Isolation and Molecular Characterization of Lactic Acid Bacteria From Raw Milk

By

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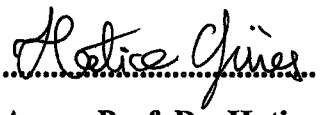

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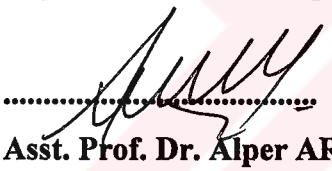

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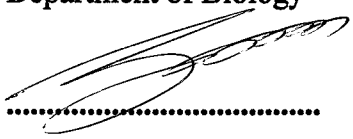

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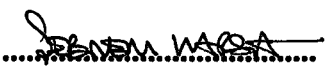

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ABSTRACT

Lactic acid bacteria are industrially important because they are used as starter cultures in food production, they produce antimicrobial compounds and they are used in the formulation of probiotic products. Several dairy products such as raw milk, traditionally fermented cheese (produced without the use of commercial starter cultures), and kefir which are produced in country are good sources of novel lactic acid bacterial strains. These lactic acid bacterial strains may have potential for the production of new fermented dairy products with characteristic aroma and flavour. Therefore, the isolation of lactic acid bacteria from natural products and their identification are important. For many years, several phenotypic methods have been used to identify lactic acid bacteria, but they are not often capable of effectively differentiating subspecies and strains within a genus. New methods based on the genotypic properties have been developed and used for the proper classification of bacteria.

The aim of this research was the isolation of lactic acid bacteria from raw milk and the identification of the lactic acid bacterial isolates by biochemical tests, polymerase chain reaction (PCR)-based methods and pulsed field gel electrophoresis (PFGE).

Lactic acid bacteria were isolated from cow's raw milk and identified by biochemical reactions. Two PCR based methods, ITS-PCR (Internal Transcribed Spacer-PCR) and PCR-RFLP (PCR- Restriction Fragment Length Polymorphism) were then used for the differentiation of reference strains of lactic acid bacteria. PCR-RFLP method, based on the amplification and restriction digestion of 16S rRNA gene, was found to be useful for the identification. Thirteen raw milk isolates were identified as *Lactococcus lactis*, 24 as *Enterococcus* spp., and 2 as *Lactococcus lactis* subsp. *cremoris* by PCR-RFLP method.

Pulsed field gel electrophoresis was also optimized for the identification of reference strains. Restriction profiles obtained by digesting the genomic DNA with *Sma* I enabled differentiation of the reference strains of *Lactococcus*, *Enterococcus*, and *Streptococus thermophilus*.

ÖZ

Laktik asit bakterileri, çeşitli gıdaların üretiminde starter kültür olarak kullanılmaları, antimikrobiyal maddeleri üretmeleri ve probiyotik ürünlerin formülasyonlarında yer almaları bakımından endüstriyel açıdan büyük öneme sahiptir. Çiğ süt, geleneksel yöntemlerle, ticari starter kültür kullanılmadan üretilmiş peynirler ve kefir gibi ülkemizde üretilen birçok süt ürünü yeni laktik asit bakteri suşları için iyi bir kaynak teşkil etmektedir. Bu laktik asit bakteri suşları, tat ve aroma özelliği bakımından farklı fermente süt ürünlerinin elde edilmesinde büyük potansiyele sahip olabilirler. Bu nedenle laktik asit bakterilerinin doğal kaynaklardan izolasyonu ve tanımlanması büyük önem taşımaktadır. Uzun zamandan beri, laktik asit bakterilerinin tanımlanmasında çeşitli fenotipik yöntemler kullanılmaktadır. Ancak, bu metotlar, bir genus içindeki alt türleri ve suşları etkin bir şekilde ayırt etmede çoğu zaman yetersiz kalmaktadır. Bu yüzden genotipik özelliklere dayanan yeni metotlar geliştirilmiş ve bakterilerin etkin bir şekilde tanımlanmasında kullanılmaya başlanmıştır.

Bu çalışmada, laktik asit bakterilerinin çiğ inek sütünden izolasyonu yapılarak, biyokimyasal testler, polimeraz zincir reaksiyonuna (PCR) dayanan metotlar ve "pulsed field" jel elektroforezi (PFGE) uygulanarak tanımlanması amaçlanmıştır.

Laktik asit bakterileri çiğ inek sütünden izole edilmiş ve ilk önce biyokimyasal testlerle tanımlanmıştır. Daha sonra polimeraz zincir reaksiyonuna dayanan iki metot, ITS-PCR ("Internal Transcribed Spacer-PCR") ve PCR-RFLP ("PCR- Restriction Fragment Length Polymorphism"), referans laktik asit bakteri suşlarının tanımlanması için kullanılmıştır.16S rRNA geninin çoğaltılması ve restriksiyon enzimiyle kesilmesine dayanan PCR-RFLP metodunun tanımlama için uygun bir metot olduğu kanısına varılmıştır. PCR-RFLP metoduyla, çiğ sütten elde edilen 13 izolat *Lactococcus lactis*, 24 izolat *Enterococcus* spp. ve 2 izolat da *Lactococcus lactis* subsp. *cremoris* olarak belirlenmiştir.

Ayrıca PFGE, referans kültürlerin tanımlanması için optimize edilmiştir. Genomik DNA' nın *Sma* I restriksiyon enzimi kesilmesi sonucu elde edilen fragman profilleri *Lactococcus*, *Enterococcus* ve *Streptococcus thermophilus* referans suşlarının tanımlanmasını mümkün kılmıştır.

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ABBREVIATIONS

A	Adenine
ARDRA	Amplified Ribosomal DNA Restriction Analysis
bp	Base pair
BSA	Bovine Serum Albumin
Cit$^+$	Citrate utilizing
C	Cytosine
CHEF	Clamped Homogeneous Electrical Field
DNA	Deoxyribonucleic Acid
dNTP	Deoxynuclotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
ERIC	Extragenic Repetitive Intergenic Consensus
G	Guanine
ITS	Internal Transcribed Spacer
kb	Kilo base
LAB	Lactic Acid Bacteria
LB	Luria Bertani
m	minute
Mb	Mega base
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulfonyl Floride
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
REP	Repetitive Extragenic Palindromic
Rep-PCR	Repetitive Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
subsp.	Subspecies
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA

TE	Tris EDTA
U	Unit
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

For thousands of years, fermentation has been used to preserve perishable food materials and to produce new foods. After the fermentation processes, new food materials with their own characteristic aroma and flavour are formed, the shelf life of raw materials is prolonged and the growth of pathogenic and spoilage organisms is inhibited. There are various types of fermented foods consumed around the world. One of the main classes of fermented foods is the products of lactic acid fermentation. Lactic acid fermentation is performed by bacteria, which are called as lactic acid bacteria (LAB). In lactic acid fermented foods, the pathogenic or spoilage organisms are inhibited due to the (Jay, 1992):

(i) competition for the nutrients
(ii) decrease in pH
(iii) acid formation (lactic, acetic acid and others)
(iv) production of antimicrobial compounds

The genera of lactic acid bacteria (LAB) associated with foods are *Lactobacillus*, *Leuconostocs*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Oenococcus*, *Weisella*, *Tetragenococcus*, *Carnobacterium*, *Bifidobacterium* (Stiles and Holzapfel, 1997). They are generally known as Gram positive, catalase and oxidase negative rods or cocci. They ferment carbohydrates into lactic acid either by homofermentation or heterofermentation processes. The main product of homofermentation is the lactic acid. In heterofermentation on the other hand, together with the lactic acid, some other products such as ethanol and carbondioxide are also formed.

The industrial importance of lactic acid bacteria can be summarized as follows: i) Several strains are used as starter cultures for the production of various kinds of fermented foods (yogurt, cheese, etc.). ii) Several strains of LAB have been used as probiotic cultures. iii) Bacteriocin production. Lactic acid bacteria have been known to show bacteriocidal effects on closely related species. Recently, it has been shown that

they also possess antifungal properties (Magnusson and Schnürer, 2001). It is therefore obvious that the isolation of lactic acid bacteria from different kinds of environments and their precise characterization is important.

LAB represent a very diverse group and they are found in various kinds of sources. Traditional dairy products are generally produced from raw milk without the use of starter cultures. They rely on the activities of lactic acid bacteria naturally occuring in milk as adventitious contaminants (Wouters *et al.*, 2002). Raw milk is therefore a good source of lactic acid bacteria, especially *Lactococcus lactis*, which is used as starter culture for cheese production. This means that raw milk may harbour novel lactococcal strains (Wouters *et al.*, 2002).

Another important characteristic of lactic acid bacteria is the production of antimicrobial compouds called as bacteriocins. These bacteriocins inhibit closely related species. Bacteriocins of lactic acid bacteria are generally regarded as safe since lactic acid bacteria themselves are also considered to be safe. Bacteriocins of lactic acid bacteria have the potential to be used to control pathogenic and spoilage bacteria in food products (Wouters *et al.*, 2002). Raw milk is also a good source of novel strains of lactic acid bacteria for use in biopreservation of dairy products (Rodríguez *et al.*, 2000).

After the isolation of lactic acid bacteria from any kind of source, it is needed to unequivocally identify cultures and determine their physiological and biochemical properties. Until recent years, the most common methods used to identify bacteria have been mainly based on the phenotypical and biochemical characteristics. Using these methods however, it is not often possible to reliably identify lactic acid bacteria. Because phenotypic methods generally fail to identify closely related isolates, there has recently been great attention to the use of genotypic characterization methods. Several genotypic methods (RAPD, plasmid typing, ribotyping, PFGE) have been applied to the lactic acid bacteria. Today it is possible to identify closely related species at the strain level, by the use of genotypic methods.

This dissertation focuses on the isolation of wild strains of lactic acid bacteria from raw cow's milk and the identification of these isolates. Isolates were first identified by biochemical reactions. PCR –RFLP method based on the amplification and restriction digestion of 16S rRNA genes were used to confirm the results of the biochemical identification. In addition, pulsed field gel electrophoresis method was optimized, using lactic acid bacterial reference cultures.

CHAPTER 2

LACTIC ACID BACTERIA

A typical lactic acid bacterium is Gram-positive, non-spore forming, catalase-negative, facultatively anaerobe requiring complex media, acid tolerant, fermentative and it lacks cytochromes and produces lactic acid as the major end product (Axelsson, 1998).

By the description of new genera and taxonomic revisions of the known members, lactic acid bacteria have been grouped into the following genera: *Aerococcus*, *Alloicoccus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella* (Axelsson, 1998). However, among these groups the important lactic acid bacteria associated with foods are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* (Stiles and Holzapfel, 1997). The G+C content of the genome of lactic acid bacteria is below 50% and they are included within the phylum *Clostridium* branch. In addition, the genus *Bifidobacterium* are sometimes included in the lactic acid bacteria, however according to their G+C content of the DNA which is above 50%, they are much closer to *Actinomycetes* branch rather than *Clostridia* (Holzapfel and Wood, 1995).

Lactic acid bacteria can either be heterofermentative or homofermentative when glucose is the main carbon source (Holzapfel and Wood, 1995).

2.1 Important Genera of Dairy Associated Lactic Acid Bacteria

Although lactic acid bacteria include eleven different genera, five of them are important in dairy technology; *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Streptococcus* and *Lactobacillus* since only these five genera include dairy starter lactic acid bacteria; (Cogan, 1996).

2.1.1 *Lactobacillus*

The members of the genus *Lactobacillus* are rods or coccobacilli, aerotolerant or anaerobic but growth is enhanced in the presence of 5 % CO_2. Some are anaerobic on isolation, and are aciduric or acidophilic. Even they are generally known as catalase- and oxidase-negative and do not reduce nitrates, some strains of some species exhibit activities of catalase, nitrate reduction or even contain cytochromes (Hammes and Vogel, 1995).

According to glucose fermentation, *Lactobacillus* spp. can either be classified as homofermentative or heterofermentative. In homofermentation the major end product is lactic acid (> 85 %). In contrast, the end products of heterofermentation are equimolar amounts of lactic acid, carbondioxide and ethanol (and/or acetic acid).

In terms of nucleic acid composition they have 33-55 % G + C in their chromosomes. However it has been generaly suggested that in a well-defined genus there must be no more than 10% difference in G-C content. Therefore 33-55 % G+C content among lactobacilli indicates a wide range of diversity (Stiles and Holzapfel, 1997).

Species of *Lactobacillus* are widely distributed in the environment (Holt *et al.* 1994). They are often found in habitats rich in carbohydrates (Hammes and Vogel, 1995). Major environments for *Lactobacillus* are listed in Table 2.1.

Species of *Lactobacillus* produce an acidic environment (pH 4.0) in foods containing a carbohydrate source. Therefore other coexisting bacteria are often killed or their growth is inhibited at low pH (Stiles and Holzapfel, 1997).

Several species of *Lactobacillus* are used as starter cultures for the production of fermented foods for example cheese, fermented vegetables, silage, fermented meats, fermented drinks (wine and beer) and sourdough-bread production (Stiles and Holzapfel, 1997).

Lactobacilli have been divided into three groups: *Thermobacterium*, *Streptobacterium* and *Betabacterium*.

Table 2.1 Habitats of the *Lactobacillus* spp. (Stiles and Holzapfel, 1997)

Human
Oral cavity
Intestinal tract
Vagina
Other habitats
Plants and plant materials
Soil, water, sewage and manure
Food fermentations (milk, meat and vegetable)
Cereal products
Silage
Food spoilage
Beer
Fruit and grain mashes
Sugar processing
Milk
Meat and meat products
Fermented beverages

Thermobacterium includes homofermentative lactobacilli, which are able to grow at 45 °C but not ≤ 15 °C. They are generally seen as long rods and rarely in chains under the light microscope. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*, *Lactobacillus acidophilus* are dairy starters included in this group. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Lactobacillus helveticus* are used for the production of cheese, which requires high temperatures. *Lactobacillus acidophilus* is used for probiotic yogurt and dietetic products (Yaygın and Kılıç, 1993).

Streptobacterium includes homofermentative lactobacilli, which are able to grow ≤ 15°C. This group include *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus buchneri*, which are found in dairy products. Only however *Lactobacillus casei* and *Lactobacillus plantarum* are used as starter cultures for the production of several types of cheese (Yaygın and Kılıç, 1993).

Betabacterium group includes the heterofermentative lactobacilli. They ferment lactose and produce lactic acid together with acetate, ehtyl alcohol and CO_2. *Lactobacillus brevis* and *Lactobacillus fermenti* are found in dairy products, but they are not used as dairy starters (Yaygın and Kılıç, 1993).

Grouping of lactic acid bacteria as *Thermobacterium*, *Streptobacterium* and *Betabacterium* is not valid anymore since this grouping was based on morphology and growth temperatures and many recently described species did not fit into this classification scheme (Hammes and Vogel, 1995). Lactobacilli have been then physiologically subdivided into three groups (Axelsson, 1998, Hammes and Vogel, 1995):

- obligately homofermentative,
- facultatively heterofermentative, and
- obligately heterofermentative.

Several species of obligately homofermentative and facultatively heterofermentative groups and some of the obligately heterofermentative group are used in the food fermentation, but obligately heterofermentative group is often involved in food spoilage.

Lactobacillus spp. have also been phylogenetically clustered into three groups (*Lactobacillus delbrueckii* group, *Lactobacillus casei-Pediococcus* group and *Leuconostoc* group) (Hammes and Vogel, 1995, Schleifer and Ludwig, 1995).

In recent grouping by Hammes and Vogel (1995), the physiological grouping (obligately homofermentative, facultatively heterofermentative, and obligately heterofermentative) has been kept. *Lactobacillus* spp. have been group into these three groups denoted by three letters (**A** for obligately homofermentative lactobacilli, **B** for facultatively heterofermentative lactobacilli and **C** for obligately heterofermentative lactobacilli). They have also assigned three suffixes to each species in order to reflect their positions in phylogenetic clusters. (**a** for affiliation to *Lactobacillus delbrueckii* group, **b** for *Lactobacillus casei-Pediococcus* group, and **c** for *Leuconostoc* group). For example **Aa** have been used to define obligately homofermentative lactobacilli, which were affilited to *Lactobacillus delbrueckii* group.

2.1.2 *Streptococcus*

The members of the genus *Streptococcus* are homofermentative and they are seen as spherical or ovoid cells under light microscope. They are arranged in chains or pairs, and because of such typical morphological arrangements they have been named with the term of streptococcus (Hardie and Whiley, 1995). Some species of *Streptococcus* are encapsulated (Holt *et al.* 1994 and Hardie and Whiley, 1995). The metobolism is homofermentative and lactate is the major end product. No gas is produced. Their growth is usually limited between 25-45°C and optimum temperature is 37°C (Holt *et al.* 1994).

Many known species are parasitic to human (Hardie and Whiley, 1995) and animals and some are pathogenic (Hardie and Whiley, 1995 and Stiles and Holzapfel 1997). Especially *Streptococcus pnemoniae*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* are the highly pathogenic species (Stiles and Holzapfel 1997). They possess complex nutritional requirements (Hardie and Whiley, 1995 and Stiles and Holzapfel, 1997) and they are especially associated with tissues of the intestinal tract of animals, milk, dairy products, and vegetables (Stiles and Holzapfel, 1997).

Streptococcus thermophilus is the only important species in food fermentation. It plays an important role as a starter culture for the production of yogurt and cheese (Stiles and Holzapfel 1997). It grows at 45 °C and up to 50 °C, but not at 15 °C. It is relatively resistant to heat (Stiles and Holzapfel 1997). It withstands to 60°C for 30 minutes, therefore it is rather thermoduric than thermophilic.

2.1.3 *Lactococcus*

Lactococcus spp. are coccoid shaped lactic acid bacteria. Their morphology is characterized as spheres of ovoid cells occuring singly, in pairs or in chains and often they are elongated in the direction of the chain. Chain length depends mainly on strain. Growth medium also influences the chain length (Teuber, 1995).

Lactococci ferment lactose to mainly L (+)-lactic acid without gas formation (Holt *et al.*1994).

Their optimum growth temperature is 30 °C. They are found in dairy and plant products. They can also grow at 10 °C but not at 45 °C (Holt *et al.*, 1994).

Lactococci are commonly called as mesophilic lactic streptococi since they have been included in the genus *Streptococcus* by Orla Jensen and then they have been differentiated according to their serological group N antigen from the pathogenic streptococci (group A, B, C, and D). Finally as a result of taxonomic studies, they have been separated from true streptococci and enterococci and named as *Lactococcus* (Teuber, 1995).

The genus *Lactococcus* has five known species; *Lactococcus lactis*, *Lactococcus garviae*, *Lactococcus plantarum*, *Lactococcus raffinolactis* and *Lactococcus piscium*. *Lactococcus lactis* has two subspecies *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* is considered as citrate utilising variant of *Lactococcus lactis*.

Subspecies of *Lactococcus lactis* are important in the production of several fermented dairy products (Cogan, 1996). Another lactococci associated with raw milk is *Lactococcus raffinolactis*. But it has been rarely isolated (Garvie, 1984).

2.1.4 *Leuconostoc*

Leuconostoc species are spherical and they sometimes exist as short rods with rounded ends in long chains. Their fermentation capability is restricted to the mono- and disaccharides. They are widely associated with plants, dairy and other food products. They ferment glucose by heterofermentation and produce mainly D-lactate and ethanol and usually gas. They play an important role in changing the organoleptic quality and texture of fermented food products such as milk, butter, cheese, and meat. They also play an important role in the fermentation of sourdough (Dellaglio *et al.* 1995). Their optimum growth temperature is 20-30 °C (Holt *et al.*, 1994 and Dellaglio *et al.*, 1995).

The cells appear to be elongated when cultured with glucose medium and grown on solid media. Thus they resemble lactobacilli but most strains are coccoid when grown in milk (Dellaglio *et al.*, 1995).

Leuconostocs are found in the composition of starter cultures and they produce diacetyl from citrate and this may be important for flavour formation (Garvie, 1984). The species *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc lactis* are dominantly associated with milk and fermented milk products (Dellaglio *et al.*, 1995; Marshall, 1987).

2.1.5 *Enterococcus*

The genus *Enterococcus* includes members, which are seen as spherical and ovoid cells. They ferment wide range of carbohydrates and produce mainly L (+)-lactic acid but no gas. Final pH ranges between 4.2 and 4.6. Their optimum growth temperature is 37 °C. They usually grow at both 10 °C and 45 °C, at pH 9.6, and in the presence of 6.5% NaCl and 40 % bile. They usually ferment lactose. They are usually found in feaces of vertabrates. The type species is *Enterococcus faecalis* (Holt *et al.*, 1994).

Enterococci are generally not considered as important in food technology. Some species of enterococci are found in local cheese types in Southern Europe (Axelsson, 1998). They are often present in artisanal starters; *Enterococcus feacalis* and *Enterococcus faecium* (Cogan, 1996). They have ideal starter culture properties because of the rapid acid production, resistance to higher cooking temperatures of hard cheeses

made with mesophilic cultures, and good tolerance to salt concentrations (Cogan, 1996). In addition, several strains have been used as probiotics and as silage inoculants (Axelsson, 1998).

Like *Lactococcus*, the genus *Enterococcus* was also initially classified within the genus *Streptococcus*. Later they have been separated from other streptococci and classified into the Lancefield's group D because of their serological group D antigen. Then the genus has been revised and named as *Enterococcus* and serological grouping the *Enterococcus* has not been valid any more.

2.2 The Other Genera of Lactic Acid Bacteria

2.2.1 *Pediococcus*

The genus Pediococcus is the tetrad-forming lactic acid bacteria, since during cell division, two perpendicular planes are formed. They are often heterofermentative and they all produce DL-lactate with the exception of *Pediococcus dextranicus* (Stiles and Holzapfel, 1997). They are used as starter cultures for the production of fermented sausages in some regions. Especially dry, semi-dry sausages are produced by starter cultures, developed from some strains of *Pediococcus acidilactici* (Simpson and Taguchi, 1995). They exhibit poor growth in milk since they cannot easily metabolize lactose (Stiles and Holzapfel, 1997). But several species of pediococci have been employed in cheese production (Simpson and Taguchi, 1995).

2.2.2 *Tetragenococcus*

The genus *Tetragenococcus* like *Pediococcus* is another tetrad-forming bacteria. *Pediococcus halophilus* growing in the presence of 18 % NaCl has been classified into a new genus *Tetragenococcus* (Stiles and Holzapfel, 1997). *Pediccoccus halophilus* is important in the production of soya sauce (Stiles and Holzapfel, 1997; Simpson and Taguchi, 1995).

2.2.3 *Oenococcus*

The genus *Oenococcus* has only one species; *Oenococcus oeni*, and it is generally found in wine. It is important in the malolactic fermentation in wines (Delaglio *et al.*, 1995). This species had first been included in the leuconostocs as *Leuconostoc oenos*, but later it has been transferred to new genus *Oenococcus* (Axelsson, 1998).

2.2.4 *Vagococcus*

This genus has been formed by the transfer of motile strains of group N streptococci, isolated from chicken feaces and river according to 16S ribosomal nucleic acid sequence data by Collins *et al* (1989). They have been found as phylogenetically unrelated to lactococci, but related to *Enterococcus*.

Its members are homofermentative and produce L (+)-lactate. However, not all species of this genus are motile.

2.2.5 *Carnobacterium*

Carnobacteria are associated with meat, poultry and fish (Klaenhammer *et al.*, 1993; Schillinger and Holzapfel, 1995). They are rod shaped lactic acid bacteria resembling lactobacilli but they are not able to grow on acetate media. They produce L (+)-lactic acid and are heterofermentative. They can grow at high pH (pH 9.5) (Stiles and Holzapfel, 1997). This genus has been created for the reclassification of *Lactobacillus piscicola* and *Lactobacillus divergens* as *Carnobacterium piscicola*, and *Carnobacterium divergens*, respectively and for incorporating the poultry originated isolates, which had not been allocated into the previously described genera, into new species *Carnobacterium gallinarum* and *Carnobacterium mobile* (Schillinger and Holzapfel, 1995).

2.2.6 *Weissella*

This genus has been proposed by the transfer of some heterofermentative species of *Lactobacillus* together with several species of *Leuconostoc* in to a new genus (Axelsson, 1998).

2.3 Importance of Dairy Lactic Acid Bacteria

2.3.1 Starter Cultures

Transformation of milk into organoleptically acceptable products by fermentation requires rapid acid production from lactose and the development of volatile compounds such as diacetyl and acetaldehyde in suitable amounts (Marshall, 1987).

The most important characteristic of lactic acid bacteria in dairy fermentations is the production of lactic acid by completely or partially metabolizing the milk sugar, lactose (Yaygın and Kılıç, 1993). They are called as starter because they start or initiate the lactic acid formation in milk. Several dairy products; such as cheese, ripened cream, lactic butter, sour cream, yogurt with standart and desired flavour are produced by using starter lactic acid bacterial cultures. In dairy industry starter cultures have to be used to produce cheese, yogurt and butter with desired flavour and aroma (Yaygın and Kılıç, 1993). The use of starter cultures enables the production of microbiologically safe products with reproducible organoleptic and structural properties (Wouters *et al.* 2002).

The starter cultures used in dairy products can be divided into three groups: mesophilic, thermophilic (Mäyra-Mäkinen and Bigret, 1998, Cogan, 1996, Yaygın and Kılıç, 1993) and artisanal (Cogan, 1996). Mesophilic cultures have an optimum temperature of around 26°C and thermophilic cultures have around 42°C. Therefore, they contain different bacteria, and they are each further divided into undefined and defined cultures. Undefined cultures are the subcultures of milk which were soured during the late nineteenth century and early twenteeth century (Cogan, 1996). They were found to produce good-quality cheese and butter. Up to now they have been transferred several times, and now their composition is different than their first use. Defined cultures are composed of known strains (Table 2.2).

Table 2.2 Some examples of starters and their use in different products (Cogan, 1996)

Starter Type	Organisms	Product
Mesophilic		
O	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	Cheddar cheese; Feta cheese
L	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis* *Leuconostoc* sp.	Lactic butter; Feta cheese; Cheddar cheese
D	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis* *Cit*$^+$ lactococci	Lactic butter
DL	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis* *Cit*$^+$ lactococci *Leuconostoc* sp.	Edam and Gouda cheese; Cheddar cheese; lactic butter; Cultured buttermilk
Thermophilic		
	Streptococcus thermophilus *Lactobacillus delbrueckii* subsp. bulgaricus	Yogurt; Mozarella cheese;
	Streptococcus thermophilus *Lactobacillus helveticus*	Emmental cheese; Grana cheese
	Streptococcus thermophilus *Lactobacillus acidophilus* *Bifidobacterium bifidum*	Mild yogurt

The third class of starter cultures is artisanal cultures, which have been produced by incubating milk or whey under predetermined conditions. In a cheese manufactoring plant they are produced daily and therefore the number and types of lactic acid bacteria vary and thus they are also undefined.

Mesophilic starter cultures include *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis subsp. lactis* biovar. *diacetylactis* (Mäyra-Mäkinen and Bigret, 1998; Cogan, 1996), *Leuconostoc* spp. (Cogan, 1996), *Leuconostoc lactis* and *Leuconostoc cremoris* (Mäyra-Mäkinen and Bigret, 1998) (Table 2.3.). While *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* are acid-producing organisms, *Lactococcus lactis subsp. lactis* biovar. *diacetylactis* and *Leuconostoc* spp. are the citric acid fermenting bacteria. The substrates for lactic acid and diacetyl are lactose and citrate, respectively. The only difference between *Lactococcus lactis subsp. lactis* biovar. *diacetylactis* and *Lactococcus lactis* subsp. *lactis* is that *Lactococcus lactis subsp. lactis* biovar. *diacetylactis* is able to metabolise citrate to acetoin/diacetyl and CO_2 (Garvie, 1984). It possesses the plasmid encoding a citrate-transporting molecule (Garvie, 1984; Cogan, 1996). Therefore, this organism is considered as citrate utilizing (Cit^+) variant of *Lactococcus lactis subsp. lactis* (Cogan, 1996). Diacetyl is an important metabolite in flavour of dairy products. Some strains however may overproduce diacetyl or CO_2 and may cause several problems. Therefore it is important to distinguish citrate utilising strains (Garvie, 1984).

The acid producers constitute 90-99% of the mixed cultures and they are the dominant organisms. The flavour producers make up the remaining, 1-10 %.

Mesophilic cultures are mainly divided into different groups due to the nature of cit^+ strains (Cogan, 1996):

- D-types with Cit^+ *Lactococcus lactis* subsp. *lactis* (D stands for its old species name of diacetylactis)
- L-types with *Leuconostoc* spp. as flavour producers (L stands for the first letter of *Leuconostoc*)
- DL-types include both Cit^+ *Lactococcus lactis* subsp. *lactis* and *Leuconostoc* spp. as flavour producers.
- O-types, which include no flavour producers

Table 2.3 Lactoccocci as components of starter cultures for fermented dairy products (Teuber, 1995)

Type of product	Composition of starter culture
1. Cheese types without eye formation (Cheddar, Camembert, Tilsit)	*Lactococcus lactis* subsp. *cremoris*, 95-98% *Lactococcus lactis* subsp. *lactis*, 2-5%
2. Cottage cheese, fermented milk, cheese types with few or small eyes (e.g. Edam)	*Lactococcus lactis* subsp. *cremoris*, 95%; *Leuconostoc mesenteroides* subsp. *cremoris*, 5%; or *Lactococcus lactis* subsp. *cremoris*, 85-90%; *Lactococcus lactis* subsp. *lactis* 3%; *Leuconostoc mesenteroides* subsp. *cremoris*, 5%
3. Cultured butter, fermented milk butter milk, cheese types with round eyes (e.g. Gouda)	*Lactococcus lactis* subsp. *cremoris*, 70-75%; *Lactococcus lactis* subsp. '*diacetylactis*', 15-20%; *Leuconostoc mesenteroides* subsp. *cremoris*, 2-5%
4. Casein	*Lactococcus lactis* subsp. *cremoris*
5. Kefir	Kefir grains containig lactose-fermenting yeasts (e.g. Candida kefir) *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, *Lactococcus lactis* subsp. *lactis*

Thermophilic starters include both coccal and rod shaped bacteria or a mixture of them (Cogan, 1996). The genera involved are *Streptococcus* and *Lactobacillus* (Mäyra-Mäkinen and Bigret, 1998). Coccus-shaped microorganisms are invariably *Streptococcus thermophilus* and rod shaped ones are *Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *lactis* (Mäyra-Mäkinen and Bigret, 1998; Cogan, 1996), which are used for the production of cheeses requiring high cooking temperatures (Mäyra-Mäkinen and Bigret, 1998) or *Lactobacillus delbrueckii* subsp. *bulgaricus* (Cogan, 1996). *Lactobacillus delbrueckii* subsp. *bulgaricus* is used in the production of yogurt together with *Streptococcus thermophilus*. As a result of symbiosis (Kılıç, 2001) between these two bacteria, yogurt can be produced (Mäyra-Mäkinen and Bigret, 1998). Some strains of *Lb. casei* produce diacetyl from citrate; they are only used in the Japaneese fermented milk, Yakult (Mäyra-Mäkinen and Bigret, 1998).

2.3.2 Bacteriocin Production

Another important property of lactic acid bacteria is the production of bacteriocins. Bacteriocins are defined as a heterogenous group of antimicrobial proteins varying in activity, mode of action, molecular weight, genetic origin, and biochemical properties (Abee *et al.*, 1995).

In recent years, there has been considerable attention on the use of microorganisms and their metabolites to preserve food products (to prevent spoilage and prolong the shelf life).

Bacteriocins are divided into four distinct classes (Klaenhammer, 1993):

- lantibiotics,
- small, heat stable, non-lantionine containing membrane-active peptides,
- large heat labile proteins,
- complex bacteriocins composed of protein plus one or more chemical moieties (lipid, carbohydrate).

The well-known bacteriocin is nisin and it is produced by many strains of the *Lactococcus lactis* subsp. *lactis* and it is the only bacteriocin used as a food preservative (Teuber, 1995). Several desirable properties of nisin as a food preservatives are that (Jay, 1992):

- It is non-toxic
- It is naturally produced by *Lactococcus lactis*
- It is stable to heat and it has excellent storage stability
- It can easily be destroyed by digestive enzymes
- It does not produce off-flavors or off-odors
- It exhibits narrow spectrum of activity

Two commercial formulation of nisin has been introduced; one is Nisaplin™ from Aplin & Barret with a nisin content of 25 mg/g and the other is Chrisin™ from Danish firm Chr. Hansen (Broughton-Delves, 1998).

Nisin is effective against a range of Gram-positive bacteria and, especially against those, which form heat resistant spores, however it exhibits little or no activity on Gram- negative bacteria, yeasts or moulds. Nisin has been used in the preservation of processed cheese, hard cheese, desserts, milk, yogurt, cottage cheese, fermented beverages, meat products (e.g. bacon, frankfurters, smoked fish), and canned vegetables (Teuber, 1995).

As well as nisin, several other bacteriocins are also produced by different lactic acid bacteria.

2.3.3 Probiotics

A probiotic can be defined as the single or a mixture of live cultures of microorganisms, which improve the properties of the indigenous microflora (Klaenhammer and Kullen, 1990).

The important benefits of probiotic cultures are (Klaenhammer and Kullen, 1990):

- pathogen interference, exclusion and antagonism
- immunostimulation and modulation
- anticarcinogenic and antimutagenic activities
- alleviation of the symptoms of lactose intolerance
- reduction in serum cholesterol
- reduction in blood pressure

- decreased incidence and duration of diarrhoea (antibiotic associated diarrhoea, *Clostridium difficile*, travelers and rotaviral)
- prevention of vaginitis
- maintenance of vaginal integrity

Lactic acid bacteria play an important role in the formulation of probiotic products. *Lactobacillus* and *Bifidobacterium* are the main genera important in probiotic concept. As many as eigthteen species of *Lactobacillus* have been of some interest to be used as probiotics and several species of Bifidobacteria have been considered as probiotics (Table 2.4).

The development of molecular methods has provided new tools for the identification of probiotic strains. Besides colony morphology, fermentation patterns, serotyping and some combinations of these methods, genetic based techniques have been used successfully (Klaenhammer and Kullen, 1990). Especially it is important to monitor gastrointestinal survival and passage of a fed probiotic strain in humans (Klaenhammer and Kullen, 1990). Nowadays molecular methods enable us to type a given isolate at the strain level and they make it possible to identify a probiotic strain in gastrointestinal system.

Table 2.4 Primary lactic acid bacterial species used as human probiotics (Klaenhammer and Kullen, 1990)

Lactobacillus species	*Bifidobacterium*	*Streptococcus*	*Enterococcus*
acidophilus	*animalis*	*thermophilus*	*faecium*
amylovorus	*bifidum*		
casei	*breve*		
crispatus	*infantis*		
gallinarum	*longum*		
gasseri	*lactis (animalis)*		
johnsonii			
plantarum			
reuteri			
rhamnosus			
salivarus			

CHAPTER 3

IDENTIFICATION METHODS FOR BACTERIA

3.1 Phenotypic or Biochemical Methods

Until recent years, methods used to identify microorganisms have been based on the morphological, physiological and biochemical methods. In this chapter, several phenotypic methods are described.

3.1.1 Morphological Methods

The first important step in the identification of an isolate is the morphological examination. First of all, bacterial cells are stained and their appearance is observed under light microscope. There are several staining methods. They give information on the cellular morphology, Gram status, sporulation and capsulation. Motility can also be tested by the microscopic observation. Morphological examination also gives us information on the purity of an isolate.

3.1.2 Phenotypical Methods

Normally, different phenotypical characteristics give information on the taxonomical status of the isolates. For example, according to reactions with different chemicals and reactions at different conditions, bacteria can be classified into groups, genera and into species (e.g. catalase, oxidase, oxidation-fermentation tests, fermentation of carbohydarets, etc.). However, sometimes, phenotypical results may be confusing.

There are several phenotypic methods: Biotyping, antibiotic susceptibility testing, phage typing, serotyping, protein profiling/immunoblotting and multilocus enzyme electrophoresis, and bacteriocin typing.

Biotyping includes a set of biochemical reactions in order to classify an isolate at species or genus level. Biochemical reactions however may not always differentiate bacteria at subspecies or strain level.

Antibiotic susceptibility testing is performed by analyzing the growth of an organism in the presence of a specific antibiotic. Even it is one of the most standardized phenotyping methods, its discriminatory power is low (Bush and Nitschko, 1999).

Phage typing depends on the infection of a particular bacterium with a specific phage. Different phages infect different isolates. Therefore bacterial isolates can be identified depending on the nature of the infection.

Serotyping is another method by which the isolate is characterized using the specific antibodies.

Protein profiling depends on the separation of whole cell proteins by polyacrylamide gel electrophoresis (PAGE). Comparison of the protein profiles of isolates can be used to type and classify different strains (Bush and Nitschko, 1999).

Since phenotypic methods are generally affected by the environmental conditions (i.e. variable alterations in gene expression), it is not often possible to obtain reproducible and reliable results for a given isolate. Phenotypical methods are also restricted by the limited number of characteristics and every species exhibit different fermentation patterns, different reactions with different antigens and different susceptibility levels to different antibiotics, different enzymatic profiles. Therefore they are also time consuming.

An ideal typing method should therefore give reproducible results. Discriminatory power of the method is also another important factor. A method should have a high discriminatory power, in the sense that unrelated and closely related strains could easily be differentiated. The method should also be applicable to a wide range of microorganisms.

3.1.2 Phenotypic Identification of Lactic Acid Bacteria

For the identification of lactic acid bacteria, Gram reaction, microscopic morphology, catalase test, carbohydrate fermentation and homo- or heterofermentation tests are generally used (Sharpe and Fryer, 1966; Hammes and Vogel, 1995). Oxidase test can also be used to define LAB members. However several strains can give catalase positive reaction (Sharpe and Fryer, 1966). For example strains of *Lactobacillus mali* produce pseudocatalase, consequently catalase positive reaction is observed (Hammes and Vogel, 1995). Lactic acid bacteria can be classified into rods and cocci. Only

lactobacilli and carnobacteria are rod shaped and all the other genera are cocci. However, the new genera *Weissella* including heterofermentative species previously classified into either *Lactobacillus* and *Leuconostoc*, contains both coccus and rod shaped bacteria (Axelsson, 1998). The cell morphology is generally used for the primary step for the phenotypical identification of lactic acid bacteria; however, it may result in misidentification. For example, due to the ovoid shape of lactococci and elongation of the cells in the direction of the chain, it is difficult to interpret their morphology. For example, *Lactococcus lactis* forms coccoid cells after cultivation in milk but has an elliptical morphology after the growth in broth culture (Garvie, 1984). This can also be seen in the example of *Lactobacillus xylosus* and *Lactobacillus hordinae*; first they had been classified as species of *Lactobacillus*, but now they have been classified as *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *hordinae* (Stiles and Holzapfel, 1997). Leuconostocs are also elongated and resemble lactobacilli when grown in a glucose-containing medium although they are coccoid shaped, but most strains are coocoid if they are cultured in milk (Dellaglio *et al.*, 1995). The coccus shaped genera of lactic acid bacteria can be separated according to tetrad formation (cell division into perpendicular planes), and tetrad forming genera are *Pediococcus*, *Tetragenococcus* and *Aerococcus* (Axelsson, 1998). Another important character used in the differentiation of lactic acid bacteria is the mode of glucose fermentation under standard conditions. The lactic acid bacteria are divided into two groups, heterofermentative or homofermentative. Leuconostocs, oenococci, weissellas and a subgroup of lactobacilli are heterofermentative genera of lactic acid bacteria (Holzapfel and Wood, 1995). Test for gas production from glucose can be used to distinguish between homofermentative and heterofermentative groups.

Mainly growth temperature is used to distinguish some of the cocci. Lactococci and vagococi grow at 10° but not at 45° C. Classical enterococci grows at both 10° and 45° C. Streptococci generally grow well at 45°C (Axelsson, 1998). *Streptococcus thermophilus*, only one species important in foods, can grow at 45 °C but do not grow at 10° C.

Salt tolerance is another important character (6.5%), which can be used to differrentiate *Lactococcus/Vagococcus*, *Enterococcus* and *Streptococcus*. Enterococci grow at this salt concentration but lactococci/vagococci cannot. Among the members of

Streptococcus, growth at 45° C is dependent on the species. *Streptococcus thermophilus* cannot grow at 6,5 % NaCl (Axelsson, 1998).

The genus *Tetragenococcus* is able to grow at extreme salt concentrations and and grows in 18 % NaCl. These characteristics can be used to differentiate this genus from the others (Axelsson, 1998; Stiles and Holzapfel, 1997).

The different isomeric forms of lactic acid produced from glucose are useful in distinguishing most heterofermentative lactobacilli (racemic mixture; DL-lactic acid) and leuconostocs (only D-lactic acid). Members of the genus *Weissella* produce D- or DL- lactic acid (Axelsson, 1998).

A summary of the tests used to distinguish the genera of lactic acid bacteria, can be seen in Table 3.1.

In addition, the ability to grow at pH 9.0 and inability to grow on acetate media can be used to differentiate carnobacteria from lactobacilli. Also, acid and ethanol tolerance of *Oenococcus* are used to differentiate between *Oenococcus* and *Leuconostocs* (Axelsson, 1998).

The fermentation of large numbers of carbohydarates, arginine hydrolysis, acetoin formation (Voges-Proskauer test), bile tolerance, extracellular polysaccharide production, requirements for growth factors, presence of several enzymes (β-galactosidase and β-glucoronidase), growth characteristics and serological typing have been used to identify the species of lactic acid bacteria (Axelsson, 1998).

3.2 Molecular Characterization Methods

Due to the disadvantages of phenotypic and biochemical procedures mentioned above, some alternative characterization methods have been developed in order to classify microorganisms much more effectively. These are mainly based on the genotypic variations. Plasmid profile analysis, restriction endonuclease analysis, ribotyping, pulsed-field gel electrophoresis, polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping, and RAPD), nucleotide sequence analysis have been widely used for this purposes (Farber, 1996).

Table 3.1 Main characteristics used to distinguish lactic acid bacteria (Axelsson, 1998)

Character	RODS		COCCI						
	Carnobacteria	*Lactobacillus*	*Enterococcus*	*Lactococcus* *Vagococcus*	*Leuconostoc* *Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*[a]
Tetrad formation	-	-	-	-	-	+	-	+	-
CO_2 from glucose	-[b]	±	-	-	+	-	-	-	+
Growth at 10°C	+	±	+	+	+	±	-	+	+
Growth at 45°C	-	±	+	-	-	±	±	-	-
Growth at 6.5% NaCl	Not Determined	±	+	-	±	±	-	+	±
Growth in 18% NaCl	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	Not Determined	±	+	±	±	+	-	-	±
Growth at pH 9.6	-	-	+	-	-	-	-	+	-
Lactic acid from glucose	L	D, L, DL	L	L	D	L, DL	L	L	D, DL

a *Weisella* includes rod or coccus shaped strains
b Depending on media, CO_2 can be produced in small amounts

The main advantages of these methods are as follows (Table 3.2):

- They have a high discriminatory power (two closely related strains can be distinguished).
- It is always possible to extract DNA from bacteria therefore all strains can be typed.
- Since the analytical strategies of the methods are similar, all methods can be applied to DNA from any source.

- Because the genomic DNA is stable and the method is not affected by cultural conditions and preparation procedures, more reliable and reproducible results can be obtained.
- Results can be further improved by the statistical analyses.
- They are suitable for automation.
- Databases enabling the classification of newly isolated strains can also be developed or constructed (Farber, 1996; Olive and Bean, 1999).

3.2.1 Plasmid Profile Analysis

Plasmids are extrachromosomal, self-replicating small and usually supercoiled, double-stranded DNA. They are often responsible for encoding products or functions, which modify the phenotype of the harbouring strain. In plasmid profile analysis (plasmid typing), plasmids of the isolates are extracted and separated by agarose gel electrophoresis and the differences in plasmid number and size between the plasmid profiles are used to differentiate the isolates. Plasmid profile analysis is the oldest and simplest of the genotype-based methods. It is relatively fast and easy. However the loss of plasmids or transfer of the plasmid between the strains, and between the species (known as horizontal gene transfer) are the main disadvantage of the method.

In addition, the presence of a plasmid with a similar molecular weight does not always refer to the same plasmid. In this case restriction endonuclease digestions can be used. It is expected that a particular restriction enzyme cuts plasmids at different sites and resulting fragment patterns will show different mobility during electrophoresis.

Table 3.2 Characteristics of bacterial typing systems (Farber, 1996)

Typing System	Proportions of strain typeable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
I. Phenotypic Methods					
Biotyping	All	Poor to fair	Poor	Excellent	Excellent
Antimicrobial susceptibility testing	All	Fair	Poor	Excellent	Very good to excellent
Serotyping	Most	Good	Fair	Good to excellent	Fair to good
Bacteriophage typing	Variable	Fair	Fair	Fair to good	Poor to fair
Multilocus enzyme electrophoresis	All	Excellent	Good	Excellent	Fair to good
II. Genotypic Methods					
Plasmid profile analysis	Variable	Fair to good	Good	Good	Excellent
Restriction endonuclease analysis	All	Very good	Good	Poor	Excellent
Ribotyping	All	Excellent	Fair to good	Very good to excellent	Fair to good
Pulsed-field gel electrophoresis	All	Excellent	Excellent	Excellent	Fair to good
PCR ribotyping	All	Very good to excellent	Good	Excellent	Very good to excellent
PCR restriction digest	All	Excellent	Good	Excellent	Very good to excellent
RAPD	All	Good	Very good to excellent	Very good	Very good to excellent
Nucleotide sequence analysis	All	Excellent	Excellent	Excellent	Fair

3.2.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism is also known as chromosomal DNA restriction analysis or DNA microrestriction analysis. In this method the chromosomal DNA from bacteria is isolated, and it is treated with a frequent cutting restriction enzyme and the resulting fragments are separated on an agarose gel by electrophoresis. The fragments obtained are usually 1,000 to 20,000 bp in length (Farber, 1996). The fragments are then visualized by staining with ethidium bromide and under UV light. After photographing the gels, the patterns are compared and the differences in the banding patterns are used to differentiate each isolate.

This method is universally applicable, rapid, inexpensive and relatively easy to perform. However, because numerous fragments are obtained and these are closely spaced on the agarose, interpretation of profiles is not easy. Therefore it is often required to use several restriction endonucleases in order to obtain interpretable results.

3.2.2 Ribotyping

The ribosomal RNA (rRNA) constitute nearly 82 % of the total RNA in a typical bacteria and consists of three species: 23S, 16S and 5S rRNA. The genes endoding ribosomal RNAs are highly conserved. On the other hand, numbers of rRNA genes varies among bacteria, between 2 to 11 copies. Ribotyping depends on the use of nucleic acid probes complementary to the rRNA genes. Therefore, if there are more copies of rRNA genes in a bacterium, the method becomes more discriminatory. In ribotyping, the genomic DNA of bacteria is first isolated and then restricted by restriction endonucleases. Fragments are separated by agarose gel electrophoresis. DNA on the gel is then transferred onto a nylon or nitrocellulose membrane by a capillary system or electrophoresis (Farber, 1996). Then fragments are hybridized with labelled probes, which are specific to 23S, 16S and 5S rRNA sequences. After hybridization, each fragment containing rRNA genes is exposed on an X-ray film. The film is developed and RNA banding patterns are compared. A disadvantage of this technique is that the small number of RNA specific bands limit the ability to distinguish between closely related strains (Olive and Bean, 1999). In ribotyping the use of universal probes is the major advantage and the reproducibility of the method is high (Farber, 1996)

3.2.3 Polymerase Chain Reaction Based Methods

Polymerase chain reaction is basically the in vitro amplification of DNA. First, template DNA is extracted from the isolates. Thermostable DNA polymerase enzymes (usually *Taq* polymerase isolated from *Thermus aquaticus*) are used for the amplification. In a PCR reaction, depending on the base composition of the primer used, the region of interest in the genome of a bacterium can be amplified.

Following restriction digestion or sequence analysis of the amplified DNA (amplicon), a given bacterium can be identified at the strain level. There are several PCR-based methods, which are explained below.

3.2.3.1 Randomly amplified polymorphic DNA (RAPD)

In a RAPD assay primers of 8-9 bases in length are used in the amplification reaction. These primers anneal randomly to the genomic DNA (template). There is therefore no complete homology between the primers and the bacterial DNA.

This method is easy to perform. It does not require isotopic labelling nor the use of restriction endonucleases. Because of the random priming, prior knowledge on the template DNA is not necessary.

Problems however may arise in the reproducibility of the method and because a large number of amplicons is often obtained, the comparison of differrent patterns can be very difficult (Bush and Nitschko, 1999; Olive and Bean, 1999).

3.2.3.2 PCR – Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR- PFLP is a rapid method. The target regions in the genome are amplified by the use of specific primers. The resulting amplicon is then digested with a frequent cutting restriction enzyme. The restriction enzyme can be chosen on the basis of the known base composition of the target region. Finally restriction fragments obtained are separated in an agarose gel by electrophoresis. Restriction patterns are then compared (Figure 3.1).

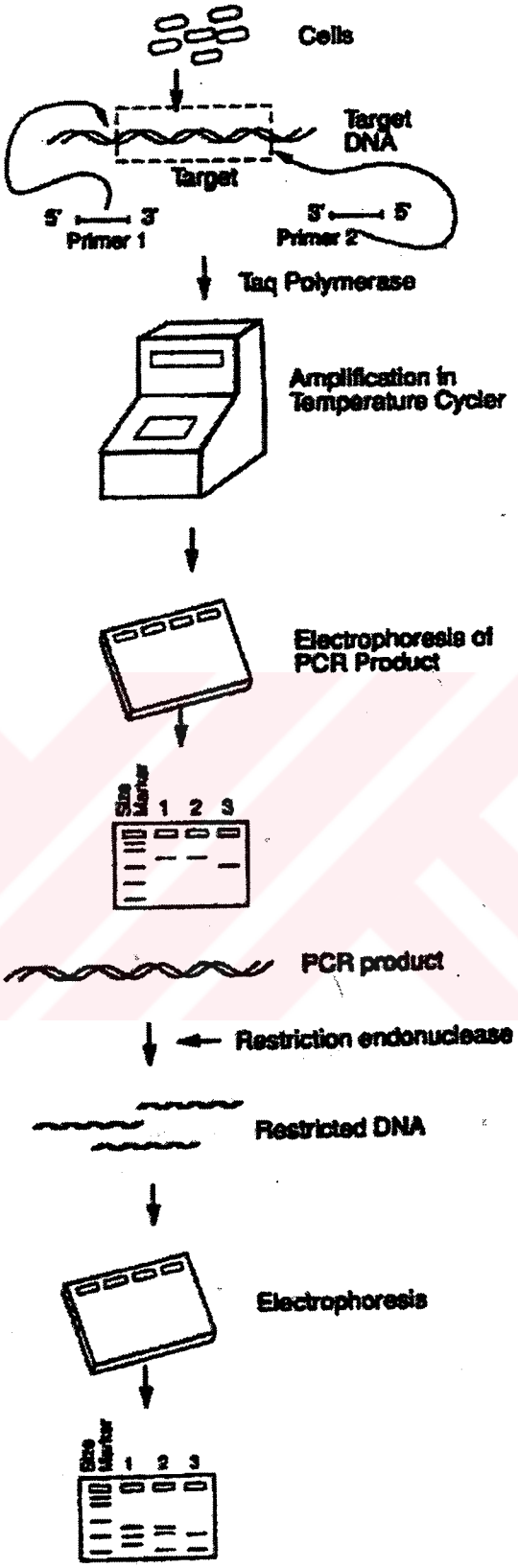

Figure 3.1 Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (Farber, 1996)

In PCR-RFLP method, the choice of the region of interest depends on the nature of isolates. Generally, known target regions are amplified, e.g. virulence genes and genes coding for flagellar proteins. A need for a prior knowledge of target regions is the main disadvantage of this method. But, amplification of target regions by using universal primers can overcome this disadvantage.

A method generally known as ARDRA, (amplified ribosomal DNA restriction analysis) is also a PCR-RFLP based method. It is also often considered as a ribotyping method. This method is based on the amplification of 16S ribosomal genes of isolates. 16S rRNA is important taxonomic tool in the classification of microorganisms since it is composed of highly conserved and variable sequences. Here 16S ribosomal RNA genes can be amplified by using universal primers (Andrighetto *et al.*, 1998) or species-specific primers (Andrighetto *et al.*, 1998; Bouton *et al.*, 2002; and Drake *et al.*, 1996a).

After the amplification of 16S ribosomal RNA gene, restriction endonuclase digestion is performed. The choice of restriction endonuclase is based on the nucleotide composition of the amplified region.

After restriction digestion, the fragments are separated on an agarose gel and the profiles of each isolates are compared.

3.2.3.3 Repetitive-Polymerase Chain Reaction (Rep-PCR)

There are repeating elements in bacterial genomes called repetitive elements. There are two main elements in bacterial genome commonly used for DNA typing; the Repetitive extragenic palindromic (REP) elements and extragenic repetitive intergenic consensus (ERIC) sequences. While REP elements are about 38 kb consisting of six degenerate positions and 5 bp variable loops, ERIC sequences are 126 bp elements containing a highly conserved central inverted repeat. REP or ERIC amplifications may enable good discrimination at the strain level (Olive and Bean, 1999).

The third element is the BOX sequence, which has been used to differentiate *Streptococcus pneumoniae*. They are mosaic repetitive elements consisting of various combinations of three subunits sequences known as box A, box B, box C with 59, 45 and 50 bp in length respectively. BOX elements have also been found in a number of other bacterial species (Olive and Bean, 1999).

Rep-PCR has been shown to be superior to other typing methods. For example, it has higher discriminatory power than restriction analysis of 16S rRNA genes or the 16S-23S spacer regions. However it has been shown that its discriminatory power is slightly lower than PFGE (Olive and Bean, 1999).

3.2.3.4 Internal Transcribed Spacer Region – Polymerase Chain Reaction (ITS-PCR) or PCR-Ribotyping

In prokaryotes, there are three genes coding for ribosomal RNA; 16S, 23S, and 5S (Figure 3.2). They are separated by the spacer regions and they show higher variations in sequence and length at both genus and species levels (Farber, 1996).

Amplification of spacer regions between the 16S and 23S ribosomal DNA is generally known as internal transcribed spacer- polymerase chain reaction (ITS-PCR). Because the target regions here is the spacer between ribosomal RNA genes, the method is also called as polymerase chain reaction-ribotyping.

The amplification products separated on an agarose gel can be compared. Restriction analysis or sequencing of the amplification product can increase discriminatory power of the method.

In this method, generally the spacer region between 16S-23S rRNA is amplified. However, amplification of 23S-5S spacer region gives increased discrimination (Farber, 1996).

Primers specific to 16S-23S spacer regions of bacteria can easily be designed. Besides, universal primers are also available and this is the major advantage of the method. In ITS typing, it is possible to obtain stable, easily detectable amplification in a rapid manner and this makes the method valuable for the molecular epidemiology. However, ITS-PCR has lower discriminatory power than PFGE and RAPD (Farber, 1996).

Amplification of internal transcribed spacer region of 16S and 23S rRNA genes has been reported for the identification of lactic acid bacteria (Tilsala- Timisjärvi and Alatossava, 1997; Warda *et al.*, 2001; and Drake *et al.*, 1996b).

Moschetti *et al.* (2001) has also used universal primers specific for 16S and 23S rRNA genes in combination with nisin gene-specific primers in order to identify bacteriocin-producing microorganisms. ITS-PCR has also been used for differentiation

of strains of *Lactobacillus helveticus* (Drake *et al.*, 1996b). They have suggested the use of ITS-PCR for grouping the strains. This method has also been used for grouping of lactic acid bacterial isolates (Warda *et al.*, 2001).

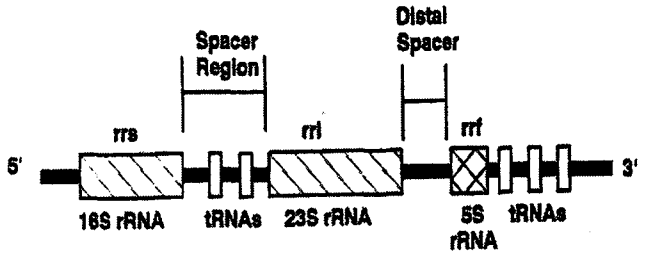

Figure 3.2 A typical ribosomal operon (Farber, 1996)

3.2.5. Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis is considered as the gold standard of molecular typing methods. In this method, the genomic DNA is cut with a restriction enzyme having 6 or 8 bp in its recognition site and the fragments are separated on an agarose gel. PFGE is a very discriminatory and reproducible method and it is applicable to all microorganisms. It resolves the diversity at subspecies and strain level. The main advantage of this method is that the restriction enzyme cleavage patterns resolved by PFGE easily demonstrate heterogeneity or homogeneity of isolates within one diagnostic group without the need for various probes (Bush and Nitschko, 1999).

In PFGE typing, live cells are embedded in agarose and then they are lysed in the agarose making their genomic DNA accessible to restriction enzymes. Embedding the DNA in agarose avoids the random shearing of DNA into several fragments by mechanical forces generated during DNA extraction. After obtaining the genomic DNA embedded in agarose (Figure 3.3), infrequent cutting restriction enzymes are used to digest the DNA. The choice of infrequent cutting restriction enzyme depends on the

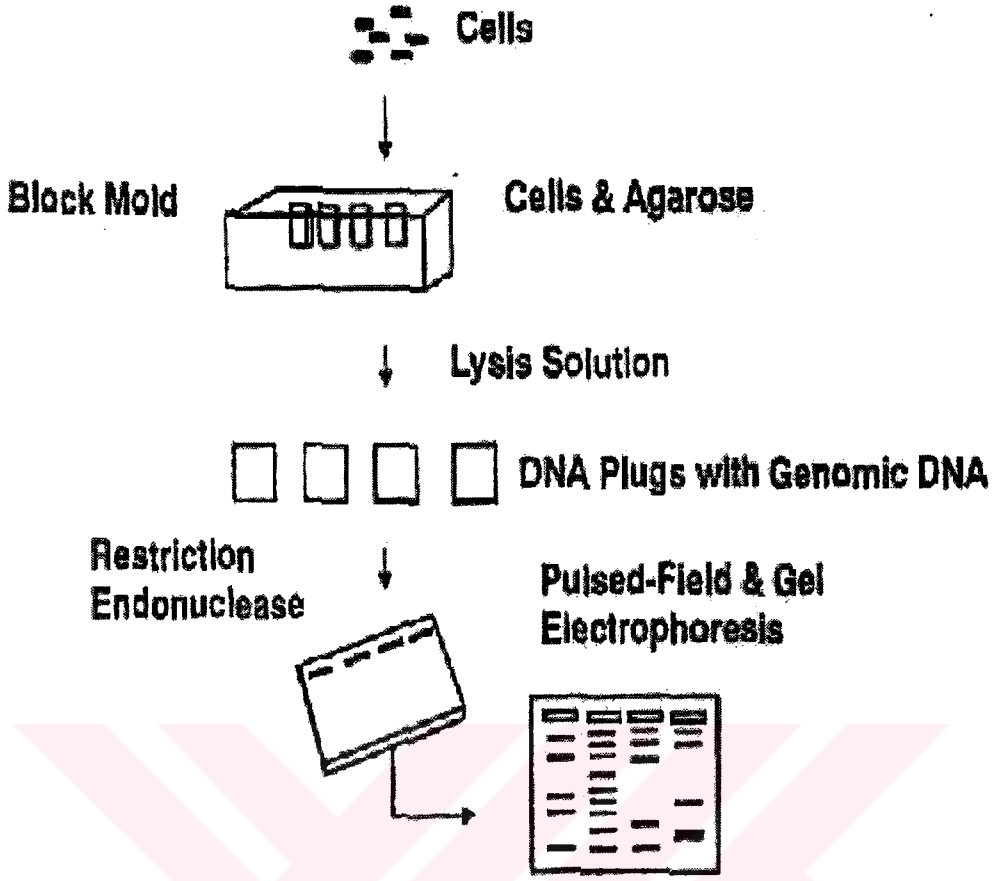

Figure 3.3 Schematic illustration of PFGE (Farber, 1996)

bacterial genome. For example, for bacteria having G+C content below 50-mol %, those restriction enzymes having rich guanine and cytosine in their recognition sites are used. After the restriction enzyme digestion, a small number of large DNA fragments ranging from 10 to 800 kb are obtained (Olive and Bean, 1999).

Electrical property of PFGE system is based on the alternating electrical field with predetermined intervals. Direction of electrical field is changed at these intervals. These intervals are called switch times or pulse times. When the first electrical field is applied, DNA fragments reorient and migrate in the gel. After a pulse time, new electrical field with different direction is applied. DNA molecules therefore change their direction and reorient themselves in the gel matrix. A change in the direction of electrical field allows the DNA fragments to migrate to the direction of the new electrical field. This principle allows the separation of higher molecular weight DNA fragments.

After the separation of fragments, the gel is stained with ethidium bromide. Ethidium bromide is a fluorescent dye intercalating between the two DNA strands of the double helix. It makes DNA fragments possible to be visualized over the UV light. After photographing the gels, restriction banding patterns specific to each isolate are compared by statistical analysis or visual inspection.

Clamped homogeneous electric field electrophoresis (CHEF) is the latest type of the PFGE system. This method was modified according to an observation that straight lines could be obtained by the amplification of homogeneous electrical fields using multiple electrodes (Birren and Lai, 1993). CHEF is one of the most commonly used PFGE systems. CHEF DR II system consists of twenty-four electrodes arranged in a hexagonal array (Bio-Rad Manual, 2001, Birren and Lai, 1993). It has an orientation angle of 120°, which eliminate lane distortions during electrophoresis (Bio-Rad Manual, 2001, Birren and Lai, 1993).

Several parameters affect the separation of high molecular weigth DNA fragments by pulsed field gel electrophoresis (Bio-Rad Manual, 2001):

- agarose concentration
- buffer concentration
- pulse times
- voltage
- electrophoresis run time

Agarose concentration is effective on the size range of DNA to be separated. It also affects the sharpness or tightness of the bands. When concentration of agarose is decreased, DNA migration rate increses. Higher molecular weight fragments can therefore be separated, but sharpness of DNA bands decreases. Typical agarose concentration used to separate DNA fragments up to 3 Mb is 1% (Bio-Rad Manual, 2001). If the fragments greater than 3 Mb will be separated, 0.5-0.9% agarose can be used (Bio-Rad Manual, 2001). When the band tightness to be increased, agarose concentration can be increased to 1.2-1.5%, however in this case, electrophoresis time is also needed to be increased (Bio-Rad Manual, 2001).

Buffer concentration, buffer temperature and buffer type also affect the mobility of DNA molecules. When the buffer temperature increases, the mobility of DNA increases but band sharpness and resolution decrease. In order to maintain band sharpness and dissipate the heat generated, it is recommended to chill buffer to 14°C for an acceptable compromise between speed and resolution (Bio-Rad Manual, 2001, Birren and Lai, 1993). In PFGE, the most commonly used buffers are 0.5X TBE or 1X TAE (Bio-Rad Manual, 2001). The latter provides increased migration when compared with 0.5X TBE (Bio-Rad Manual, 2001, Birren and Lai, 1993).

Voltage or field strength is also an important factor in the separation of DNA molecules. Selection of voltage affects the final gel results (Birren and Lai, 1993). When voltage is increased, DNA migration also increases but band sharpness decreases (Bio-Rad Manual, 2001). Although a high voltage increases DNA migration rate, for the high molecular weight DNA fragments (> 2Mb), field strength should be decreased (Birren and Lai, 1993). When the voltage will be selected, a compromise between run time and resolution has to be made (Bio-Rad Manual, 2001).

Electrophoresis run time affects the resolution of fragments. When the migration rate of fragments is low, longer electrophoresis period is required to adequately separate the fragments (Bio-Rad Manual, 2001). However, pulse times rather than electrophoresis time is effective on the resolution of fragments (Birren and Lai, 1993). Increasing the electrophoresis time do not separate unresolved higher molecular weight fragments, therefore different switch times have to be used (Birren and Lai, 1993).

The most important factor in PFGE is the pulse time. When DNA size increases, higher switch times are required to resolve the fragments since the time required for reorientation of larger DNA molecules is high (Birren and Lai, 1993). Therefore, pulse

times, which should be increased for the resolution of high molecular weight fragments (Bio-Rad Manual, 2001).

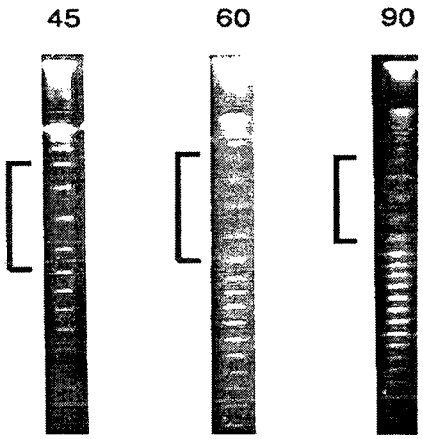

Figure 3.4 Effects of pulse times on separation of DNA fragments (Birren and Lai, 1993).

Effect of pulse times on separation of DNA fragments can be observed in Figure 3.4 (Birren and Lai, 1993). Three different constant pulse times have been used to separate DNA fragments. First lane represents 45s pulse time and the bracket contains 350-550 kb. When pulse time is increased to 60s, fragment sizes in the bracket are 550-750. With 90s pulse time, fragment sizes increase to 650-900kb. It is clear that as the pulse time increases, higher molecular weight fragments can be separated. Although an increase of pulse time enables us to resolve larger DNA fragments, resolution of the lower molecular weight fragments decrease as it is seen from the fragments below the bracket.

Pulsed field gel electrophoresis has been widely used for strain differentiation among the genera of lactococci (Tanskanen *et al.*, 1990; Vela *et al.*, 2000; and Moschetti *et al.* 2001).

A reliable strain identification protocol for lactococci has been reported by Tanskanen *et al.* (1990). They have analysed *Sma* I digestion patterns of 29 strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. cremoris. A 16h PFGE

run with pulse times increasing linearly from 1 to 20s, which separated fragments between 50 and 240 kbp, have been found valuable for strain differentiation.

Twelve nisin producing *Lactococcus lactis* strains have also been analysed by PFGE (Moschetti *et al.*, 2001). *Sma* I patterns have enabled the differentiation of nine strains due to their unique patterns where three strains yielded the same restriction profile.

PFGE has also been used for strain differentiation of another member of the genera of *Lactococcus*, *Lactococcus garviae*, which is important as an emerging pathogen in veterinary and human medicine (Vela *et al.*, 2000). They have found 19 different types of *Lactococcus garviae* indicating a high diversity of its strains.

PFGE method has been found useful for typing of *Streptococcus thermophilus* strains (O'Sullivan and Fitzgerald, 1998; Roussel *et al.*, 1997). O'Sullivan and Fitzgerald (1998) have used *Sfi* I, *Sma* I, *Bss* HIII and *Not* I restriction enzymes for comparison of the genomes of *Streptococcus thermophilus* strains. *Sma* I has been found as valuable for strain differentiation and determining strain relatedness. Construction of physical maps of *Streptococcus thermophilus* strains and comparison of those strains has also been performed by the use of PFGE (O'Sullivan and Fitzgerald, 1998). *Sma* I restriction digestion profiles of two strains have been compared with that of strain A054. One strain has exhibited a slight genetic polymorphism involved in a few regions in the chromosome. In contrast, a more important polymorphism related with numerous regions in the chromosome has been found between the other strain and strain A054.

Several strains of *Lactobacillus acidophilus* (Roussel *et al.*, 1993), *Lactobacillus casei* (Ferrero *et al.*, 1996), *Lactobacillus helveticus* (Lortal *et al.* 1997), and *Lactobacillus rhamnosus* (Tynkkynen *et al.*, 1999) have been analysed by PFGE.

Lactobacillus casei strains isolated from Grana cheese together with two reference strains (*Lactobacillus casei* ATCC 394 and *Lactobacillus paracasei* ATCC 334) have been analysed by their restriction profiles obtained by *Sma* I, *Sfi* I and *Bgl* I (Ferrero *et al.* 1996). *Sma* I has revealed differences between two species *Lactobacillus casei* and *Lactobacillus paracasei*, whereas *Sfi* I revealed marked polymorphism among the strains of the same species.

Nineteen different restriction enzymes have also been used for the differentiation of *Lactobacillus helveticus* strains (Lortal *et al.*, 1997). They reported

that more informative patterns could be obtained by *Sma* I, *Sgr*AI and *Rsr* II. Comparison of *Sma* I restriction patterns of 22 *Lactobacillus helveticus* strains yielded 18 different profiles.

Several enzymes have also been tested for the differentiation of *Lactobacillus acidophilus* strains (Roussel *et al.* 1993). *Sma* I has been found suitable for use in the differentiation of strains examined. Four closely related type strains, *Lactobacillus acidophilus* IP7613, *Lactobacillus crispatus* IP102990, *Lactobacillus gasseri* IP102991, and *Lactobacillus* species IP7134 have also been analysed with *Sma* I (Roussel *et al.* 1993). They have displayed specific macrorestriction patterns. This has proved that closely related strains could easily be differentiated by PFGE.

The use of one restriction enzyme may not reveal the diversity within the strains of lactic acid bacteria. In this case, a second restriction enzyme may enable us to reveal diversity of strains. For example, Tynkkynen *et al.* (1999) have used two restriction enzymes (*Not* I and *Sfi* I) for the identification of *Lactobacillus casei* strains. *Not* I has revealed 15 genotypes whereas *Sfi* I 16 genotypes over 24 strains. Combination of the results of digestions with two restriction enzymes has differentiated 17 genotypes. Bértrand *et al.* (2000) have used *Apa* I to differentiate two *Enterococcus faecalis* strains, one from clinical sources, and the other from cheese. They have not been differentiated due to *Sma* I digestion pattern, but *Apa* I has revealed the diversity between these two isolates.

Pulsed field gel electrophoresis protocols normally takes 3-7 days to complete. Therefore, there have been several reports on the evaluation of short PFGE protocols for lactic acid bacteria (Turabelidze *et al.*, 2000; Benson and Ferrieri, 2001). Turabelidze *et al.* (2000) have reported a simple reproducible and cost effective system for vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* strains. They have shortened the standart procedure from 3-7 days to nearly 28h. Benson and Ferrieri (2001) have also reported a rapid method with increased reproducibility, higher image quality and reduction of time for *Streptococcus* isolates.

PFGE has also been used for enterococci isolated from several cheese (Mannu *et al.*, 1999; Bértrand *et al.*, 2000) and from clinical sources and culture collections (Murray *et al.*, 1990, Kühn *et al.*, 1995; Bértrand et al., 2000).

3.2.4 DNA Sequencing

DNA sequencing is the determination of the nucleotide composition of a DNA molecule. DNA typing methods are normally based on the differentiation of bacteria by the differences in DNA. Although, it seems that it is the best way to use DNA sequencing in order to discriminate isolates, sequencing the whole genome of each isolate is not practical. Therefore generally either, the 16S rRNA gene or the 16S rRNA itself sequenced since it consists of variable and conserved regions within bacterial species. Databases of 16S rRNA sequences are constructed and comparison of these sequences may enable the identification of bacterial isolates. Indeed 16S rRNA sequences are very useful for taxomomic studies of bacteria. According to 16S rRNA sequences, evolutionary trees are constructed and phylogenetic relationships of bacterial species are determined.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

Chemicals used in this study are shown in Appendix A.

4.1.2 Raw Milk Samples

Ten raw milk samples (nearly 100 ml) representing the mixture of different cow's milks from İzmir and Balıkesir regions were aseptically taken. These samples were used for the isolation of lactic acid bacteria.

4.1.3. Reference Strains Used

Lactobacillus curvatus DSM 8768,

Lactobacillus casei CH1,

Lactobacillus plantarum DSM 1954, and

Lactobacillus casei subsp. *casei* NRRL B-441 were kindly provided by Professor Dr. Şebnem Harsa, Biotechnology and Bioengineering Department, Izmir Institute of Technology

Lactococcus lactis A216 was kindly provided by Professor Dr. Sevda Kılıç, Department of Dairy Technology, Ege University

Following strains were kindly provided by Prof Dr. L. K. Nakamura (Microbiologist Emeritus, Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture)

Lactobacillus rhamnosus NRRL B-442

Lactobacillus delbrueckii subsp. *lactis* NRRL B-735

Lactobacillus casei subsp. *casei* NRRL B-1922

Lactobacillus plantarum NRRL B-4496

Lactobacillus fermentum NRRL B-4524

Lactobacillus brevis NRRL B-4527

Lactobacillus reuteri NRRL B-14170

Following strains were kindly provided by Professor Dr. Frederico Uruburu Director Coleccion Española de Cultivos Tipo (CECT), Edificio de Investigacion, Universidad de Valencia, Campus de Burjassot, Burjassot (Valencia), Spain

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* CECT 4431

Lactococcus raffinolactis CECT 988T

Streptococcus thermophilus CECT 986T

Leuconostoc mesenteroides subsp. *mesenteroides* CECT 219T

Finally, the following strains were kindly provided by Ömre Sıkılı and Prof. Dr. Mehmet Karapınar Food Engineering Department, Ege University, İzmir

Lactococcus lactis subsp. cremoris CECT 697T

Enterococcus faecium CECT 4102

Enterococcus faecalis CECT 184

Enterococcus gallinarum CECT 970T

Enterococcus mundtii CECT 972T

Pediococcus damnosus CECT 4671

Pediococcus parvulus CECT 813T

Pediococcus dextrinicus CECT 4791T

In addition *Lactococcus lactis* 1403 was also used in this study.

4.2 Methods

4.2.1. Culture Media and Growth Conditions

One mililiter aliquots from each milk sample were aseptically transferred to 9 ml of $^1/_4$ strength Ringers' solutions and further dilutions were obtained. One mililiter aliquots from the 4^{th}, 5^{th}, 6^{th} and 7^{th} dilutions were plated on MRS agar (pH 6.2 and 5.4) (Yaygın and Kılıç, 1993, see App. B.1) and M17 agar plates (pH 7.15) (Yaygın and

Kılıç, 1993, see App. B.2) by the pour plate method. Double layer method was used for MRS agar plates.

All plates were incubated at 30°C for 3 days.

4.2.2. Isolation of Lactic Acid Bacteria

Individual colonies on agar plates of appropriate dilutions (Section 4.2.1) were randomly taken. They were transferred into 10 – 12 % sterile skimmed milk broths. All broths were incubated at 30 °C for 24 h and the proper coagulation of milk samples was checked. Isolates, which were unable to coagulate milk, were incubated for additional 24 h. Cultures giving a homogenous and proper coagulation were taken as lactic acid bacteria.

4.2.3. Phenotypic Identification Lactic Acid Bacterial Isolates

Samples giving a desirable coagulation were stained by simple staining method. A loopfull culture was transferred onto the microscope slide. After drying, they were fixed by exposure to the flame. They were stained with methylene blue (Appendix E.1) and washed with water. Morphology of isolates was determined under a light microscope. Isolates were classified as cocci and transferred to M17 broth.

4.2.3.1 Identification of Cocci

Coccus shaped lactic acid bacteria presumptively identified as lactococci, enterococci and *Streptococcus thermophilus* were transferred into *Streptococcus* cultivation broth (Appendix C.1). For the identification of lactic acid bacteria, overnight cultures were tested for growth at 10 °C, 40 °C and 45 °C, in 4% and 6.5 % NaCl, at pH 9.2, fermentation of maltose, saccharose and salicin, hydrolysis of arginine and CO_2 production from citrate (see Table 4.1).

Table 4.1 Characteristics used for the identification of coccus shaped lactic acid bacteria in this study (Garvie, 1984, Teuber, 1995)

Character	*Lactococcus lactis* subsp.			*Streptococcus*	*Enterococcus*	*Enterococcus*	*Lactococcus*
	lactis	*diacetylactis*	*cremoris*	*thermophilus*	*faecalis*	*faecium*	*raffinolactis*
Growth at 10° C	+	+	+	-	+	+	+
40° C	+	+	-	+	+	+	-
45° C	-	-	-	+	+	+	-
Growth in 4 %NaCl	+	+	-	-	+	+	-
6.5 %NaCl	-	-	-	-	+	+	-
Growth at pH 9.2	+	+	-				
Acid formed from							
maltose	+	+	rarely	-	+	+	+
saccharose	±	-	-	+	+	±	+
salicin	+	+	-	-	+	+	+
Arginine hydrolysis	+	+	-	-	+	+	variable
CO_2 from citrate	-	+	-				

4.2.3.1.1 Tests for Growth at Different Temperatures

0.1 ml of overnight cultures were transferred into the tubes containing 5 ml of broth given in Appendix C.2 and incubated at 10°C, 40°C and 45 °C for 7 days. Change of the color of the broth to yellow was considered as positive reaction.

4.2.3.1.2 Test for Growth at Different NaCl Concentrations

Ability of isolates to grow in 4% and 6.5 % NaCl was tested in the test tubes containing 5 ml of broths given in Appendix C.3.

0.1 ml of overnight grown cultures were inoculated into the broths and incubated at 30 °C for 7 days. Yellow color formation was recorded as positive reaction.

4.2.3.1.3 Test for Growth at pH 9.2

In order to test growth at pH 9.2, broth given in Appendix C.4 were used. 0.1 ml of overnight grown cultures were inoculated into 5 ml of broths and incubated at 30°C for 7 days. Change of the color of the broth to red was taken as positive reaction.

4.2.3.1.4 Test for Fermentation of Carbohydrates

Ability of isolates to ferment maltose, saccharose and salicin was tested. 0.1 ml of overnight grown cultures were inoculated into test tubes containing 2.5 ml of broths (Appendix C.5) with a desired carbohydrate and incubated at 30°C for 7. Change of color of the broth was considered as positive reaction.

4.2.3.1.5 Test for Arginine Hydrolysis and CO_2 Production From Citrate

Ability of isolates to hydrolyse arginine and to produce CO_2 from citrate was tested in tubes containing nearly 8 ml of Reddy broth and inverted Durham tubes (App. C.6). Isolates were inoculated into the broth and incubated at 30°C for 5 days. Isolates, which were able to change the color were considered as non-arginine hydrolising strains

and gas accumulation in the Durham tubes was taken as gas production from citrate.

4.2.4. Storing the Isolates

Reference cultures of lactic acid bacteria and coccus-shaped lactic acid bacterial isolates were stored as described by Kelly *et al.* (1998), but in LB broth containing 20 % glycerol. Cultures were grown overnight in LB broth (App. B3). 0.5 ml of each was transferred into the cryotubes and 0.5 ml broth containing 40% glycerol was added. Then tubes were mixed gently but thoroughly. Cultures were stored at –80 °C.

4.2.4 Identification of Lactic Acid Bacteria by PCR-based Techniques

4.2.5.1 Identification of Lactic Acid Bacteria by ITS-PCR

For the identification of lactic acid bacteria, two PCR- based methods (ITS-PCR and restriction analysis of amplified 16S rRNA gene) were used to differentiate reference strains of lactic acid bacteria. Results obtained from two different methods were compared and the most suitable method was choosen in order to identify lactic acid bacterial isolates originated from raw milk.

4.2.5.1.1 Amplification of Internal Spacer Region between 16S and 23S Ribosomal RNA Genes

A method based on the amplification of internal transcribed spacer region between 16S and 23 rRNA genes was applied to the reference strains of lactic acid bacteria. Universal primers targeting 16S-23S spacer region, were described by Jensen *et al.* (1993).

Primers:

G1: 5'-GAAGTCGTAACAAGG-3'

L1: 5'-CAAGGCATCCACCGT-3'

Primer G1 has been selected from a highly conserved region adjacent to the 16S-23S spacer and it was located nearly 30 to 40 nucleotides upstream from the spacer boundry. Primer L1 has been selected from five bacterial and four plant chloroplast 23S

sequences. It was the most conserved 23S sequence following spacer and located at 20 bases downstream from the spacer boundary. They have limited the sequences of primers to a length of 15 bases since there are sequence variations beyond these highly conserved regions.

All lactic acid bacterial isolates were grown in tubes containing nearly 5 ml of Luria Bertani (LB) broth (App. B3). They were then streaked twice on LB agar plates. Single colonies were transferred into 0.2 ml PCR tubes containing 47 µl PCR mixtures (App. F1) until sufficient turbidity was observed. All PCR mixtures were then overlaid with 60 µl mineral oil. PCR amplifications were performed in a thermocycler, PTC-0150 Mini Cycler (MJ Research Inc., USA) using following program:

Step 1: 95 °C for 5 m
Step 2: 95 °C for 1 m (denaturation)
Step 3: 44 °C for 1 m (annealing) } 40 cycles
Step 4: 72 °C for 1 m (elongation)
Step 5: 72 °C for 10 m (final extension)

At the end of the first step, reaction was paused and samples were taken into ice in order to extract genomic DNA to be used as template. Samples were then centrifuged for 3 s at 6000 rpm.

Three microliters of *Taq* DNA polymerase enzyme dilution (App. F.2) were added to each sample. All samples were mixed gently after the addition of the enzyme dilution and kept on ice. They were centrifuged again for 3 s at 6000 rpm and placed into the wells of thermocycler. Amplification reaction was then continued.

4.2.5.1.2 Separation of Amplified ITS Fragments

Amplified ITS fragments were separated in 1 % agarose. For this purposes, 0.5 g agarose was dissolved in 1xTBE buffer by boiling. After cooling the agarose solution to 40°C, 5 µl of ethidium bromide solution (10mg/ml) were added. Agarose solution was poured into gel casting stand and combs were placed. After casting the gel, the combs were removed. 10 µl of samples below the mineral oil was taken and mixed with 2 µl of gel loading buffer (Appendix F.3). Samples were then loaded into the wells of agarose

gel starting from the second well. Five microliter of DNA molecular weight marker was loaded into the first gel. After the loading, PCR products were electrophoresed in 1x TBE buffer at 40 mA constant voltage in an agarose gel apparatus until bromophenol blue reached the end of the gel. Fragments were illuminated on an UV illuminator (Vilber Lourmat, France) and photographed by using polaroid films.

4.2.5.2 Identification of Lactic Acid Bacteria by PCR-RFLP Method

In order to identify lactic acid bacterial isolates by PCR-RFLP methods, 16S ribosomal RNA (rRNA) genes were amplified by using the primers targeting 16S rRNA genes (Mora *et al.* 1998) and then restriction fragment profiles of 16S rRNA genes, which were obtained after digestion with restriction enzymes *Taq* I and *Hae* III were compared.

4.2.5.2.1 Amplifications of 16S Ribosomal RNA Genes

All lactic acid bacterial isolates were grown in tubes containing nearly 5 ml of Luria Bertani (LB) broth. They were then streaked twice on LB agar plates. Single colonies were transferred into 0.2 ml PCR tubes containing 47 µl PCR mixtures (Appendix F.1) until sufficient turbidity was observed. All PCR mixtures were then overlaid with 60 µl mineral oil. PCR amplifications were performed in a thermocycler, PTC-0150 Mini Cycler (MJ Research Inc., USA) using following program:

Step 1: 95 °C for 5 min
Step 2: 95 °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 (final extension)

At the end of the first step, reaction was paused and samples were taken into ice. They were then centrifuged for 3 s at 6000 rpm.

Three microliters of *Taq* DNA polymerase enzyme dilution (see App. F.2) were added to each sample. All samples were mixed gently after the addition of the enzyme

dilution and kept on ice. They were centrifuged again for 3 s at 6000 rpm and placed into the wells of thermocycler. Amplification reaction was then continued.

4.2.5.2.2 Electrophoresis of Amplified 16S Ribosomal RNA Genes

At the end of the reaction, amplifications of 16S rRNA genes were controlled by separating the PCR products in 1 % agarose gels. For this purpose, 0.5 g agarose were dissolved in 50 ml of 1x TAE or 1x TBE buffer by boiling. Gel was cooled to nearly 40 °C and 5 µl of ethidium bromide solution (10mg/ml) were added. Gel was then poured into gel casting stand and combs were placed. After casting the gel, 10 µl of samples below the mineral oil was taken and mixed with 2 µl of gel loading buffer. Samples were then loaded into the wells of agarose gel starting from the second well. First lane was loaded with 5 µl of DNA molecular weight marker. PCR products were electrophoresed in 1x TAE or 1x TBE buffer at 40 mA constant voltage in agarose gel apparatus until bromophenol blue reached the end of the gel. Fragments were illuminated on an UV illuminator (Vilber Lourmat, France). Presence of a 1400 bp fragment indicated the amplification of 16S rRNA gene.

4.2.5.2.3 Chloroform Extraction of Amplified 16S Ribosomal RNA Genes

In order to extract PCR amplification products, the volume of the samples were adjusted to 100 µl by the addition of 60 µl of 1x TE buffer. They were then centrifuged for 5 s 10000 rpm. Phase below the mineral oil was removed and transferred into 1.5 ml tubes. Two hundred microliters of chloroform-isoamyl alcohol solution (App. D19) were added to the samples and samples were mixed thoroughly. They were then centrifuged for 2 m at 10 000 rpm. Upper phase was taken and mixed with 200 µl of choloroform-isoamyl alcohol solution. They were shaken thoroughly again and centrifuged for 2 m at 10 000 rpm. Upper phase (100 µl) was then taken and transferred into the tubes containing 10 µl of 3 M sodium acetate and mixture was mixed thoroughly. Two hundred and fifty microliters of 99 % ethanol were added to the mixtures. They were mixed throughly. Samples were stored at –20 °C for 30 m. They were then centrifuged for 15 m at 10 000 rpm. After that, the liquid phase was removed without disturbing the pellet. Pellets were then washed with 300 µl, 70 % ethanol and

mixed well. They were centrifuged for 5 m at 10 000 rpm. Ethanol was removed without disturbing the pellets. Pellets were again washed with 70 % ethanol and centrifuged for 5 m at 10 000 rpm. Ethanol was removed. Finally pellets were dried at room temperature.

4.2.5.2.4 Restriction Enzyme Digestion of 16S Ribosomal RNA Genes

DNA pellets were dissolved in 10 µl 1x TE buffer and centrifuged for 3 s at 6000 rpm. Five microliters of each sample was transferred to 0.5 ml PCR tubes containing 15 µl restriction enzyme mixtures including all ingredients shown in App. F4 except DNA samples. Samples were digested with *Taq* I and *Hae* III at 65 °C for 2 h and 37 °C overnight respectively. Additionally, samples to be restricted with *Taq* I were overlaid with mineral oil in order to avoid evaporation at 65 °C. After the restriction enzyme digestion, samples were stored at –20 °C until electrophoresis.

4.2.5.2.5 Electrophoresis of Restriction Fragments

For the separation of restriction fragments, 1.3 % agarose gel was prepared. 1.95 g agarose was dissolved in 150 ml 1x TBE or 1x TAE by boiling. After it was cooled to nearly 42°C, 15 µl ethidium bromide (10 mg/ml) was added and it was mixed well. Gel was poured to gel casting stand and combs were placed. Twenty microliters of samples was mixed with 4 µl gel loading buffer and samples were loaded into the agarose gel by starting from the second well. Electrophoresis was performed in 1250 ml 1x TBE or 1xTAE buffer at constant voltage of 60 miliampere. At the end of the electrophoresis, the gel was removed and fragments were visualised by placing the gel into the UV illuminator. Finally, agarose gel was photographed by GelCam (0,4x electrophoresis hood GH20, UK) by using Polaroid film.

4.2.6 Pulsed Field Gel Electrophoresis – Restriction Fragment Length Polymorphism

Method used for pulsed field gelectrophoresis of lactic acid bacterial isolates was the modification of the method given in Bio-Rad application manual (Bio-Rad Manual, 2001).

4.2.6.1 Preparation of Agarose Embedded Bacterial DNA

For the preparation of agarose embedded bacterial DNA, reference cultures of lactic acid bacteria were inoculated into 5ml LB broths and grown with gentle agitation. After sufficient turbidity was observed, 5 µl of chloramphenicol stock solution (App. D15) was added to give a 180 µg/ml final concentration and incubation was continued for up to 1 hour.

Three mililiters of bacterial culture were taken and centrifuged for 5 m at 10000 rpm in a microcentrifuge. The supernatant was removed and cells were resuspended in 50 µl of Cell Suspension Buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA). The cell suspension was then equilibrated to 50° C.

Two percent of low melting point agarose was prepared by using sterile water, and melted. The solution was then equilibrated to 50°C.

Fifty microliters of cell suspension buffer combined with 50 µl of low melting point agarose by mixing gently but thoroughly. By keeping the cell-agarose mixture at 50°C, the mixture was transferred to plug molds using sterile pipettes and it was then allowed to solidify.

Five hundred microliters of lysozyme buffer (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2 % sodium deoxycholate, 0.5 % sodium lauryl sarcosine, 10 mg / ml lysozyme) were added into microcentrifuge tube in order to perform lysis of the cell wall. Agarose plugs were transferred to the microcentrifuge containing lysozyme buffer. The plugs were incubated for 1 hour at 37 °C without agitation.

The lysozyme buffer was removed and the plugs were rinsed with 2.5 ml of 1x wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA) by incubating for 45 m at room temperature with agitation. The wash buffer was then removed and 0.5 ml of Proteinase K buffer (100 mM EDTA, pH 8.0, 0.2 % sodium deoxycholate, 1 % sodium lauryl

sarcosine, 1 mg/ ml Proteinase K) was added to plugs in order to remove all proteineceous materials. The plugs were then incubated for 18 h at 50° C without agitation.

Proteinase K buffer was then removed. The plugs were washed two times with 5 ml of wash buffer including 100 mM NaCl (20 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl) for 45 m at room temperature with gentle agitation. Plugs were then washed with 5 ml of wash buffer including 1 mM PMSF (20 mM Tris, pH 8.0, 50 mM EDTA, 1mM PMSF) to remove all residual activity of proteinase K. Plugs were finally washed with wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA).

For subsequent enzymatic reactions, plugs were washed with 0.1x wash buffer for 30 m.

4.2.6.2. Restriction Enzyme Digestion of Agarose Plugs

The plugs were transferred into the sterile 1,5 ml microcentrifuge tubes containing 1 ml of the 1x restriction enzyme (supplied with restriction enzyme) buffer and were then incubated about 1 hour with gentle agitation at room temperature.

The restriction enzyme buffer was aspirated off and 300 µl of fresh 1x enzyme buffer was added. Twenty units of the restriction enzyme *Sma* I were added and incubated at 30°C for 16h. After digestion, the buffer was removed and digest was incubated in 1 ml of 1x TAE for approximately 30 m with gentle agitation at room temperature.

4.2.6.3. Casting the Gel and Loading the Plugs

Agarose gel for pulsed field gel electrophoresis was cast with 14 cm x 13 cm gel casting stand provided with CHEF DRII equipment. 15 well 1.5 mm thick comb was attached to comb holder. Comb holder was then placed into one of two positioning slots on each side of the casting stand in the way that the bottom of the comb would be 2mm above the platform.

One-gram molecular biology certified agarose was weighed and it then was dissolved in 100ml 1x TAE buffer by boiling. After the gel was cooled (< 60°C), it was

poured onto platform in the casting stand with a thickness of 5-6 mm. The gel was allowed to solidify for 30 m at room temperature.

In order to load samples into the gel, plugs were placed into the well by using a spatula in a way that height of the plugs would be less than 90% of the height of the wells. The plugs were firmly pressed against the front walls of the wells.

Fifty miligram low melting point agarose was dissolved in 5ml 1x TAE buffer by boiling. After it was cooled to proper temperature, each well was filled with this low melting point agarose solution.

4.2.6.4 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis of lactic acid bacterial isolates was performed in CHEF DR II with model 1000 Mini Chiller (Bio-Rad, USA). 1x TAE buffer was used as electrophoresis buffer and buffer temperature was 14 °C.

After loading samples to agarose gel, gel was removed from the casting stand together with the platform. They were placed into the electrophoresis cell. Two liters of 1x TAE buffer was poured into the electrophoresis cell in order to cover the gel surface 2mm above. Different electrophoretic conditions were used to optimize PFGE.

4.2.6.5 Staining the PFGE Gels

Gels were stained in 0.5 mg/ml ethidium bromide solution in water for 20-30 m with gentle agitation. After staining with ethidium bromide, gel was destained with deionized water. Destaining was performed in deionized water for 1-3 h with gentle shaking. The patterns of restricted DNA were then visualized on a UV transilluminator (Vilber Lourmat, France) and the gel was photographed by Polaroid film.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Isolation of Lactic Acid Bacteria

Ten raw milk samples representing the mixture of different cow's raw milk from the local regions of İzmir and Balıkesir were used. They were plated on MRS (pH6.2 and pH 5.4) and M17 agar plates (pH 7.15) and incubated at 30°C. After random sampling of the colonies from agar plates, they were inoculated into sterile milk broths perapared using skimmed milk. In total 39 isolates coagulating the milk samples were taken as lactic acid bacteria. By determining the colony morphology under the light microscope, all isolates were found as cocci. They were presumptively identified as *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Lactococcus* since they were coccus shaped and isolated from raw milk. Although *Leuconostoc* spp. can be found in raw milk and they are coccus shaped lactic acid bacteria, they exhibit poor growth in sterile milk broths since milk has lower levels of citrate. Their acidification rate lower, it was therefore assumed that they could not coagulate milk broths at incubation time used. As a result 39 isolates were presumptively identified as *Enterococcus*, *Streptococcus* and *Lactococcus*.

5.2 Identification of Lactic Acid Bacteria

5.2.1 Phenotypic Identification of Lactic Acid Bacteria

For the identification of coccus shaped isolates, characteristics shown in Table 4.1 were used. Only 27 of 39 isolates were taken for biochemical identification and the other 12 were used to test whether they could be identified by only PCR-RFLP method.

Isolates were first classified according to their growth at 45 °C and at 6.5 % NaCl concentration, they were therefore identified as *Enterococcus*. In total eleven isolates were able to grow at 6.5% NaCl and 45 °C and they were identified as *Enterococcus* spp. According to citrate utilization, *Enterococcus* spp. were divided into two groups; 9 out of 11 isolates (A6, A7, A8, A9, A10, A11, A12, A13, and A14) could

produce gas from citrate where two isolates (A15 and A16) could not. Only one enterococcal isolate (A6) could not grow at 10°C.

Six isolates (A17, A18, A19, A20, A21, and A22) were able to grow at 45°C but not at 6.5% NaCl concentrations. They were also able to grow at 10 °C and 40 °C, at 4% NaCl concentration and at pH 9.2. They could produce gas from citrate; hydrolyse arginine and ferment maltose, saccharose and salicin. One isolate (A23) was also able to grow at 10°C, 40 °C, 45 °C, in 4 % NaCl, and and at pH 9.2. It could ferment maltose and salicin. However it was not able to grow at 6.5% NaCl and it could not ferment saccharose and produce gas from citrate. According to these results, these seven isolates could not be confined into any known genus or species. It has been known that new species of *Enterococcus* are not able to grow at 6.5 % NaCl concentration. In contrast to classical enterococci, especially *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus avium* and related species often give negative results (Devriese and Pot, 1995). It was therefore concluded that these 7 isolates might be *Enterococcus* spp.

Five of 39 isolates (A1, A2, A3, A4, and A5) could grow at 10°C, 40 °C, in 4 % NaCl, and at pH 9,2 and were able to ferment maltose, salicin and/or saccharose. They were therefore identified as *Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. In order to test the production of CO_2 from citrate and arginine hydrolysis, Reddy broths were used. According to the reactions observed in Reddy broth, it was found that they were not able to produce gas from citrate. They were therefore identified as *Lactococcus lactis* subsp. *lactis*.

Isolate A24 was only able to grow at 10°C and pH 9.2 and ferment saccharose. It was not able to hydrolyse arginine and to produce gas from citrate. This isolate was tentatively identified as *Lactococcus raffinolactis* but normally this species can utilize three sugars, maltose, saccharose and salicin. In contrast, A24 was not able to ferment either maltose or salicin.

One isolate (A25) was able to grow at 10°C and ferment maltose and saccharose. This isolate was also not able to hydrolyse arginine and to produce gas from citrate. It was concluded that this isolate might be *Lactococcus lactis* subsp. *cremoris*, which is able to ferment saccharose.

Two isolates (A26 and A27) could not be identified by using biochemical tests. The isolate (A26) was able to grow only at 10°C and 40°C and ferment maltose. It was also not able to hydrolyse arginine and to produce gas from citrate. The isolate (A27)

Table 5.1 Results of biochemical identifications

No	Isolate	Results of Biochemical Tests
1	A1	*Lactococcus lactis* subsp. *lactis*
2	A2	*Lactococcus lactis* subsp. *lactis*
3	A3	*Lactococcus lactis* subsp. *lactis*
4	A4	*Lactococcus lactis* subsp. *lactis*
5	A5	*Lactococcus lactis* subsp. *lactis*
6	A6	*Enterococcus* spp.
7	A7	*Enterococcus* spp.
8	A8	*Enterococcus* spp.
9	A9	*Enterococcus* spp.
10	A10	*Enterococcus* spp.
11	A11	*Enterococcus* spp.
12	A12	*Enterococcus* spp.
13	A13	*Enterococcus* spp.
14	A14	*Enterococcus* spp.
15	A15	*Enterococcus* spp.
16	A16	*Enterococcus* spp.
17	A17	-
18	A18	-
19	A19	-
20	A20	-
21	A21	-
22	A22	-
23	A23	-
24	A24	*Lactococcus raffinolactis*
25	A25	*Lactococcus lactis* subsp. *cremoris*
26	A26	-
27	A27	-

was able to grow at 10, 40, and 45°C and pH 9.2. It fermented both maltose and salicin but not saccharose. It was able to hydrolyse arginine but not able to produce gas from citrate. It failed to grow at 4% and 6.5% NaCl concentrations. Results of biochemical identification were given in Table 5.1

Biochemically identified 27 isolates together with 12 isolates, which were not identified biochemically were taken for characterization by PCR-RFLP method.

5.2.2 PCR- based Methods for the Identification of Lactic Acid Bacteria

In order to identify lactic acid bacteria by polymerase chain reaction based techniques, following primers specific to 16S ribosomal RNA (rRNA) genes (Mora *et al.* 1998) and universal primers specific to internal transcribed spacer region (ITS) between 16S and 23S rRNA genes (Jensen *et al.*, 1993) were used.

Primers specific to 16S rRNA genes:

EGE 1 (forward): 5'- AGAGTTTGATCCTGGCTCAG -3'

EGE 2 (reverse): 5'- CTACGGCTACCTTGTTACGA –3'

Primers specific to ITS region:

G1 (forward): 5'- GAAGTCGTAACAAGG –3'

L1 (reverse): 5'- CAAGGCATCCACCGT –3'

5.2.2.1 Internal Transcribed Spacer (ITS) Region Amplifications of Reference Strains of Lactic Acid Bacteria

Amplifications of internal transcribed spacer region (ITS) between 16S rRNA and 23S rRNA genes of *Lactobacillus* reference strains and *Leuconostoc mesenteroides* subsp. *mesenteroides* strain gave two to three fragments with molecular weights ranging between 275 and 825 bp (Figure 5.1, Figure 5.2 and Table 5.2). Since rRNA genes can be found from 2 to 11 copies per bacterial cell in prokaryotes (Farber, 1996), more than one band can be obtained in some cases by the amplification of ITS region.

Lactobacillus plantarum DSM 1954, and *Lactobacillus curvatus* DSM 8768 could not be differentiated since they both had similar patterns (Figure 5.1, lanes 4, and 5). Their patterns included three fragments with molecular weights approximately of 350, 450, 575 bp and they were classified in a single group.

Second group included *Lactobacillus plantarum* NRRL B- 4496 and *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T. Two similar patterns with two fragments (350 bp and 450 bp) were obtained (Figure 5.1, lanes 3 and 6).

All casei species gave similar patterns (including three fragments with 300, 500, 825 bp, Figure 5.2, lanes 2,3,and 4) and it was possible to differentiate them from all other strains of lactobacilli and *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T (Figure 5.1 and Figure 5.2).

Other strains; *Lactobacillus reuteri* NRRL B-14170 (Figure 5.1, lane 2), *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735 (Figure 5.1, lane 7), *Lactobacillus fermentum* NRRL B-4524 (Figure 5.2, lane 5), and *Lactobacillus rhamnosus* NRRL B-442 (Figure 5.2, lane 6) gave their unique ITS patterns and it was therefore possible to differentiate all from the others (see Table 5.2 for fragment sizes obtained after ITS amplifications).

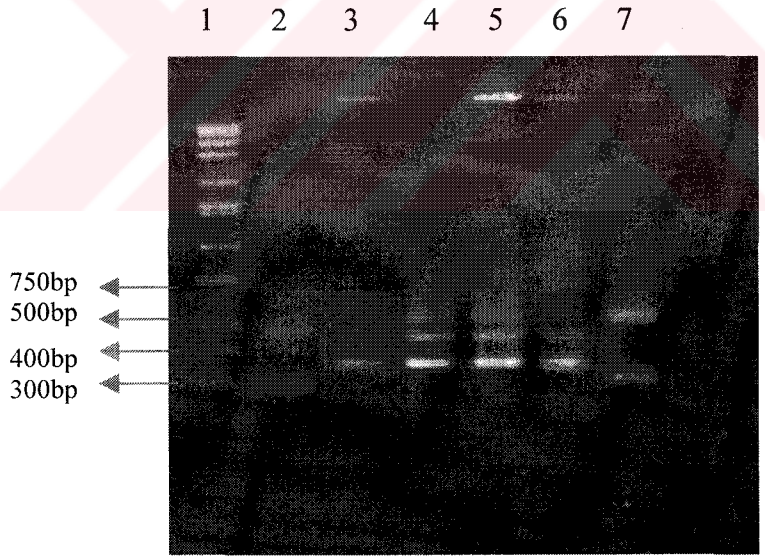

Figure 5.1 ITS amplification products of *Lactobacillus* reference strains

Lanes 1. Direct Load™ Wide-Range DNA Marker 2. *Lactobacillus reuteri* NRRL B-14170, 3. *Lactobacillus plantarum* NRRL B- 4496, 4. *Lactobacillus plantarum* DSM 1954, 5. *Lactobacillus curvatus* DSM 8768, 6. *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T, 7. *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735

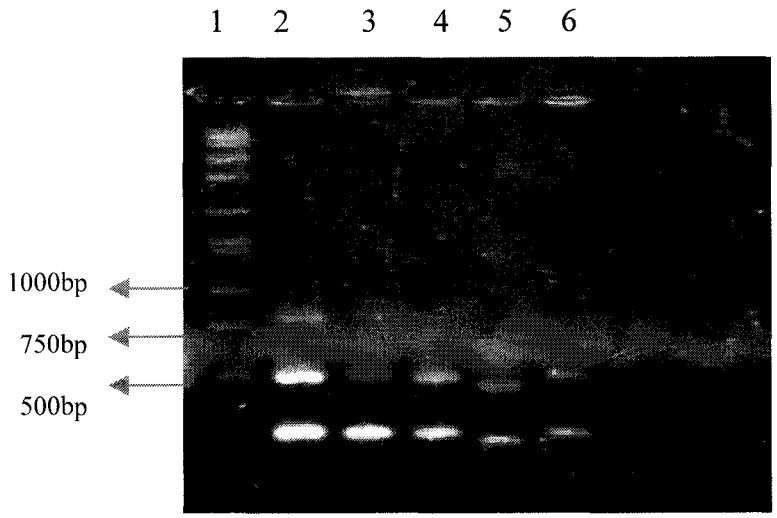

Figure 5.2 ITS amplification products of *Lactobacillus* reference strains Lanes 1. Direct Load™ Wide-Range DNA Marker, 2. *Lactobacillus casei* subsp. *casei* NRRL B-441, 3. *Lactobacillus casei* subsp. *casei* NRRL B-1922, 4. *Lactobacillus casei* CH1, 5. *Lactobacillus fermentum* NRRL B-4524, 6. *Lactobacillus rhamnosus* NRRL B-442

Table 5.2 Fragment sizes obtained by ITS amplifications of *Lactobacillus* reference strains and *Leuconostoc mesenteroides*

Strain	**Fragment sizes (basepairs)**
Lactobacillus reuteri NRRL B-14170	300, 400, 475
Lactobacillus plantarum NRRL B-4496	350, 450
Lactobacillus plantarum DSM 1954	350, 450, 575
Lactobacillus curvatus DSM 8768	350, 450, 575
Leuconostoc mesenteroides subsp. *mesenteroides* CECT 219T	350, 450
Lactobacillus delbrueckii subsp. *lactis* NRRL B-735	325, 575
Lactobacillus casei subsp. *casei* NRRL B-441	300, 500, 825
Lactobacillus casei subsp. *casei* NRRL B-1922	300, 500, 825
Lactobacillus casei CH1	300, 500, 825
Lactobacillus fermentum NRRL B-4524	275, 475
Lactobacillus rhamnosus NRRL B-442	300, 500

According to the ITS amplifications of *Streptococcus thermophilus* CECT 986T and *Lactococcus* strains, all strains yielded a single band (Figure 5.3). *Lactococcus lactis* 1403, *Lactococcus lactis* A216, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 and *Streptococcus thermophilus* CECT 986T gave an identical band patterns and clustered together (Figure 5.3, lanes 3, 4, 5 and 7 respectively) whereas *Lactococcus raffinolactis* CECT 988T (Figure 5.3, lane 6) and could be differentiated from others according to its characteristic ITS fragment. All *Lactococcus lactis* strains and *Streptococcus thermophilus* CECT 986T gave a approximately 200 bp fragment whereas *Lactococcus raffinolactis* CECT 988T gave an ITS fragment with a molecular weight of 225 bp (Table 5.3).

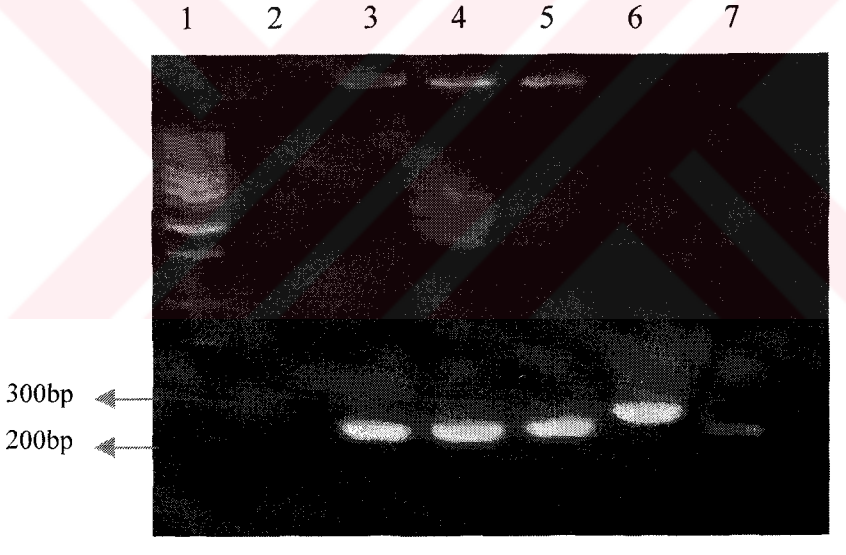

Figure 5.3 ITS amplifications of reference strains of *Lactococcus* and *Streptococcus thermophilus* strain Lanes 1. 1kb DNA ladder Gene Ruler™ 2. empty, 3. *Lactococcus lactis* 1403, 4. *Lactococcus lactis* A216, 5. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431, 6. *Lactococcus raffinolactis* CECT 988T, 7. *Streptococcus thermophilus* CECT 986T

Table 5.3 Fragment numbers and sizes of reference strains of *Lactococcus* and *Streptococcus thermophilus* strain after ITS amplifications

Reference Strains	**Spacer (ITS fragment)**	
	Number of Spacers	**Molecular weights of spacers (bp)**
Lactococcus lactis 1403	1	200
Lactococcus lactis A216	1	200
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* CECT 4431	1	200
Lactococcus raffinolactis CECT 988T	1	225
Streptococcus thermophilus CECT 986	1	200

Moschetti *et al.* (2001) used primers described by Jensen *et al.* (1993) in combination with nisin specific primers in order to identify several bacteriocin producing lactic acid bacteria in a multiplex-polymerase chain reaction. They have found that *Lactococcus lactis* isolates and two *Lactococcus lactis* subsp. *lactis* reference strains gave a fragment with a molecular weight of 380 bp and *Streptococcus thermophilus* strains have yielded a 350 bp fragment.

In this study, three *Lactococcus lactis* strains gave a fragment with a molecular weight of approximately 200 bp. *Streptococcus thermophilus* CECT 988T gave also an ITS fragment with a molecular weight of 200 bp and could not be differentiated from *Lactococcus lactis* strains. These results were not in accordance with those of Moschetti *et al.* (2001).

5.2.2.2 PCR-RFLP Profiles of Reference Strains of Lactic Acid Bacteria

In order to identify lactic acid bacteria, 16S rRNA genes were also amplified. After the amplification of 16S ribosomal rRNA genes, two restriction enzymes, *Taq* I and *Hae* III were used to identify lactic acid bacteria according to their restriction profiles.

First, several reference strains of lactic acid bacteria were used and their restriction profiles were obtained. All reference strains of lactic acid bacteria gave an amplification product with molecular weigth of approximately 1400 basepairs (Figure 5.4 and Figure 5.5).

Restriction digestion with *Taq* I did not reveal considerable difference within the reference strains of *Lactobacillus* (Figure 5.6). It was therefore concluded that restriction digestion of 16S rRNA by *Taq* I was not suitable for differentiation of *Lactobacillus* reference strains.

When the *Taq* I restriction profiles of 16S rRNA genes of reference strains of *Lactococcus* and *Streptococcus thermophilus* were analysed (Fig 5.8), *Lactococcus lactis* 1403, *Lactococcus lactis* A216, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 gave similar profiles. Restriction profiles of *Lactococcus raffinolactis* CECT 988T and *Streptococcus thermophilus* CECT 986T were also similar.

Restriction enzyme analysis of 16S ribosomal RNA genes of reference strains of lactobacilli with *Hae* III gave the fragments ranging from 50 to 625 base pairs (Figure 5.7). All strains except *Lactobacillus reuteri* NRRL B-14170 gave 5 bands whereas *Lactobacillus reuteri* NRRL B-14170 yielded 6 bands.

Hae III profiles clustered 11 reference strains of *Lactobacillus* strains into 5 groups. First group included *Lactobacillus curvatus* DSM 8768, *Lactobacillus plantarum* NRRL 4496 and *Lactobacillus plantarum* DSM 1954 (Figure 5.7, lanes 3,8 and 9, respectively). They gave similar profiles with five bands with molecular weights of 600, 450, 350, 75, and 50 bps. Together with two *plantarum* strains, *Lactobacillus curvatus* DSM 8768 was also clustered with this group and it could not be differentiated from *Lactobacillus plantarum* strains.

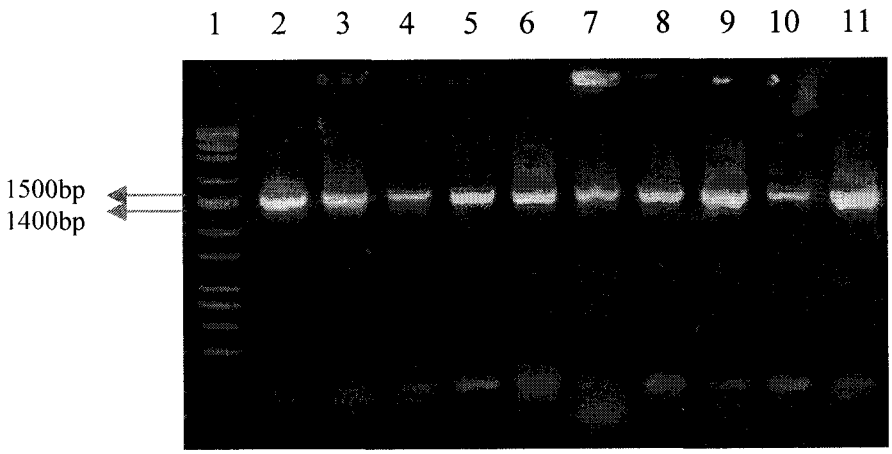

Figure 5.4 16S rRNA genes of reference strains of *Lactobacillus* Lanes 1. Direct Load™ Wide-Range DNA Marker, 2. *Lactobacillus reuteri* NRRL B 14170, 3. *Lactobacillus plantarum* NRRL B-4496, 4. *Lactobacillus plantarum* DSM 1954, 5. *Lactobacillus curvatus* DSM 8768, 6. *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735, 7. *Lactobacillus casei* subsp. *casei* NRRL B-441, 8. *Lactobacillus casei* subsp. *casei* NRRL B-1922, 9. *Lactobacillus casei* CH1, 10. *Lactobacillus brevis* NRRL B-4527, 11. *Lactobacillus fermentum* NRRL B-4524

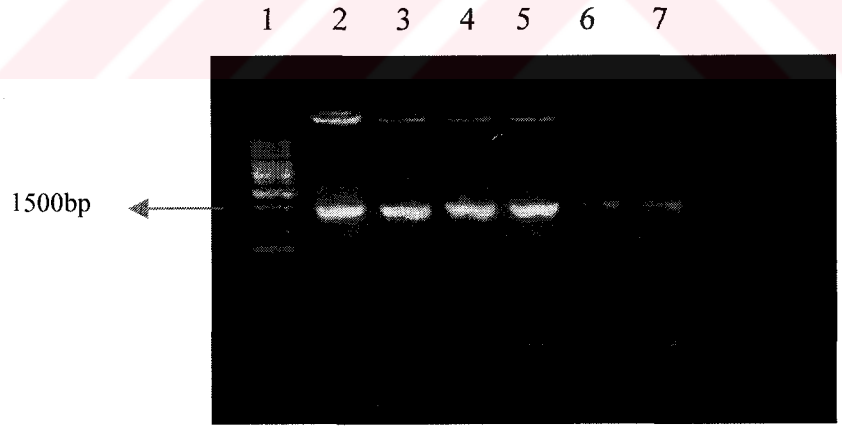

Figure 5.5 16S rRNA genes of reference strains of *Lactococcus* and *Streptococcus thermophilus* Lanes 1. 1kb DNA ladder Gene Ruler™ 2. empty, 3. *Lactococcus lactis* 1403, 4. *Lactococcus lactis* A216, 5. *Lactococcus lactis* subsp. *diacetylactis* CECT 4431, 6. *Lactococcus raffinolactis* CECT 988T, 7. *Streptococcus thermophilus* CECT 986T

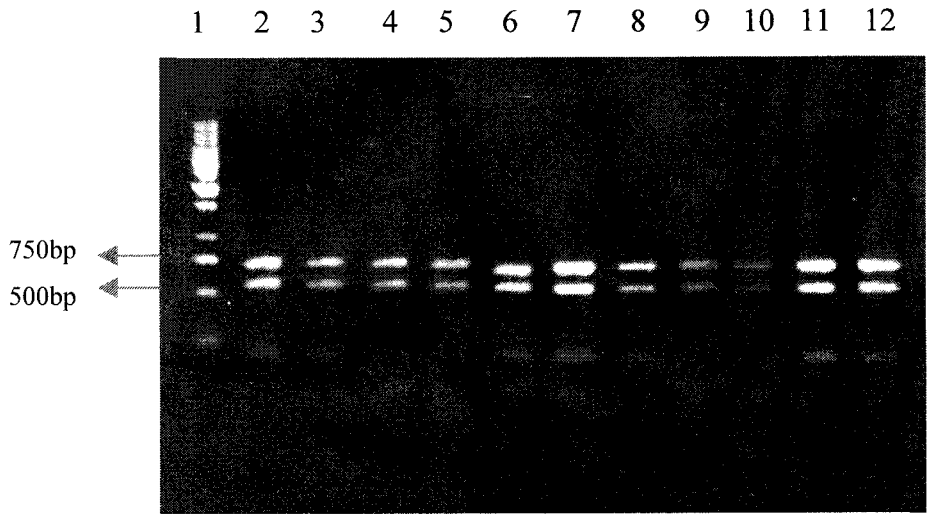

Figure 5.6. *Taq* I digests of 16S rRNA genes of *Lactobacillus* reference strains Lanes 1. 1kb DNA ladder Gene Ruler™, 2. *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735 3. *Lactobacillus curvatus* DSM 8768, 4. *Lactobacillus rhamnosus* NRRL B-442 5. *Lactobacillus casei* subsp. *casei* NRRL B-441, 6. *Lactobacillus casei* subsp. *casei* NRRL B-1922, 7. *Lactobacillus casei* CH1, 8. *Lactobacillus plantarum* NRRL 4496, 9. *Lactobacillus plantarum* DSM 1954, 10. *Lactobacillus brevis* NRRL B-4527, 11. *Lactobacillus reuteri* NRRL B-14170, 12. *Lactobacillus fermentum* NRRL B-4524

Another group consisted of *Lactobacillus rhamnosus* NRRL B-442, *Lactobacillus casei* subsp. *casei* NRRL B-441, *Lactobacillus casei* subsp. *casei* NRRL B-1922 and *Lactobacillus casei* CH1. Three *casei* strains together with *Lactobacillus rhamnosus* NRRL B-442 gave similar profiles. (Figure 5.7, lanes 4, 5, 6, and 7, respectively). They all had five bands with molecular weights of 625, 450, 350, 75 and 50 bps.

Third group included *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735 and *Lactobacillus brevis* NRRL B-4527. These two reference strains gave the similar patterns and could not be differentiated (Figure 5.7, lanes 2 and 10, respectively).

Lactobacillus reuteri NRRL B-14170, and *Lactobacillus fermentum* NRRL B-4524 gave unique patterns (Figure 5.7, lanes 11 and 12, respectively). They were clustered in a single group for their characteristics patterns and it was possible to differentiate them from the other *Lactobacillus* strains. Fragment sizes obtained after digestion with *Hae* III are shown in Table 5.4.

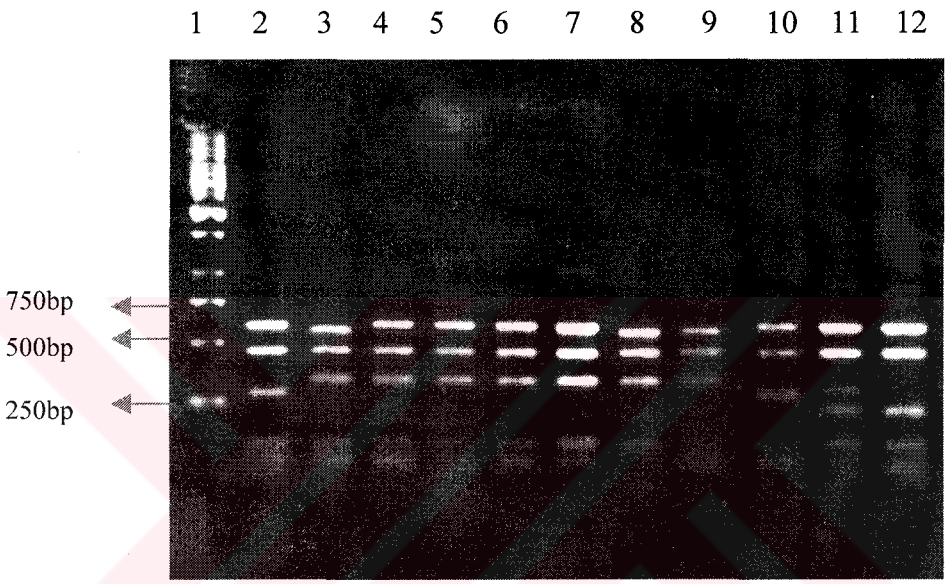

Figure 5.7. *Hae* III digests of 16S rRNA genes of *Lactobacillus* reference strains Lanes 1. 1kb DNA ladder Gene Ruler™, 2. *Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735 3. *Lactobacillus curvatus* DSM 8768, 4. *Lactobacillus rhamnosus* NRRL B-442 5. *Lactobacillus casei* subsp. *casei* NRRL B-441, 6. *Lactobacillus casei* subsp. *casei* NRRL B-1922, 7. *Lactobacillus casei* CH1, 8. *Lactobacillus plantarum* NRRL B-4496, 9. *Lactobacillus plantarum* DSM 1954, 10. *Lactobacillus brevis* NRRL B-4527, 11. *Lactobacillus reuteri* NRRL B-14170, 12. *Lactobacillus fermentum* NRRL B-4524

Table 5.4 Fragment sizes obtained by *Hae* III restriction analysis of 16S rRNA genes of several *Lactobacillus* reference strains

Strain	**Fragment sizes (basepairs)**
Lactobacillus delbrueckii subsp. *lactis* NRRL B-735	50, 75, 275, 450, 625
Lactobacillus curvatus DSM 8768	50, 75, 350, 450, 600
Lactobacillus rhamnosus NRRL B-442	50, 75, 350, 450, 625
Lactobacillus casei subsp. *casei* NRRL B-441	50, 75, 350, 450, 625
Lactobacillus casei subsp. *casei* NRRL B-1922	50, 75, 350, 450, 625
Lactobacillus casei CH1	50, 75, 350, 450, 625
Lactobacillus plantarum NRRL 4496	50, 75, 350, 450, 600
Lactobacillus plantarum DSM 1954	50, 75, 350, 450, 600
Lactobacillus brevis NRRL B-4527	50, 75, 275, 450, 625
Lactobacillus reuteri NRRL B-14170	50, 75, 200, 275, 450, 625
Lactobacillus fermentum NRRL B-4524	50, 75, 200, 450, 625

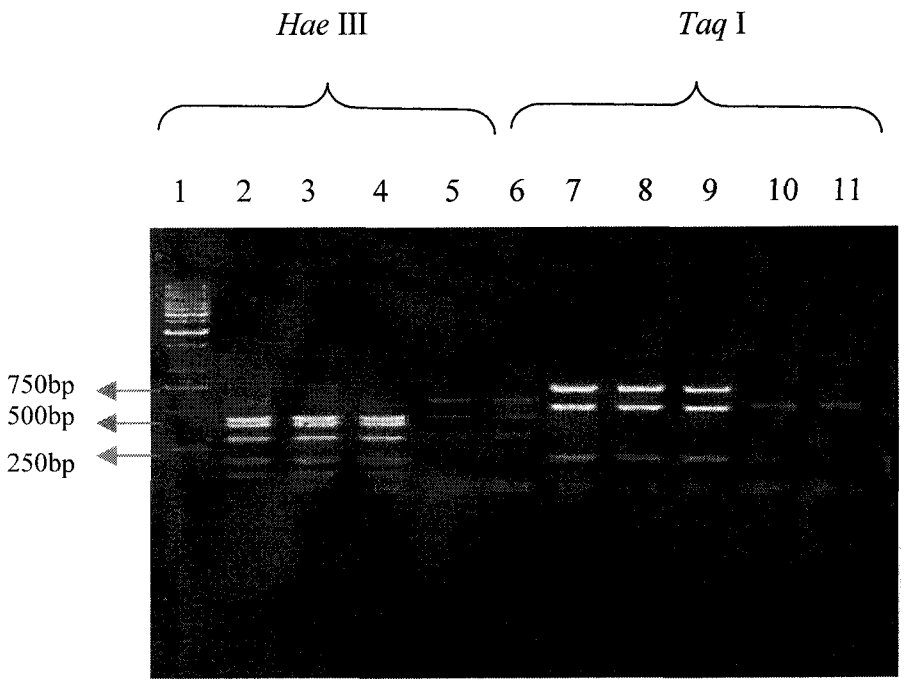

Figure 5.8. *Hae* III and *Taq* I digests of 16S rRNA genes of *Lactococcus* and *Streptococcus* reference strains. Lanes 1. 1kb DNA ladder Gene Ruler™, 2. *Lactococcus lactis* 1403, 3. *Lactococcus lactis* A216, 4. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 5. *Lactococcus raffinolactis* CECT 988T, 6. *Streptococcus thermophilus* CECT 986T, 7. *Lactococcus lactis* 1403, 8. *Lactococcus lactis* A216, 9. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431, 10. *Lactococcus raffinolactis* CECT 988T, 11. *Streptococcus thermophilus* CECT 986T

When *Hae* III was used for the identification of several reference strains of *Lactococcus* and *Streptococcus*, two groups were found. *Lactococcus lactis* 1403, *Lactococcus lactis* A216, and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 gave similar restriction profiles. They had 5 restriction fragments (Figure 5.8 and Table 5.5). *Hae* III restriction pattern of 16S rRNA gene of *Lactococcus raffinolactis* CECT 988T were similar with that of *Streptococcus thermophilus* CECT 986T (Figure 5.8, lanes 5 and 6, respectively). They were therefore clustered into a second group.

Table 5.5 Fragment sizes obtained by *Hae* III restriction analysis of 16S rRNA genes of several *Lactococcus and Enterococcus* reference strains and *Streptococcus thermophilus* strain

Strain	**Fragment sizes (basepairs)**
Lactococcus lactis 1403	175, 200, 300, 450, 475
Lactococcus lactis A216	175, 200, 300, 450, 475
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* CECT 4431	175, 200, 300, 450, 475
Lactococcus raffinolactis CECT 988T	300, 475, 625
Streptococcus thermophilus CECT 986	300, 475, 625
Enterococcus gallinarum CECT 970T	125, 300, 475, 625
Enterococcus faecium CECT 4102	125, 300, 475, 625

With the help of ITS results, *Hae* III restriction analysis of 16S ribosomal RNA genes of reference strains could differentiate closely related species (Table 5.6).

Two *Lactobacillus plantarum* strains (NRRL B- 4496 and DSM 1954 were not distinguished by *Hae* III analysis (Figure 5.7, lanes 8 and 9) but ITS amplification was able to separate these two different strains (Figure 5.1, Lanes 3 and 4).

Lactobacillus rhamnosus NRRL B-442 produced similar *Hae* III profile with those of three *Lactobacillus casei* strains (Figure 5.7, lanes 4, 5, 6, and 7). When it was analysed by ITS amplification, *Lactobacillus rhamnosus* NRRL B-442 could be differentiated from *Lactobacillus casei* strains (Figure 5.2, lanes 6, 2, 3, and 4).

Lactobacillus reuteri NRRL B-14170, and *Lactobacillus fermentum* NRRL B-4524 were distinguished from all other reference strains according to their unique amplification patterns obtained by both ITS amplifications and *Hae* III digestion of 16S ribosomal RNA genes.

Lactococcus lactis 1403, *Lactococcus lactis* A216, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* CECT 4431,and *Streptococcus thermophilus* CECT 986T gave similar ITS fragments. On the other hand, ITS amplification was not able to distinguish *Streptococcus thermophilus* CECT 986T from *Lactococcus lactis* strains.

Table 5.6 Comparison of groups obtained by *Hae* III restriction analysis and ITS-PCR

***Hae* III groups**	**ITS-PCR Groups**
Lactobacillus curvatus DSM 8768 *Lactobacillus plantarum* NRRL B-4496 *Lactobacillus plantarum* NRRL B-1954	*Lactobacillus plantarum* NRRL B-4496 *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T
	Lactobacillus plantarum DSM 1954 *Lactobacillus curvatus* DSM 8768
Lactobacillus rhamnosus NRRL B-14170 *Lactobacillus casei* CH1 *Lactobacillus casei* subsp. *casei* NRRL B-1922 *Lactobacillus casei* subsp. *casei* NRRL B-441	*Lactobacillus rhamnosus* NRRL B-14170
	Lactobacillus casei CH1 *Lactobacillus casei* subsp. *casei* NRRL B-1922 *Lactobacillus casei* subsp. *casei* NRRL B-441
Lactobacillus reuteri NRRL B-14170	*Lactobacillus reuteri* NRRL B-14170
Lactobacillus delbrueckii subsp. *lactis* NRRL B-735 *Lactobacillus brevis* NRRL B-4527	*Lactobacillus delbrueckii* subsp. *lactis* NRRL B-735
Lactobacillus fermentum NRRL B-4524	*Lactobacillus fermentum* NRRL B-4524
Lactococcus lactis A216 *Lactococcus lactis* 1403 *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431	*Lactococcus lactis* A216 *Lactococcus lactis* 1403 *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 *Streptococcus thermophilus* CECT 986T
Streptococcus thermophilus CECT 986T *Lactococcus raffinolactis* CECT 988T	*Lactococcus raffinolactis* CECT 988T

Only *Lactococcus raffinolactis* CECT 988T could be differentiated from these strains (Figure 5.3). In contrast, by the analysis of fragment profiles obtained by *Hae* III digestion of amplified 16S ribosomal RNA genes, *Streptococcus thermophilus* CECT 986T could be differentiated from *Lactococcus lactis* strains, but in this case restriction profile of this isolate was the same with that of *Lactococcus raffinolactis* CECT 988T (Figure 5.8). According to the results obtained by ITS amplification and PCR-RFLP method, the latter method was found as much more suitable for the identification of lactic acid bacterial isolates. Two restriction enzymes were used to identify reference strains. Both *Hae* III and *Taq* I digestion of 16S rRNA yielded the same clusters when used for the identification of reference strains of *Lactococcus* and *Streptococcus thermophilus* but *Hae* III digestion was much more suitable for the differentiation of reference strains of *Lactobacillus*. It was therefore choosen as a restriction enzyme of choice for the identification of raw milk isolates.

5.2.2.3 Identification of Raw Milk Isolates by PCR-RFLP

Lactic acid bacteria isolated from raw milk were identified by restriction profiles obtained by *Hae* III digestion of 16S rRNA genes. Results of *Hae* III restriction analysis were given in Table 5.7. Also, comparisons of the biochemical identification and PCR-RFLP results were given in Table 5.8

In total, twenty seven biochemically identified isolates and twelve isolates which were not tested by biochemical reactions were taken for the identification by *Hae* III restriction analysis of their 16S rRNA.

Five isolates (A1, A2, A3, A4, and A5), which were biochemically identified as *Lactococcus lactis* subsp. *lactis,* and one isolate identified as *Enterococcus* spp. (A9) according to phenotypic characterization, gave similar restriction patterns (Figure 5.9, lanes 2, 3, 4, 5, 6, and 13, respectively). Two isolates (A19 and A23), which were not confined into any described species, also yielded similar patterns with those of isolates identified as *Lactococcus lactis* subsp. *lactis* (Figure 5.10, lanes 4 and 12).

A23 differed from A19 according to biochemical reactions since it could not ferment saccharose and could not produce gas from citrate. All of these isolates had 5 bands with molecular weights approximately of 475, 450, 300, 200 and 175 base pairs. The restriction profiles of these isolates were similar with those of three reference

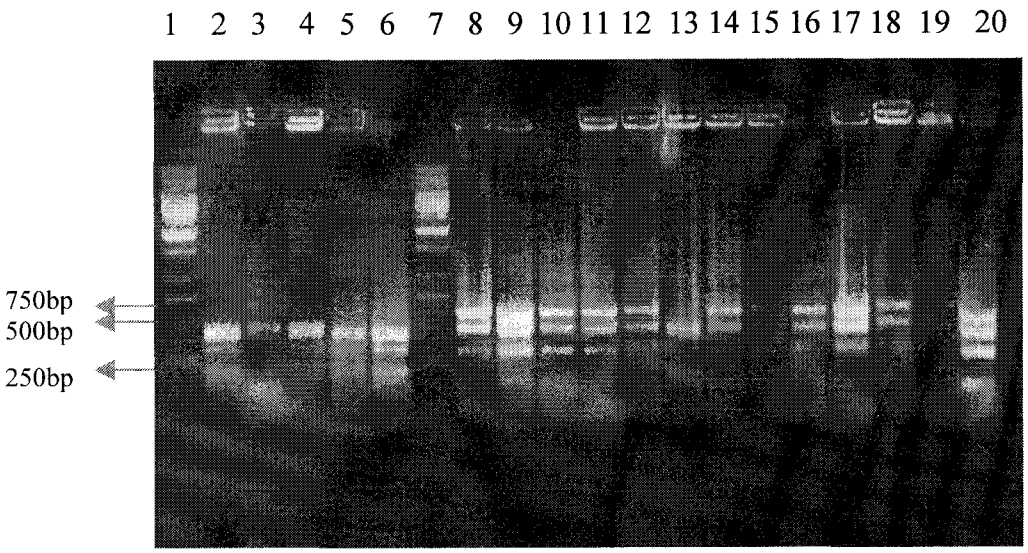

Figure 5.9 *Hae* III digests of 16S rRNA genes of raw milk isolates, Lanes 1. 1kb DNA ladder Gene Ruler™ 2. A1, 3. A2, 4. A3, 5. A4, 6. A5, 7. 1kb DNA ladder Gene Ruler™, 8. *Enterococcus gallinarum* CECT 970T, 9. *Enterococcus faecium* CECT 4102, 10. A6, 11. A7, 12. A8, 13. A9, 14. A10, 15. A11, 16. A12, 17. A13, 18. A14, 19. A15, 20. A16

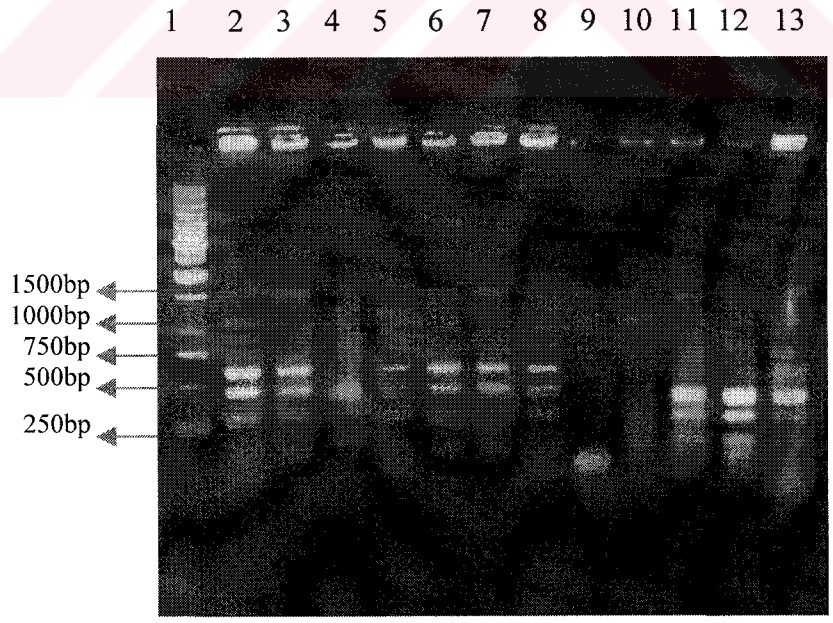

Figure 5.10 *Hae* III digests of 16S rRNA genes of raw milk isolates, Lanes 1. 1kb DNA ladder Gene Ruler™ 2. A17, 3. A18, 4. A19, 5. A20, 6. A21, 7. A22, 8. A28, 9. A24, 10. A25, 11. A26, 12. A23, 13. A27

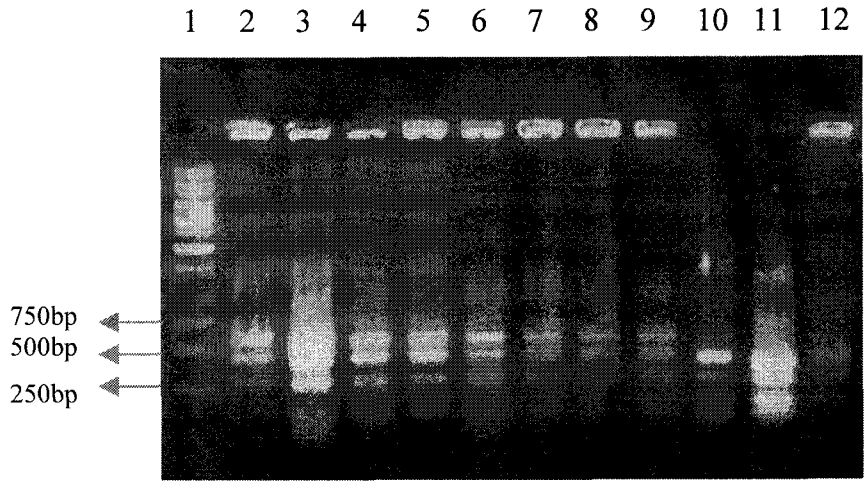

Figure 5.11 *Hae* III digests of 16S rRNA genes of raw milk isolates, Lanes 1. 1kb DNA ladder Gene Ruler™, 2. A29, 3. A30, 4. A31, 5. A32, 6. A33, 7. A34, 8. A35, 9. A36, 10. A37, 11. A38, 12. A39

Lactococcus lactis strains (Figure 5.8, Lanes 2, 3, 4). They were therefore identified as *Lactococcus lactis*.

Isolate A26 was only able to grow at 10°C and 40°C and ferment maltose. It was unable to hydrolyse arginine and produce gas from citrate. According to these biochemical results, it was not possible to confine this isolates into any species. When its restriction pattern was analysed (Figure 5.10, lane 11), it yielded similar banding pattern with those of isolates, which were biochemically identified as *Lactococcus lactis* subsp. *lactis* and three *Lactococcus lactis* reference strains.

Another isolate (A27) could not also be identified according to biochemical reactions. This isolate could grow at 10°C, 40°C, 45°C and at pH 9.2. It could only ferment maltose and salicin and it was unable to utilize saccharose. It could hydrolyse arginine but could not produce gas from citrate. PCR-RFLP method was useful to identify this isolate. It had characteristic patterns similar to those of the isolates biochemically identified as *Lactococcus lactis* subsp. *lactis* and with *Lactococcus lactis* reference strains (Figure 5.10, lane 13).

Here it was surprising that isolates biochemically identified as *Enterococcus* spp. and isolates, which could not be confined into any described species, were

identified as *Lactococcus lactis* subsp. *lactis* by PCR-RFLP method. This might also indicate that it is often difficult to interpret the results of biochemical tests.

Three isolates (A37, A38, and A39), which were not subject to biochemical identification, gave similar restriction profiles with *Lactococcus lactis* subsp. *lactis* isolates (Figure 5.11, lanes 10, 11, and 12, respectively). This result showed that, it was possible to identify isolates without using biochemical reactions. PCR-RFLP was useful to identify *Lactococcus lactis* subsp. *lactis* isolates according to their characteristic *Hae* III restriction digestion of 16S rRNA profiles.

Enterococcus gallinarum CECT 970T and *Enterococcus faecium* CECT 4102 were also analysed by the restriction digestion of their 16S ribosomal DNA (Figure 5.9, lanes 8 and 9, respectively). Indeed, they gave similar RFLP patterns and their restriction profiles were characteristic for enterococci (four fragments with molecular weight of 125, 300, 475, and 625 bp). Ten isolates (A6, A7, A8, A10, A11, A12, A13, A14, A15, and A16), which were biochemically identified as *Enterococcus* gave similar profiles with those of reference strains of *Enterococcus* (Figure 5.9, lanes 10, 11, 12, 14, 15, 16, 17, 18, 19 and 20, respectively). Five isolates (A17, A18, A20, A21, A22) could not grow at 6.5 % NaCl. This was surprising because growth at 6.5% NaCl is a characteristic property of *Enterococcus* spp. When their *Hae* III restriction profiles were analysed, they also give similar restriction patterns with those of reference strains of *Enterococcus* (Figure 5.10, lanes 2, 3, 5, 6, and 7, respectively). The use of growth at 6.5% NaCl may be useful for the separation of *Enterococcus* spp. from the other genera of lactic acid bacteria but newly identified enterococcal isolates especially *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus avium* and related species have failed to grow at 6.5 % NaCl (Devriese and Pot, 1995).

Isolates A28, A29, A30, A31, A32, A33, A34, A35, and A36 were not biochemically identified. They were only characterised by PCR-RFLP method. Restriction profiles of these bacteria (Figure 5.10, lane 8 for A28 and Figure 5.11, lanes 2, 3, 4, 5, 6, 7, 8, and 9 respectively for other isolates) were similar with those of *Enterococcus gallinarum* CECT 970T, and *Enterococcus faecium* CECT 4102. They were therefore identified as *Enterococcus* spp.

Hae III digests of 16S rRNA genes of *Lactococcus lactis* subsp. *cremoris* CECT 697 have produced two fragments with molecular weigths of 250 and 1200bp (Sıkılı, 2002). Two isolates (A24 and A25) (Figure 5.10, lane 9 for A24 and lane 10 for A25)

gave two fragments with 1200bp and 250bp and they were therefore identified as *Lactococcus lactis* subsp. *cremoris*. According to biochemical identification, the isolate A24 was identified as *Lactococcus raffinolactis* because it was only able to grow at 10°C and pH 9.2 and to ferment saccharose. It was not able to hydrolyse arginine and to produce gas from citrate. *Lactococcus lactis* subsp. *cremoris* only grow at 10°C and rarely ferment maltose. It neither hydrolyses arginine nor produces gas from citrate. This isolate did not fit into description of *Lactococcus lactis* subsp. *cremoris* since it was able to grow at pH 9.2 and ferment saccharose. In contrast to its biochemical reactions, it was identified as *Lactococcus lactis* subsp. cremoris by *Hae* III restriction analysis. The other isolate A25, which was identified as *Lactococcus lactis* subsp. *cremoris*, was also characterized as *Lactococcus lactis* subsp. *cremoris* according to *Hae* III analysis of 16S rRNA.

Hae III restriction analysis of amplified 16S ribosomal RNA genes of lactic acid bacterial isolates were useful to identify dairy originated lactic acid bacterial strains. According to the results of restriction analysis, thirteen of thirty nine isolates were identified as *Lactococcus lactis*. Twenty four isolates were identified as *Enterococcus* spp. whereas 2 isolates were characterised as strains of *Lactococcus lactis* subsp. *cremoris*.

In a recent work on the isolation of bacteriocin producing lactic acid bacteria from ewes', goats' and cows' raw milk (Rodríguez *et al.*, 2000), 82 selected strains having broad inhibitory activity have been phenotypically and genotypically identified. According to phenotypic results, 67 have been identified at the genus level as *Lactococcus*, 8 as *Enterococcus*, 5 as *Lactobacillus* and 2 as *Leuconostoc*. By a PCR method based on the amplification of histidine operon, most of the lactococci have been identified as *Lactococcus lactis* subsp. *lactis*, 2 as *Lactococcus lactis* subsp. *cremoris* and 6 as *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Seven *Enterococcus* species have been identified as *Enterococcus faecalis* based on their growth on KF agar, as typical colonies, and on bile agar and their ability to ferment glycerol and mannitol. The other enterococcal isolate have been characterised as *Enterococcus faecium* by 16S rRNA gene sequencing. *Lactobacillus* isolates have been identified as *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus plantarum*.

The results of this study were in accordance with the work of Rodríguez *et al.* (2000). In this study lactic acid bacterial isolates were identified as *Lactococcus lactis*

subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus* spp. Indeed, we could not test the differentiation power of the PCR based method for the discrimination of *Lactococcus lactis* subsp. *lactis* from *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. These two are known as phenotypically and genotypically indistinguishable from each other except the ability of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* to metabolise citrate (Cogan, 1996). But there have been reports on differentiation of subspecies of *Lactococcus lactis* (Rodríguez *et al.*, 2000). *Hae* III restriction patterns of 16S rRNA genes of *Lactococcus lactis* subsp. *cremoris* CECT 697 (Sıkılı, 2002) was different from *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 (Figure 5.8, lane 4). This shows *Hae* III analysis can be useful to discriminate between the *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 and *Lactococcus lactis* subsp. *cremoris* CECT 697. But further work must be performed for whether the method can discriminate *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*.

Table 5.7 PCR-RFLP identification results of raw milk originated lactic acid bacteria based on *Hae* III digestions of 16S rRNA genes

PCR-RFLP Groups		
1 (475, 450, 300, 200, 175 bp) *Lactococcus lactis*	**2** (625, 475, 300, 125 bp) *Enterococcus* spp.	**3** (1200, 250 bp) *Lactococcus lactis* subsp. *cremoris*
A1, A2, A3, A4, A5, A9 A19, A23, A26, A27, A37, A38, A39	A6, A7, A8, A10, A11 A12, A13, A14, A15 A16, A17, A18, A20 A21, A22, A28, A29 A30, A31, A32, A33 A34, A35, A36	A24, A25

Table 5.8 Comparison of results of biochemical identification and PCR-RFLP

Isolates	Biochemical Identification Results	PCR-RFLP Results
A1, A2, A3, A4, A5	*Lactococcus lactis* subsp. *lactis*	*Lactococcus lactis*
A9	*Enterococcus* spp.	*Lactococcus lactis*
A19, A23	-	*Lactococcus lactis*
A26, A27	-	*Lactococcus lactis*
A37, A38, A39	Not determined	*Lactococcus lactis*
A6, A7, A8, A10, A11, A12, A13, A14, A15, A16	*Enterococcus* spp.	*Enterococcus* spp.
A17, A18, A20, A21, A22	-	*Enterococcus* spp.
A28, A29, A30, A31, A32, A33, A34, A35, A36	Not determined	*Enterococcus* spp.
A24	*Lactococcus raffinolactis*	*Lactococcus lactis* subsp. *cremoris*
A25	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris*

5.2.3 PFGE-RFLP of Reference Strains of Lactic Acid Bacteria

The first pulsed field gel electrophoresis experiment in our laboratory was performed on reference strains of lactic acid bacteria in order to optimize PFGE-RFLP conditions for LAB (voltage gradient, pulse times and electrophoresis time).

Sma I digested genomic DNA of reference strains were separated at a voltage gardient of 4 V/cm with pulse times 5 s to 30 s for 24 h. The image of this first experiment of PFGE on different genera of lactic acid bacteria was shown in Figure 5.12.

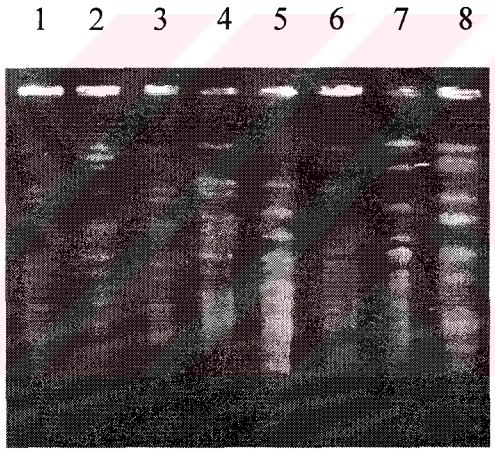

Figure 5.12 Pulsed field gel electrophoresis of *Sma* I digests of *Enterococcus*, *Pediococcus*, and *Lactococcus* reference strains. Migration conditions: 1% molecular biology certified agarose, 1x TAE running buffer, 4V cm^{-1} during 24h. Pulse times: 5-30s, electrophoresis time: 24h, Lanes 1. *Enterococcus mundtii* CECT 972T, 2. *Enterococcus faecalis* CECT 184, 3. *Pediococcus parvulus* CECT 813T, 4. *Pediococcus damnosus* CECT 4671, 5. *Lactococcus lactis* subsp. *cremoris* CECT 697, 6. *Pediococcus dextrinicus* CECT 4791T, 7. *Lactococcus lactis* A216, 8. *Lactococcus lactis* 1403

Pediococcal genomic DNA digested with *Sma* I was separated at these conditions. It was found that *Sma* I digested genomic DNA of *Pediococcus parvulus* CECT 813T, *Pediococcus damnosus* CECT 4671 and *Pediococcus dextrinicus* CECT 4791T (Figure 5.12, lanes 3,4 and 6, respectively) could be succesfully separated under these conditions, but pulse times should be lowered and electrophoresis run time should be increased to obtain interpretable fragment profiles.

In Figure 5.12, lanes 1 and 2 represent, two enterococcal strains, *Enterococcus mundtii* CECT 972T and *Enterococcus faecalis* CECT 184, respectively. Their *Sma* I digested fragments could be separated at these conditions, but low molecular weight fragments were poorly separated. It was therefore concluded that the pulse times should be decreased and electrophoresis time should be increased.

Three strains of *Lactococcus* were also analysed at these conditions. *Lactococcus lactis* 1403, *Lactococcus lactis* A126, *Lactococcus lactis* subsp. *cremoris* CECT 697 were digested with *Sma*I and their fragements were also separated at 4V/cm, with pulse times 5-30 s for a 24 h-electrophoresis time. All strains of *Lactococcus lactis* yielded *Sma* I digestion patterns, which could be separated by PFGE. By visual inspection of this first PFGE gel, three *Lactococcus lactis* strains could be distinguished (Figure 5.12, lanes 5, 7, and 8). At these PFGE conditions, higher molecular weight fragments could be separated well, but lower molecular weight fragments were poorly resolved. It was therefore concluded that the electrophoresis time must be increased. Whereas increasing the electrophoresis time, it was also thought that decrease in pulse time should help to separate those fragments.

According to the results obtained from the first pulsed field gel electrophoresis experiment (Figure 5.12), it was concluded that reduction in pulse time and increase in electrophoresis time would be necessary for typing the strains of *Lactococcus*, *Pediococcus*, *Enterococcus*. Pulse times were therefore reduced to 5-25 s and electrophoresis run time were increased to 28 h.

At these conditions, four *Lactococcus lactis* strains were analysed. *Lactococcus lactis* A216, *Lactococcus lactis* 1403, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 and *Lactococcus lactis* subsp. *cremoris* CECT 697 gave distinct and unique restriction patterns (Figure 5.13, lanes 1, 2, 3, and 4, respectively). When the restriction profiles of *Lactococcus lactis* strains were analysed, their distinct and characteristic banding patterns suggested that *Sma* I digestion patterns could be

succesfully used for the identification of *Lactococcus lactis* at the subspecies and strain level. Tanskanen *et al.* (1990) have reported that *Sma* I restriction patterns separated by PFGE could be used for reliable strain identification in *Lactococcus*. However, they used PFGE conditions of 200 V, pulsed times 1-20 s and 16 h electrophoresis time.

In Figure 5.13, lane 5 represents *Sma* I digested genomic DNA of *Streptococcus thermophilus* CECT 988T. Its restriction profile was different from all reference strains used. It could therefore be differentiated from all other lactic acid bacteria.

Three reference strains of *Enterococcus* were also analysed according their *Sma* I restriction patterns. These three different enterococcal strains; *Enterococcus faecium* CECT 4102, *Enterococcus faecalis* CECT 184, and *Enterococcus mundtii* CECT 972T

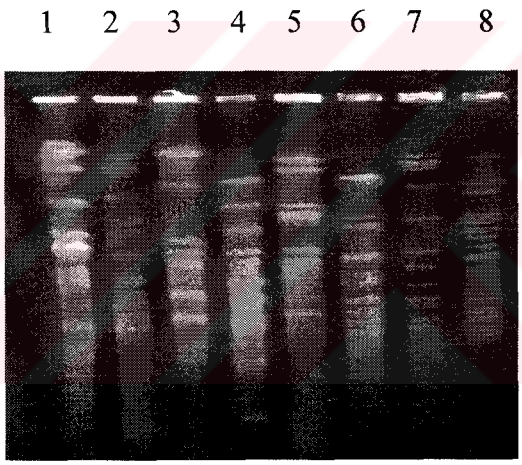

Figure 5.13 Pulsed field gel electrophoresis of *Sma* I digests of *Lactococcus* and *Enterococcus* reference strains. Migration conditions: 1% molecular biology certified agarose, 1x TAE running buffer, 4V/cm, pulse times: 5-25 s, electrophoresis time: 28h. Lanes 1. *Lactococcus lactis* A216, 2. *Lactococcus lactis* 1403, 3. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431, 4. *Lactococcus lactis* subsp. *cremoris* CECT 697, 5. *Streptococcus thermophilus* CECT 986T, 6. *Enterococcus faecium* CECT 4102, 7. *Enterococcus faecalis* CECT 184, 8. *Enterococcus mundtii* CECT 972T

yielded distinct and unique PFGE patterns. They could therefore be differentiated according their restriction profiles (Figure 5.13, lanes, 6, 7, and 8 respectively).

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, lactic acid bacteria isolated from cow's raw milk were characterized by phenotypic and PCR-based techniques. Two PCR-based techniques, ITS-PCR and restriction analysis of amplified 16S rRNA genes, were performed by using several reference strains of lactic acid bacteria. In addition pulsed-field gel electrophoresis was also used to differentiate the genera of *Lactococcus*, and *Enterococcus*.

In total 39 coccus-shaped lactic acid bacteria were isolated from cow's raw milk. Only 27 of 39 isolates were subjected to identification by biochemical tests. According to the results of biochemical tests, five isolates were identified as *Lactococcus lactis* subsp. *lactis*, 11 isolates as *Enterococcus* spp., 1 isolate as *Lactococcus raffinolactis* and 1 isolate as *Lactococcus lactis* subsp. *cremoris*. Nine isolates could not be confined into any known species.

Amplification and restriction enzyme digestion of 16S rRNA genes provided useful information for the identification of isolates. Combination of the results of two PCR methods increased the differentiation power among the reference strains. In restriction analysis of amplified 16S rRNA genes of reference strains, two restriction enzymes, *Taq* I and *Hae* III were used. *Hae* III was found to be much more suitable enzyme for the differentiation of lactic acid bacteria. When *Hae* III digestion profiles of 39 lactic acid bacteria isolates were compared with those of reference strains, 13 of 39 were identified as *Lactococcus lactis*, 24 of 39 isolates were identified as *Enterococcus* spp and 2 as *Lactococcus lactis* subsp. *cremoris*. Here 12 isolates, which were not biochemically tested, could also be identified by restriction analysis of 16S rRNA genes. This proved that PCR- RFLP was suitable for the identification. For example, the isolates, which were identified as *Enterococcus* spp. by biochemical tests were found to be *Lactococcus lactis* by PCR-RFLP. Isolates, which could not be confined into any known species could succesfully be identified by PCR-RFLP method.

In this study, pulsed field gel electrophoresis method was also optimized by using reference strains of lactic acid bacteria. Fragments obtained by *Sma* I restriction enzyme could be successfully separated at 4 V/cm with pulse times 5-25 s, for 28 h. At

these conditions, reference strains of *Enterococcus*, *Lactococcus*, and *Streptococcus thermophilus* could be succesfully differentiated. In the future, all the lactic acid bacterial isolates from raw milk will be analysed by PFGE.

In dairy products, starter cultures, which contain several different lactic acid bacteria affect the characteristics of each product. Isolation of lactic acid bacteria from different kinds of raw milk and traditionally fermented dairy products is important since they may harbour novel lactic acid bacteria that may contain or harbour different technological properties for the production of different fermented dairy products.

After the isolation and identification of lactic acid bacteria from a given source, strains have to be classified according to their origins and biotypes.

There are also several physiological functions important for the selection of starter cultures (Kılıç, 2001):

- Lactic acid production
- Aroma and flavour production
- Proteolytic activity
- Gas production
- Resistance to bacteriophages
- Synthesis of texturing agents
- Production of inhibitory compounds
- Resistance to inhibitors
- Dietetic properties (L-lactic acid production, aminoacid production, assimimilation of minerals, probiotic properties, etc.)

When a strain is to be used as starter culture, above-mentioned properties of the strain have to be evaluated. Starter cultures are generally composed of more than one strain. In this case, physiological balance between strains has also to be tested. In addition, it is also important to use strains that minimize risks of phage infection. After, the formulation of any starter culture, suitability of strains to industrial production and industrial use has to be evaluated (Kılıç, 2001). It is therefore necessary to test the production of cultures and to test the production of fermented products in pilot scale (Kılıç, 2001).

Characterization of technological and physiological functions of lactic acid bacterial isolates is the most important step in screening of the strains with challenging technological properties of starter cultures.

In this study, several lactic acid bacteria were isolated from cow's raw milk and they were identified by biochemical and genotypic methods. In future physiological and technological of cultures may also be evaluated.

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APPENDICES

APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Microbiological Experiments

NO	CHEMICAL	CODE
1	Agar	Merck 1.01613
2	Bacteriological pepton	Oxoid LP037
3	Lab-Lemco Meat Extract	Oxoid LP029
4	D-Glucose	AppliChem A3666
5	Yeast Extract	Merck A 1.03753
6	Skimmed milk	Pınar and Ova
7	Maltose	BDH 29131
8	Sucrose	Difco 0176-17
9	Salicin	BDH 38060
10	Arginine monohydrocholoride	BDH 6548390
11	Lactose	Sigma L3750
12	Glycerol	AppliChem A2926
13	NaCl	Merck 6400.100
14	Triammonium citrate	Sigma A1332
15	Sodium citrate	AnalaR 10242,
16	Bromcresol purple	Merck 3025
17	Sodium acetate	Sigma S2889
18	K_2HPO_4	Sigma P8281
19	Glycocoll	Riedel-De Haën 652296
20	Bromtymol blue	Riedel-De Haën 35088
21	Sodium phosphate di basic	Merck 926870
22	$MgSO_4.7H_2O$	Merck 1.05886
23	$MnSO_4.4H_2O$	Merck 1.02786
24	Ascorbic acid	Merck 5.00074
25	Phenol red	BDH 20091

Table A.2 PCR Reagents

NO	CHEMICAL	CODE
1	Taq DNA polymerase	Promega M1865
2	Primers: Ege 1 and Ege 2	Promega
3	Primers: G1 and L1	Promega
4	dNTP set	MBI, Fermentas, R0181
5	Standard agarose (low electroendoosmosis)	AppliChem A2114
6	*Taq* I	Promega, R6151
7	*Hae* III	Promega, R6171
8	Chloroform	AppliChem A3633
9	Sodium acetate	Sigma S 2889
10	Isoamyl alcohol	AppliChem A2610
11	Mineral oil	Sigma M5904
12	Bromophenol blue	Merck 1.08122
13	Glycerol	AppliChem A2926
14	1 kb DNA ladder Gene Ruler ™	Fermentas, SM0311
15	DirectLoad ™ Wide-Range DNA Marker	Sigma D-7058
16	Boric acid	AppliChem A2940
17	Polaroid Films	Sigma F3390
18	BSA	Promega R396D
19	Glacial acetic acid	Merck 1.00056

Table A.3 PFGE Reagents

NO	CHEMICAL	CODE
1	NaCl	AppliChem A2942
2	Sodium hydroxide	Merck 1.06498
3	Hydrogen chloride	Merck 1.00317
4	*SmaI*	MBI, Fermentas, ER0662
5	Low melting point agarose	AppliChem A3762
6	Molecular Biology Certified Agarose	Bio-Rad 162-0134
7	Tris Base	Sigma T6066
8	EDTA	AppliChem A2937
9	Isopropanol	AppliChem A3928
10	Lysozyme	AppliChem A3711
11	Sodium lauryl sulfate	AppliChem A1163
12	Sodium deoxycholate	AppliChem A1531
13	Chloramphenicol	AppliChem A1806
14	Glacial acetic acid	Merck 1.00056
15	Proteinase K	AppliChem A3830
16	Phenyl methyl sulfonyl floride	AppliChem A0999
17	Ethidium bromide	AppliChem A1151
18	Ethanol	AppliChem A3678
19	Boric acid	AppliChem A2940
20	Polaroid Films	Sigma F3390

APPENDIX B

RECIPIES FOR CULTURE MEDIA

B.1 MRS BROTH AND MRS AGAR

MRS BROTH	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7\ H_20$	0.2
$MnSO_4.4\ H_2O$	0.05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121° C for 15 minutes.

MRS AGAR	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7\ H_20$	0.2
$MnSO_4.4\ H_2O$	0.05
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.2 M17 BROTH AND M17 AGAR

M17 BROTH	**g/l**
Polypepton	5.0
Phytone pepton	5.0
Yeast extract	2.5
Meat extract	2.5
Lactose	5.0
Ascorbic acid	0.5
β-disodium glycerophosphate	19.0
$MgSO_4$ (0.1M) 7 H_2O	1.0 ml
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water in a water bath for 20 min. pH was adjusted to 7.15 ± 0.1. Medium was autoclaved at 121 °C for 15 minutes.

M17 AGAR	**g/l**
Polypeptone	5.0
Phytone pepton	5.0
Yeast extract	2.5
Meat extract	2.5
Lactose	5.0
Ascorbic acid	0.5
β-disodium glycerophosphate	19.0
$MgSO_4$ (0.1M) 7 H_2O	1.0 ml
Agar	12.0
Deionized water	1000.0 ml

All the ingredients except lactose were dissolved in 900 ml deionized water by holding in a water bath for 20 min. pH was adjusted to 7.15 ± 0.1. Medium was sterilized by autoclaving at 121 °C for 15 minutes.

Lactose was dissolved in 100 ml deionized water, autoclaved at 121°C for 15 minutes. After sterilization lactose solution was added to medium.

B.3 LURIA-BERTANI (LB) BROTH AND LB AGAR

LB BROTH

	g/l
Trypton	10
Yeast Extract	5
Glucose	5
NaCl	5
Deionized water	1000 ml

All ingredients were dissolved in1 liter deionized water. Medium was sterilized by autoclaving 121° for 15 min.

LB AGAR

	g/l
Trypton	10
Yeast Extract	5
Glucose	5
NaCl	5
Agar	15
Deionized water	1000 ml

All ingredients were dissolved in 1 liter with deionized water. Medium was sterilized by autoclaving 121° for 15 min.

APPENDIX C

MEDIA USED IN THE IDENTIFICATION OF COCCI

C.1 STREPTOCCOCUS CULTIVATION BROTH

	g/l
Special peptone	15.6
Yeast extract	2.8
NaCl	5.6
Glucose	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 7.5. Medium was autoclaved at 121 °C for 15 minutes.

C.2 MEDIA FOR TESTING THE GROWTH AT DIFFERENT TEMPERATURES

	g/l
Special peptone	15.6
Yeast extract	2.8
NaCl	5.6
Glucose	10.0
Deionized water	1000 ml

Indicator: Bromtymol blue solution (Appendix E.2) 10 ml was added to 1000 ml of broth.

All ingredients were dissolved in deionized water and pH was adjusted to 7.5. Medium was autoclaved at 121 °C for 15 minutes.

C.3 MEDIA FOR TESTING THE GROWTH AT DIFFERENT NaCl CONCENTRATIONS

	g/l
Peptone	10.0
Lab-Lemco meat extract	10.0
Glucose	10.0
Deionized water	1000 ml

Indicator: Bromtymol blue solution (Appendix E.2) 10ml was added to 1000 ml of broth.

For testing the growth at 4% and 6.5% NaCl, 40 g/l and 65 g/l NaCl was added to medium respectively.

All ingredients were dissolved and pH was adjusted to 7.5. Medium was autoclaved at 121 °C for 15 minutes.

C.4 MEDIA FOR TESTING THE GROWTH AT pH of 9.2

SOLUTION A	**g/l**
Lab-Lemco meat extract	10.0
Peptone	10.0
Glucose	10.0
NaCl	5.0
Deionized water	1000 ml

Indicator: Phenol red solution (Appendix E.3) 10ml was added to 1000 ml of broth.

SOLUTION B (Buffer solution)	**g/l**
Glycocoll	7.505
NaCl	5.850
Deionized water	1000 ml

A hundred ml of Solution B was added to 900 ml of Solution A. pH was adjusted to 9.35 by N/10 NaOH. After the overnight holding period, it was filtered. It was then sterilized by autoclaving at 121 °C for 20 min. (Final pH of this medium should be 9.2 and medium should be used with in 2 days)

C.5 MEDIA FOR TESTING THE FERMENTATION OF CARBOHYDRATES

	g/l
Lab-Lemco meat extract	10.0
Peptone	10.0
NaCl	3.0
Na_2HPO_4	2.0
Deionized water	1000 ml

Indicator: Bromtymol blue solution (Appendix E.2) 20 ml was added to 1000 ml of broth.

Maltose, saccharose and salicin were added at concentration of 1% into the medium.

All the ingredients were dissolved in deionized water, pH was adjusted to 7.5 and media was autoclaved at 121 ° C for 15 minutes.

C.6 REDDY BROTH

	g/l
Peptone	5.0
Yeast extract	5.0
K_2HPO_4	1.0
Arginine hydrochloride	5.0
Sodium citrate	20.0
Bromcresol purple	0.002
Skim milk	35.0 ml
Deionized water	1000 ml

All ingredients were dissolved in deionized water. pH was adjusted to 6.2. It was distributed into tubes containing inverted Durham tubes. Media was then autoclaved at 121 °C for 15 minutes.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1 1M Tris-HCl pH 7.2

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 7.2 with concentarted HCl. Volume is brought to 1L with deionized water.

D.2 1M Tris-HCl pH 8.0

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 8.0 with concentrated HCl. Volume was brought to 1L with deionized water.

D.3 0,5 M EDTA pH 8.0

186.12 g EDTA was dissolved in 800 ml of deionized water and pH is adjusted to 8.0 with 10 N NaOH. Volume was brought to 1000ml with deionized water.

D.4 50 X TAE

242 g Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

D.5 3 M NaCL

175.32 g NaCl was dissoled in deionized water and the volume was brought to 1000 ml with deionized water.

D.6 10 X TBE

108 g Tris Base and 55 g boric acid were weighed. They were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1 L with deionized water.

D.7 1X TBE

100 ml 10X TBE was taken and the volume was brought to 1 liter with deionized water to obtain 1liter 1X TBE buffer.

D.8 1X TAE

20 ml of 50X TAE buffer was taken and the volume was adjusted to 1 liter with deionized water to obtain 1 liter 1X TAE buffer.

D.9 CELL SUSPENSION BUFFER

10 mM Tris, pH 7.2

20 mM NaCl

50 mM EDTA

D.10 LYSOZYME BUFFER

10 mM Tris, pH 7.2

50 mM NaCl

0.2 % sodium deoxycholate

0.5 % sodium lauryl sarcosine

10 mg / ml lysozyme

D.11 1X TE BUFFER

10 mM Tris pH 8.0

1 mM EDTA pH 8.0

D.12 WASH BUFFER

20 mM Tris, pH 8.0

50 mM EDTA

D.13 PROTEINASE K BUFFER

100 mM EDTA, pH 8.0

0.2 % sodium deoxycholate

1 % sodium lauryl sarcosine

1 mg/ ml Proteinase K

D.14 PHENYL METHYL SULFONYL FLORIDE (PMSF) STOCK SOLUTION (100 mM)

17,4 mg PMSF was dissolved in 1 ml isopropanol and stored at –20 °C.

D.15 CHLORAMPHENICOL STOCK SOLUTION

180 mg chloramphenicol was dissolved in 1 ml 95% ethanol.

D.16 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

0.5 g ethidium bromide was dissolved in 50 ml of deionized water.

D.17 BSA (BOVINE SERUM ALBUMIN) (10x)

10mg/ml, 150 µl BSA was diluted with 1.5 ml TE buffer. It was divided into three aliquots (each at10x concentration) and stored at –20 °C.

D.18 SODIUM ACETATE (3 M, pH 5.2)

408.1 g sodium acetate (3 H_2O) was dissolved in 800 ml deionized water and pH was adjusted to 5.2 by glacial acetic acid. Volume was brought to 1000 ml.

D.19 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION

48 ml of chloroform was mixed with 2 ml of isoamyl alcohol.

APPENDIX E

STAINS AND INDICATORS

E.1 METHYLENE BLUE STAIN

0,3 g methylene blue was dissolved in 30 ml of 95 % ethyl alcohol. 0.01 g KOH was dissolved in deionized water and volume was brougth to 100 ml with deionized water. Two solutions were combined and mixed thoroughly. It was allowed to stand several days and filtered before use.

E.2 BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

E.3. PHENOL RED SOLUTION

Phenol red	1 g
N/10 NaOH	40 ml
Deionized water	460 ml

APPENDIX F

PCR RECIPIES

F.1 PCR MIXTURE

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

F.2 TAQ DNA POLYMERASE ENZYME DILUTION

Mg free Taq DNA polymerase buffer	0.3 µl
Sterile deionized water	2.4 µl
Taq DNA polymerase	0.3 µl (1.5 U)

F.3 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

F4. RESTRICTION ENZYME MIXTURE

Restriction enzyme buffer	2 µl
Sterile deionized water	11 µl
Bovine serum albumin (10x)	2 µl
DNA	5 µl
Restriction enzyme (10u/µl)	0.2 µl (2 U)

F5. dNTP (10X)

10 µl of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at –20 °C.

APPENDIX G

OLIGONUCLEOTIDE PRIMERS

G.1 PRIMERS FOR ITS AMPLIFICATIONS

G1: 5'- GAAGTCGTAACAAGG -3'

350 µg primer G1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliter of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at –20 °C.

L1: 5'- CAAGGCATCCACCGT -3'

350 µg primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliter of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at –20 °C.

G.2 PRIMERS FOR 16S rRNA GENE AMPLIFICATIONS

EGE 1: 5'- AGAGTTTGATCCTGGCTCAG -3'

590 µg primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Five microliter of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at –20 °C.

EGE 2: 5'- CTACGGCTACCTTGTTACGA -3'

680 µg primer EGE 2 was dissolved in 340 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Five microliter of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at –20 °C.

APPENDIX H

RESTRICTION ENZYMES AND THEIR RECOGNITION SITES

H1. *Taq* I

5'- T ▼CG A -3'

5'- A GC ▲T -3'

H2. *Hae* III

5'- GG ▼CC- 3'

5'- CC ▲GG- 3'

H3. *Sma* I

5'- CCC ▼GGG- 3'

5'- GGG ▲CCC- 3'

APPENDIX I

Table I.1 Identification Results Of Coccus Shaped Lactic Acid Bacteria

Isolate	TEST IN REDDY BROTH			IDENTIFICATION TESTS								
	Growth	Change in color	Gas Production	Growth at 10 °C	Growth at 40 °C	Growth at 45 °C	Growth in % 4 NaCl	Growth in % 6.5 NaCl	Growth at pH 9.2	Maltose Ferment	Saccharose Ferment	Salicin Ferment
A1	+	±	-	+	+	-	+	-	+	+	+	+
A2	+	-	-	+	+	-	+	-	+	+	-	+
A3	+	-	-	+	+	-	+	-	+	+	-	+
A4	+	-	-	+	+	-	+	-	+	+	-	+
A5	+	-	-	+	+	-	+	-	+	+	-	+
A6	+	-	+	-	+	+	+	+	+	+	+	+
A7	+	-	+	+	+	+	+	+	+	+	+	+
A8	+	-	+	+	+	+	+	+	+	+	+	+
A9	+	-	+	+	+	+	+	+	+	+	+	+
A10	+	-	+	+	+	+	+	+	+	+	+	+
A11	+	-	+	+	+	+	+	+	+	+	+	+
A12	+	-	+	+	+	+	+	+	+	+	+	+

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Table I.1 Identification Results Of Coccus Shaped Lactic Acid Bacteria (cont.)

Isolate	TEST IN REDDY BROTH			IDENTIFICATION TESTS								
	Growth	Change in color	Gas Production	Growth at 10 °C	Growth at 40 °C	Growth at 45 °C	Growth in % 4 NaCl	Growth in % 6.5 NaCl	Growth at pH 9.2	Maltose Ferment	Saccharose Ferment	Salicin Ferment
A13	+	-	+	+	+	+	+	+	+	+	+	+
A14	+	-	+	+	+	+	+	+	+	+	+	+
A15	+	-	-	+	+	+	+	+	+	+	+	+
A16	+	-	-	+	+	+	+	+	+	+	+	+
A17	+	-	+	+	+	+	+	-	+	+	+	+
A18	+	-	+	+	+	+	+	-	+	+	+	+
A19	+	-	+	+	+	+	+	-	+	+	+	+
A20	+	-	+	+	+	+	+	-	+	+	+	+
A21	+	-	+	+	+	+	+	-	+	+	+	+
A22	+	-	+	+	+	+	+	-	+	+	+	+
A23	+	±	-	+	+	+	+	-	+	+	-	+
A24	+	+	-	+	-	-	-	-	+	-	+	-
A25	+	+	-	+	-	-	-	-	-	+	+	-
A26	+	+	-	+	+	-	-	-	-	+	-	-
A27	+	-	-	+	+	+	-	-	+	+	-	+