Isolation and Molecular Characterization of Lactic Acid Bacteria From Cheese

By

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A Dissertation Submitted to the
Graduate School in Partial Fulfillment of
the Requirements for the Degree of

MASTER OF SCIENCE

Depertmant: Biotechnology and Bioengineering
Major: Biotechnology

İzmir Institute of Technology
Izmir, Turkey

September, 2003

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Assistant Prof. Ali Fazıl Yenidünya for his endless encouragement, support and patience during this reseach. I also wish to express my thanks to my co-advisors Prof. Dr. Şebnem Harsa and Assoc. Prof. Hatice Güneş for their all kind of support and help.

I would also like to thank Prof. Dr. Sevda Kılıç from Department of Dairy Technology at Ege University for her guidance and the valuable information she provided.

I want to add thanks to Prof. Dr. Mehmet Karapınar and Ömre Sıkılı from Food engineering Department at Ege University for their support.

I want to express my thankfulness to my friends; Burcu Okuklu, H. Sevgi Çoban, Elif Yavuz, Güney Akbalık, Seda Elmacı, Çelenk Çınar and Ayça Çakın. Additionaly, special thanks for Mert Sudağıdan due to sharing all kinds of experience with me. Also, I would like to thank Gönül Yenidünya for special help and kindness.

I would like to thank managers and employers of Ministry of Agriculture in Nevşehir and Avanos; Mustafa Titrek, Ali Çopur and the Mayor of the Avanos; M. Seyhan Duru for their kindness and supports on collecting of cheese samples.

Finally, I offer sincere thanks to my family members for their support and love.

ABSTRACT

Specially selected starter cultures are required for the industrial production of cheese. These starter cultures are mainly composed of lactic acid bacteria (LAB). Starter LAB have many functions in cheese production. They produce lactic acid during the fermentation process and provide formation of the curd. Futhermore, they show proteolytic activity and also they play a role in the production of aroma compounds and antimicrobial substances. In order to prevent loss of LAB biodiversity and loss of traditional cheese diversity, it is important to identify novel LAB from traditional cheese.

The aim of this project was to isolate and identify natural LAB flora involved in traditional "Çömlek Peyniri" fermentation. In order to achive this goal, LAB were isolated and characterized by using phenotypic (cell morphology, Gram staining, physiological and biochemical tests) and genotypic methods (PCR- Restriction Fragment Length Polymorphism). Moreover, technological characterization was performed by monitoring the acid production profiles of the isolates.

At the end of the study, a total of 113 coccal and 21 mesophilic lactobacilli were obtained and maintained for future use. It was found that cocci shaped isolates included 54 lactococci and 59 enterecocci. Further identification at the species level indicated that all of the lactococci isolates were *L. lactis* ssp. *lactis*. Thirty of the enterecocci were *E. faecium*, 8 of them were *E. faecalis* , 3 of them were *E. avium*, 2 of them were *E. durans* and 16 of them were other *Enterococcus* ssp. Lactobacilli isolates were identified as *Lb. paracasei* ssp. *paracasei* (3 isolate), *Lb. casei* (3 isolate) and other *Lactobacillus* spp (15 isolate) . PCR-RFLP method which is based on the amplification of 16S rRNA- ITS genes and restriction digestion with *Hae*III and *Taq*I endonucleases was found to be useful for further identification. Finally, acid production profiles of isolates indicated that 35 of the isolates could lower the pH of UHT skim milk below 5.3 for 6 h incubation at 30 °C and these isolates were therefore the best starter candidates for industrial applications.

ÖZ

Endüstriyel peynir üretimi özel olarak seçilmiş starter kültürleri gerektirmektedir. Bu starter kültürler başlıca laktik asit bakterilerinden (LAB) oluşmaktadır. Starter LAB peynir üretiminde pek çok fonksiyona sahiptir. Fermentasyon prosesi boyunca laktik asit üretirler ve pıhtının oluşmasının sağlamaktadırlar. Ayrıca, proteolitik aktivite göstermekte, aroma bileşikleri ve de antimikrobiyal madde üretiminde rol oynamaktadırlar. LAB bioçeşitliliğinin ve de geleneksel peynir çeşitliliğinin kaybolmasını önlemek için, geleneksel peynirlerden yeni laktik asit bakterilerinin tanımlanması önemlidir.

Bu projenin amacı, geleneksel "Çömlek Peyniri" fermentasyonuna dahil laktik asit bakterilerinin izolasyonu ve tanımlanmasıdır. Bu amacı gerçekleştirmek üzere, laktik asit bakterileri izole edilmiştir ve fenotipik (hücre morfolojisi, Gram boyama, fizyolojik ve biyokimyasal testler) ve de genotipik ("PCR- Restriction Fragment Length Polymorphism") metodlar kullanılarak karakterize edilmiştir. Ayrıca , izolatların laktik asit üretme profilleri monitor edilerek teknolojik karakterizasyon gerçekleştirilmiştir.

Çalışmanın sonunda, toplam 113 kok ve 21 mezofil laktobasil elde edilmiştir. Kok şeklindeki izolatların 54 adedi laktokok ve 59 adedi enterokok olarak bulunmuştur.Tür düzeyindeki ileri tanımlama göstermiştir ki; tüm laktokok izolatlar *L. lactis* ssp. *lactis*'dir ve enterekokların 30'u *E. faecium*, 8'i *E. faecalis*, 3' ü *E. avium*, 2' si *E. durans* ve 16 'sı *Enterococcus* ssp. olarak tanımlanmıştır. Laktobasil izolatları *Lb. paracasei* ssp *paracasei* (3 izolat) , *Lb. casei* (3 izolat) ve *Lactobacillus* ssp. (15 izolat) olarak tanımlanmıştır. 16S rRNA-ITS genlerinin amplifikasyonuna ve *Taq* I ve *Hae* III endonükleazları ile restriksiyonuna dayalı PCR- RFLP ("PCR- Restriction Fragment Length Polymorphism") metodu, ileri tanımlama için faydalı bulunmuştur. Son olarak izolatların asit üretme profilleri göstermiştir ki; 35 izolat, UHT yağsız sütün pH sını 30 °C de 6 saat inkubasyon sonunda 5.3 ün altına düşürebilmiştir ve bu izolatlar endüstriyel uygulama için en iyi starter adayları olmuştur.

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CHAPTER 1

INTRODUCTION

The production of cheese from milk is a very ancient process. Cheese manufacturing started about 8 000 years ago in the "Fertile Crescent" between Tigris and Euphrates rivers (Hayaloğlu *et al.*, 2002).

Production of cheese is essentially achieved by bringing four ingredients together: milk, rennet, microorganisms, and salt. The process includes the following steps: gel formation, acid production, whey expulsion, salt addition, and finally ripening period. The main biochemical changes that occur in cheese manufacture is the production of lactic acid from lactose. This is achieved by different species of lactic acid bacteria (LAB). The responsible flora that form acid development during cheese production are starter cultures that cause decrease in the pH, formation of curd, expulsion of whey (Beresford *et al.*, 2001).

Since the early 1900s there has been a remarkable increase in the industrial production of cheese. Because, hygiene is the most important criterion in the large scale production (Mäyra-Mäkinen *et al.*,1998). For this reason, pasteurized milk is used. Therefore, natural LAB flora contained in the milk is lost. Consequently, in order to make cheese from pasteurized milk, an external LAB source is needed. This source includes predefined strains of LAB and are called starter strains. In Turkey there has been no starter strain developed as yet that could represent our native LAB flora. This prompted us to collect LAB from the regions where traditional cheese making is still dominating. Traditionally, raw milk of cow, goat and sheep are fermented by the help of naturally occurring indigenous LAB. Beside the technological parameters like curd handling and cooking temperature, the quality of cheese is mainly dependent on the microbial associations within the respective region. Therefore, the LAB flora of traditional cheese making can be taken as the basis of starter strains with unique characteristics. In order to prevent the loss of microbial diversity and loss of wide range of cheese variety, it is very important task to build up LAB collections.

"Çömlek Peyniri" is one type of traditional cheese that is very common in central Anatolia. It has been produced from raw cow or sheep milk for many millennia. Although production recipes change from one village to another, and even among

personal applications, generally Çömlek Peyniri making process include the following steps below (Figure 1.1.). On the other hand, industrial production of cheese (Figure 1.2.) relies exclusively on the use of specially selected cultures which are carefully maintained and subcultured.

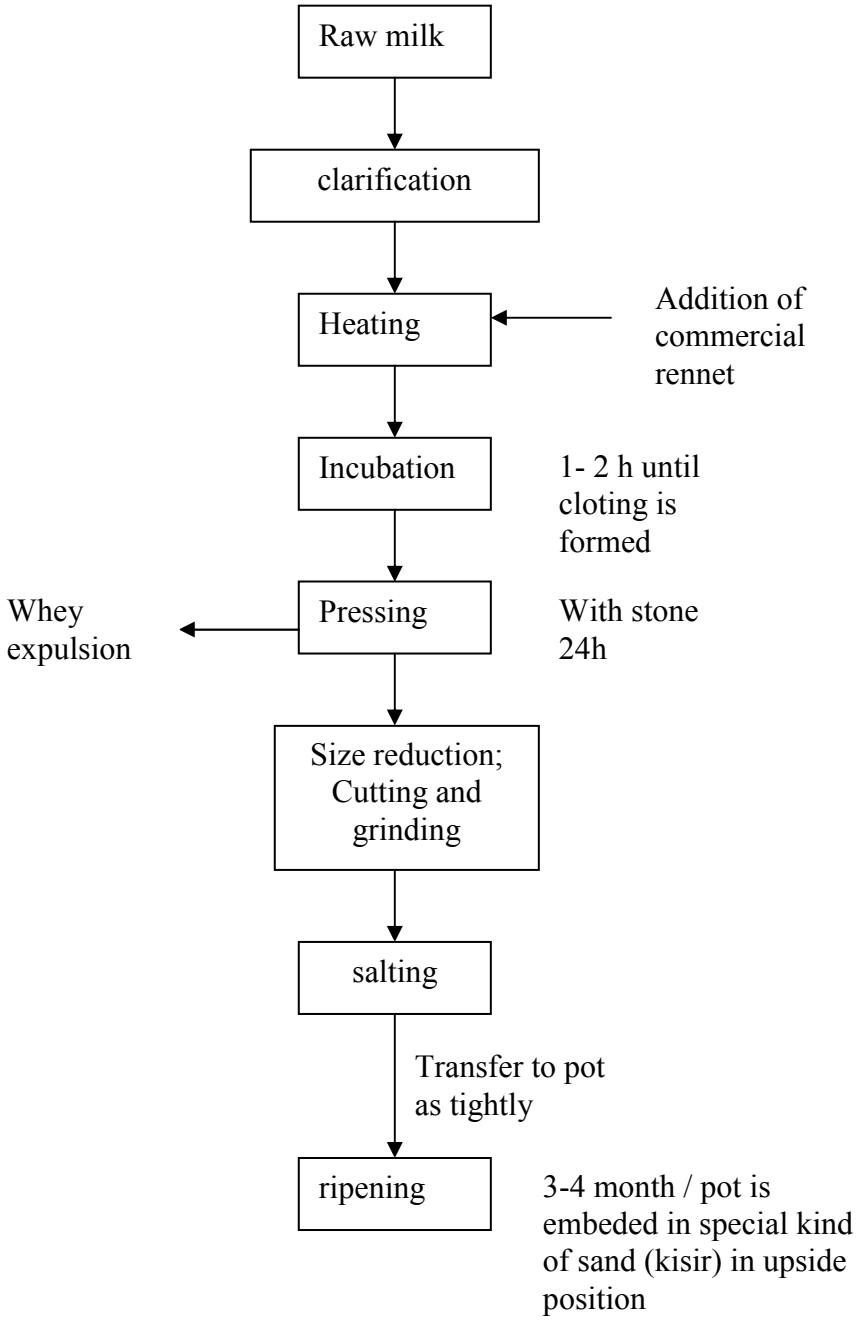

Figure1.1. Flow sheet of "Çömlek peyniri" making process

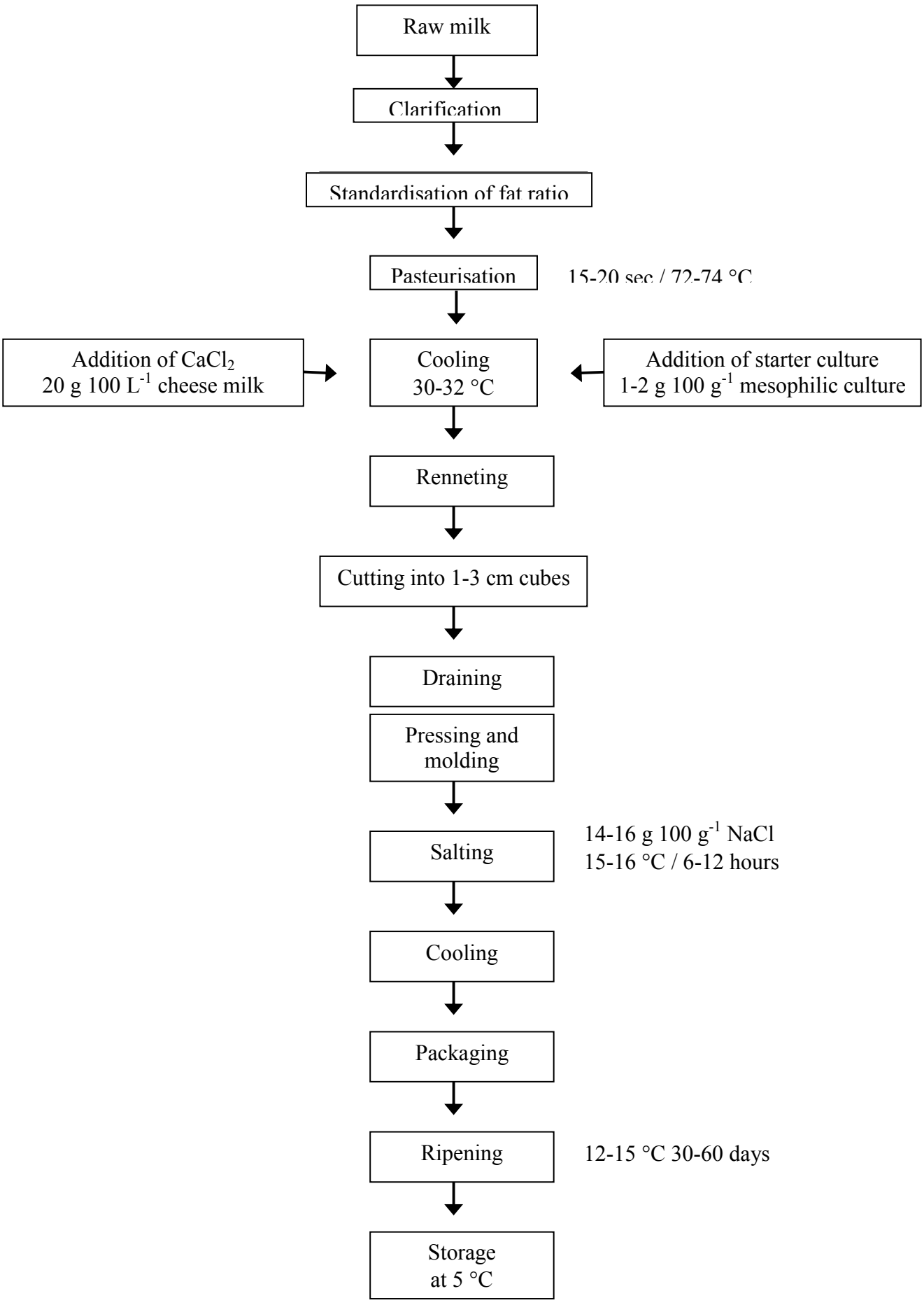

Figure 1.2. Flowsheet of "Beyaz peynir" manufacturing (Hayaloglu *et al*., 2002).

For the identification of novel starter strains, working with fresh cheese is very important because fermentation occurs at the beginning. Strains participate in fermentation process diminish immediately after fermentation. It is reported that at the fermentation step, starter strain amount may reach up approximately 10^9 colony forming units (cfu) per g of cheese. During ripening, however, the number of starter cells decreases about two orders of magnitude (Beresford *et al.,* 2001).

There have been many reports about the isolation of starter LAB from traditional cheese (Requena *et al.,*1991; Parente *et al.,*1997; Cogan *et al.,* 1997; Giraffa *et al*., 1999; Pérez *et al.,* 2000; Lopez Diaz *et al.,* 2000; Coppala *et al*.,2000; Menéndez *et al*., 2001; Proudromou *et al.,*2001 ;Alonso-Calleja *et al.,* 2002; Manopulou *et al.,* 2002; Mannu *et al.,*2002; Bouton *et al.,* 2002).

European Union has developed a project under ECLAIR Programme, AGRE-0064 for the isolation of new starter cultures from traditional cheese and fermented milk accross Europe. This project involved 10 laboratories in 7 countries (Portugal, Spain, Italy, Greece, France, The Netherlands and Ireland). One of the assumptions which was made before this project began was that the LAB in these cheeses would be different from those that have been in use as starters. A total of 4,379 strains of LAB were isolated and characterized from 33 products. More than 90% of these isolates have been characterized and cataloged (Cogan *et al.,* 1997) .

Traditional LAB flora of Turkey still waits for scientific attention and because of uncontrolled industrialization, the folkloric knowledge in cheese making could be lost in the near future. Therefore, biodiversity of LAB must be characterized and the isolates should be preserved for long term use. In this study it was thus aimed to isolate LAB in Capadoccia region.

CHAPTER 2

STARTER LACTIC ACID BACTERIA

LAB are widespread in nature, their nutritional requirements are very complex. Hence, they predominate habitats that rich in carbohydrates, protein breakdown products, vitamins and environments with low oxygen. This confirms the prevalence in dairy products (Stiles and Holzapfel, 1997).

Generally, lactic acid bacteria (LAB) can be defined as Gram positive, non-spore forming, catalase negative, devoid of cytochromes, acid tolerant, and facultative anaerobe group that produce lactic acid as the major end-product during fermentation of carbohydrates. According to carbohydrate metabolism, they can be divided into two main groups:

1. Homofermentative LAB (produce mainly lactic acid).

2. Heterofermentative LAB (produce lactic acid, carbon dioxide, ethanol and/or acetic acid).

This classification is originated from metabolic routes that organisms used and resulting end product (Figure 2.1). While homofermentives use glycolysis (Embden-Meyerhof Pathway), heterofermentives use the 6-phosphogluconate/phosphoketolase Pathway (Garvie, 1984).

Although LAB are comprised of 11 genera, only 6 of them are dairy associated. Theese are *Lactococcus, Enterococcus, Streptococcus, Leuconostoc, Pediococcus,* and *Lactobabillus.* (Axelsson, 1998; Garvie , 1984)

Cheese microflora is further divided into two groups. Primary group includes starter flora which refer to stater LAB and secondary group includes non starter lactic acid bacteria (NSLAB), propionic acid bacteria (PAB), smear bacteria, moulds and yeasts (Beresford *et al.,* 2001). In this study, it was focused on starter LAB. Starter strains in industrial terms can be defined as isolates which produce sufficient acid to reduce the pH of milk to <5.3 in 6 h at 30- 37 °C (Beresford *et al.,* 2001).

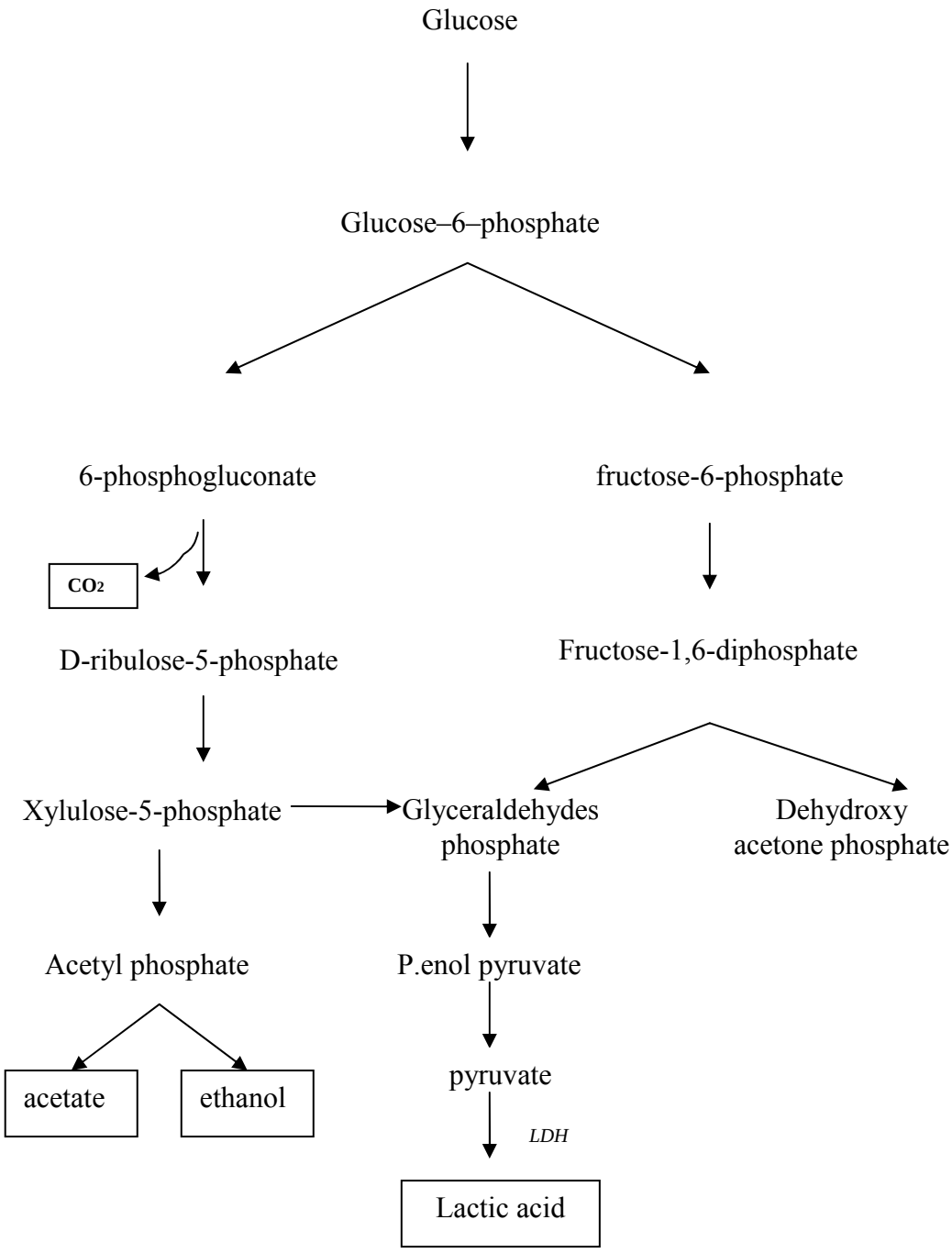

Figure 2.1. Glucose utilization metabolic pathways of LAB

2.1. Main Groups of Lactic Starters in Cheese Industry

In dairy industry, starter cultures can be divided into three groups as;

1. Mesophilic starter cultures
2. Thermophilic starter cultures
3. Artisanal starter cultures

Each of them can be divided futher: undefined cultures, in which number of strains is unknown, and defined cultures which are composed of a known number of strains (Axelsson, 1998). On Table 2.1. some defined starter species were shown.

2.1.1. Mesophilic Starter Cultures

Mesophilic group of LAB have an optimum growth temperature at 30 °C. They are composed of species which belong to the two genera, *Lactococcus* and *Leuconostoc. Lactococcus lactis* ssp *lactis*, *Lactococcus lactis* ssp. *cremoris* which are acid producers and *Lactococcus lactis* ssp. *lactis* var. *diacetylactis*, *Leuconostoc lactis* , *Leuconostoc cremoris* which are flavor producers. According to the nature of flavor produced (citrate positive strains), mesophilic cultures can be divided into 4 group;

1. O type, containing only *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris.*
2. D type, containing citrate fermenting species as flavor producers only *L. lactis* ssp. *lactis* var. *diacetylactis.*
3. B (or L) type, containing citrate fermenting species as flavor producers only *Leuconostoc.*
4. BD (or LD) type, containing both flavor producers as *L. lactis* ssp. *lactis* var. *diacetylactis* and *Leuconostoc*.

Table 2.1. Examples of starters used for specific type of cheeses (Marshall *et al.*,1984).

	CHEESE PRODUCT	MICROORGANISMS ADDED
1	Parmesan, Romano	Mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*
2	Cheddar	*L. lactis* ssp. *lactis; L. lactis* ssp. *cremoris L. lactis* ssp. *lactis* var. *diacetylactis.*
3	Swiss, Emmental	Mixture of *Lactobacillus bulgaricus* (or *Lactobacillus lactis* or *Lactobacillus helveticus*) and *Streptococcus thermophilus* and *Propiani bacterium shermanii*
4	Provolone	Mixture of heat-resistant *Lactobacillus* species and *Streptococcus thermophilus*
5	Blue, Gogonzola, Roquefort, Stilton	*L. lactis* ssp. *lactis* plus *Penicillium roqueforti*
6	Camembert	*Lactococcus spp*. plus *Penicillium camembert* (caseirolum)
7	Brick, Limburger	Mixture of *Streptococcus thermophilus* and *L. lactis* ssp. *cremoris*; mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*; mixture of *L. lactis* ssp. *lactis* and *Streptococcus thermophilus*
8	Muenster	Mixture of *Streptococcus thermophilus* and *Lactobacillus species*
9	Gouda, Edam	*L. lactis* ssp. *lactis* , *L. lactis* ssp. *cremoris Leuconostoc*
10	Mozzeralla	Mixture of heat-resistant *Lactobacillus* species and *Streptococcus thermophilus*
11	Cottage cheese, Cream cheese	*L. lactis* ssp. *lactis* or *L. lactis* ssp. *cremoris* ; mixture of *L. lactis* ssp. *lactis* or *L. lactis* ssp. *lactis* and *L. lactis* ssp. *lactis* var. *diacetylactis.* (or *Leuconostoc* species)

Mesophilic starter cultures are used in the manufacture of broad range of cheese type. It was estimated that two thirds of the milk fermentation is mesophilic type. The dairy industry is concerned with strains which ferment milk as rapidly as possible. This appears to be a property of Lactococci. Strains of other species will lower the pH of milk at a much slower rate (Cogan, 1996).

After fermentation of milk, the autolysis of the lactococci is occured during following ripening times. Autolysis of starter cells is due to a muraminidase(Beresford *et al.,* 2002). This could be originated by NaCl concentration and associated salt in moisture values of cheese(Beresford *et al.,* 2002). Autolysis of cells results release of intracellular enzymes into cheese and cause free amino acid production. So, this initiates a number of flavor reactions. If starter bacteria reach too high a population or survive too long, flavor defects such as bitterness could become a problem.

2.1.2. Thermophilic Starter Cultures

They are used in making cheese types where high cooking temperatures are required (Emmental, Gryere, Grana, Comte). The thermophilic LAB belong to two genera which are *Lactobacillus* and *Streptococcus*. Although *Lactobacillus* is a large group that consists of 64 species with both homo- and heterofermentative characteristic. Only a few of them are involved in milk fermentation. The commercial lactobacilli starters mainly consist of *Lactobacillus delbrueckii* ssp *bulgaricus*, *Lactobacillus delbrueckii* ssp *lactis* and *Lactobacillus helveticus* which are obligate fermenters.

On the other hand, *Streptococcus thermophilus* is only one dairy and food associated species among 27 *Streptococcus* species. Until recent times, it was described as *Streptococcus salivarus* ssp. *thermophilus* because it was observed to have a very close relationship with *Streptococcus salivarus.* After a more detailed DNA hybridization analysis, raised it to species level again (Schleifer *et al.,* 1991).

Str. thermophilus, *Lb lactis, Lb. bulgaricus* do not metabolize galactose and so lactose metabolism by *Str. thermophilus* results in the galactose accumulation in medium. Therefore, it is suggested that only the galactose fermenting lactobacilli should be used as starter together with *Str. thermophilus* (Mäyra-Mäkinen and Bigret ., 1998).

2.1.3. Artisanal or "Natural" Starter Cultures

Artisanal cultures are derived from using part of a previous batch of fermented product to inoculate a new batch. For instance, Kopanisti which is a Greek cheese variety, is produced by mixing cheese from a previous batch with drained curd of new batch. For some Italian and Swiss cheese varieties, whey from the previous day's manufacture is incubated in selective conditions such as high incubation temperature and low pH to obtain "whey starter". It is obvious that their composition is very complex, relatively variable and often undefined. Several types of species may be present. Although their variable performance contrasts with current trends in starter technology where consistent performance is required, their replacement by defined starter systems has sometimes results in less flavor (Axelsson,1998)

In this group, beside the classical starters, *Enterococcus* species are often present in significant numbers. They have some advantages, because they produce acid rapidly, withstand the cooking temperatures, have high tolerance to salt. The main disadvantages are that they are fecal origin and some strains are considered to be pathogenic. However, it was concluded that enterococci represent the dominant microflora of raw milk traditional cheeses and should be used as starter in order to produce the typical characteristic (Lopez-Diaz *et al*., 2000). *Enterococcus feacalis* has been used to accelerate ripening and improve organaleptic characteristics of cheeses (Neviani *et al*., 1982; Hegazi , 1989a; Villani and Coppala,1994; Tzanetakiset *et al*., 1995). It has been found that they have beneficial effects on the growth of other LAB species because of their intense proteolytic activity (Lopez-Diaz *et al*., 2000).

So far, Pediococci have not been used in any dairy starters although they may be found in ripened cheese samples. Only two species *Pediococcus pentosaous* and *Pediococcus acidilactici* are found in dairy products. Recently, lactose positive pediococci have been used instead of *streptococcus thermophilus.* Because *streptococcus thermophilus* is much more susceptible to bacteriophage infections (Garvie,1984)

Table 2.2. Starter bacteria used in Turkish "beyaz peynir" (Hayaloğlu *et al.*, 2002)

Microorganisms added	References
Lactococcus lactis subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis* + *Leuconostoc cremoris*	Ucuncu (1971); Celik(1982)
Enterococcus durans 41770 + *Lactobacillus delbrueckii* subsp.*bulgaricus* CH2	Tunail (1978)
Lactococcus lactis subsp. *cremoris* +*Lactobacillus casei* + *Lactobacillus plantarum*	Ergullu (1980)
Lactococcus lactis subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis*	Kaymaz (1982); Tekinsen (1983), Kurt(1991)
Lactococcus lactis subsp. *cremoris* +*Lactococcus lactis* subsp. *lactis* + *Lactococcus lactis* subsp. *lactis* +*Lb .casei*	Yildiz,Kocak,Karacabey and Gursel(1989)
Lactococcus lactis subsp. *lactis* +*Lactococcus lactis* subsp. *cremoris* + *Lactobacillus sake*	Akgun(1995)
Lactococcus lactis subsp. *lactis* +*Lb .casei* and/or *Lb.plantarum*	Ucuncu(1999)
Lactococcus lactis subsp. *lactis* +*Lactococcus lactis* subsp. *cremoris* + *Lactobacillus helveticus*	Gursoy,Gursel,Senel, Deveci, and Karademir(2001)

2.2. Starter Functions

2.2.1. Acid Production

LAB use carbohydrate fermentatively and produce lactic acid. Lactic acid production leads decrease in pH . Fermentation of sugars that cause leading to pH decrease is important for clotting of milk. Beside, increasing acidity initiates following desirable reactions and changes such as whey expulsion. Because there is a correlation

between pH and whey expulsion from curd. Additionally, acid production has beneficial effect on formation of texture, aroma and flavor (Ross *et al.*,2000)

2.2.2 Proteolytic Activity

Proteolysis is an important event that occurs during cheese ripening. The lactic acid bacteria use the polypeptides. These polypeptides are generated by milk clotting enzymes and by bacterial cell-wall proteins. Rennet which is the milk clotting enzyme, is responsible for casein degradation. Because of the casein degradation peptides are produced which are transported into the cell. In the cell, peptidases continue degradation to produce smaller peptides and amino acids. It has been known that aminoacid composition plays an essential role in the aroma of cheese (Wouters *et al.,* 2002)

2.2.3. Flavor Formation

The quality of cheese and other fermented food products is dependent on the ability of flavor and aroma production of microorganisms which include starter culture. Flavor compounds produced by LAB can be divided into two groups;

- The compounds in fermented milk.
- The compounds present mostly in maturated cheese.

First group consists of organic acids such as lactic acid and acetic acid, which are produced by *L. lactis* ssp *lactis* and *L. lactis* ssp *cremoris.* Second group consists of acetaldehyde, diacetyl, acetoin, and 2-3 butylene-glycol which are produced by *L. lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc* species from citrate present in milk. It has been reported that these aroma compouns might be produced to avoid to pyruvate accumulation in the cell. Moreover, improved knowledge of proteolysis and peptidolysis in cheese, analysis on enzymatic systems of LAB and evaluation of different strains, will provide better understanding between flavor development and starter activity. A number of different LAB have been evaluated for their ability to degrade amino acids to aroma compounds. *L. lactis* subsp. *lactis* and *L. lactis* supsp. *cremoris*, *Lactobacillius lactis*, *Lactobacillius helveticus*, *Lb. bulgaricus*, *Lb. casei* are capable of degrading methionine to methonethiol, dimethyledisulphide (DMDS) and dimethyltrisulphide (DMTS)(Yvon *et al.,* 2001)

2.2.4. Exopolysaccharide Formation

Many strains of LAB produce exopolysaccharides (EPS). These compounds can be produced as capsules(Cps) which are tighyly attached to the bacterial cell wall ,or as a loose slime (ropy polysaccharide) which is liberated into the medium (Mayra, Makinen and Bigret, 1998). EPS could be composed of one type of sugar monomer (homopolysaccharides) or consist of multi type of monomers (heteropolysaccharides) and coud be substituted organic or inorganic molecules(Broadbent *et al.,*2001).While several species of LAB (*L. lactis* ssp. *lactis*, *Lb*. *delbrueckii* ssp. *bulgaricus*, and *S. thermophilus*) produce heteropolysaccharides, homopolysaccharides are produced by a few organisms such as *Leu. mesenteroides.*

Due to the viscocity enhancing and stabilizing properties, EPS producing starter cultures are beneficial for industrial usage. They contribute texture development. It has been reported EPS producing starter strains that enhance the functionality of low fat cheese (Broadbent *et al.,* 2001).

2.2.5. Antimicrobial Property

LAB have been used as natural preservatives because of their antimicrobial capacity

a. Through fermentation products: Antimicrobial activity can be exerted through the reduction of pH or production of organic acids (lactic acid, acetic acid), CO_2, reuterin, diacetyl, 2-pyrorelidone, 5-carboxylic acid (PCA) (Mayra, Makinen and Bigret, 1998). Effective starter culture activity can prevent the pathogen and contaminant growth that may occur during cheese making process.

b. Through bacteriocins: Bacteriocins can be defined as protein antibiotics of relatively high molecular weight and mainly affecting the same or closely related species. Bacteriocins of LAB are classified into four group (Table 2.3.). It is known that LAB are generally regarded as safe microorganisms and so are their bacteriocins. Thus, these bacteriocins can potentially be used to control the growth of spoilage and pathogenic organisms in food (Cardinal *et al.,*1997). Bacteriocin producing lactococcal

strains have been used successfully as starter cultures for cheesemaking in order to improve the safety and quality of the cheese. In recent work, 79 wild lactococci have been studied and 32 of theese have been found to be antimicrobially active (Wouters *et al.,* 2002). In 17 of these strains, the well-known antimicrobial peptide nisin has been found, whereas the others produced diplococcin , lactococcin or a unidentifed bacteriocin-like compound. Moreover, the use of nisin as an effective preservative in processed cheese has been widely accepted.

Table 2.3. Classes of bacteriocins Produced by LAB.

Class	**Subclass**	**Description**
Class I		Labntibiotics
Class II		Small(<10kDa),heat stable, non-lanthionine containing membrane-active peptides
	II a	Listeria- acttive peptides
	II b	Two- peptide bacteriocins
	II c	Thiol-activated peptides
Class III		Large (>30 kda) heat-labileproteins
Class IV		Complex bacteriocin:protein with lipid and /or carbohydrate

2.3. Commercial Production of Dairy Starter Cultures

Starter cultures are essential for industrial production of all kinds of cheese. Before adding to milk, cultures have been pre-grown in milk or milk-based media. Depending on the cheese type, the inoculation volume varies from 0.2% to 2% of volume of milk (Cogan,1996). Each year ~12.5 x 10^{10} tons of milk are used in order to produce ~12.5 x 10^{6} tons of cheese worldwide. If it is assumed that 0.5% (v/v) in ratio inoculums used for each type of cheese, it indicates that ~6.3 x 10^{8} L starter is required. When it is considered bulk starter cultures contain ~1 x 10^{9} cells/ml, it can be easily understood that ~6.3 x 10^{20} cells are required worldwide. Commercial production of dairy starter cultures refers this mother culture.

1. Liquid Starter Culture. This is a traditional method which is based on the following procedure. First the starter is cultivated as a liquid stock culture, and then sufficient volume is obtained by subculturing. This method has advantages, if the production area and laboratory that provide culture is close. On the other hand, contamination risk due to the number of inoculations, phage infection could be faced. Strains which are kept in liquid media can easily loose its starter properties also.

2. Air Dried Culture. This method is based on the principle of adsorption of liquid cultures on a special material and drying by pulverization under the vacuum. However, during the vacuum pulvarization most of the cells can die.

3.Freeze Dried Starter Cultures (Lyophilization). Lyophilization is a process in which the product is first frozen so that a matrix is formed in which the solvent is crystallized and separated from its solute. Solvent is then removed by sublimation and desorption. Despite the advantages as easy to use, significant amounts of cell injury and death occur.

4. Freeze-Dried Concentrated Starter Culture. This method aims of direct inoculation of the milk. Freeze-dried concentrated cultures are being used extensively in Europe while in US has limited usage. This is because of that this type of starters require more time to reach log phase of growth.

5. Frozen-Concentrated Starter Cultures. Frozen concentrated starters usually contain 10^{10} to 10^{11} cfu/g. In this method, the most critical point is rate of thawing in order to minimize cell injury. It is important to thaw the samples rapidly as possible.

2.4. Genetically Modified Lactic Acid Bacteria and Culture Improvement

Due to the considerable economical importance of LAB, culture improvement studies have been accelerated in recent years. Progress in gene technology has allowed this development. Modification has been achieved by introducing new genes to improve bacteria that better fitted to technological processes or enhanced organoleptic properties. It is expected that better understanding of the genetics and physiology of LAB will give rise to better strain use, selection and improvement(Ross *et al.*,2000)

Construction of bacteriophage resistant strains is very important. The resistance mechanisms are often carried out by plasmids and transposons. Some high level resistance plasmids were shown to carry more than one resistance mechanisms (Coffey *et al.,1994*).

In some cases , the starter strains have been engineered for autolysis. These cells will lyse at an appropriate moment during cheese making. Lysis allows the release of many enzymes into cheese matrix that leads to degradation of peptide to amino acids. These free amino acids are the precursors of aromatic substances (Renault, 2003).

Moreover, it is possible to modify genes or transfer genes from food LAB into other lactic starter strains. For instance, a heterologous catabolic glutamate dehydrogenase (GDH) gene from *Peptostreptococcus asaccharolyticus* has been introduced into *L. lactis* to allow the production of alpha-ketoglutarate from glutamate. This is an amino acid present at high levels in cheese. Amino acid degradation requires alpha-keto acid as the amino group receptor. So that, availability of alpha-keto acid is limiting factor for conversion of amino acids to aroma compounds. On the other hand GDH-producing strains produced a higher proportion of carboxylic acids that are major aroma compounds. Therefore, they not need alpha-ketoglutarate supplementation (Renault, 2003).

CHAPTER 3

IDENTIFICATION METHODS FOR DAIRY BACTERIA

3.1. Phenotypic Methods

In order to identify LAB, phenotypic methods which include morphological examinatinations, physiological and biochemical tests are widely used.

3.1.1. Morphological Methods

Microscopic examination is first criteria that provide information about genus level, purity of lactic acid bacteria. There are some staining methods in order to differentiate the cells such as simple stain, gram stain, acid fast stain, endospore stain, capsule stain. The most important and widely used method is Gram staining. On the basis of the reaction to Gram stain, bacteria can be divided into two large groups; Gram positive organisms and Gram negative organisms. LAB belong the Gram positive group. Rounded or spherical cells are called cocci, elongoted rod shaped cells are called bacilli, ovoid cells, intermediate in shape between cocci and bacilli are called cocobacilli, cell division in two perpendicular directions in a single plane that lead to tetrad formation are called tetracocci (Garvie, 1984).

3.1.2. Physiological and Biochemical Tests

Orla-Jensen (1919) achieved basis of classification of LAB. Their work has had a large impact on the systematic of LAB. It is obvious that there have been several revisions as considerable extent for classification of LAB. However, the basis of classification is remarkable unchanged and includes following physiological and biochemical criteria (Stiles and Holzapfel, 1997).

1.Mode of glucose fermentation (homo or heterofermentation)

2.Growth at certain “cardinal” temperatures (e.g. 10 °C and 45 °C)

3. Range of sugar utilization

Therefore, these characters are still very important in current classification of LAB.

An important character used in differentiation is mode of glucose fermentation under standard conditions. Standard conditions refer to nonlimiting concentration of glucose, growth factors, limited availability of oxygen .Under these conditions, LAB can be divided into two groups.

1. Homofermentative. Glucose is converted almost quantitatively to lactic acid

2. Heterofermentative. Glucose is fermented to lactic acid, ethanol, acetic acid, and carbon dioxide.

Another characteristic for primarily identification is growth at certain temperatures. At this point, lactococci can not grow at 45 ºC while enterococci can grow both at 45 °C and 10 °C. On the other hand, Steptococci do not grow at 10 ºC.

Additionally, growth in different salt concentration provide differentiation especially cocci shaped starter lactic acid bacteria. As it is presented Table 3.1, this character is used as useful tool in order to differentiate *Lactococci, Streptococci* and *Enterococci*. Moreover, other characteristics which are arginine hydolysis, acetoin formation, bile tolerance, type of hemolysis, production of exopolysaccharides, growth factor requirements, presence of certain enzymes, growth characteristic in milk and serelogical typing are used biochemical characterization. Relatedly, *Lc. lactis* subsp. *cremoris* is dintiqueshed from *L. Lactis* ssp. *lactis* by inability to grow at 40 ºC, growth in 4% salt, hydrolyse arginine, ferment ribose (Axelson, 1998).

Table 3.1. Differential characteristics of the Cocci shaped LAB found in starter Cultures (Cogan, 1996).

	Isomer of lactate	Growth		
		10 ºC	45 ºC	6.5% NCl
Lactococcus sp.	L	+	-	-
Leuconostoc sp.	D	+	-	-
S. thermophilus	L	-	+	-
Enterecoccus sp.	L	+	+	+

3.2. Genotypic Methods

Identification of bacteria isolated from natural microflora involved in cheese fermentation has been limited by the complexity of the bacterial associations (Garvie, 1984). Additionaly, bacterial population involved has smilar nutritional and environmental requirements. At this point, the applications of molecular methods resolve identification problems. Nucleic acid probe technology could be an alternative for faster and more reliable differentiation. Several species-specific probes have also been designed.Furthermore, 16S or 23S rRNA targeted oligonucleotides have been used for the specific identification of LAB. It is important point that it is now possible identify various LAB in fermented food without cultivation step at species level within one working day (Scheifer, 1995). Additionally, DNA restriction fragment analysis and ribotyping have been used to distinguish LAB. Especially, polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR Ribotyping, and RAPD), pulsed-field gel electrophoresis can be used as main new molecular tools (Farber,1996 and Olive, 1999). Comparison of procedural steps on Table 3.2 provided better understanding of their principle.

Table 3.2. Procedural steps of main genotypic methods.

RAPD	PFGE	REP-PCR	AFLP	DNA Sequencing
PCR Amplification with a single primer	Embed organisms in agarose plug	PCR Amplification with REP or ERIC primers	R.E. Digestion	PCR Sequencing Reactions
↓	↓	↓	↓	↓
Gel Electrophoresis	Protease Digestion	Gel Electrophoresis	Linker Ligation	Gel Electrophores
↓	↓	↓	↓	↓
Gel Staining	R.E. Digestion	Gel Staining	Selective PCR	Computer aided sequence analysis
↓	↓	↓	↓	↓
Interpretation	Electro-phoresis	Interpretation	Gel E. Through an Automated DNA Sequencer	Interpretation
	↓		↓	
	Interpreta-tion		Gel Interpretation	

In the last years, characterization of LAB from various sources is maintained by combining of phenotypic methods (physiological and biochemical tests) with molecular methods. Therefore, characterization of starter culture at the strain level could be possible. This is very useful from technological aspects of dairy industry. Hebert *et al.*, has been characterized natural isolates of *Lactobacillus* by respectively, physiological and biochemical test , SDS- PAGE of whole cell proteins, and sequencing of variable region (V1) of the 16S ribosamal DNA in 2000.

For another recent work, lactic acid bacteria from artisanal Italian cheese have been characterized by combining use of PCR 16S-23S rDNA and sequencing. In this study, also a few main phenotypic methods such as salt tolerance, growth at different temperatures and production gas from glucose tests have been performed (Ayad, *et al*., 2001).

For another work, DNA fingerprints of thermophilic lactic acid bacteria generated by repetitive sequence based polymerase chain reaction have been applied (Uriaza *et al*. 2000). They described BOX- PCR base method for characterization.

Ventura *et al*., studied *Lactobacillus johnsonii* by using PCR based methods and pulsed field gel electrophoresis (PFGE). They used enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), Rep-PCR, amplified fragment length polymorphism, PFGE. It was considered that PCR fingerprinting methods such as ERIC-PCR, Rep-PCR and TAP-PC are rapid and easy to perform.

3.2.1. Randomly Amplified Polymorphic DNA (RAPD)

RAPD refers to randomly amplified polymophic DNA which is one of the genotypic methods that based on the polymerase chain reaction. In the PCR, two synthetic oligonucleotide primers are needed to initiate synthesis of new DNA strand .RAPD is very simple and quick genotypic method (Farber, 1996). The PCR that is used for RAPD differs from common PCR because of the some aspects,

1. The primers that used are very short.
2. Sequences are chosen at random.
3. Annealing temperature for RAPD is lower than a normal PCR.

3.2.2. PCR Ribotyping

In prokaryotes, the three genes coding for rRNA (16S, 23S and 5S rRNA) are separated by spacer region. When it is considered that most bacterial genera contain multiple copies of the operon for rRNA, hence the spacer regions within a single strain may differ in length and or sequence.

3.2.3. PCR -RFLP

It is based on the principle that related sequences of nucleotides can be compared by exposing them to the same restriction endonucleases. Electrophoresis and staining of fragments from a given sequence yields a characteristic fingerprint, sot that different sequences can be compared by comparing their fingerprints (Figure 3.1)

3.2.4. Rep-PCR

In this method, PCR is used to generate fingerprints by copying particular sequences in chromosome (rather than random sequences). Rep PCR refers to repetitive

extragenic palindromic sequence that occurs in different strains. Rep-PCR yields DNA molecules of various sizes. When they are separated by gel electrophoresis, these molecules give a characteristic fingerprint.

3.2.5. Pulsed Field Gel Electrophoresis

In this method, the genomic DNA is cutted with a restriction enzyme then fragments are separated on an agarose gel. It provides resulation at subspecies and strain level. Firstly, live cells are embedded in agarose and then lysis is achieved. Hence, genomic DNA is digested with infrequent cutting restriction enzymes. This method provide alternative electrical field with predetermined intervals. Direction of electrical field is changed at these intervals. These intervals are called pulse times. So, based on this property higher molecular weights DNA fragments could be separated with this method.

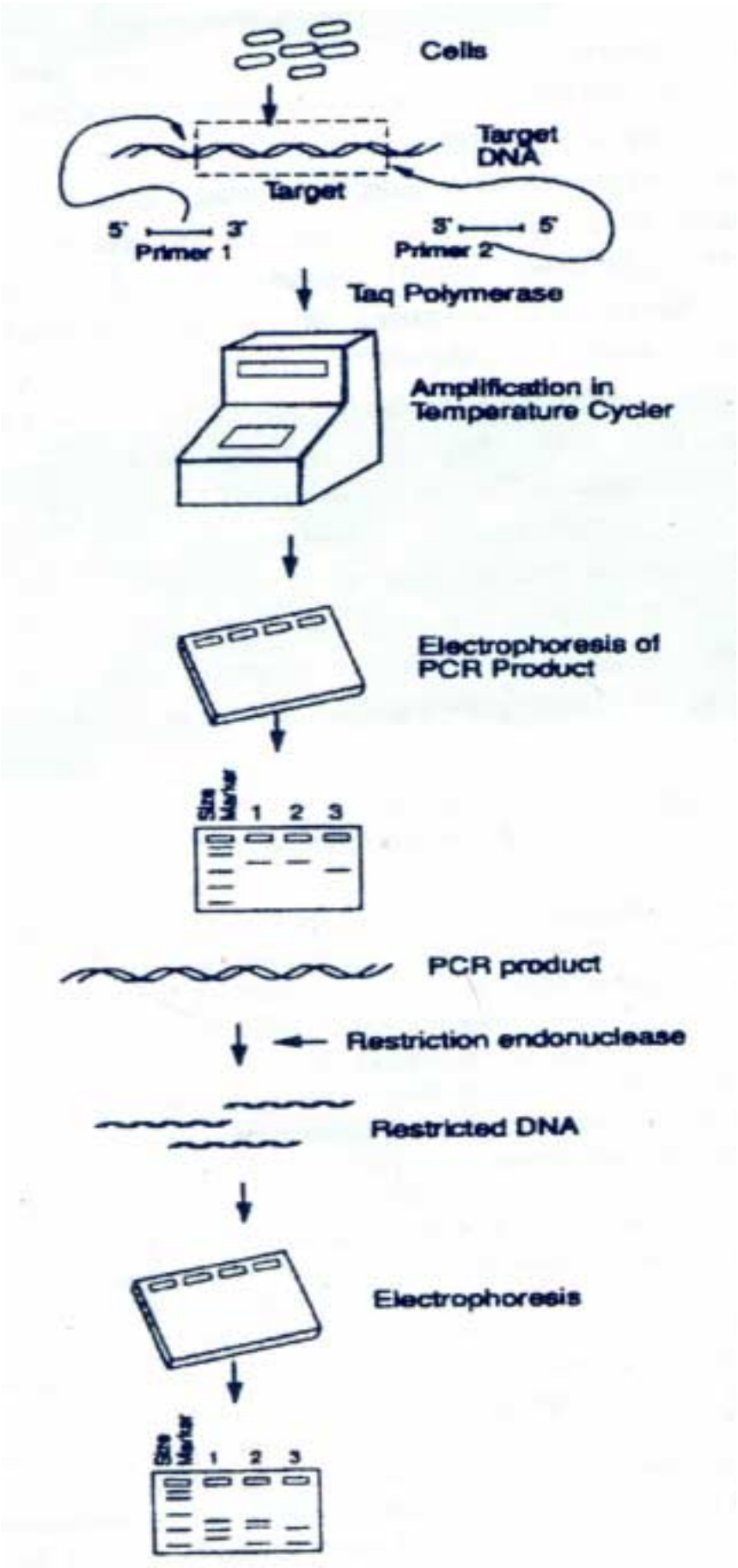

Figure 3.1. Polymease Chain Reaction-Restriction Fragment Length Polymorphism (Farber, 1996)

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

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

4.1.2. Samples

In this study, twenty cheese samples were analysed. Although nineteen of these were collected from seven different villages of Nevşehir, only one sample was obtained from Dinar/Afyon. Majority of the samples were fresh cheese and the rest of them were ripened cheese. Moreover, some samples were obtained from different steps of cheese making process. When it is considered the type of cheese, "Çömlek Peyniri" was focus of the study. Beside, two of traditional "beyaz peynir" was also be analysed (Table 4.1).

Table 4.1. Sample types and location

Sample No	Location	Age of sample	Source	Type of milk
S1	Sulusaray	2 months	pot	cow
S2	Sulusaray	3 months	pot	cow
S3	Sulusaray	4 days	Pressing step	cow
S4	Sulusaray	1 day	Pressing step	ewe
S5	Çat	3 months	pot	cow
S6	Çat	3 months	pot	cow
S7	Çat	15 days	pot	cow
S8	Çat	2 days	After salting	cow
S9	Çat	2 months	pot	cow
S10	Nar	3 months	pot	cow
S11	Üçhisar	2 months	White brined cheese	cow
S12	Üçhisar	1 day	From pot	pot
S13	Üçhisar	2 months	White brined cheese	cow
S14	Üçhisar	2 months	pot	cow
S15	Ayhanlar	1 week	After salting	cow
S16	Göre	3 months	pot	ewe
S17	Çardak	1 day	Before pressing	ewe
S18	Çardak	2 months	pot	cow
S19	Çardak	1.5 months	From plastic storage material	cow
S20	Dinar	3 months	From nylon storage material	cow

4.1.3. Reference Strains

Reference strains used in the study as fallows:

1. *Lactococcus lactis* subsp. *lactis* CECT 4432 which was provided by Professor Dr. Frederico Uruburu, Collecion Espanola de Cultivos Tipo (CECT).

2. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CECT 4431 provided by Professor Dr. Frederico Uruburu, Collecion Espanola de Cultivos Tipo (CECT).

3. *Lactococcus lactis* A 216 provided by Professor Dr. Sevda Kılıç, Department of Dairy Technology, Ege University.

4. *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T provided by Collecion Espanola de Cultivos Tipo (CECT).

5. *Lactobacillus curvatus DSM 8768* was provided by Professor Dr. Şebnem Harsa, Biotechnology and Bioengineering Department, Izmir Instute ofTechnology.

6. *Lactobacillus casei* subsp. casei NRRL-B 1922 provided by Professor Dr. L. K. Nakamura, National Centre for Agricultural Utilization Reseach.

7. *Lactobacillus delbrueckii subsp. lactis* NRRL-B 735 provided by Professor Dr. L. K. Nakamura, National Centre for Agricultural Utilization Reseach.

Finally, following strainswere provided by Dr. Ömre Sıkılı and Prof. Dr. Mehmet Karapınar, Foog Engineering Department, Ege University.

8. *Enterococcus faecium* CECT 4102
9. *Enterococcus faecalis* CECT 184
10. *Enterococcus gallinarum* CECT 970
11. *Enterococcus casselifavus* NRRL- B 3502

4.2. Methods

4.2.1. Isolation of Lactic Acid Bacteria

4.2.1.1. Culture Media and Growth Conditions

Samples were analysed by the dilution pour plate method. For this purpose, 10 grams of each sample were weighed aseptically and homogenised in 90 ml of sterile quarter-strength Ringer's solution in a hand made stomacher. Then, sequential decimal

dilutions of the homogenate were obtained. One ml aliquot of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were used for the isolation of LAB. Following culture media and conditions (pH and incubation temperatures) were used. Every sample were plated into these three media.

1. MRS agar (pH 6.2-6.8) ; isolation and enumeration of lactobacilli
2. M17 agar (pH 7.15) ; isolation and enumeration of streptecocci and lactococci
3. Slanetz Bartley Medium (pH 7.2) ; isolation and enumeration of enterecocci

MRS plates were incubated under microaerophilic conditions using gas generating kit (Oxoid). All plates were incubated for 3 days at 30 ºC. After the incubation, the plates with colony forming units (CFU) ranging from 30 and 300 were selected for enumeration. After the colony counting , the numbers were expressed in logaritmic scales ($logCFUg^{-1}$). Standart deviations and mean values were calculated using Microsoft excel computer programme.

4.2.2. Phenotypic Identification

4.2.2.1. Selection of the Isolates According to Their Fermentative Properties

Colonies were taken from the plates at 10^{-6} and 10^{-7} dilutions and were transferred into 10-12% sterile skim milk broths. For this purpose, 120 g skim milk powder were weighed and the volume adjusted to 1 L with deionized water and the pH was adjusted to 6.7-6.8. The solution was strelized for 10 min at 113 ºC. Before use, skim milk broths were then preincubated for 24h at 37 ºC for control of sterility. Selected individual colonies were inoculated into the skim milk broths and incubated for 24 h at 30 ºC. During the incubation, changes in the appearence of the skim milk were monitored periodically. When proper coagulation was achived, the incubation was terminated. Another 24 h incubation was allowed for those cultures which did not coagulate milk during the first 24 h. Isolates, which were able to coagulate milk even after 48 h incubation, were taken presumptively as homofermentative LAB. Others which show undesirable texture defects such as serum expulsion, and gas bubbles were eliminated.

4.2.2.2. Morphological Examination

4.2.2.2.1. Simple Staining

Isolates which produced coagulation were examined morphologically under the light microscope. At this point, simple staining method was used. This staining technique was consisted of following steps;

1. Transferring a loopfull culture broth onto microscope slide.
2. Drying.
3. Fixation by exposure to flame 2-3 times for 1-2 sec.
4. Staining with methylene blue for 1-2 min.
5. Washing under the tap water.
6. Drying by blotting onto cotton towels and drying.

After simple staining, cell shape and arrangements were examined. Additionaly, the purity was checked. Samples showing heterogenous cell morphology were eliminated. Isolates which have homogenous cell morphology were classified as cocci shaped and rod shaped. Cultures which were grown Slanetz Bartley medium were then transferred into *Streptococcus* cultivation broth. Cultures grown in M17 medium were transferred into M17 broths and cultures grown in MRS medium, transferred into both M17 and MRS broths. They were thus activated by subculturing at least for 3 times.

4.2.2.2.2. Colony Morphology

Isolates were further purified by streaking repeatedly on MRS agar plates, and the colony morphologies (color, shape and size) were examined by eye.

4.2.2.2.3. Gram Staining

The Gram status of the isolates was determined by light microscopy after the Gram staining. LAB are known to be Gram positive and the blue-purple color indicates the Gram positive nature of the bacteria.

Cells from fresh cultures have to be used for Gram staining. For this reason, 24 h cultures were grown in MRS broths at 30 ºC. Cultures were then transferred aseptically into 1.5ml eppendorf tubes. After centrifugation for 5 min at 6 000 rpm, and most of the

supernatant was removed. The cells were resuspended in the remaining approximately 10 µl phase and cells were then Gram stained by following procedure:

1. Transferring 10 µl cell suspension onto microscope slide.
2. Drying in open air.
3. Fixation by exposure to flame 2-3 times for 1-2 sec.
4. Primary straining with crystal violet for 1 min.
5. Washing excess stain under the tap water.
6. Applying of Gram's iodine mordant for 1 min.
7. Washing excess mordant.
8. Applying acetone /or alcohol (95%) decolorizing agent for 6 sec.
9. Applying counter stain (safranin) for 30 sec.
10. Washing excess safranin.
11. Drying by blotting onto cotton towels.

4.2.2.3. Catalase Test

Catalase is an enzyme produced by many organisms and therefore the lack of catalase is a significant diagnostic characteristic. The enzyme breaks down hydrogen peroxide into water and oxygen as below and gas bubbles are observed. The formation of gas bubbles therefore indicates the presence of catalase enzyme

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

LAB were known as catalase negative. Hence, in order to confirm catalase status of the isolates, catalase test was performed. For this purpose, overnight cultures of isolates grown on MRS agar plates at 30 ºC were used. Catalase activity was investigated by dropping 3% hydrogen peroxide solution (one drop) onto randomly chosen colony.

4.2.2.4. Long Term Preservation of the Isolates

Isolates showing homofermentative, Gram positive and catalase negative characteristics were preserved in MRS broth medium which contained 20% (v/v)

gycerol as frozen stocks at -80°C. Glycerol stock samples were prepared by mixing 0.5 ml of overnigh cultures, and 40% glycerol.

4.2.2.5. Physiological and Biochemical Identification

4.2.2.5.1. Identification of Cocci

Each isolate was activated in 5 ml MRS broth for 24 h at 30 ºC before use. Therefore, overnight cultures were used during all the identification procedures. Physiological and biochemical identifications were performed according to the methods and criteria of Sharpe and Fryer, (1969); Garvie (1984), Devriese *et al.*,(1995); Teuber (1995). The characteristic used for the identification of cocci shaped LAB in this study were presented (Table 4.2.). For all tests a negative control (non-inoculated media) was also used and respective reference strains were also included in the experiments as positive controls.

4.2.2.5.1.1. Gas Production from Glucose

In order to further define homofermentative isolates, CO_2 production from glucose test was performed. For this purpose, citrate lacking MRS broths and inverted Durham tubes were used. Fifty µl of overnight cultures were transferred into the 8 ml test media. After incubation for 5 days at 30 ºC, gas accumulation in Durham tubes was taken as the evidence for CO_2 production from glucose.

4.2.2.5.1.2. Growth at Different Temperatures

Fifty µl of overnight cultures were transferred into the tubes which contain 5 ml temperature test media (Appendix C1). After inoculation , they were incubated for 7 days at 10 ºC, 40 ºC or 45 ºC . Cells growth at any of these temperatures was dedected by the change in the color of the cultures, from purple to yellow.

4.2.2.5.1.3. Growth at Different NaCl Concentrations

Fifty µl of overnight cultures were transferred into the tubes which contain 5 ml NaCl test media (Appendix C2). Isolates were tested for growth at 2%, 4% or 6.5% NaCl concentrations. They were incubated for 7 days at 30ºC. The change of the color from purple to yellow taken as the evidence for cell growth.

4.2.2.5.1.4. Arginine Hydrolysis and Gas Production From Citrate

In order to perform this test, 8 ml of Reddy broth (Appendix C4) and inverted Durham tubes were used. Fifty µl of overnight cultures were inoculated into the Reddy broth and were then incubated for 5 days at 30 ºC.

1. Arginine Hydrolysis : The cultures which utilize arginine, change the color of the broth first to yellow due to the lactic acid production and to violet because of the ammonia production (Cardinal *et al.,*1997). On the other hand, the cultures which do not utilize arginine assume a deep-yellow color by producing lactic acid only.

2. Gas Prpduction from Citrate: The breakdown of the citrate results in production of carbon dioxide. Gas accumulation in inverted Durham tubes indicated citrate utilization.

4.2.2.5.1.5. Carbohydrate Fermentations

Isolates were also characterized on the basis of their sugar fermentation profiles. All the reactions were performed by using 96-well microtitre plates. (Parante *et al.,* 1997 ; Urraza *et al.,* 2000, López Díaz *et al.,* 2000). Fifteen different sugars were used (Appendix D1). For each test, strains were inoculated in 5 ml MRS broth (50ml/L), and were then incubated for 24 h at 30 ºC in order to obtain overnight cultures. After this, cultures were centrifuged for 10 min at 10 000 rpm. Pelleted cells were washed and resuspended in MRS (without glucose) containing bromocresol purple as the pH indicator. Forty µl of filter sterilized (0,22µm, Millipore) 10% sugar solutions were pipetted into each well . On to the sugar solutions , 160 µl of suspended cells were added. Thus, 2% final sugar concentration was obtained. Dublicate reactions were prepared for each of the sugar fermentations experiment. After 24 h incubation at 30 ºC, the results were read at 690 nm absorbance in an automated microtitre plate reader(Bio-

tek Instruments, Inc.). When the sugar fermentation was taken place, the color changed from purple to yellow and turbidity was inceased. Glucose fermentation included to positive control, and samples without sugar were used as negative control.

Table4.2. Differential characteristic of cocci shaped LAB(Garvie,1984,Teuber,1995,Devriese *et al.*,1995)

Characteristics	*L. lactis* ssp *lactis*	*L. lactis* ssp. *diacetylactis*	*L. lactis* ssp. *cremoris*	*L. raffinolactis*	*E. faecalis*	*E. faecium*	*E. avium*	*E. durans*	*S. thermophilus*
Growth at 10 °C	+	+	+	+	+	+	+	+	-
Growth at 40 °C	+	+	-	-	+	+	+	+	+
Growth at 45 °C	-	-	-	-	+	+	+	+	+
Growth in 2% NaCl	+	+	+	+	+	+	+	+	±
Growth in 4% NaCl	+	+	-	-	+	+	+	+	-
Growth in 6.5% NaCl	-	-	-	-	+	+	+	+	-
Hydolysis of arginine	+	±	-	-	+	+	+	+	-
CO_2 from citrate	-	+	-						
Acid formed from									
arabinose	±	-	-	±	-	±	-	+	-
ribose	+			+			+	+	-
xylose	±	-	-	±			-	-	-
lactose	±	+	+	+	+	+	+	+	+
maltose	+	+	-	+	+	+	+	+	-
sucrose	±	-	-	+	+	±		-*	+
trehalose	±	±	-	+	+	+	+	+	-
raffinose	±	±	-	+	-	±	-	D-	
mannitol	±	±	-	±	+	±	+	-	-
sorbitol	-	-	-	±	+	±	+	-	-
salicin	+	+	-	+	+	+	+	+	-
glycerol					+*	-	+	-	
galactose					+	+	+	+	
mannose					+	+	+	+	
glucose	+	+	+	+	+	+	+	+	+

D- means usually negative

* means in anaerobic condition

4.2.2.5..2. Identification of Lactobacilli

For the identification of rod shaped isolates, following tests were applied.

1. Gas poduction from glucose
2. Growth at different temperatures (15ºC, 45ºC)
3. Growth at 6.5% NaCl concentration
4. Arginine hydrolysis
5. Carbohydrate fermentation

Except for the arginine test, all the other tests were the same as those for cocci shaped LAB. In the arginine hydrolysis, arginine MRS broth (Appendix C6) was used instead of Reddy broth. Fifty µl overnight cultures were inoculated into 5 ml arginine test media, and were then incubated for 5 days at 30 ºC. After the incubation, ammonia production was detected by using Nessler reagent. For this purpose, 100 µl of culture broth were pipetted into each well of the microtitre plates and 100 µl of Nessler reagent were added. Immediate orange color formation was taken as the indication for ammonia production. No color change indicated that the strain could not hydrolyse arginine.

4.2.3. Genotypic Identification by PCR-RFLP

4.2.3.1. Genomic DNA Isolation

Genomic DNA was prepared by using the following procedure (Cardinal *et al.,* 1997). Ten ml overnight cultures were prepared in MRS broths. Cells were harvested in a microcentrifuge for 5 min at 6000 rpm. After this, they were suspended in 200 µl 1xTE buffer (pH8) containing 25% sucrose and 30 mg/ml lysozyme. The cell suspensions were then incubated for 1 h at 37 ºC. After the incubation, 370 µl, 1x TE (pH 8) containing Proteinase K (1mg/ml) 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% cetytrimethylammonium bromide, 0.7 M NaCl) , respectively. Lysed samples were incubated for 10 min at 65 °C. Chloroform extraction was performed twice using one equal 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 6000 rpm. The aqueous phase was transferred into a new eppendorf tube. Chloroform extractions were

performed twice. The aqueous phase was transferred into a clean eppendorf tube and the genomic DNA was precipitated by the addition of isopropanol (one equal volume). After that, precipitated DNA was transferred into a fresh eppendorf tube which contained 500 µl 70% ethanol, and washed. When DNA precipitate was not visible, isopropanol containing samples were centrifuged for 10 min at 6000 rpm to pellet genomic DNA. After washing, DNA was pelleted by centrifugation for 10 min at 6000 rpm. Ethanol was removed and the pellets were dried for 10 min at 37 °C. Dried pellets were dissolved in 100 µl 1xTE containing 100 µg/ml RNase. After incubation for 1h at 37°C, the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shock (80 °C 10 min to -20°C 20 min 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 6000 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 6000g. 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 6000 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 1x TE. 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.

4.2.3.2. Amplification of 16S rDNA and ITS(Internally Transcribed Spacer) Region by PCR Reaction

This method was based on the amplification of internally transcribed spacer region (ITS) situated between 16S and 23S ribosomal RNA(rRNA) genes, plus 16 S ribosomal DNA. For the amplification following primers were used;

1. Forward primer; EGE1: 5'-AGAGTTTTGATCCTGGCTCAG-3' (Mora *et al.,*1998)

2. Reverse primer ; L1: 5'-CAAGGCATCCACCGT-3' (Jensen *et al.,*1993)

While the forward primer is complementary to the 5´- end of 16S rRNA genes,the reverse is complementary to the 3´- end of ITS.

Two µl template DNA was used (500ng). Amplification was performed in a 50 µl total reaction volume (Appendix F1). PCR reactions were performed in a thermocycler, PTC-0150 minicycler (MJ Research Inc., USA) using the following program;

Step1: 94 °C for 5 min
Step2: 94 °C for 1 min (denaturation) } 40 cycles
Step3: 42 °C for 1 min (annealing)
Step4: 72 °C for 1 min (elongation)
Step5: 72 °C for 10 min (final extension)

4.2.3..3. Separation of amplified Fragments

After the completion of PCR reaction, amplified products were separated in a 0.8 % agarose gel. For this purpose, 0.8 g agarose was dissolved in 100 ml 1x TAE buffer by boiling. Agarose solution was cooled to nearly 40 °C. After cooling, 15µl ethidium bromide solution (10mg/ml) were added. The agarose gel was poured into the gel casting stand and the combs were placed. When the gel was solidified, the combs were removed. For loading, 10 µl of amplification products were taken below the mineral oil 2 µl of gel loading buffer (Appendix F4) were added. Starting from the second well, samples were loaded. After the loading of samples, 500 ng of DNA molecular weight marker (Gene Ruler, Fermentas) were loaded into the first well. Finally, electrophoresis was performed at 40 mA. Amplification products were visualised in a gel documentation system (Vilber-Lourmat, France). The presence of DNA fragments sized between 1500-2000 bp indicated that targeted amplification was achieved.

4.2.3.4. Purification of PCR Products

PCR amplification products were purified by chloroform extraction. Firstly, 100 µl 1xTE buffer was added to adjust the volume of the samples to 150 µl. Two hundred µl chloroform/isoamyl alcohol solution (Appendix E10) were then added and mixed thoroughly. After this, thesamples were centrifuged for 5 min at 4000 rpm. The aqueous phase was taken into a fresh eppendorf tube and chloroform extraction was repeated once more. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH5.2) and mixed thoroughly. After that, 450 µl of 99%

ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. After obtaining the DNA pellet, liquid phase was removed. Pellets were washed in 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm , ethanol was removed and pellets were dried for 10 min at 37 °C 10 min. Finally, pellets were dissolved in 55 µl 1xTE, and stored at -20°C.

4.2.3.5. Restriction Fragment Length Polymorphism (RFLP)

Ten µl of the purified amplification products were used for each of the restriction enzyme digestions. Digestion was performed in a 50 µl final reaction volume (Appendix F2). Two endonucleases were used. These were *Taq* I and *Hae* III. While *Taq* I digestion was performed at 65°C in a water bath , *Hae* III digestion was performed at 37 °C. Both digestion were performed overnight. Additionaly , in order to avoid evaporation at 65°C, *Taq* I restriction reactions were overlaid with mineral oil.

4.2.3.6. Purification of Digested DNA Fragments

After the restriction, the volumes of the samples were adjusted to 100 µl with 1x TE. Chloroform extraction was performed twice using two volume of chloroform/isoamyl alcohol solution. After the second extraction , aqueous phase was transferred into a new eppendorf tube containing 1/10 volume of 3 M (pH5.2) Sodium acetate solution and mixed. Two and half volume of 99% ethanol were added and mixed thoroughly. After centrifuging for 15 min at 7 000 rpm, liquid phase was removed, and DNA pellets were washed in 300 µl 70% ethanol. Ethanol was removed and samples were dried for 10 min at 37 °C. Finally , samples were dissolved in 15 µl 1xTE and 3 µl of 6x gel loading buffer were added. All samples were stored at -20°C until the electrophoresis.

4.2.3.7. Electrophoresis of Restriction Fragments

Restricted fragments were seperated in a 2.5% agarose gel. For this purpose, 2.5 g agarose were dissolved in 100 ml 1x TAE by boiling. After cooling nearly to 45°C, 20 µl of ethidium bromide (10 mg/ml) were added .The gel was poured into the gel casting stand and comb was placed. When the gel solidified, the combs were removed.

Before electrophoresis, agarose gel apparatus was washed with tap water and deionized water respectively. The tank was filled with 1x TAE buffer . Ten µl of the samples were loaded into each well and 500 ng of DNA molecular weight marker were loaded into the first well. Finally, electrophoresis was started using the following conditions . For the first 30 min, electrophoresis was performed at 60 mA, and the current was adjusted to 80 mA and the electrophoresis was allowed to last for about 2 h.

4.2.3.8. Interpretation of Results

After the elecrtrophoresis , RFLP patterns were observed in gel documentation system. Fragments were stored in Adobe Photo shop 7.0 and analysed by using BIO-ID ++ software(Vilber-Lourmat, France). The smilarity between strains was determined automatically by specifying the formula of Jaccard. Strain clustring was performed by the un-weighed pair group method with arithmetic averages, UPMGA, BIO-ID++.The dendogram was built up by using 15 % homology coafficient.

4.2.4. Technological Characterization of Isolates

4.2.4.1. Acidifying Activity of Isolates

Acid production ability in milk is one of the most important technological characteristic of LAB (Cogan *et al.,*1997, Herreros *et al.,* 2003). In order to determine acidifying activity, potantiometric (pH measurement) and titrimetric methods were applied (Sarantinopoulos *et al.*, 2001). For this purpose, isolates were activated from frozen stocks in MRS broth for 24 h at 30°C and 0.1 ml overnight cultures were inoculated in 10 ml of sterile UHT skim milk broths. Duplicate inoculations were prepared. After the $3h^{th}$, $6h^{th}$,$9h^{th}$ and 24 h^{th} incubations at 30 °C, 2 ml aliquots were taken aseptically, and used for the procedures

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4.2.4.1.1. Monitoring of pH

pH of samles were determined according to the potentiometric method. For this reason, pH meter with glass electrode was used (Hanna insturements). Before using, pH

meter was calibrated with buffer (pH7) 1 and (pH4) buffer 2. After the calibration, the glass electrode was soaked into each of the samples and pH values were recorded.

4.2.4.1.2. Monitoring Lactic Acid Production

Onto the 2 ml of aliquots, 1-2 drops of phenolphatlein solution were added as indicator. Samples were then titrated by using standardized (F = 0,8816) 0.1 N NaOH solutions. When the first trace of pink color was observed ,titration was terminated. Consumption of the 0.1 N NaOH was recorded. Each ml of 0.1 N NaOH equals to 9.008 mg of lactic acid. Finally, the results were expressed in mg/ml.

4.2.4.1.2.1. Standardization of 0.1 N NaOH and determination of factor value

Sodium hydroxide was standardized with standardized HCl. Firstly, 1N HCl was standardized with sodium bicarbonate. For this purpose; 0.2 g, 0.3g and 0.4 g sodium bicarbonate were weighed and dissolved in 100 ml deionized water. These solutions were titrated by using 1-2 drops of phenyl orange indicator. When the colorless solution was turned into pink ,titration was terminated and the volumes of HCl consumed were recorded as 4.6 ml for m_1=0.2 g, 6.6 ml for m_2=0.3g and 8.6 ml for m_3=0.3g. Samples were heated to boiling 1-2 s in order to remove carbon dioxide and then were cooled. If the color of the samples was steady, the volume of HCl used was taken as necessary amount for titration. If the color disappeared, titration was continued. Finally, F1, F2 anf F3 factors of HCl were calculated using the following formula.

$T = m \times S \times F \times N$

$F1_{HCl} = 0.8647$

$F2_{HCl} = 0.9335$

$F3_{HCl} = 0.9144$

$\Big\} F_{meanHCl} = 0.9042$

In order to obtain factor values of 0.1 N NaOH, standardized 1 N HCL solution was titrated by using 1-2 drops of phenol phenolphtalein solution as indicator. For this reason, 50 ml and 100 ml of 1 N HCl (F=0.9042) were used. After adding the indicator, 0.1 N NaOH added onto 1 N HCl. When the color changed from colorless to pink, the volumes of 0.1 N NaOH were recorded as 5ml (for V_1=50 ml) and 9.5 ml (for

V_2=100ml). As a result, factor value of 0.1 N NaOH was obtained using following equation.

$F_{meanHCl} \times N_1 \times V_1 = N_2 \times V_2$ $F1_{NaOH}$= 0.9042 } $F_{meanNaOH}$ = 0.8816

$F2_{NaOH}$= 0.8589

4.2.4.1.3. Evaluation of Results

First, the results of isolates (pH and lactic acid mg/ml values corresponded to 0, 3, 6, 9, 24 h) were grouped into 3 genera as Lactococci, Enterococci and Lactobacilli. Lactic acid production versus time and pH versus time graphs were drawn by using Microsoft Excell programme. Beside , standart deviations and mean values were calculated for each genus by using Excell programme.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Isolation of Lactic Acid Bacteria

Twenty cheese samples, were used in the study (section 4.2.1.). LAB were first grown on agar plates containing MRS (pH6.2-6.8), M17 (pH7.15) and Slanetz and Bartley media for 48 h at 30 ˚C and the colonies were counted (Table 5.1.).

The arithmetic means LAB counts of fresh samples on MRS, M17 and on Slanetz and Bartley medium were found to be higher than those of ripened cheese samples. Higher NaCl concentrations in ripened cheese disfavoured sensitive LAB and caused a reduction in their growth levels. For different types of cheese (Valdeteja raw milk cheese, Parmigiano Reggiano cheese, Orinotyri ewe's milk cheese, Kefalotyri cheese, and etc.) enumeration of LAB was achived by using media (Coppala *et al.*,2000; Alonso-Calleja *et al.*,2001; Prodromou *et al.*,2001). When our results were compared with those of authors, it was found similarities with Orinotyri ewe's milk cheese. Moreover, these studies indicated that mean log cfu/g counts of LAB had been increased from milk to curd and slightly decreased during the ripening. For this reason, isolation was achieved from mainly fresh cheese and curd in our study.

Table 5.1. Logarithmic Microbial Count (log cfu/g) and Standard Deviations

Type of media	Fresh cheese samples	Ripened cheese samples
MRS agar	9,05 (±0,51)	8,027 (±0,27)
M17 agar	9,11 (±0,50)	7,97 (±0,75)
Slanetz and Bartley agar	8,82 (±0,64)	6,67(±1,082)

5.2. Phenotypic Identification

5.2.1. Examination of Homofermentative Properties

Initially, 661 isolates were obtained and tested for coagulation of skim milk broths. Among these, 246 isolates showed better coagulation properties. Remaining isolates which could not coagulate skim milk and isolates causing textural defects such as gas bubbles were eliminated. Presumptively homofermentative LAB cultures were thus selected at this step.

5.2.2. Morphological Examination

Isolates were analyzed under the light microscope. At this step, cell shape (like cocci, ovoid, rod) and arrangements (like diplo form, chains form, tetrad form) were examined after simple staining. Eighty-two of the isolates showing heterogeneous cell shape were eliminated. Finally, 164 pure isolates which include 143 of cocci and 21 of bacilli were obtained.

5.2.3. Subculturing of Isolates

Subculturing is necessary to activate LAB. After three consecutive subculturing 20 of isolates could not be activated and therefore they were eliminated.

5.2.4. Gram Staining and Catalase test

Cultured cells of the isolated strains were subjected to Gram staining. It was found that all the isolated strains were Gram positive. All the strains also showed catalase activity. Thus, from 20 cheese samples, in total 134 LAB strains were isolated. On the figure 5.1. Gram staining of representative cocci shaped and bacilli shaped isolates were demonstrated.

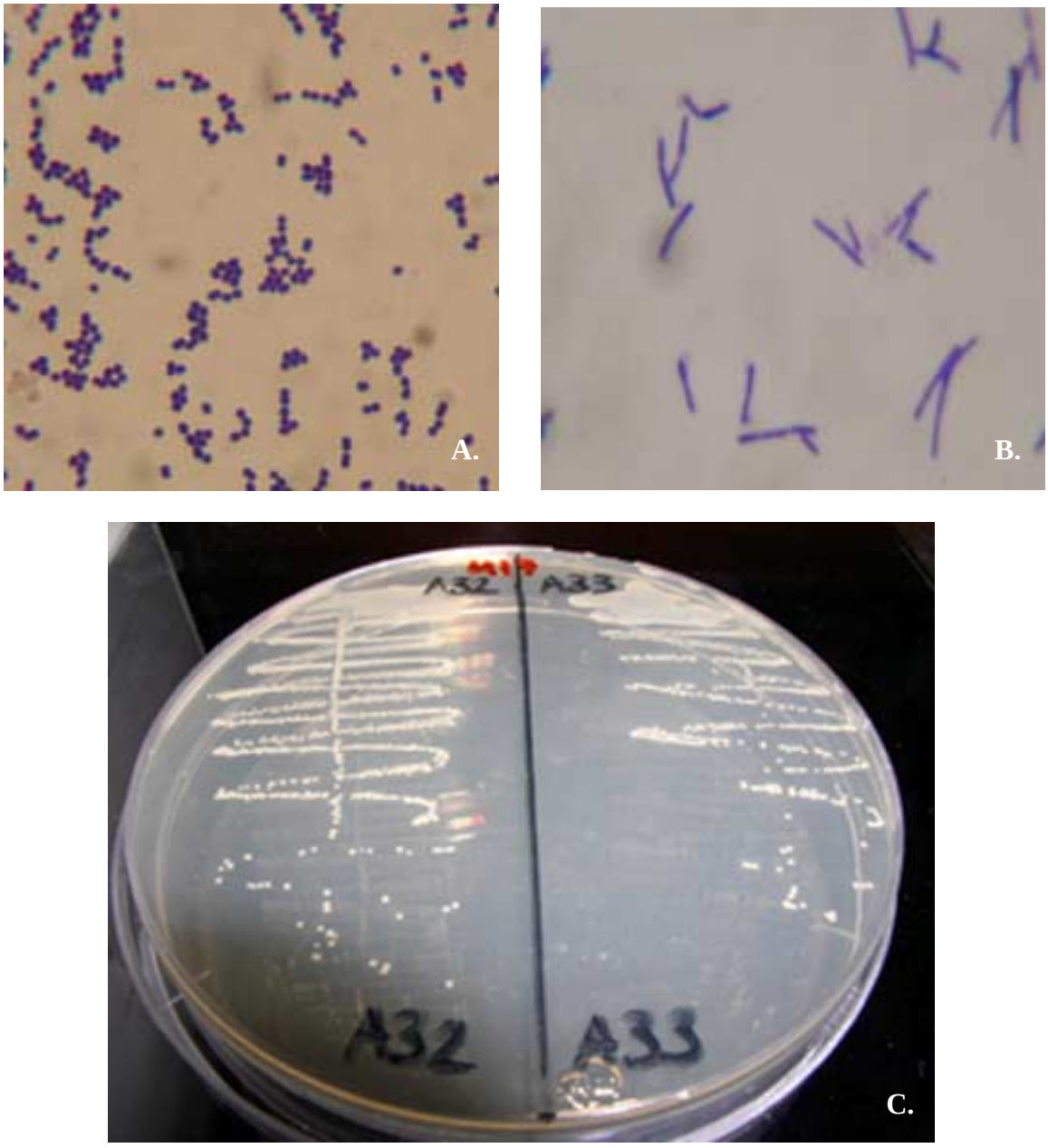

Figure 5.1. A. Gram staining of *L. lactis ssp. lactis* (C22**)** showing cocci shaped **, B.** Gram staining of *Lb.* casei (D2) showing rod shaped, **C.** colony morphology of typical LAB.

5.2.5. Physiological and Biochemical Tests

In order to identify coccus shaped isolates characteristics presented in Table 4.2. was used. The results of physiological and biochemical tests of the cocci and bacilli shaped LAB were given (Table5.4 and table 5.6). Furthermore, results were summarized (Table 5.3 and 5.5). Upon evaluating the results, 44% of the strains (59 isolates) were enterecocci, 40% of the strains were lactococci and 16 % strains (21 isolates) were lactobacilli.

5.2.5.1. Physiological and Biochemical Differentiation of Cocci shaped Isolates

In order to identify of cocci shaped isolates following tests were performed. Reference strains were subjected to similar tests.(Appeendix I1)

1. Carbon dioxide production from glucose
2. Growth at 10˚C, 40 ˚C, and 45˚C and
3. Growth at 2%, 4% and at 6.5% NaCl concentration
4. Arginine hydrolysis
5. Citrate utilization
6. Carbohydrate fermentations

None of the cocci shaped isolates produced carbon dioxide from glucose and this finding confirmed previously found homofermentative characteristics of them in milk.

According to their growth at 10˚C, 45˚C and 6.5% NaCl concentration, LAB were clearly dominated by two genera as *enterococcus* and *lactococcus* in our samples. For the lactococci; arginine hydrolysis, citrate utilisation and sugar utilisation characteristics of isolates indicated that all of the 54 isolates were identified as *L. lactis* ssp. *lactis*. *L. lactis* ssp. *lactis* is considered the most important *lactococcus* species in cheese (López-Díaz *et al.,* 2000). The abundance of *L. lactis* ssp *lactis* isolation from many cheese types confirmed by many other studies. (Prodroou *et al*., 2001, Parante *et al*., 1997, Menéndez *et al*., 2001).

Surprisingly, some *L. lactis* ssp. *lactis* isolates showed atypical properties such as growth at 45 °C and growth in 6.5% NaCl concentration (A26, A30, A47, A48, B10, B21, B28, C4, C15, C16, C28, C32 , C35). These atypic properties can be an advantage in dairy industry. Similarly, in a recent work lactococci isolates which grow in the presence of 6.5% NaCl and/or at 10 °C has been reported (Fortina *et al.,* 2003). In our

study, it was possible to identify lactococcal isolates at subspecies level by phenotypic methods. On the contrary, atypic characteristic of some lactococcal strains caused difficulties to distinguish them from *E. faecium* because they displayed similar sugar fermentation profiles. This difficulty however was overcome by genotypic methods.

As was seen the above the most abundant strains among the 134 isolates belonged to *Enterecoccus* genus. The presence of enterococci in cheese has been questioned due to the potential health problems; especially endocarditis and urinary tract infection. Numbers of enterecocci in Mediterrian type cheese curds can be ranged from 10^4 to 10^6cfu/g and in the fully ripened cheese from 10^5 to 10^7cfu/g (Franz *et al.,* 2003). Enterecocci can grow in this restrictive environment of high salt concentration and at low pH. Therefore, their abundance in such large numbers in our cheese samples should not be suprising. However they have beneficial effects on flavor developments in many type of cheese (Lopéz- diaz *et al.,* 2000). For this reason enterecocci with desirable technological and metabolic properties have been suggested as part of defined starter cultures for different European cheeses (Franz *et al*., 2003). Besides, their bacteriocin production abilities are also beneficial to control some food pathogens. It was suggested that enterecocci play a role in flavor development probably due to citrate utilization (Sarantinopoulos *et al.,* 2001). When it is considered our enterecoccal isolates, 19 out of 59 of them could produce gas from citrate. Hence, breakdown of citrate results in the production of carbon dioxide and also some flavor compounds like diacetyl, acetaldehyde and acetoin. The isolates which were able to metabolize citrate were listed (Table 5.2.).

Sugar fermentation profiles provided identification at species level for our typical enterecoccal isolates. Arabinose, sorbitol and glycerol provided discrimination between *E. faecium* and *E. faecalis*. Moreover, *E. durans* was distinguished from others due to the mannitol, sorbitol, and trehalose negative sugar fermentation characteristics. On the other hand, *E. avium* can ferment all sugars that were tested except for arabinose, raffinose, and xylose. It was found that 30 of them was *E. faecium*, 8 of them *E. faecalis*, 2 of them *E. durans*, 3 of them *E. avium*. On the other hand, 16 of them could not be determined by using available phenotypic characteristics.

Table 5.2. Enterococcal isolates which produced carbon dioxide from citrate.

No	Isolate no	Species description
1	A39	N.D.
2	A43	N.D.
3	A53	N.D.
4	A56	N.D.
5	A60	N.D
6	A69	N.D.
7	B32	*E. faecalis*
8	B33	*E. avium*
9	B35	*E. faecalis*
10	B36	*E.faecalis*
11	B37	*E. faecalis*
12	C30	N.D.
13	C36	*E. faecium*
14	C38	*E. faecalis*
15	C39	N.D
16	C40	N.D.
17	C41	*E. faecalis*
18	C42	N.D.
19	C43	*E. faecium*

Table 5.3. Biochemical results of cocci shaped isolates.

Group1 *L lactis* ssp *lactis* (total=54)	**Group 2. *E. faecium* (total=30)**
A1, A2, A3, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A16, A19, A20, A21, A22, A23, A25, A26, A27, A28, A29, A30, A35, A37, A40, A44, A45, A46, A47, A48, B8, B10, B11, B15, B20, B21,C1, C4, C10,C11, C15, C16, C18, C19 I, C19 II, C22, C24,C28, C34, C35	A17, A18, A31, A32, A33, A34, A38, A41, A42, A49, A50, A57,A58, A59, A66, A67, A68, A70, A71, B17, B22, B23, B24, B25, B26, B27 ,B28 ,B29 , C36 , C43
Group 3. *E. faecalis* (total=8)	**Group 4. *E. avium* (total=3)**
B19, B30, B32, B35, B36, B37, C38, C41	B33,B34, B46
Group 5. *E.durans* (total=2)	**Group 6. Not determined (total=16)**
B16, B31	A39, A43, A53, A56, A60, A61, A62, A63,A64,A65, A69, C30, C31,A39,A40,C42.

Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5 % NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
1	A1	-	+	+	-	+	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	-	+	-	+	+	+
2	A2	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
3	A3	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
4	A5	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
5	A6	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+/-	-	+	-	+	+	+
6	A7	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+/-	-	+	-	+	+	+
7	A8	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
8	A9	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
9	A10	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+/-	+	-	+	+	-	-	+	-	+	+	+
10	A11	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+/-	+	-	+	+	-	-	+	-	+	+	+
11	A12	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
12	A13	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
13	A14	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+

(cont. on next page)

Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
14	A16	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+/-	-	+	+/-	+	+	+
15	A17	-	+	+	+	+	+	+	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
16	A18	-	+	+	+	+	+	+	-,+	-	+	-	-	+	+	+/-	+	-	+	+/-	-	-	+	-	+	+	+
17	A19	-	+	+	-	+	+	-	-,+	-	+	-	-	+	-	-	+	-	+	+	+	-	+	-	+	+	+
18	A20	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+/-	+	-	+	+	+	-	+	-	+	+	+
19	A21	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
20	A22	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
21	A23	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
22	A25	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
23	A26	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+/-	+	-	+	+	-	-	+	-	+	+	+
24	A27	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
25	A28	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
26	A29	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+

(cont. on next page)

Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
27	A30	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	+	+
28	A31	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+
29	A32	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
30	A33	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
31	A34	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
32	A35	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
33	A37	-	+	+	-	+	+	-	-	-	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	+	+
34	A38	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+/-	-	-	+	+/-	+	+	+
35	A39	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+
36	A40	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+/-	+	-	+	-	+	+	+
37	A41	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+/-	+	+	+
38	A42	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	+	+/-	+	+	+
39	A43	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+

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(cont. on next page)

Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
40	A44	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
41	A45	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-	+	-	+	+/-	-	-	+	-	+	+	+
42	A46	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
43	A47	-	+	+	+	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
44	A48	-	+	+	+	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
45	A49	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+/-	-	+	-	+	+	+
46	A50	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	+	+,-	+	+	+
47	A53	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+
48	A56	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+/-	-	-	+	+	+	+	+
49	A57	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
50	A58	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+/-	-	+	-	+	+	+
51	A59	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+/-	-	+	+/-	+	+	+
52	A60	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+/-	+/-	-	+	+/-	+	+	+

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Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
53	A61	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
54	A62	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
55	A63	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
56	A64	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
57	A65	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
58	A66	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	+/-	+	+	+
59	A67	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	+/-	+	+	+
60	A68	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+/-	-	+	-	-	-	+	+/-	+	+	+
61	A69	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	-	-	-	+	+/-	+	+	+
62	A70	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+/-	-	+	+	-	-	+	-	+	+	+
63	A71	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	+/-	+	+	+
64	B8	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
65	B10	-	+	+	-	+	+	+	-,+	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+

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Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
66	B11	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
67	B15	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
68	B16	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
69	B17	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+/-	+	+	+
70	B19	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+/-	-	+	+	-	+	+	+
71	B20	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	+	+
72	B21	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
73	B22	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	+	+
74	B23	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+/-	-	-	+	+/-	+	+	+
75	B24	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+
76	B25	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+
77	B26	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	+	-	+	+	+
78	B27	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+

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Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5 % NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
79	B28	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
80	B29	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+/-	+	+	+
81	B30	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	+	+	+/-	+	+	+
82	B31	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
83	B32	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+/-	+	+	+
84	B33	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	-	+	+/-	+	+	+
85	B34	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+/-	+	+	+
86	B35	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+
87	B36	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+
88	B37	-	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+
89	C1	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
90	C4	-	+	+	-	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
91	C10	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+

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Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
92	C11	-	+	+	-	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
93	C15	-	+	+	-	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
94	C16	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	+	+	-	+	+	+
95	C18	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
96	C19I	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
97	C19II	-	+	+	-	+	+	-	-,+	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+
98	C22	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
99	C24	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+/-	+	+	+	-	+	-	+	+	+
100	C28	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+
101	C30	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
102	C31	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	+	+	+	+	+
103	C32	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+
104	C34	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+

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Table 5.4. Physiological and Biochemical Test Results of Cocci Shaped LAB (cont.)

No	Isolate No	Catalase	2% NaCl	4% NaCl	6.5% NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
									Color	Gas	Growth																
105	C35	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+
106	C36	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+
107	C38	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+
108	C39	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+/-	+	-	+	+	+	+	+
109	C40	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	-	+	+	+	+	+
110	C41	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
111	C42	-	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+/-	-	+	+	+	+	+
112	C43	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+
113	C46	-	+	+	+	+	+	+	-,+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+

5.2.5.2. Identification of Bacilli Shaped Isolates

When it was considered Lactobacillus isolates, following tests were performed. Moreover, reference strains were used to corroborate the phenotypic methods.

1. Carbon dioxide from glucose
2. Growth at 15 ˚C and 45˚C.
3. Growth at 6.5% NaCl concentration
4. Arginine Hydrolysis
5. Carbohydrate Fermentations

The number of the Lactobacilli isolates is 21. Phenotypic tests indicated that all of them were mesophilic type. Interestingly, only 3 of them shown heterofermentative properties (C17I, C17II, C27) which was not confirmed previously homofermentative behaviour in skim milk.

Moreover physiological tests indicated that all of them were mesophilic type. Because all lactobacilli isolates could grow at 15 ˚C. The physiological and biochemical test results were presented (Table 5.6.) and summarized (Table 5.5.).

Table 5.5. Biochemical Identification Results of Lactobacilli.

No	Isolate code	Identification
1	C3	*Lactobacillus* spp.
2	C5	*Lb. paracasei* ssp. *paracasei*
3	C7	*Lactobacillus* spp.
4	C8	*Lb. paracasei* ssp. *paracasei*
5	C9	*Lactobacillus* spp.
6	C12	*Lb. paracasei* ssp. *paracasei*
7	C17 I	*Lactobacillus* spp.
8	C17II	*Lactobacillus* spp.
9	C27	*Lb. casei*
10	C29	*Lactobacillus* spp
11	C37	*Lactobacillus* spp
12	C47	*Lactobacillus* spp
13	D1	*Lactobacillus* spp
14	D2	*Lb. casei*
15	D3	*Lactobacillus* spp
16	D4	*Lb. casei*
17	D5	*Lactobacillus* spp
18	D6	*Lactobacillus* spp
19	D7	*Lactobacillus* spp
20	D8	*Lactobacillus* spp
21	D9	*Lactobacillus* spp

Table 5.6. Physiological and Biochemical Test Results of Lactobaccilli

No	Isolate No	Catalase	6.5% NaCl	15 °C	45 °C	NH_3 from arginine	$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
1	C3	-		+	-	-	-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
2	C5	-		+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
3	C7	-		+	-	-	-	-	+	+	+	+	-	+	-	-	-	+	-	+	+	+
4	C8	-		+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
5	C9	-		+	-	-	-	-	+	+	+	+	-	+	-	-	+/-	+	-	+	+	+
6	C12	-		+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	-	+	+
7	C17 I	-		+	-	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
8	C17II	-		+	-	+	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	+
9	C27	-		+	-	-	+	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+
10	C29	-		+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
11	C37	-		+	+	-	-	+	+	+	+	+	-	+	-	+/-	-	+	-	+	+	+
12	C47	-		+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+
13	D1	-	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+/-	+	-	+	+	+

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Table 5.6. Physiological and Biochemical Test Results of Lactobaccilli

No	Isolate No	Catalase	6.5% NaCl	15 °C	45 °C	NH_3 from arginine	$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
14	D2	-	+	+	+	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
15	D3	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+
16	D4	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+
17	D5	-	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
18	D6	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+
19	D7	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+
20	D8	-	+	+	-	-	-	-	+	+	-	-	-	+	-	+/-	-	+	-	-	+	+
21	D9	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+

5.3. Genotypic Identification

5.3.1. Amplification of 16 S rRNA and ITS region

In order to identify lactic acid bacteria by PCR-RFLP technique, 16 S rRNA and ITS regions were amplified. The length of amplification products varied from 1500 to 2 000 bp (Figure 5.2).

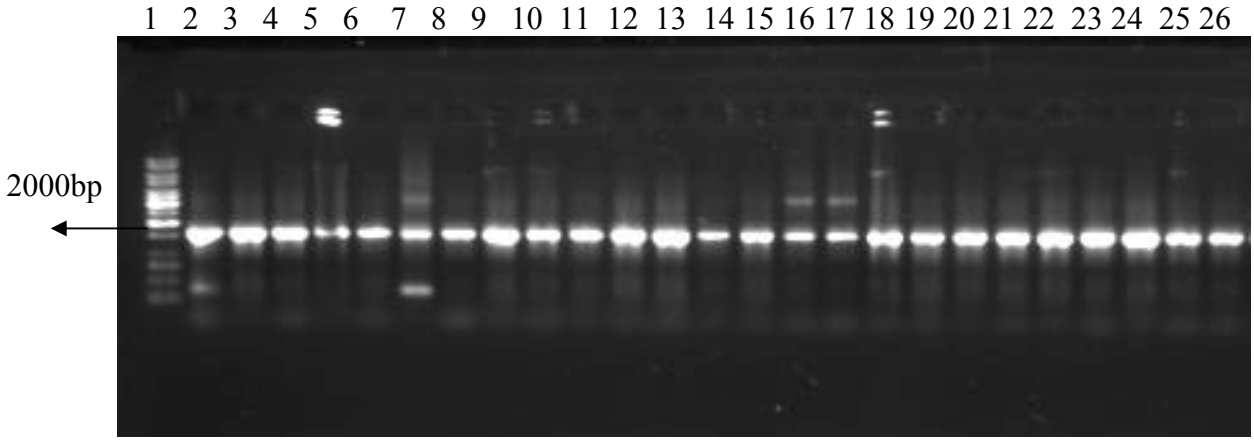

Figure 5.2. Represantative 16 S and ITS amplification products of isolates.
Lanes **1.** 1kb DNA ladder Gene Ruler^{TM} **2.** A1, **3.** A2, **4.** A3, **5.** A5, **6.** A7, **7.** A17, **8.** A18, **9.** A19, **10.** A20, **11.** A21, **12.** A35, **13.** A37, **14.** A38, **15.** A39, **16.** A40, **17.** A41, **18.** A42, **19.** B10, **20.** B15, **21.** B16, **22.** B17, **23.** B19, **24.** B20, **25.** B21, **26.** C1 **.**

5.3.2. Digestion of Amplified 16 S rRNA and ITS region

Isolates were identified according to genotypic characteristics by using restriction profiles. Amplification products were purified and digested with *Hae* III and *Taq* I restriction enzymes. One hundred and twenty seven of the isolates were subjected to genotypic identification. After digestion with two endonucleases, different gel fragments were obtained. For both enzyme profiles, isolates which represent different restriction band patterns were selected and grouped for final gel electrophoresis (Figure 5.3. and figure 5.4.).Then, their dendograms were built up (figure 5.5 and figure 5.6). Moreover fragment sizes were presented (Figure 5.7 and figure 5.8)

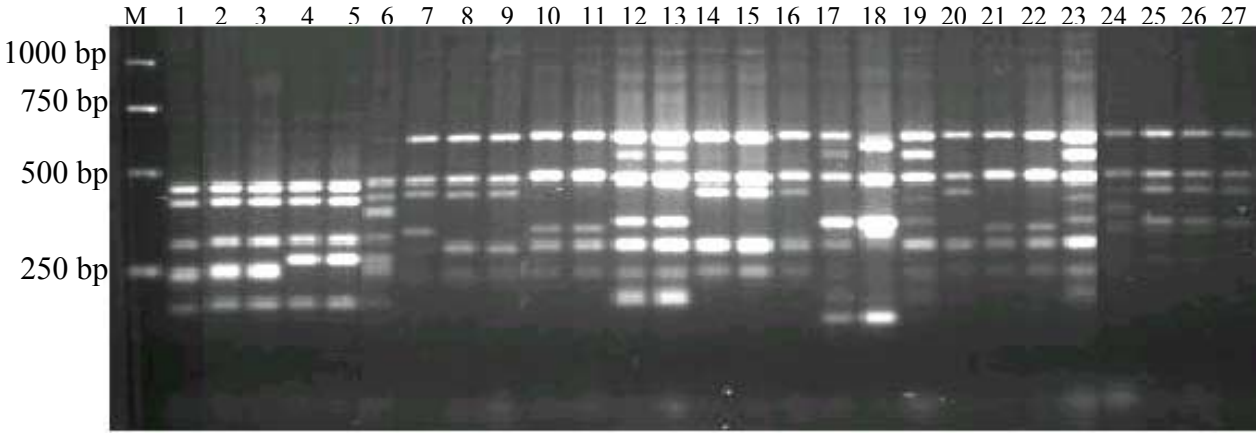

Figure 5.3. *Hae* III digests of 16S-ITS rRNA genes of represantative isolates and reference strains, Lanes **M.** 1kb DNA ladder Gene Ruler^{TM}; **1.** *L. lactis* ssp. *lactis* CECT 4432; **2.** A16, *L. lactis* ssp. *lactis*; **3.** B 10, *L. lactis* ssp. *lactis*; **4.** A 35, *L. lactis* ssp. *lactis*; **5.** A37, *L. lactis* ssp. *lactis*; **6.** C 28, *L. lactis* ssp. *lactis*; **7.** *E. casseliflavus* NRRL-B 3502; **8.** *E. faecalis* CECT 184; **9.** *E. gallinarum* CECT 970; **10.** A 17, *E. faecium*; **11.** A 18, *E. faecium*; **12.** A 31, *E. faecium*; **13.** A 32, *E. faecium*; **14.** A 39, Not determined; **15.** A 53, Not determined;**16.** B 31, *E. durans*; **17.** A 41, *E. faecium*; **18.** A 56, Not determined; **19.** A 42, *E. faecium*; **20.** A 43, Not determined; **21.** A 60, Not determined; **22.** A 61, Not determined; **23.** B24 , *E. faecium*; **24.** *Lb. casei* ssp *casei* NRRL-B 1922; **25.** C 3, Not determined; **26.** C 5, *Lb. paracasei* ssp. *paracasei*; **27.** D 4, Not determined.

5.3.2.1. *Hae* III digestion

In total 27 representative isolates and reference strains were used for evaluation of *Hae* III digestion profiles. Thirteen genotypic groups were obtained (Figure 5.5). When lactococcal isolates were examined by comparing with all other isolates ,they were clustured together. They could be easily differentiated from other enterecocci and lactobacilli isolates.

Although these all lactococcal isolates were previously identified as *L. lactis* ssp. *lactis* according to phenotyic methods , 3 different genotypic groups were obtained. First group which comprises of A16 and B10 represented 49 of the *L. lactis* ssp. *lactis* isolates. It was found that this group yielded similar restriction enzyme profile with reference strains of *L. lactis* ssp. *lactis* (100% homology).

Second group which comprises of A 35 and A 37 represented 4 of the *L. lactis* ssp. *lactis*. It was found that this group yielded restriction profile slightly different from reference strain of *L. lactis* ssp. *lactis* (80% homology).

Interestingly, only one isolate which was represanted as C 28 gaved distinct digestion profile.

Finally, it could be concluded that PCR-RFLP method by using *Hae* III enzyme indicated good correlation with phenotyic methods for *L. lactis* ssp. *lactis.* Moreover, further differentiation was achived by using this genotypic method.

For enterococci , isolates (A39, A53, A43) which could not be identified by using phenotyic methods gaved similar band patterns with *E. faecalis.* But. It was the handicap that *Hae* III digestion could not differentiate reference strains of *E. faecalis* from *E. gallinarum.*When sugar fermentation of these isolates were examined, it was seen that these were more closely *to E. faecalis*. The typical characteristic of the *E . gallinarum* is positive xylose and raffinose fermentation. On the other hand, *E. faecalis* species could not ferment these sugars.

Also it was found that isolates of A17, A18, A 60, A61 were clustured in the same group. Two of them (A17, A18) could be previously biochemicaly identified as E. facecium and therefore similarity at genotypic base (100% homology) provided identification. Other isolates (A 60 and A 61) which could not identified by using phenotypic methods , might be identified as *E. faecium* also.

One enterococci isolate (B 31) which was previously identified as E. durans gaved distinct digestion profiles.

Other E. faecium isolates (A31, A32, A42, B24, and A41) were clustred into 4 group . It might be considered that undefined isolate of A56 might be identified as *E. faecium* also.

For lactobacilli, evaluation was more difficult due to the unsufficient type of the reference strains. Lactobacilli isolates were clustured in one genotypic group which yielded different restriction profile from reference strain of *Lb. casei* ssp *casei* NRRR-B 1922.

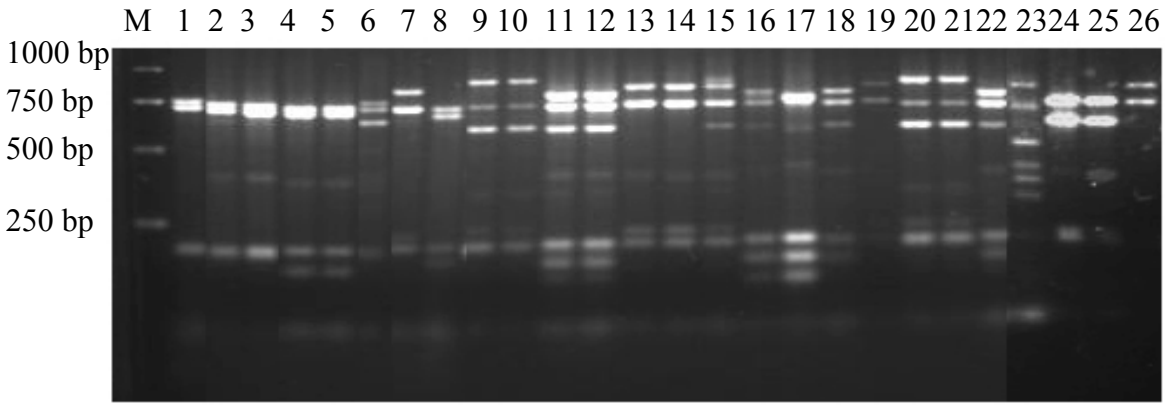

Figure 5.4. *TaqI* digests of 16S-ITS rRNA genes of representative isolates and reference strains, Lanes **M.** 1kb DNA ladder Gene Ruler^{TM}; **1.** *L. lactis* ssp. *lactis* CECT 4432; **2.** A16, *L. lactis* ssp. *lactis*; **3.** B 10, *L. lactis* ssp. *lactis*; **4.** A 35, *L. lactis* ssp. *lactis*; **5.** A37, *L. lactis* ssp. *lactis*; **6.** C 28, *L. lactis* ssp. *lactis*; **7.** *E. faecalis* CECT 184; **8.** *E. gallinarum* CECT 970; **9.** A 17, *E. faecium*; **10.** A 18, *E. faecium*; **11.** A 31, *E. faecium*; **12.** A 32, *E. faecium*; **13.** A 39, Not determined; **14.** A 53, Not determined;**15.** B 31, *E. durans*; **16.** A 41, *E. faecium*; **17.** A 56, Not determined; **18.** A 42, *E. faecium*; **19.** A 43, Not determined; **20.** A 60, Not determined; **21.** A 61, Not determined; **22.** B24, *E. faecium*; **23.** *Lb. casei* ssp *casei* NRRL-B 1922; **24.** C 3, Not determined; **25.** C 5, *Lb. paracasei* ssp. *paracasei*; **26.** D 4, *Lb. casei*

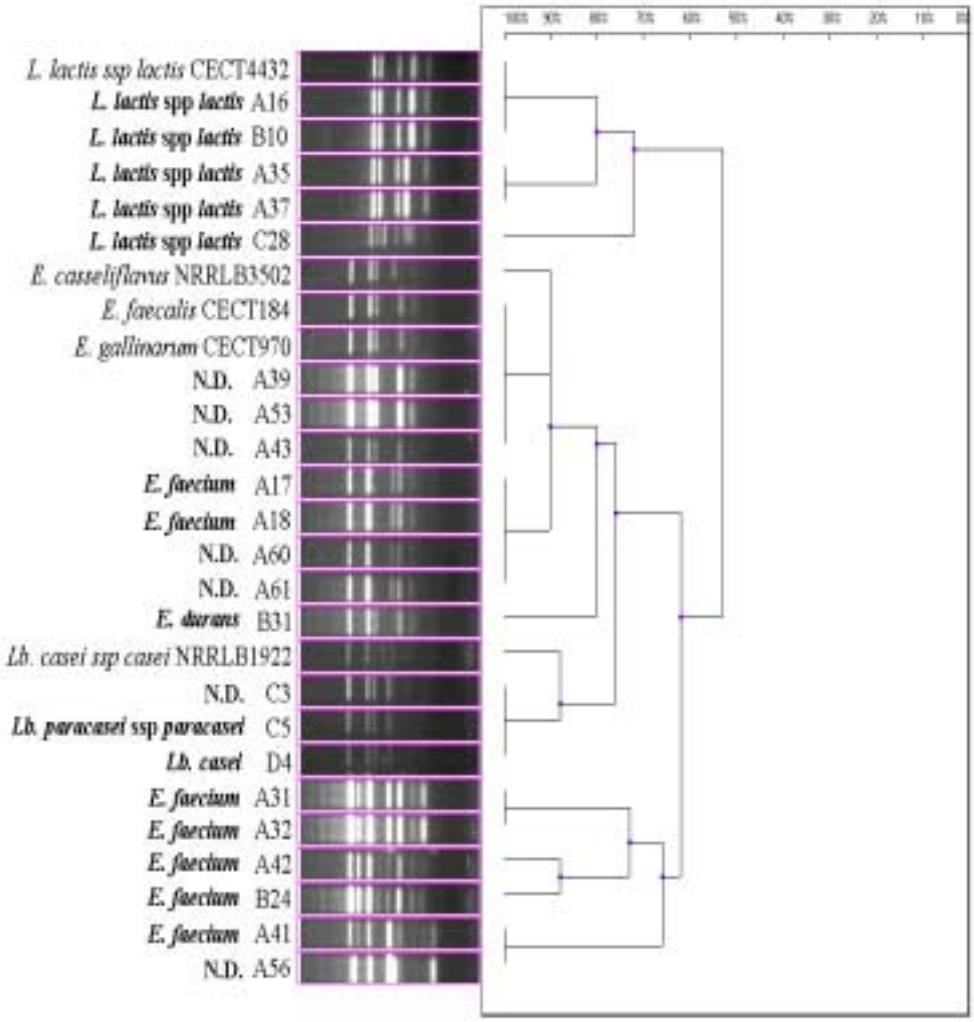

Figure 5.5. Dendogram of *Hae* III digest of representative isolates and reference strains. N.D. means not determined.

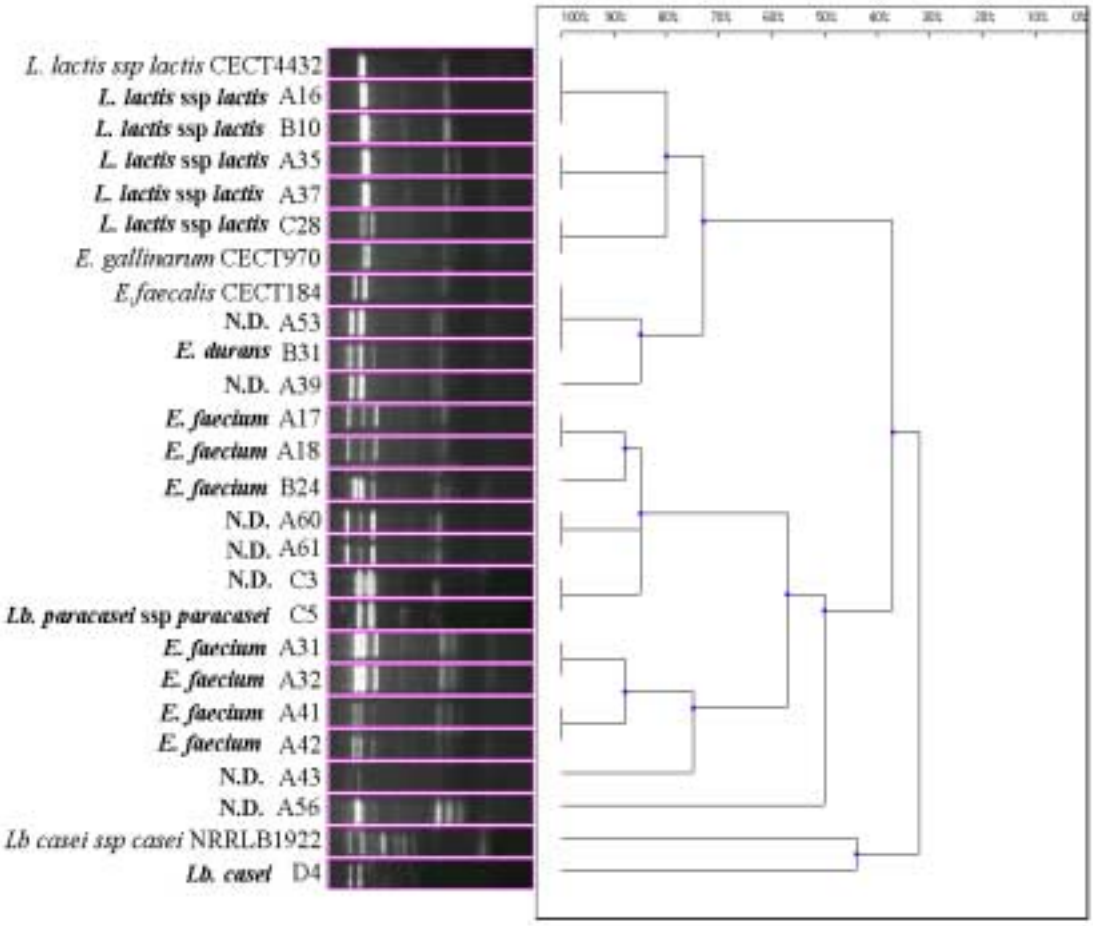

Figure 5.6. Dendogram of *Taq* I digest of representative isolates and reference strains. N.D. means not determined.

Table 5.7. Fragment sizes of *Hae* III digests of the isolates and reference strains

	M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L25	L26	L27	L28	L29
1	1.000	0.459	0.465	0.465	0.465	0.465	0.479	0.618	0.618	0.627	0.636	0.636	0.609	0.601	0.618	0.618	0.636	0.627	0.584	0.636	0.636	0.636	0.636	0.636	0.646	0.636	0.646	0.636
2	0.750	0.413	0.420	0.426	0.426	0.432	0.439	0.479	0.486	0.486	0.500	0.486	0.486	0.486	0.493	0.486	0.493	0.486	0.486	0.560	0.493	0.500	0.500	0.500	0.500	0.500	0.500	0.500
3	0.500	0.312	0.323	0.323	0.323	0.329	0.395	0.445	0.445	0.445	0.353	0.353	0.370	0.382	0.452	0.445	0.312	0.370	0.370	0.493	0.452	0.358	0.358	0.459	0.459	0.459	0.459	0.452
4	0.250	0.238	0.244	0.250	0.284	0.289	0.329	0.347	0.306	0.306	0.312	0.312	0.312	0.312	0.318	0.312	0.250	0.128	0.128	0.312	0.312	0.318	0.318	0.364	0.407	0.376	0.370	0.370
5		0.159	0.165	0.165	0.171	0.171	0.278	0.295	0.244	0.250	0.244	0.250	0.183	0.183	0.250	0.250					0.256	0.256	0.256	0.323	0.353			
6							0.256	0.238																0.261				

Table 5.8. Fragment sizes of *Taq* I digests of the isolates and reference strains

	M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27
1	1.000	0.736	0.736	0.710	0.698	0.698	0.698	0.808	0.698	0.885	0.885	0.778	0.793	0.854	0.854	0.854	0.808	0.793	0.823	0.869	0.918	0.918	0.808	0.710	0.778	0.764	0.869
2	0.750	0.698	0.686	0.662	0.662	0.662	0.629	0.686	0.651	0.578	0.588	0.723	0.723	0.723	0.723	0.736	0.736	0.204	0.750	0.764	0.608	0.608	0.736	0.533	0.618	0.618	0.750
3	0.500	0.158	0.158	0.158	0.158	0.158	0.151	0.171	0.171	0.224	0.224	0.588	0.588	0.224	0.191	0.191	0.204	0.145	0.204	0.204	0.204	0.204	0.211	0.432	0.224	0.237	0.339
4	0.250				0.092	0.092				0.171	0.178	0.184	0.184	0.191			0.138	0.079	0.138				0.158	0.391			
5												0.125	0.125											0.352			

5.3.2.2. *Taq* I digestion

In total 26 representative isolates and reference strains were examined. Fifteen different genotypic groups were obtained (Figure 5.6).

For *L. lactis* ssp. *lactis* isolates, *Taq* I digestion indicated good correlation with *Hae* III digestion. Isolates of A16 and B10 yielded similar restriction profile with reference strain (100% homology). Other *L. lactis* ssp. *lactis* isolates of A35 and A37 showed slightly different profiles typical isolates.

Interestingly , *L. lactis* ssp. *lactis* isolates of C 28 gaved quitely different profile and grouped with enterococci.

It was observed that *Taq* I could discriminate reference strains of *E. gallinarum* and *E. faecalis.*

Only two of enterococcal isolates were clustured with in the same group for both of the enzyme digestion.These isolates were A31 and A32 and both were biochemically identified as *E. faecium.*

It was concluded that PCR- RFLP method was beneficial especially for *L. lactis* ssp. *lactis* isolates. It might be provided better evaluation for enterococcal and lactobacilli isolates, if much more reference strains were included in this study.

5.4. Acid Production

Acid production properties of LAB are main technological characteristics for dairy industry (Berresford *et al.*,2001). For this aim, pH and lactic acid production were monitored for 130 isolates. Results were given on appendix. After results were grouped according to the genera, graphs were obtained (Figure 5.7 and figure 5.8). Interestingly, it was observed that isolates which belonged to each genus yielded similar acidifiying profile.

It was obvious that 35 of isolates could manage to lower pH below 5.3 for 6h incubation at 30 °C. It was clear that 34 of them was *L. lactis* ssp. *lactis* and only one of them was *E. faecium* (A31). Solely this isolate of enterococci cause to pH almost 5.3 in6 h incubation. On the other hand other 34 of the *L. lactis* ssp *lactis* species showed the highest acidifying capacity. Moreover , when these isolates were compared with others, it was obtained the most amount of lactic acid production in 6 h incubation. Rapid acid production abilities indicated that they were the most suitable starter candidates for dairy applications.

From lactic acid production view, it was also suggested that strains of *L. lactis* ssp. *lactis* whose acidifying capacity gave an acidity >2.5 mg/ml of lactic acid after 6 h incubation can be used as starter culture in cheese manufacture (Herreros *et al.*, 2003). At this mean 39 of our *L. lactis* ssp. *lactis* isolates could be considered for cheese manufacturing.

Table 5.9. Acidification characteristics of of isolates

Group		0h	3h	6h	9h	24h
Lactococcus n=54	pH	6,6 (0)	6,113 (±0,207)	5,271 (±0,580)	4,826 (±0,673)	4,397 (±0,480)
	L.A. (mg/ml)	1,587 (0)	2,388 (±0,355)	4,371 (±1,379)	5,787 (±1,832)	5,950 (±0,906)
Enterecoccus n=58	pH	6,6 (0)	6,354 (±0,075)	5,779 (±0,199)	5,347 (±0,222)	4,671 (±0,242)
	L.A. (mg/ml)	1,587 (0)	2,092 (±0,202)	2,952 (±0,307)	4,053 (±0,698)	5,950 (±0906)
Lactobacillus n=18	pH	6,6 (0)	6,283 (±0,097)	5,973 (±0,245)	5,768 (±0,334)	5,046 (±0,639)
	L.A. (mg/ml)	1,587 (0)	2,434 (±0,192)	2,808 (±0,459)	3,321 (±0,686)	6,247 (±2,539)

L.A. Titration value of lactic acid production
Mean±standard deviation

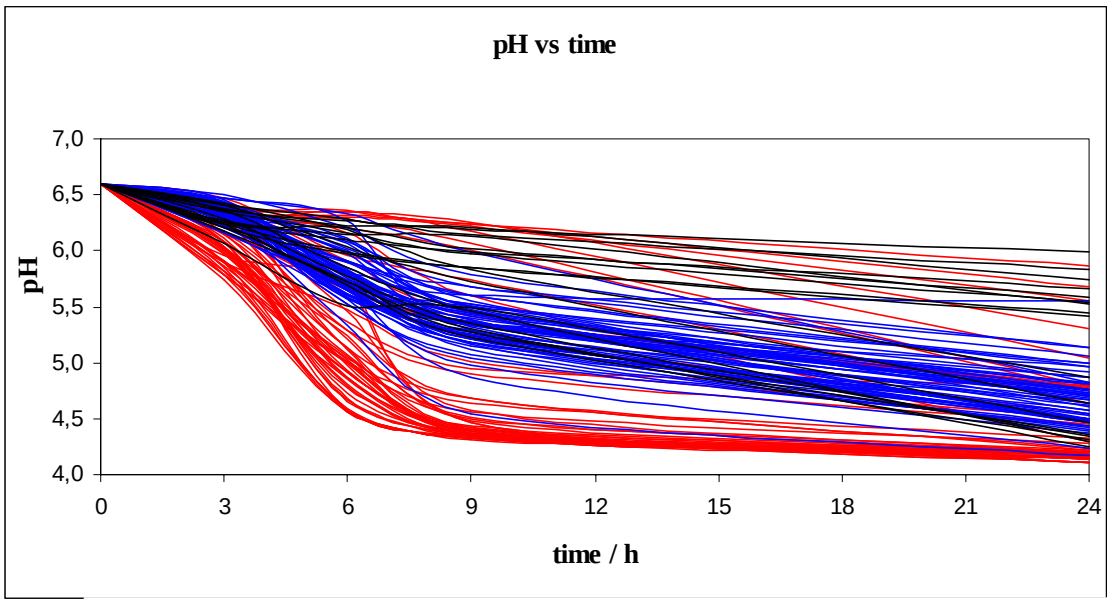

Figure 5.7. pH changes in UHT skim milk broths during incubation for 3 h, 6 h, 9 h ,24 h at 30 °C.
Red color-lactococci, blue color-enterococci, and black color-lactobacilli

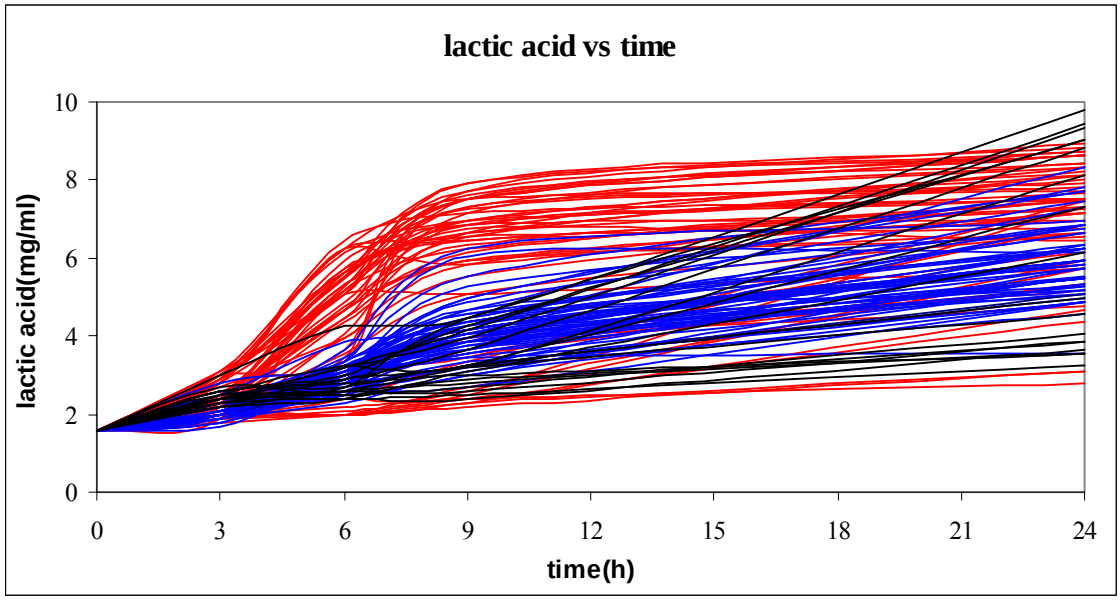

Figure 5.8. Lactic acid production in UHT skim milk broths during incubation for 3 h, 6 h, 9 h ,24 h at 30 °C.
Red color-lactococci, blue color-enterococci, and black color-lactobacilli

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVE

Isolation of starter LAB was the focus of this study. In order to achive this aim , phenotypic methods (morphology, phsiologicaland biochemical tests) and genotypic methods (16S-ITS PCR - RFLP) were performed.The following goals were achived:

1. All isolates were identified as LAB
2. One hundred and thithy one of the 134 isolates were identified as homofermentative LAB.
3. Genotypic methods were beneficial for futher identification because isolates which could not be determined according to the phenotypic methods were identified by genetic mean.
4. Technological characterization of isolates was performed according to the acid activity tests. The results of these tests indicated that 35 isolates have significant importance as starters for industrial cheese production. Because these isolates could lower the pH of UHT skim mik below 5.3 after 6 h incubation at 30 °C, and candidates as good staters for dairy industry.

Starter cultures which were isolated from different sources have important contributions in quality and in other characteristics of industrially produced cheese. Isolation of LAB with challenging technological properties have a gerat importance in industry and science. In the future, it will be beneficial to determine the following characteristics of the isolated strains.

1. Bacteriophage resistance.
2. Proteolytic activity.
3. Lipolytic activity.
4. Production of aroma and flavor compounds.
5. Antimicrobial properties.
6. Dietetic properties (L- Lactic acid produced, probiptic properties).

Finally, the isolated strains might also be tried for new fermented food formulations.

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APPENDICES

APPENDIX A

CHEMICALS USED

Table A.1. Chemicals Used In Microbiolgical Experiments

NO	CHEMICAL	CODE
1	Agar	Merck 1.01613
2	Bacteriological pepton	Oxoid LP037
3	Lab-Lemco Meat Extract	Oxoid LP029
4	D-Glucose	AppliChem A3666
5	Yeast Extract	Merck A 1.03753
6	Skim milk	Pınar and Ova
7	Maltose	Merck Art 5911
8	Sucrose	Difco 0176-17
9	D (-) Salicin	Fluka 84150
10	Arginine monohydrocholoride	Merck Art 1543
11	Lactose	Sigma L3750
12	D-Mannitol	DIFCO 0171-13
13	D- Mannose	DIFCO 0175-13
14	Raffinose	Merck 7419
15	D (+) -Xylose	Merck 8689
16	D (-) - Ribose	Flula 83860
17	D (+) - Galactose	Aldrich 11259-3
18	L (+) - Arabinose	Aldrich A9190-6
19	D (+) - Trehalose	Sigma T 9531
20	Glycerol	AppliChem A2926
21	NaCl	Merck 6400.100
22	Triammonium citrate	Sigma A1332
23	Sodium citrate	AnalaR 10242
24	Bromcresol purple	Merck 3025
25	Sodium acetate	Sigma S2889

NO	CHEMICAL	CODE
26	K_2HPO_4	Sigma P8281
27	Glycocoll	Riedel-De Haën 652296
28	Bromtymol blue	Riedel-De Haën 35088
29	Sodium phosphate di basic	Merck 926870
30	$MgSO_4.7H_2O$	Merck 1.05886
31	$MnSO_4.4H_2O$	Merck 1.02786
32	Ascorbic acid	Merck 5.00074
33	Phenol red	BDH 20091
34	Crysal violet	Sigma C3886
35	Potassium loidide	Sigma C6757
36	Safranine	Merck 1.15948

Table A.1. Chemicals Used in PCR-RFLP

NO	CHEMICAL	CODE
1	Taq DNA polymerase	PromegaM1835
2	Primers: Ege 1 and L1	Promega
3	dNTP set	MBI, Fermentas, R0181
4	Standart agarose (low electroendoosmosis)	AppliChem A3666
5	Taq I	Promega R6151
6	Hae III	Promega R6171
7	Chloroform	AppliChem A3633
8	Sodium acetate	Sigma S 2889
9	Isoamyl alcohol	AppliChem A2610
10	Mineral Oil	Sigma M5904
11	Bromophenol blue	Merck 1.08122
12	Glycerol	AppliChem A2926
13	1kb DNA ladder Gene Ruler^{TM}	Fermentas SM0311
14	Phenol	AnalaR 10242
15	Rnase	Merck 3025
16	Lysozyme	Sigma S2889
17	K_2HPO_4	Sigma P8281
18	Proteinase K	Riedel-De Haën 652296
19	Ethidum bromide	Riedel-De Haën 35088
20	Ethanol	Merck 926870

APPENDIX B

RECIPIES FOR CULTURE MEDIA

B.1 MRS BROTH AND MRS AGAR

MRS BROTH	**g/l**
Pepton	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
D (-) Glucose	20,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

MRS AGAR	**g/l**
Pepton	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
D (-) Glucose	20,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Agar	15,0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.2 M17 BROTH AND M17 AGAR

M17 BROTH	**g/l**
Polypepton	5,0
Phyton pepton	5,0
Yeast extract	2,5
Meat extract	2,5
Lactose	5,0
Ascorbic acid	0,5
ß-disodium glycerolphospate	19,0
$MgSO_4$(0,1M).$7H_2O$	1 ml
Deionized water	1000 ml

All ingredients were dissolved in deionized water in a water bath for 20min. pH was adjusted to 7,15 ±0,1. Medium was autoclaved at 121°C for 15 minutes.

M17 AGAR	**g/l**
Polypepton	5,0
Phyton pepton	5,0
Yeast extract	2,5
Meat extract	2,5
Lactose	5,0
Ascorbic acid	0,5
ß-disodium glycerolphospate	19,0
$MgSO_4$(0,1M).$7H_2O$	1 ml
Agar	12,0
Deionized water	1000 ml

All ingredients except lactose were dissolved in 900ml deionized water by holding in a water bath for 20min. pH was adjusted to 7,15 ±0,1. Medium was autoclaved at 121°C for 15 minutes.

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

APPENDIX C

MEDIA FOR IDENTIFICATION

C.1. MEDIA FOR TESTING THE GROWTH AT DIFFERENT TEMPERATURES

	g/l
Pepton	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
D (-) Glucose	20,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Bromecresol purple	0,04
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

C.2. MEDIA FOR TESTING THE GROWTH AT DIFFERENT NACL CONCENTRATIONS

	g/l
Pepton	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
D (-) Glucose	20,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Bromecresol purple	0,04
NaCl	20, 40,65 for each concentration of 2%, 4% and 6,5% NaCl
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

C.3 MEDIA FOR GAS FROM GLUCOSE

	g/l
Pepton	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
D (-) Glucose	20,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6..It was distrubuted into tubes containing inverted Durham tubes. Medium was sterilized by autoclaving at 121°C for 15 minutes.

C.4 REDDY BROTH

	g/l
Peptone	5,0
Yeast extract	5,0
K_2HPO_4	1,0
Arginine hyrochloride	5,0
Sodium citrate	20,0
Bromcresol purple	0,002
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2. It was distrubuted into tubes containing inverted Durham tubes. Medium was sterilized by autoclaving at 121°C for 15 minutes.

C.5 MODIFIED MRS FOR CHO FERMENTATIONS

	g/l
Peptone	10,0
Lab-Lemco meat extract	10,0
Yeast extract	5,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Bromecresol purple	0,04
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

C.6 ARGININE MRS

	g/l
Peptone	10,0
Yeast extract	5,0
Tween 80	1 ml
K_2HPO_4	2,0
Sodium acetate	5,0
Sodium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Arginine	1.5 g
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6,2 – 6,6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

APPENDIX D

CARBOHYDRATES

D 1. Sugars which were used for carbohydrate fermentations

1. L(+)-arabinose,
2. D(+) galactose,
3. Lactose,
4. Maltose,
5. D-mannitol,
6. Raffinose,
7. Sucrose,
8. D(-)salicin,
9. Sorbitol,
10. D(+) trehalose,
11. D(+)xylose,
12. Glycerol,
13. D(+)mannose,
14. D(-)ribose,

APPENDIX E

BUFFERS AND STOCK SOLUTIONS

E.1. 1 M Tris-HCl pH 7.2

121.1 g Tris base was dissolved in 800 ml of deionized water. PH was adjusted to 7.2 with concentrated HCl. Volume was brought to 1000 ml with deionized water.

E.2. 1 M Tris-HCl pH 8.0

121.1 g Tris base was dissolved in 800 ml of deionized water. PH was adjusted to 8.0 with concentrated HCl. Volume was brought to 1000 ml with deionized water.

E.3. 0.5 M EDTA pH 8.0

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

E.3. 50 X TAE

242 g Tris base was dissolved in deionized water. After this, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 5.8) were added. Volume was brought to 1000 ml with deionized water.

E.5. 3 M NaCl

175.32 g NaCl was dissolved in deionized water and the volume was adjusted to 1000 ml.

E.6. 5 M NaCl

292.2 g NaCl was dissolved in deionized water and the volume was adjusted to 1000 ml.

E.7. 1 X TAE

20 ml of 50 X TAE buffer was taken and the volume was adjusted to 1000 ml with deionized water.

E.8. ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

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

E.9. SODIUM ACETATE (3 M, pH 5.2)

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

E.10. CHLOROFORM- ISOAMYL ALCOHOL SOLUTION

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

E.11. PHENOL

First, phenol was melted. An equal volume of 0.5 M Tris HCl pH 8.0 was added and mixture was stirred for 15 min. After turning off the strirrer , separated two phases were obtained. Upper aqueous phase was removed and then an equal volume of 0.1 M Tris HCl pH 8.0 was added. After strirring for 15 min. Upper phase was removed. Extraction was repeated until the pH of phenolic phase is > 7.8. After the phenol was equilibrated and the final upper phase was removed, 0.1 M Tris HCl pH 8.0 containing 0.2 % β- mercaptoethanol. The phenol solution was distributed into 50 ml falcon tubes and covered with aluminium papers to avoid light. It was stored in this form at – 20 °C. Before using, 40 mg hydoxyquinoline was added for 40 ml phenol solution (0.1 %) then yellow color was appeared. Yellow color provides a convenient way to identify the phenol phase.

E.12. 1 XTE BUFFER

10 mM Tris pH 8.0

1 mM EDTA

E. 13. BOVIN SERUM ALBUMIN (BSA) 10 X

10 mg /ml , 150 µl BSA was diluted with 1.5 ml TE buffer. It was divided into aliquots and stored at-20°

APPENDIX F

PCR RECIPIES

F.1. PCR MIXTURE

Mg free Taq DNA polymerase buffer	5 µl
Mg Cl_2 (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward 10 picomole/µl	1 µl
Oligo reverse 10 picomole/µl	1 µl
d NTP (2 mM each) 10 X	5 µl

F.2. RESTRICTION ENZYME MIXTURE

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

F.3. TAQ DNA POLYMERASE ENZYME DILUTION

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

F.4. 6 x GEL LOADING BUFFER

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

Bromophenol blue was added with toothpick to obtain sufficient color.

F.5. d NTP (10 X)

10 µl of each 100 mM dATP, dCTP, dGTP and dTTP were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added and mixed.Hence, 2 mM concentration was obtained for each of them.

APPENDIX G

OLIGONUCLEOTIDES AND RESTRICTION ENZYMES

G.1. PRIMER OF EGE 1

EGE 1: 5'-AGAGTTTGATGATCCTGGCTCAG-3'

590 µg primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain 2 µg/ml stock solutions. Five µl of stock solution was mixed with 95 µl sterile deionized water. Hence, 100 µl, 10 picomole/µl working solution was obtained. Both working and stock solutions were stored at -20°.

G.2. PRIMER OF L1

L1: 52-CAAGGCATCCACCGT-3'

350 µg primer EGE 1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg/ml stock solutions. Four µl of stock solution was mixed with 96 µl sterile deionized water. Hence, 100 µl, 10 picomole/µl working solution was obtained. Both working and stock solutions were stored at -20°.

G.3. *Taq* I

5'-T▼GC A-3'

5'-T GC▲T -3'

G.4. *Hae* III

5'-GG▼CC-3'

5'-CC▲GG-3'

APPENDIX H

ACID ACTIVITY TEST RESULT

H1. pH MEASUREMENT RESULTS

Table H1.1. pH Changes in UHT skim milk during incubation at 30 °C for *Lactococcus* genus.

No	Isolate No	The genus *Lactococcus*				
		0h	3h	6h	9h	24h
1	A1	6,600	6,325	6,280	6,075	4,785
2	A2	6,600	6,000	4,815	4,360	4,190
3	A3	6,600	6,026	5,250	4,480	4,190
4	A5	6,600	5,985	4,790	4,345	4,185
5	A6	6,600	6,030	5,045	4,630	4,330
6	A7	6,600	6,320	6,350	6,250	5,310
7	A8	6,600	5,905	5,025	4,360	4,185
8	A9	6,600	5,975	4,775	4,370	4,205
9	A10	6,600	5,915	5,060	4,370	4,180
10	A11	6,600	6,035	4,675	4,365	4,185
11	A12	6,600	5,890	4,575	4,345	4,170
12	A13	6,600	6,265	5,295	4,690	4,240
13	A14	6,600	5,745	4,565	4,330	4,140
14	A16	6,600	6,330	6,310	6,240	5,685
15	A19	6,600	6,350	6,215	6,000	4,765
16	A20	6,600	5,780	4,595	4,315	4,150
17	A21	6,600	5,855	4,580	4,330	4,160
18	A22	6,600	5,820	4,555	4,330	4,160
19	A23	6,600	6,030	4,650	4,355	4,115
20	A25	6,600	6,025	4,730	4,350	4,145
21	A26	6,600	6,080	4,575	4,325	4,110
22	A27	6,600	5,980	5,010	4,560	4,285
23	A28	6,600	6,120	4,865	4,335	4,135
24	A29	6,600	6,335	6,305	6,220	5,555
25	A30	6,600	6,040	4,915	4,420	4,205
26	A35	6,600	6,285	5,640	5,360	4,775
27	A37	6,600	6,170	5,660	5,365	4,795
28	A40	6,600	6,385	6,355	6,170	5,040
29	A44	6,600	6,085	4,680	4,345	4,150
30	A45	6,600	6,155	4,925	4,435	4,190
31	A46	6,600	6,155	5,375	4,955	4,785
32	A47	6,600	6,040	4,865	4,400	4,205
33	A48	6,600	6,150	5,295	4,900	4,455
34	B8	6,600	6,350	6,160	5,605	4,290
35	B10	6,600	6,275	5,140	4,685	4,215

No	Isolate No	The genus *Lactococcus*				
		0h	3h	6h	9h	24h
36	B11	6,600	6,370	6,345	6,235	5,860
37	B15	6,600	6,085	5,140	4,420	4,135
38	B20	6,600	6,405	5,905	5,410	4,755
39	B21	6,600	6,375	5,645	5,245	4,550
40	C1	6,600	5,790	4,910	4,395	4,170
41	C4	6,600	5,895	5,470	5,005	4,490
36	B11	6,600	6,370	6,345	6,235	5,860
42	C10	6,600	5,900	4,920	4,400	4,170
43	C11	6,600	5,815	4,990	4,390	4,175
44	C15	6,600	5,780	5,005	4,395	4,145
45	C16	6,600	6,330	5,460	4,570	4,180
46	C18	6,600	5,860	4,855	4,395	4,175
47	C19I	6,600	6,085	5,070	4,505	4,215
48	C19II	6,600	6,310	5,215	4,510	4,200
49	C22	6,600	6,385	5,120	4,400	4,135
50	C24	6,600	6,290	4,975	4,365	4,115
51	C28	6,600	6,465	5,955	5,740	4,630
52	C32	6,600	6,335	5,675	4,465	4,110
53	C34	6,600	6,435	6,370	6,315	4,435
54	C35	6,600	5,960	5,705	4,465	4,105

Table H1.2. pH Changes in UHT skim milk during incubation at 30 °C for *Enterecococcus* genus

No	Isolate No	The genus *Enterococcus*				
		0 h	3 h	6 h	9 h	24 h
1	A17	6,600	6,325	5,530	5,070	4,420
2	A18	6,600	6,335	5,520	5,040	4,440
3	A31	6,600	6,295	5,330	4,550	4,175
4	A32	6,600	6,365	5,710	4,865	4,240
5	A33	6,600	6,345	5,585	5,270	4,470
6	A34	6,600	6,385	5,705	5,210	4,505
7	A38	6,600	6,415	5,970	5,325	4,505
8	A39	6,600	6,285	5,720	5,370	4,745
9	A41	6,600	6,425	5,845	5,520	4,755
10	A42	6,600	6,320	5,910	5,595	4,990
11	A43	6,600	6,300	5,735	5,425	4,820
12	A49	6,600	6,215	5,675	5,515	4,920
13	A50	6,600	6,230	5,905	5,530	4,990
14	A53	6,600	6,225	5,640	5,225	4,575
15	A56	6,600	6,380	6,085	5,680	5,060
16	A57	6,600	6,225	5,900	5,675	5,140
17	A58	6,600	6,270	5,820	5,600	5,560
18	A59	6,600	6,395	5,670	5,340	4,755
19	A60	6,600	6,320	5,580	5,175	4,545
20	A61	6,600	6,360	5,620	5,155	4,475
21	A62	6,600	6,395	5,650	5,185	4,490
22	A63	6,600	6,360	5,810	5,250	4,525
23	A64	6,600	6,370	5,700	5,245	4,545
24	A65	6,600	6,335	5,655	5,260	4,645
25	A66	6,600	6,435	5,755	5,365	4,720
26	A67	6,600	6,420	5,805	5,410	4,700
27	A68	6,600	6,390	5,680	5,360	4,690
28	A69	6,600	6,170	5,605	5,335	4,705
29	A70	6,600	6,400	5,795	5,465	4,875
30	A71	6,600	6,380	5,730	5,440	4,810
31	B16	6,600	6,370	5,565	5,370	4,805
32	B17	6,600	6,365	5,825	5,345	4,960
33	B19	6,600	6,340	5,640	5,280	4,675
34	B22	6,600	6,375	5,835	5,385	4,575
35	B23	6,600	6,500	5,465	5,360	4,725
36	B24	6,600	6,395	5,605	5,340	4,870
37	B25	6,600	6,410	5,800	5,340	4,700
38	B26	6,600	6,445	6,015	5,605	4,970
39	B27	6,600	6,500	6,205	5,790	5,135
40	B29	6,600	6,420	5,660	5,245	4,485
41	B30	6,600	6,415	5,660	5,175	4,405

No	Isolate No	The genus *Enterococcus*				
		0 h	3 h	6 h	9 h	24 h
42	B31	6,600	6,380	5,725	5,215	4,460
43	B32	6,600	6,375	5,615	5,295	4,630
44	B33	6,600	6,455	5,805	5,450	4,710
45	B34	6,600	6,370	5,810	5,210	4,615
46	B35	6,600	6,265	5,785	5,165	4,385
47	B36	6,600	6,280	5,570	4,980	4,380
48	B37	6,600	6,265	5,765	5,125	4,435
49	C30	6,600	6,470	6,275	5,295	4,460
50	C31	6,600	6,380	6,065	5,500	4,735
51	C36	6,600	6,300	5,710	5,390	4,495
52	C38	6,600	6,310	6,105	5,440	4,465
53	C39	6,600	6,360	5,985	5,505	4,760
54	C40	6,600	6,395	6,120	5,555	4,600
55	C41	6,600	6,305	6,000	5,465	4,485
56	C42	6,600	6,365	5,890	5,470	4,665
57	C43	6,600	6,165	5,715	5,395	4,735
58	C46	6,600	6,465	6,325	5,995	4,830

Table H1.3. pH Changes in UHT skim milk during incubation at 30 °C for *Lactobacillus* genus.

No	Isolate No	**The genus *Lactobacillus***				
		0 h	3 h	6 h	9 h	24 h
1	C3	6,600	6,250	6,105	5,990	5,665
2	C5	6,600	6,245	6,215	6,020	5,590
3	C7	6,600	6,350	6,155	5,975	5,545
4	C8	6,600	6,275	6,145	6,125	5,530
5	C9	6,600	6,245	5,990	5,850	5,415
6	C12	6,600	6,375	6,220	6,200	5,735
7	C27	6,600	6,070	5,505	5,490	4,460
8	C29	6,600	6,210	5,970	5,855	5,450
9	C37	6,600	6,215	5,730	5,305	4,255
10	C47	6,600	6,280	5,770	5,355	4,345
11	D1	6,600	6,405	6,035	5,725	4,645
12	D2	6,600	6,370	5,855	5,460	4,295
13	D3	6,600	6,180	5,730	5,275	4,355
14	D5	6,600	6,235	5,695	5,235	4,310
15	D6	6,600	6,370	6,270	6,195	5,985
16	D7	6,600	6,190	5,640	5,795	4,530
17	D8	6,600	6,405	6,285	6,145	5,835
18	D9	6,600	6,425	6,195	5,830	4,875

H2. LACTIC ACID PRODUCTION RESULTS

Table H2.1. Lactic acid production in UHT skim milk during incubation at 30 °C for *Lactococcus* genus.

No	Isolate No	The genus *Lactococcus*				
		0 h (mg/ml)	3 h (mg/ml)	6 h (mg/ml)	9 h mg/ml)	24 h (mg/ml)
1	A1	1,587	1,887	2,081	2,477	5,951
2	A2	1,587	2,477	5,554	7,238	7,941
3	A3	1,587	1,887	4,064	6,647	8,331
4	A5	1,587	2,971	5,651	6,647	8,031
5	A6	1,587	2,380	4,461	6,145	7,538
6	A7	1,587	1,781	1,984	2,380	4,364
7	A8	1,587	2,477	4,858	6,938	8,128
8	A9	1,587	2,477	5,554	6,938	8,234
9	A10	1,587	2,380	4,761	6,541	8,128
10	A11	1,587	2,283	5,351	6,841	7,670
11	A12	1,587	2,874	6,444	7,141	7,538
12	A13	1,587	2,178	3,967	5,854	7,141
13	A14	1,587	3,077	6,251	7,538	7,732
14	A16	1,587	2,081	1,984	2,283	3,086
15	A19	1,587	2,081	1,984	2,680	5,748
16	A20	1,587	2,971	6,145	6,938	7,538
17	A21	1,587	2,777	6,251	6,444	7,441
18	A22	1,587	2,777	6,251	5,854	7,538
19	A23	1,587	2,380	5,748	6,444	7,238
20	A25	1,587	2,380	5,351	6,647	7,238
21	A26	1,587	2,380	5,951	6,841	6,938
22	A27	1,587	2,477	4,664	5,951	6,647
23	A28	1,587	2,178	5,157	6,251	7,335
24	A29	1,587	1,984	1,984	2,178	3,077
25	A30	1,587	2,380	4,664	5,951	6,541
26	A35	1,587	2,178	3,077	3,870	4,761
27	A37	1,587	2,178	2,971	3,967	5,157
28	A40	1,587	1,887	1,984	2,283	4,664
29	A44	1,587	2,380	5,748	6,541	6,744
30	A45	1,587	2,178	4,664	6,348	6,938
31	A46	1,587	2,178	3,570	5,060	6,647
32	A47	1,587	1,887	5,157	6,744	6,444
33	A48	1,587	2,178	5,060	5,060	5,748
34	B8	1,587	1,984	2,380	3,368	7,441
35	B10	1,587	2,178	4,364	5,951	7,141
36	B11	1,587	1,984	2,178	2,380	2,777
37	B15	1,587	2,380	4,461	6,841	7,538
38	B20	1,587	1,984	2,680	3,570	5,316
39	B21	1,587	1,984	3,174	4,161	6,145
40	C1	1,587	2,874	5,554	7,934	8,631

No	Isolate No	The genus of *Lactococcus*				
		0 h (mg/ml)	3 h mg/ml)	6 h (mg/ml)	9 h (mg/ml)	24 h (mg/ml)
41	C4	1,587	2,680	3,967	5,554	7,141
42	C10	1,587	2,874	5,457	7,732	8,922
43	C11	1,587	3,077	5,157	7,538	8,728
44	C15	1,587	2,971	5,060	7,934	8,825
45	C16	1,587	2,283	3,667	7,141	8,428
46	C18	1,587	2,874	5,651	7,538	8,631
47	C19I	1,587	2,777	5,060	7,335	7,837
48	C19II	1,587	2,477	4,664	7,335	8,331
49	C22	1,587	2,380	5,254	7,635	8,631
50	C24	1,587	2,477	5,651	7,538	8,631
51	C28	1,587	2,178	3,077	3,474	6,841
52	C32	1,587	2,283	3,474	7,740	8,728
53	C34	1,587	2,283	2,380	2,574	7,335
54	C35	1,587	2,971	3,368	7,538	8,428

Table H 2.2. Lactic acid production in UHT skim milk during incubation at 30 °C for *Enterecoccus* genus.

No	Isolate No	The genus *Enterococcus*				
		0 h (mg/ml)	3 h (mg/ml)	6 h (mg/ml)	9 h (mg/ml)	24 h (mg/ml)
1	A17	1,587	1,984	3,368	4,655	6,251
2	A18	1,587	1,984	3,368	4,558	6,251
3	A31	1,587	1,984	3,667	6,251	6,938
4	A32	1,587	1,984	2,971	5,254	6,744
5	A33	1,587	1,984	2,971	3,474	3,570
6	A34	1,587	1,984	2,971	4,558	5,157
7	A38	1,587	1,984	2,574	4,364	6,251
8	A39	1,587	2,178	2,777	3,967	5,351
9	A41	1,587	1,984	2,574	3,271	4,955
10	A42	1,587	1,887	2,574	3,271	4,858
11	A43	1,587	2,081	2,874	3,870	5,157
12	A49	1,587	2,081	2,971	3,474	5,254
13	A50	1,587	2,081	2,574	3,368	4,858
14	A53	1,587	2,081	2,777	3,271	6,348
15	A56	1,587	1,887	2,283	2,971	4,858
16	A57	1,587	2,081	2,574	3,077	5,351
17	A58	1,587	2,081	2,574	2,698	4,858
18	A59	1,587	1,984	2,971	3,570	5,457
19	A60	1,587	1,984	3,174	4,161	5,951
20	A61	1,587	1,984	3,174	4,267	5,951
21	A62	1,587	1,984	2,971	4,064	5,854
22	A63	1,587	1,984	2,874	4,161	5,951
23	A64	1,587	1,984	2,971	4,161	5,951
24	A65	1,587	1,887	2,874	3,870	5,748
25	A66	1,587	1,781	2,777	3,764	5,457
26	A67	1,587	1,781	2,680	3,570	5,457
27	A68	1,587	1,684	2,874	3,870	5,457
28	A69	1,587	2,275	3,174	4,064	5,951
29	A70	1,587	1,984	2,971	3,667	5,457
30	A71	1,587	1,984	2,971	3,764	5,254
31	B16	1,587	1,984	3,271	3,667	5,951
32	B17	1,587	1,984	2,777	3,870	5,060
33	B19	1,587	1,984	3,077	4,064	5,457
34	B22	1,587	2,178	3,077	4,267	6,647
35	B23	1,587	1,887	3,077	3,967	5,554
36	B24	1,587	2,081	3,077	4,064	5,060
37	B25	1,587	2,081	2,971	4,161	5,854
38	B26	1,587	1,984	2,680	3,271	5,060
39	B27	1,587	1,984	2,477	3,174	4,558
40	B29	1,587	2,081	3,271	4,364	6,348
41	B30	1,587	1,984	3,174	4,461	6,647

No	Isolate No	The genus *Enterococus*				
		0h (mg/ml)	3h (mg/ml)	6h (mg/ml)	9h (mg/ml)	24h (mg/ml)
42	B31	1,587	2,081	3,174	4,461	6,841
43	B32	1,587	2,081	3,368	4,364	5,854
44	B33	1,587	2,081	2,971	4,161	5,748
45	B34	1,587	2,178	2,971	4,858	6,841
46	B35	1,587	2,380	3,077	4,955	7,837
47	B36	1,587	2,380	3,474	5,554	7,732
48	B37	1,587	2,380	3,077	4,955	7,732
49	C30	1,587	2,178	2,477	4,664	7,441
50	C31	1,587	2,283	2,777	3,967	6,251
51	C36	1,587	2,477	3,870	4,267	8,331
52	C38	1,587	2,477	2,777	4,064	7,335
53	C39	1,587	2,283	2,874	3,667	6,251
54	C40	1,587	2,283	2,777	3,667	6,841
55	C41	1,587	2,574	2,874	3,870	6,841
56	C42	1,587	2,380	3,077	3,967	6,251
57	C43	1,587	2,777	3,368	6,048	6,145
58	C46	1,587	2,283	2,380	2,971	5,748

Table H 2.3. Lactic acid production in UHT skim milk during incubation at 30 °C for *Lactobacillus* genus.

No	Isolate No	**The genus *Lactobacillus***				
		0 h (mg/ml)	3 h (mg/ml)	6 h (mg/ml)	9 h (mg/ml)	24 h (mg/ml)
1	C3	1,587	2,574	2,777	2,971	3,570
2	C5	1,587	2,680	2,477	2,874	3,570
3	C7	1,587	2,283	2,574	2,777	3,870
4	C8	1,587	2,380	2,574	2,574	3,870
5	C9	1,587	2,380	2,874	3,174	4,558
6	C12	1,587	2,380	2,477	2,680	4,064
7	C27	1,587	2,971	4,258	4,461	9,821
8	C29	1,587	2,574	2,680	3,271	4,955
9	C37	1,587	2,380	3,174	4,161	9,424
10	C47	1,587	2,380	3,174	3,967	9,323
11	D1	1,587	2,178	2,574	3,271	7,335
12	D2	1,587	2,283	2,874	3,667	8,825
13	D3	1,587	2,574	2,971	4,267	9,028
14	D5	1,587	2,477	2,680	4,461	9,028
15	D6	1,587	2,380	2,380	2,477	3,271
16	D7	1,587	2,477	3,271	3,271	8,128
17	D8	1,587	2,283	2,380	2,380	3,667
18	D9	1,587	2,178	2,380	3,077	6,145

APPENDIX I

Table I.1 Reference strains subjected to physiological and biochemical tests

Species name	2% NaCl	4% NaCl	6.5 % NaCl	10 °C	40 °C	45 °C	Test in Reddy Broth			$CO_2$from glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
							Color	Gas	Growth																
L lactis ssp. *lactis* CECT 4432	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
L lactis ssp. *lactis* A 216	+	+	-	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	+
L.lactis ssp.*diacetylactis* CECT 4432	+	+	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	-	+	-	+	+	+
E. facecium CECT 4102	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+
E. faecalis CECT 184	+	+	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+	+/-	+	+	+	+	+	+
E. gallinarum CECT 4102	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+/-	+	+	-	+	+	+

Species Name	6.5% NaCl	15 °C	45 °C	Arginine	CO_2 glucose	Xylose	Ribose	Galactose	Arabinose	Trehalose	Raffinose	Maltose	Mannitol	Sucrose	Sorbitol	Lactose	Gycerol	Salicin	Mannose	Glucose
Lactobacillus curvatus DSM 8768		+	-		-	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
Lactobacillus casei subsp. casei NRRL-B 1922		+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+