to tolerate yoghurt better than other dairy products containing the same amount of lactose. One possibility for this phenomenon is that the viable cells of *S. thermophilus* survive in the stomach and are lysed in the gastrointestinal tract. The intracellular β-galactosidases are released and hydrolyze lactose. Lactose does not reach the large intestine and the symptoms of lactose intolerance does not occur (Zirnstein and Hutkins 1999). It has been reported that *Lb. delbrueckii* ssp. *bulgaricus* has a preservative effect on the product not only because of the production of lactic acid and hydrogen peroxide, but also by the help of the antimicrobial compound (bacteriocin) it produces. The compound, namely Bulgarican,

is inhibitory towards both Gram-positive and Gram-negative bacteria. Some Inhibitory compounds against *Staphylococcus* and *Clostridium* have also been found (Teixeira 1999).

1.6. Starter Culture Systems Used for Yoghurt Production in Dairy Industry

Yoghurt starter cultures are mass-produced in fermenters under aseptic conditions for dairy industry. Milk or whey based media are most often used for commercial production but molasses and corn-syrup can also be used as the basal media. Vitamins, especially vitamin B, and some specific amino acids are also required for optimum growth of starter cultures. Acids produced during fermentation can inhibit the further increase in cell density, so the pH is controlled by the addition of alkalines, usually gaseous NH_3, NH_4OH, Na_2CO_3, or KOH. Lactic acid bacteria (LAB) are harvested at late log phase or early stationary phase, but the optimum harvest time should be determined for each specific organism. Cells are grown at a cell density of 10^9-10^{10} and are then packaged in liquid form, frozen or freeze-dried (lyophilized). For both frozen and lyophilized cultures, cryoprotectants should be used to maintain cell viability. Common cryoprotectants are glycerol, lactose, sucrose, trehalose, ascorbate and glutamate (Durso and Hutkins 2003).

Liquid daily propagated cultures are prepared from the mother culture and purchased with some time intervals, depending on the success of factory for the culture maintenance. Starter cultures are activated and propagated for production by daily subculturing. The use of liquid cultures is now restricted to small factories (Wigley 1999).

Frozen cultures can be manufactured as normal, concentrated or direct set type. Normal frozen cultures are supplied in bottle , which has been frozen after inoculation. After shipping in insulation, bulk starter is propagated from the overnight thawed culture. With respect to normal frozen cultures, frozen concentrated cultures include 10^{10}-10^{11}cfug^{-1} which allows 70ml of culture for 1 ton medium for bulk propagation. Frozen concentrated cultures are transported in insulated aluminum cans containing solid CO_2 to maintain the temperature at -70°C. The shelf-life of culture is usually 3 months at -50°C. For the production of frozen direct-to-vat cultures, cells are produced

with high-cell-density fermentations, concentrated prior to freezing and washed to remove the spent medium. Cell densities can be as high as 10^{11}-10^{12} and a single can can be sufficient to ferment 2000 kg of milk for direct yoghurt fermentation. In general, frozen starter cultures are immediately active on thawing. They do not need lag phase after inoculation.

Lyophilized cultures are also manufactured for both bulk starter propagation and direct-to vat propogation. For lyophilization the grown and harvested cells are freeze-dried under vacuum. In freeze-drying water is removed by sublimation. It not only concentrates culture but also preserves cell by lowering water activity. Lyophilized cells are stored at +4°C and some are also stable at room temperature. The cells are usually packaged in oxygen-impermeable materials. A major disadvantage of freeze-dried culture is the longer lag phase. An additional 30-60 min is required to complete fermentation.

Direct-vat-set (DVS) type cultures are more popular. Because DVS cultures eliminate the risk of phage contamination and maintain the strain balance in mixed strain cultures (Surono and Hosono 2002).

1.7. Factors Leading to Inhibition of Yoghurt Starters

Starters should able to propagate and produce lactic acid very fast for yoghurt fermentation. However some factors may lead the fermentation to fail or slow acid production. In addition to substandard product and economic losses, pathogenic organisms found in raw material can grow in the end-products at high pHs.

Bacteriophages are the major cause of slow acid production or complete inhibition in dairy fermentations. Until recently, phages of mesophilic lactic acid bacteria which are used for cheese production took the most attention. However due to the huge increase in world production of yoghurt, and the centralization of yoghurt production, the phage problems of yoghurt starters become more common and they lead to serious economic losses. Both *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains are susceptible to virulent phages. Lysogenic phages of both strains have also been reported (Josephsen and Neve 2004). Problems are observed especially when starters contain a single strain or when the same culture is re-used over an extended period. Some of the solutions in order to overcome phage problems include good

aseptic techniques, rotating the starter cultures, using phage-resistant starter cultures or using multiple strain starters, each of which is resistant to different host-specific phages (Teixeira 1999). Phage inhibitory media are also used as a means of phage protection. In 1956, the US Department of Agriculture introduced the idea of using substances to chelate Ca^{++} and Mg^{++} required for successful phage adsorption to the bacterial cell. For example, the phosphate salts have buffering capacity for fermentation (Durso and Hutkins 2003).

Many antibiotics are used to control mastitis in milk-producing cows which lead to excretion of antibiotics to milk. An effect of antibiotic residues in yoghurt is to cause a breakdown in symbiotic relationship or a slow down in acid development (Tamime and Robinson 1985). *S. thermophilus* is sensitive to antibiotics like penicillin, streptomycin, neomycin and ampicillin. Levels of contamination of 0.004 International Units of penicillin can inhibit the cell wall development of *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* is more tolerant to penicillin (0.02 IU). The presence of antibiotics can easily delay the fermentation, hence it is essential to check the milk for the presence of antibiotics. The residues of detergents and disinfectants like quaternary ammonium compounds, iodophors, hypochlorites and hydrogen peroxide used for cleaning of dairy equipment can also reduce the activity of starter cultures (Surono and Hosono 2002).

Growth of starters can be inhibited by natural compounds such as lactins and agglutinins present in milk. However these compounds are heat sensitive and destroyed during pasteurization. Phagocytosis occuring as a result of leucocytes in mastitic milk and environmental pollutants like insecticides have also crucial effect on inhibition (Teixeira 1999).

Changes in the activity of a culture such as the rate of acid production or of aroma-flavour production occur as a result of the routine subculturing or numerical imbalance between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus.* The incompatibility between strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* may also result in complete destruction of protocooperation between these species (Robinson 2003).

1.8. Genetics of Yoghurt Starter Cultures

Recently, complete physical maps of *S. thermophilus* ST1 and A054 chromosomes has revealed genome sizes of 1.75 and 1.82 megabase respectively. The nearest relative of *S. thermophilus* in lactic acid bacteria is *Lactococcus lactis* but its genome is significantly smaller than the 2.35 genome size of *Lactococcus lactis*. Mapping number of clones of *S. thermophilus* A054 after serial subculturing showed the instability of its genome. One of the *rrn* operon was lost. Genes coding for peptidase utilization, restriction-modification systems have been identified but the most studied genes are the ones coding for carbohydrate catabolism. These studies have revealed that the expression of the *lac* and *gal* genes is regulated at the transcription level and repressed by glucose (Zinstein and Hutkins 1999). Ten percent of *S. thermophilus* genes are pseudogenes (not functional) and one third of these pseudogenes are for sugar metabolism (Vadeboncoeur and Moineau 2004).

S. thermophilus have fewer and smaller plasmids than other lactic acid bacteria. Actually the plasmid-free state is more common. In mesophilic lactococci, plasmids determine the metabolic activity crucial in fermentation; however the plasmids of *S. thermophilus* do not posses the genes coding for metabolic functions. In a study of 23 *S. thermophilus* strains, five of them were found to posses 4-18 copies of a single, small (2.2-3.5kb) plasmid and the remaining strains were plasmid-free. The largest *S. thermophilus* plasmid studied is 25.5 kb. Altering this plasmid did not affect the utilization of carbohydrate or antibiotic resistance profile (Pearce and Flint 1999).

A recent genomic study on *Lactobacillus delbrueckii* subspecies revealed the genetic heterogeneity of the subspecies. 16S rDNA sequence variations (cytosine to thymine oriented mutations) suggested that all three *delbrueckii* subspecies evolved from a common ancestor and *L. delbrueckii* ssp. *lactis* is still very close to this ancestor and *L. delbrueckii* ssp. *bulgaricus* is quite different, having accumulated the maximum number of mutations. Among the mutations occurring during the speciation of *delbrueckii* subspecies, cytosine to thymine conversion at position 686, generated an *Eco*RI site, which allows the differentiation of this species from *delbrueckii* group (Germond et al. 2003).

Another study on the complete genome sequence of *L. delbrueckii* ssp. *bulgaricus* indicates the ongoing reductive evolution of bacteria. Several unique

features of its genome support this hypothesis. Exceptionally high numbers of rRNA and tRNA genes may indicate that *L. bulgaricus* genome is in the recent phase of size reduction, in agreement with the observed high frequency of gene inactivation and elimination. A much higher GC content at codon position 3 than expected on the basis of overall GC content suggests that the genome is evolving to a higher GC content. The presence of a 47.5 kbp inverted repeat in the replication termination region, an extremely rare feature in bacterial genomes, may be interpreted as a transient stage in genome evolution (Guchte et al. 2007).

The studies on lactose metabolism showed that only two enzymes namely lactose antiport permease and β-galactosidase for the uptake and hydrolysis are involved in the *lac* operon. However, the phospho-β-galactosidase, regulating sugar uptake and hydrolysis, has never been identified for *bulgaricus* (Lapierre et al. 2001). In *L. delbrueckii ssp. lactis*, the regulation of the *lac* operon expression was shown to be under the control of a repressor encoded by the *lacR* gene . However, in *L. delbrueckii* ssp. *bulgaricus,* control of *lac* gene expression was lost and and β-galactosidase is produced constitutively. Sequencing of *lacR* gene from *bulgaricus* revelaed that it was inactivated by several insertion/ deletion events. Besides, the studies on the *lac* operon of *L. delbrueckii* ssp. *delbrueckii* revealed that the entire *lac* operon was deleted (Germond et al. 2003).

1.9. Identification Methods for Yoghurt Starters

Taxonomy includes three interrelated topics: classification nomenclature and identification. Classification is the grouping of organisms into taxa on the basis of phylogenetic relationships. Nomenclature is the assignment of names to taxa according to the rules of international community. Identification determines to which of the taxonomic groups a new isolate belongs. Bacteria are classified only according to properties shown in type strains as the reference. The type strains are authentic but they may not all the time be typical. During identification reference strains are very important to reveal the comparisons. These strains are preserved in and available from culture collections (Morata et al.1999).

Identification needs both the use of biochemical and molecular biology methods.

1.9.1. Biochemical Identification

In traditional identification, most commonly used characteristics are morphology, staining reactions, nutritional requirements, cell wall chemistry, ability to use different energy sources, fermentation byproducts, gas requirements, temperature and pH tolerance, antibiotic sensitivity, pathogenicity, immunological characteristics and habitat (Morata et al.1999).

Some of these characters are used to identify specific bacterium or a group of bacteria but the difficulty here is to determine which attribute should be used for the identification of a given organism.

1.9.1.1. Biochemical Identification of *Lb. delbrueckii* ssp. *bulgaricus*

Lb. delbrueckii. ssp. *bulgaricus* is a member of the genus *Lactobacillus.* A number of traits distinguish members of the *Lactobacillus* genus from other lactic acid bacteria (Table 1.2).

Table 1.2. Classification of Lactic Acid Bacteria, (Source: Batt 1999)

Character	Carno	Lactob	Aeroc	Enteroc	Lacto/ Vagno	Leuco/ Oenoc.	Pedio	Strepto	Tetragen	Weissella
Tetrad formation	–	–	+	–	–	–	+	–	+	–
Co_2 from glucose	–	+/–	–	–	–	+	–	–	–	+
Growth at 10°C	+	+/–	+	+	+	+	+/–	–	+	+
Growth at 45°C	–	+/–	–	+	–	–	+/–	+/–	–	–
Growth at 6.5% NaCl	ND	+/–	+	+	–	+/–	+/–	–	+	+/–
Growth at 18% NaCl	–	–	–	–	–	–	–	–	+	–
Growth at pH 4.4	Ns	+/–	–	+	+/–	+/–	+	–	–	+/–
Growth at pH 9.6	–	–	+	+	–	–	–	–	+	–
Lactic acid	L	D,L,DL	L	L	L	D	L,DL	L	L	D, DL

Lactobacillus is the largest genus and it is also reflected by the wide range of the G-C content of DNA from 32-53 mol% which is a span twice as large as it is usually accepted for a single genus. The traditional Orla-Jensen classification scheme with three sub-grouping is still in use for the biochemical characterization of lactobacilli although some modifications in the definitions of the subgroups have been made (Axelsson 2004).

Orla and Jensen have divided the lactobacilli into three sub-groups namely, *Thermobacteria, Streptobacteria,* and *Betabacteria*. This classification is based on the homo and heterofermenting ability during glucose fermentation.

- *Thermobacteria* are obligate homofermenters and hexoses are fermented to lactic acid by the Embden-Mayerhof pathways. However pentoses or gluconate are not fermented.
- *Streptobacteria* are facultative heterofermenters, and hexoses are fermented to lactic acid by the Embden-Mayerhof pathways but pentoses are fermented to lactic acid, acetic acid and via an inducible phosphoketolase
- *Betabacteria* are obligate heterofermenters, both hexoses are fermented to lactic acid, acetic acid (ethanol) and CO_2 and pentoses are fermented to lactic and acetic acid. Both pathways involve phosphoketolase (Kandler and Weiss 1992). Some special characteristics of these sub-groups are given below.

Table 1.3. Classification of the *Lactobacillus* Subgroups, (Source: Carr et al. 2002)

The Lactobacillus Subgroups (Orla and Jensen)
Streptobacteria, Betabacteria, and Thermobacteria

	Growth Temperatures		Gas from glucose	Gas from gluconate	Arginine hydrolysed	Ferment pentose substrates	Thiamine requirement	Lactic acid from glucose
	15°C	45°C						
Thermobacteria	-	+	-	-	-	-	-	+
Streptobacteria	(+)	(V)[25,40]	-	+	(-)[3]	+	-	+
Betabacteria	(+)	(V)[25,40]	+	+	+	+	+	+

	Predominant Lactic acid type	Fructose fermented & reduced to mannitol
Thermobacteria	L(+), D(-), DL,	-
Streptobacteria	L(+), D(-), DL,	-
Betabacteria	DL	+

*+ indicates a positive test result; -. indicates a negative test result; (+/-) indicates most strains positive, an occasional strain negative; (-/+) indicates most strains negative, an occasional strain positive; V indicates variable, some produce (+) or (-) results; L(+) indicates levo-lactic acid; D(-) indicates dextro-lactic acid; DL indicates a mixture of D and L lactic acid; ND indicates no data is available.

Lactobacillus delbrueckii is a *thermobacterium* and it is the type species of the genus *Lactobacillus.* It includes three subspecies: *delbrueckii, bulgaricus* and *lactis*. They present different fermentation patterns and require different ecological nitche. Although *bulgaricus* and *lactis* grow in milk, *delbrueckii* colonizes vegetable sources. It is unable to ferment lactose and degrade casein, so it cannot grow in milk (Germond et al. 2003).

Phylogenetically the nearest *Thermobacteria* to *L. delbrueckii* ssp. *bulgaricus* different from the subspecies are *L. helveticus,* and *L. acidophilus.* The Taxonomic Subcommittee of the Lactobacilli has merged *Lactobacillus jugurti* into *L. helveticus. L. leichmanii* and *Lactobacillus lactis* have been reclassified as a single subspecies, namely *L. delbrueckii* ssp *lactis* (Carr et.al. 2002).

Most *thermobacteria* are long straight bacilli, however lactis and bulgaricus may palisade. *L. bulgaricus* has interesting microscopic morphology like "corkscrew form". Metachromic granules are observed for lactis and bulgaricus strains after methylene blue staining. *L. delbrueckii* ssp *bulgaricus* is known to catabolize a much lower number of carbohydrates than *L. delbrueckii* ssp *lactis*. In a study, it was shown that growth on lactose was faster than on glucose, mannose, and fructose for the 22 tested strains (Chervaux, C. et.al. 2000). Sugar fermentation patterns of the most related bacteria are presented (Table 1.4). Different authors have given different sugar fermentation profiles, which reflect the instability of biochemical traits.

Table 1.4. Classification Scheme for *L. delbrueckii* Subspecies

(Source: Frank Catt et al. 2002, [1]:Tamime 1985, [2]:Robinson 2002, [3:] Teixeira 1999, [4]: Crow 2002)

Strain	Peptidoglycan type	G-C Content(mol%)	Lactic Acid Isomer	Growth (15/45°C)	NH_3 from Arginine	Amygdalin	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Mellibiose	Raffinose	Salicin	Sucrose	Trehalose	Fructose	Glucose	Sorbitol	Esculin
L. delbrueckii ssp.*bulgaricus*	Lys-Dasp	49-51	D	-/+	-	-	-	-[3,4]/d[1,2]	+[1,2,3,4]	-	-	-[2,3,4]/+[1]	-	-	-	-	-	+[2,3,4]/d[1]	+[1,2,3,4]	+/-[1,2,3,4]	+/-[1]
L. delbrueckii ssp. *lactis*	Lys-Dasp	49-51	D	-/+	d	+/-[1]	d	d	+	+	-	+	-	-/d[1]	+	+	+	+	+	+/-[1]	+/-[1]
L. delbrueckii ssp. *delbrueckii*	Lys-Dasp	49-51	D	-/+	d	-	d	-	-	d	-	+	-	-	-	+	d	+	+	+/-[1]	-
L. helveticus	Lys-Dasp	38-40	DL	-/+	-	-	-	+	+	d	-	d	-	-	-	-	d	+/d[1]	+	+/-[1]	+/-[1]
L. acidophilus	Lys-Dasp	34-37	DL	-/+	-	+	+	+	+	+	-	+	d	d	+	+	d	+	+	+/-[1]	+[1]

Symbols: (+): 90% or more strains are positive, (-): 90% or more strains are negative, (d):11-89% of strains are positive.

1.9.1.2. Biochemical Identification of *S. thermophilus*

The genus *Streptococcus* includes Gram positive bacteria with similar metabolic properties but they live in different habitats and have many physiological differences. In the past two decades, several important *Streptococcus* species have been reclassified as members of recently named genera *Enterococcus* and *Lactococcus*. The only dairy *streptococcus* remained is *S. thermophilus.*

Streptococci grouped as "oral", "pyogenic" and "other streptococci". "Oral" streptococci is also subdivided into four groups; *S. mutans*, *S. mitis*, *S. anginosus* and *S. thermophilus* groups (Gobbetti and Corsetti 1999). Although *S. thermophilus* is a member of "*S. thermophilus* group" phylogenetically, it is the only bacterium in Streptococci with dairy origin. The Gram positive and cocci genera sharing the same habitat with *S. thermophilus* includes enterococci, lactococci, pediococci and leuconostocs. The pediococci is readily distinguished from other genera by the tetrad morphology in broth media. Some of the physiological differences which are helpful for the first grouping at the genus level are given in the table below.

Table 1.5. Classification of Cocci Lactic Acid Bacteria, (Source: Carr et al. 2002)

Microorganism	Growth at 10°C	45°C	Growth in 6.5 % NaCl	Type Lactate Formed	Gas from Glucose	Growth in Broth at pH 9.6	Arginine hydrolysis	0.10% Methylene Blue Reduction
Enterococcus	+	+	+	ND	-	+	+	+
Lactococcus	+	-	-	L	-	-	V	V
Streptococcus	-	+	-	L	-	-	V	-
Leuconostoc	+	+	-	D	+	ND	-	ND

*+ indicates a positive test result; -. indicates a negative test result; (+/-) indicates most strains positive, an occasional strain negative; (-/+) indicates most strains negative, an occasional strain positive; V indicates variable, some produce (+) or (-) results; L(+) indicates levo-lactic acid; D(-) indicates dextro-lactic acid; DL indicates a mixture of D and L lactic acid; ND indicates no data is available.

S. thermophilus is highly adapted to the dairy environment, and in the wild. It can only be isolated from dairy products. *S. waius* is a recently identified thermophilic *Streptococcus* isolated from stainless still pasteurization machinery of milk. It shares many phenotypic characteristics with *S. thermophilus* but can be distinguished by the

fermentation of galactose, salicin, cellobiose, maltose, melibiose and D-raffinose. *S. waius* is also tolerant up to 7% NaCl (Pearce and Flint 1999).

Unlike other streptococci, *S. thermophilus* does not possess a group- specific antigen. The peptidoglycan structure is identical to that of *Enterococcus faecalis*; however it is distinguished from enterococci and lactococci by its sensitivity to salt. It does not grow in the presence of 4% salt, and some strains will not grow in as little as 2% salt (Zirnstein and Hutkins 1999).

The sugar fermentation profiles of *S. thermophilus* strains studied were highly variable. Even the very basic sugar metabolisms, like galactose metabolism, displays variable phenotypes (Vin et al. 2005). The sugar fermentation profile of *S. thermophilus* from different sources is given in the table 1.5.

Table 1.6. Classification Scheme for *S. thermophilus*,

(Source: [1]: Marco Gobbetti 1999, [2]: L Pearce 2002, [3]: Gerald Zirnsein 1999, [4]: Robinson 2002)

Peptidoglycan type	G-C Content(mol%)	Lactic Acid Isomer	Growth (15/45°C)	NH_3 from Arginine	Amygdalin	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Mellibiose	Raffinose	Sucrose	Fructose	Glucose	Sorbitol
Lys-Ala	37-40		-/+	-[1,2]	-[1]	-[1]	d[4]	+[1,2,3,4]	-[1]/d[4]	-[1]	+[4]/-[1,2,3]	d[1]	d[1]	+[2,3]/d[4]	+[2,3,4]	+[2,3,4]	-[1]

Symbols: (+): 90% or more strains are positive, (-): 90% or more strains are negative, (d):11-89% of strains are positive

1.9.2. Molecular Characterization Methods

Although the phenotypic techniques have proven to be useful, there is a general awareness that strains with similar phenotypes do not necessarily have closely related genotypes. Phenotypic methods have also poor reproducibility, ambiguous, and poor discriminatory power. Wild type strains isolated from natural habitats show phenotypic variability and are often classified as "atypical" (Milliere et al. 1996).

Genotypic techniques have different levels of discrimination, from species level to individual strain level. Most of them are based on Polymerase Chain Reaction (PCR), which enables the amplification of targeted DNA fragments by the use of designed primers under controlled reaction conditions. The most powerful and most extensively used phylogenetic marker is 16S ribosomal RNA and the genes code for it. The advantages result from the fact that the ribosomes must have been present in the earliest prokaryotic cells, the components of the ribosomes have not changed their function and the presence of multiple genes coding for rRNA makes horizontal gene transfer unlikely. That is why there is a high degree of conservation within the tRNA genes' sequences. More than 12.000 sequences of 16S rDNA are available for prokaryotic strains in gene banks (Morata et al. 1999).

PCR-RFLP (Restriction Fragment Length Polymorphism) is the most commonly used method to identify isolates at intra-species level. Method is based on the digestion of amplicons resulting from PCR with restriction enzymes. When RFLP is applied to ribosomal genes, the method is called amplified ribosomal DNA restriction analysis (ARDRA). PCR-ARDRA with *EcoR* I has been to be a reliable and rapid method for identifying *L. delbrueckii* isolates at the subspecies level and for differentiating this species from *L. helveticus* and *L. acidophilus* (Andrighetto et al. 1998, Bouton et al. 2002, Coeuret et al. 2003, Giraffa et al.1998,). This was also justified by another study, which differentiated the subspecies of L. delbrueckii and also reclassified some of the ATCC type strains known to be *L. delbrueckii* ssp. *bulgaricus* as *L. delbrueckii* ssp. *lactis* (Delley and Germond 2002).

DNA fingerprinting method that solely relie on PCR include Randomly Amplified Polymorphic DNA (RAPD). This analysis makes use of short arbitrary primers and low-stringency conditions to amplify DNA randomly. Fragments obtained are then separated electrophoretically to produce a fingerprint. The great flexibility in

primer choice enables it to be used to differentiate LAB at different taxonomic levels, from genus to intra-specific level. However, since the primers are random and not directed to a specific region, reproducibility is major problem. RAPD-PCR was shown to be superior in distinguishing individual *L. delbrueckii* strains (Torriani et al. 1999). On the other hand, *S. thermophilus* strains showing phenotypic anomalisms were not easy to locate within *S. thermophilus* clusters using this method (Moschetti et al. 1998).

Species-specific PCR is another method which relie on PCR for rapid and accurate identification for *S. thermophilus* isolates. Differentiation of *S. thermophilus* strains from other *Streptococcus* species such as *S. mutans, S. salivarius*, and other bacteria such as *L. delbrueckii* subspecies, *Lactococcus lactis* subspecies, *L. acidophilus, L. brevis, L. fermentum, L. helveticus, L. plantarum, Enterococcus* subspecies and *E. coli* was accomplished clearly using primers homologous within the *lacZ* gene (Lick et al. 1995). This method was also justified by a recent study which makes use of the polyphasic approach to show the genotypic and phenotypic heterogeneity of *S. thermophilus* strains (Giraffa et al. 2001) and also with the study, which investigates the species composition of commercial dairy starters (Giraffa and Rossetti 2004).

Pulsed Field Gel Electrophoresis (PFGE) is another molecular typing method. It has an alternating field of electrophoresis to separate large DNA fragments resulting from restriction with rare cutting enzymes. The crucial point in PFGE is the extraction of intact chromosomal DNA, which is more time consuming than other fingerprinting techniques. Since large DNA fragments, representing the whole genome, are analyzed with PFGE, it has a superior discriminatory power at subspecies and strain levels.

Ribotyping combines an enzymatic restriction digestion and the detection of the restriction fragments by means of rDNA probes. Fluorescent or radioactively labeled probes can be used for hybridization. Discriminatory power of this method is dependent on the number and type of endonucleases and probes. In a study, where ribotyping has been applied to 30 different *L. delbrueckii* strains, it has been found that only ribotyping with *EcoR* I allowed the differentiation of three subspecies on the basis of a typical hybridization pattern (Miteva 2001). Differentiation at strain level has also been achieved for *S. thermophilus* strains both by restriction endonucleases digestion combined with pulsed field gel electrophoresis (Salzano et al. 1993) and by ribotyping (Salzano et al 1994).

16S or 23S rDNA sequencing is another useful method, when unknown isolates are to be identified. Obtained sequences are compared with the sequences previously deposited in a database. Stackobrandt and Goobel stated that strains that are more than 3% divergent in 16S rRNA are nearly always members of different species, as determined by DNA-DNA hybridization studies (Stackebrandt and Goebel 1994). Whereas the strains with less than 3% divergency are generally members of the same species. A cutoff of 3% is a recommended limit as a conservative criterion (Cohan 2002).

DNA-DNA hybridization has a higher discrimination power than 16S sequencing. Various approaches such as nitrocellulose filter methods, free-solution methods, and recently the microarray technology have been used. As a general rule, strains, which have a DNA-DNA relatedness of more than 70% and 5°C or less difference in melting point, belong to the same species. However, use of isotopes and lack of database affect the popularity of method negatively.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemicals and their catalog codes were given in the Appendix A.

2.1.2. Yoghurt Samples

In total 14 artisanal yoghurt samples were used for the isolation of yoghurt starter bacteria. Thirteen of them were collected from the “Toros” region of Turkey and 1 was gathered from Adana. Yoghurts had been made in a traditional way and they were selected according to their organoleptic properties such as aroma, viscosity, and acidity. The sample codes were given in the Table 2.1.

Table 2.1. The Origin of the Yoghurt Samples and Their pH Values

Yoghurt Sample Code	Location	pH
M3	Mersin	3,90
M5	Mersin	3,91
M7	Mersin	3,92
M8	Mersin	3,98
TY1	Güneyli	3,96
TY2	Kızılgeçit	3,94
TY4	Karaahmetli	3,94
TY5	Yağcılar	3,97
TY6	Sarıaydın	3,97
TY7	Elbeyli	3,96
TY9	Albeyli	3,96
TY10	Çukurbağ Silifke	4,01
TY11	Seydi	3,98
TY12	Adana	4,1

2.1.3. Reference Strains:

The reference strains used in the study were as fallows:

1. *Lb. delbrueckii* ssp. *bulgaricus* CCM 7190
2. *Lb. delbrueckii* ssp. *delbrueckii* CCM 7191
3. *Lb. delbrueckii* ssp. *delbrueckii* NRRL-B 763
4. *Lb. delbrueckii* ssp. *lactis* NRRL-B 1924
5. *Lb. delbrueckii* ssp. *lactis* NRRL-B 735
6. *Lb. delbrueckii* ssp. *lactis* CCM 2772
7. *Lb. helveticus* NRRL-B 4526
8. *Lb. acidophilus* CCM 4833
9. *S. thermophilus* CCM 4757

2.2. METHODS

2.2.1. Isolation of Bacteria

Bacteria were isolated from natural yoghurt samples using the pour plate technique. Briefly, 10ml of each sample was taken and homogenized in 90 ml of Peptone water. Dilutions up to 10^{-10} were prepared and 1 ml aliquots from 10^{-5}, 10^{-6}, 10^{-7} dilutions were used for isolation. Each sample was plated twice.

2.2.1.1. Selective Media and Growth Conditions

For *S. thermophilus*, M17 Agar (pH 6.9) at 42°C

For *L. delbrueckii* ssp. *bulgaricus*, MRS Agar (pH 6.2) at 42°C

All plates were incubated for 3 days in microaerophilic conditions using Anerogen kit, which reduces the oxygen level in the jar below 1% with a resulting CO_2 level between 9% and 13%. After the incubation, plates with colony forming units (cfu) between 30 and 300 were counted and results were recorded as viability of yoghurt bacteria.

Bacteria were isolated from the incubated plates and transferred into the MRS or M17 medium for enrichment. The enriched bacteria were purified using streak plate technique. Purification was terminated when the appearance of colonies on the plate was homogeneous.

2.2.2. Long Term Preservation of the Isolates

The purified bacteria with homogeneous cell morphology were stored in a -80°C deep freezer in MRS or M17 medium in 20% (v/v) glycerol for preservation. The glycerol stocks were prepared by mixing 0,5 ml of active cultures with 0,5 ml of fresh, sterile MRS or M17 medium with 40% glycerol in eppendorf tubes. Hence, the resulting suspension included 20% glycerol. The frozen stocks were prepared in triplicate and only one set was used for the test for avoiding any contamination or loss of activity.

2.2.3. Biochemical Identification

2.2.3.1. Gram Staining

Lb. delbrueckii ssp. *bulgaricus* and *S. thermophilus* are Gram positive bacteria and the Gram status of the isolated bacteria were determined by the microscopic examination of Gram-stained isolates.

One ml of overnight activated broth culture was aliquated into an eppendorf tube and centrifuged at 6.000 rpm for 5 min. Supernatant was removed and cells were resuspended in sterile water. Ten µl of cell suspension was pipetted to a microscopic lam and they were Gram stained after drying and fixation by exposure to a flame. The main steps in Gram Staining procedure were as follows:

- Crystal violet staining for 1 min
- Removing the excess stain by washing under tap water
- Staining with Gram's iodine mordant for 1 min
- Washing under tap water
- Fixation with 95% alcohol for 15 s
- Counter staining with safranine for 30 s
- Washing under tap water
- Drying with cotton towels gently

Gram positive bacteria became blue-purple after Gram staining; however Gram negative bacteria became pink-red.

2.2.3.2. Catalase Test

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and oxygen production is observed by the generation of O_2 bubbles. The generation of gas bubbles indicates the presence of the enzyme, hence the catalase positive nature of the bacterium.

Lb. delbrueckii ssp. *bulgaricus* and *S. thermophilus* are both catalase negative and no O_2 production (gas bubbles) should be observed when 3% H_2O_2 solution is dropped on top of the colonies grown overnight on agar medium. In order to determine the catalase nature of isolates, all were screened using 3% H_2O_2 solution.

2.2.3.3. Gas Production from Glucose

CO_2 production from glucose is the major criterion for the determination of homofermentative or heterofermentative nature of an isolate. Homofermentatives ferment lactose into lactic acid, while heterofermentatives ferment lactose to lactic acid (or acetic acid, ethanol) and CO_2. *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are both homofermentative bacteria. MRS broths containing inverted Durham tubes were utilized to determine CO_2 production. Some ingredients such as citrate can also undergo a reaction yielding CO_2; hence citrate was excluded from the MRS test medium.

Fifty microliters of overnight activated cultures were inoculated into 8 ml MRS containing inverted Durham tubes and incubated for 5 days at 42°C. CO_2 production was observed as an accumulation of gas in the inverted tubes.

2.2.3.4. Growth at Different Temperatures

Growth at 15°C and 45 °C are most frequently used criteria for the classification of bacilli (Hammes and Vogel 1995). *Lb. delbrueckii* ssp. *bulgaricus* cannot grow at 15°C, however can grow at 45°C. For the classification of cocci isolates, the growth temperatures of 10°C and 45°C were used. *S. thermophilus* cannot grow at 10°C, but grow well at 45°C.

In order to determine the growth at given temperatures, the modified MRS and M17 media given in the Appendix B were used. Basically, all ingredients were the same, except for Bromecresol purple. Bromecresol purple was used to determine the color change in acidity with from purple to yellow, indicating lactic acid production and cell growth. Fifty microliters of overnight activated cultures were inoculated into 5 ml test media and the incubation period at the given temperatures was observed for 7 days to determine the growth and color change.

2.2.3.5. Growth at Different NaCl Concentrations

Unlike other bacilli and cocci, *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are highly sensitive to NaCl. *S. thermophilus* does not grow even at 2% NaCl concentration, but there is no data available for *Lb. delbrueckii* ssp. *bulgaricus.* The most frequently used NaCl concentration for the identification of bacilli are 4% and 6.5% salt concentrations. Hence growth at 2% and 4% NaCl concentrations were used for cocci isolates and the growth at 4% and 6.5% NaCl concentrations were used for bacilli isolates in NaCl test medium (Appendix B).

Fifty microliters of overnight activated cultures were inoculated into 5ml NaCl test media and the incubation at 42°C was observed for 7 days to determine the growth with the indication of color change from purple to yellow.

2.2.3.6. Determination of Carbohydrate Fermentation Profiles

Isolates were screened for their ability to ferment 19 different carbohydrates (Appendix C), and all the reactions were performed by using 96-well microtitre plates (Bulut et al. 2003). The procedure includes two steps. The fist step is the preparation of active cells free from sugar residues and the preparation of sterile sugar solutions. Second step includes combining the prepared active cell culture free from sugar with the test sugar solutions to give a final sugar concentration of 2% and allowing them to grow at 42°C in microtitre plates.

Preparation of active cell culture free from sugar:

- Overnight activation of isolates in 10ml MRS and M17 media at 42°C
- Centrifugation 10 min at 10.000 rpm
- Resuspending the pelleted cells in 5 ml MRS without glucose and containing bromecresol purple. The modified MRS medium used for carbohydrate fermentation (Appendix B).
- Centrifugation 10 min at 10.000 rpm
- Resuspending the pelleted cells in actual volume of 10 ml MRS without glucose and containing bromecresol purple.

Preparation of Test Sugar Solution:

- Dissolving each sugar in deionized water at a final concentration of 10% (w/v).
- Filter sterilization of sugar solutions by filters with 0,22 µm pore diameter

Combination of Sugar Solutions and Active Cell Culture without sugar:

- Forty microliters of sugar solution was pipetted into the well
- One hundred and sixty microliters of active cell solution without sugar was added to the sugar solution pipetted wells.
- Two negative controls and 1 positive control were used for the indication of any contamination coming from basal media or any activity problem with culture.

  1st Negative Control: 200µl of active cell solution without sugar

  2nd Negative Control: 160µl of MRS solution without sugar and without active cells + 40µl of sugar solution

  Positive Control: 160µl of active cell solution without sugar + 40µl of glucose solution
- All the reactions were performed twice.
- After overnight incubation at 42°C, the turbidity and the color change from purple to yellow with respect to negative and positive controls was recorded as positive fermentation result.

2.2.4. Molecular Characterization

2.2.4.1. Molecular Characterization of Bacilli Isolates by Amplified Ribosomal DNA Restriction Analysis (ARDRA)

16S-23S-5S sequences are the coding sequences for ribosomal RNAs. They are highly conserved and most frequently used for identification. ITS (Internal Transcribed Spacer), which is located between 16S and 23S sequences, includes both conserved and highly variable sequence motifs and it is useful for identification at species level. Hence for the molecular characterization of the bacilli isolates, amplified ribosomal DNA restriction analysis based on16S-ITS region of rDNA was used. The basic steps of the procedure were as follows;

- Genomic DNA Isolation from strains

- Determination of the most discriminative endonucleases for restriction fragment length polymorphism
- Amplification of 16S-ITS region of rDNA with Polymerase Chain Reaction (PCR)
- Purification of PCR products
- Restriction of PCR products with endonucleases
- Purification of restriction products
- Electrophoresis of restriction fragments
- Determination of length polymorphism and interperation of results

2.2.4.1.1 Genomic DNA Isolation

Genomic DNA was isolated using the following procedure (Cardinal et al. 1997).

- Overnight activation of 10ml MRS or M17 cultures
- Harvesting cells at 6.000 rpm for 5 min.
- Removing the liquid phase, washing pellet with 500µl 1xTE buffer (pH 8) and then centrifugation at 6.000 rpm for 5 min
- Suspending cells in 200µl 1xTE buffer (pH 8) containing 25% sucrose and 30mg/ml lysozyme and mixing gently
- Incubation for 1 h at 37°C.
- Addition of 370µl 1xTE buffer (pH 8) containing 1mg/ml Proteinase K
- Addition of 30 µl SDS
- Incubation for 1 h at 37°C.
- Addition of 100µl 5M NaCl solution and 80µl CTAB/NaCl solution (10% cetytrimethylammonium bromide, 0,7M NaCl)
- Incubation for 10 min at 65°C
- Adding 750 µl chloroform/isoamyl alcohol (24/1, v/v) and mixing effectively
- Centrifugation at 6.000 rpm for 5 min
- Transferring the upper aqueous phase into a new eppendorf tube.
- 2nd Chloroform/isoamyl alcohol extraction and centrifugation at 6000 rpm for 5 min
- Transferring the upper aqueous phase into a new eppendorf tube.
- DNA precipitation by the addition of an equal volume (750µl) of isopropanol

- If DNA wool is observed, taking the wool with a yellow tip and transferring into a new eppendorf tube containing 500 µl 70% ethanol
- If DNA wool is not observed, centrifugation at 6.000 rpm for 10 min to pellet DNA and washing with 500 µl 70% ethanol
- Pelleting DNA with centrifugation at 6.000 rpm for 10 min after washing
- Removing the ethanol and drying the pellet at 37°C for 10 min
- Dissolving pellet in 100µl 1xTE buffer (pH 8) containing 100µg/ml RNase
- Incubation for 1 h at 37°C
- Adjusting the volume to 400µl with 1xTE buffer (pH 8)
- Dissolving DNA with alternating cold-heat shock twice (80°C for 10 minutes, and -20° C for 20 min)
- Addition of one volume (400µl) of phenol, mixing efficiently
- Centrifugation at 6.000 rpm for 5 min
- Transferring the upper aqueous phase into new eppendorf tube
- Addition of one volume (400µl) of chloroform/isoamyl alcohol (24/1, v/v) , mixing efficiently
- Centrifugation at 6.000 rpm for 5 min
- DNA precipitation with the addition of 1/10 volume (40 µl) of 5M NaCl and 2 volumes (800µl) of 99% ethanol.
- Centrifugation at the maximum spin for 10 min
- Washing pellet with 500µl 70% ethanol
- Centrifugation at 6.000 rpm for 5 min
- Removing all the ethanol and drying DNA for 10 min at 37°C
- Dissolving DNA in 50µl, 100µl or 150µl according to pellet size
- Dissolving DNA with alternating cold-heat shock (80°C for 10 min, and -20° C for 20 min)
- Preservation of dissolved genomic DNA samples at -20°C

At the end of the procedure, it has to be checked whether genomic DNAs were isolated. It is visualized by agarose gel electrophoresis using the procedure (Section: 2.2.4.1.4).

2.2.4.1.2. Determination of the Most Discriminative Endonucleases for Restriction Fragment Length Polymorphism

The most frequently used restriction enzyme in the studies which made use of ARDRA for identification of *L. delbrueckii* ssp. *bulgaricus* isolates is *EcoR* I. However there could be any other enzyme that has not been studied yet, so the discrimination capability of other restriction enzymes should be determined and the discriminative power of *EcoR* I should be evaluated.

- For this purpose, many 16S complete sequences of the most related 5 lactobacilli, namely *Lactobacillus delbrueckii* subspecies, *Lactobacillus helveticus* and *Lactobacillus acidophilus* were taken from NCBI Gene Bank (Their accession numbers were given in the Appendix G).
- These sequences were digested theoretically using *EcoR* I, *Taq* I, *Hae* III, *Apa* I, *Alu* I, *Hind* III, *Rsa* I, *Dra* I *and Not* I with the help of Ape-A Plasmid Editor v1.10.4.
- According to theoretical digestion patterns, discriminative enzymes were determined.
- For proving these results, 16S and 16S-ITS products of the genomic DNA's of reference strains were obtained using PCR (conditions in section 2.2.4.1.3., and purified with the procedure given in section 2.2.4.1.4).
- Purified PCR Products were digested with the selected restriction enzymes using the procedure in section 2.2.4.1.5.
- Restriction fragments were separated by agarose gel electrophoresis using the method given in 2.2.4.1.7., and the polymorphism in digestion products of reference strains was determined with BIO-ID++ software (Vilber-Lourmat) (section 2.2.4.1.8).
- The most discriminative enzyme was determined for both digestion of 16S and 16S-ITS products.
- Lastly, the isolated strains were identified by the restriction fragment length polymorphism of 16S-ITS region.

2.2.4.1.3. Amplification of 16S-ITS (Internal Transcribed Spacer) rDNA Region by Polymerase Chain Reaction

For the amplification of 16S-ITS rDNA, EGE1 and L1 primers were used. Forward primer is complementary to the 5'end of 16S rDNA, and the reverse primer is complementary to the 3' end of ITS region.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: L1: 5'CAAGGCATCCACCGT-3'

The PCR Conditions:

Step 1: 94°C for 5 min

Step 2: 94°C for 1 min (denaturation)

Step 3: 42°C for 1 min (annealing) } 40 cycles

Step 4: 72°C for 1 min (elongation)

Step 5: 72°C for 10 min

For the amplification of 16S rDNA of reference strains, EGE1 and EGE2 primers were also used. Forward primer is complementary to the 5'end of 16S rDNA, and the reverse primer is complementary to the 3' end of 16S rDNA region.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACCA-3'

The PCR Conditions:

Step 1: 94°C for 5 min

Step 2: 94°C for 1 min (denaturation)

Step 3: 56°C for 1 min (annealing) } 40 cycles

Step 4: 72°C for 1 min (elongation)

Step 5: 72°C for 10 min

The final reaction volume of polymerase chain reaction was 50µl and the amount of DNA template used was 500ng. The recipe of the reaction mixture was given in the Appendix E.

2.2.4.1.4. Separation of Amplification Products

Preparation of Agarose Gel:

0.8g of agarose were dissolved in 100µl 1x TAE buffer by boiling. The recipe of TAE buffer was given in Appendix D. After boiling, it was cooled to 45°C. 15µl ethidium bromide solution (10mg/ml) was added and stirred. The agarose gel was poured into the gel casting stand and the combs were placed.

Loading of Agarose Gel:

Five microliters of PCR products were mixed with 2µl of gel loading dye (Appendix D). The samples were loaded into the wells, starting from the second well on the gel. A DNA size-marker (1 kb, Fermentas) was loaded into the fist well.

Electrophoresis of the Products

PCR products were electrophoresed at 80V for 30 min. Amplification products were visualized in a gel documentation system (Vilber-Lormat). The presence of DNA fragments with the size of 1500-2000 bp indicated that the amplification was achieved.

Purification of PCR Products

PCR Products were purified before digestion with restriction enzymes:

- Adjusting the volume of PCR products to 100µl by adding 50µl 1xTE buffer
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 5.000 rpm for 10 min
- Transferring the upper aqueous phase into a new eppendorf tube
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 5.000 rpm for 10 min
- Transferring the upper aqueous phase into a new eppendorf tube containing 0.l volume of 3M sodium acetate (pH 5.2) and mixing effectively
- Addition of 2 volumes (220µl) of 99% ethanol
- Centrifugation at 8.000 rpm for 10 min and removing the liquid phase
- Washing the pelleted DNA with the addition of 500µl of 70% ethanol
- Centrifugation at 5.000 rpm for 10 min
- Removing the ethanol and drying pellet at 37°C for 10 min
- Dissolving DNA in 50µl 1xTE solution

- Storing DNA solution at -20°C

2.2.4.1.5. Restriction of PCR Products with Endonucleases

Three different endonucleases were used for the restriction of the purified PCR products; *Taq* I, *Hae* III, and *EcoR* I. Ten microliters of purified PCR product was used for each of the restriction reaction and digestion was performed in 50µl of final reaction volume (Appendix F). *Hae* III, and *EcoR* I digestions were performed at 37°C, and *Taq* I digestion was performed at 65°C . Samples were covered with a mineral oil layer to prevent evaporation. All the reactions were performed overnight.

2.2.4.1.6. Purification of Restriction Products

- Adjusting the volume of restriction products to 100µl with the addition of 90µl 1xTE buffer
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 8.000 rpm for 5 min
- Transferring the upper aqueous phase into the new eppendorf tubes containing 0.1 volume of 3 M sodium acetate (pH 5.2) and mixing effectively
- Addition of 250µl of 99% ethanol
- Centrifugation at 8.000 rpm for 15 min and removing the all liquid phase
- Washing pellet with 300µl of 70% ethanol
- Centrifugation at 8.000 rpm for 5 min
- Removing the ethanol and drying pellet at 37°C for 10 min
- Dissolving the pellet in 10-15µl 1xTE solution
- Storing DNA solution at -20°C

2.2.4.1.7. Electrophoresis of Restriction Fragments

Restricted fragments were separated in 1.6 % agarose gel.

Preparation of Agarose Gel:

2.4g of agarose were dissolved in 150ml 1x TAE buffer by boiling. After boiling, it was cooled to almost 45°C. 22.5 µl ethidium bromide solution (10mg/ml) was added and stirred. The agarose gel was poured into the gel casting stand and the combs were placed. The combs were removed, when the gel was solidified.

Loading of Agarose Gel:

The solidified agarose gel was placed into the electrophoresis tank and 1,5lt TAE buffer containing 300µl of ethidium bromide was poured into the tank. 10-12µl of the digestion products were mixed with 2µl of gel loading dye. The samples were loaded into wells, starting from the second well on the gel. The fist well on the gel was loaded with the 2µl (500ng) of DNA molecular weight marker for 100 bp., and the last well on the gel was loaded with the 2µl (500ng) of DNA molecular weight marker for 1kb.

Electrophoresis of the Products

The samples were electrophoresed at 60mA for 30 min and at 80mA for 4 h. Amplification products were visualized in a gel documentation system (Vilber-Lormat).

2.2.4.1.8. Interperation of RFLP Results

RFLP paterns were visualized in gel documentation system. The images were modified in Adobe Photoshop 7.0 and analyzed by using BIO-ID++ software (Vilber-Lourmat). The similarities between strains were determined automatically by specifying the formula of Jaccard. Strain clustering was performed by the un-weighed pair group method with arithmetic averages, UPGMA, BIO-ID++. The dendrogram was prepared using 13% homology coefficient.

2.2.4.1.9. 16S rRNA Sequencing of One Representative Bacilli Isolate

The 16S rRNA gene sequence of one representative isolate, bTY30, was sequenced and basic steps of the procedure are given below.

- 16S rRNA product was obtained as explained previously (Section 2.2.4.1.4).

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACCA-3'

The PCR Conditions:
Step 1: 94°C for 5 min
Step 2: 94°C for 1 min (denaturation)
Step 3: 56°C for 1 min (annealing) } 40 cycles
Step 4: 72°C for 1 min (elongation)
Step 5: 72°C for 10 min

The total reaction volume of polymerase chain reaction was 50µl and the amount of DNA template used was 500ng (Appendix E).

- Purified PCR products were sequenced in a ABI 310 DNA Sequencer at Middle East Technical University. Two forward and 2 reverse primers were used during sequencing. The sequence of the primers;

Forward primer 1: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse primer 1: L1: 5'-CAAGGCATCCACCGT-3'
Forward primer 2: 515F: 5'-GTGCCAGCAGCCGCGGTAA-3'
Reverse primer 2: 1115R: 5'-AGGGTTGCGCTCGTTG-3'

- Obtained sequences were compared with the sequences deposited to NCBI database with BLAST Analysis.

2.2.4.2. Molecular Characterization of Cocci Isolates

2.2.4.2.1. Identification by ARDRA

For the identification of cocci isolates with ARDRA, *Taq* I *and Hae* III enzymes were used. These enzymes are found to be the most effective enzymes for the

identification of cocci lactic acid bacteria in previous studies (Bulut et al. 2005). The procedure was the same with the one given in part 2.2.4.1.

2.2.4.2.2. Identification by Species Specific PCR

The results of ARDRA was also verified by species specific PCR. To increase detection sensitivity, a species-specific PCR amplification procedure has been developed and primers have been used for the amplification of an intragenic fragment of 968bp within the *lacZ* gene sequence of *S. thermophilus* (Lick et al. 1995)..

The procedure includes the following steps;

- **Genomic DNA Isolation of Cocci Isolates**

  The procedure was the same with the given one for bacilli isolates in section 2.2.4.1.1.

- **Species Specific PCR**

  For the amplification of the intragenic fragment of 968bp from lacZ gene IYTE1 and IYTE2 primers were used.

Forward Primer: IYTE1: 5'-CAC TAT GCT CAG AAT ACA-3'
Reverse Primer: IYTE2: 5'-CGA ACA GCA TTG ATG TTA-3'

The PCR Conditions:
Step 1: 94°C for 5 min
Step 2: 94°C for 1 min (denaturation)
Step 3: 46°C for 1 min (annealing) } 40 cycles
Step 4: 72°C for 1 min (elongation)
Step 5: 72°C for 10 min

- **Separation of Amplification Products**

  The procedure includes 3 basic steps; Preparation of agarose gel, loading of the gel, and the electrophoresis of the products. The details of the procedure were given in section 2.2.4.1.4.

- **Visualizing and Interpretation of Results**

The PCR products were visualized in a gel documentation system. The images were modified with Adobe Photoshop 7.0 and analyzed by using BIO-ID++ software (Vilber-Lourmat).

2.2.4.2.2. 16S rRNA Sequencing of One Representative Cocci Isolate

The 16S rRNA gene sequence of one representative isolate, cTY17, was sequenced for the verification of the results obtained with both ARDRA and species specific PCR. Basic steps of the procedure are given below.

- 16S rRNA product was obtained using the given primers and PCR conditions described in section 2.2.4.1.4.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACCA-3'

The PCR Conditions:
Step 1: 94°C for 5 min
Step 2: 94°C for 1 min (denaturation)
Step 3: 56°C for 1 min (annealing) } 40 cycles
Step 4: 72°C for 1 min (elongation)
Step 5: 72°C for 10 min

The total reaction volume of polymerase chain reaction was 50µl and the amount of DNA template used was 500ng. The recipe of the reaction mixture is given in the Appendix E.

- Purified PCR products were sequenced in a ABI 310 DNA Sequencer at Central Laboratory of Middle East Technical University. Two forward and 2 reverse primers were used during sequencing. The sequence of the primers;

Forward primer 1: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse primer 1: L1: 5'-CAAGGCATCCACCGT-3'
Forward primer 2: 515F: 5'-GTGCCAGCAGCCGCGGTAA-3'

Reverse primer 2: 1115R: 5’-AGGGTTGCGCTCGTTG-3’

- Sequencing results obtained with forward and reverse primers were matched and compared with primer walking and the gaps were completed. The obtained sequences were compared with the sequences previously deposited in NCBI database with BLAST Analysis.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation of Lactic Acid Bacteria

In total 14 artisanal yoghurt samples were used for the isolation of yoghurt starter bacteria. Bacteria were isolated with MRS and M17 agar media at 42°C under anaerobic conditions. MRS was used for the isolation of *L. delbrueckii* ssp. *bulgaricus* and M17 was used for the isolation of *S. thermophilus* .Counts of bacteria after 3 days of incubation were given (Table 3.1). The counts are the arithmetic mean of the colony forming units of -7, -6 and -5 dilutions.

Table 3.1. The Counts of Isolates from M17 and MRS Plates

Code of Yoghurt Sample	cfu of MRS plates	cfu of M17 plates	Ratio (M17/MRS)
M2	-	6.0*exp8	
M5	4.3* exp8	2.3*exp8	0.5
M7	1.6*exp8	3.1*exp8	1.9
M8	1.0*exp8	1.1*exp8	1.1
TY1	2*exp5	5.5*exp8	2750
TY2	4.5*exp7	5.3*exp8	11.8
TY4	9*exp5	1.2*exp8	133.3
TY5	7.2*exp6	1.1*exp9	152.8
TY6	2.7*exp7	4.4*exp8	16.3
TY7	1.9*exp7	6.9*exp8	36.3
TY9	4.3*exp7	3.2*exp8	7.4
TY10	3.6*exp7	4.7*exp8	12.2
TY11	5.4*exp7	3.5*exp8	6.5
TY12	2.5*exp8	-	

The arithmetic means of M17 counts were found to be higher than the counts of MRS. The expected ratio of M17 counts to MRS counts was between 1-10 (Tamime, 1985). However the ratio of TY1, TY4, TY5 and TY7 was much higher than the expected value. Isolation was performed with samples stored at -80°C. These extreme ratios would result from the intolerance of MRS isolates to cryopreservation. The amount of lactic acid bacteria for a satisfactory yoghurt fermentation should be between 10^8 and 10^9 for both of the species. The amount of 10^7 is evaluated as doubtful. When these values were compared with the colony counts, the counts of MRS colonies seemed to be low, but the M17 colony counts were optimum.

In total 400 bacteria were isolated from both M17 and MRS plates. 137 isolates remained at the end of isolation and purification, after the loss of unstable isolates during purification and subculturing steps and also because of the loss of intolerant strains to -80°C cryopreservation. Sixty six of them were M17 isolates, and 71 of them were MRS isolates.

3.2. Biochemical Identification

All of the isolates were subjected to Gram staining and they were examined under light microscope. All the strains gave blue- purple color with staining; hence they all were Gram positive bacteria. The isolates coming from MRS plates were all bacilli with long and rounded ends. They appeared mostly as a chain of 3-4 cells or single. The isolates coming from M17 isolates were all cocci with spherical or ovoid morphology. They appeared mostly as pairs or forming chains. An electron microscopic image of one representative cocci and bacilli isolate were given in Figure 3.1.

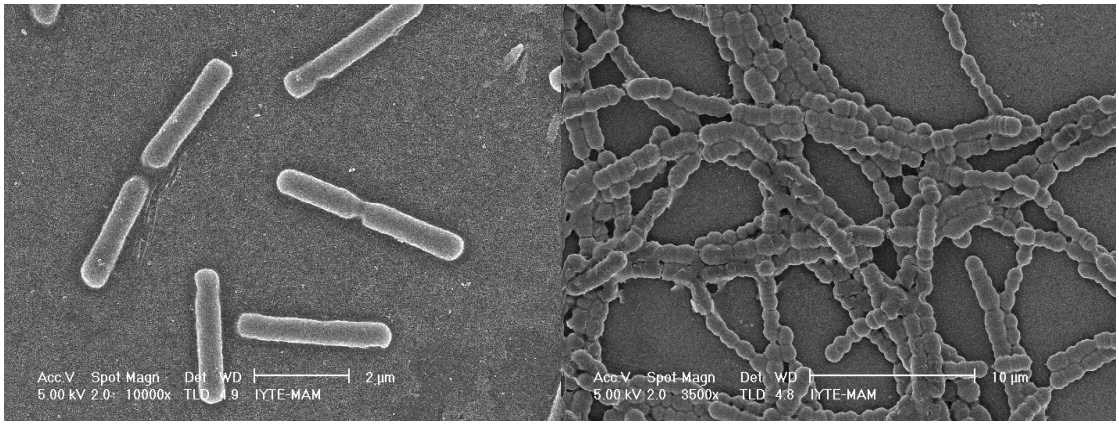

Figure 3.1. Scanning Electron Microscopic Image of Bacilli (bTY30) and Cocci (cTY17) Isolate

All of the isolates were tested for catalase activity. None of them showed catalase activity.

All of the isolates were also subjected to the test for gas production from glucose and the test tubes were observed for five days. There was no gas accumulation in Durham tubes, although the cultures were well grown. This result confirmed the homofermentative behavior of the isolates.

Regarding the ability to grow at different NaCl concentrations, there was no stated record for the ability of growth even at 2% NaCl concentration for *S. thermophilus.* Ability to grow at 2% and 4% NaCl concentrations were tested for all cocci isolates and only two of them were found to be resistant to 2% NaCl concentration but sensitive to 4% concentration. These were c97-2 and cTY44 isolates. For bacilli isolates the most frequently used NaCl concentration for classification was 4% and 6.5%. However there is no data for the resistance of *L. delbrueckii* ssp. *bulgaricus* to NaCl in literature. Hence the bacilli isolates were tested at 4% and 6.5% NaCl concentrations. Only one isolate, bTY45, showed resistance to 4% salt concentration, but it was not resistant to 6.5% NaCl concentration.

Another criterion for the identification was the ability to grow at different temperatures. For the identification of bacilli isolates, the growth at 15°C and 45 °C criteria were used. *Lb. delbrueckii* ssp. *bulgaricus* cannot grow at 15°C, however can grow at 45°C. In the same manner, for the classification of cocci isolates, the growth abilities at 10°C and 45°C were tested. *S. thermophilus* cannot grow at 10°C, but grow

well at 45°C. All of the isolates showed the expected results. All of them were able to grow at 45°C. None of the bacilli isolates were able to grow at 15°C, and none of the cocci isolates were able to grow at 10°C.

The most useful test for the determination of strain differences is carbohydrate fermentation. Nineteen different carbohydrates were used for the identification purpose. According to the test results, all of the MRS isolates gave positive results with the sugars, glucose, fructose, lactose and mannose and there was no atypical sugar fermentation pattern (Table 3.2).

However the results of M17 isolates were much more confusing with different sugar fermentation profiles. The identification scheme, which included data from different studies (given in "Biochemical Identification of *S. thermophilus"* in Introduction), also indicates the instability of *S. thermophilus* for sugar fermentations. Comparing the literature information on sugar fermentations, it was seen that all of the scientists agree on positive fermentation of glucose, lactose and fructose. On the other hand mannose, galactose, sucrose, maltose, melibiose and raffinose fermentations were found to be variable. *S. thermophilus* CCM 4757 reference strain was also shown to be glucose, lactose, fructose, mannose, galactose, and sucrose positive. Hence, in the evaluation of the data, lactose, glucose, fructose positive strains were determined to be *S. thermophilus*. The isolate numbers, which were determined to be *S. thermophilus,* were cTY72, c60, c62, c74, c78, c90b, c94. Others gave atypical fermentation patterns (Table 3.4).

The cocci isolates were also grouped according to their sugar fermentation profiles (Table 3.5). Their atypical characteristics and species assignments were also indicated in the table. In total 21 different groups were obtained from the sugar fermentation results. Interesting fermentation profiles were also seen in the table in the groups 7, 8, 9, and 10. The common feature of these groups was the glucose (-) nature of the bacteria. They all fermented lactose but not glucose. This could be the result of metabolic failures. In normal conditions, yoghurt starters synthesize both the enzyme β-D-galactosidase (β-gal) and β-Pgal. The former enzyme is normally more active. In commercial practice the production of lactic acid is more likely to occur via the glycolysis of glucose and to a lesser degree via the D-tagatose-6P pathway. It is possible however, that β-Pgal becomes more active under certain conditions, for example on synthetic media. It is possible that the lactose and galactose is catabolized via D-tagatose-6P pathway with β-Pgal (Tamime and Robinson 1985). Hence the failure to

utilize lactose but not glucose suggests that strains could be devoid of some metabolic functions in glycolytic pathway.

Table 3.2. Biochemical Test Results of Reference Strains

No Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Fructose	Mannose	Lactose	Sorbitol	Ribose	Salicin	Trehalose	Mellibiose	Sucrose	Mannitol	Arabinose	Melezitose	Maltose	Raffinose	Rhamnose	Galactose	Xylose	Glycerol
L. delbrueckii ssp. *delbrueckii* CCM 7191	+	-	-	+	-	-		-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
L. delbrueckii ssp. *delbrueckii* NRRL 763	+	-	-	+	-	-		-	+	+	+	+	-	-	-	+	-	-	+	+	-	-	+	-	-	+	-	-
L. delbrueckii ssp. *lactis* NRRL 1924	+	-	-	+	-	-		-	+	+	+	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
L. delbrueckii ssp. *lactis 735*	+	-	-	+	+	+		-	+	+	+	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
L. delbrueckii ssp. *lactis CCM 2772*	+	-	-	+	-	-		-	+	+	+	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
L. delbrueckii ssp. *bulgaricus* CCM 7190	+	-	-	+	-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. helveticus NRRL 4526	+	-	-	+	+	-		-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	-	-
L. acidophilus CCM 4833	+	-	-	+	-	-		-	+	+	+	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
S. thermophilus CCM 4757	+	-	-	-	-	-	-		+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-

Table 3.3. Biochemical Test Results of Bacilli Shaped Isolates

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Fructose	Mannose	Lactose	Sorbitol	Ribose	Salicin	Trehalose	Mellibiose	Sucrose	Mannitol	Arabinose	Melezitose	Maltose	Raffinose	Rhamnose	Galactose	Xylose	Glycerol
1	b16	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	b22	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	b22b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	b24	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	b25	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	b26	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	b30	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	b30b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	b33	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	b33b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	b34	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	b44	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	b48	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	b49	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	b51	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	b53	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	b54	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	b57	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	b62	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	b64	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table 3.3. (cont.)

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Fructose	Mannose	Lactose	Sorbitol	Ribose	Salicin	Trehalose	Mellibiose	Sucrose	Mannitol	Arabinose	Melezitose	Maltose	Raffinose	Rhamnose	Galactose	Xylose	Glycerol
21	b69	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	b71	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	b76	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	b77	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	b79	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	bTY5	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	bTY5b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	bTY6	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	bTY7	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	bTY8	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	bTY9a	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	bTY9b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	bTY11	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	bTY14a	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	bTY14b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	bTY16	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	bTY17	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	bTY20	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	bTY21	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	bTY22a	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table 3.3. (cont.)

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Fructose	Mannose	Lactose	Sorbitol	Ribose	Salicin	Trehalose	Mellibiose	Sucrose	Mannitol	Arabinose	Melezitose	Maltose	Raffinose	Rhamnose	Galactose	Xylose	Glycerol
41	bTY22b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	bTY23	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	bTY24	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	bTY27a	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	bTY27b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	bTY30	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	bTY34	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	bTY36b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	bTY39	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	bTY40	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51	bTY41	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52	bTY42	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	bTY43	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	bTY45	+	-	-		+	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	bTY68	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	bTY69	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	bTY70	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
58	bTY71	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
59	bTY73	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60	bTY77a	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table 3.3. (cont.)

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Fructose	Mannose	Lactose	Sorbitol	Ribose	Salicin	Trehalose	Mellibiose	Sucrose	Mannitol	Arabinose	Melezitose	Maltose	Raffinose	Rhamnose	Galactose	Xylose	Glycerol
61	bTY77b	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	bTY79	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63	bTY80	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64	bTY83	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65	bTY85	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66	bTY86	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67	bTY87	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
68	bTY88	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
69	bTY90	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
70	bTY91	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
71	bTY92	+	-	-		-	-		-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.4. Biochemical Test Results of Cocci Shaped Isolates

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Lactose	Sucrose	Galactose	Fructose	Mannose	Melezitose	Maltose	Mellibiose	Ribose	Mannitol	Arabinose	Salicin	Trehalose	Raffinose	Rhamnose	Sorbitol	Xylose	Glycerol
1	cTY8	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	cTY 9	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	cTY10	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	cTY12	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	cTY14	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	cTY15	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	cTY17	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	cTY20	+	-	-	-	-		-		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	cTY21	+	-	-	-	-		-		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	cTY23	+	-	-	-	-		-		+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	cTY24	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	cTY25	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	cTY26	+	-	-	-	-		-		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	cTY27	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	cTY29	+	-	-	-	-		-		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	cTY30	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	cTY31	+	-	-	-	-		-		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	cTY32	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	cTY38	+	-	-	-	-		-		+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	cTY41	+	-	-	-	-		-		+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(cont. on next page)

Table 3.4. (cont.)

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Lactose	Sucrose	Galactose	Fructose	Mannose	Melezitose	Maltose	Melliibiose	Ribose	Mannitol	Arabinose	Salicin	Trehalose	Raffinose	Rhamnose	Sorbitol	Xylose	Glycerol
21	cTY44	+	-	-	+	+		-		+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
22	cTY45	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	cTY47	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	cTY53	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	cTY55	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	cTY57	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	cTY61	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	cTY62	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	cTY63	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	cTY63/2	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	cTY67	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	cTY69	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	cTY70	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	cTY71	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	cTY72	+	-	-	-	-		-		+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	cTY75	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	cTY77	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	cTY78	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	cTY79	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	cTY81	+	-	-	+	+		-		+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-

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Table 3.4. (cont.)

No	Strain	Gram Test	Catalase	Gas from Glucose	2% NaCl	4% NaCl	6,5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Lactose	Sucrose	Galactose	Fructose	Mannose	Melezitose	Maltose	Melliibiose	Ribose	Mannitol	Arabinose	Salicin	Trehalose	Raffinose	Rhamnose	Sorbitol	Xylose	Glycerol
41	cTY82	+	-	-	+	-		-		+	+	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-
42	c28	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	c38c	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	c39a	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	c47	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	c50	+	-	-	-	-		-		+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	c52	+	-	-	-	-		-		+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	c60	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	c62	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	c65	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51	c66a	+	-	-	-	-		-		+	+	+	+	+	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	+
52	c66b	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	c71	+	-	-	-	-		-		+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
54	c74	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	c77a	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	c77b	+	-	-	-	-		-		+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	c78	+	-	-	-	-		-		+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
58	c79	+	-	-	-	-		-		+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
59	c85	+	-	-	-	-		-		+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60	c90b	+	-	-	-	-		-		+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table 3.4. (cont.)

No	61	62	63	64	65	66
Strain	c94	c94a	c95	c95-2	c97-1	c97-2
Gram Test	+	+	+	+	+	+
Catalase	'	'	'	'	'	'
Gas from Glucose	'	'	'	'	'	'
2% NaCl	+	'	'	'	'	+
4% NaCl	'	'	'	'	'	'
6,5% NaCl						
Growth at 10°C	'	'	'	'	'	'
Growth at 15°C						
Growth at 45°C	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Galactose	'	'	+	'	'	'
Fructose	+	'	+	'	'	'
Mannose	'	'	+	'	'	'
Melezitose	'	'	+	'	'	'
Maltose	'	'	'	'	'	'
Mellibiose	'	'	'	'	'	'
Ribose	'	'	'	'	'	'
Mannitol	'	'	'	'	'	'
Arabinose	'	'	'	'	'	'
Salicin	'	'	'	'	'	'
Trehalose	'	'	'	'	'	'
Raffinose	'	'	'	'	'	'
Rhamnose	'	'	'	'	'	'
Sorbitol	'	'	'	'	'	'
Xylose	'	'	'	'	'	'
Glycerol	'	'	'	'	'	'

Table 3.5. Grouping of Cocci Shaped Isolates According to Fermentation Patterns

Group	Isolate Number	Fermented Sugars	Atypical Character	Biochemical Species Assignment
1	c51	Glucose, Lactose, Sucrose, Mannose	Fructose(-)	*Streptococcus* sp.
2	cTY8, cTY30, cTY44, cTY45, cTY53, cTY55, cTY62, cTY69, cTY70, cTY75, cTY77, cTY78, c28, c38C, c50	Glucose, Lactose, Sucrose, Galactose	Fructose(-)	*Streptococcus* sp.
3	cTY9, cTY10, cTY47, cTY57, cTY61, cTY63, cTY67, cTY71, c77a, c77b, c94a, c95, c97-1, c97-2	Glucose, Lactose, Sucrose	Fructose(-)	*Streptococcus* sp.
4	cTY12, cTY14, cTY15, cTY17, cTY24, cTY25, cTY27, cTY32, cTY41	Glucose, Lactose, Galactose	Fructose(-)	*Streptococcus* sp.
5	cTY72, c90b, c94	Glucose, Lactose, Sucrose, Fructose	-	*S. thermophilus*
6	cTY20, cTY21, cTY26, cTY29, cTY31	Glucose, Lactose	Fructose(-)	*Streptococcus* sp.
7	cTY23	Lactose, Fructose	Glucose (-)	*Streptococcus* sp.
8	cTY38	Lactose, Galactose	Glucose(-), Fructose(-)	*Streptococcus* sp.
9	cTY63-2	Lactose, Sucrose, Galactose	Glucose(-), Fructose(-)	*Streptococcus* sp.
10	cTY79, c65	Lactose, Sucrose	Glucose(-), Fructose(-)	*Streptococcus* sp.

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Table 3.5. (cont.)

Group	Isolate Number	Fermented Sugars	Atypical Character	Biochemical Species Assignment
11	C85	Lactose, Sucrose, Fructose	Glucose(-)	*Streptococcus* sp.
12	cTY81	Glucose, Lactose, Fructose, Sucrose, Galactose, Melezitose, Maltose	Melezitose(+)	*Streptococcus* sp.
13	c39a	Glucose, Lactose, Sucrose, Galactose, Melezitose	Fructose(-), Melezitose(+)	*Streptococcus* sp.
14	c47	Glucose, Lactose, Sucrose, Galactose, Mannose	Fructose(-)	*Streptococcus* sp.
15	c60, c62, c78	Glucose, Lactose, Sucrose, Galactose, Fructose, Mannose	-	*S. thermophilus*
16	cTY82	Glucose, Lactose, Sucrose, Galactose, Melezitose, Melibiose, Mannitol	Fructose(-), Melezitose(+), Mannitol(+)	*Streptococcus* sp.
17	c95-2	Glucose, Lactose, Sucrose, Galactose, Fructose, Mannose, Melezitose	Melezitose (+)	*Streptococcus* sp.
18	c79	Glucose, Lactose, Sucrose, Galactose, Fructose, Melezitose, Glycerol	Melezitose(+), Glycerol(+)	*Streptococcus* sp.
19	c74	Glucose, Lactose, Sucrose, Galactose, Fructose	-	*S. thermophilus*
20	c71	Glucose, Lactose, Sucrose, Galactose, Fructose, Mannose, Melezitose, Glycerol	Melezitose(+), Glycerol(+)	*Streptococcus* sp.
21	c66b	Glucose, Lactose, Sucrose, Galactose, Mannose	Fructose(-)	*Streptococcus* sp.
22	c66a	Glucose, Lactose, Sucrose, Galactose, Fructose, Melezitose, Ribose, Salisin, Trehalose, Glycerol	Melezitose(+), Ribose(+), Salisin(+), Trehalose(+), Glycerol(+)	*Streptococcus* sp.

3.3. Molecular Characterization

3.3.1. Molecular Characterization of Bacilli Isolates

3.3.1.1. Genomic DNA Isolation

The isolation of genomic DNAs was verified with agarose gel electrophoresis. Genomic DNAs were seen as a smear of different molecular weight DNAs under UV light. Some genomic DNA patterns of isolates were shown in Figure 3.2.

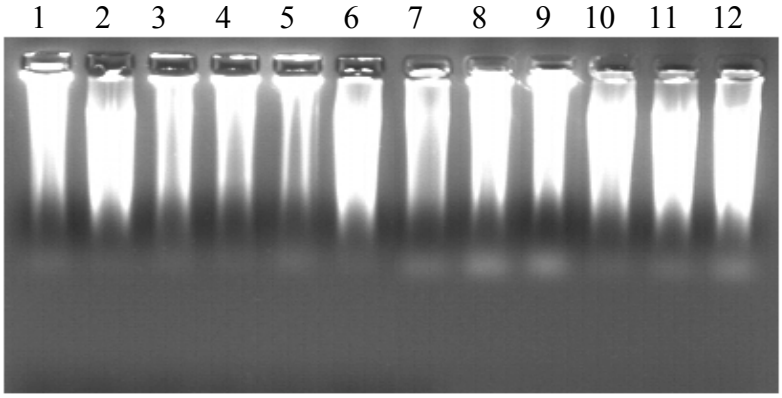

Figure 3.2. Representative genomic DNAs of isolates
Lanes: **1**. cTY70, **2**. cTY10, **3**. cTY67, **4**. cTY57, **5**. cTY55, **6**. cTY71, **7**. cTY78, **8.** cTY47, **9**. cTY45, **10**. cTY77, **11**. cTY82, **12**. cTY62

3.3.1.2. Determination of the Most Discriminative Endonucleases for Restriction Fragment Length Polymorphism

Fourteen different 16S complete sequences of *Lactobacillus delbrueckii* subspecies, *Lactobacillus helveticus* and *Lactobacillus acidophilus* were taken from NCBI Gene Bank and their accession numbers were given in the appendix. The

sequences were theoretically digested with *EcoR* I, *Taq* I, *Hae* III, *Apa* I, *Alu* I, *Hind* III, *Rsa* I, *Dra* I *and Not* I with the help of Ape-A Plasmid Editor v1.10.4.

According to theoretical digestion patterns *EcoR* I was found to be discriminative at the subspecies level between *Lactobacillus delbrueckii* subspecies. It differentiates *Lb. delbrueckii* ssp. *bulgaricus* from other two subspecies, although it does not differentiates *lactis* and *delbrueckii* subspecies. *Taq*I and *Hae*III enzymes were discriminative at species level but did not differentiate *Lb. delbrueckii* subspecies.

For the experimental verification, 16S and 16S-ITS regions of the genomic DNA's were amplified for the reference strains and given in Figure 3.3.

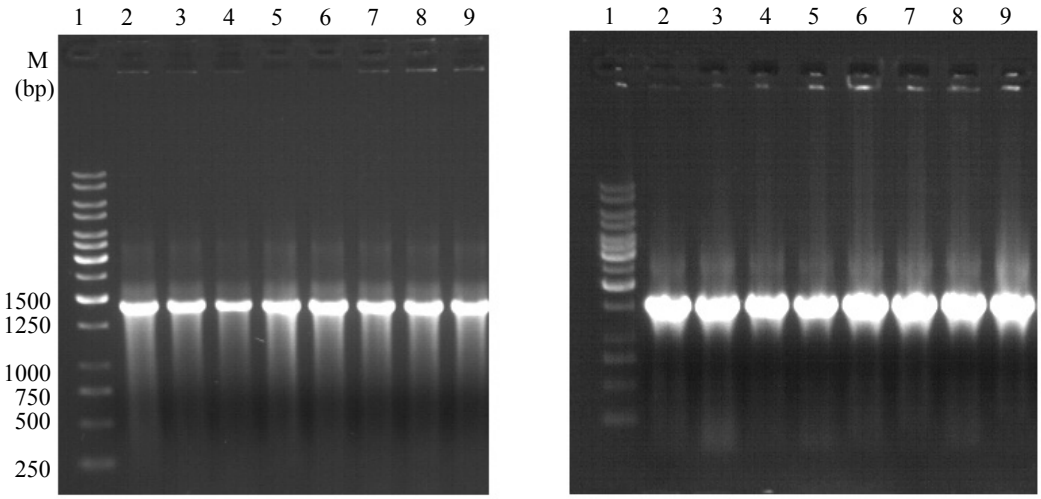

Figure 3.3.a. Representative 16S-ITS Amplification Products of Reference Strains,
b. Representative 16S Amplification Products of Reference Strains
Lanes: **1**.1kb DNA ladder Gene Ruler^{TM} **2**. CCM 4833 *Lb. acidophilus*, **3**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii*, **4**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii*, **5**. CCM 2772 *Lb. delbrueckii* ssp *lactis*, **6**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis,* **7**. NRRL 735 *Lb. delbrueckii* ssp. *lactis*, **8.** NRRL 4526 *Lb. helveticus* , **9**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*

The purified PCR products were then digested with the *EcoR*I, *Taq*I or *Hae*III restriction enzymes. The agarose gel electrophoresis paterns of restricted fragments were given in figures below.

3.3.1.2.1. *EcoR*I Restriction Profiles of Reference Strains

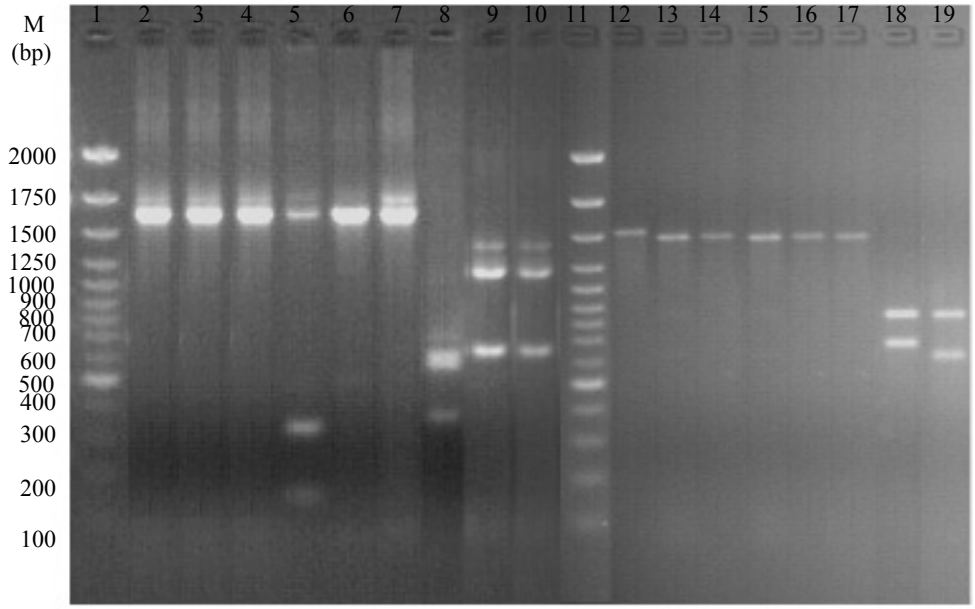

Figure 3.4. *EcoR*I Digests of both 16S-ITS and 16S rRNA genes of Reference Strains Lanes: **1**.100bp DNA ladder Gene Ruler^{TM} **2**. CCM 4833 *Lb. acidophilus* (16S-ITS), **3**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **4**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **5**. NRRL 1927 *Lb. delbrueckii* ssp *lactis* (16S-ITS), **6**. CCM 2772 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **7**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **8.** NRRL 4526 *Lb. helveticus* (16S-ITS), **9**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S-ITS), **10**. b22; **11**.1kb DNA ladder Gene Ruler^{TM} **12**. CCM 4833 *Lb. acidophilus* (16S), **13**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **14**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **15**. CCM 2772 *Lb. delbrueckii* ssp *lactis* (16S), **16**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis* (16S), **17**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S), **18.** NRRL 4526 *Lb. helveticus* (16S), **19**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S)

The theoretical digestion profiles were verified by the experimental *EcoR*I restriction results. According to 16S digestion profiles, *Lb. delbrueckii* ssp. *bulgaricus* was differentiated from other subspecies with two 600 and 800 bp digestion fragments; however for other subspecies only one digestion product, with almost 1.500 kb, was seen. *Lb. delbrueckii* ssp. *bulgaricus* was also differentiated from *Lb. helveticus* with its smaller digestion product. According to 16S-ITS digestion profiles, the similar digestion patterns were obtained for *delbrueckii* subspecies but the digestion pattern of *L. helveticus* was more clearly differentiated from the pattern of *Lb. delbrueckii* ssp.

bulgaricus for the 16S-ITS products. One more digestion product was also seen with slight brightness for all of the 16S-ITS products. This resulted from the ITS regions. Not all of the ITS regions contains only t-RNA genes as insert and some of them also contains t-RNA-Ala genes. This phenomenon leads to more than one product for the same digestion. It also leads to more than one PCR products with similar molecular weight as well.

3.3.1.2.2. *Taq*I Restriction Profiles of Reference Strains

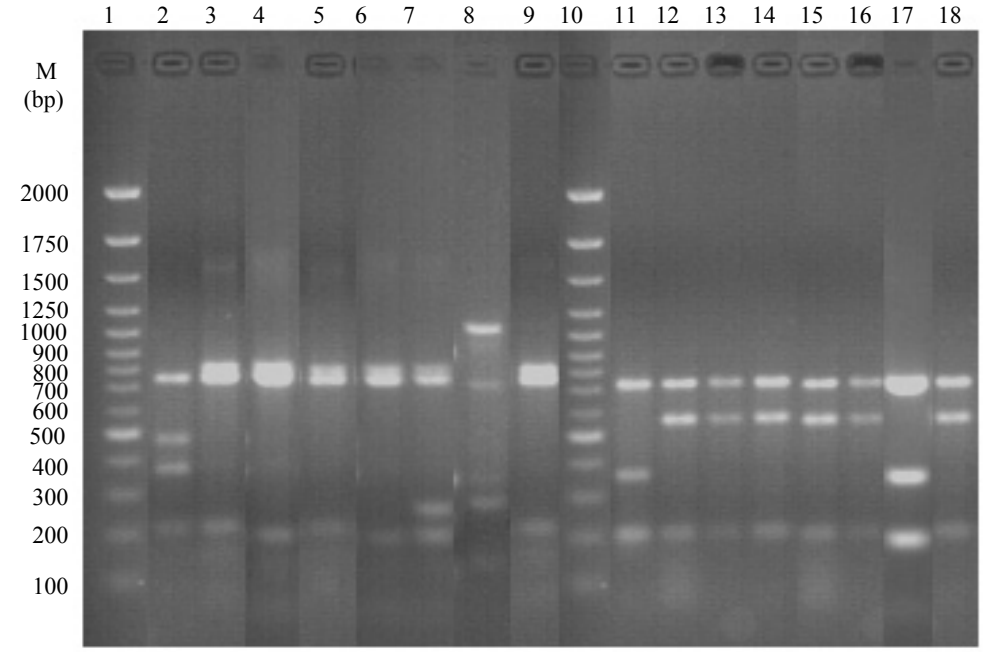

Figure 3.5. *Taq*I Digests of both 16S-ITS and 16S rRNA genes of Reference Strains Lanes: **1**.100bp DNA ladder Gene Ruler^{TM} **2**. CCM 4833 *Lb. acidophilus* (16S-ITS), **3**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **4**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **5**. CCM 2772 *Lb. delbrueckii* ssp *lactis* (16S-ITS), **6**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **7**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **8.** NRRL 4526 *Lb. helveticus* (16S-ITS), **9**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S-ITS), **10**.1kb DNA ladder Gene Ruler^{TM} **11**. CCM 4833 *Lb. acidophilus* (16S), **12**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **13**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **14**. CCM 2772 *Lb. delbrueckii* ssp *lactis* (16S), **15**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis* (16S), **16**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S), **17.** NRRL 4526 *Lb. helveticus* (16S), **18**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S)

Both 16S-ITS and 16S digestion profiles provided differentiation at species level but not between delbrueckii subspecies at the subspecies level.

3.3.1.2.3. *Hae*III Restriction Profiles of Reference Strains

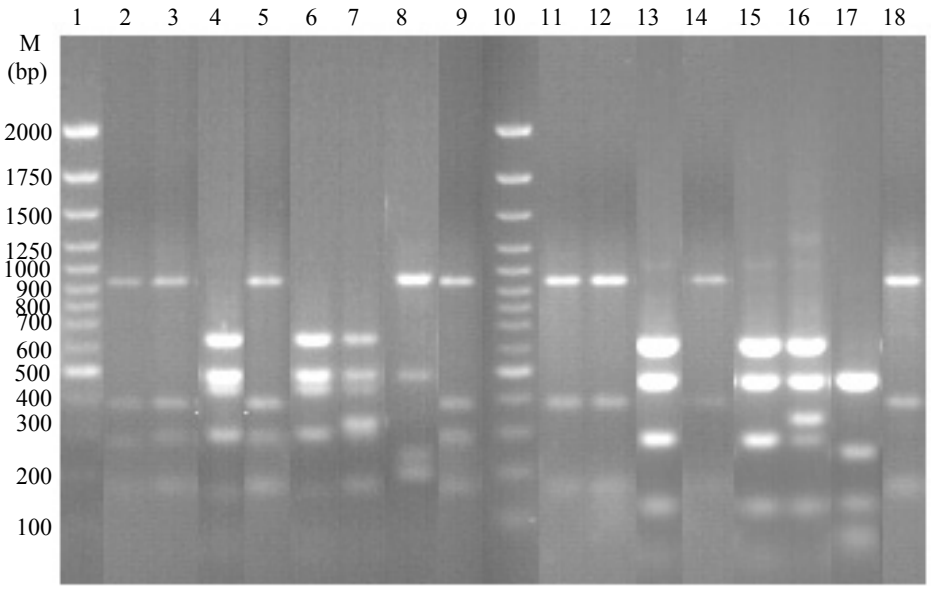

Figure 3.6. *Hae*III Digests of both 16S-ITS and 16S rRNA genes of Reference Strains Lanes: **1**.100bp DNA ladder Gene Ruler^{TM} **2**. CCM 4833 *Lb. acidophilus* (16S-ITS), **3**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **4**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S-ITS), **5**. CCM 2772 *Lb. delbrueckii* ssp *lactis* (16S-ITS), **6**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **7**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S-ITS), **8.** NRRL 4526 *Lb. helveticus* (16S-ITS), **9**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S-ITS), **10**.1kb DNA ladder Gene Ruler^{TM} **11**. CCM 4833 *Lb. acidophilus* (16S), **12**. CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **13**. NRRL 753 *Lb. delbrueckii* ssp. *delbrueckii* (16S), **14**. CCM 2772 *Lb. delbrueckii* ssp *lactis* (16S), **15**. NRRL 1927 *Lb. delbrueckii* ssp. *lactis* (16S), **16**. NRRL 735 *Lb. delbrueckii* ssp. *lactis* (16S), **17.** NRRL 4526 *Lb. helveticus* (16S), **18**. CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*(16S)

*Hae*III restriction profiles of reference strains did not provide differentiation at species level.Comparing the results of experimental digestion patterns, *EcoR* I enzyme was found to be the most effective. It could differentiate *Lb. delbrueckii* ssp. *bulgaricus* from other two subspecies. Both 16S and 16S-ITS regions gave valuable results but the use of 16S-ITS region was preferable. Because the digestion pattern of *L. helveticus* was more clearly differentiated from the pattern of *Lb. delbrueckii* ssp. *bulgaricus* for the 16S-ITS products and also there were fewer studies on the RFLP of 16S-ITS genes. As a result, the genotypic identification of newly isolated strains was based on the RFLP of 16S-ITS region with *EcoR*I restriction.

3.3.1.3. *EcoR*I Restriction Profiles of 16S rRNA and ITS Region for Bacilli Isolates

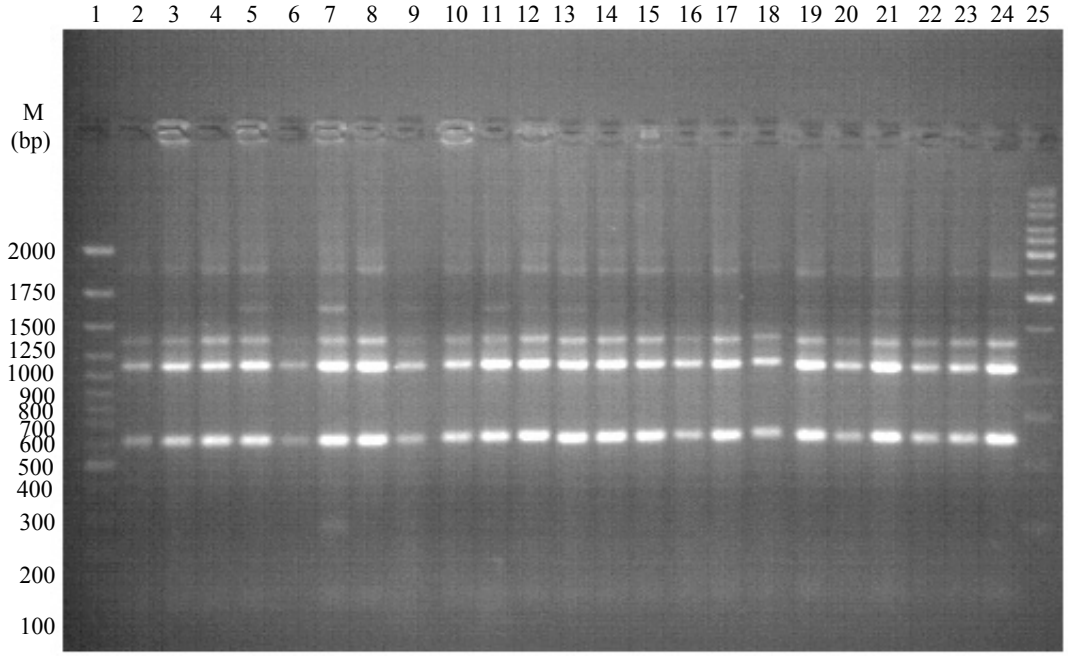

Figure 3.7. *EcoR*I Digests of 16S-ITS rRNA Genes of Representative Bacilli Isolates Lanes: **1**. 100bp DNA ladder Gene Ruler^{TM}, **2**. bTY77a, **3**. bTY79,**4**. bTY80, **5**. bTY83, **6**. bTY85, **7**. bTY86, **8**. bTY87, **9.** bTY88, **10**. bTY91 **11**. bTY92, **12**. b26, **13**. b33a, **14**. b33b, **15**. b44, **16**. b48, **17**. b51, **18**. b54, **19**. b62, **20**. b64, **21**. b71, **22**. b76, **23**. b77, **24**. b79, **25**. 1 kb DNA ladder Gene Ruler^{TM}

In total 71 bacilli isolates and reference strains were identified by *EcoR*I digestion. The restriction fragment patterns of all isolates were similar and they yielded %100 homology with the pattern of *L. delbrueckii* ssp. *bulgaricus* reference strain. Hence only one genotypic group was obtained. Two representatives from the bacilli isolates were selected and grouped with the reference strains for final gel electrophoresis. Lastly, their dendrograms were obtained. *L. delbrueckii ssp. bulgaricus* isolates and the reference showed 30% homology to the restriction profile of *L. helveticus* and %0 homology to the restriction profiles of other *delbrueckii* subspecies and *L. acidophilus*. ARDRA with *EcoR*I was found to be effective method for the differentiation of *L. delbrueckii* ssp. *bulgaricus* at the subspecies level.

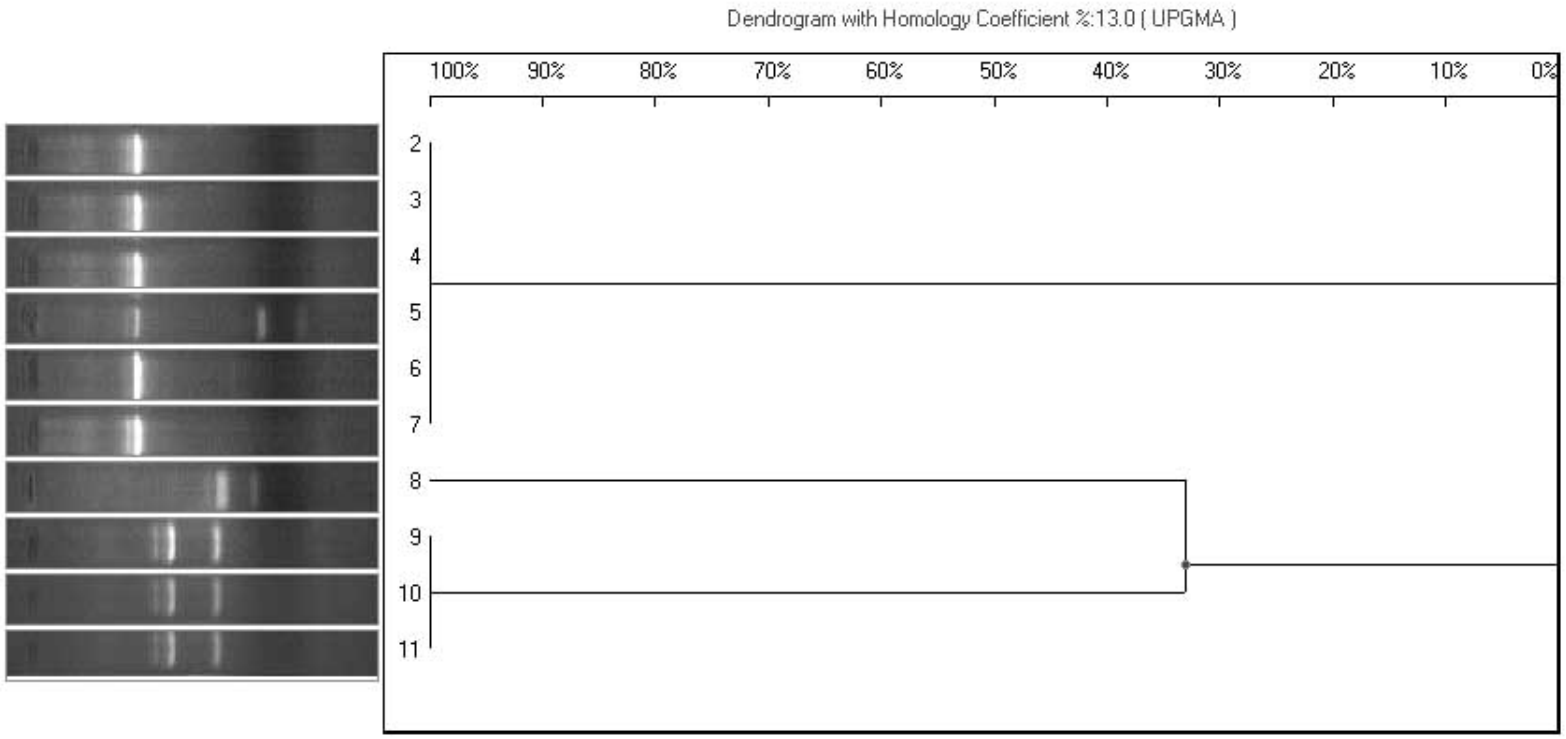

Figure 3.8. Dendogram of *EcoR* I Digests of Representative Bacilli Isolates and the Reference Strains

Lanes: **2** *Lb. delbrueckii* ssp. *delbrueckii* CCM 7191, **3.** *Lb. delbrueckii* ssp. *delbrueckii* NRRL-B 763; **4.** *Lb. delbrueckii* ssp. *lactis* NRRL-B 1924; **5**. *Lb. delbrueckii* ssp. *lactis* NRRL-B 735; **6**. *Lb. delbrueckii* ssp. *lactis* CCM 2772; **7**. *Lb. acidophilus* CCM 4833; **8.** *Lb. helveticus* NRRL-B 4526; **9.** *Lb. delbrueckii* ssp. *bulgaricus* CCM 7190; **10.** bTY30; **11.** bTY86**.**

3.3.1.4. Sequencing of a Representative Bacilli Isolate bTY30

Although ARDRA with *EcoR*I gave clear differentiation of *L. delbrueckii* ssp. *bulgaricus* from other subspecies, the identification results were also verified by sequencing. The 16S rRNA product of one representative isolate, bTY30, was sequenced in two directions with four primers. Sequences with forward and reverse primers were matched and compared with primer walking and the gaps were completed. The sequence of the 16S rRNA product of bTY30 was 1450bp and given below. The BLAST Analysis at the NCBI gene bank gave 99% homology to *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC BAA-365, 98% homology to *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842, and 99% homology to *Lactobacillus delbrueckii* ssp. *bulgaricus* strain BCRC10696.

5'-CGCTGGCGGCGTGCCTATACATGCCNNCGAGCGAGCTGAATTCAAAGATT
NNNNCCGGGATGATTTGTTGGACGCTAGCGGCGGATGGGTGAGTAACACGT
GGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAACAGGTGCTAATAC
CGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGTAAGCTG
TCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCC
TACCAAGGCAATGATGCGTAGCCGAGTTGAGAGACTGATCGGCCACATTGG
GACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
ACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTT
CGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGCAGTAACTGGT
CTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA
GCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAA
TTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCG
AAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAG
CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGCGCTAG
GTGTTGGGGACTTTCCGGTCCTCAGTGCCGCAGCAAACGCATTAAGCGCTCC
GCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTGTGCTACACCTAGAGATAGGTGGTTCCCTTCGG
GGACGCAGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCATCATTA
AGTTGGGCACTCTAAAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA
TGGGCAGTACAACGAGAAGCGAACCCGCGAGGGTAAGCGGATCTCTTAAA
GCTGTTCTCAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGCTGGAAT
CGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGAAGTCTGCAATGCCCAAAGTGAAAGTG
GGATAACCTTTATAGGAGTCA-3'

3.3.2. Molecular Characterization of Cocci Isolates

3.3.2.1. Identification by Amplified Ribosomal DNA Restriction Analysis

For the identification of cocci isolates by ARDRA, *Taq* I *and Hae* III enzymes were used for restriction fragment length polymorphism based on 16S rRNA and ITS genes. These enzymes were found to be the most effective enzymes for the identification of cocci lactic acid bacteria in previous studies (Bulut et al. 2005). The procedure was the same with the one given in section 2.2.4.1.

3.3.2.1.1. *Taq*I Restriction Profiles of Reference *S. thermophilus* Strain and the Cocci Isolates

In total 66 cocci isolates were digested with *Taq*I. Although only 6 of cocci isolates were determined to be *S. thermophilus* with phenotypic methods, the restriction fragment patterns of 63 isolates were the same and have good corelation with the pattern of *S. thermophilus* reference strain. Only 3 of the cocci isolates, cTY81, cTY21, cTY77b, gave different restriction fragment paterns. The isolates; cTY81, cTY21, cTY77b and one representative cocci (cTY55) from the remaining 63 isolates and also the reference strain were grouped for the final gel electrophoresis. Restriction profiles were then used for the dendrogram analysis. *Taq*I digests of 16S-ITS genes of some of the cocci isolates were shown in Figure 3.9 and The RFLP of representative strains and their dendogram was shown in Figure 3.10.

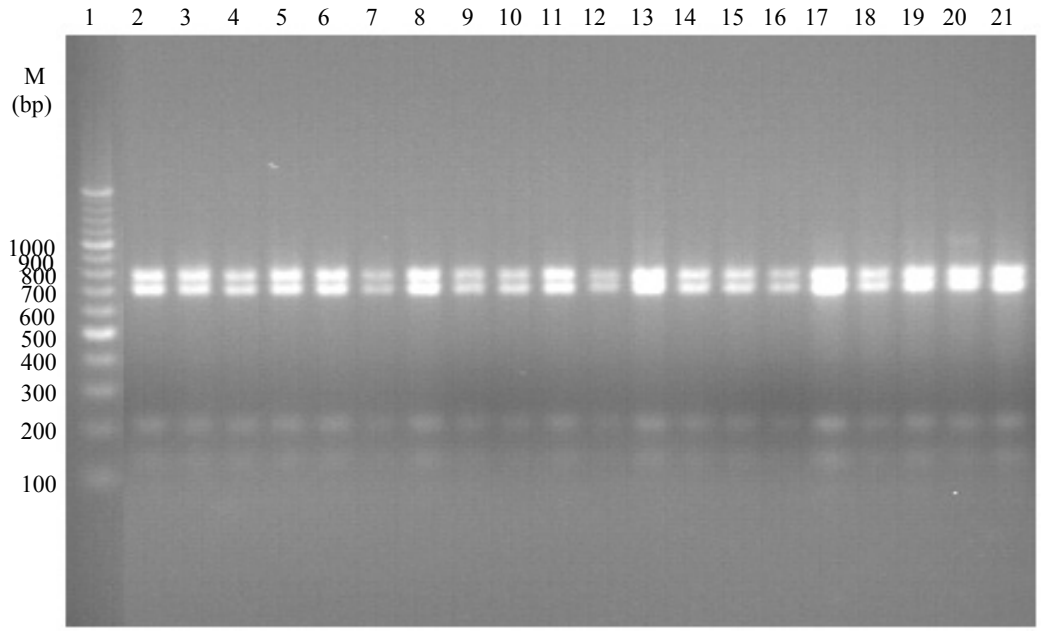

Figure 3.9. *Taq*I Digests of 16S rRNA and ITS genes of Some Cocci Isolates Lanes: **1**.100bp DNA ladder Gene Ruler^{TM} **2**. *S. thermophilus CCM 4757*; **3.**c29; **4.** c39; **5.** c47; **6.** c50; **7.** c60; **8.** c66A; **9.** c*70*; **10.** c*79*; **11.** c*85*; **12.** c90b; **13.** c94; **14.** c94c; **15.** c95-1; **16.** c95-2;**17.** c97-1; **18.** cTY9; **19.** cTY12; **20.** cTY14; **21.** cTY15;

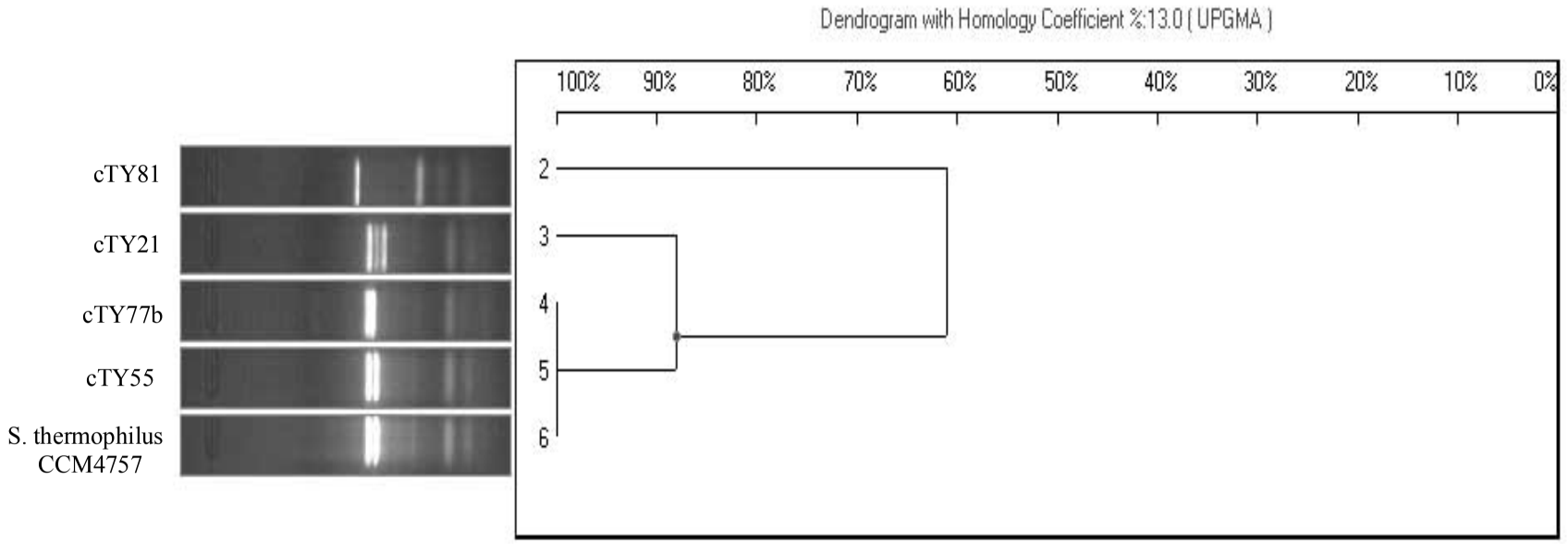

Figure 3.10. Dendrogram of *Taq* I Digests of 16S-ITS genes of Representative Cocci Isolates and Reference Strains

3.3.2.1.2. *Hae*III Restriction Profiles of Reference *S. thermophilus* Strain and the Cocci Isolates

All of the 66 isolates gave the same restriction pattern for *Hae*III digestion. It was also highly correlated (100% homology) with the pattern of reference strain. The restriction patterns and dendrogram of some of the isolates with the reference *S. thermophilus* strain was given in the Figure 3.11. and the dendRogram was given in the Figure 3.12.

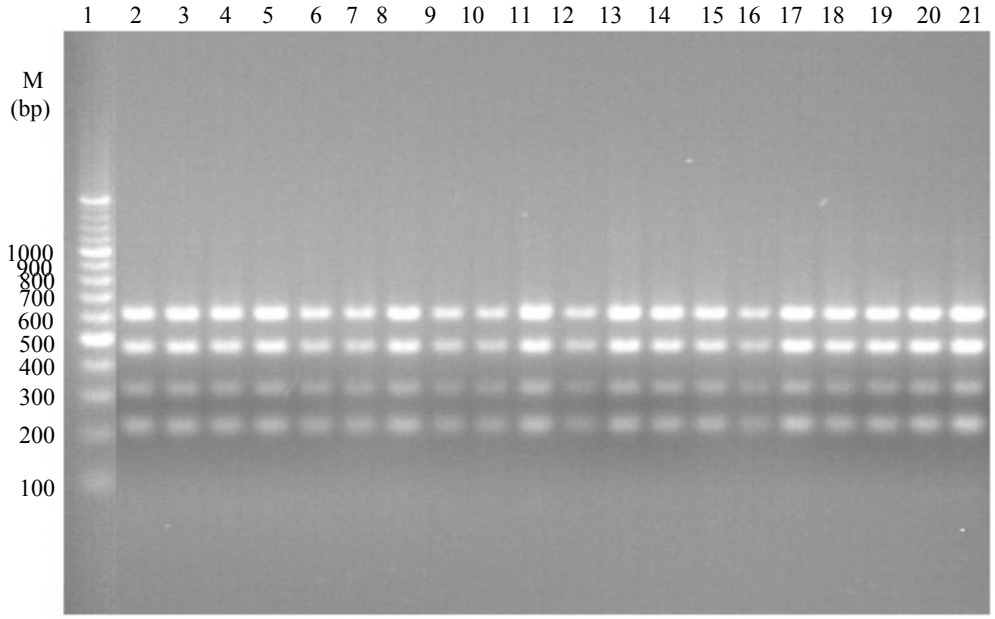

Figure 3.11. *Hae*III Digests of 16S-ITS genes of Some Cocci Isolates
Lanes: **1**.100 bp DNA ladder Gene Ruler™ **2**. *S. thermophilus CCM 4757;* **3.**c29; **4.** c39; **5.** c47; **6.** c50; **7.** c60; **8.** c*66A*; **9.** c*70*; **10.** c*79*; **11.** c*85*; **12.** c*90b*; **13.** c94; **14.** c94c; **15.** c95-1; **16.** c95-2; **17.** c97-1; **18.** cTY9; **19.** cTY12; **20.** cTY14; **21.** cTY15;

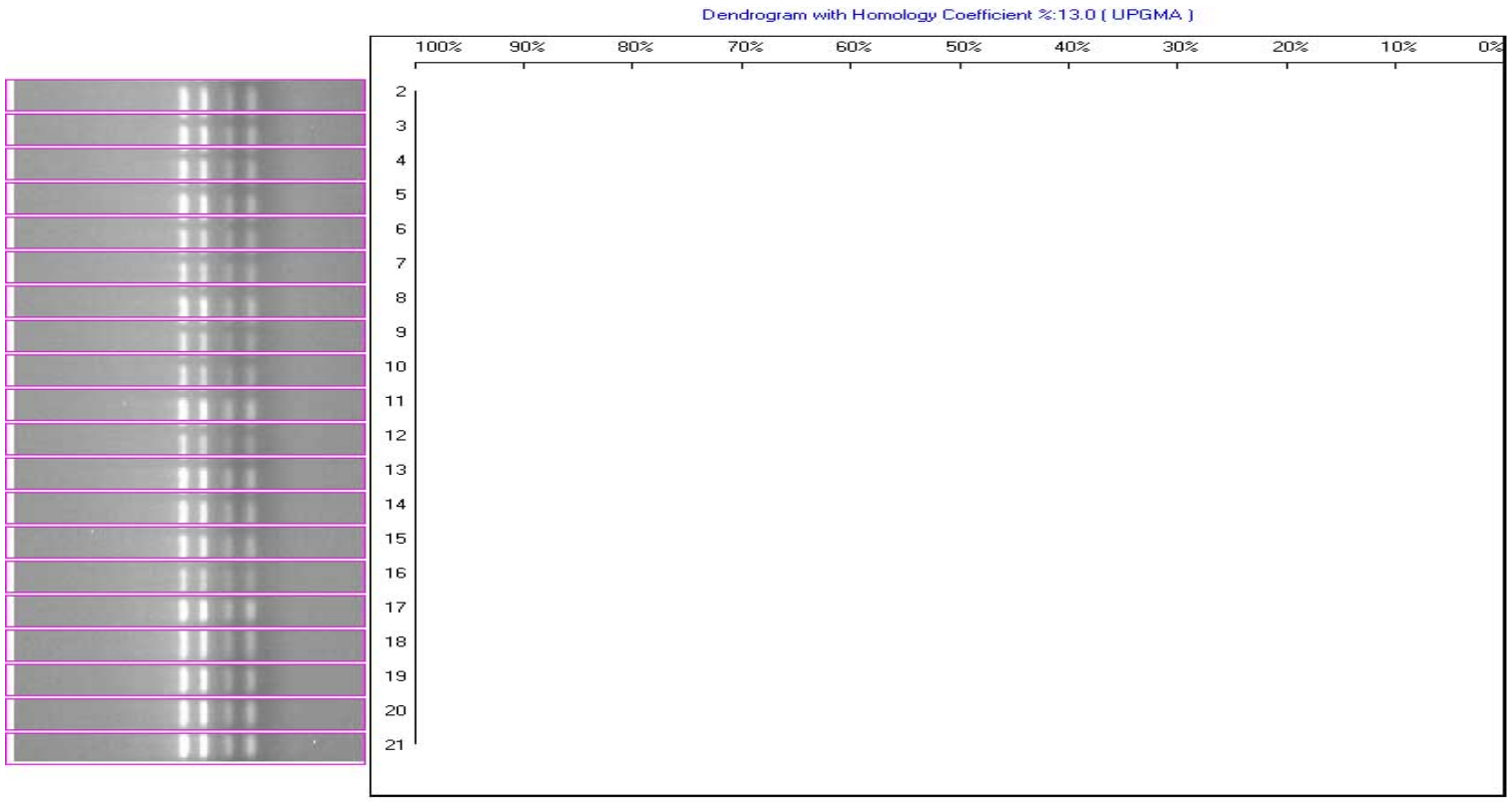

Figure 3.12. Dendrogram of *Hae* III Digests of Representative Cocci Isolates and the Reference *S. thermophilus* Strain

Lanes: **2**. *S. thermophilus CCM 4757;* **3.**c29; **4.** c39; **5.** c47; **6.** c50; **7.** c60; **8.** c*66A*; **9.** c*70*; **10.** c*79*; **11.** c*85*; **12.** c*90b*; **13.** c94; **14.** c94c; **15.** c95-1; **16.** c95-2; **17.** c97-1; **18.** cTY9; **19.** cTY12; **20.** cTY14; **21.** cTY15.

Comparing *Taq*I and *Hae*III RFLP results, *Taq*I seemed to be more effective at the strain level.

3.3.2.2. Identification by Species Specific PCR

The results of ARDRA was also verified by species specific PCR. To increase detection sensitivity, a species-specific PCR amplification procedure was developed (Lick et al. 1995). Primers were shown to be specific to amplify an intragenic fragment of 968bp within the *lacZ* gene sequence of *S. thermophilus*.

All of the 66 cocci isolates gave the expected size of PCR products and they were also correlated with the reference strain. The PCR products was shown in the Figure 3.13 below.

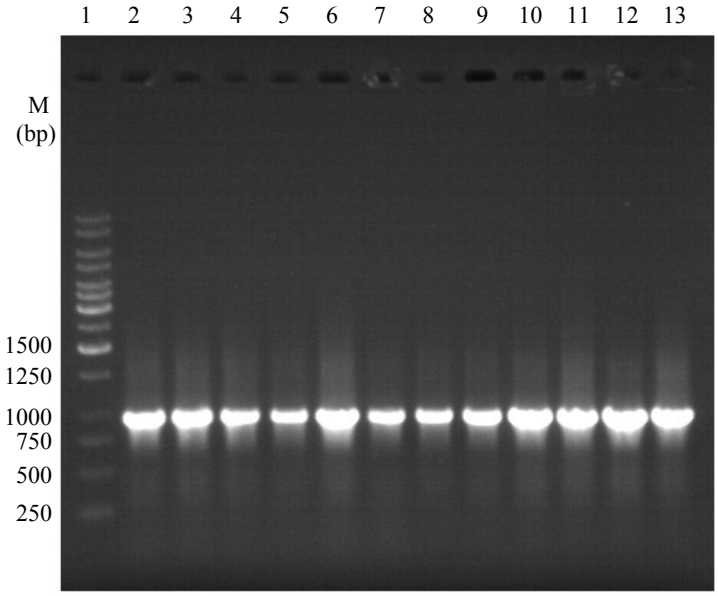

Figure 3.13. Species Specific PCR Products of Some of the Cocci Isolates
Lanes: **1**.1kb DNA ladder Gene Ruler™ **2**. *S. thermophilus CCM 4757;* **3.**c7; **4.** c38c; **5.** c62; **6.** c90b; **7.** c94c; **8.** c*97-1*; **9.** cTY23; **10.** cTY31; **11.** cTY32; **12.** cTY45; **13.** cTY65.

3.3.2.3. Sequencing of a Representative Cocci Isolate cTY17

The results of ARDRA with *Taq* I and *Hae* III showed 100% homology of cocci isolates to *S. thermophilus* CCM 4757. However, use of only one reference strain and the absence of other representative cocci species was considered to be insufficient for reliable identification. Thus, the isolates were characterized by the species-specific

PCR. The results were also verified by sequencing of 16S rRNA of one representative isolate. The 16S rRNA product of one representative isolate, cTY17, was sequenced in both directions using two external and two inner primers. Sequences with four primers were matched and compared with primer walking and the gaps were completed. The sequence of the 16S product of cTY17 was 1460bp and given below. The BLAST Analysis at the NCBI gene bank gave 98% homology to *Streptococcus thermophilus* LMD-9, 98% homology to *Streptococcus thermophilus* LMG 18311, and 98% homology to *Streptococcus thermophilus* CNRZ1066.

5'_GGCGGCGTGCCTAATACATGAAGTTAGAACGCTGAAGAGAGGAGCTTGC
TCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTG
TAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGAT
GACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCT
GCGTTGTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACA
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTG
ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGT
TGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGC
TTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTT
GATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAAACT
GTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAA
ATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTG
TAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGGA
TTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC
AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCG
ATGCTATTTCTAGAGATAGAAAGTTACTTCGGTACATCGGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGAGAC
TGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCGA
GTCGGTGACGGCGAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGG
CTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCAC
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACG
AGAGTTTGTAACACCCGAGAANNGGTGAGGTAACNTTTTGGAGCCAAATAT
NNNTAAGGGGGACAGATGATTGGGGA_3'

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVE

L. delbrueckii ssp. *bulgaricus* and *S. thermophilus* are the well-known yoghurt starter bacteria. Isolation and identification of these yoghurt starters by molecular techniques was the object of the study. Different phenotypic tests such as morphological examination, resistance to different salt concentrations and temperatures, gas production from glucose, determination of sugar fermentation patterns were applied for phenotypic identification. ARDRA based on 16S rRNA and ITS genes, species-specific PCR and 16S rRNA sequencing were also the methods of choice for genotypic identification. At the end of the study;

- All cocci isolates were identified as *S. thermophilus.* Genotypic method was more effective than the phenotypic methods for species assignment. Only 6 of the isolates could be identified as *S. thermophilus* with phenotypic methods.
- Phenotypic methods, especially determination of sugar fermentation patterns, were found to be more effective than genotypic methods for the differentiation of cocci isolates at the strain level. 21 different sugar fermentation profiles, so 21 different groups of cocci isolates were obtained with phenotypic methods.
- All bacilli isolates were identified to be *L. delbrueckii ssp. bulgaricus*. Both phenotypic and genotypic methods were found to be effective for identification at species level. No atypical behaviour was observed among bacilli isolates. However the phenotypic and genotypic methods did not give conclusive results for the differentiation at strain level.

Identification of yoghurt starter bacteria is actually the first step for the use of these starter microorganisms in dairy industry. Therefore, they should also be screened for their technological parameters. For yoghurt starters, these technological parameters mainly includes acidifying activity, production of aroma compounds especially acetaldehyde and diacetyl, exopolysaccharide production, and bacteriophage resistance. Determination of such characteristics would be helpful for industrial applications in the future.

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APPENDICES

APPENDIX A

Chemicals For Microbiological Experiments and Molecular Characterization

Table A.1 Chemicals Used In Microbiological Experiments

No	Chemical	Code
1	MRS Broth	Merck 1.10661
2	M17 Broth	Merck 1.15029
3	Bacteriological Pepton	Oxoid LP037
4	Yeast extract	Merck A 1.03753
5	Lablemco Meat Extract	Oxoid LP029
6	Sodium Acetate	Sigma S2889
7	Agar	AppliChem A0949
8	D(-) Mannitol	AppliChem A1903
9	D(+) Sucrose	AppliChem A2211
10	Fructose-	AppliChem A3688
11	D(-) Salicin	Fluka 84150
12	Esculin	AppliChem A1537
13	Mannose	Aldrich 11,258-5
14	(D+) Raffinose	AppliChem A6882
15	Arabinose	Aldrich A,9190-6
16	Trehalose	Merck 1.08216
17	(D-) Ribose	Merck 1.07605
18	L-Arginine hydrochloride	AppliChem A3709
19	D(+) Glucose	AppliChem A3666
20	D(+) Lactose	Sigma L3750
21	D(+) Maltose Monohydrate	AppliChem A3891
22	D(+) Galactose	Aldrich 11259-3

(cont. on next page)

Table A.1 (cont.)

No	Chemical	Code
23	D(+) Xylose	Merck 1.08689
24	D(+)Melesitose	Sigma M5375
25	L Rhamnose	AppliChem A4336
26	Melibiose	Sigma M5500
27	Triammonium citrate	Sigma A1332
28	Sodium Citrate trisodiumsalt	Sigma S4641
29	$MgSO_4.7H_2O$	Merck 1.05886
30	$MgCl_2$	Merck 1.4733
31	$MnSO_4.4H_2O$	Merck 1.02786
32	NaCl	AppliChem A2942
33	K_2HPO_4	Sigma P8281
34	Sodium hydroxide	Merck 1.06498
35	Glyserol-2-phosphate disodium salt	Sigma G6376
36	Tween 80	AppliChem A1390
37	Glycerol	AppliChem A2926
38	Anaerogen	Oxoid AN0025A
39	Safranine	Merck 1.15948
40	Crystal Violet	Sigma C3886
41	Potasium iodide	Sigma C6757
42	Methylene blue	AppliChem A1402
43	Bromcresol purple	Merck 1.03025
44	Mineral oil	Sigma M5904
46	Hyrogen peroxide (%30)	Merck 1.07209

Table A.2 Chemicals Used For Molecular Characterization

1	Agarose (standard)	AppliChem A2114
2	Chloroform	AppliChem A3830
3	Isoamyl alcohol	AppliChem A2610
4	PCR tubes	Greiner
5	EDTA	AppliChem A2937
6	Tris Base	Sigma T6066
7	SDS	AppliChem A0805
8	2-mercaptoethanole	Merck 8.05740
9	8-hydroxy chilonine	AppliChem
10	Phenol crystalline	AppliChem A1594
11	Isopropanol	AppliChem A3928
12	Ethanol (Molecular Biology Grade)	AppliChem A1151
13	*Taq* DNA Polymerase	MBI, Fermentas EP0401
14	Primers: Ege1 and L1	-
15	dNTP Set	MBI, Fermentas R0181
16	Sodium acetate	Sigma S-2889
17	D(+) Sucrose	AppliChem A3935
18	Ethidium Bromide	AppliChem A1151
19	*Taq* I	Fermentas ER0671
20	*Hae* III	Fermentas ER0151
21	*EcoR* I	Fermentas ER0271
22	Lysozyme	AppliChem A3711
23	Ribonuclease A	AppliChem A3832
24	Bromophenol Blue	Merck 1.08122
25	Mineral Oil	Sigma M5904
26	1kb DNA Ladder Gene Ruler™	Fermentas SM0313
27	100bp DNA Ladder Gene Ruler™	Fermentas SM 0328
28	Glycerol	AppliChem A2926
29	Boric Acid	AppliChem A2940
30	Cetyltrimethylammonium bromide	AppliChem A0805

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Table A.2 (cont.)

31	Proteinase K	AppliChem A3830
32	Sodium dodecyl suplhate	AppliChem A2263
33	Sodium hydroxide	Merck 1.06498
34	Hydrochloric Acid	Merck 1.00317
35	Ammonium acetate	AppliChem A2936
36	Glycial acetic acid	Merck 1.00056

APPENDIX B

Recipes For Culture Media And Biochemical Tests

B.1 MRS Broth

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.2 MRS Agar

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0

$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15.0
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

B.3 M17 Broth

Ingredients	**g/l**
Peptone from soymeal	5.0
Peptone from meat	2.5
Peptone from casein	2.5
Yeast extract	2.5
Meat extract	5.0
Lactose monohydrate	5.0
Ascorbic acid	0.5
Sodium β-glycerophosphate	19.0
Magnesium sulfate	0.25

All the ingredients were suspended into deionized water, and pH was adjusted to 6.9. Then solution was dispensed to the test tubes and autoclaved at 121 °C for 15 min.

B.4 M17 Agar

Ingredients	**g/l**
Peptone from soymeal	5.0
Peptone from meat	2.5
Peptone from casein	2.5
Yeast extract	2.5
Meat extract	5.0
Lactose monohydrate	5.0
Ascorbic acid	0.5
Sodium β-glycerophosphate	19.0

Magnesium sulfate	0.25
Agar-agar	12.75.

All the ingredients were suspended into deionized water, and pH was adjusted to 6.9. Then solution was autoclaved at 121 °C for 15 min.

B.5 Modified MRS Broth For Testing The Growth At Different Temperatures

Ingredients	**g/l**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.6 Modified M17 Broth For Testing The Growth At Different Temperatures

Ingredients	**g/l**
Peptone from soymeal	5.0
Peptone from meat	2.5
Peptone from casein	2.5
Yeast extract	2.5

Meat extract	5.0
Lactose monohydrate	5.0
Ascorbic acid	0.5
Sodium β-glycerophosphate	19.0
Magnesium sulfate	0.25
Bromcresol purple	0,04

All the ingredients were suspended into deionized water, and pH was adjusted to 6.9. Then solution was dispensed to the test tubes and autoclaved at 121 °C for 15 min.

B.7 Modified MRS Broth For Testing The Growth At Different NaCl Concentrations

Ingredients	**g/l**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
NaCl	20,40,65 for the concentration of 2%, 4% and 6.5%
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.8 Modified M17 Broth For Testing The Growth At Different NaCl Concentrations

Ingredients	g/l
Peptone from soymeal	5.0
Peptone from meat	2.5
Peptone from casein	2.5
Yeast extract	2.5
Meat extract	5.0
Lactose monohydrate	5.0
Ascorbic acid	0.5
Sodium β-glycerophosphate	19.0
Magnesium sulfate	0.25
Bromcresol purple	0.04
NaCl	20,40 for the concentration of 2% and 4%

All the ingredients were suspended into deionized water, and pH was adjusted to 6.9. Then solution was dispensed to the test tubes and autoclaved at 121 °C for 15 min.

B.9 Modified MRS Broth For Gas Production From Glucose

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and inverted durham tubes were distributed to each test tube, and lastly sterilized by autoclaving at 121°C for 15 min.

B.10 Modified MRS For Carbohydrate Fermentations

Ingredients	**g/l**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

APPENDIX C

Carbohydrates Used For Carbohydrate Fermentation Tests

1. L(+) Arabinose
2. D(+) Galactose
3. Lactose
4. Maltose
5. Mannitol
6. Raffinose
7. Sucrose
8. D(-) Salicin
9. Sorbitol
10. D(+) Trehalose
11. D(+) Xylose
12. Glycerol
13. D(+) Mannose
14. D(-) Ribose
15. Melibiose
16. Melezitose
17. Glucose
18. Fructose
19. Rhamnose

APPENDIX D

Buffers and Stock Solutions for Molecular Characterization

D.1 1M Tris-HCl pH 7.2 and pH 8.0

121.1 g of Tris base was dissolved in 800 ml of deionized H_2O. pH was adjusted to the desired value by adding concentrated HCl. The approximate values of the amount of HCl required for the desired pH values are given below.

pH	*HCl*
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool to room temperature before making final adjustments to the pH, and the volume of the solution was adjusted to 1 L with H_2O. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. It was dispensed into aliquots and sterilized by autoclaving. If the 1 M solution had a yellow color, it was discarded and obtained Tris of better quality.

D.2 0.5M EDTA pH 8.0

186.1 g of disodium EDTA•$2H_2O$ was added to 800 ml of deionized H_2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with 10N of NaOH (or approx. 20 g of NaOH pellets). Volume was adjusted to 1 L with deionized water. It was dispensed into aliquots and sterilized by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

D.3 50X TAE

242 g of Tris base was dissolved in deionized H_2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Lastly volume was adjusted to1 L with deionized water.

D.4 1X TAE

20ml of 50 X TAE buffer was taken and the volume was adjusted to 1 L with deionized water. The 1x working solution was 40 mM Tris-acetate/1 mM EDTA.

D.5 3M NaCl

175.32g NaCl was dissolved in deionized water and the volume was adjusted to 1L.

D.6 5M NaCl

292.2g NaCl was dissolved in deionized water and the volume was adjusted to 1L.

D.7 Ethidium Bromide Stock Solution (10mg/ml)

0.5g ethidium bromide was dissolved in 50ml deionized water and the solution was storred in dark bottle at room temperature.

D.8 3M Sodium Acetate pH 5,2

408.3 g of sodium acetate•$3H_2O$ was dissolved in 800 ml of deionized H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 L with deionized H_2O.

D.9 Chloroform-Isoamyl Alcohol Solution

96ml of chloroform was mixed with 4ml of isoamyl alcohol.

D.10 Phenol

Most batches of commercial liquefied phenol are clear and colorless and can be used in molecular techniques without redistillation, however some batches of liquefied phenol are pink or yellow, and these should be rejected. Crystalline phenol was preferred in experiments. First, it was allowed to warm at room temperature and then it was melted at 68°C. Before use, phenol must be equilibrated to a pH of >7.8 because the DNA partitions into the organic phase at acid pH. Gloves, full face protection, and a lab coat should be weared when carrying out this procedure.

To the melted phenol, an equal volume of 0.5 M Tris-Cl (pH 8.0) buffer was added at room temperature. The mixture was stirred on a magnetic stirrer for 15 minutes. When the two phases have separated, the aqueous phase (upper phase) was removed with separation funnel. Then an equal volume of 0.1 M Tris-Cl (pH 8.0) was added to the phenol. The mixture was stirred on a magnetic stirrer for 15 min. The upper aqueous phase was removed as described before. The extractions were repeated until the pH of the phenolic phase is >7.8 (as measured with pH paper).

After the phenol was equilibrated and the final aqueous phase has been removed, phenol was divided to aliquots and 0.1 volume of 0.1 M Tris-Cl (pH 8.0) was added on top of each aliquot. The phenol solution should be stored in this form under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at -20°C. When needed, phenol was melted at room temperature. Hydroxyquinoline (to a final concentration of 0.1%), and ß-mercaptoethanol (to a final concentration of 0.2%) were added before use. Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.

D.11 1 X TE BUFFER

100mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) was mixed and the buffer was stored at room temperature.

D.12 CTAB/NaCl Solution

4.1g NaCl was dissolved in 80ml deionized water. 10g CTAB was added slowly while heating and stirring. The solution can be heated to 65°C to increase the dissolution. Lastly, the final volume was adjusted to 100ml.

D.13 10% Sodium Dodecyl Sulfate (SDS)

100g of SDS was dissolved in 900ml of deionized water. Solution was heated to 68°C to dissolve. The pH was adjusted to 7.2 with the addition of a few drops of concentrated HCl. The volume was adjusted to 1L with deionized water.

D.14 10X Bovin Serum Albumin (BSA)

150µl of Bovine Serum Albumin (10mg/ml) was diluted with 1.5ml TE buffer. It was divided into aliquots and stored at -20°C.

D.15 Gel Loading Dye

2ml of 10XTBE and 6ml of glycerol was mixed in a falcon and the volume was adjusted to 20ml with sterile deionized water. Bromohenol blue was added until the adequate color was obtained.

D.16 10X TBE Buffer

108g of Tris base and 55g of boric acid are mixed and dissolved in 800ml of deionized water. 40ml o 0.5M EDTA (pH 8) was added. The volume was adjusted to 1L with deionized water.

APPENDIX E

PCR RECIPES

E.1 PCR Mixture

Mg free Taq DNA polymerase buffer	5µl
$MgCl_2$ (25Mm)	3µl
Sterile deionized water	32µl
Oligo forward 10 picomole/µl	1µl
Oligo reverse 10 picomole/µl	1µl
dNTP (2 mM each)	5µl

E.2 dNTP (10X)

Twenty microliters of each 100mM dATP, dCTP, dGTP, and dTTP are taken and mixed in an eppendorf tube. 920µl of sterile deionized water was added to a final concentration of 2mM. Solution was mixed gently and stored at -20°C.

E.3 PRIMER OF EGE1

590µg primer EGE1 was dissolved in 295 µl of sterile deionized water to obtain 2µg/µl stock solution. 5µl of stock solution were then taken and mixed with 95µl sterile deionized water. The resulting solution had 100µl, 10 picomole /µl concentration. Stock and working solutions were stored at -20°C.

E.3 PRIMER OF L1

350µg primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2µg/µl stock solution. 5µl of stock solution were then taken and mixed with 95µl sterile deionized water. The resulting solution had 100µl, 10 picomole/µl concentration. Stock and working solutions were stored at -20°C.

E.4 PRIMER OF IYTE-1

453.8 µg primer IYTE-1 was dissolved in 832.3 µl of sterile deionized water to obtain 100 pmol/µl stock solution. Ten microliters of stock solution were then taken and mixed with 90µl sterile deionized water. The resulting solution was 10 pmole/µl concentration. Stock and working solutions were stored at -20°C.

E.5 PRIMER OF IYTE-2

281.4 µg primer IYTE-2 was dissolved in 509.4 µl of sterile deionized water to obtain 100 pmol/µl stock solution. Ten microliters of stock solution were then taken and mixed with 90µl sterile deionized water. The resulting solution was 10 pmole/µl concentration. Stock and working solutions were stored at -20°C.

APPENDIX F

Restriction Enzymes and Other Enzymes Used for Molecular Characterization

F.1 Restriction Enzyme Reaction Mixture

Restriction enzyme buffer	5µl
Sterile deionized water	34.5µl
Restriction Enzyme	0.5µl (from 5U)
DNA	10µl

APPENDIX G

A.3. Accession Numbers of 16S Sequences from NCBI Database

No	Definition	Length	Accesion Number	Version
1	*L.acidophilus* 16S rRNA gene	1379 bp	X61138	X61138.1 GI:43957
2	*Lactobacillus acidophilus* 16S ribosomal RNA	1569 bp	M58802	M58802.1 GI:175004
3	*Lactobacillus acidophilus johnsonii* 16S ribosomal RNA	1568 bp	M99704	M99704.1 GI:397798
4	*L.helveticus* 16S rRNA gene	1333 bp	X61141	X61141.1 GI:43998
5	*Lactobacillus helveticus* strain LMG 11445 16S ribosomal RNA gene	1503 bp	DQ123572	DQ123572.1 GI:74099608
6	*Lactobacillus helveticus* strain R0052 16S ribosomal RNA gene	1457 bp	DQ123580	DQ123580.1 GI:74099620
7	*Lactobacillus delbrueckii* 16S ribosomal RNA	1512 bp	M58814	M58814.1 GI:175022
8	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain BCRC10696 16S ribosomal RNA gene	1494 bp	AY773948	AY773948.1 GI:55418400
9	*Lactobacillus delbrueckii* subsp. *delbrueckii* strain BCRC12195 16S ribosomal RNA gene	1518 bp	AY773949	AY773949.1 GI:55418401
10	*Lactobacillus delbrueckii* subsp. *lactis* strain BCRC11051 16S ribosomal RNA gene	1516 bp	AY773950	AY773950.1 GI:55418402
11	*Lactobacillus lactis* 16S ribosomal RNA	1569 bp	M58823	M58823.1 GI:175029
12	*Streptococcus thermophilus* 16S ribosomal RNA gene	1508 bp	DQ176426	DQ176426.1 GI:74148898
13	*Streptococcus thermophilus* strain ATCC 19258 16S ribosomal RNA gene	1539 bp	AY188354	AY188354.1 GI:28274378
14	*Streptococcus thermophilus* strain ST3 16S ribosomal RNA gene	1540 bp	AY675258	AY675258.1 GI:53766380