INVESTIGATION OF CHROMOSOMAL AND PLASMID DNA PROFILES OF *Lactococcus lactis* ssp. *lactis*

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ABSTRACT

Lactic acid bacteria used in the manufacturing of cheese and other fermented milk products are known as starter cultures. Starters play an important role in the sensory properties of fermented milk products by lactic acid production which influences desirable quality characteristics. In dairy industry, diversity of starter strains of *Lactococcus lactis* is very limited. There is a considerable demand for novel starters with technologically desirable properties. Gene products responsible for lactose fermentation are encoded on plasmid DNA. Therefore, the plasmid stability of lactic acid bacteria is an important industrial property for starter culture.

In the previous study, *Lc. lactis* ssp. *lactis* strains were isolated from an artisanal "comlek peyniri" from Cappodocia and these strains were selected as good acid producers. The aim of this study was to investigate intraspecific diversity of 54 *Lc. lactis* ssp. *lactis* by choromosomal and plasmid profiling. In addition, the plasmid stability of 35 artisanal starters was determined by the examination β-galactosidase activity. First of all, partial DNA sequencing of the 16S rRNA genes was carried out in order to confirm that the previously identified isolates were *Lc. lactis* ssp. *lactis*. The partial DNA sequencing and blast search results indicated that representative isolates showed 90 % homology with *Lactococcus lactis.* Based on chromosome and plasmid profiling results, 35 *Lactococcus lactis* artisanal starters exhibiting good acidifying activities were classified into ten different genotypes and nine plasmid groups. None of the reference strains of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* were included into these genotypes and plasmid groups. The rest of the 17 isolates which were defined as low acid producers by technological methods were clustered into thirteen unique genotypes and seven plasmid groups. Only four of the isolates were found to contain the most stable plasmids by instability studies and these kept their lactase activities during prolonged subculturing.

As a result, chromosome and plasmid DNA profiling allowed us to classify artisanal starter strains in certain groups. The strains with stable and instable plasmids will serve us to characterize and improve technological features of these artisanal starter strains in future studies.

ÖZET

Peynir ve diğer fermente süt ürünlerinin üretiminde kullanılan laktik asit bakterileri "starter kültür" olarak bilinir. Starterler istenilen kalite kriterlerini etkileyen laktik asit üretimi ile fermente süt ürünlerinin duyusal özelliklerinde önemli rol oynarlar. Süt ve süt ürünleri endüstrisinde *Lactococcus lactis* starter suşlarının çeşitliliği sınırlıdır. Teknolojik olarak istenen özellikteki yeni starterler için önemli ölçüde talep duyulmaktadır. Laktoz fermentasyonundan sorumlu gen ürünleri plazmit DNA üzerinde kodlanır. Bu nedenle, laktik asit bakterilerinin plazmit stabilitesi starter kültürler için önemli bir endüstriyel özelliktir.

Önceki çalışmada, , *Lc. lactis* ssp. *lactis* suşları Kapodokya bölgesi artisanal "çömlek peynirinden" izole edildi ve bu suşlar iyi asit üreticileri olarak seçildi. Bu çalışmanın amacı, 54 *Lc. lactis* ssp. *lactis* suşunun kromozomal ve plazmit profilleri ile intraspesifik çeşitliliğini araştırmaktır. Buna ek olarak, 35 artisanal starterin plazmit stabilitesi β-galaktozidaz aktivesitesinin incelenmesi ile belirlendi. İlk olarak, önceden *Lc. lactis* ssp. *lactis* olarak tanımlanan izolatların doğrulanması için 16S rRNA genlerinin kısmi DNA dizi analizleri gerçekleştirildi. Kısmi DNA dizi analizleri ve blast araştırma sonuçları temsili izolatların *Lactococcus lactis* ile % 90 homolojiye sahip olduğunu gösterdi. Kromozom ve plazmit profil sonuçlarına göre iyi asit aktivitesi gösteren 35 *Lactococcus lactis* artisanal starteri 10 farklı genotip ve 9 farklı plazmit gruplarına ayrıldı. *Lactococcus lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris* ve *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* referans suşları bu genotip ve plazmit grupları içinde yer almadı. Teknolojik yöntemlerle düşük asit aktivitesine sahip olduğu belirlenen diğer 17 izolat 13 farklı genotip ve 7 plazmit grubuna ayrıldı. Plazmit stabilitesi çalışmaları ile sadece dört izolatın stabil plazmit içerdiği ve bu izolatların sürekli kültürleme süresince laktaz aktivitelerini koruduğu bulundu.

Sonuç olarak, kromozom ve plazmit DNA profili analizleri artisanal starter suşlarının belli gruplara ayrılmasını sağladı. Stabil ve stabil olmayan plazmit içeren suşlar ileriki çalışmalarda artisanal starterlerin teknolojik özelliklerini karakterize etmek ve geliştirmek için kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

bp : Base pair

DNA : Deoxyribonucleic Acid

dNTP : Deoxynucleotide triphosphate

EDTA : Ethylenediamine tetra acetic acid

h : Hour

ITS : Internally Transcribed Spacer

kb : Kilo base

LAB : Lactic Acid Bacteria

Lc. : *Lactococcus*

Mb : Mega base

min : Minutes

µl : Microliter

mM : Milimolar

MRS : de Man, Rogosa and Sharpe Medium

NCBI : National Center for Biotechnology Information

PCR : Polymerase Chain Reaction

PFGE : Pulsed Field Gel Electrophoresis

PMSF : Phenyl Methyl Sulforyl Floride

rRNA : Ribosomal Ribonucleic Acid

ssp. : Subspecies

s : Second

TAE : Tris Acetate EDTA

TBE : Tris Borate EDTA

TE : Tris EDTA

U : Unit

UPGMA : Unweighted Pair-Group Mean Arithmetic Method

UV : Ultra Violet

CHAPTER 1

INTRODUCTION

1.1. History

The microbial nature of lactic fermentation was recognized by Louis Pasteur in 1857. The first isolation, identification, and description of a chemical entity from sour milk by Carl Wilhelm Scheele in 1780, was actually L (+) lactic acid produced by lactococci. The first pure culture of a bacterium obtained and scientifically described by Joseph Lister (1873), was *Lactococcus lactis*, then called "*Bacterium lactis*". Mesophilic lactic streptococci which were responsible for the spontaneous fermentation of sour milk, sour cream and cheese, had been used as an industrial starter culture in 1890. Orla-Jensen has divided the mesophilic lactic streptococci as "*Streptococcus lactis*" and "*Streptococcus cremoris*" in 1919 (Table 1.1). According to the serological differentiation of the streptococci, lactic streptococci have been classified as group N which are clearly differentiated from pathogenic streptococci (group A, B, C) by Lancefield (1933). The serological typing of Lancefield has been very important for the classification of streptococci. Although this classification has lost its importance during the time, it is still very useful for the rapid identification of major pathogens. The first systematic classification of the streptococci was proposed by Sherman (1937) and lactic streptococci had been divided into four groups (Table 1.2). Based on the molecular characteristics, Schleifer has separated the mesophilic lactic streptococci from the true streptococci (genus *Streptococcus*) and enterococci (genus *Enterococcus*) so the new genus *Lactococcus* has been generated (1985). *Streptococcus thermophilus* is the only streptococcal species associated with dairy industry especially in yoghurt manufacturing.

Lactococci are the most important organisms for dairy technology. They are divided into five species (Williams *et al*. 1990) and only one species, *Lactococcus lactis* is used for cheese fermentation.

Table 1.1. Key for the differentiation of lactic acid bacteria (Stiles and Holzapfel 1997).

Genus	**Shape**	**Catalase**	**Nitrite Reduction**	**Fermentation**	**Current Genera**
Betabacterium	Rod	-	-	Hetero-	*Lactobacillus* *Weissella*
Thermobacterium	Rod	-	-	Homo-	*Lactobacillus*
Streptobacterium	Rod	-	-	Homo-	*Lactobacillus* *Carnobacterium*
Streptococci	Coccus	-	-	Homo-	*Streptococcus* *Enterococcus* ***Lactococcus*** *Vagococcus*
Betacoccus	Coccus	-	-	Hetero-	*Leuconostoc* *Oenococcus* *Weissella*
Microbacterium	Rod	+	+	Homo-	*Brochotorix*
Tetracoccus	Coccus	+	+	Homo-	*Pediococcus* *Tetragenococcus*

Table 1.2. Sherman's classification system for streptococci (Stiles and Holzapfel 1997).

Sherman's Group				
	Pyogenic	Viridans	Lactic	*Enterococcus*
Lancefield's Group	A,B,C,G	-	N	D (Q)
Haemolysis	β	-	-	-
Growth at 10 ˚C	-	-	+	+
Growth at 45 ˚C	+	+	-	+
Growth at pH 9.6	-	-	-	+
Survive 60 ˚C for 30 min	-	+	Variable	+
Methylene blue (0.1%)	-	-	+	+,-

1.2. Lactic Acid Bacteria

The term of "lactic acid bacteria" is used synonymously with "milk souring microorganisms". The lactic acid bacteria (LAB) consisting of a number of diverse genera are grouped as either homofermenters or heterofermenters on the basis of fermentation end products (Carr *et al*. 2002). Homolactic fermentation is known as Embden-Meyerhof-Parnas (EMP) pathway for glycolysis (Teuber 1995). The homofermenters produce only lactic acid as the major end-product by the fermentation of carbohydrates (Carr *et al*. 2002, Axelsson 1998). The homofermentative group of LAB includes *Streptococcus*, *Pediococcus*, *Lactococcus*, and *Lactobacillus* species (Table 1.3). The heterofermenters on the other hand produce a number of other products such as carbondioxide, acetic acid, ethanol besides lactic acid (Carr *et al*. 2002, Axelsson 1998).

Homofermentation and heterofermentation are differentiated by the production of aldolase which is the key enzyme for glycolysis. The homofermenters possess aldolase which ferments glucose to lactic acid. The heterofermenters do not produce this enzyme and follow pentose-monophosphate pathway instead (Figure 1.1). They can not breakdown fructose 1, 6 diphosphate to triose phosphates, and only oxidize glucose-6-phosphate to 6-gluconate by phosphoketolase.

Table 1.3. Differentiation of the lactic acid bacteria according to the carbohydrate metabolism (Carr *et a*l. 2002).

Genus	**Gram stain morphology**	**Type of lactic acid**	**CO_2 production from glucose**	**Ribose fermentation**	**Gluconate fermentation**	**Arginine hydrolysis**
Homofermenters						
Streptococcus	Cocci/chains	L+	-	-,+	ND	V
Pediococcus	Cocci/tetrad	DL, L+	-	V	-	V
Lactococcus	Cocci/chains	L+	-	-	-	V
Lactobacillus	Bacilli/pairs	D-, L+	-	+,-	+,-	-
Heterofermenters						
Betabacteria (*Lactobacillus)*	Pair/chains	DL	+	+	+	+
Leuconostoc	Pair/chains	D-	+	V	-,+	-

Abbreviations: +, - indicates most positive strains, an occasional negative strain; -,+ indicates most negative strains, an occasional positive strain; V indicates variable; ND indicates no data is available.

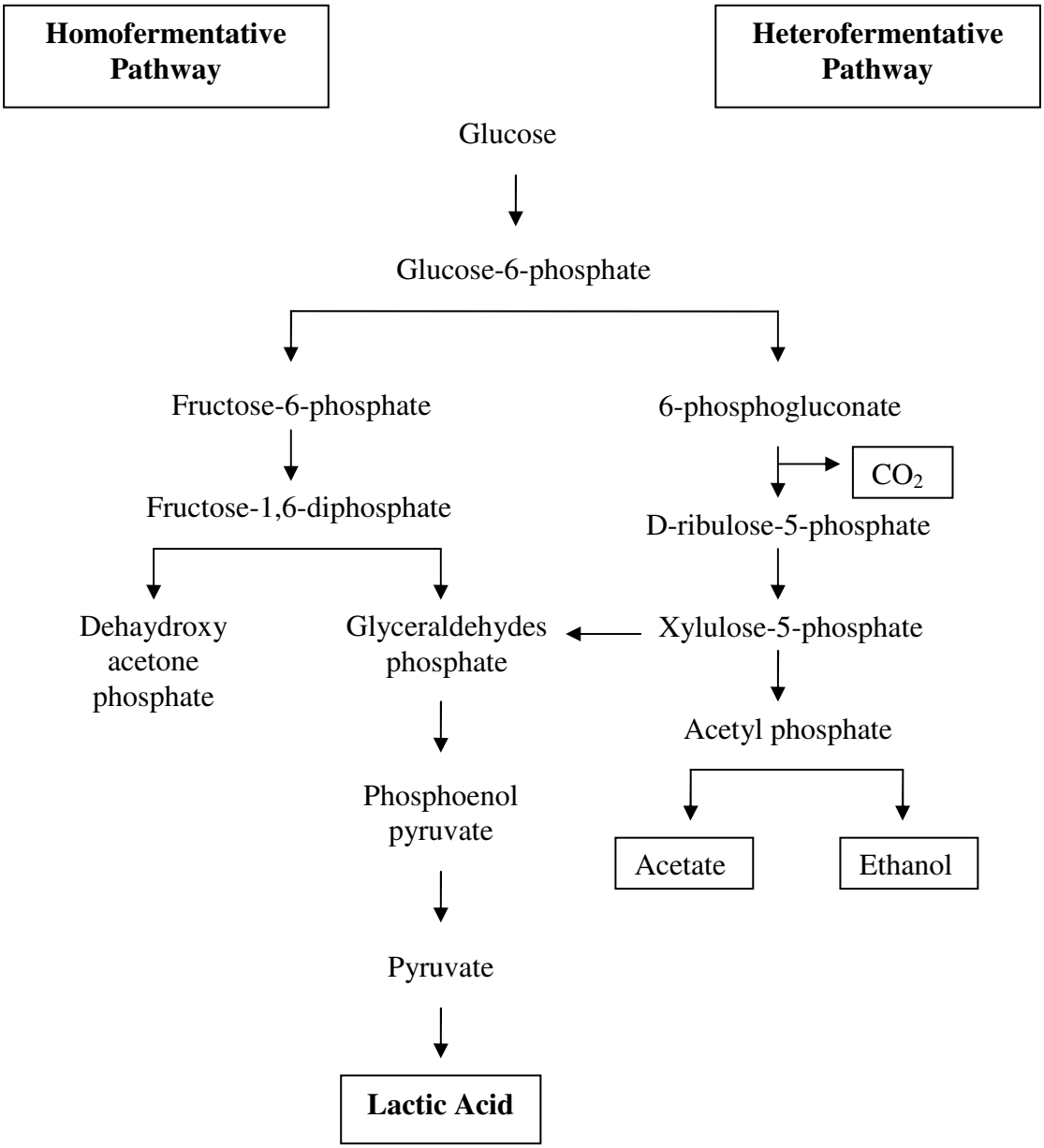

Figure 1.1. Two different pathways for glucose fermentation (Bulut 2003).

1.2.1. Phylogeny of the Lactic Acid Bacteria

The prokaryotes have a simple morphology so phylogenetic relationships can only be deduced by comparative sequence analysis of conserved macromolecules (Shliefer and Kilpper-Bälz 1987). Based on the comparative sequence analysis of 16S rRNA (16S ribosomal ribonucleic acid) genes, bacteria can be divided into 12 major phyla (Figure1.2).

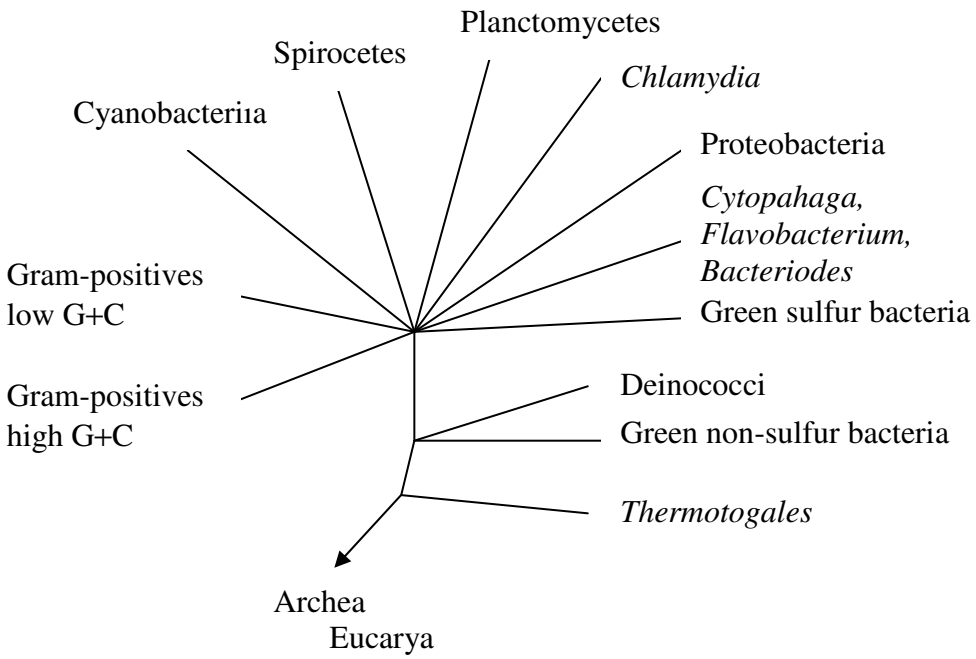

Figure 1.2. Phylogenetic tree of bacteria based upon 16S rRNA sequence comparison (Schlifer and Ludwig 1995).

Lactic acid bacteria constitute phylogenetically related groups of anaerobic Gram-positive bacteria, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (De Vos 1999). Gram-positive bacteria can be further divided into two main groups, the high G+C (guanine plus cytosine) and the low G+C content which refers to the mol % G+C in the DNA. The typical lactic acid bacteria have a G+C content less than %50 and belong to the low G+C content group (Figure 1.3). The G+C content of *Lactococcus lactis* ranges from 34.4 to 36 mol % (Galtier and Lobry 1997; Stiles *et al*. 1997).

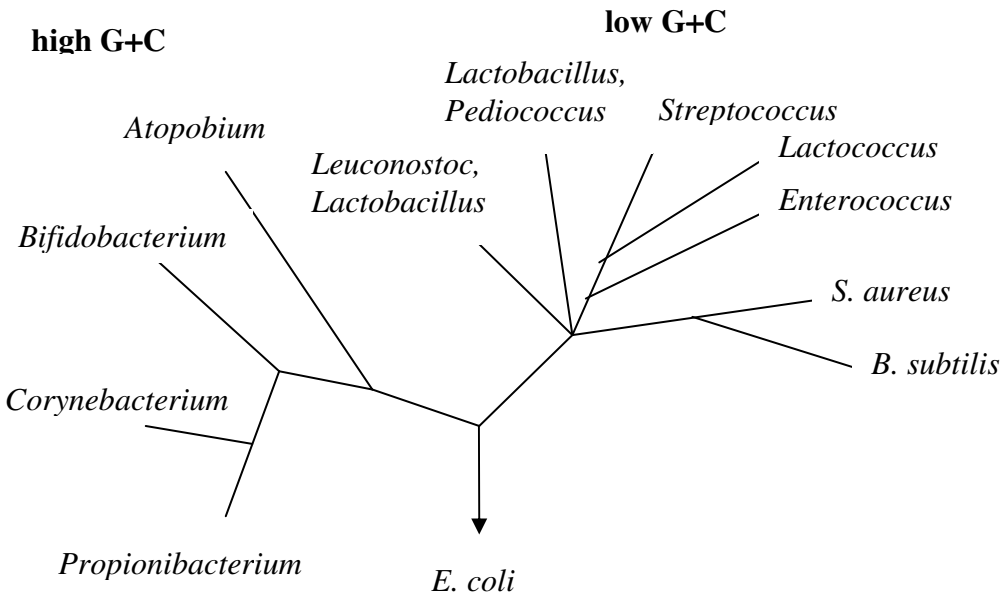

Figure 1.3. Phylogenetic tree of Gram-positive bacteria (Schlifer and Ludwig 1995).

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1.2.2. Lactococci

Most of the Lancefield N group lactic streptococci have been transferred to the genus *Lactococcus* (Schleifer *et al.* 1985). Lactococci are coccoid, Gram-positive homofermentative bacteria which produce L (+) lactic acid from lactose (Figure 1.4.). Lactococci is characterized by spheres of ovoid cells (0.5 μm n diameter) occurring as single cells, in pairs or in chains, and being often elongated in the direction of the chain (Figure 1.5). They do not form endospores and they are non-motile microorganisms. Lactococci do not carry out electron transport phosphorylation, therefore they provide energy only by substrate level phosphorylation. Lactococci grow anaerobically. Unlike many anaerobes, lactic acid bacteria are not sensitive to oxygen. They can grow in the presence of oxygen, thus they are known as aerotolerant anaerobes (Madigan *et al*. 1997).

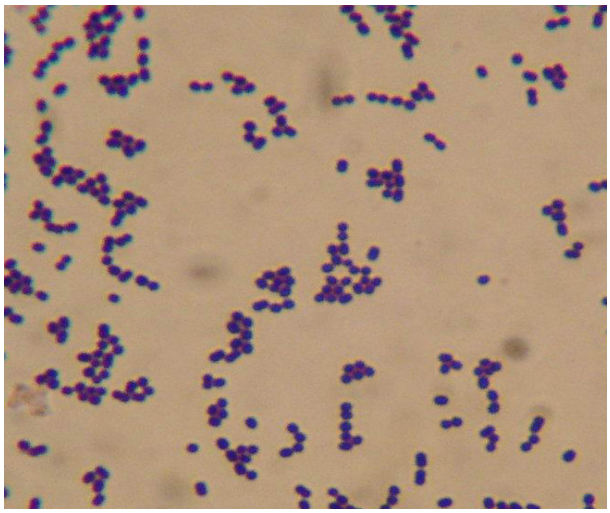

Figure 1.4. *Lactococcus lactis* ssp. *lactis*, isolate C22
(Source : Bulut 2003).

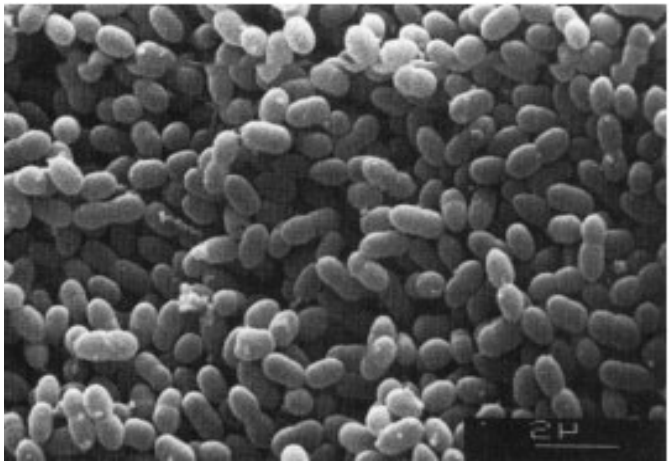

Figure 1.5. Scanning electron micrograph of *Lactococcus lactis* growing in pairs of ovoid cells.
(Source: WEB_1)

Currently, five species are included in *Lactococcus* genus: *Lactococcus lactis*, *Lactococcus garvie*, *Lactococcus plantarum*, *Lactococcus piscium*, and *Lactococcus raffinolactis.* The maximum growth temperatures, sugar fermentation characteristics, the type of peptidoglycan and lipoteichoic acid in the cell wall, and arginine hydrolysis are the key physicochemical parameters to differentiate these species. *Lactococcus lactis* is further divided into two subspecies; *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*.

1.3. Starter Lactic Acid Bacteria

All of the fermented dairy products are produced by the use of microorganisms, including lactic acid bacteria, molds and yeasts. The lactic acid bacteria are the most important ones among these microorganisms and are normally added to the milk as milk grown culture; also called as "Starter Culture". The primary function of starter bacteria is acid production during the fermentation processes (Beresford *et al*. 2001). In industry, starter cultures can be defined as strains which produce sufficient acid to reduce pH of the milk to < 5.3 in 6 h at 30 ^{o}C (Beresford *et al*. 2001). Lactococci are widely used as a starter culture for large scale production of fermented dairy products. For this purpose, *Lactococcus lactis* ssp. *lactis* and *cremoris* are approved as food grade organisms (Liu *et al.* 1996). The nature of fermented dairy products mainly depends on regional factors such as local indigenous microflora, and climatatic conditions. Traditional fermented milk in regions with colder climate contained mesophilic starter bacteria especially lactococci, but the thermophilic starter flora prevailed in regions with much warmer or tropical climates (Savadogo *et al.* 2004).

Thus, the starters used in dairy industry can be divided into three groups (Table1.4). These are mesophilic, thermophilic and artisanal starter cultures (Mayra-Makinen and Bigret 1998).

1.3.1. Mesophilic Starter Cultures

Mesophilic starter cultures can grow at temperatures ranging from 10 to 40 ^{o}C, with the optimum temperature around 30 ^{o}C. They are used for the production of many types of cheese.

Mesophilic starters include *Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, *Leuconostoc lactis* and *Leuconostoc cremoris* (Table 1.4). The subspecies of *Lc. lactis* (*lactis, cremoris,* biovar. *diacetylactis*) are known as acid producers and *Leuconostoc* species are known as flavor producers.

Mesophilic cultures can be classified into four groups based on the flavor production;

- O Type: Contain non-flavor producing starters, *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris.*

- D Type: Contain citrate fermenting bacteria such as, *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* as a flavor producer.

- B or L Type: Only contain *Leuconostoc* species which also ferment citrate as flavor.

- BD or LD Type: Contain both aroma-forming species as *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc* species (Mayra-Makinen and Bigret 1998).

A combination of single or multiple strains of mesophilic starter cultures can be used for cheese production. The acid producers are the dominant microorganisms in mixed cultures and comprise 90-99 % of the flora and flavor producers comprise the remaining 1-10 %.

Table 1.4. Starter microflora of fermented milks (Cogan 1996).

Producer(s)	**Product(s)**
Mesophilic Starters	
Lactococcus lactis ssp. *lactis*	Butter milk, Cottage cheese
Lactococcus lactis ssp. *cremoris*	Butter milk, Cottage cheese
Lactococcus lactis ssp. *lactis* biovar. *diacetylactis*	Butter milk, Cottage cheese
Leuconostoc mesenteroides ssp. *lactis*	Butter milk, Cottage cheese
Leuconostoc cremoris	Butter milk, Cottage cheese
Thermophilic Starters	
Streptococcus thermophilus	Yoghurt
Lactobacillus delbrueckii ssp. *bulgaricus*	Yoghurt
Lactobacillus delbrueckii ssp. *lactis*	Yoghurt
Lactobacillus acidophilus	Health products
Lactobacillus casei	Health products

1.3.2. Thermophilic Starter Cultures

Thermophilic starters contain two genera, *Lactobacillus* and *Streptococcus* (Table 1.4) and they grow optimally at 45 ^{o}C. The genus *Lactobacillus* are a large group containing both homofermentative and heterofermentative species but only a few of them are involved in milk fermentation (Marshall 1987). *Lactobacillus delbrueckii* ssp. *lactis*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* are often used as starter lactobacilli in dairy industry (Table 1.4).

Streptococcus thermophilus is the only food associated streptococci and used in combination with lactobacilli. There is a symbiotic relationship between *Lactobacillus bulgaricus* and *Sreptococcus thermophilus.*

1.3.3. Artisanal Starter Cultures

Artisanal or natural starter cultures are undefined mixtures of lactic acid bacteria. They are often derived directly from the previous batch of the fermented product. These cultures are produced each day and they also relly on the selective pressure or competetion of the original flora.

Artisanal starter culture composition is very complex, and variable. They are classified according to the raw materials, and the incubation temperatures. On the basis of the raw materials, they can be differentiated as milk, whey and whey with rennet cultures. They can also be classified as thermophilic and mesophilic cultures according to the incubation temperature.

1.4. Starter Functions

Starter cultures have a great importance on the production of cheese. Lactococci can influence the final organoleptic qualities of the product. The most important function is production of lactic acid from carbohydrates that are resulting in pH decrease. The other important properties of lactoccci are protein hydrolysis, flavor compound synthesis and inhibitory substances production.

Thus, lactic acid production, proteolytic activity, bacteriocin, and exopolysaccharide production are the major attributes of starter bacteria. The ability of

rapid acid production is probably the most important property among all these activities. It reduces the pH which in turn increases the expulsion of whey from the curd and the moisture content. Acid production also initiates desirable reactions and changes such as curd and texture formation, aroma and flavor production (Mayra-Makinen and Bigret 1998).

Milk is deficient in many of the amino acids essential for the growth of starter bacteria. These bacteria have a proteolytic system to hydrolyze the milk proteins to their amino acids and peptides required for growth. Proteolysis is also important for the development of cheese flavor (Cogan *et al*. 1997).

1.4.1. Acid Production

Two different sugar transport systems across the cell membrane have been found in lactococci during fermentation. There are two important systems; the permease and the phosphoenol pyruvate-phosphotransferase (PEP-PTS) system. Generally permease system is found in *Leuconostoc* and thermophilic species. PEP-PTS system is found in lactococci. In this system, lactose is transported into the cell and is phosphorylated to lactose phosphate, then hydrolyzed to glucose and galactose-6-phospate by phospho-β-galactosidase (P-β-gal). It is hydrolyzed to glucose and galactose by β-galactosidase (β-gal) inside the cell. Lactose can also be transported into the cell by a permease system and this system requires energy.

1.4.2. Carbohydrate Metabolism

After transportation, sugar molecules can be metabolized by different pathways (Figure 1.6). The lactose-6P is hydrolyzed by a phospho-β-galactosidase into glucose and galactose-6P. Then, glucose is catabolized through EMP pathway. Galactose-6P is metabolized by D-tagatose-6P pathway. Pyruvate is the key compound of the carbohydrate metabolism of lactococci. During glycolysis, under aerobic conditions pyruvate is reduced to lactate in order to regenerate NAD^+ which serves as an electron acceptor for the substrate level phosphorylation.

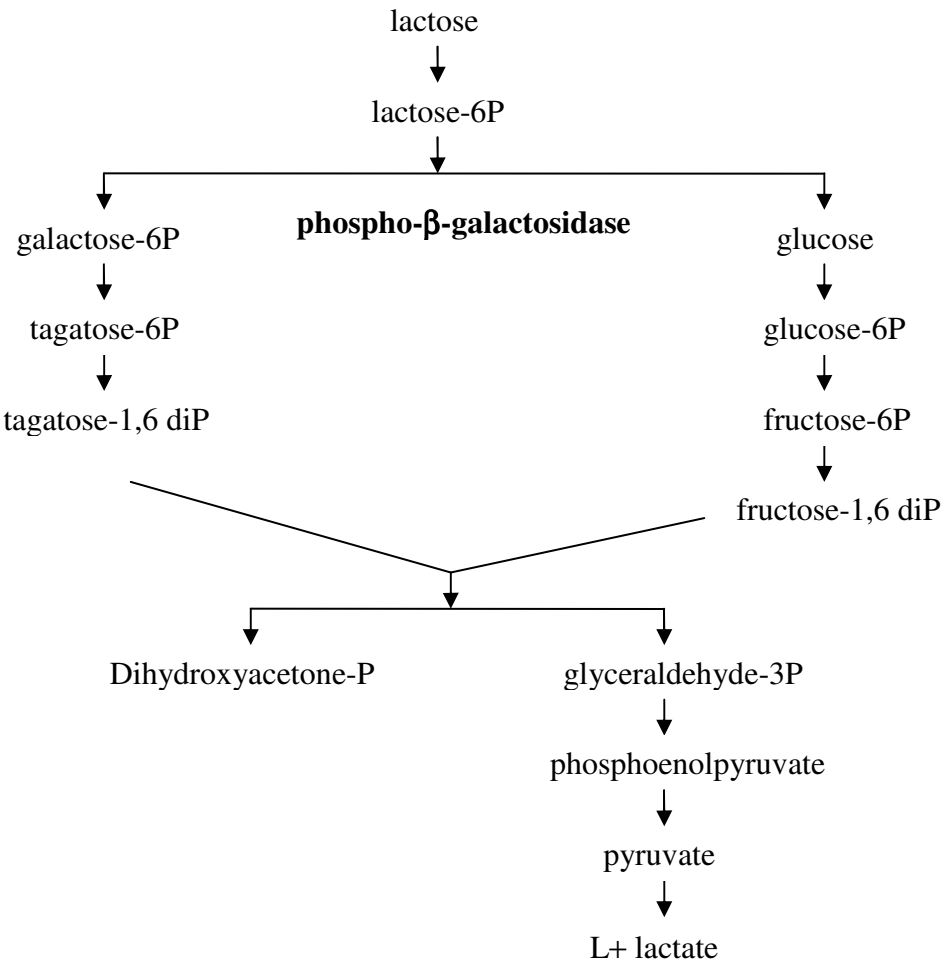

Figure 1.6. Pathway of lactose utilization by *Lactococcus lactis* strains (Teuber 1995).

1.4.3. Proteolytic Activity

LAB metabolize polypeptides by their milk-clotting enzymes. However, cell wall proteases are used by LAB for the casein degradation. Thus, both proteinases and peptidases provide the cell peptides and free amino acids in combination.

Starter strains hydrolyze all the milk proteins including whey proteins. The first step is the casein degradation and is performed by the cell surface enzymes of lactococci. A proteinase is located on the cell surface, associated with the cell envelope (Cogan 1996).

Milk clotting enzymes and proteinases initiate the casein degradation and produce large peptides. These peptides are then hydrolyzed with peptidases to smaller

peptides and amino acids. There are a variety of peptidases such as aminopeptidase, dipeptidases and tripeptidases.

1.4.4. Aroma Production

Aroma and flavor production properties of the starter cultures affect quality of the final product. The flavor compounds produced by lactococci are divided in two groups. These are aroma compounds in fermented milk and matured cheese.

The former includes mainly organic acids such as lactic and acetic acids produced by *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*. The latter group includes acetaldehyde, diacetyl, acetoin and 2-3 butylene-glycols from the citrate of milk. These are mainly produced by *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc* species. These functions are technologically important in the fermentation of milk. (Axelson 1998). Diacetyl is known as butter aroma compound (Mayra-Makinen and Bigret 1998).

Determination of the aromatic compound production by lactococci is very difficult due to indirect role in aroma development because lactococci produce some important flavor compounds at nano-gram concentrations.

1.4.5. Exopolysaccharide Formation

Most of the Gram-positive bacteria, including lactic acid bacteria, produce exopolysaccharides (EPS). EPS are either excreted into the growth medium (slime) or attached to the bacterial cell wall (capsules) (Zambou *et al*. 2004). They are long-chain, high molecular carbohydrate polymers (De Vuyst and Degeest 1999). In dairy industry, EPS producing cultures provide the fermentation produced with viscosity, stability and water binding functions (Smitinont *et al*. 1999). EPS from lactic acid bacteria can be divided into two categories (De Vuyst and Degeest 1999, Marshall *et al*. 2001); homopolysaccharides and heteropolysaccharides. Homopolysaccarides are generally produced by non-starter lactic acid bacteria and consist of four subgroups; α-D- glucans, β-D- glucans, fructans and polygalactans**.** Heteropolysaccharides are produced by mesophilic (*Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lb. casei*, *Lb. sake*, etc.) and

thermophilic (*Lb. acidophilus*, *Lb. bulgaricus*, *Lb. helveticus*, *S. thermophilus*) lactic acid bacteria.

Chemical composition of the EPS is strain dependent. The EPS from lactic acid bacteria are generally neutral heteropolysaccharides that are composed of galactose, glucose, rhamnose and mannose polymers (Petry *et al*. 2003).

1.4.6. Antimicrobial Activity

Lactic acid bacteria can produce antimicrobial substances with the capacity to inhibit the pathogenic and food spoiling microorganisms. Organic acids, hydrogen peroxide, diacetyl, and bacteriocins are included among these compounds (Herreros *et al*. 2005). These antimicrobial activities can improve the quality of fermented foods (O'Sullivan *et al*. 2002).

Lactic acid is produced by homofermentative process. On the other hand, equimolar amounts of lactic acid, acetic acid/ethanol, and carbondioxide are produced heterofermentatively. Organic acids are good natural preservatives (Hansen 2002). The weak organic acids have much better antimicrobial activity than low pH and neutral pH. Acetic acid is a much stronger inhibitor than lactic acid and it inhibits the growth of yeasts, molds and other bacteria.

Lactic acid bacteria are able to produce hydrogen peroxide (H_2O_2) through the action of flavoprotein-containing oxidase, superoxide dismutase, and NADH oxidases, in the presence of oxygen. H_2O_2 has a strong bactericidal effect since sulfhydryl groups of cell proteins and cell membrane lipids can be oxidized by H_2O_2.

Heterofermentative lactic acid bacteria produce carbondioxide (CO_2). The effect of CO_2 is not known, but it probably inhibits the enzymatic decarboxylation. High concentrations of CO_2 can inhibit the growth of some microorganisms, whereas low concentration of CO_2 can stimulate the growth (Mayra-Makinen and Bigret 1998).

Diacetyl is generally produced by *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* species. Because hexose metabolism represses diacetyl production. However, if citrate is metabolized, diacetyl can be produced. Diacetyl is more active against Gram-negative bacteria, yeasts, and molds than against Gram-positive bacteria. A relatively large amount is needed for inhibition (Mayra-Makinen and Bigret 1998).

Diacetyl affects arginine utilization in Gram-negative bacteria by inhibiting the activity of arginine-binding protein.

1.4.6.1. Bacteriocins

Bacteriocins are often small proteins and affecting unrelated or closely related microorganisms. According to the aminoacid composition and structural elements, bacteriocins are classified into four groups (Abee *et al*. 1995);

- Low-molecular weight lantibiotics (<5 kDa): They contain lanthoinine residues such as nisin and lacticin 481
- Low-molecular weight peptides (<10 kDa): They do not contain lanthoinine such as diplococcin, lactococcin A, lacticin F.
- Large heat-labile proteins (>30 kDa): Helviticin J and caseicin 80.
- Complex bacteriocins: They are composed of protein and one or more chemical moieties (carbohydrate, lipid) such as plantaricinS, lactocin 27.

Nisin is the first characterized bacteriocin of lactic acid bacteria. Also, it is the most extensively described one and is produced by *Lactococcus lactis* ssp. *lactis* (Harris *et al*. 1992). It is the prototype of lantibiotics that is used commercially in the food industry. Its use is now permitted as a food additive in at least 47 countries. It particularly inhibits *Clostridium* species (*botulinum* and *tyrobutyricum*). The host range of nisin includes most Gram-positive bacteria such as some staphylococci, listeria, and *Mycobacterium tuberculosis*. This bacteriocin breaks down the electrochemical potential of bacterial membranes (Teuber 1995).

Lactococcin is a small heat stable non-lanthonine bacteriocin mainly produced by *Lactococcus lactis* ssp. *cremoris*. There are three different types of lactococcins, A, B, and M. All types of lactococcins are coded by plasmid DNA. Their host range is rather narrow affecting only lactococcal strains. Their functions are on the cytoplasmic membranes of susceptible organisms (Teuber 1995).

1.5. Genetics of Lactic Acid Bacteria

DNA is normally organized within the bacterial cell in the form of a single large molecule called chromosome. It carries all the genes required for the production of enzymes and other proteins essential for cell survival. However, most of the bacteria possess small circular DNA molecules carrying some other genes. These molecules are called plasmid DNA. Plasmids are relatively much more unstable molecules than the bacterial chromosome.

The most important biosynthetic and catabolic pathways in lactic acid bacteria are chromosomally encoded. However, other properties such as carbohydrate metabolism and proteolytic activities may involve both plasmid-linked and chromosomal genes. For example, some of the the lactococcal proteinases are plasmid encoded. The key enzyme of lactose metabolism in lactococci is encoded by plasmid DNA but the pathway from glucose to lactate is directed by chromosomal DNA.

1.5.1. The *Lactococcus lactis* Chromosome

The development of DNA based techniques such as pulsed field gel electrophoresis (PFGE), has enabled the investigation and elucidation of genome size and organization of the chromosome of *Lactococcus lactis*. Chromosome size of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* varies between 2.0 and 2.7 Mb. The first complete genome of the lactic acid bacteria group has been published for *Lactococcus lactis* ssp. *lactis* IL1403. It has been sized to be 2.365.589 bp and encode approximately 2310 proteins (Bolotin *et al*, 2001; Klaenhammer *et al*. 2002). The genome size of the *Lactococcus lactis* is one of the smallest within Eubacteria. *Sma*I and *Apa*I endonucleases which cleave the DNA at G+C rich sequences are generally used for mapping of *Lactococcus lactis* genome.

1.5.2. The Plasmid Biology of Lactic Acid Bacteria

Plasmid biology has become an important area of investigation in dairy starter cultures since it now appears that some properties, vital for successful milk fermentation, are coded by genes residing in plasmid DNA (Ward *et al*. 2004). The

following functions are plasmid-linked; Plasmid-linked function are as follows; fermentation of lactose, sucrose, galactose, mannose, xylose, and even glucose, proteinase activity, phage resistance, bacteriocin production, citrate utilization, exopolysaccharide production, DNA restriction and modification.

1.5.2.1. The Plasmids of *Lactococcus lactis*

Lactococcus lactis might include a number of plasmids. In different strains, for example, between one and twelve plasmids of different sizes (ranging in size from 2 to over 80 kbp) can be found (Sanchez and Mayo, 2004). There are two important findings about lactococcal plasmids. Many of the functions encoded on plasmids and they are related to growth in milk. Most strains could exchange plasmids by natural conjugation or transduction. The following factors identified as plasmid encoded properties in *Lactococcus lactis* (McKay 1983, Sanchez and Mayo 2004);

- Lactose transport and metabolism
- Casein degradation by cell wall protease
- Citrate and oligopeptide transport from outside the cell
- Bacteriophage resistance
- Exopolysaccharides formation
- Antibiotic resistance

Among them, the most technologically important properties are those involved in lactose metabolism and proteinase activity. Lactose is used as a source of energy to grow rapidly in milk. Furthermore, *Lactococcus lactis* must digest the milk proteins to provide its essential aminoacids (Gasson and Davies, 1980). The *lac* genes for the lactose specific enzymes of the PTS, the phospho-β-galactosidase and the enzymes of the tagatose 6-phosphate pathway are found on several indigenous plasmids (Figure 1.6) (Teuber 1995). In the lactococci, there are two proteolytic sytems, PI and PIII. The proteinase enzyme is encoded by a single gene on the plasmid DNA (Cogan 1996).

In dairy industry, the other important function is aroma production and the diacetyl is an important aroma compound which is produced from citrate by *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*. The key enzyme in this pathway is citrate permease and it is also encoded by plasmid DNA (Cogan 1996). Besides bacteriocin production, resistance to ultraviolet irradiation is also provided by plasmids

(Wrigth and Sibakov 1998). Three basic phage resistance mechanisms have been identified in lactococci: inhibition of phage adsorption, restriction modification system and intracellular inhibition of phage development (Hansen 2002). All three mechanisms are encoded by plasmid DNA (Cogan 1996).

Isolation and characterization of these plasmids have provided a good opportunity to improve the performance and stability of cheese starter cultures (Lee and Moon 2003).

1.6. Molecular Characterization Methods for Lactic Acid Bacteria

The classical approach to bacterial taxonomy is mainly based on morphological and physiological properties. Various revisions and improvements have been made by additional physiological and chemotaxonomical data such as major fermentation pathways, carbohydrate patterns, configuration of lactic acid produced or analysis of peptidoglycan. But due to their phenotypic nature, their taxonomic usefulness is limited in the discrimination of closely related organisms. The uncertainties are raised by their variability and instability of certain phenotypic characters and culturing conditions (Ehrmann and Vogel 2005). Thus, phenotypic methods alone have been repeatedly shown to be insufficient (Fortina *et* al. 2003; Delgado and Mayo 2004).

Molecular characterization methods involve the analysis of genetic materials which include both chromosomal and plasmid DNA (Farber 1996). Molecular classification methods have resulted in dramatic changes in the taxonomy of lactic acid bacteria (Stiles and Holzapfel 1997). These techniques generally allow a better understanding of the genetic relationships of bacteria (Stiles and Holzapfel 1997). They are much more adventegous with reproducibility, typeability, discriminatory power, and ease of interpretation and performance (Farber 1996).

The first-generation of DNA-based typing methods have been whole-genome restriction fragment analysis and plasmid DNA analysis (Vadamme *et al*. 1996). These include the analyses of fragment length polymorphisims (REA, RFLP, Ribotyping), pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and culture independent strategies [Denaturating gel gradient electrophoresis (DGGE), length heterogenity-PCR (LH-PCR), terminal-restriction fragment length

polymorphism (T-RFLP)], and plasmid profiling (Farber 1996; Olive and Bean 1999; Coppola *et al*. 2001; Lazzi *et al*. 2004; Ehrmann and Vogel 2005).

1.6.1. Pulsed Field Gel Electrophoresis (PFGE)

PFGE technique has been known to be an accurate and reproducible molecular typing method for closely related bacterial strains at the species level (Tanskanen *et al*. 1990; Tenover *et al*. 1995).

Standard gel electrophoresis can resolve DNA fragments up to 75 kb. Genomic DNA analysis on the other hand often requires resolution of megabasepairs fragments. DNA migrating through a gel must find pores large enough to pass through. The principle of the PFGE separations as follows: When the first electrical field is applied to the gel, DNA molecules elongate in the direction of the field and begin to migrate in the gel. After the first electric field is removed the second field at some angle to the first field is activated. The DNA must change confirmation and reorient before it can migrate in the direction of the second electric field.

PFGE nomenclature remains confusing because of the large number of acronyms used. The original term pulsed field gradient gel electrophoresis (PFGE) was used by Schwartz and Cantor (1984) to any gel which has been using alternating multiple electric field (Birren and Lai 1993). The PFG abbreviation is taken to mean pulsed field gel, not pulsed field gradient. There are some different PFGE systems (Table 1.5). These often involve some variations in original electrode geometry, homogeneity, reorientation of the electric field. These different systems however rely on the same phenomenon for separation.

Table 1.5. Pulsed field gel acronyms (Brain and Lei 1993).

Acronym	**Electrophoresis System**
PFGE	Pulsed Field Gradient Gel Electrophoresis
FIGE	Field Inversion Gel Electrophoresis
TAFE	Transverse Alternating Field Electrophoresis
CHEF	Contour Clamped Homogenous Electric Field
OFAGE	Orthogonal Field Alternation Gel Electrophoresis

FIGE is a special case of PFGE, in which the fields are oriented at 180^{o} and directly opposing each other. FIGE is easier to use than PFGE, since it can be carried out in a standard horizontal gel apparatus. Its range of size resolution is more limited: up to 2Mb as compared to 5Mb in PFGE.

A vertically oriented gel apparatus is used for TAFE system and allows the limitation in size of gel. CHEF system is the most commonly used PFGE system. CHEF system provides highly uniform, homogenous electric fields within the gel and it generates a 120^{o} reorientation angle for separating DNA molecules ranging from 100 kb to 6 Mb in size (Bio-Rad Manual 2001). The principle of CHEF system is the homogeneous electric field. Twenty-four electrodes are used and the voltage from the power supply is divided between these electrodes so the voltage gradient is constant across the gel. These electrodes have been arranged in a hexagonal array and clamped. The main advantage of this system is its ability to separate a large number of different DNA molecules in size, along a straight lane.

PFGE gels are run in a constantly changing electric field. Current protocols generally employ fields at 120^{o} angles. In all cases, the effect is to force the DNA to continuously re-configure itself to migrate in a new direction. Larger molecules take longer to reorient themselves and therefore make less overall progress through the gel.

Before PFGE, bacterial cells are embedded into agarose plugs because conventional techniques for genomic DNA isolation and purification often shear the DNA molecule. The embedded cells are lysed within the agarose plugs by a suitable lytic agent such as lysozyme. Contaminating cellular proteins and other cell debris are then digested with proteinase K. After the protein digestion, the plugs are treated by PMSF (phenylmethylsulfonnyl fluoride) to remove residual proteinase K activity. Before the restriction enzyme digestion, the plugs are washed several times with Tris-EDTA buffer to eliminate compounds that might inactivate the specific endonuclease activity. For the restriction enzyme digestion, rare cutter enzymes which recognize 6 bp are used. The plugs are then inserted into the PFGE grade agarose gel and then subjected to the electrophoresis. After the electrophoresis, genomic DNA fragments are visualized with ethidium bromide (Figure 1.7).

Pulsed field gel electrophoresis is sensitive to changes in many electrophoretic parameters such as voltage gradient, agarose concentration, running temperature, buffer type and electrophoresis time.

Switch interval is the amount of time during which each of the alternating electric field is active. Switch interval is also referred to as switch time or pulse time. As stated above each time the field is switched the DNA molecules must change its direction or reorientate itself in the gel matrix. Thus, larger DNA molecules require longer switch times to reorient with each type of electrical field.

A voltage gradient is the difference between the electrical potential of the electrodes in a gel box. This difference represents the force that runs DNA molecules through the gel. The voltage gradient is not only affect the migration rate, but also affect the size of the separation. Although, high voltage can reduce the resolution capacity of the system, DNA migration will increase by increasing the voltage gradient. Voltage gradient and switch interval together affect the resolution of a gel. Running at lower voltage gradient requires longer switch interval to produce comparable resolutions (Birren and Lai 1993).

Pulsed field agarose is used for separation of larger DNAs because it reduces run times. For plug preparation, low melting gel agarose is used because its pore size smaller than regular agarose types and it also reduces gel strength. The agarose concentration affects the speed of separation and the size of fragments resolved. Agarose concentration of 1% is optimum concentration for separating DNA molecules up to 3 Mb. When the agarose concentration is decreased, migration rate of the DNA molecule is increased, a larger size range of DNA can be separated but sharpness of DNA bands is decreased. Gel concentration below 1% (0.5-0.9%) are useful in separation of high molecular weight DNA (>3 Mb). 1.2-1.5% agarose concentrations are used for improved band tightness, however, this agarose concentration increase the run times.

The mobility of the DNA molecule is also sensitive to changes in the temperature of electrophoresis buffer. When the buffer temperature increases, DNA molecule migration will increase but the band sharpness and resolution will decrease. The temperatures ranging from 4ºC to room temperature are normally used but the optimum temperature is 12-15 ºC. A 14ºC running temperature is commonly used and this temperature provides compromise between speed and resolution. When the running temperature is reduced, longer switch intervals are needed for similar resolutions.

Buffer concentration and type also affect the mobility and resolution. In the lower ionic strength buffer, DNA migrates quickly. The buffering capacity of the

diluted buffers is reduced. For example, fragments run faster in 0.5×TBE than in 1×TAE.

Pulsed field gels do not contain ethidium bromide during the electrophoresis for several reasons. Intercalation of ethidium bromide slows DNA migration through the gel and also changes the pulse condition for separation.

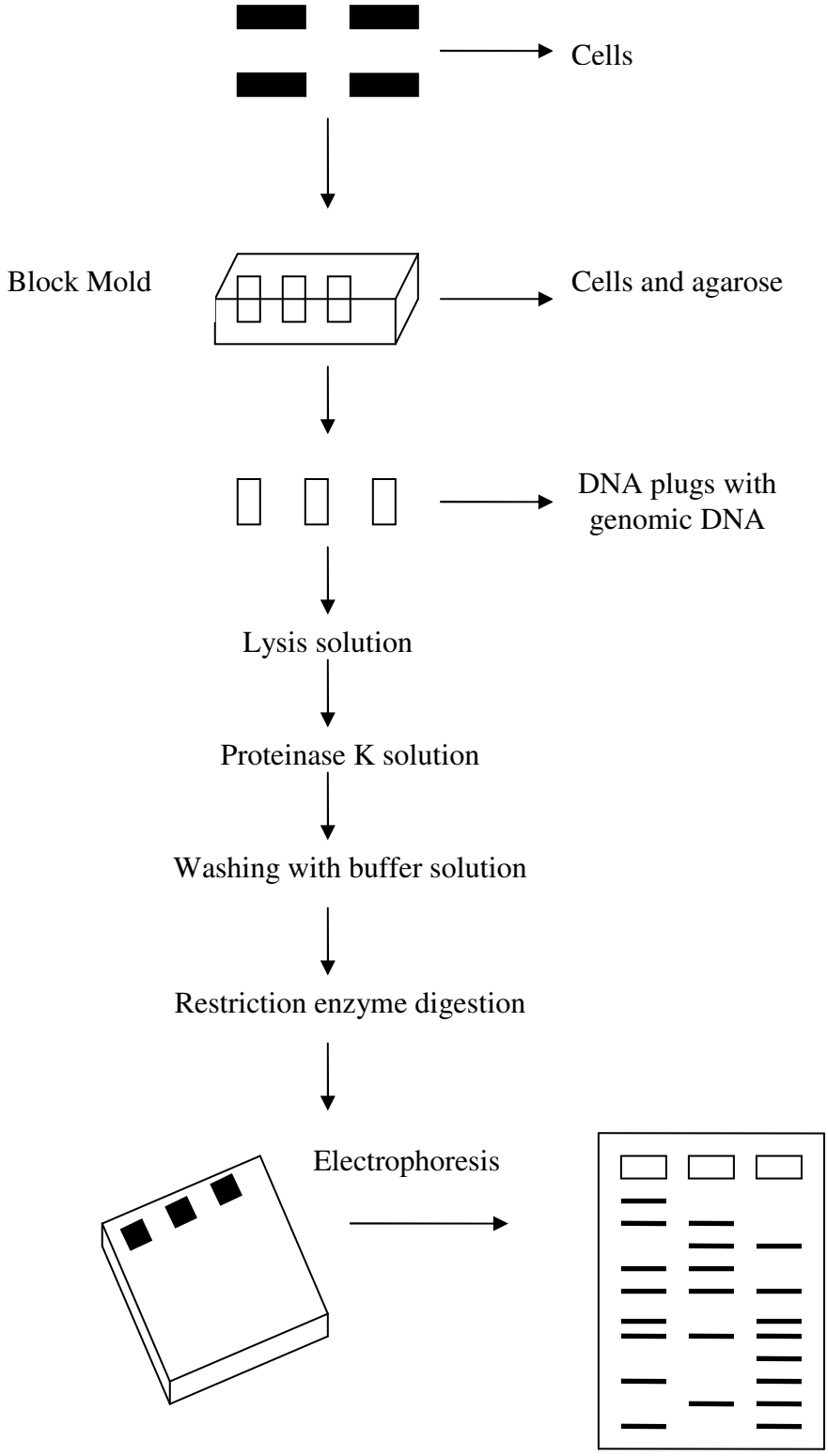

Figure 1.7. Schematic illustration of PFGE (Farber 1996).

1.6.2. Plasmid Profiling

Plasmids are typically small genetic elements that exist and replication the cell independent of the chromosome. They are often double-stranded DNA molecules. Many prokaryotes contain one or more plasmids in addition to their chromosome.

Although plasmids encode defined properties, lactococci often contain plasmids with no known function. These plasmids are named as cryptic plasmids and they have been used as the basis of cloning systems for analyzing non-lactococcal genes (Cogan 1996).

In plasmid typing method, first plasmids are isolated from strains and these plasmids are then digested with a specific restriction enzyme. The restriction fragments are separated in an agarose gel by electrophoresis. In order to determine different plasmid contents, fragments are analyzed using a suitable computer program.

Plasmid profiling is an excellent tool for strain characterization and identification and for the characterization of starter culture composition (Mayra-Makinen and Biget 1995). Typing based on plasmid analysis is also particularly useful for preliminary screening if it is used in conjunction with other techniques (Mannu *et al*. 2002; Mannu and Paba 2002). Although this method has many advantages, plasmids are unstable compared with the chromosome, and therefore the encoded characteristics can be lost on repeated subculture (Cogan 1996).

1.6.2.1. Plasmid Instability

The stability of plasmids in the cells is an important industrial property for starter cultures (Beal *et al*. 1998). Plasmid DNA stability is also very important in developing genetically improved starter strains (Lee and Moon 2003). The correlation between plasmids and technologically important characteristics is usually confirmed in a series of studies involving plasmid loss or curing procedures (Cogan 1996). Stability of a plasmid also depends on environmental conditions (Beal *et al*. 1998; Lee and Moon 2003) and they can be lost by growth without lactose, at high sub-lethal temperatures, by continuous sub-culturing, and by treatment with some mutagenic chemicals such as ethidium bromide. Such conditions are often strain dependent (Teuber 1995; Lee and Moon 2003). Plasmid instability can be studied as follows: initially, variants that had

lost key metabolic traits are identified and plasmid profile of parent and variants are compared. For example, a parental strain that is lactose positive will be compared with a number of strains that have lost this ability (lactose negative). If this loss is always accompanied by the absence of a particular plasmid, it will be taken as the presumptive evidence that genes involved could be located on their plasmid.

1.6.3. DNA Sequencing

DNA sequencing is the gold standard for bacterial typing. There are two different procedures for sequencing: the Maxim and Gilbert method and the Sanger dideoxy method. Both of the methods generate DNA fragments that end at each of the four bases. These fragments are then subjected to gel electrophoresis and molecules with one nucleotide difference in length are then separated on the gel. The Maxim and Gilbert method uses hydroxyl-radicals that break the DNA preferentially at some of the four nucleotides. In the Sanger dideoxy method, the sequence is determined by making a copy of the single stranded DNA, using the enzyme DNA polymerase.

DNA sequencing generally begins with PCR amplification of a DNA region of interest, and followed by sequencing reactions with the PCR products (Olive and Bean, 1999). The four fluorescent dyes represent one of the four nucleotides which make up the DNA molecule and four separate annealing and extension reaction must be performed for each sample to be analyzed. After the sequencing reactions are completed, the four sets of reaction products are loaded separately the wells on a polyacrylamide gel. During the electrophoresis, these fluorescently labeled products are excited by an argon laser and detected automatically.

Whole genome sequencing represents the most powerful approach to the identification of genomic diversity among closely related strains (Fraser *et al*. 2002). One method is the sequencing of the entire genome, called map-directed sequencing. This method consists of producing an ordered library of overlapping or contiguous molecular clones. These clones are mapped using restriction enzymes and are then sequenced. Constructing restriction maps and ordering the clones in a gene library can be laborious and time consuming. However, once an ordered library is available, sequencing becomes a straightforward process. Shotgun sequencing is another method. This method involves shotgun cloning the entire genome and the sequencing the clones

randomly and made by automated sequencing. The clones are sequenced without knowing the order or orientation of the cloned DNA. The sequences obtained will then be alligned by computer program.

1.7. Thesis Objective

Lactic acid bacteria are essential for the manufacture of fermented dairy products. Studies on the biochemistry, physiology and genetics of these microorganisms over the last century have contributed considerably to the improvement of fermentation processes and have resulted in better and safer products. Due to the industrial interest, metabolic engineering of lactic acid bacteria has been carried out mainly with *Lactococcus lactis*. The diversity of the industrially used starter *Lactococcus lactis* is very limited. Therefore there is a considerable demand for novel starters with technologically desirable properties. Therefore, the purpose of this study was to investigate intraspecies-biodiversity of *Lactococcus lactis* ssp. *lactis* artisanal starters. Chromosome profiles of starter strains and three reference strains of comlek peyniri obtained from oue previous study (Bulut 2003), *Lactococcus lactis* ssp. *lactis* (CECT 4432), *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* (CECT 4431) and *Lactococcus lactis* ssp. *cremoris* (NRRL B 632), were studied by pulsed field gel electrophoresis (PFGE) in order to see if there is a similarity between the chromosomal DNA pattern of starter strains and reference strains. These isolates and reference strains were also differentiated by plasmid profiling. In addition plasmid profiles of starter strains and refernce strains investigated to detect if there is a diversity among the strains based on plasmid content.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemicals used in the study were listed in Appendix A.

2.1.2. Media

Media used in this study were listed in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions were presented in Appendix C.

2.2. Methods

2.2.1. Isolates

In this study, 54 *Lactococcus lactis* ssp. *lactis* strains which have been isolated from Cappadocia, and identified as good starter candidates (Bulut *et al*, 2005) were analysed by molecular techniques. Moreover, *Lactococcus lactis* ssp. *lactis* strains were investigated for technological characteristics and 30 of the isolates which isolated from one cheese sample from Sulusaray of Nevsehir were also investigated by plasmid stability.

2.2.2. Reference Strains

Reference strains used in this study were obtained from Collecion Espanola de Cultivos Tipo (CECT) and Agricultural Research Service Culture Collection (NRRL).

1. *Lactococcus lactis* subsp. *lactis* CECT 4432
2. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431
3. *Lactococcus lactis* subsp. *cremoris* NRRL-B 634

2.3. Genotypic Characterization

2.3.1. Partial DNA Sequencing on 16S rRNA Gene

2.3.1.1. Isolation of Genomic DNA

Genomic DNA was isolated by using the following procedure (Cardinal *et al,* 1997) with some minor modifications. Ten ml of overnight cultures were grown in MRS (De Man *et al*, 1960) broth medium. Bacterial cells were harvested in a microcentrifuge for 5 min at 8.000 rpm. They were then suspended in 200 µl of 1× TE buffer (pH 8.0) containing 25% sucrose and 30 mg/ml lysozyme. The cell suspensions were incubated for 1 h at 37 ºC. After the incubation, 370 µl of 1× TE (pH 8.0) containing 1mg/ml Proteinase K and 30 µl 10% SDS were added. The samples were then incubated for 1 h at 37 °C. Cells were lysed by the addition of 100 µl 5M NaCl and 80 µl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7 M NaCl), respectively. Lysed samples were incubated for 10 min at 65 °C. Chloroform extraction was performed twice using one sample volume of chloroform (chloroform/isoamyl alcohol: 24/1). First, one equal volume of chloroform/isoamyl alcohol was added and the samples were centrifuged for 5 min at 8,000 rpm. The aqueous phase was transferred into a sterile eppendorf tube and the genomic DNA was precipitated by the addition of isopropanol (one sample volume). After that, precipitated DNA was transferred into a fresh eppendorf tube which contained 500 µl of 70% ethanol and washed. When DNA precipitate was not visible, isopropanol containing samples were centrifuged for 10 min at 8,000 rpm to pellet genomic DNA. After washing, DNA was

pelleted by centrifugation for 10 min at 8,000 rpm. Ethanol was removed and the pellets were dried for 10-20 min at 37 °C. Dried pellets were dissolved in 100 µl 1× TE containing 100 µg/ml RNase. After incubation for 1h at 37°C, the sample volume was adjusted to 400 µl with 1× TE. DNA was dissolved by alternating cold-heat shock (for 10 min at 80°C and for 20 min at -20°C as twice). DNA was further purified by phenol/chloroform extraction. One volume of phenol was added, and mixed well. After the centrifugation for 5 min at 8000 rpm, the aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform/isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 8,000 rpm. After that, DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 8000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. Finally, according to pellet size, it was dissolved in 50 µl, 100 µl or 150 µl 1× TE. Alternating cold-heat shock (for 20 min at 80°C and 20 min at -20°C) was performed in order to dissolve the samples. Dissolved genomic DNA samples were stored at -20°C.

2.3.1.2. Amplification of 16S rRNA Gene

16S rDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers;

EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3' (Jensen *et al,* 1993).

EGE2: 5'-CTACGGCTACCTTGTTACGA-3' (Jensen *et al,* 1993).

PCR amplifications were performed in 50 µl final reaction volumes containing 500 ng of genomic DNA as the template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 picomoles each of the primers, 1× PCR buffer (Fermentas) and 1.25 U *Taq* DNA polymerase (Fermentas). Forty-eight µl of the PCR mixture were distributed into the tubes. All the manipulations for PCR reactions were performed on ice. The tubes were then overlaid with 60 µl mineral oil, centrifuged for 3-5 s at 8,000 rpm. Finally; they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program;

An initial denaturation at 94 ˚C for 5 min; followed by 40 cycles of 94 ˚C for 1 min, 56 ˚C for 1 min (annealing) and 72 ˚C for 1 min (elongation); and a final extention at 72˚C for 10 min.

2.3.1.3. Extraction of DNA Fragments from Agarose Gels

DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel using a razor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55 ˚C, then mixed well and further incubated for 2 min. Ten µl of well mixed glass powder suspension was added and mixed well. They were incubated for 5 min at 55 ˚C by occasional mixing. The samples were then centrifuged for 10 s at 10,000 rpm and the supernatant was discarded. The glass pellet was rinsed with 500 µl wash buffer and resuspended by pipetting or gently flicking the tubes. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the final wash, the supernatant was removed and then the pellet was resuspended in 25 µl of 1×TE buffer. The samples were eluted for 5 min 55 ˚C with occasional mixing then centrifuged for 45 s at 10,000 rpm. The supernatant was transferred into new eppendorf tubes and the samples were stored at – 20 ˚C until use.

2.3.1.4. Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were first labelled as "A", "C", "G", and "T" for termination reactions. Two µl of corresponding Cy5.5 ddNTP termination mix were dispensed into the labelled tubes. The master mix was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7 µl of the mix were dispensed into the each labelled "A", "C", "G", and "T". The tubes were then overlaid with one drop of mineral oil, centrifuged for 3-5 s at 8,000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) and sequencing reaction were performed using the following program;

An initial denaturation at 95 ˚C for 1 min; followed by 40 cycles of 95 ˚C for 30 s, 56 ˚C for 30 s and 72 ˚C for 30 s.

The primer EGE1 was used for sequencing of the PCR products.

EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3' (Jensen *et al,* 1993).

2.3.1.5. Purification of Sequencing Reactions

The volume of the sample was adjusted to 50 µl by adding 1× TE buffer. The samples under the mineral oil were transferred into the new eppendorf tubes and then 13.5 µl of 7.5 M ammonium acetate and 40 µl of chilled 100% ethanol were added onto each sample. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. The samples were centrifuged at 12,000 rpm for 30 min at 4 ˚C. The supernatants were removed carefully and the pellets were washed with 200 µl 70% ethanol. After the centrifugation for 5 min, the supernatant was removed and the pellet was dried. Each pellet was resuspended in 6 µl of formamide loading dye and vortexed vigorously for 3 min. Before loading the samples onto the gel, each sample was heated for 3 min at 70 ˚C for denaturation and then placed on ice. Two µl of the denaturated samples were loaded onto the lanes of the sequencing gel.

2.3.1.6. Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amersham Pharmacia Biotech). The system consist of SEQ personal sequencer, SEQ Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL- 6% cartridge, filling gun, and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerazing the acrylamide in the polymerizer. After the polymerization, the comb was removed from cassette and then the cassette was carefully cleaned. The clean cassette was placed into the buffer chamber. At this step pre-run was performed to measure the background reading. Once the gel cassette was aligned and the pre-run was complete, 1× TBE buffer was poured into the upper and lower buffer chambers. Two µl of sequencing samples were loaded into the gel and the analysis was started. After the run was finished, the data were analysed by SEQ software. The sequences were obtained and evaluated and then submitted to GenBank.

2.3.2. Pulsed Field Gel Electrophoresis RFLP

2.3.2.1. Preparation of Agarose Embedded Bacterial DNA

Bacterial cultures were inoculated into MRS broth from frozen glycerol stocks and were then incubated overnight at 30 ºC. The re-usable 15 welled plug mould holder (Bio-Rad) was unscrewed and agarose plugs were prepared. The cells were embedded into the agarose as follows: Overnight bacterial cultures (16-18h), 50 µl, were harvested by centrifugation for 5 min at 10,000 rpm and washed with 300 µl cell suspension buffer (10 mM Tris, pH 7.0, 20 mM NaCl, 50 mM EDTA, pH 8.0). The cells were first suspended in 50 µl of cell suspension buffer, mixed with 50 µl of 2% low melting temperature agarose (in sterile water, at 50 ºC) , and were then pipetted into re-usable plug moulds. The plugs were then allowed to solidify at room temperature.

The cells were lysed by transferring the plugs into 1.5 ml eppendorf tubes containing 1 ml of lysozyme solution (30 mM Tris, pH 8.0, 50 mM NaCl, 5mM EDTA, pH 8.0, 10 mg/ml lysozyme) and were then incubated for 4 h at 37 ºC without agitation. The plugs were transferred into the 50 ml falcon tubes containing 5 ml of 1× wash buffer (20 mM Tris pH 8.0 and 50 mM EDTA pH 8.0) with gentle agitation for 45 min at room temperature on an orbital shaker. The plugs were then transferred into 1.5 ml eppendorf tubes containing 1 ml proteinase K buffer (100 mM EDTA pH 8.0, 0.2% sodium deoxycholate, 1% sodium N-laurylsarcosinate, 1 mg/ml proteinase K) and were incubated overnight at 50 ºC without agitation.

Before the restriction enzyme digestion, the plugs were washed 5 times in 5 ml wash buffer by gentle agitation for 45 min at room temperature on an orbital shaker. First and second washes were in 1× wash buffer (20 mM Tris pH 8.0 and 50 mM EDTA pH 8.0) plus 1 mM NaCl. Third wash was in 1× wash buffer plus 1 mM PMSF (phenyl methyl sulfonyl floride). Fourth wash was in 1× wash buffer and the final wash was in 0.1× wash buffer.

2.3.2.2. Restriction Enzyme Digestion of Agarose Plugs

*Sma*I restriction endonuclease (5'-CCC GGG-3') (Fermentas) was used for the restriction enzyme digestion of genomic DNA.

After the washing steps, the plugs were equilibrated in 1ml of 1× *Sma*I restriction enzyme buffer for 1h at room temperature with gentle agitation on an orbital shaker. The restriction enzyme was then removed after the agarose plugs were digested with 30 units of *Sma*I in 300 µl reaction volume overnight at 30 ºC. Before the electrophoresis the plugs were equilibrated in 1 ml of 0.5× TBE buffer for 1 h at room temperature with gentle agitation on an orbital shaker.

2.3.2.3. Pulsed Field Gel Electrophoresis

The electrophoresis was performed in 1% PFGE grade agarose (Bio-Rad) gel. One gram of agarose was dissolved in 100 ml of 0.5× TBE buffer by boiling. After the gel was cooled, it was poured into the platform of casting stand provided by CHEF DRII equipment (Bio-Rad). The 15-wells comb was placed into the comb holder and the gel was then allowed to solidify at room temperature. After the gel was solidified, the comb was removed gently. The agarose plugs were sliced to a comb size and loaded into the wells by using a spatula. The plugs were placed into the front walls of the agarose wells and care was taken to ensure that the height of the plugs should not be more than 90% height of the wells.

One percent low melting temperature agarose was dissolved in 0.5× TBE by boiling. After it was cooled to approximately 40 ºC, the agarose wells were then covered with low melting temperature agarose. The gel was allowed to solidify for 15-20 min. Two liters of 0.5× TBE at 4 ºC were poured into the electrophoresis chamber. The chiller and the pump were switched on 60 min before the electrophoresis to adjust the buffer temperature to 14 ºC. After the chiller and the pump were switched off, the gel removed from the casting stand with a platform and placed into the electrophoresis cell.

Electrophoresis was performed in a CHEF DRII system with 5-25 pulse times, for 24 h at 4V/cm at 14 ºC.

2.3.2.4. Staining the PFGE Gels

When the electrophoresis was complete, the gel was removed from the CHEF DRII system and stained in 200 ml dH_2O containing 200µl (10 mg/ml) ethidium bromide for 45 min with gentle agitation. The gel was then destained with deoinized water for 1 h with gentle agitation. The image of the gel was analysed in a gel documentation system (Vilber Lourmat, Torcy, France).

2.3.3. Plasmid Profiling

2.3.3.1. Plasmid DNA Isolation

The method of Q'Sullivan and Klaenhammer (1993) was used with some modifications, for the isolation of plasmid DNA from *L. lactis* ssp. *lactis* isolates.

Bacterial cultures were inoculated into 10 ml MRS broth and were then incubated overnight at 30 ºC. They were centrifuged for 10 min at 5,000 rpm. The supernatant was discarded and the pellet was resuspended in 500 µl 1× TE buffer. After that, the samples were for centrifuged 10 min at 5,000 rpm and the supernatant was discarded completely. The pellet was suspended in 200 µl lysozyme buffer (1×TE containing 25% sucrose and 20 mg/ml lysozyme). The solution was incubated for 1 h at 37 ºC. After the incubation, 400 µl alkaline SDS solution (3% SDS, 0.2N NaOH) was added and incubated at room temperature for 7 min. After that, 300 µl of ice cold 3M sodium acetate (pH 4.8) were added. The solution was mixed immediately and centrifuged for 20 min at 10,000 rpm at 4ºC. The supernatant was transferred into the new eppendorf tubes and 650 µl isopropanol was added and mixed. The solution was centrifuged for 20 min at 10,000 rpm at 4ºC, then the supernatant was removed and the pellet was suspended in 300 µl 1×TE. Two hundred µl of 7.5 M ammonium acetate containing 0.5 mg/ml ethidium bromide were added into the solution and mixed well. After this step, 350 µl phenol solutions were added and centrifuged for 10 min at 10,000 rpm at 4ºC. The aqueous phase was transferred into a new eppendorf tube and then 350 µl chloroform/isoamylalcohol (24:1) was added, mixed well and centrifuged for 10 min at 10,000 rpm at 4ºC. The aqueous phase was again transferred into the new eppendorf tube and 1ml 99% cold ethanol was added and mixed. After centrifugation for 10 min at

10,000 rpm at 4ºC the aqueous phase was removed and the pellet was washed in 500 µl of 70% ethanol. After the washing step, plasmid DNA was pelleted and dried at 37 ºC and then resuspended in 50 µl 1×TE containing 0.1 mg/ml RNase, incubated for 1 h 37 ºC and stored at -20ºC.

2.3.3.2. Electrophoresis of Plasmid DNA

Plasmid DNA was electrophoresed in 0.8% agarose gel. Eight hundred mg of standard agarose was dissolved in 100 ml of 1× TAE buffer by boiling. Fifteen µl of ethidium bromide (10 mg/ml) were added after cooling the agarose solution to 40-45 ºC. After cooling, the agarose solution was poured into the gel casting stand and combs were placed. After the gel was cooled, the combs were removed gently. The casting tray carrying the agarose gel was placed into the electrophoresis tank and 1× TAE buffer was added until the buffer cover the gel. Ten µl of plasmid DNA sample were taken and mixed with 2 µl of 6× gel loading buffer. After that, the samples and 3 µl of DNA (500ng) molecular weight marker were loaded into the wells. The electrophoresis was performed for 1h at 60mA. Images of the gel were visualised in a gel documentation system (Vilbert-Lourmant, Torcy, France) and for further analysis Bio-ID++ computer program was used.

2.3.3.3. Plasmid Instability

Plasmid DNA was isolated from *Lactococcus lactis* ssp. *lactis* strains according to the protocol of Q'Sullivan and Klaenhammer (1993).

For biochemical detection of plasmid instability, isolates were grown in M17 broth (Terzaghi and Sandine 1975) and then continuous subculturing was performed. In order to determine plasmid loss biochemically, M17 agar medium with some modification was used. Addition of a pH indicator (bromcresol purple) and reduction of β-disodium glycerophosphate (Sinha 1990; Trotter *et al*. 2002) allowed to an easy differentiation between lactose fermenting and non-fermenting strains. This indicator gives yellow and blue/purple colours under acid and alkaline conditions respectively. If the isolates contain lactose associated plasmid, they utilize the lactose and produce lactic acid. That is why utilization of lactose was observed by the change the medium

colour from purple to yellow. When they lose lactose associated plasmids, the medium colour do not change and the colonies appear in white.

After biochemical detection of plasmid instability, a molecular detection assay was also applied. First, plasmid DNAs were isolated from parental Lac (+) strains (yellow colony) and then Lac (-) isolates (white colony) were monitored by biochemical tests. If the isolates gave white colonies, they are harvested by centrifugation and plasmid DNA isolation was performed. Lac (+) and Lac (-) strains' plasmids were separated by electrophoresis and the results were compared.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Partial DNA Sequencing on the 16S rRNA Gene

Fifty four *Lactococcus lactis* ssp. *lactis* strains which have been isolated from Cappadocia, and identified as good starter candidates (Bulut *et al*. 2005) were investigated for biodiversity of *Lactococcus lactis* ssp. *lactis* artisanal starters in this study. Partial DNA sequencing on 16S rRNA gene of starter strains was performed to support the previous 16S-ITS RFLP results (Bulut 2003). One representative isolate which was randomly picked among the members of the 16S-ITS RFLP homology groups (Bulut 2003) was used for 16S rRNA gene partial sequence analysis. Thermo sequenase Cy 5.5 Dye Terminator Cycle Sequencing Kit and 5'-AGAGTTTGATCCTGGCTCAG -3' forward primer (Jensen *et al.* 1993) were used for the reactions. This primer was complementary to the 5'- end of 16S rRNA region. Sequencing results were obtained in a SEQ 4X4 personal sequencer system and they were analyzed using BLAST programme in NCBI nucleotide database. The following five accession numbers were obtained from GenBank: AY604868, AY604869, AY514788, AY514787 and AY513725 for the isolates B20, B21, C28, A35 and A40. Each of the sequence results showed more than 90% homology with the 16S rRNA gene sequences of *Lactococcus lactis.* In other words, these results confirmed that the strains belong to *Lactococcus lactis* species.

3.2. Chromosome Profiling by Pulsed Field Gel Electrophoresis

Lactic acid bacteria are important starter cultures for traditional cheese fermentation. "Comlek peyniri" is one of the typical traditional cheeses and is very common in Cappodocia region. Industrial production of the cheese manufacture requires defined starter strains with desirable technological characteristics and most of the commercial starters are homofermentative and also developed from *Lactococcus lactis* (Beresford *et al*., 2001).

PFGE-RFLP with *Sma*I restriction on chromosomal DNA was carried out to differentiate 54 isolated strains. Isolates producing similar restriction chromosome profiles were grouped together. The groups were named as PFGE-RFLP genotypes (Figure3.2). Further analyses were performed on the dendogram using BioID++ computer program.

In order to enhance resolution capacity of PFGE-RFLP, the following conditions were optimized such as bacterial cell, amount of lysozyme, incubation time, pulse time, running time and running buffer. In preliminary experiments 5-25 pulse time, 20U of *Sma* I enzyme, 5 mg/ml lysozyme and overnight incubation of agarose plugs and 1× TAE as running buffer were used for PFGE-RFLP. Unfortunately, interpretable DNA profiles could not be observed (Figure 3.1). To solve these problems, the amount of lysozyme was increased to 10 mg/ml and the sodium lauryl sarcosinate and sodium deoxycholate were not added into this lysis solution. And, the pulse times of 5-30 and 5-40 were tried to separate the DNA fragments. These pulse times failed to separate the small fragments so 5-25 pulse time were chosen. Different running times such as 24 h, 30 h and 36 h were also tested to obtain better gel resolution. The type buffer used for running also affected the discriminative power of the DNA fragments. 0.5× TBE buffer was found to be more effective than 1× TAE buffer to separate fragments. Using low number of cells resulted in more distinct patterns because the restriction enzyme was not able to digest the chromosomal DNA prepared from high number of cells. After these trials, 150µl culture for plug preparation, 4 h lysis by solution containing 10 mg/ml lysozyme, 20U of restriction enzyme and digestion overnight, 24 h running time, 5-25 pulse time for electrophoresis were used for all PFGE-RFLP experiments.

Different PFGE conditions were used to separate the chromosomal DNA of lactic acid bacteria strains. Similar to this study, Tanskanen *et al*. (1990) analyzed 29 starins of *Lactoccus lactis* ssp. *lactis* and *Lactoccus lactis* ssp. *cremoris* were analyzed by PFGE. They concluded that 16 h run time, 1-20 pulse time conditions and 10U of *Sma* I digestion gave rise to good separation to identify *Lactococcus* species. However, Delgado and Mayo (2004) proposed that *Apa* I digestion gave better separated bands than *Sma*I for both lactococci and entrococci strains isolated from starter free farmhouse cheeses in Northern Spain. Mannu and Paba (2002) have used plasmid profiling and PFGE to type the milk cheese strains isolated from home made Pecorino Sardo cheese at different periods of ripening. Similar to our conditions, they have digested genomic DNA by two fold *Sma*I and separated DNA fragments in 0.5 × TBE. According to the

combined results of plasmid profiling and PFGE, *Lactococcus lactis* genotypes were detected at the beginning of the ripening. Similarly, most strains isolated from unripened cheese by Bulut *et al*. (2005) were found to show different genotypes in our study as well.

Ward *et al*. (2004) have also characterized nine strains of *Lactococcus lactis* used in the dairy industry by PFGE and plasmid profiling. It was proposed that these nine strains were found to be very closely related after *Sma*I digestion of chromosomal DNA.

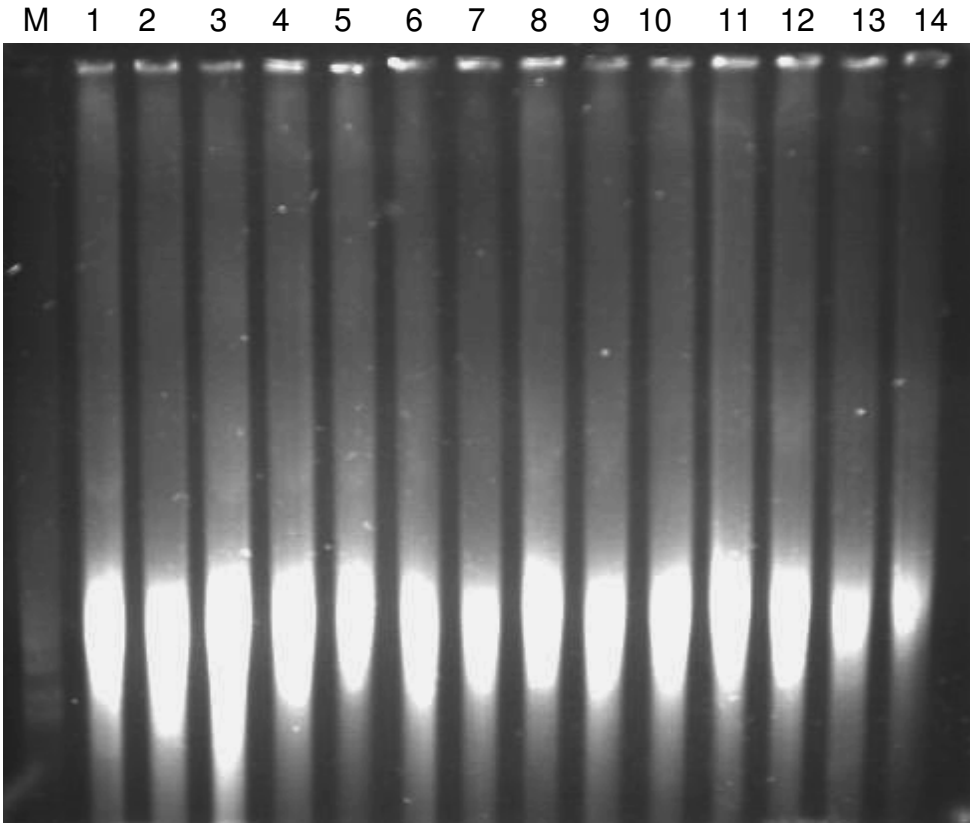

Figure 3.1. Pulsed-field gel electrophoresis of isolates with low concentration of lysozyme and 36 h of run time at 5-30 pulse time.

In this study, artisanal starter strains which decreased the pH of the milk 5.3 after 6 h incubation resulted in 10 distinct restriction patterns by PFGE-RFLP analysis of the chromosomal DNA. Two main clusters were obtained by the dendogram (Figure 3.2) and named as cluster A and B. The pattern homology between the two clusters appeared to be *ca* 50%. Three reference strains, *Lactococcus lactis* ssp. *lactis* (CECT

4432), *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* (CECT 4431) and *Lactococcus lactis* ssp. *cremoris* (NRRL-B 634) exhibited quite different PFGE-RFLP genotypes and none of the artisanal starters yielded these digestion profiles. These results indicate that our artisanal starter strains are different from reference strains and suggest that they may be new *Lactococcus lactis* strains specific for Comlek cheese.

The PFGE-RFLP cluster A1 contained four different genotypes together with two reference strains (Figure 3.2). The pattern homology between two clusters (A1 and A2 genotypes) was *ca* 57%. Restriction pattern homology between A1.2 and A1.3 genotypes was *ca* 87%. The reference strain *Lactococcus lactis* ssp. *lactis* (CECT 4432) belonged to this cluster. A1.2 genotype contained only one artisanal starter strain, A3 but A1.3 genotype contained three strains, A44, A45 and A47. These three isolates had also similar acidifying activities, they decreased the pH of milk 4.6-4.8 after 6h incubation at 30 °C but acidifying activity of isolate A3 differed from other isolates in this group (Table 3.1). The genotype A1.5 contained six of the isolates (A10, A14, A20, A23, A26 and A27) and the pattern homology between A1.5 and *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* (CECT 4431) was 80%. Isolate C24 had a different PFGE-RFLP genotype, A1.6, and the pattern homology between A1.5 and A1.6 genotypes together with reference strain *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* (CECT 4431) was *ca* 72%. The cluster A2 contained also four different genotypes and the pattern homology was 72% (Figure 3.2). Fifteen artisanal starters belonged to the genotype A2.1 containing 43% of the total isolates. The genotype A2.2 contained only one isolate (A9). The genotype A2.3 consisted of two of the artisanal starters (C10 and C11). The pattern homology between the genotype A2.2 and A2.3 was 90%. The acidifying activities of these isolates were similar (Table 3.1). Isolate C15 and C22 had a same PFGE-RFLP genotype (A2.4) and the pattern homology with A2.2, A2.3 and A2.4 was 80 %. C15 and C22 decreased the pH of milk 5.0-5.1 after 6h incubation. Thus, acidifying activity of the isolates C10 and C11 (genotype A2.3) was very similar to the genotype A2.4 (Table 3.1). The other main cluster B contained two different genotypes (B1 and B2) and the pattern homology between clusters A and B was 50% (Figure 3.2). Cluster B contained three isolates together with the reference strain *Lactococcus lactis* ssp. *cremoris* (NRRL-B 634). The pattern homology within the cluster B was *ca* 65 %. Isolate A30 and B15 showed B2.1 genotype whereas isolate A48 gave a different pattern (B2.2). The pattern similarity between genotype B2.1 and B2.2

was 85% (Figure 3.2). Acidifying activity of these genotypes was very similar (pH 4.9 to pH 5.1) to each other (Table 3.1).

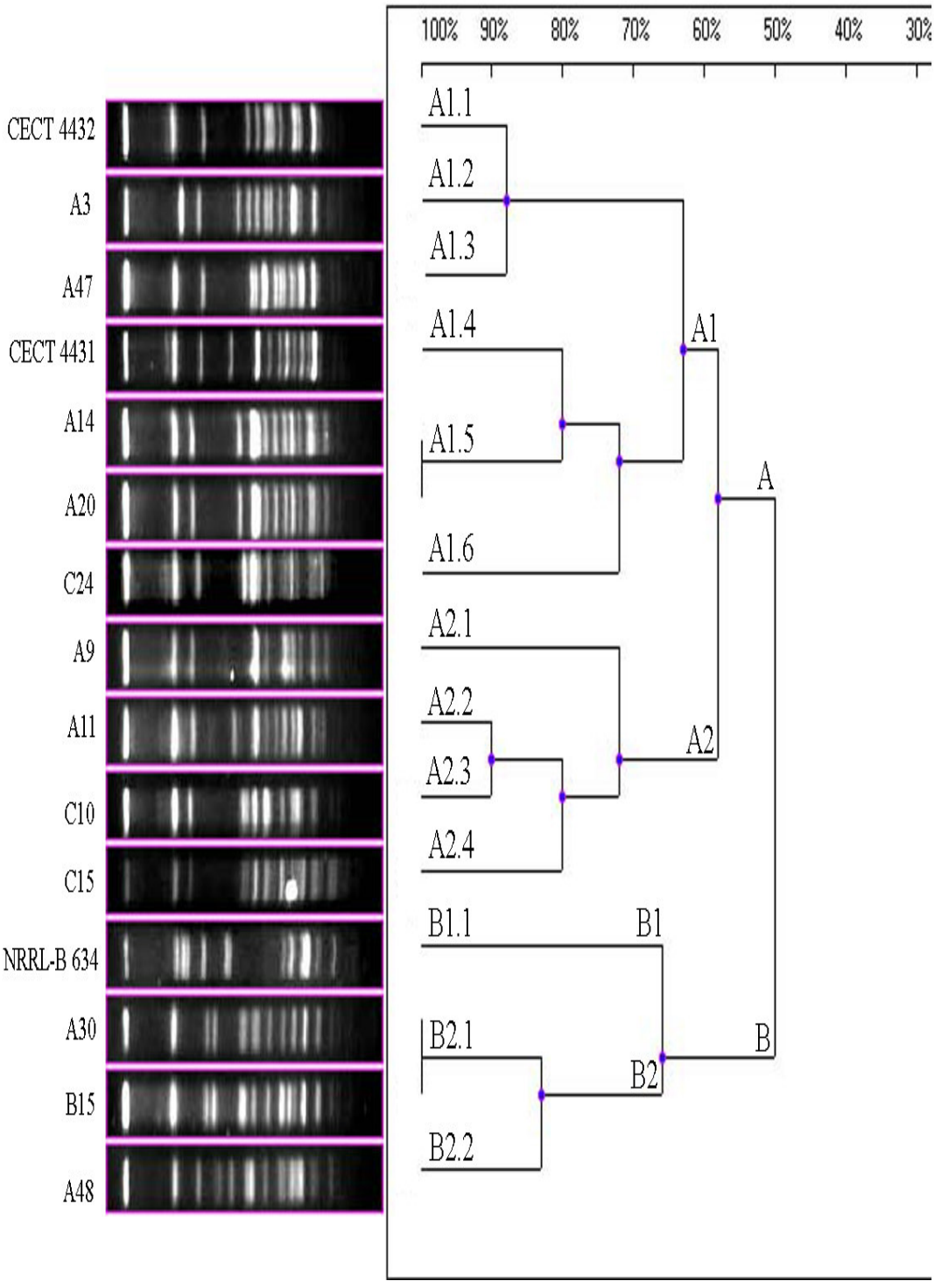

Figure 3.2. Dendogram of isolates showing high acidifying activities. Restriction patterns of each of the genotypes were normalized against two DNA size markers (8-48 kb and 5 kb ladder CHEF DNA size standards; Bio-Rad, USA), and analysed with 15% homology coefficient in a Bio-1D++ computer programme (Vilber-Lourmat).

Table 3.1. PFGE-RFLP, plasmid groups, stability of the beta-galactosidase and range of acid activity.

PFGE-RFLP Genotypes	Isolate Name	Plasmid Groups	Plasmid Stability (d)	Acidifying Activity
A1.2	A3	pG2	55	4.480
A1.3	A44	pG6	21	4.345
	A45	pG7	21	4,435
	A47	pG3	21	4.400
A1.5	A10	pG3	11	5.060
	A14	pG5	14	4.565
	A20	pG5	45	4.595
	A23	pG1	11	4.650
	A26	pG5	52	4.575
	A27	pG5	36	5.010
A1.6	C24	pG5	20	4.975
A2.1	A2	pG1	40	4.815
	A5	pG1	20	4.790
	A6	pG1	50	5.045
	A8	pG5	14	5.025
	A9	pG1	14	4.775
	A12	pG1	55	4.575
	A13	pG1	14	5.295
	A21	pG1	14	4.580
	A22	pG1	30	4.555
	A25	pG1	20	4.730
	A28	pG1	36	4.865
	B10	pG1	40	5.140
	C1	pG1	55	4.910
	C19I	pG1	45	5.070
	C19II	pG1	*	5.215
A2.2	A11	pG3	14	4.675
A2.3	C10	pG4	45	4.920
	C11	pG4	*	4.990
A2.4	C15	pG4	*	5.005
	C18	pG4	20	4.855
	C22	pG4	52	5.120
B2.1	A30	pG6	21	4.915
	B15	pG9	*	5.140
B2.2	A48	pG8	21	5.295

*: Strains retaining the beta-galactosidase activity beyond 55 d incubation with consecutive sub-culturing.

In addition to the isolates with high acidifying activity, the isolates with low acidifying activity were also differentiated by PFGE-RFLP and thirteen genotypes were obtained (Figure 3.3). They could not decrease the milk pH of the below 5.3 after 6 h incubation (Bulut 2003). The acidifying activity of these isolates ranged from pH 5.4 to 6.3 (Table 3.2). These isolates constituted two main clusters named as C and D. The pattern homology between them was ca 60% (Figure 3.3). Cluster C was subdivided into three main genotypes C1, C2 and C3 and 72% cluster homology was obtained. The genotype C1 contained three different genotypes and two of them consisted of reference strains, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* (C1.1) and *Lactococcus lactis* ssp. *lactis* (C1.2). The genotype C1.3 included two strains, B20 and B21. The pattern similarity within the C1 cluster was 75%. The genotype C2 gave four different patterns (C2.1, C2.2, C2.3, and C2.4) and pattern homology within the C2 clusters were also 75%. The genotypes obtained from C2.1, C2.2, C2.3 were different from others (isolate A7, A16, and B8 respectively) but the isolates A37 and C34 had the same genotype, C2.4. The acidifying activity of the isolate A37 (pH 5.3) was less than the isolate C34 (pH 6.3). Third main cluster of the genotype C was C3 that gave only one unique PFGE-RFLP pattern (isolate A35). Acidifying activity of the isolate A35 was as good as isolate A37 (Table 3.2).

The cluster D included three major genotypes (D1, D2 and D3). The pattern homology within the D clusters was 66%. The reference strains *Lactococcus lactis* ssp. *cremoris* was clustered into the genotype D1. The cluster D2 contained three different genotypes. The genotypes D2 and D3 shared ca 70% homology. The genotype D2.1 contained two of the isolates (A1 and A19) and their acidifying capacity was very low (pH 6.2). The genotype D2.2 was differed from other classes and contained only one isolate, C16. Also, the genotype D2.3 contained only one isolate (C4). The acidifying activity of the isolate C4 and C16 was better (pH 5.4) than isolates belonging to the genotype D2.1. The pattern similarity within the D2 genotypes was 83% and the pattern homology between the D2.1 and D2.2 was 88%. The other cluster, D3, contained four different genotypes which shared 76% homology. D3.1 gave a distinct PFGE-RFLP profile and contained the isolate A40. D3.2 also had one isolate, B11. The acidifying activity of the isolate A40 and B11 was very low (pH6.3). The pattern similarity between these two genotypes was ca 78%. The genotype D3.3 contained isolate C28 and the genotype D3.4 had two of the isolate, C32 and C35. Their acidifying activity of

the two genotypes was very similar (pH 5.6-5.9) the pattern homology between the genotype D3.3 and D3.4 was 88%.

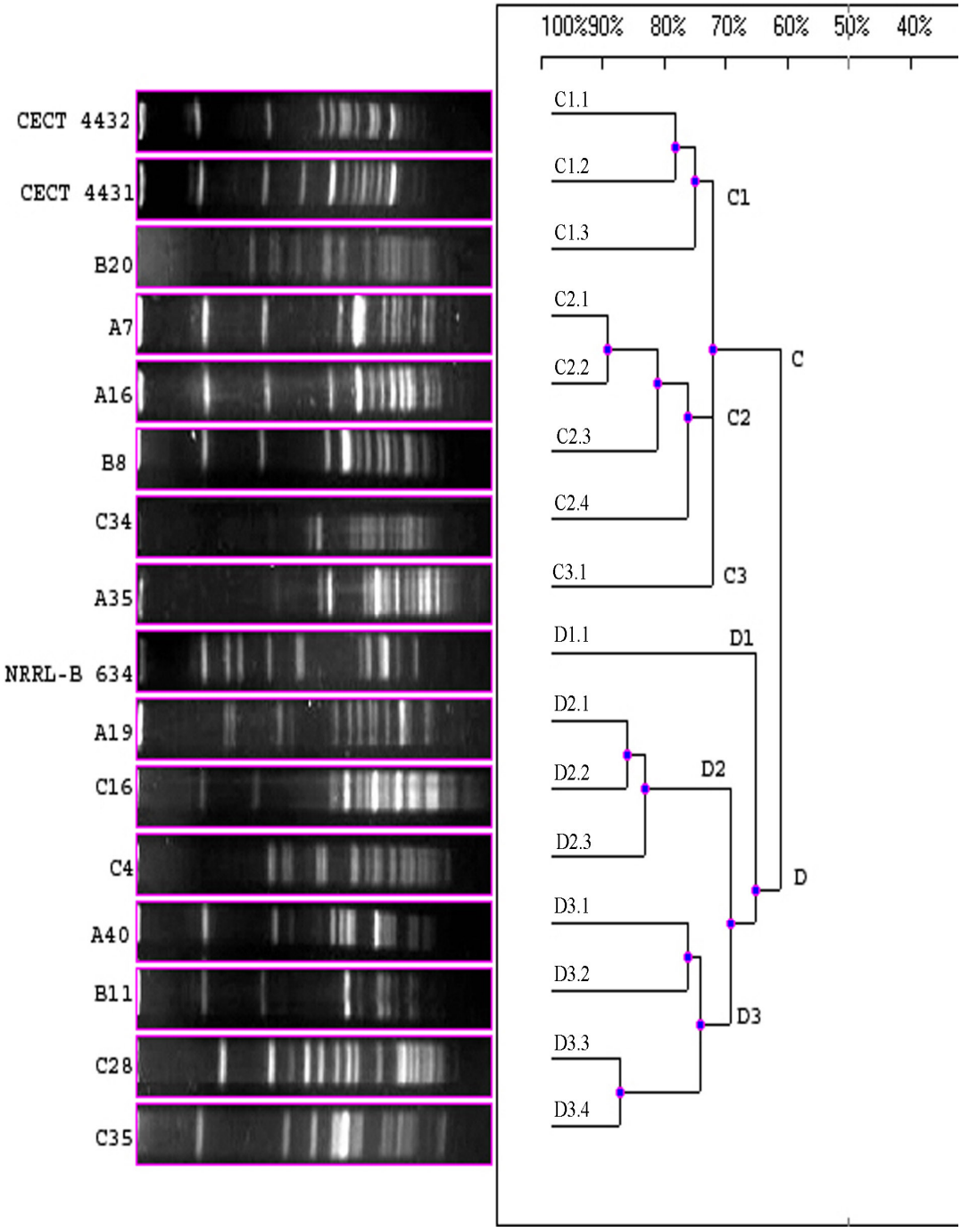

Figure 3.3. Dendogram of the isolates with low acidifying activity. Restriction patterns of each of the genotypes were normalized against two DNA size markers (8-48 kb and 5 kb ladder CHEF DNA size standards; Bio-Rad), and analysed with 15% homology coefficient in a Bio-1D++ computer programme (Vilber-Lourmat).

Table 3.2. PFGE-RFLP, plasmid groups and range of acidifying activities.

PFGE-RFLP Genotypes	Isolate Name	Plasmid Groups	Acidifying Activity
C1.3	B20	pGd	5.905
	B21	pGd	5.645
C2.1	A7	pGb	6.350
C2.2	A16	PGc	6.310
C2.3	B8	pGd	6.160
C2.4	A37	pGe	5.660
	C34	pGe	6.370
C3	A35	pGa	5.640
D2.1	A1	pGa	6.280
	A19	pGa	6.215
D2.2	C16	pGd	5.460
D2.3	C4	pGd	5.470
D3.1	A40	pGf	6.355
D3.2	B11	pGd	6.345
D3.3	C28	pGg	5.955
D3.4	C32	pGa	5.675
	C35	pGa	5.705

3.3. Plasmid Profiling

In order to differentiate *Lactococcus lactis* ssp. *lactis* isolates, plasmid profiling studies were also performed. Isolates which showed desirable acidifying activities constituted nine different plasmid groups (Table 3.1, Figure 3.4). However, seven plasmid groups were obtained from the isolates producing low acidifying activity. Figure 3.4 and 3.7 showed the plasmid patterns of the representative isolates and the three reference strains. None of the 54 isolates shared a similar plasmid profiles with reference strains of the *Lactococcus*. Three of the reference strains were replaced into different plasmid groups. These results show that, our artisanal starter strains exhibit a great diversity based on the origin of the comlek cheese samples.

The most common plasmid group named plasmid group1 (pG1), contained 15 isolates (Figure 3.5). Plasmid group 2 (isolate A3) indicated a distinct plasmid profile. Plasmid group 3 contained three of the isolates. Five artisanal starters were found in plasmid group 4 (C10, C11, C15, C18 and C22). Plasmid group 5 contained six of the isolates (A8, A14, A20, A26, A27 and C24). Isolates A30 and A44 were found in

plasmid group 6. Plasmid patterns of group 7, 8 and 9 were also quite different from other groups (isolate A45, A48 and B15 respectively).

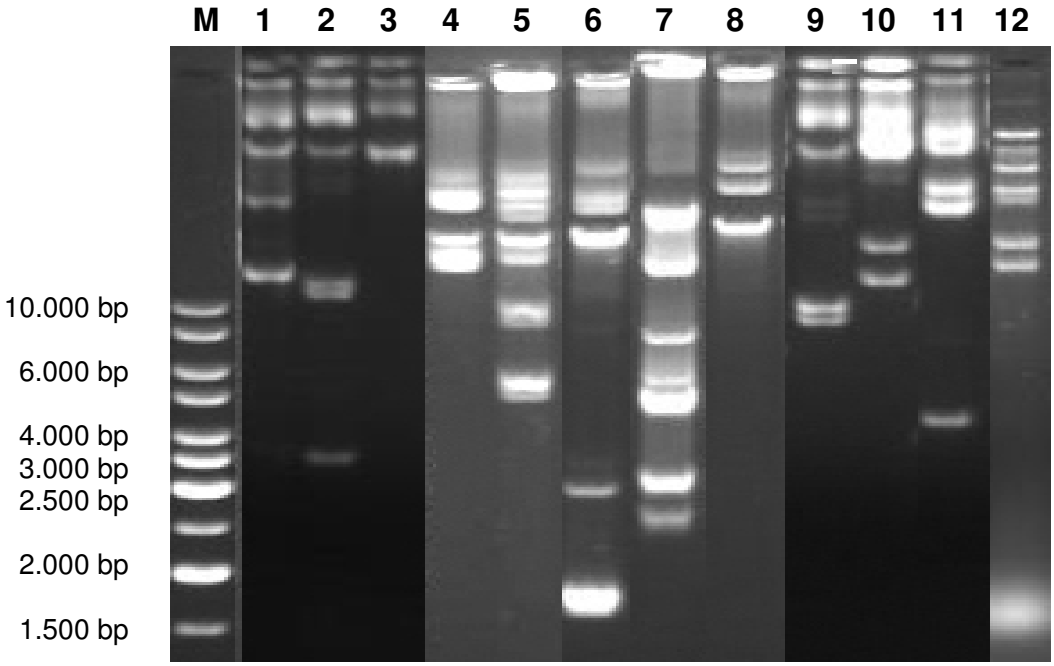

Figure 3.4. Plasmid profiles of representative isolates with high acidifying activity. Lane 1, *Lc*. *lactis* ssp. *lactis* biovar. *diactetylactis* (CECT4431); Lane 2, *Lc*. *lactis* ssp. *lactis* (CECT 4432) ; Lane 3, *Lc*. *lactis* ssp. *cremoris* (NRRL-B 634); Lane 4, pG1; Lane 5, pG2; Lane 6, pG3; Lane 7, pG4; Lane 8, pG5; Lane 9, pG6; Lane 10, pG7; Lane 11, pG8; Lane 12, pG9; M was a DNA size marker.

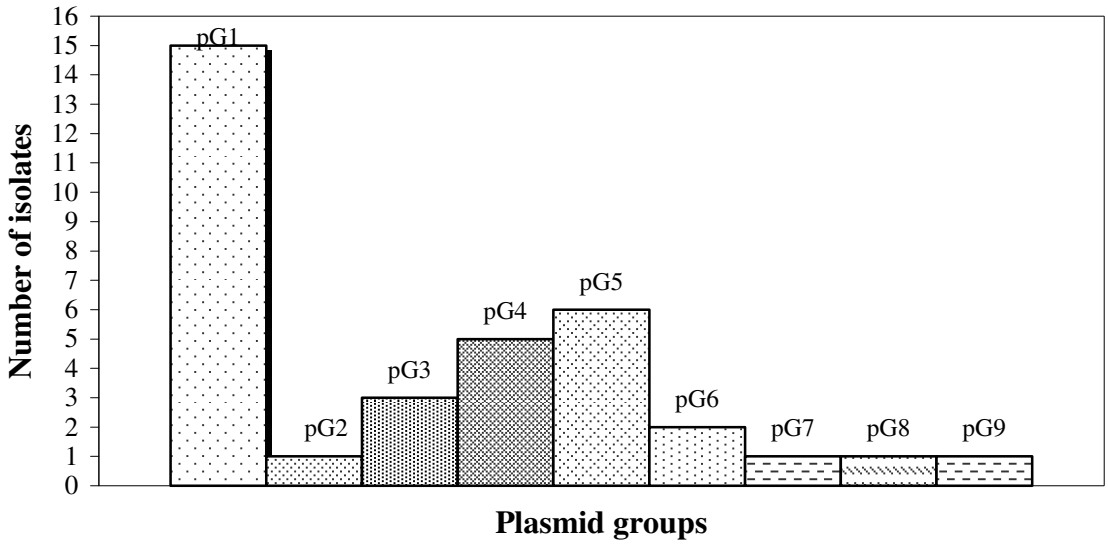

Figure 3.5. Plasmid groups of isolates showing high acidifying activities.

Isolates which had acidifying activity above pH 5.3 were classified into seven different plasmid profiles (Figure 3.6). Group b, c, f and g resulted in unique patterns (isolates A7, A16, A40, and C28 respectively). The most common profile, group d contained seven isolates (A35, B8, B11, B20, B21, C15 and C16, Figure 3.6). Four of the other isolates (A1, A19, C32 and C34) included in plasmid group a. Plasmid group e contained two of the isolates (A37 and C34).

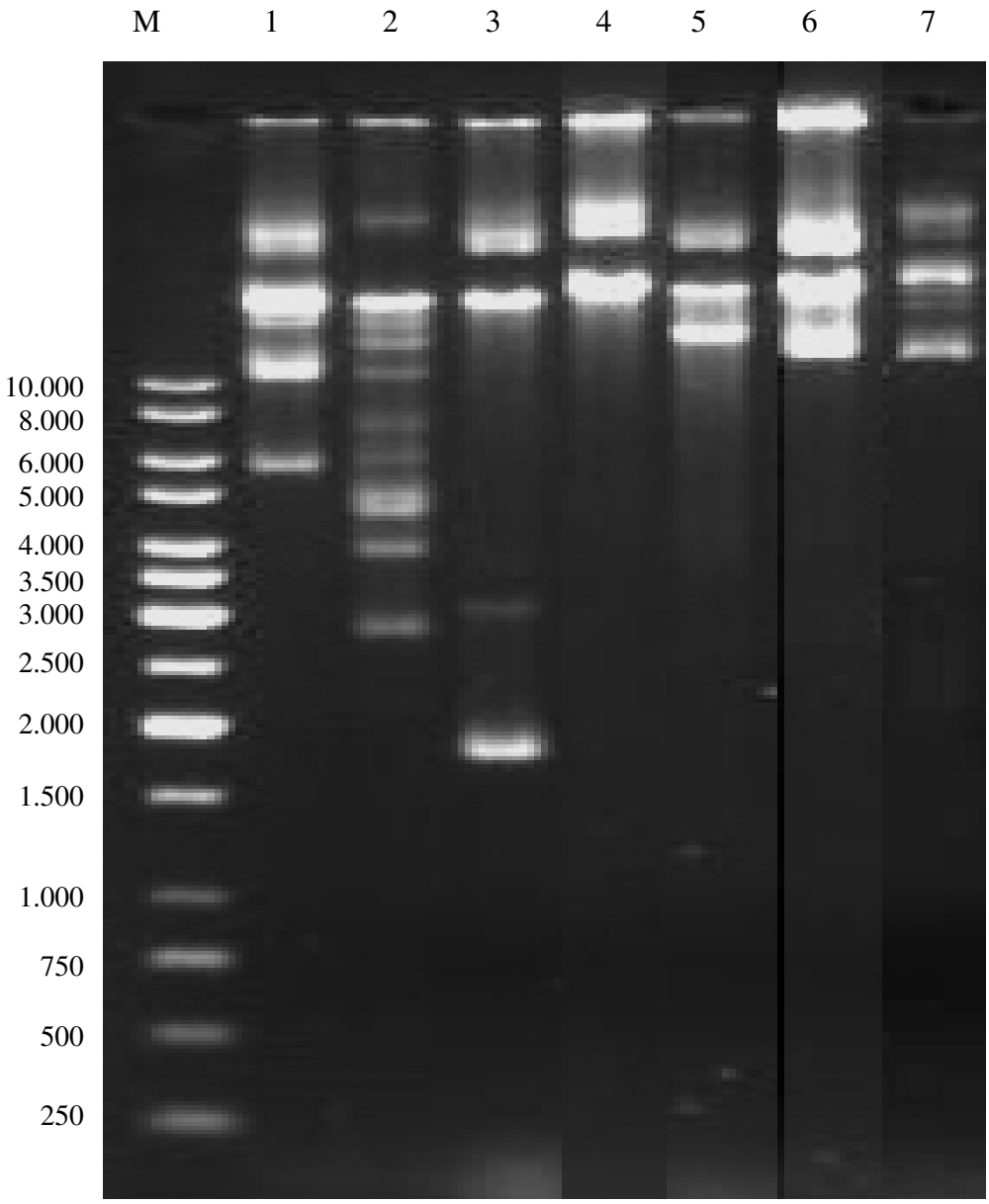

Figure 3.6. Plasmid profiles of representative isolates with low acidifying activity. Lane1, pGa; lane 2, pGb; lane 3, pGc; lane 4, pGd; lane 5, pGe; lane 6, pGf; lane 7, pGg; M was a DNA size marker.

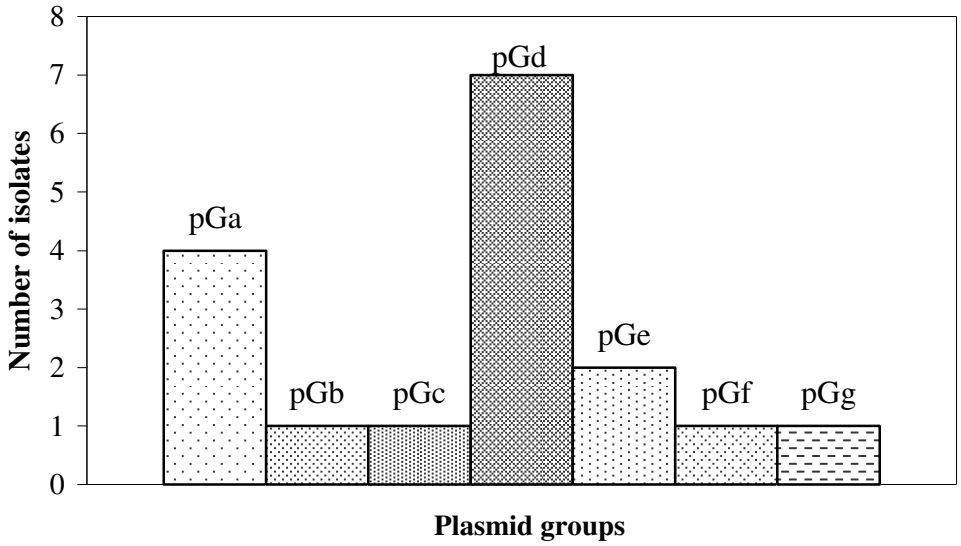

Figure 3.7. Plasmid groups of isolates showing low acidifying activities.

3.4. Combined Analysis of Chromosome and Plasmid Profiling Studies

Isolates within the A2.1 genotypes were the most common PFGE-RFLP genotypes and showed the same plasmid profile (Table 3.3). All isolates except isolate A8 in genotype A2.1 gave the same plasmid pattern named as pG1. Isolate A3 in genotype A1.1 was differed from other isolates on the basis of both chromosomal and plasmid profiling (pG2). Plasmid group 5 contained six isolates, four of them were included in the genotype A1.5. Other two isolates gave different chromosome profiles; isolate A8 was in the genotype A2.1 whereas isolate C24 belonged to the genotype A1.6 (Table 3.3). Five isolates of the plasmid group 4 were found in the A2 genotypes (A2.3 and A2.4). Isolate A48 (B2.2 genotype and pG8) and isolate A11 (A2.2 genotypes and pG3) gave distinct chromosome and plasmid profiles. Isolates A30 and B15 having the same PFGE-RFLP genotype exhibited different plasmid patterns (pG6 and pG9, respectively). Three isolates of the genotype A1.3 showed quite different patterns. Also, three different plasmid profiles were observed for the genotype A1.5. The most common plasmid group in genotype A1.5 was pG5. In addition, the genotype A1.5 contained plasmid patterns of pG1 and pG3 as well (Table 3.3).

Table 3.3. Combined results of PFGE-RFLP genotypes and plasmid groups showing high acidifying activities.

Plasmid Groups	PFGE-RFLP Genotypes	Isolate Code
pG1	A1.5	A23
	A2.1	A2
		A5
		A6
		A9
		A12
		A13
		A21
		A22
		A25
		A28
		B10
		C1
		C19I
		C19II
pG2	A1.2	A3
pG3	A1.3	A47
	A1.5	A10
	A2.2	A11
pG4	A2.3	C10
		C11
	A2.4	C15
		C18
		C22
pG5	A1.5	A14
		A20
		A26
		A27
	A2.1	A8
pG6	A1.3	A44
	B2.1	A30
pG7	A1.3	A45
pG8	B2.2	A48
pG9	B2.1	B15

Plasmid group d (pGd) was the most common plasmid cluster within the second group of the isolates which showed less acidifying activity. This group contained seven isolates and all of the isolates except B20 and B21 showed different PFGE-RFLP genotypes from each other (Table 3.4). The second abundant plasmid profile was pGa containing four isolates. This plasmid group had two different genotypes and these genotypes replaced in cluster D (Table 3.4). Plasmid group e (pGe) contained two isolates and these isolates had the same PFGE-RFLP genotypes, C2.4. Other plasmid groups (pGb, pGc, pGf, and pGg) were differed from other chromosomal and plasmid profiles.

Table 3.4. Combined results of PFGE-RFLP genotypes and plasmid groups showing less acidifying activities.

Plasmid Groups	**PFGE-RFLP Genotypes**	**Isolate Code**
pGa	C3	A35
	D2.1	A1
		A19
	D3.4	C32
		C35
pGb	C2.1	A7
pGc	C2.2	A16
pGd	C1.3	B20
		B21
	C2.3	B8
	D2.2	C16
	D2.3	C4
	D3.2	B11
pGe	C2.4	A37
		C34
pGf	D3.1	A40
pGg	D3.3	C28

3.5. Plasmid Instability Studies on *Lactococcus lactis* ssp. *lactis*

The use of *Lactococcus lactis* ssp. *lactis* in dairy industry as a starter culture makes this strain the most important lactic acid bacteria. Production of lactic acid from milk sugar, lactose, is the major biochemical change in cheese fermentation. This technological characteristic is dictated by plasmid DNA and stability of this plasmid is very important for dairy industry. Stable lactase activity is a desirable characteristic for cheese manufacturing.

Stability of the lactase activity was investigated by biochemical and molecular methods. Plasmid instability studies were only investigated for starter candidates (35, high acidifying isolates) because stable acid activities were desirable for industry.

To determine the stability of the lactose related plasmids, lactase activity was initially investigated by biochemical techniques. For biochemical characterization, the media, M17 containing the pH indicator (bromocresol purple) and reduced amount of the β-disodiumglycerol phosphate were used. Lactose utilizing isolates produced lactic acid and changed the color of the medium from purple to yellow (Figure 3.8). Thus these isolates were named as Lac (+). These strains were sequentially subcultured into the M17 broth and also spotted onto M17 agar medium with bromocresol purple. When the isolate produced white colony, Lac (-), incubation was terminated and plasmid DNA of strains were isolated (Figure 3.8).

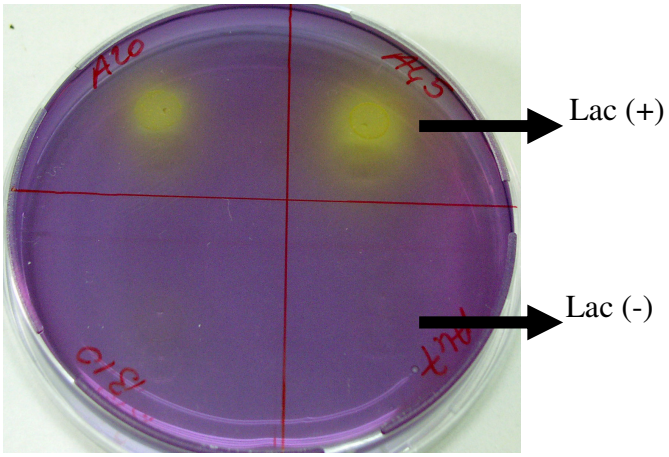

Figure 3.8. Biochemical detection of plasmid instability on M17 agar medium containing bromocresol purple.

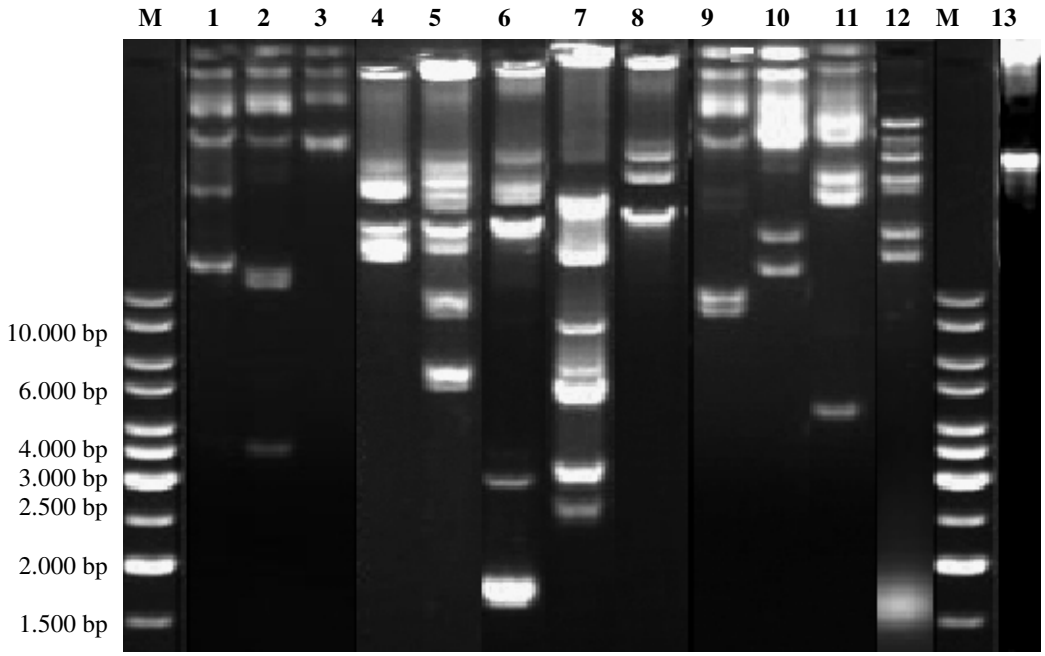

Figure 3.9. Plasmid groups and lactose associated plasmid molecule. Lane 1, *Lc. lactis* ssp. *lactis* biovar. *diactetylactis* (CECT4431); Lane 2, *Lc. lactis* ssp. *lactis* (CECT 4432) ; Lane 3, *Lc. lactis* ssp. *cremoris* (NRRL-B 634); Lane 4, pG1; Lane 5, pG2; Lane 6, pG3; Lane 7, pG4; Lane 8, pG5; Lane 9, pG6; Lane 10, pG7; Lane 11, pG8; Lane 12, pG9; Lane 13, the lactose associated plasmid molecule; M was a DNA size marker (1 kb DNA ladder, GeneRuler; Fermentas, Vilnius, Lithuania).

Four isolates (B15, C11, C15 and C19II) retained their lactase activity after 55 days of incubation through a series of sequential subculturing (Table 3.1; Figure 3.9, lane 13). Other isolates lost lactose related plasmids. Three reference strains of the lactococci lost also lactose activities after continuous subculturing. B15 had a unique plasmid profile and was included in plasmid group 9 and gave the same chromosomal profile with isolate A30 (genotype B2.1). However, other two isolates containing stable plasmids (C11 and C15) gave the same plasmid profiles (pG4) but their PFGE-RFLP genotypes were different (genotypes A2.3 and A2.4 respectively). Isolate C19II had also stable plasmid and included in most abundant plasmid group, pG1 and PFGE-RFLP genotype A2.1. Isolate A3, A12 and C1 lost their plasmids at 55^{th} days. Except the isolate A3, other two isolates showed the same chromosomal and plasmid profiles (genotype A2.1 and pG1). A3 had distinct chromosomal and plasmid DNA profile. As a

result, plasmid instability studies indicated that four of the isolates retained lactase activity; however, the rest of the isolates lost their lactase activity after continious subculturing. Because the lactase activity is carried out on the plasmid DNA, it is most probable that most of the isolates lost their plasmid. Hybridization experiments with isolate DNA and lactase gene (β-galactosidase) will further confirm the plasmid instability. The isolates which lost plasmids will be valuable tool to improve the industrial features of the lactic acid bacteria by introducing new plasmids into bacteria with desirable genes.

Trotter *et al*. (2002) achieved plasmid curing by repeatedly transfer of culture in M17 without the buffering agent β-glycerophosphate and individual Lac^- isolates have been compared on the basis of plasmid profiles. This study involved plasmid curing *Lactococcus lactis* ssp. *lactis* to generate plasmid-free variants. In our study, M17 containing reduced amount of buffering agent, β-disodiumglycerol phosphate was used to determine the lactase activity.

Sinha (1990) also studied the stability of plasmids in *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* strains by extended incubation using low nutrient media under acidic conditions. Lac- phenotypes have been detected in M17 agar medium containing bromocresol purple. Lactose utilizing isolates changed the color of the medium from purple to yellow due to acid production. Then, plasmid DNA of parental strains and their Lac- variants were extracted. This study suggested that larger plasmids were sensitive to sequential sub-culturing, low pH, and low nutrient media.

These studies and our study could be important tools for the dairy industry to obtain starter cultures with desired properties. For the development of the new genetic engineering strategies, plasmid instability studies of certain strains could also be useful.

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVE

Lactic acid bacteria are functionally related group of non-pathogenic, phylogenetically diverse bacteria that produce lactic acid as a primary metabolic end product from glucose and are often associated with food fermentations. The cultures of bacteria used in the manufacture of cheese and other fermented milk products are known as starters. They play a vital role in the manufacture of these products; they produce the lactic acid that influences important quality characteristics such as texture, moisture content, and taste. *Lactococcus lactis* strains are widely used as starter cultures for several types of cheese, and fermented milk products.

In this study, intraspecific biodiversity of *Lactococcus lactis* ssp. *lactis,* the artisanal starters of Comlek Peyniri, isolated from Cappadocia region and the stability of their β-galactosidase activity were investigated. Firstly, partial sequencing of the 16S rRNA genes was accomplished. These results showed that representative isolates had 90 % homology with *Lactococcus lactis.* After that, 54 isolates were characterized at strain level by chromosomal and plasmid DNA profiling. Results indicated that, 35 *Lactococcus lactis* artisanal starters having good acidifying activities were divided into ten different genotypes and nine plasmid groups. None of the reference strains of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* were included into these genotypes and plasmid groups. These results suggest that high level of diversity is present among *Lactococcus lactis* ssp. *lactis* strains obtained from comlek peyniri. In addition, the rest of the isolates which were defined as less acid producers by technological tests were classified into thirteen genotypes and seven plasmid groups based on chromosomal and plasmid DNA analysis. Moreover, plasmid instability studies were carried out by biochemical and molecular methods. The results showed that, only four of the isolates contained the most stable plasmids and they protected their lactase activities during consecutive subculturing for 55 d. These findings allowed us to classify the artisanal starter strains by genotyping methods.

In the light of these results, it will be useful to determine other technological characteristics such as bacteriophage resistance, proteolytic and lipolytic activity, aroma

and flavor compound production, antimicrobial activities and exopolysaccharide production as well as bioamines. Genes residing within the plasmids related to such functional properties could also be investigated. Function and expression of these genes should be carried out to provide an improved understanding. Technological methods could be developed to characterize these strains in order to provide the use of them in the dairy industry. Finally, strains that were genotypically characterized in this study could be selected by technological methods and used as starter cultures for the fermentation of foods in dairy industry.

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APPENDIX A

CHEMICALS USED IN EXPERIMENTS

CHEMICAL	CODE
Agar-Agar	Applichem A0949
Peptone from Casein	Applichem A2210
D (+)-Glucose	Applichem A3666
Yeast Extract	Merck 1.03753
Glycerol	Applichem A2926
Sodium Chloride	Applichem A2942
$MgSO_4.7H_2O$	Merck 1.05886
Cetyltrimethylammonium bromide	Applichem A0805
Tris Base	Sigma T6066
EDTA	Applichem A2937
Isopropanol	Applichem A3928
Proteinase K	Applichem A3830
Ethidium bromide	Applichem A1151
Ethanol	Applichem A1151
Taq DNA polymerase	MBI, Fermentas EP0401
dNTP set	MBI, Fermentas R0181
*Taq*I	Fermentas ER0671
*Hae*III	Fermentas ER0151
Agarose (Standard)	Applichem A2114
Lysozyme	Applichem A3711
Chloroform	Applichem A3830
Isoamyl alcohol	Applichem A2610
Bromophenol blue	Merck 1.08122
Sodium dodecyl sulphate	Applichem A2263
Sodium hydroxide	Merck 1.06498
Boric acid	Applichem A2940
Hydrochloric acid (HCl)	Merck 1.00317
Ammonium acetate	Applichem A2936

Phenol	Applichem A1594
Ribonuclease A	Applichem A3832
1 kb DNA Ladder Gene Ruler^{TM}	Fermentas SM0313
D (+)-Sucrose	Applichem A3935
Bromcresol purple	Merck 1.03025
Sodium acetate	Sigma S-2889
Lab-Lemco	Oxoid LP0029
Tween 80	Applichem
K_2HPO_4	Applichem
Ammonium citrate tribasic	Sigma A-1332
$MnSO_4.4H_2O$	Merck 1.02786
Agarose low melt	Applichem A3762
Molecular biology certified agarose	Bio-Rad 162-0134
N-laurylsarcosine	Applichem A1163
Sodium deoxycholate	Applichem A1531
L (+) Ascorbic acid	Merck 5.00074
β-glyrecophosphate disodium salt	Sigma G-6376
PMSF	Applichem A0999
Meat extract	Merck 1.03979
Sma I	Fermentas ER0661
D (+)-Sucrose	Applichem A3935
β-Lactose	Sigma L-3750
Glacial acetic acid	Merck 1.00056

APPENDIX B

MEDIA

B.1. MRS BROTH MEDIUM

	g/l
Peptone	10
Lab-Lemco	10
Yeast Extract	5
D-Glucose	20
Tween 80	1 ml
K_2HPO_4	2
Sodium acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

All ingredients are dissolved in distilled water by stirring with gentle heating. pH is adjusted to 6.2 - 6.6. Medium is sterilized by autoclaving at 121°C for 15 min

B.2. MRS AGAR MEDIUM

	g/l
Peptone	10
Lab-Lemco	10
Yeast Extract	5
D-Glucose	20
Tween 80	1 ml
K_2HPO_4	2
Sodium acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15
Deionized water	1000 ml

All ingredients are dissolved in distilled water by stirring with gentle heating. pH is adjusted to 6.2 – 6.6. Medium is sterilized by autoclaving at 121°C for 15 min.

B.3. M17 BROTH MEDIUM

	g/l
Peptone	10
Lab-Lemco	5
Yeast Extract	5
β-Lactose	5
Ascorbic acid	1
β-Glycerolphosphate disodium salt	19
$MgSO_4$(0.1M).$7H_2O$	1 ml
Deionized water	1000 ml

Ingredients are dissolved in distilled water by stirring with gentle heating. pH is adjusted to 7.15 ± 0.1. Medium is sterilised by autoclaving at 121°C for 15 min.

B.4. M17 AGAR MEDIUM

	g/l
Peptone	10
Lab-Lemco	5
Yeast Extract	5
β-Lactose	5
Ascorbic acid	1
β-Glycerolphosphate disodium salt	19
$MgSO_4.7H_2O$	1 ml
Agar	12
Deionized water	1000 ml

Ingredients except lactose are dissolved in 900 ml distilled water by stirring with gentle heating. pH is adjusted to 7.15 ± 0.1. Medium is sterilized by autoclaving at 121°C for 15 min.

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

B.5. M17 AGAR MEDIUM WITH BROMCRESOL PURPLE

	g/l
Peptone	10
Lab-Lemco	5
Yeast Extract	5
β-Lactose	5
Ascorbic acid	1
β-Glycerolphosphate disodium salt	5
$MgSO_4.7H_2O$	1 ml
Agar	12
Bromcresol purple (1%)	1 ml

Ingredients except lactose are dissolved in 900 ml distilled water by stirring with gentle heating. pH adjusted to 7.15 ± 0.1. Medium is sterilised by autoclaving at 121°C for 15 min.

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

APPENDIX C

REAGENTS AND SOLUTIONS

C.1. BROMCRESOL PURPLE SOLUTION

Bromcresol purple	1 g
Distilled water	100 ml

Bromcresol purple is added into the water and solution is mixed thoroughly.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1. 50× TAE

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

D.2. 1× TAE

20 ml of 50× TAE buffer is taken and 980 ml of deionized water is added to obtain 1× TAE buffer.

D.3. 10× TBE

108 g of Tris base and 55 g boric acid are mixed and dissolved in 800 ml of deionized water. 40 ml of 0.5 M EDTA (pH 8.0) is added. The volume is brought to 1000 ml with deionized water.

D.4. 0.5× TBE

50 ml of 10× TBE buffer is taken and 950 ml of deionized water is added to obtain 0.5× TBE buffer.

D.5. 1M Tris-HCl (pH 7.2/ pH 8.0)

121.1 g of Tris base is dissolved in 800 ml of deionized water. The desired pH is obtained by adding concentrated HCl. The volume of the solution is brought to 1000 ml with deionized water.

D.6. 1× TE (pH 8.0)

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) is mixed.

D.7. 0.5 M EDTA pH 8.0

186.1g of EDTA are added to 800 ml of deionized water. Solution is stirred vigorously and the pH is adjusted to 8.0 with NaOH pellets (~ 20 g). Volume is completed to 1000 ml with deionized water. Solution is dispensed into aliquots and sterilized by autoclaving.

D.8. 3 M SODIUM ACETATE (pH 5.2)

408.1 g sodium acetate.$3H_2O$ is dissolved in 800 ml of deionized water and the pH is adjusted to 5.2 with glacial acetic acid. The volume is brought to 1000 ml. Solution is sterilized by autoclaving.

D.9. ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionized water by stirring on a magnetic stirrer to dissolve the dye completely. Solution is transferred to a dark bottle and stored at room temperature.

D.10. CHLOROFORM-ISOAMYL ALCOHOL SOLUTION

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

D.11. PHENOL

Phenol is allowed to warm at room temperature, and melted at 68 ^{o}C. Equal volume of buffer (usually 0.5 M Tris-Cl pH 8.0, at room temperature) is added to the melted phenol. The mixture is stirred for 15 minutes. When the two phases have

separated, the aqueous (upper) phase is removed using a separation funnel. Equal volume of 0.1 M Tris-Cl pH 8.0 is then added to the phenol. The mixture is again stirred for 15 minutes. The aqueous phase is removed as described below. The extractions are repeated until the pH of the phenolic phase is > 7.8. The pH is measured by using pH paper slips. After the phenol is equilibrated, the mixture is divided into aliquots and they are stored under 100 mM Tris-Cl (pH 8.0) at -20 ^{o}C. When needed, the phenol is melted at room temperature. Hydroxyquinoline and β-mercaptoethanol are added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at -20 ^{o}C.

D.12. CTAB/NaCl SOLUTION

4.1 g NaCl is dissolved in 80 ml water. 10 g CTAB is added slowly while heating and stirring. To increase dissolution, the solution is heat to 65 ^{o}C. The final volume is adjusted to 100 ml.

D.13. 10 M AMMONIUM ACETATE

770 g of ammonium acetate is dissolved in 800 ml of distilled water and the volume is adjusted to 1000 ml. Solution is filtered-sterilized.

D.14. 10% SODIUM DODECYL SULFATE (SDS)

100 g of SDS is dissolved in 900 ml of deionized water. Solution is heat to 68 ^{o}C to dissolve. The pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume is brought to 1000 ml with water.

D.15. GEL-LOADING DYE (6×)

2 ml of 10× TBE, 6 ml of glycerol is mixed in a falcon and the volume is adjusted to 20 ml with sterile deionized water. Bromophenol blue is added until the adequate color is obtained.

D.16. 100 mM PHENYL METHYL SULFORYL FLORIDE STOCK SOLUTION (PMSF)

17.4 mg of PMSF is dissolved in 1 ml isopropanol by stirring.

D.17. 5M NaCl

292.2 g of NaCl is dissolved in 800 ml of deionized water by stirring. The volume of the solution is adjusted to 1 liter with water. Solution is dispensed into aliquots and sterilized by autoclaving.

APPENDIX E

PCR RECIPIES

E.1. PCR MIXTURE

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

E.2. dNTP (10X)

20 µl of each 100mM dATP, dCTP, dGTP and dTTP are taken and mixed in an eppendorf tube. 920 µl of sterile deionised water is added to dilute the solution to a final concentration of 2 mM. Solution is mixed gently and stored at –20 °C until its needed.

APPENDIX F

OLIGONUCLEOTIDES AND RESTRICTION ENZYMES

F.1. PRIMERS OF EGE 1

EGE1: 5'-AGAGTTGATGATCCTGGCTCGA-3'

590 µg primer EGE1 was dissolved in 295 µl of sterile deinonized water to obtain 2 µg/ µl stock solution. Five microliter of stock solution were then taken and mixed with 95 µl sterile deinized water. Therefore 100 µl, 10 picomole/ µl working solution was obtained. Stock and working solutions were stored at – 20 °C.

F.2. *Sma*I RESTRICTION ENZYME

*Sma*I is a six cutter endonuclease which digests the DNA at this restriction site;

5'- CCC ↓ GGG- 3'

3'- GGG ↑ CCC- 5'

APPENDIX G

16S PARTIAL DNA SEQUENCING RESULTS

G.1. ISOLATE B21

Lactococcus lactis, isolate B21, 16S ribosomal RNA gene, partial sequence result (286 bp). Accession number AY604869.

ggagtgctag atagcatgca tacgctcgga tcgacgtgta ctgttttcac accggagact
tcgctagagc cgtggataac cataggtacg tggcgaatcg gagtgagtac acgtgaggtg
aatctaccat cagacaggtg gatcacactt ggaaacaggt gctaataccg tcataacaat
cgaaatcgca tggtttgatt tgaaaggcgc tttcgggtgt tcgctgatgg atggacccgc
gtgcattagc tcacgttggt gaggtatcag ctcaccaggc cacgat

G.2. ISOLATE B20

Lactococcus lactis, isolate B20, 16S ribosomal RNA gene, partial sequence result (724 bp). Accession number AY604868.

tgctatacag caagtcgacg cttctttttc accgagcttg ctccaccgga aaaagagagt
ggcaacgggt gagtacacgt gggtaactgc ccatcagaag gggataacac ttggaaacag
gtgctaatac cgtataacaa tcaaacgcat ggttttgatt tgaaaggcgc tttcgggtgt
cgctgatgga tggacccgcg gtgcattagc tagttggtga ggtacggctc accaggccac
gatgcatagc cgacctgaga gggtgatcgc cacattggga ctgagacacg gcccaactcc
tacgggagca gcagtaggga tcttcggcaa tggacgaaag tctgaccgag cacgccgcgt
gagtgaagaa ggttttcgga tcgtaaactc tggtggtaga gaagaacagg atgagagtac
tgtcatccct gacggtatct acccgaagca cgctaactac gtgcagcagc ccggtaatcg
taggtggcag cttgtccgat taatgggcgt aagcagccag cggtttctaa tgtctgatgt
gaagccccgt caccgggagg tcattgaact ggaacttgat gcgaaaggaa ttgatctatg
ttagcgagat cacgtcgaaa tgggaaccct gcaacggtct tgctgaatta cactagctgc

cacatggaca cagttgatat atctattagc tacaccaaca atatgaatga ggttcctaca
gagg

G.3. ISOLATE C28

Lactococcus lactis, isolate C28, 16S ribosomal RNA gene, partial sequence result (430 bp). Accession number AY514788.

ctatacatgc agttgagcgc tgaggttggt acttgtacca ctggatgagc agcgacgggt
gagtacgcgt gggatctgcc tttgagcggg gacacatttg gaacgaatgc taataccgca
taaaaacttt aacacaagtt ttagtttgaa gatgcattgc atcactcaag atgatcccgc
gttgtattag ctagttggtg aggtaaagct caccaaggcg atgatacatt agccgacctg
agagggtgat ccgcacaatt gggactgaga cacggcccaa attctacggg aggcgcgtag
ggaatcttcg gcaatgacga agtctgaccc agcacgcccc gtgagtgaag aaggttttcg
gatcgtaaac tctgtggtag agaaagaaca tcggtgagag tggaagctcc tccagtgacg
taactacccc

G.4. ISOLATE A35

Lactococcus lactis, isolate A35, 16S ribosomal RNA gene, partial sequence result (255 bp). Accession number AY514787.

aacacaagtt ttaagtttga agatgcaatt gcatcactca agatgatccc gcgttgtatt
agctagttgg tgaggtaaag ctcaccaggc gatgatacat agccgacctg agaggtgatc
gcacatggat gagaccgccc caactctacg gaggcgcgta ggaatcttcg gcatgacgaa
agtctgaccc agcacgcccg tgagtgaaga agttttcgga tgtaaactcc gtggtagaga
agaactcggt gagag

G.5. ISOLATE A40

Lactococcus lactis, isolate A40, 16S ribosomal RNA gene, partial sequence result (450 bp). Accession number AY513725.

acatgcagtt gagcgctgag gttggtactt gtaccactgg atgagcagcg acgggtgagt
aacgcgtggg gaatctgcct ttgagcgggg gacacatttg gaacgaatgc taataccgca
taaaacttta aacacaagtt ttagtttgaa agatgcattg catcactcaa gatgatcccg
cgttgtatta gctagttggt gaggtaagct caccacggcg atgatacata gccgacctga
gaagggtgat cgcacaattg ggactgaaga cacggcccaa aatcctacag gaggcagcag
tagggaatct tcgcaatgac gaagatctga ccgagcacgc cccgtgagtg aagaaggttt
ccggatctaa actccgttgg tagagaaaga acatctggtg agagtggaag ctcctcacgt
gacgtgaact acccgaaagg gacggtaaca