CHARACTERIZATION OF *LACTOBACILLUS DELBRUECKII* SUBSPECIES *BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* AS LACTIC CULTURES ISOLATED FROM TRADITIONAL TURKISH YOGURTS AND SUBTYPING OF *STREPTOCOCCUS THERMOPHILUS* USING CRISPR ANALYSIS AND MLST

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ABSTRACT

CHARACTERIZATION OF *LACTOBACILLUS DELBRUECKII* SUBSPECIES *BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* AS LACTIC CULTURES ISOLATED FROM TRADITIONAL TURKISH YOGURTS AND SUBTYPING OF *STREPTOCOCCUS THERMOPHILUS* USING CRISPR ANALYSIS AND MLST

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Yogurt is a characteristic fermented dairy product of Turkey and Bulgaria and its popularity has been increasing all over the world. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) are used together as starter culture in production of yogurt. The objective of this study was to isolate and characterize yogurt cultures from traditionally produced yogurts (i.e. produced without using commercial starter cultures) and to search the genotypic diversity within traditional *S. thermophilus* isolates.

Yogurt cultures were isolated from traditionally produced yogurts collected from different regions of Turkey and identified biochemically. Acidification ability of the isolates was examined and the cultures giving best acidifying rates were further subjected to a selection in terms of their acetaldehyde production ability. Then, phage resistance and proteolytic activity of chosen isolates were tested. Finally, twenty-five *L. bulgaricus* and twenty-two *S. thermophilus* isolates were selected as cultures having best technological properties.

Furthermore, subtyping studies were carried out to indicate strain diversity among isolates. *S. thermophilus* was selected as target organism for subtyping in this study. Clustered regularly interspaced short palindromic repeats (CRISPR) loci are highly polymorphic genetic regions, which are composed of partially palindromic direct repeats interspaced by sequences called spacers. In order to characterize *S. thermophilus* isolates genotypically, CRISPR1 locus of the isolates were analyzed. Additionally, nineteen isolates selected after CRISPR1 analysis were characterized using multilocus sequence typing (MLST). This provided to compare CRISPR1 analysis with MLST as a typing method. According to CRISPR1 analysis *S. thermophilus* isolates were grouped into 6 main clusters with a total of 15 sub-clusters. MLST results demonstrated an evolutionary relationship among these strains compatible with that derived from the CRISPR1 analysis.

Keywords: Yogurt, starter culture, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, clustered regularly interspaced short palindromic repeats (CRISPR) and multilocus sequence typing (MLST)

ÖZ

GELENEKSEL TÜRK YOĞURTLARINDAN LAKTİK KÜLTÜR OLARAK İZOLE EDİLEN *LACTOBACILLUS DELBRUECKII* SUBSPECIES *BULGARICUS* VE *STREPTOCOCCUS THERMOPHILUS* KARAKTERİZASYONU VE *STREPTOCOCCUS THERMOPHILUS*'LARIN CRISPR ANALİZİ VE MLST KULLANILARAK ALTTİPLENDİRMESİ

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Yoğurt, Türkiye ve Bulgaristan'ın karakteristik bir fermente süt ürünüdür ve populeritesi dünya çapında artmaktadır. Yoğurt üretiminde *Streptococcus thermophilus* ve *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) birarada starter kültür olarak kullanılmaktadır. Bu çalışmanın amacı, geleneksel olarak üretilen yoğurtlardan (ticari starter kültür kullanılmadan üretilen) yoğurt kültürleri izole etmek, bu kültürleri karakterize etmek ve geleneksel S. *thermophilus* izolatları arasındaki genetik çeşitliliği araştırmaktır.

Türkiye'nin değişik bölgelerinden toplanan geleneksel olarak üretilmiş yoğurtlardan yoğurt kültürleri izole edilmiş ve biyokimyasal tanıları yapılmıştır. İzolatların asidifikasyon yetenekleri incelenmiş ve en iyi asidifikasyonu veren kültürler asetaldehit üretimlerine göre yeniden bir seçime tabi tutulmuşlardır. Ardından, seçilen izolatların faj dirençlilikleri ve proteolitik aktiviteleri incelenmiştir. Son olarak, 25 adet *L. bulgaricus* ve 22 adet *S. thermophilus* izolatı en iyi teknolojik özelliklere sahip kültürler olarak seçilmiştir.

Ayrıca, izolatlar arası farklılıkları göstermek için alt tipleme çalışmaları gerçekleştirilmiştir. Bu çalışma için *S. thermophilus* hedef organizma olarak seçilmiştir. Clustered regularly interspaced short palindromic repeats (CRISPR) bölgesi oldukça polimorfik bir genetik bölgedir ve kısmi palindromic direkt tekrarların spacer denilen sekanslarla bölünmesinden oluşmuştur. *S. thermophilus* izolatlarını genotipik olarak karakterize etmek için izolatların CRISPR1 lokusları analiz edilmiştir. Ek olarak CRISPR1 analiz sonuçlarına göre seçilen 19 adet izolat multilocus sequence typing (MLST) kullanarak da karakterize edilmişlerdir. Bu durum CRISPR1 analizinin bir karakterizasyon metodu olarak MLST ile kıyaslanmasını sağlamıştır. CRISPR analizine göre *S. thermophilus* izolatları toplamda 15 adet alt grup içeren 6 adet ana grupta toplanmışlardır. MLST sonuçları, bu suşların CRISPR1 analizi sonuçları ile uyumlu bir evrimsel ilişki ortaya koymuştur.

Anahtar Kelimeler: Yoğurt, starter kültür, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, clustered regularly interspaced short palindromic repeats (CRISPR) ve Multilocus sequence typing (MLST)

To My Familly

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LIST OF ABBREVIATIONS

AFLP: Amplified Fragment Length Polymorphism

bp: basepair

Cas genes: CRISPR-associated genes

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DVS: Direct Vat Set, direct inoculation of starter

DVI: Direct Vat Inoculation, direct inoculation of starter

EPS: Exopolysaccharide

ITS: Intergenic Transcribed Spacer

LAB: Lactic Acid Bacteria

MLST: Multilocus Sequence Typing

MLVA: Multilocus Variable-Number Tandem Repeats Analysis

OD: Optical Density

PCR: Polymerase Chain Reaction

PFGE: Pulsed-Field Gel Electrophoresis

RFLP: Restriction Fragment Length Polymorphism

rRNA: ribosomal RNA

RSM: Reconstituted Skim Milk

snp: single nucleotide polymorphism

TES: N−tris(hydroxymethyl)methyl−2−aminoethanesulfonic acid

CHAPTER 1

INTRODUCTION

1.1. Yogurt

Yogurt, a fermented dairy product, was likely originated in the Middle East (Tamime and Robinson, 2007) and may have firstly made by Turks (Hayaloglu, et al., 2007). It has been always very popular in Turkey since traditional cuisine including yogurt as an ingredient in some recipes or a topping and dressing for lots of traditional meals besides consuming plain yogurt by itself. Nowadays, production of different types of yogurt such as fruit added yogurt and probiotic yogurts makes it a very attractive snack and health beneficial food not only in Turkey and Balkans but also in all over the world.

Yogurt is made by the action of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. These two cultures transform milk sugar (lactose) to lactic acid which decreases pH and hence is responsible for coagulation. Yogurt starter cultures produce also some byproducts during growth in milk, which contribute to the specific aroma and flavor of yogurt (De Brabandere and De Baerdemaeker, 1999).

In the yogurt production, one of the most important factors which determine the quality of the product is the quality of raw material to be true for all other food products and another is the type of the starter culture.

1.1.1 Yogurt Types and Manufacture

At present, there are many types of yogurt available on the markets worldwide with different classifications. Tamime and Robinson (2007) prepared a generalized scheme for the classification of yogurts (Figure 1.1) based on the following features:

- Regulations that apply to chemical composition of the final product (specifically fat content)
- Physical property (such as solid or liquid)
- Flavors used
- Processes applied after fermentation (such as addition of vitamins or heat treatment)

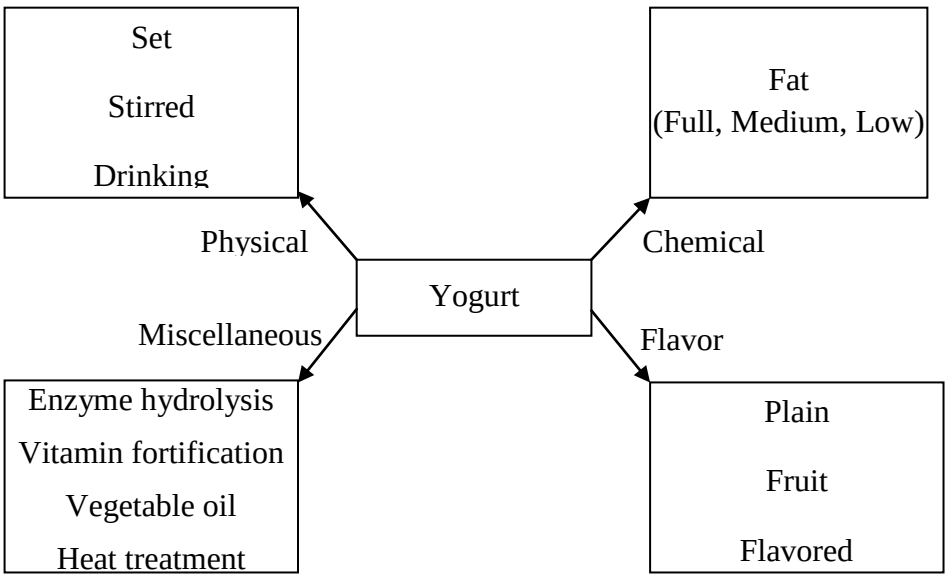


Figure 1.1 Classification of yogurts (Tamime and Robinson, 2007)

Even if there are different kinds of yogurts available on the market, basic production steps of these yogurts are similar. Firstly, the milk must be tested for

bacterial quality, fat and total solid content, antibiotic residues or any other inhibitory compounds and the convenience for yogurt production by a lab-scale fermentation test. If the milk is within the limits defined by legal authorities and by company itself, it is accepted for processing. The accepted milk can be directly send to yogurt processing line or to pasteurization if it is needed to wait before processing. The first step of yogurt processing is standardization of milk in terms of fat and total solid content. After that, this standardized milk is homogenized and heated by applying slightly severe conditions than pasteurization. Then, it is cooled to inoculation temperature of the starter bacteria before inoculation of starter culture. After incubation is completed, the temperature is decreased in order to stop fermentation and the product is stored at refrigeration temperature. A flow chart showing basic steps of set and stirred yogurt prepared by Duboc and Mollet (2001) is given in Figure 1.2.

Standardization of milk fat depends on the desired fat content in the final product such as full, medium or low fat yogurt. It should be also emphasized that fat content of yogurt affects creaminess in yogurt and also expands the mouthfeel (Lucey and Singh, 1998). Adjusting total solid content in milk to higher levels (14-16 g/100 g) than 13 g/100 g improves the viscosity of final product (Tamime and Robinson, 2007).

It is known that homogenization of milk influences the yogurt quality by changing color of milk to a whiter color, by improving mouthfeel of the product and by increasing milk viscosity and also by preventing fat from separation especially during fermentation period (Tamime and Robinson, 2007; Kopanos et al., 2010). The pressures within 10-20 MPa at temperatures between 55-65 ºC are used for homogenization of milk (Lucey and Singh, 1998).

Heating of milk prior to yogurt production is another important factor for quality of the final product. The standardized milk for yogurt production is heated to 85 ºC for 30 min or 90-95 ºC for 5-10 min (Tamime and Robinson, 2007). The effect of heat treatment in yogurt manufacturing was summarized by Tamime and Robinson (2007) as follows;

- Increasing sanitary quality of milk by destruction of pathogens and some other undesirable microorganisms
- Production of some compounds act as stimulator and/or inhibitor to starter cultures
- Changing the properties of some compounds such as proteins in milk, which have an important role in yogurt gel formation.

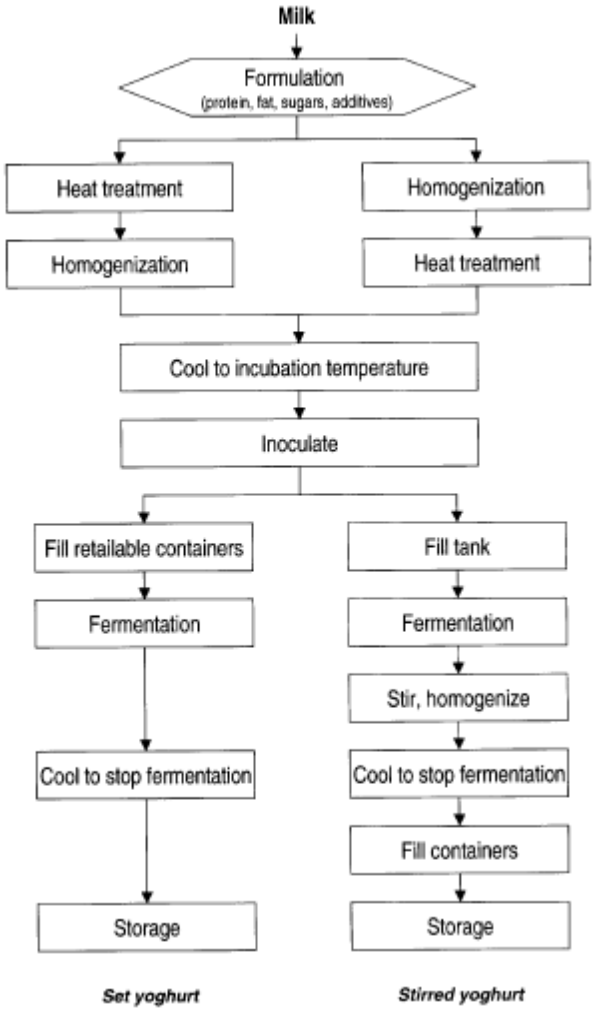


Figure 1.2 Flow chart for manufacturing of set and stirred yogurts (Duboc and Mollet, 2001)

Addition to process conditions, milk and starter culture used in the production play a very important role for quality of yogurt.

1.2 Milk as a Raw Material

Average chemical composition of cow's milk is given in Table 1.1 (Jay et al., 2005). However it should also be noted that the chemical composition of milk is variable and shows some differences according to geographical location, the origin of cow, lactation state, age of the cow, milking intervals, season of the year, climate temperature and nutrition (Mayra-Makinen and Bigret, 1998; Kopanos et al., 2010) and these differences influences the technological properties of yogurt culture and the properties of yogurt produced. Milk obtained from different kinds of animal shows also differences and causes variations in the product properties. Abu-Tarboush (1996) examined growth and proteolytic characteristics of *L. bulgaricus* and *S. thermophilus* in pasteurized whole camel's and cow's milk and detected a higher growth of both of yogurt bacteria in cow's milk than in camel's milk. However a higher proteolysis was observed in camel's milk than in cow's milk for both *L. bulgaricus* and *S. thermophilus*.

Table 1.1 Average composition of whole cow's milk (Jay et al., 2005).

Components	**%**
Water	87.0
Protein	3.5
Fat	3.9
Carbohydrate	4.9
Ash	0.7

There can be found some inhibitory compounds in milk such as antibiotic residues (i.e. resulted from antibiotics improperly used to treat the infectious diseases of dairy cows (Albright et al., 1961)), which would slow down or stop yogurt fermentation. Therefore, before processing to yogurt, milk should be tested for those inhibitory compounds.

1.3 Yogurt Starter Culture

In yogurt production a starter culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, termed *L. bulgaricus* hereafter, is used.

1.3.1 *Lactobacillus bulgaricus*

Species of Genus *Lactobacillus* are rod-shaped, Gram-positive, nonsporing and facultatively anaerobic. They are usually long rods making commonly short chains. They require rich media and 5% CO_2 generally enhances their growth. (Holt et al., 1994). The type species of this genus is *Lactobacillus delbrueckii* which has three subspecies, namely *Lactobacillus delbrueckii* subsp. *delbrueckii*, subsp. *lactis* and subsp. *bulgaricus*.

Genus *Lactobacillus* can be classified into three groups according to their fermentation features. These groups are obligately homofermentative (Group 1), facultatively heterofermentative (Group 2) and finally obligately heterofermentative (Group 3). Obligately homofermentative lactobacilli, which include *L. bulgaricus*, ferment glucose solely to lactic acid (Axelsson, 1998; Stiles and Holzapfel, 1997). D (-) lactic acid is the isomer produced by *L. bulgaricus*. Majority of the strains of *L. bulgaricus* can ferment fructose, glucose and lactose (Teixeira, 2000).

Genomes of *Lactobacillus bulgaricus* ATCC 11842 (van de Guchte et al., 2006) and *Lactobacillus bulgaricus* ATCC BAA-365 (Makarova et al., 2006) are sequenced and publicly available.

1.3.2 *Streptococcus thermophilus*

Species of Genus *Streptococcus* are nonmotile, nonsporing, Gram positive, having spherical or ovoid cells occurring in pairs or chain in liquid media, facultatively anaerobic and catalase negative. They also require nutritionally rich growth media. Fermentation product of *Streptococcus* spp. is mainly composed of lactate with no gas (Holt et al., 1994). As a species of *Streptococcus*, *S. thermophilus* possesses the general properties of this genus given above.

Streptococcus thermophilus belongs to viridans group of non-beta-hemolytic streptococci. Viridans Streptococci classified into 5 group including Mutans group, Salivarius group, Anginosus group, Sanguinus group, and Mitis group. *S. thermophilus* is a member of Salivarius group together with *Streptococcus salivarius* and *Streptococcus vestibularis*. Some investigators prefer the term "oral streptococci" instead of "viridans streptococci" however, it should also be noted that not all Streptococcus spp. in this group is originated from oral sources (Facklam, 2002).

Streptococcus thermophilus was reclassified as a subspecies of *Streptococcus salivarius* based on research by Farrow and Collins (1984) and named as *Streptococcus salivarius* subsp. *thermophilus* for several years. However, *S. thermophilus* was then showed as a distinct species by Schleifer et al. (1991) based on DNA–DNA hybridization studies under stringent conditions.

Streptococcus thermophilus is the only species in genus *Streptococcus* used in food fermentation. It is used in production of hard Italian and Swiss cheeses and yogurt (Gobbetti and Corsetti, 2000). In yogurt production it is inoculated together with *L. bulgaricus*.

Habitat of *S. thermophilus* is milk. Therefore, it can be isolated from milk, dairy utensils, pasteurized dairy products and pasteurization equipment as well as yogurt and cheese (Zirnstein and Hutkins, 2000).

S. thermophilus has the ability to ferment a few carbohydrates with a preference for lactose and sucrose. Additionally, it can also grow on glucose, galactose and fructose with a slower manner (Zirnstein and Hutkins, 2000).

S. thermophilus strains which can ferment free galactose does not ferment it if there is also glucose in the growth medium (Zirnstein and Hutkins, 2000).

Recently, genomes of three *S. thermophilus* strains were completely sequenced, that is, *S. thermophilus* LMG 18311(Bolotin et al. 2004), *S. thermophilus* CNRZ 1066 (Bolotin et al. 2004) and *S. thermophilus* LMD9 (Makarova et al. 2006).

1.3.3 Associative Growth of *S. thermophilus* and *L. bulgaricus*

The relationship between *S. thermophilus* and *L. bulgaricus* are quite often named as "symbiosis". However some researchers prefer other terms instead of symbiosis such as protocooperation (Fredrickson, 1977) or associative growth since both *S. thermophilus* and *L. bulgaricus* can grow in milk inoculated as single culture (Tamime and Robinson 2007). The term associative growth will also be used in this text hereafter to define the relationship between two yogurt starter bacteria.

Associative growth between *S. thermophilus* and *L. bulgaricus* have been studied in detail and well characterized. It is known that *L. bulgaricus* is more proteolytic than *S. thermophilus* (Rajagopal and Sandine, 1990). Therefore, *L. bulgaricus* stimulates *S. thermophilus* by providing peptides and free amino acids critical for growth of *S. thermophilus* like valine, histidine, tyrosine, methionine and isoleucine. *S. thermophilus* is also produce some compounds which stimulates growth of *L. bulgaricus* such as formic acid and CO_2 (Tamime and Robinson, 2007). Courtin and Rul (2004) examined the both sides of the association in terms of proteolysis and formic acid production. They detected that *L. bulgaricus* provided more free amino acids and peptides than *S. thermophilus* and the amounts of free amino acids and peptides were decreased in mixed culture, which could be explained by consumption of these compounds by *S. thermophilus*. The similar observation was made by the same author for formic acid. They detected that the

formic acid in mix culture was lower than the amount produced by *S. thermophilus* in pure culture and explained the reason of this decrease as the consumption of formic acid by *L. bulgaricus.* Suziki et al. (1986) studied the role of formic acid on growth of *L. bulgaricus* and concluded that formate stimulate *L. bulgaricus* by being a precursor of purine base.

It is a well known fact that, technological properties of yogurt starter bacteria are affected by their associative growth. Production of acid and acetaldehyde is greater in mixed culture compared with pure cultures (Jay et al., 2005; Tamime and Robinson, 2007; Ray and Bhunia, 2008).

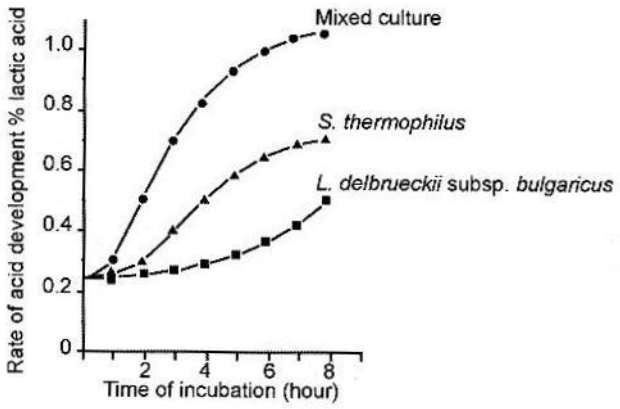


Figure 1.3 The acidification rate of yogurt bacteria in pure cultures and mixed culture i.e. the effect of associative growth on acidification rate.
Cultures inoculated in autoclaved skim milk including10% total solid with a 2% inoculation rate and incubated at 40 ºC (Tamime and Robinson, 2007)

Temperature is known as an effective factor on associative growth and also on technological properties of yogurt bacteria such as acidification rate. When acidification rate of *S. thermophilus, L. bulgaricus* and mixed culture are measured

while incubating at different temperatures, the maximum acidification rate is obtained at 40 ºC for *S. thermophilus* and 45 ºC for *L. bulgaricus* (Figure 1.4). 45 ºC is also the optimum temperature for activity of mixed culture. However 42 ºC is the recommended temperature for mixed culture to maintain 1:1 ratio of *S. thermophilus* to *L. bulgaricus* (Tamime and Robinson, 2007).

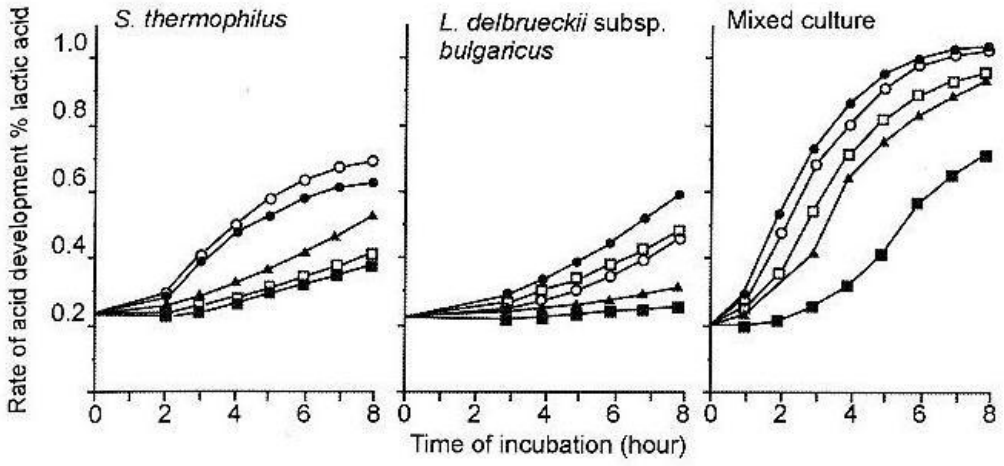


Figure 1.4 The effect of temperature on acidification rate of pure and mixed cultures of yogurt bacteria.
Cultures inoculated in autoclaved skim milk including10% total solid with a 2% inoculation rate and incubated at different temperatures which are 30 ºC (■), 35 ºC (▲), 40 ºC (○), 45 ºC (●) and finally 50 ºC (□) (Tamime and Robinson, 2007).

Although preparing of yogurt cultures containing *L. bulgaricus* and *S. thermophilus* in a ratio of 1:10-1000 is the new approach (Tunail, 2009), classical yogurt starter culture includes *S. thermophilus* and *L. bulgaricus* in a ratio of 1:1. During incubation, *S. thermophilus* grows fast at the beginning and provide initial acidification at the presence of dissolved oxygen and produce formic acid and CO_2. Then, growth of *L. bulgaricus* accelerates by anaerobic condition, formic acid and CO_2 and after about 3 h incubation the ratio of two bacteria is again

approximately 1:1. Changing the growth temperature influences the balance between the two bacteria. Increasing temperature causes predominating of *L. bulgaricus*. Contrarily, decreasing it predominates the growth of *S. thermophilus* (Jay et al., 2005; Ray and Bhunia, 2008).

1.3.4 Starter Culture and Use of Starter Culture in Dairy Industry

Since starter cultures are the most important factors which determine the final features and quality of the product and different forms of starters are available on the market, selection of starter type and form is crucial (Mayra-Makinen and Bigret, 1998).

Starter cultures can be composed of pure culture of a defined strain or by combination of different strains of a species or even of different species. Therefore different types of starter culture are available on the market and they can be grouped as following (Mayra-Makinen and Bigret, 1998; Wigley, 2000);

- Single-strain starter: composed of pure culture of a certain strain. Since the risk of phage attack, single-strain cultures are used in pairs or triples. It can use with or without rotation.
- Multiple-strain starter (defined strain starter): composed of different strains of a species. It is used alone and it can be used with or without rotation.
- Multiple-mixed-strain starter: composed of different defined strains of distinct species.
- Raw mixed-strain starter: naturally occurred cultures which are composed of partly or all unknown species and strains. These kinds of cultures are generally used in small traditional producers. Since they are composed of mostly unknown bacteria, the control of fermentation is difficult when using this type of culture.

Yogurt starter cultures generally contain *Streptococcus thermophilus* and *L. bulgaricus* together and hence called as mixed strain starter cultures (Tamime and Robinson, 2007)

There are different methods available to produce starter cultures and according to the production technique, storage and usage of starters vary. Essentially, it is possible to divide starter cultures into two main groups i.e. direct-to-vat cultures and cultures which need presteps to propagate the bacteria and to increase their activity (Gurakan and Altay, 2010). Different starter systems are shown in Figure 1.5 prepared by Mayra-Makinen and Bigret (1998). Direct-to-vat cultures are concentrated cultures by starter producers. Therefore they can be used by adding directly to processing tank without any preliminary steps. However, the non-concentrated cultures need sequential inoculation steps by increasing media volume at each step (Figure 1.5).

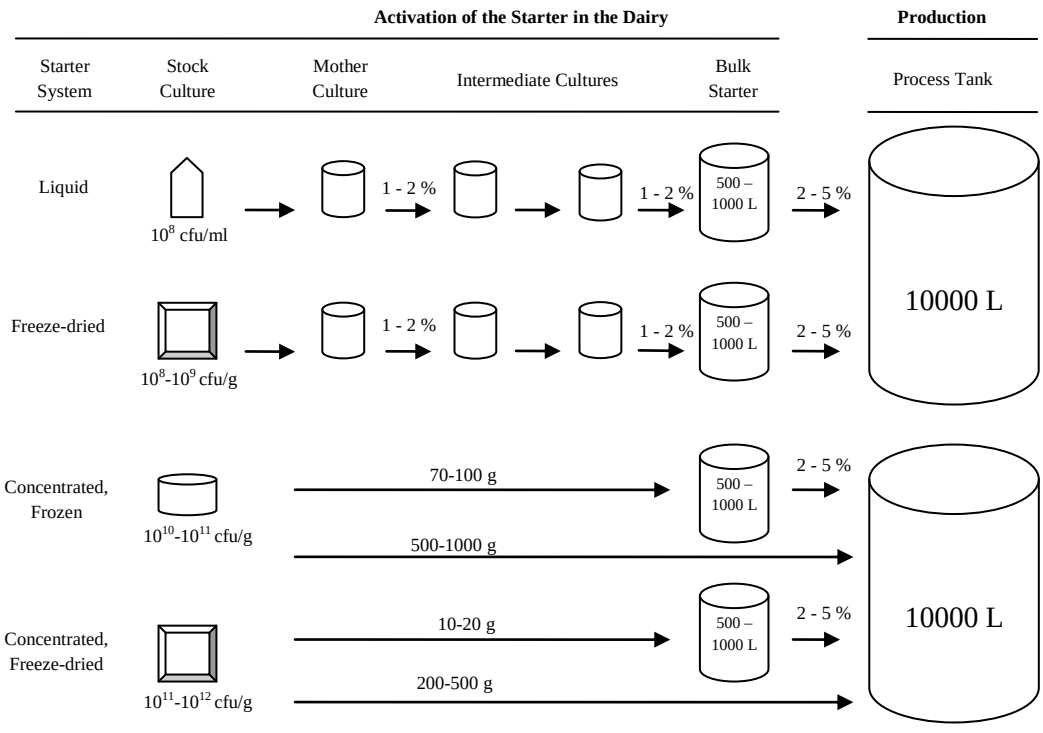


Figure 1.5 Various starter systems (Mayra-Makinen and Bigret, 1998)

It is possible to see all the starter systems (i.e. liquid, freezed dried, frozen and concentrated cultures for DVI) on the market. Main features of distinct starter system were given in Table 1.2 and as seen in the table each of these systems has some positive and negative aspects. Therefore, even liquid cultures is still available in the product lists of some companies such as Danisco (for only ripening cultures) (http://www.danisco.com) and Intermak (for yogurt culture) (B. A. Değirmenci, Intermak Makina İmlalat- İthalat Sanayi Ticaret A. Ş., Konya, 2010, personal communication). However, the general tendency within starter producers is to produce freeze-dried and frozen cultures including for yogurt starter cultures (Table 1.3). These cultures are mostly concentrated cultures to be used as DVS cultures.

Main starter producers thought the world are given in Table 1.3. The most of them contains yogurt starter cultures in their product lists. In Turkey, there is only one starter culture producer (Intermak, http://www.intermak.com.tr). Intermak has a history of 5-6 years of starter production. They produce and sell yogurt starter culture and their consumer group consists of small local dairy plants. Their culture collection is composed of isolated yogurt bacteria from traditional yogurt, cheese, raw milk collected from different regions of Turkey. They produce freeze dried, frozen and liquid culture, although their freeze dried culture is produced in a very limited amount. Their liquid yogurt cultures are their most popular yogurt starter culture in present times, since its non-expensive price and they can transport it easily to their consumers in Turkey (B. A. Değirmenci, Intermak Makina İmlalat-İthalat Sanayi Ticaret A. Ş., Konya, 2010, personal communication).

Table 1.2 Properties of different starter systems

	Bulk Starter Cultures				Direct to Vat Cultures	
Characteristic	Daily propagated cultures	Deep-frozen cultures	Deep-frozen concentrated cultres	Lyophilized concentrated cultures	Deep-frozen direct vat cultures	Lyophilized direct vat cultures
Cost of culture per vat	Low	Medium	Medium	Medium	High	Medium
Level of technical skill required	High	High	Medium	Medium	None	None
Cost of storage of cultures	Low	Low	High	Low	High	Low
Level of phage relationship data available	None	High	High	High	High	High
Period of planning required to manufacture starter	72 h	48 h	24 h	25 h	None	None
Level of technical support provided for system	None	High	High	High	High	High

Table 1.3 Starter producer and starter systems

Producer	Country	Yogurt[a]	Starter System[b]	References
Chr Hansen	Denmark	Yes	DVS	http://www.chr-hansen.com
Danisco	Denmark	Yes	Freeze dried/Frozen	http://www.danisco.com
DSM	The Netherlands	Yes	Freeze dried/Frozen	http://www.dsm.com
Alce	Italy	No	DVS	http://www.alce.eu
CSL	Italy	Yes	Freeze Dried concentrated	http://www.csl.it
BioSource Flavors, Inc	USA	Yes	Freeze dried/Frozen can	http://www.biosourceflavors.com
CSK Food Enrichment	The Netherlands	Yes	Deep frozen pellets	http://www.cskfood.com
BIOPROX	France	Yes	Freeze dried	http://www.bioprox.com

[a] Production of yogurt starter culture

[b] DVS: Direct Vat Set cultures, Forzen and/or Freeze dried; not specified; Frozen/Freeze Dried ; not specified whether concentrated

Producers and webpages compiled from Gurakan and Altay, 2010

1.3.5 Starter Culture Production

Production of concentrated frozen and freeze dried cultures is focused on in this chapter because they are the most common starter cultures available on the market and additionally their production steps mainly include the production steps of the other starter systems. Using concentrated cultures to prepare bulk cultures or to inoculate into directly processing tank has also advantages like; saving time,

eliminating tedious steps in propagating culture for inoculation, and decreasing the risk of undesirable bacteria and/or bacteriophage contamination (Gilliland, 1977).

Production steps of concentrated cultures can be summarized as follows (Mayra-Makinen and Bigret, 1998);

- Inoculum preparation
- Growth media preparation
- Fermentation
- Collecting the culture
- Cryoprotective agent addition
- Freezing
- Freeze-drying
- Packaging and storage

1.3.5.1 Propagation of Starter Cultures and Concentration

Batch fermentations are used in industrial production of starter cultures (Mayra-Makinen and Bigret, 1998). It can be faced with some problems during continuous culture production such as bacteriophage contamination, unfavorable mutations and complexity in equipments (Northrop, 1966; Lloyd and Pont, 1973; Gilliland, 1977; Mayra-Makinen and Bigret, 1998).

One of the important factors affecting fermentation is the composition of the growth media. The main features of a good medium for propagation of bacteria to prepare concentrated cultures were defined by Gilliland (1977) as follows;

- the medium must contain all necessary nutrients to provide optimum growth of the starter bacteria
- the growth medium must be designed such a way that the propagated cells must have the enzymes and biological activity which will be essential during production of fermented milk products. Therefore, containing milk

solids is a preferable property in a growth media to produce starters for dairy processes

- the medium should not have any negative effect on harvesting process

Addition to growth medium, some other factors such as temperature, pH, agitating, type of neutralizer used and harvesting time is important to control growth and activity of the starter culture (Mayra-Makinen and Bigret, 1998).

It is crucial to prevent inhibitory effect of the acid produced by the starter culture itself during fermentation. This can be achieved by controlling the pH by adding some neutralizers. The pH of growth medium can be kept between at a range which is optimum for the culture and this application increases the total number of cells obtained after fermentation (Peebles et al., 1969; Gilliland 1977, Mayra-Makinen and Bigret, 1998). Additionally, the kind of the neutralizer used was effective on the amount of cell obtained (Peebles et al., 1969; Gilliland 1977, Mayra-Makinen and Bigret, 1998). The optimum pH range for *L. bulgaricus* is reported as 5.4-5.8 and ammonium hydroxide is claimed as the neutralizer giving highest yield for this starter bacterium (Mayra-Makinen, unpublish data, Mayra-Makinen and Bigret, 1998).

In order to maintain constant pH during fermentation, agitation of the growth medium is necessary. Decrease in the amount of produced starters has been observed in fermentations sparged with air (Cogan et al., 1971).

The resistance of the cultures against concentration, freezing or drying processes can be enhanced depending on strains by adding specific nutrients or additives to growth media (Mayra-Makinen and Bigret, 1998). Smittle et al. (1972) reported that the addition of Tween 80 (polyoxyethylene sorbitan monooleate) into the growth medium of *L. bulgaricus* strains enhance the storage stability of the *L. bulgaricus* strains after freezing in liquid nitrogen. Similar improvement of resistance of *L. bulgaricus* strains to freezing has been also observed by adding only sodium oleate into the growth medium instead of Tween 80 (Smittle et al., 1974). Smittle et al. (1974) additionally claimed that C_{19} cyclopropane fatty acid

was correlated with the resistance of *L. bulgaricus* to freezing. Goldberg and Eschar (1977) also noted an increased viability of lactic cultures frozen at -17ºC if oleic acid or Tween 80 was added to their growth medium and reported a change of cellular fatty acid composition when the bacteria were grown in media supplemented with Tween 80. These researches concluded that the resistance of lactic acid bacteria to freezing can be related with composition of the cellular fatty acids (Smittle et al., 1974; Goldberg and Eschar, 1977). The cell membrane of Gram positive bacteria contains the majority of the lipids in these cells and hence composition of the fatty acids of the cell membrane is important to the resistance of the streptococci to freezing (Gilliland, 1977).

Although optimum growth temperature of the bacteria were suggested as fermentation temperature for starter culture production by some researchers (Tamime and Robinson, 2007), a decreased or increased growth temperature was reported as having an effect on process stability during freezing and freeze-drying in thermophilic starter cultures by affecting the dechaining of bacteria (Mayra-Makinen, unpublished data, Mayra-Makinen and Bigret, 1998).Therefore, before processing, optimum temperature for the process need to be determined (Mayra-Makinen and Bigret, 1998).

Although pH control by using some neutralizers (such as sodium hydroxide and mostly ammonium hydroxide) increases the yield of starter obtained after fermentation, the cell production stops even if there is enough available nutrients and acid production is continuing (Gilliland, 1977). This inhibition of growth is caused by some inhibitory compounds such as lactate accumulated in media and D-leucine produced by certain lactic streptococci (Gilliland, 1968; Tamime and Robinson, 2007). Lactic acid produced by the starter reacts with the neutralizer and form lactate which inhibits the starter bacteria after a specific level (Tamime and Robinson, 2007). Therefore, starter bacteria obtained from pH controlled fermentors still need to be concentrated to reach higher concentrations (i.e. 10^{10}-10^{12}cfu/g) (Tamime and Robinson, 2007). Centrifugation or membrane concentration can be used to separate culture from the medium (Mayra-Makinen and Bigret, 1998).

Activity during storage of the starter culture is affected by the pH of the cell concentrate (Mayra-Makinen and Bigret, 1998). Optimum pH of the concentrate has been determined as 5.4-5.8 for lactobacilli. However, lower pH values for lactobacilli do not change the activity after freeze-drying. *S. thermophilus* is oppositely affected very much by pH values lower than the optimum concentrate pH (i.e. 6.2-6.6) (Mayra-Makinen, unpublish data, Mayra-Makinen and Bigret, 1998).

Another factor affecting viability after freeze drying is harvesting time of the cells. The early stationary phase of the bacteria such as *Streptococcus cremoris* or *L. bulgaricus* was pointed out the time that the bacteria were in the most resistant stage (Morichi, 1974). *S. thermophilus* is however suggested to be harvested before stationary phase (Mayra-Makinen, unpublish data, Mayra-Makinen and Bigret, 1998).

Starter suspending material must be selected with a great care to minimize damages caused by freezing (Gilliland, 1977). Cryoprotectants have been used in order to enhance the resistance of cultures against freezing, frozen storage and freeze drying (Mayra-Makinen and Bigret, 1998). Different cryoprotectants were studied to enhance the survival rate of starter cultures (Tamime and Robinson, 2007). In industrial production of starter cultures, lactose or sucrose (7%), monosodium glutamate (5%) and ascorbate in milk or water base are the cryoprotectants which are commonly chosen (Mayra-Makinen and Bigret, 1998).

Frozen yogurt starter cultures are produced by either deep or subzero freezing (-30 to -80 ºC) or ultra-low temperature freezing (-196 ºC) using liquid nitrogen (Tamime and Robinson, 2007).

Concentrated starter cultures in frozen form are stored at -40 ºC for at least 6 months with a good activity while freeze dried starter cultures are stored at between -20 ºC and -40 ºC and additionally at refrigeration temperature for a short time without any activity loss (Mayra-Makinen and Bigret, 1998).

1.3.6 Effects of Starter Culture on Yogurt Production and Characteristics

Starter culture is one of the most important factors influencing yogurt quality. For this reason, technologically important properties of cultures should be examined carefully. The functions listed below are the key functions of starter cultures for production and determining the characteristic properties of yogurt (Mayra-Makinen and Bigret, 1998; Tamime and Robinson, 2007).

1. Acid production
2. Proteolytic activity
3. Aroma formation
4. Exopolysaccharide (EPS) formation
5. Phage resistance

1.3.6.1 Acid Production

Acid production rate, in other words acidification rate, is probably the most important technological characteristic of a yogurt starter culture, since acidification causes coagulation of casein and hence production of yogurt. Lactic acid produced by yogurt starter culture during fermentation also gives yogurt its characteristic acidic taste and contributes its aromatic flavor (Tamime and Robinson, 2007).

Different lactic acid isomers are produced by lactic acid bacteria. In yogurt, *S. thermophilus* produces L (+) lactic acid (Zirnstein and Hutkins, 2000) and *L. bulgaricus* produces D (-) lactic acid (Teixeira, 2000). The ratio of these isomers to each other in yogurt samples can vary depending on the factors such as rod:cocci ratio in the starter culture, incubation temperature during manufacture, incubation time, inoculation rate or storage time. Isomer type of lactic acid can be important for health concerns, since hydrolyzation of D-lactic acid in humans is very slow and may cause disease called D-Lactic acidosis. The World Health Organization suggests a maximum daily intake of 100 mg D-lactic acid /kg body weight (Zourari et al., 1992). However, L-lactic acid does not have any limitation (Holzapfel, 2002). In Turkish Standards, amounts of maximum and minimum

lactic acid are specified as 0.6% and 1.6% total titratable acidity in terms of lactic acid (Turkish Standards, TS1330/Nisan 2006). However D-lactic acid amount in yogurt is not defined in this standard.

1.3.6.2 Proteolytic Activity

Proteolytic activity of yogurt starters is less important compared to cheese starters (Tamime and Robinson, 2007). However, associative growth of *S. thermophilus* and *L. bulgaricus* based on mainly the difference of their proteolytic activity and hence proteolytic activity may play an important role in blending together of distinct strains of *S. thermophilus* and *L. bulgaricus* (Gurakan and Altay, 2010). Additionally, *S. thermophilus* and *L. bulgaricus* are known as weakly proteolytic. However, it is also reported that they may show significant proteolytic activity which may affect the physical structure and flavor of the yogurt as a result of releasing peptides and free amino acids (Tamime and Robinson, 2007).

In general, proteolytic activity of *L. bulgaricus* is higher than *S. thermophilus* even if significantly different proteolytic activities were reported within the strains of the same species. Addition to the strains used, non-fat total milk solid, incubation time and preheating of milk has also effect on proteolysis during incubation. Maximum level of proteolysis was observed when nonfat total milk solid was adjusted to 14.5%, but increasing non fat total milk solid beyond 14.5% showed a decrease in proteolysis (Slocum et al., 1988a).

1.3.6.3 Aroma Formation

There are different compounds which contribute to yogurt aroma. Some of these aroma compounds come from milk and the others are products of yogurt starter culture (Ott et al., 1997). Ott et al. (1997) identified 91 volatile compounds in yogurt flavor and 21 of these compounds were reported as having a major impact on the yogurt aroma. Even the aroma of yogurt is very complex and composed of lots of different compounds, acetaldehyde is claimed as the volatile compound which gives the typical aroma to yogurt (Tamime and Robinson, 2007).

L. bulgaricus and also *S. thermophilus* can produce acetaldehyde. According to some authors *L. bulgaricus* plays more important role in acetaldehyde production than *S. thermophilus* whereas other authors have reported the contrary. However, it seems that acetaldehyde production in milk by lactic acid bacteria vary depending on the strain (Ott et al., 2000). It was also reported that changing the amount of acetaldehyde in yogurt during storage at 4ºC was also culture dependent (Hamdan et al., 1971).

Ott et al. (2000) searched the origin of acetaldehyde during milk fermentation by *L. bulgaricus* and *S. thermophilus*. In their study, 90% or more of the acetaldehyde produced was found to originate from glucose during fermentation by *L. bulgaricus* and *S. thermophilus*. If amount of L-threonine was artificially increased to high concentration such as100 mg/l, L-threonine was the main precursor for acetaldehyde production. However, under normal condition cow's milk does not contain such a high amount of L-threonine, so glucose was claimed as the main precursor of acetaldehyde.

The production of acetaldehyde becomes apparent at pH 5.0 and increases while acidification increase and show maximum at pH 4.2 and it stabilizes at pH 4.0 (Tamime and Robinson, 2007). Beshkova et al. (1998) showed that acetaldehyde production also occurs after milk coagulation during refrigeration and storage of the starter cultures. Maximum concentration was detected between 22-31h after inoculation (Beshkova et al., 1998).

Acetaldehyde can be also related with flavor problems in plain yogurts, in which a chalky flavor is obtained by low concentrations of acetaldehyde while very high concentrations cause green flavor (Ray and Bhunia, 2008). Acetaldehyde is produced in the ranges of 2-41 μg/g by mixed yogurt starter cultures (Tamime and Robinson, 2007) and optimal acetaldehyde concentration can vary in different places, which is determined according to consumer's demands.

1.3.6.4 EPS Formation

Exopolysaccharides are polysaccharides located outside of the cell wall, which can be found either attached to the cell wall as capsules or secreted into the extracellular environment (Bubb et al., 1997).

Expolysaccharides produced by LAB have an important effect on the improvement of the rheology, texture and mouthfeel of fermented milk products, such as yogurt (De Vuyst and Degeest, 1999; Welman and Maddox, 2003). Their impacts on these structural properties of products defined by their ability to bind water, interact with proteins, and increase the viscosity of the milk serum phase (Duboc et al., 2001).

Exopolysaccharides are divided into two groups according to their composition, i.e. homopolysaccharides and heteropolysaccharides. Homopolysaccharides are composed of single kind of monosaccharide while heteropolysaccharides are composed of more than one sugar moiety (Duboc, 2001). In general, glucose, galactose and rhamnose are mainly the sugar moieties found in EPSs (Tamime and Robinson, 2007).

It was known that some strains of both *S. thermophilus* and *L. bulgaricus* produce EPS. These are heteropolysaccharides composed of either linear or branched repeating units (Tamime and Robinson, 2007). Compositions of exopolysaccharides produced by yogurt cultures have been reported, even the polymer composition may be affected by culture conditions and carbon source in growth medium (Cerning et al., 1988). Composition of EPS produced by *S. thermophilus* on skim milk was searched by Cerning et al. (1988) and galactose and glucose were detected as the major monomers found in EPS structure together with smaller amount of rhamnose, arabinose, xylose, and mannose. Composition of EPS produced by ropy strains of *L. bulgaricus* on skim milk was also identified by Cerning et al. (1986) and it was reported that this water-soluble EPS was composed of galactose, glucose and rhamnose in an approximate molar ratio of 4:1:1.Petry et al. (2000) developed a chemically defined medium and examined the

effect of medium and growth condition on EPS yield and composition using two strains of *L. bulgaricus*. They detected that carbohydrate source, temperature, pH-controlling were effective on EPS production of *L. bulgaricus*. They also observed the majority of EPS production was occurred during stationary phase.

1.3.6.5 Phage resistance

Phage infection is the major problem in dairy plants although lots of different inhibitory factors can be also effective on starter culture during fermentation (Josephsen and Neve, 1998).

It is necessary to know steps in phage multiplication for a better understanding of phage resistance mechanisms of bacteria. Phage multiplication can occur by one of the two different ways, namely lytic cycle and lysogenic cycle depending on the phage. In lytic cycle, firstly phage attaches on bacteria and inject its DNA into bacterial cell, followed by reproducing new phages using metabolic system of the bacteria by phage DNA. When the new phages mature, cell wall of the host is disturbed and new phages are released to the environment (Cogan, 2000). In lysogenic cycle, the phage DNA, after injection, integrates into host's chromosome, which needs a small homologous part between the chromosomes of host and phage (Josephsen and Neve, 1998).

There are five natural phage defense systems in lactic acid bacteria; inhibition of phage adsorption, the blocking of DNA injection, restriction-modification (R/M) systems, and abortive infection (Abi) and recently discovered Clustered regularly interspaced short palindromic repeats (CRISPR) (Jansen et al., 2002)-mediated phage resistance (Deveau et al 2008).

In the case of inhibition of phage adsorption to host cell, the relevant receptors on the cell surface might be deficient or be masked physically (Josephsen and Neve, 1998). The information about blocking of phage DNA injection into the host cell after adsorption is limited, but some evidence for its existence in LAB is available. R/M system is based on the digestion of phage DNA by host-encoded site-specific

restriction endonucleases while the host DNA is protected via modification by a methylase enzyme (Forde and Fitzgerald, 1999). Sometimes first steps of phage multiplication are occurred without facing any resistance mechanisms and the Abi systems play role at the late phage maturation. In this system, degradation of host chromosome during phage maturation results in death of host cell, so the phages are not released to environment i. e. remain in host cell (Josephsen and Neve, 1998).

Additionally, it is also possible to use modification of DNA to improve the phage resistance of a strain. Moineau et al. (1995) introduced the plasmid-encoded *Lactococcus lactis Lla*II restriction/modification system into *S. thermophilus* and this was resulted in strong phage resistance in miscellaneous industrial *S. thermophilus* strains. However, these kinds of applications are not classified as food-grade by European Union (EU). EU does not allow self-cloning and non-self cloning in food products, while conjugation and transduction are allowed in food products (Sybesma et al., 2006).

1.4 Identification of Yogurt Starter Culture

Identification of an unknown bacteria starts with classical tests such as Gram staining, catalase test, growth test at different temperatures and fermentation test of different carbohydrates. However, even if these experiments provide lots of information about the bacteria and are very helpful for identification, most of the time, genotypic identification techniques are additionally used for confirmation.

1.4.1 Phenotypic Identification

Even if genetic identification methods give very accurate results and their application is mostly time saving, traditional identification methods are also very helpful especially differentiation in genus level and hence narrow the number of isolates for genetic identification.

Conventional identification of LAB starts with using the definition of this group and followed by using characteristic features of genera within the group. Therefore, microscopic morphology, Gram staining, catalase test are the start point for identification of *S. thermophilus* and *L. bulgaricus*. The composition of end product is very crucial for differentiation of species especially in Genus *Lactobacillus* which contains obligate homofermentative, facultative heterofermentative and also obligate heterofermentative species (Batt, 2000). Therefore, checking production of CO_2 from glucose should be the next step. Determination of growth ability at various temperatures is also helpful for especially differentiation of *S. thermophilus* from *Enterococcus* subsp. and *Lactococcus* subsp. (Figure 1.6).

After completion of the experiments targeted mainly genus differentiation within LAB, determination of carbohydrate fermentation patterns of isolates is helpful for identification in species level. For this purpose, growth of isolates on various carbohydrates should be checked. However, reliability of carbohydrate fermentation tests is questionable since distinct authors reported different carbohydrate fermentation patterns for the same species. Nevertheless, checking utilization of various carbohydrates is not only helpful for identification but also enhances the knowledge about the isolate. In general, most *S. thermophilus* isolates can ferment lactose, glucose, sucrose and fructose. Strains of *S. thermophilus* generally cannot ferment galactose, but some naturally occurring strains which can ferment galactose were reported (Zirnstein and Hutkins, 2000; van den Bogaard et al., 2004). Additionally, carbohydrate fermented by *L. bulgaricus* is also very limited. 90% or more of *L. bulgaricus* strains can ferment only fructose, glucose and lactose (Teixeira, 2000). However, fermentation of galactose by *L. bulgaricus* was also reported (Badis et al., 2004).

Additional phenotypic characterization methods can be also performed for a better identification of LAB at the species level such as determination of the lactic acid configuration produced, bile tolerance, type of hemolysis, growth factor requirements, production of certain enzymes such as β-galactosidase,

electrophoretic mobility of the lactate dehydrogenase (LDH) and fatty acid composition (Axelsson, 1998)

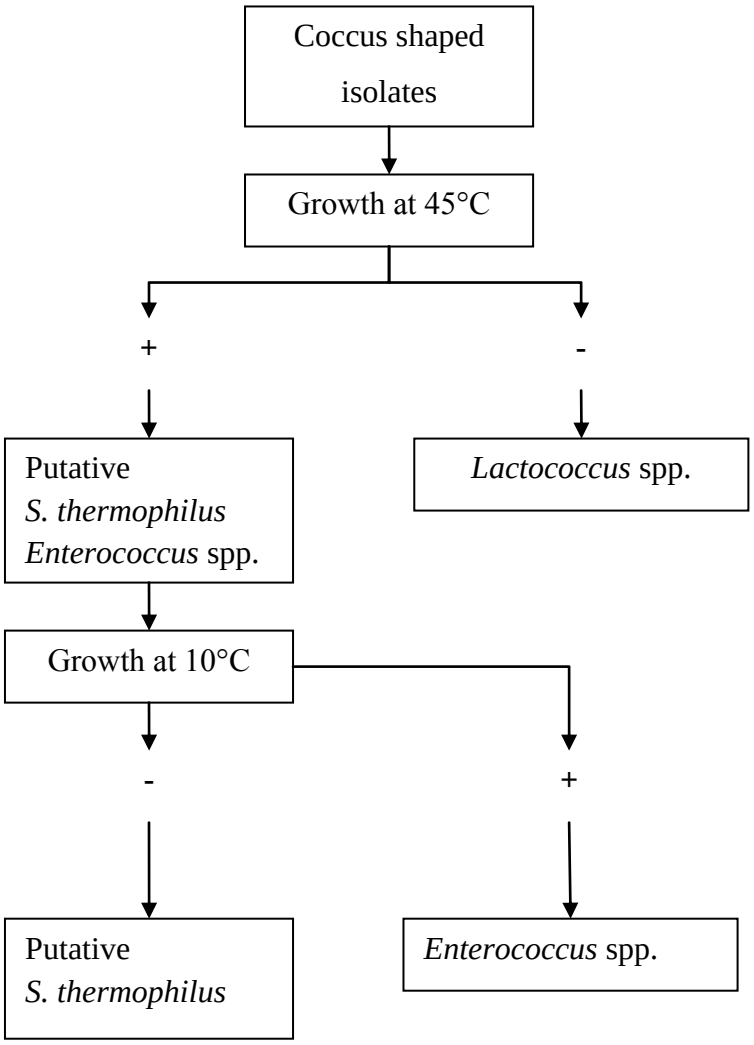


Figure 1.6 Differentiation of putative *Streptococcus thermophilus* strains from species belongs to the genera *Enterococcus* and *Lactococcus*. +:growth at related temperature, -: no growth at related temperature (Gurakan and Altay, 2010)

1.4.2 Genotypic Identification

Genetic tools are frequently used for identification of LAB, which provide more reliable results compared with biochemical identification methods. In the past determination of mole percent guanine plus cytosine (mol% G+C) content was an important tool for identification. Closely related bacteria share similar mol% G+C content, however, two organisms with similar mol% G+C contents do not have to be closely related (Gürakan,1991). On the other hand, DNA-DNA homology would confirm the identification of the isolate at species level (Gürakan, 1991). The genetic identification methods nowadays used alone or in combination with each other for identification of LAB includes 16S rRNA gene sequencing (Rossetti and Giraffa, 2005; Balca´zar et al., 2007), species-specific PCR (Rossetti and Giraffa, 2005), randomly amplified polymorphic-PCR (RAPD-PCR) (Rossetti and Giraffa, 2005; Tamang et al., 2005), amplified rDNA restriction analysis (ARDRA) (Andrighetto et al., 1998), rep-PCR (Gevers et al., 2001).

1.4.2.1 16S rRNA Gene Sequencing

In prokaryotes, ribosomes have two subunits called small subunit (30S) and large subunit (50S). Ribosomes are composed of proteins linked to rRNAs. The small subunit contains 16S rRNA while large subunit contains two RNA molecules, which are 23S rRNA and 5S rRNA. The genes of these three rRNA molecules are usually within an operon (Tourova, 2003). One of the 6 ribosomal RNA operons on *S. thermophilus* LMG 18311 was shown in Figure 1.7.

Completely or partially sequencing of 16S rRNA gene is frequently used for identification of LAB. 16S rRNA gene is a highly conserved genetic material. Dubnau et al. (1965) suggested the presence of some genes which is relatively resistant to evolutionary changes in genus Bacillus. These genes were some antibiotic resistance genes and ribosomal RNA coding genes. The reason of highly conservation of 16S rRNA gene is assumed as its being a critical component for cell function and hence mutations in this gene cannot be tolerated easily (Clarridge, 2004).

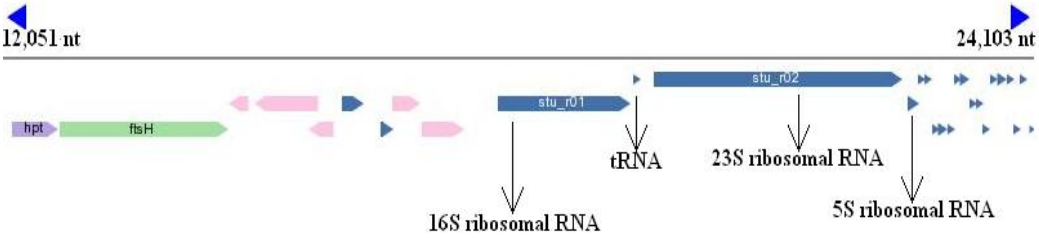


Figure 1.7 Representative ribosomal RNA operon

Figure was obtained from Overview of *S. thermophilus* LMG 18311, complete genome by NCBI-Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

The 16S rRNA gene is about 1.5 kb and composed of both variable and conserved regions. Even if sequencing of the whole gene (Sacchi et al., 2002) is sometimes necessary, partially sequencing of variable regions is mostly useful for identification. Clarridge (2004) compared 500 bp and 1500 bp long 16S rRNA gene sequences of a group composed of clinical and type strains of *Brevibacterium* and obtained not identical but similar dentograms.

In summary, phenotypic and genotypic methods should be used together for an accurate identification. For the fastest identification of yogurt starter bacteria the phenotypic identification methods such as microscopic examination, Gram staining, catalase test and gas production from glucose can be performed for genus identification and followed by determination of carbohydrate fermentation patterns both to improve the information about bacteria and to identify it at species level. After these phenotypic examinations of the bacteria, identification should be confirmed by a genotypic method preferably by sequencing of 16S rRNA gene partially or completely.

1.5 Genetic Characterization of *S. thermophilus* at Strain Level

1.5.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered regularly interspaced short palindromic repeats (CRISPR) (Jansen et al., 2002) are widely distributed repeats among prokaryotic genomes (Mojica et al., 2000). They are typically composed of 21-48 bp partially palindromic direct repeats interspaced by 20-72 bp sequences called spacers (Mojica et al., 2000, Deveau, et al. 2008, Horvath et al. 2008; Horvath and Barrangou, 2010). CRISPRs are found adjacent to some genes called CRISPR-associated (*cas*) genes. Although the architecture of locus may differ in distinct CRISPR loci (Horvath et al., 2008), a representative structure of CRISPR/Cas system is given in Figure 1.8.

The spacer sequences were reported to have homology to bacteriophage, plasmid and chromosomal sequences (Mojica et al., 2005; Pourcel et al., 2005; Bolotin et al., 2005). These homologous regions on phage genome are named as proto-spacer by Deveau et al. (2008). Recently, the function of CRISPR was established as interfering with phages and both plasmid conjugation and transformation (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). Barrangou et al. (2007) challenged a phage-sensitive wild-type *S. thermophilus* with two virulent phages and generated phage resistant mutants. The CRISPR loci of these mutants were analyzed and compared with CRISPR locus of wild type *S. thermophilus*. They concluded that integration of novel spacers into CRISPR1 locus made the mutants resistant to bacteriophages which had a 100% identical sequence to a spacer on its genome. Barrangou et al. (2007) has also demonstrated that some of the *cas* genes play role in providing CRISPR-mediated phage resistance while some is not involved in directly.

Figure 1.8 Structure of a Clustered Regularly Interspaced Short Palindromic Repeat locus (Sorek et al., 2008)

Four CRISPR loci have been identified in *S. thermophilus* chromosome and were named as CRISPR1, CRISPR2, CRISPR3 and CRISPR4 (Figure 1.9) (Bolotin et al., 2004; Bolotin et al., 2005; Horvath et al., 2008; Horvath and Barrangou, 2010).

Activity of CRISPR was defined by Horwath et al. (2008) as the property of a CRISPR locus of adding novel repeat-spacer units after exposure to foreign genetic elements to improve resistance. According to this definition, CRISPR1 were detected as the most active locus compared with CRISPR2 and CRISPR3 in *S. thermophilus* (Horvath et al. 2008). However, there is no information available on activity of CRISPR4.

These CRISPR loci can be differentiated from each other via the sequence of the repeat within the locus (Horvath et al., 2008). In Table 1.4, the typical repeats (the most frequent repeats) observed by Horvath et al. (2008) were shown for three of CRISPR loci in *S. thermophilus.* Additionally, it should be noted that even if the repeat sequences are conserved with a high frequency throughout a locus (Table 1.4), polymorphisms were also reported especially at the 3' end of terminal repeats, which become important notably for orientation of CRISPR loci and determination of all the repeats without missing the polymorphic ones (Horvath et al., 2008).

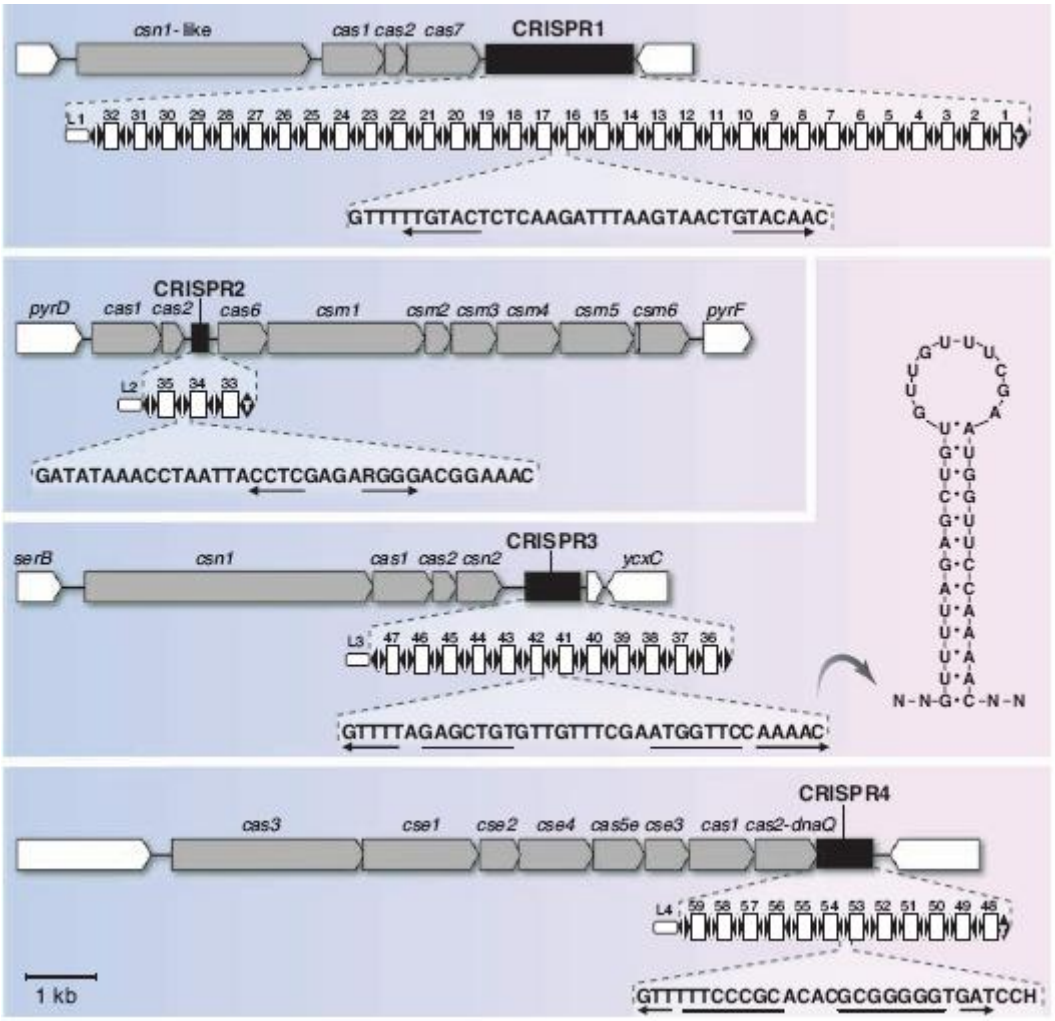


Figure 1.9 Overview of the four CRISPR/cas systems present in *Streptococcus thermophilus* DGCC7710.

For each system, gene organization is depicted on the top, with cas genes in gray, and the repeat-spacer array in black. Below the gene scheme, the repeat and spacer (captured phage or plasmid nucleic acid) content is detailed as black diamonds (T, terminal repeat) and white rectangles, respectively. Bottom line, consensus repeat sequence. L1 to L4, leader sequences. The predicted secondary structure of the CRISPR3 repeat is shown on the right. (Horvath and Barrangou, 2010)

Table 1.4 Typical repeat sequences for three CRISPR loci in *S. thermophilus* observed by Horvath et al. (2008). F: frequency

CRISPR locus	Repeat sequence (5'→3')	F (%)
CRISPR1	GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC	99.7
CRISPR2	GATATAAACCTAATTACCTCGAGAGGGGACGGAAAC	74.6
CRISPR3	GTTTTAGAGCTGTGTTGTTTCGAATGGTTCCAAAAC	99.8

Deveau et al. (2008) were further studied CRISPR1 locus in *S. thermophilus* and noted CRISPR-mediated phage resistance as a novel phage defense system. As mentioned before, integration of a new spacer to CRISPR1 locus can be resulted with resistance to the phage having 100% identical proto-spacer (Barrangou et al., 2007). It was also shown that integrating more than one spacer can enhance the overall phage resistance (Deveau et al., 2008). The proto-spacers were found in both coding and non coding stands and spreaded among phage genome without targeting any specific phage modules (Barrangou et al., 2007; Horvath et al., 2008; Deveau et al., 2008).

Although function of CRISPR loci were recently established (Barrangou et al., 2007; Marraffini and Sontheimer, 2008), the mechanism of CRISPR-mediated bacteriophage resistance is not fully characterized. However, known parts of mode of action of CRISPR/cas system is summarized in the following (Figure 1.10). It is known that CRISPR array is transcribed to a precursor RNA (pre-crRNA) (Brouns et al., 2008). After that, this pre-crRNA is cleaved to small RNAs called CRISPR RNAs (crRNAs) which are composed of a spacer adjacent to two partial repeat at each end and attend as a guide interfering with foreign genetic elements (Hale, 2008; Brouns, 2008). Even though there are strong evidences suggesting that directly DNA is the target of CRISPR/Cas system (Marraffini and Sontheimer, 2008), RNA might be additionally a target for this defense system (Horvath and Barrangou, 2010)

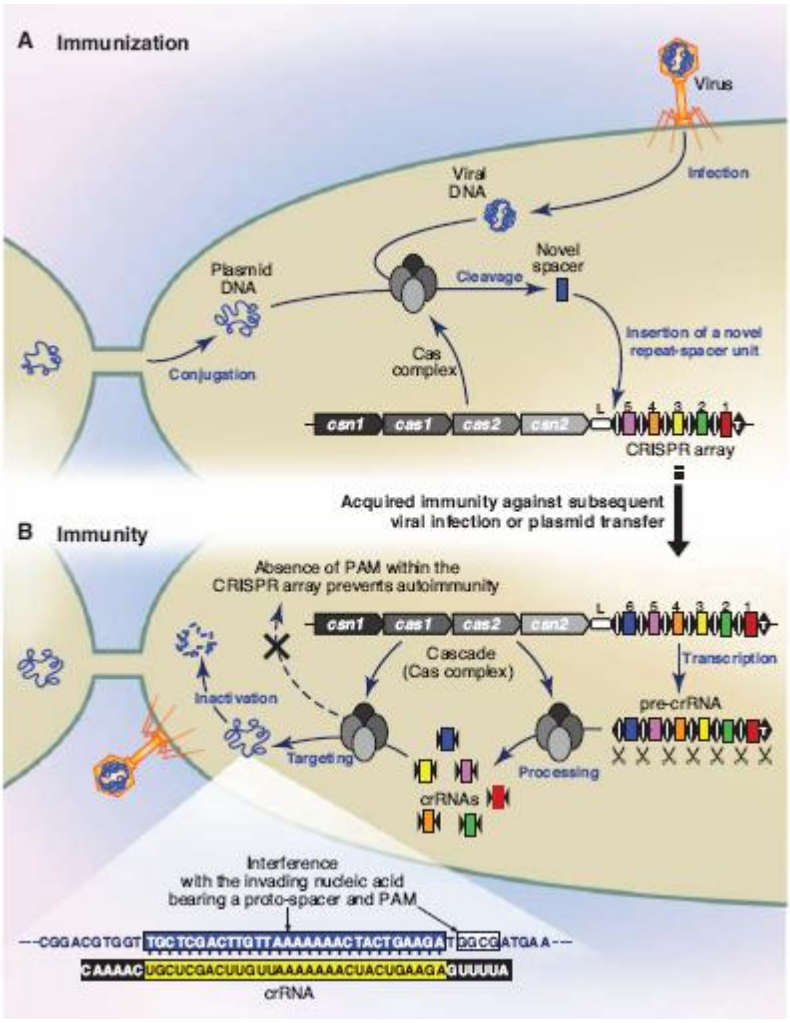


Figure 1.10 Overview of the CRISPR/Cas mechanism of action.
(A) Immunization process: After insertion of exogenous DNA from viruses or plasmids, a Cas complex recognizes foreign DNA and integrates a novel repeat-spacer unit at the leader end of the CRISPR locus. (B) Immunity process: The CRISPR repeat-spacer array is transcribed into a pre-crRNA that is processed into mature crRNAs, which are subsequently used as a guide by a Cas complex to interfere with the corresponding invading nucleic acid. Repeats are represented as diamonds, spacers as rectangles, and the CRISPR leader is labeled L. (Horvath and Barrangou, 2010)

Additionally, analyzing of CRISPR sequences might be important for typing due to its dynamic nature besides providing information about potential phage resistance of bacteria (Horvath et al., 2008; Horvath and Barrongou, 2010). CRISPR analysis can also reveal historical relationship of phages and bacteria and coevolution of them (Barrangou et al., 2007 and Horvath et al., 2008).

1.5.2 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is a molecular typing method which was firstly reported by Maiden et al. (1998) to overcome the difficulties of comparing results of multilocus enzyme electrophoresis (MLEE) from different laboratories. This method is based on sequencing internal fragments of selected housekeeping genes. Housekeeping genes is under such a selection which provides conservation of their metabolic function. These genes evolve relatively slowly and can reveal genetic relationship among bacterial isolates better than genes under positive selection (Urwin and Maiden, 2003). Therefore, it provides high level of discrimination and also facilitates the transfer of typing data between laboratories.

Urwin and Maiden (2003) listed the components necessary for designing a new MLST system as:

- Choice of the isolates
- Selection of the loci to be sequenced
- Primer design

While selecting the housekeeping genes to be sequenced or MLST analysis the following criteria should be considered (Cai et al., 2007);

- location on chromosome i. e. separately distributed on chromosome
- presence in all samples
- presence in single copy on genome
- about 1 kb long to facilitate primer design

After the fragments are sequenced the results are analyzed. In MLST analysis, different sequences observed in the same locus are designated as different alleles (even observing a single nucleotide polymorphism (snp) is enough to assign the sequence as a different allele) and the combination of alleles at each of the loci used for MLST analysis for a single isolate composes an allelic profile. The allelic profile assigns the sequence type for that isolate (Enright and Spratt, 1999).

Although MLST was firstly described for studies on bacterial epidemiology, it has been also using for identification and displaying the phylogenetic relationships of non-pathogenic bacteria, including LAB. Genotyping of LAB such as *Lactobacillus plantarum* (de las Rivas et al., 2006), *Lactobacillus casei* (Cai et al., 2007, Diancourt et al., 2007), and *S. thermophilus* (Delorme C., Bolotin A., Ehrlich S.D., Renault P., unpublished data, Hols et al., 2005, Delorme, 2008) were studied using MLST. In these studies, MLST was compared with other typing methods such as ribotyping and restriction fragment length polymorphism (RFLP) analysis of 16S-23S rDNA intergenic spacer region (de las Rivas et al., 2006), pulsed-field gel electrophoresis (PFGE) (Cai et al., 2007), amplified fragment length polymorphism (AFLP) and multilocus variable-number tandem repeats analysis (MLVA) (Diancourt et al., 2007). MLST had been found comparable or even more discriminatory than those methods except PFGE (de las Rivas et al., 2006, Cai et al., 2007, Diancourt et al., 2007).

Genetic diversity within the salivarius group was studied by analyzing of 63 strains of *S. thermophilus, Streptococcus vestibularis* and *Streptococcus salivarius* using MLST (Delorme C., Bolotin A., Ehrlich S.D., Renault P., unpublished data, Hols et al., 2005, Delorme, 2008). In their study, no clustering within *S. thermophilus* strains was revealed based on either geographic origin or product types. (Delorme C., Bolotin A., Ehrlich S.D., Renault P., unpublished data, Hols et al., 2005, Delorme, 2008). However, the scope of their study was to search genetic diversity within the salivarius group, so the housekeeping genes analyzed were principally chosen to probe genetic diversity within salivarius group, not specifically among *S. thermophilus* strains.

1.6 Aim of the Study

Yogurt is a dairy product with a high consumption in Turkey. Additionally, its industrial production is very important. There are many local dairy brands available in Turkish market. However, there is not a starter culture producing company established in Turkey, except a new small company. Absence of such a company makes these nation-wide yogurt producers to purchase starter cultures from abroad and causing more costly production. Additionally, using these starter cultures cause to produce yogurts with a taste mostly unfamiliar to native people and furthermore it could also cause losing the traditional cultures in time. Therefore, the main aim of this study is to investigate yogurt cultures in traditionally produced Turkish yogurts and to form a traditional yogurt culture collection.

This study is composed of mainly three parts. In the first part, yogurt bacteria were isolated from traditionally produced Turkish yogurts collected from mainly three cities as Antalya, Mersin and Erzincan. A strict isolation procedure was followed to eliminate adjacent flora and hence to isolate only yogurt bacteria. The isolates were identified using biochemical identification methods and then their technologically important properties were examined, forming the second part of the thesis. In the third part, the genotypic diversity and evolutionary history of traditional *S. thermophilus* isolates were investigated. For this purpose, CRISPR1 analysis and MLST methods were used. CRISPR analysis is a suggested typing method (Pourcel et al., 2005, Barrangou et al., 2007 and Horvath et al., 2008), while MLST is already an accepted method for typing of bacteria. Therefore, the aim of the third part of the study is to have information about the genotypic diversity and evolutionary history of traditional *S. thermophilus* isolates and to compare CRISPR1 analysis as a typing method with MLST.

CHAPTER 2

MATERIALS AND METHODS

2.1 Samples and Reference Bacteria

The bacteria used in this study isolated from traditionally produced Turkish yogurts. These yogurts were collected from mostly Mediterranean Region of Turkey, except K1 yogurt collected from Kemah, Erzincan. Origins of some of the yogurts were given in Table 2.1.

Table 2.1 Origins of traditionally produced Turkish yogurts

Yogurt name	Origin
K1	Kemah, Erzincan
N1	Sarıaydın, Mersin
N2	Yağcılar, Antalya
N3	Seydi, Antalya
N4	Karaahmetli, Antalya
N5	Albeyli, Antalya
N6	Güneyli, Mersin
N7	Yağda, Mersin
N8	Elbeyli, Mersin
N9	Kıca, Mersin
S1	Çukurbağ, Mersin
K2	Kızılgeçit, Mersin

Yogurt cultures were additionally isolated from commercially available starter cultures to interpret the technological properties of traditional isolates. Two *L. bulgaricus* from Danisco Yo-Mix 410 (MRS-Dan-Yo-Mix-410-1 and MRS-Dan-Yo-Mix-410-2) and 3 *L. bulgaricus* from Visby Visbyvac B 1000 (MRS-Visby-1, MRS-Visby-2 and MRS-Visby-3) were isolated. Two *S. thermophilus* from Danisco Yo-Mix 410 (M17-Dan-Yo-Mix-410-1 and M17-Dan-Yo-Mix-410-3) and 2 *S. thermophilus* from Danisco TA 040 (M17-Dan TA040-1 and M17-Dan TA040-3) were also isolated.

S. thermophilus LMG 18311 and *L. bulgaricus* DSM 20081^T were used as reference strains for biochemical identification of traditional isolates.

2.2 Growth Media and Temperature

S. thermophilus isolates were cultivated in M17 broth (Merck) and M17 agar (Merck) whose pH were adjusted from 7.2 ±0.2 to 6.8 ±0.1 at 25 °C or M17 broth (Difco) supplemented with 0.5% lactose (Difco) with a pH of 6.9±0.2 at 25 °C.

L. bulgaricus isolates were cultivated in MRS broth (Merck) and MRS agar (Merck) with a pH of 5.7 ±0.2 at 25 °C.

Both *S. thermophilus* and *L. bulgaricus* isolates were grown at 42 ºC unless otherwise noted.

2.3 Methods

2.3.1 Isolation of the Bacteria from Yogurt Samples

Dilutions of yogurt samples were carried out with sterile peptone water (0.1% w/v). A loopful of each diluted yogurt sample was streak-plated on MRS (pH 5.7) agar for isolation of Lactobacilli and M17 agar which was acidified to pH 6.8 for Streptococci. Plates were incubated at 42 ºC for 48 hours under oxygen-depleted and CO_2-enriched atmosphere conditions using gas pack (Anaerocult C, Merck)

for selective growth of *L. bulgaricus* and *S. thermophilus*. Colonies display the general characteristics of *L. bulgaricus* and *S. thermophilus* were chosen from each plate. Streak plating was performed for every isolated colony and single colony isolation was carried out to obtain pure cultures. The cultures were examined for cell morphology under microscope and rod-shaped bacteria for MRS isolates and coc-shaped bacteria for M17 isolates were selected and the stock cultures of these bacteria were prepared in 20% glycerol and stored at -80 ºC.

2.3.2. Biochemical Identification of the Cultures

2.3.2.1 Gram Staining

Gram-color staining set for Gram stain (Merck) was used and staining was performed according to the method which producer firm was suggested. This method was summarized below.

Overnight incubated liquid cultures were used for smear preparation. A loopfull of culture was transferred onto a slide and distributed. The sample was air dried and heat-fix the smear. The staining procedure was,

1. Cover the slide with crystal violet solution. Stain for 1 min, pour off.
2. Rinse the slide with Lugol's solution stabilized
3. Cover the slide with Lugol's solution stabilized. Allow the act for 1 min.
4. Rinse with distilled water
5. Apply decolorizing solution
6. Rinse with distilled water
7. Cover the slide with safranine solution. Stain for 1 min.
8. Rinse with distilled water
9. Leave to dry, examine under a microscope

2.3.2.2 Catalase Test

In a tube 1 ml 30% H_2O_2 which was at refrigerator temperature and 1 ml overnight incubated culture was added into the tube. Observing no bubbles explains negative result.

2.3.2.3 Gas Production from Glucose

Gas production from glucose was tested in MRS broth medium without meat extract and citrate, having 2% of glucose and containing Durham tube (Gürakan, 1991, Tjandraatmadja, et al., 1990).

2.3.2.4 Growth at 10°C and 45°C

Growth at different temperatures was determined in MRS broth and M17 broth having 0.04 g/l bromocresol purple. The tubes were observed during 7 days of incubation at 10 ºC and during 5 days of incubation at 45 ºC.

2.3.2.5 Carbohydrate Fermentation Test

Except esculin fermentation test, carbohydrate fermentation tests were performed using microtitre plates in duplicates as performed previously by Erkuş (2007) with some modifications. Carbohydrate fermentation profiles were determined in modified MRS broth without glucose and meat extract, containing bromocresol purple (0.04g/l) as a pH indicator (Appendix A) (Gurakan, 1991) and supplemented with each carbohydrate to a final concentration of 1%. Filter sterilized carbohydrates; arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, melibiose, ribose, saccharose, salicin, sorbitol, trehalose, xylose were used to test fermentation profiles of the isolates. Two drops of sterile mineral oil were added on each well to provide anaerobic condition and also to prevent the liquid in the well from vaporization. The color change of indicator from purple to yellow explains that the culture can ferment the carbohydrate. Test for esculin was also conducted in MRS broth without meat

extract and glucose, containing 5 g/l esculin (Tjandraatmadja, et al., 1990). This medium was used after autoclaving at 115 ºC for 15 min. Broths inoculated with bacteria are incubated at 42 ºC for 2 days. Positive results were differentiated by turbidity difference and also loss of fluorescence under UV light. *Lactobacillus casei* subsp. *casei* NRRLB 441 was used as positive control for esculin fermentation.

The bacteria which were growth for carbohydrate fermentation were incubated at 42 ºC for about 24h in 10 ml MRS or M17 broth. Prior the usage, the bacteria were centrifuged at 1220xg for 15 min. Pellets were washed by using modified MRS one or two times to eliminate any carbohydrate residues and acidity from previous media.

2.3.3 Growth of the Reference Strains

Growth of the reference strains, *L. bulgaricus* DSM 20081^{T} and *S. thermophilus* LMG 18311 were determined in triplicates using spectrophotometer according to the following procedure. Organisms were activated twice before the experiment and inoculated (0.1% v/v) from 15 hour incubated sample into the appropriate media and incubated at 42 ºC. Optical density was measured at 600 nm (OD_{600}) using spectrophotometer (Shimadzu UV1700). The first data were measured immediately after inoculation. OD_{600} was recorded at 1-hour intervals. When the OD_{600} exceeded 0.3, the sample was diluted (1:5) using sterile medium.

2.3.4 Technological Characterization of Isolates

Cultures were characterized according to some of their technologically important properties. These properties were rate of acidification, acetaldehyde production, final pH, phage resistance and proteolytic activity.

In technological characterization tests, reconstituted skim milk (RSM) was used, which was prepared by mixing skim milk powder (Fluka) with distilled water in

10% (w/v) concentration and sterilized by autoclaving at 121 ºC for 5 min. RSM was inoculated with 2% strain precultured as described following.

2.3.4.1 Standardization of Initial Load for Technological Characterization

In order to standardize the technological property tests, microbial load of preculture should also be standardized. In literature, initial load of microorganisms were generally adjusted to 10^6 cfu/ml (Badis et al., 2004, Beal et al., 1999) . Therefore the microbial load of preculture should be approximately 10^8 cfu/ml since samples are inoculated with 2% preculture. Some researchers prefer to adjust OD of preculture before usage in order to standardize. Boukseim et al. (2000) standardized the cultures to an absorbance of 0.5 at 600 nm wavelength using. According to the preexperiments performed to determine incubation time and OD_{600} combination to get approximately 10^8 cfu/ml, the precultures having the folloving properties used for all technological property analysis in order to standardize the analysis.

- Preculture incubated for 13 h at 42 ºC and OD is adjusted to 2 at 600 nm was used the technological property analysis of *S. thermophilus*.
- Preculture incubated for 18 h at 42 ºC and whose OD is adjusted to 2 at 600 nm was used the technological property analysis of *L. bulgaricus*.

Thus, culture with $OD_{600} = 2$ contains approximately 10^8 cfu/ml.

After these adjustments, the initial load at the inoculated reconstituted skim milk (RSM) was adjusted approximately to 10^6 cfu/ml.

2.3.4.2 Acidification Activity

Acidification activity was determined by pH change during time. 100 ml of RSM was inoculated 2 % isolate precultured as described before and incubated at 42 ºC. The pH was recorded by 2 hour intervals during 10 h incubation using a pH meter (WTW pH 330).

The acidification rate was calculated as $\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (Ayad et al., 2004).

2.3.4.3 Acetaldehyde Production and Final pH

The acetaldehyde level was determined after 24 h of fermentation at 42 ºC; the inoculum used was 2% preculture. Before the assay, the pH of the sample was measured (WTW pH 330) to determine final pH after 24 h incubation at 42 ºC.

Acetaldehyde production ability of the strains giving good and medium acidification activities were determined using an acetaldehyde determination kit based on the enzymatic (acetaldehyde dehydrogenese (Al-DH)) reduction of NAD to NADH (R-biopharm Roche).

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed is stochiometric to the amount of acetaldehyde. NADH was determined by means of its light absorbance at 340. The absorbance was measured at 340 nm by using spectrophotometer (Shimadzu UV1700) with quartz cuvettes and the cell temperature was adjusted to 25°C before the measurement. The acetaldehyde amount was calculated according to the following formula as described in the manual.

$$c = \left(\frac{V * MW}{\varepsilon * d * v * 1000}\right) * \Delta A \left[\frac{g}{l}\right]$$

V= Final volume (ml)
V= Sample volume (ml)
MW= Molecular weight of the substance to be assayed (g/mol)
D= Light path (cm)
E= Extinction coefficient of NADH (at 340 nm = 6.3 (l x mmol^{-}1 x cm^{-1})

For acetaldehyde;

$$c = \left(\frac{3.250 * 44.05}{\varepsilon * 1.00 * 0.222 * 1000}\right) * \Delta A \, [\frac{g}{l}]$$

$$content = \left(\frac{c \, (\frac{g}{l} sample \; solution)}{sample \; weight \; in \frac{g}{l} sample \; solution}\right) * 100 \, (\frac{g}{100g})$$

2.3.4.4 Phage Resistance

2.3.4.4.1 Phages and Media Used In Phage Resistance Experiments

The phages used to determine phage resistances of the isolates were kindly supplied by Prof. Dr. Nezihe Tunail and Dr. Esra Acar. The phages were isolated by Kaleli (2001), Acar (2002) and Acar-Soykut (2007) from raw milk, yogurt, whey and bulk culture from various dairy plants in Turkey. The detailed information on phages was available in the thesis mentioned.

In phage resistance experiments, the modified M17 mediums and MRS mediums given in Appendix A were used.

2.3.4.4.2 Phage Resistance of *S. thermophilus* Isolates

Phage resistances of *S. thermophilus* isolates were determined using spot test as performed previously by Özyurt (2005) with some modifications and explained as follows: Active *S. thermophilus* isolates were grown in modified M17 (mM17) broth for 4-5 hours at 42 ºC. Two hundreds µl of this culture were mixed with 3 ml of mM17 soft agar at about 45-50 ºC and poured onto mM17 agar plate. Since mM17 broth contains $CaCl_2$ in formulation additional $CaCl_2$ was not added into mM17 soft agar. The isolates which did not form bacterial lawn during incubation were concentrated 6 times before mixing with soft mM17 agar. Following the solidification of the soft agar layer, 10 µl of each phage lysate ($\geq 10^7$ pfu/ml) were dropped on plates. Four phages were tested on one plate. After waiting about 10

min to let the agar absorb the phage lysate, the plates were transferred to an incubator and incubated at 42 ºC for 18 h. After incubation, the plates were checked for any plaque formation.

2.3.4.4.3 Phage Resistance of *L. bulgaricus* Isolates

Phage resistances of *L. bulgaricus* isolates were determined using spot test as performed previously by Özyurt (2005) with some modifications and explained as follows: Active *L. bulgaricus* isolates were grown in MRS broth for 5-6 hours at 42 ºC and 200 µl of this culture were mixed with 3 ml of MRS soft agar at 45-50 ºC and 100 µl of sterile 1 M $CaCl_2$ to accelerate cell lysis and obtain visible plaque formation (Quiberoni et al., 2004). This mixture was then poured on MRS agar. After it solidified, 10 µl of phage lysate ($\geq 10^7$ pfu/ml) were dropped on plate. Four phages were tested on one plate. After waiting about 10 min to let the agar absorb the phage lysate, the plates were transferred to an incubator and incubated at 42 ºC for 18 h under anaerobic conditions. After incubation, the plates were checked for plaque formation.

2.3.4.5 Proteolytic Activity

Proteolytic activities were determined using the *o*-phthaldialdehyde (OPA) method described by Church et al. (1983). In this method, α-amino groups released by hydrolysis of milk proteins react with *o*-phthaldialdehyde and β-mercaptoethanol and form a compound that absorbs at 340 nm (Church et al., 1983).

The assay was performed at least duplicate for each selected strains according to their acidification and acetaldehyde production abilities. The strains were subcultured three times in appropriate medium. The preculture was prepared as defined above. To minimize carryover of free amino acids during inoculation, 5 ml of preculture were washed and resuspended to the original volume with 0.32 mM sodium phosphate, pH 7.2. Cells were inoculated (2%) into 5 ml RSM and incubated for 6 h at 42 ºC. An uninoculated RSM was also incubated at the same temperature as control. The 5 ml sample after incubation was mixed with 1 ml H_2O

and 10 ml 0.75 N trichloroacetic acid (TCA) while vortexing. The samples were filtered using a filter paper after 10 min of incubation at room temperature and frozen at -80 ºC until assayed. To assay proteolysis, using milk proteins as substrates, 150 µl aliquot was removed from the TCA filtrate and added directly to 3 ml OPA reagent prepared as described by Church, et al. (1983). The solution was mixed briefly and incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured using spectrophotometer (Shimadzu UV1700). Triplicate aliquots from each TCA filtrate were analyzed. The TCA and OPA reagent were prepared daily.

The proteolytic activity of the cultures was denoted as the absorbance of free amino groups at 340 nm. The absorbance values, measured using uninoculated RSM were subtracted from the absorbance readings to eliminate the free amino groups from RSM. Thus, ΔAbs at 340 nm was obtained as following;

$$\Delta\text{Abs 340 nm} = \text{Abs 340 nm}_{\text{cultured}} - \text{Abs 340 nm}_{\text{RSM}}$$

2.3.5 16S rRNA Gene Sequencing of *S. thermophilus* Isolates

16S rRNA gene and a small portion of internal transcribed spacer (ITS) region of putative *S. thermophilus* isolates (60) were amplified and two parts among the amplicon were sequenced to confirm biochemical identification. Type strains, *Streptococcus salivarius* ATCC 7073^T and *Streptococcus vestibularis* ATCC 49124^T was also included in the experiment as negative controls using the same primers and conditions with *S. thermophilus*.

The main steps for sequencing were as follows;

- Cell lysate preparation
- Amplification PCR
- Separating the DNA fragment using agarose gel
- DNA purification from agarose gel

- Sequencing PCR and cleaning the sequencing PCR products by magnetic beads
- Sequencing at Biotechnology Center, University of Wisconsin
- Analysis of sequenced fragments

2.3.5.1 Cell Lysate Preparation

Cell pellets were obtained by centrifugation of 250 µl of an actively growing culture and washed with 100 µl of PBS. The cell pellets were resuspended in 10 µl of 10X High Fidelity PCR Buffer (Invitrogen), 85 µl of distilled water and 4 µl of lysozyme (10 mg/ml) and incubated for 15 min at 37 ºC. After the incubation, 5 µl of proteinase K (10 mg/ml) was added and the reaction mix was incubated at 55 ºC for 1 h. Heat inactivation of the enzymes was performed by placing the samples in a boiling water bath for 10 min.

2.3.5.2 Amplification PCR

Cell lysates were used as DNA templates for the amplification. The 16S rRNA gene and a small portion of internal transcribed spacer (ITS) region were amplified using the primers; Pro-26 (5'-AGAGTTTGATCCTGGCTCAG-3') and St 4 (5'-GACCTCCTGCGTGCAAAG-3') (Table 2.2). Pro-26 was firstly described by Wilmotte et al. (1993) and also used for amplification of 16S rRNA gene of lactic acid bacteria (LAB) by Velez et al. (2007).

Table 2.2 Primers used for 16S rRNA gene sequencing of *Streptococcus thermophilus*

Primer name	Primer Sequence	Reference
Pro-26 (fwd)	5'-AGAGTTTGATCCTGGCTCAG-3'	Wilmotte et al. (1993)
St 2 (rev)	5'-ACTCTCCCCTTCTGCACTCA-3'	Designed by Altay
St3 (fwd)	5'-CAGCTCGTGTCGTGAGATGT-3'	Designed by Altay
St 4 (rev)	5'-GACCTCCTGCGTGCAAAG-3'	Designed by Altay

fwd: forward, rev: reverse

PCR amplification was accomplished using Platinum *Taq* DNA Polymerase High Fidelity (HiFi) (Invitrogen) with an iCycler Thermal Cycler (Bio-Rad). PCR amplification was performed in a volume of 50 µl reaction mixture prepared according to the producer manual with the exception of the $MgSO_4$ concentration, which was increased from 2 mM to 2.5 mM. PCR mix is given in Table 2.3.

Table 2.3 Amplification PCR mix for 16S rRNA gene sequencing (Platinum *Taq* DNA Polymerise HiFi (Invitrogen))

Reagent	Amount (µl)
dH_2O	35
10X HiFi PCR buffer	5
50 mM $MgSO_4$	2.5
dNTP mix (10 mM each)	1
Forward Primer (10 µM)	2
Reverse Primer (10 µM)	2
Templete DNA (cell lysate)	2
Platinum *Tag* DNA polymerase HiFi	0.5

Thermal Cycler program of amplification PCR for 16S rRNA gene sequencing (Platinum Taq DNA Polymerase HiFi (Invitrogen)) was:

Initial denaturation: 95 ºC for 3 min
35 cycles: 95 ºC for 45 sec
50 ºC for 30 sec
72 ºC for 1 min
Final extension: 72 ºC for 10 min
Soak: 4 ºC for ∞

2.3.5.3 Separating the DNA Fragment Using Agarose Gel

The amplification PCR products were loaded into 0.7% UltraClean agarose gel (Invitrogen Life Technologies) in TAE (Biorad), and separated by electrophoresis at 120 V for 50 min. The amplicons (~1.6 kb) were cut out from the gel.

2.3.5.4 DNA Purification from Agarose Gel

The amplicons (~1.6 kb) cut out from the gel were extracted using a PureLink Quick Gel Extraction Kit (Invitrogen Life Technologies). This kit is based on selective binding of dsDNA to silica-based membrane under adjusted conditions by mixing PCR products with Binding Buffer. After DNA bound the membrane, the impurities were removed by washing using Wash Buffer. The dsDNA were eluted in low salt Elution Buffer. The buffers mentioned were supplied with the kit.

2.3.5.5 Sequencing PCR and Cleaning the PCR Products

The amplicons of ~1.6 kb were sequenced using all four primers listed in Table 2.2. The primers, St2, St3 and St4 were designed using the PCR primers designing program Primer3 (Rozen and Skaletsky, 2000 Internet: http://frodo.wi.mit.edu/primer3/) on the basis of known gene sequence of *S. thermophilus* LMG 18311 (Bolotin et al., 2004; Accessed via Genome Project

under NCBI- Internet: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The approximate positions of the primers were shown on Figure 2.1. Primers were selected such a way that sequenced region can provide differentiation *S. thermophilus* even from closely related species (i.e. *Streptococcus salivarius* and *Streptococcus vestibularis*) (Poyart et al., 1998).

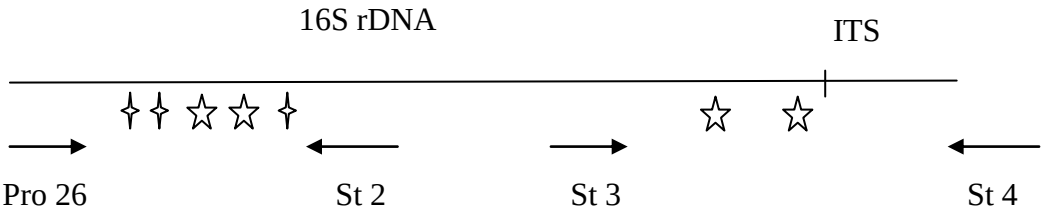


Figure 2.1 Primers for 16S rRNA gene sequencing and single nucleotide polymorphisms between *Streptococcus thermophilus* and closely related species
✦ Single nucleotide polymorphism between *S. thermophilus* and *S. vestibularis*
☆ Single nucleotide polymorphism between *S. thermophilus* and *S. vestibularis/ S. salivarius*

DNA sequencing was carried out with a Bigdye Kit (Biotechnology Center, University of Wisconsin), using the following PCR mix (Table 2.4) and thermal cycler program.

Sequencing PCR products were cleaned using magnetic beads (Beckman Coulter) according to the producer's manual and then sequences were determined by University of Wisconsin Biotechnology Center using ABI 3730xl DNA Analyzer.

Table 2.4 Sequencing PCR mix for 16S rRNA gene sequencing (Bigdye Kit - Biotech Center, University of Wisconsin)

Reagent	**Amount**
DNA template	0.2 µg
Primer	2 pmol
Bigdye buffer	2.5 µl
Bigdye	0.5 µl
Water	To a final volume of 12 µl

Thermal Cycler program for sequencing of for 16S rRNA gene:

35 cycles: 94 ºC for 30 sec
50 ºC for 20 sec
60 ºC for 4 min
Soak: 4 ºC for ∞

2.3.5.6 Analysis of Sequenced Fragments

Finch TV version 1.4.0 (http://www.geospiza.com/finchtv , Geospiza Inc., Seattle, WA, USA) was used for viewing DNA sequence chromatograms. Analysis of the nucleotide sequences was performed using online BLASTN (http://www.ncbi.nlm.nih.gov/blast).

Percent identities between the first sequenced part of *S. thermophilus* LMG 18311 and some *Streptococcus* species were calculated using the software package DnaSP (version 4.0) (Librado and Rozas, 2009). After alignment of the sequences using MEGA software version 4.0 (Tamura 2007) and saving in fasta format, the alignments were analyzed using DnaSP (version 4.0) (Librado and Rozas, 2009)

and nucleotide diversity (per site) were obtained. Percent identities were calculated by subtracting the percent value of nucleotide diversity from 100%.

2.3.6 Analyzing of CRISPR1 Locus

CRISPR1 locus of *S. thermophilus* isolates was sequenced. The main steps were given as follows:

- Cell lysate preparation
- Amplification PCR
- Separating the DNA fragment using agarose gel
- DNA purification from agarose gel
- Sequencing PCR and cleaning the sequencing PCR products by magnetic beads
- Sequencing at Biotechnology Center, University of Wisconsin
- Selecting new primers
- Successive sequencings using new primers at Biotechnology Center, University of Wisconsin
- Analysis of sequenced fragments

Cell lysates prepared for 16S rRNA gene sequencing were also used as DNA template for CRISPR1 locus amplification. CRISPR1 locus was amplified using the forward primer, yc70 (5-TGCTGAGACAACCTAGTCTCTC-3) (Bolotin, 2005) and reverse primer, CR1-rev (5-TAAACAGAGCCTCCCTATCC-3) (Horvath, 2008). The main sequencing steps which were explained detailed in 16S rRNA gene sequencing part were also used for CRISPR1 locus sequencing. Additionally, successive sequencings were performed to be able to sequenced whole CRISPR1 locus. After each sequencing step, a new primer was selected on a spacer and used as sequencing primer in the following sequencing step.

CRISPR1 spacers were given as color and shape combinations as stated previously by Barrangou et al. (2007). Unique spacers were identified and compared to

publicly available sequences using BlastN (http://www.ncbi.nlm.nih.gov/blast). Hits having identity percent above 93 were included to analysis.

2.3.7 Multilocus Sequence Typing

Nineteen isolates were selected for MLST studies according to CRISPR1 locus analysis. These isolates were selected in such a way that they contain at least one isolate from each subgroup based on CRISPR analysis and also contain a representative isolate from each yogurt if there were isolates from different yogurt samples in one subgroup.

2.3.7.1 Gene Selection

When selecting housekeeping genes for MLST analysis, 16 housekeeping genes found in one copy on *S. thermophilus* genome were selected among well known genes. The maps showing the distribution of these 16 housekeeping genes were prepared for *S. thermophilus* strains LMD-9, LMG 18311 and CNRZ 1066. Then, sequences of these genes from *S. thermophilus* strains LMD-9, LMG 18311 and CNRZ 1066 were aligned. Finally, five housekeeping genes (*pur*K, *pnc*B, *pst*S, *pro*A and *tuf*), displaying most polymorphism and separately distributed on the genomes were selected for the MLST analysis (Table 2.5). The sequences of these five genes obtained from complete genome of *S. thermophilus* LMG 18311 (Bolotin et al., 2004) were given in Appendix J. Locations of these genes on *S. thermophilus* LMG 18311 genome were given in Figure 2.2.

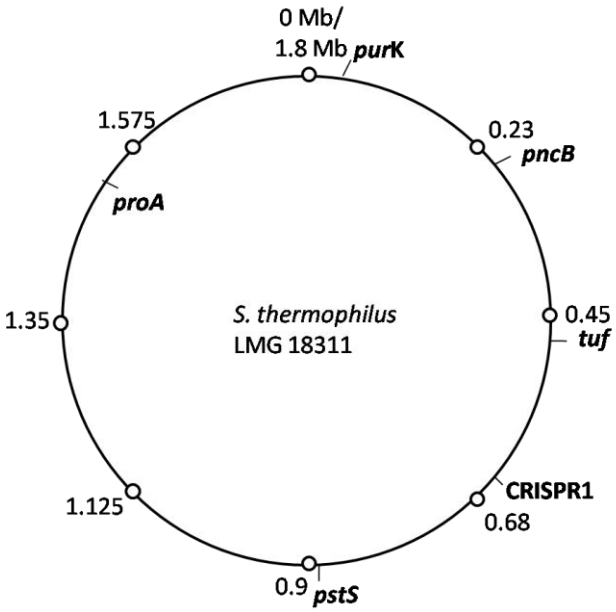


Figure 2.2 Locations of the genes analyzed for MLST and CRISPR1 locus on *S. thermophilus* LMG 18311 genome

Table 2.5 Functions of the housekeeping genes and sizes of amplicons and sequenced parts

Gene	Protein products/function	Amplicon size (bp)	sequence size (bp)
*pro*A	Gamma-glutamyl phosphate reductase	696	568
*pst*S	Phosphate-binding protein	900	620
tuf	Protein Translation Elongation Factor Tu (EF-TU)	794	557
*pnc*B	Nicotinate phosphoribosyltransferase	813	571
*pur*K	Phosphoribosylaminoimidazole carboxylase NCAIR mutase subunit	744	595

The sequencing of one of the selected housekeeping genes (*tuf* gene) was performed by Aysun Cebeci and this sequence was also included in MLST data analysis. Genomic DNA was isolated using Genemark DNA isolation kit for sequencing of *tuf* and sequences of PCR products were determined by IONTECH Company, İstanbul.

The main steps for MLST studies of *pur*K, *pnc*B, *pst*S and *pro*A were as follows;

- Gene selection and primer design
- Genomic DNA isolation
- Amplification PCR
- Separating the DNA fragment using agarose gel
- DNA purification from agarose gel
- Sequencing PCR and cleaning the PCR products by magnetic beads
- Sequencing at Biotechnology Center, University of Wisconsin
- MLST data analysis

2.3.7.2 Genomic DNA Isolation

Genomic DNA was isolated using the slightly modified method of Stahl et al. (1990). The main steps of the procedure for isolation of genomic DNA were as follows;

- Centrifuge 2 ml of culture at RoomTemperature (RT)
- Wash with 1.5 ml of ice-cold TES
- Resuspend in 250 µl TES/sucrose 25%/lysozyme 30mg/ml. Split into two 2 ml Eppendof tubes by approximately 260 µl and Incubate 1 h at 42 ºC
- Add 7 µl of DNAase-free RNAase A, 5 mg/ml, to each tube. Incubate 30 min/37ºC
- Add 15 µl of Proteinase K (10 mg/ml in water) incubate overnight at 55 ºC
- Add to each tube 550 µl of TES at RT, 50 µl of NaCl, 5% and 50 µl of SDS, 20%, incubate 15 min at 65ºC

- Cool to the RT, extract twice with equal volume of phenol-chloroform and once with chloroform
- Precipitate DNA by adding two volume of ethanol and placing the tube at -20ºC overnight
- Centrifugate at 4 ºC for 20 min, discard the supernatant; wash pellet with 70-80% ethanol. dry tubes inverted; dissolved DNA with 100 µl TE

The concentrations of genomic DNA were measured using Eppendorf Biofotometer and the quality and the concentrations of DNA were checked via loading 0.7% Agarose gel in TAE.

DNA solutions having concentration of ~1 ng/µl genomic DNA were prepared using TE buffer (pH 8.0) as diluent for amplification PCR.

2.3.7.3 Amplification PCR

Amplification PCR mixes for housekeeping genes were prepared and thermal cycler were adjusted according to Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) manual given below. DNA solutions having concentration of ~1 ng/µl genomic DNA was used as DNA template.

Table 2.6 Amplification PCR mix for MLST (Platinum *Taq* DNA Polymerise HiFi (Invitrogen))

Reagent	**Amount (µl)**
dH_2O	37.8
10X HiFi PCR buffer	5
50 mM $MgSO_4$	2
dNTP mix (10 mM each)	1
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
Templete DNA (~1 ng/µl)	2
Platinum *Tag* DNA polymerase HiFi	0.2

Thermal Cycler of amplification PCR for MLST (Platinum Taq DNA Polymerase HiFi (Invitrogen)):

Initial denaturation: 94 ºC for 2 min
30 cycles: 94 ºC for 30 sec
55 ºC for 30 sec
68 ºC for 1 min
Soak: 4 ºC for ∞

2.3.7.4 Separating DNA Fragments Using Agarose Gel

The amplification PCR products were loaded into 0.7% UltraClean agarose gel (Invitrogen) in TAE (Biorad), and separated by electrophoresis at 100 V for 1 h. 100 bp marker (Invitrogen) was used as ladder. Amplicons were cut out from agarose gel.

2.3.7.5 DNA Purification from Agarose Gel

The amplicons cut out from gel were extracted using a Pure Link Quick Gel Extraction Kit (Invitrogen) according to the producer manuel. Detailed information was given in 16S rRNA gene sequencing section.

2.3.7.6 Sequencing PCR and Cleaning the PCR Products

Sequencing PCR was run with both forward and reverse primer and additional sequencing was performed by one of the forward or reverse primer as a control (totally three sequencing). The primers for MLST analysis were designed using Primer3 (Rozen and Skaletsky, 2000 Internet: http://frodo.wi.mit.edu/primer3/) (Table 2.7).

Table 2.7 Primers used in MLST

Gene	Primers 5'→3'*	Reference
*pro*A	f-CCGTCTCATCCAAACTGTGA	Designed by Altay
	r-GATCAGAAGCGTCTTACTCTAGCA	
*pst*S	f-CTGGTTGTGCCTCTTGGATT	Designed by Altay
	r-TGCCGTCAGCATCCTTAGTA	
tuf	f-GCGCAGTTAACACACCAAAA	Designed by Altay
	r-GTGTGGCTTGATTGAACCAG	
*pnc*B	f-GCTCCTCTGTTGGAATTTGG	Designed by Altay
	r-TTGCCCCTTGTCGTAGATTG	
*pur*K	f-CATTGGTATCATCGGTGGTG	Designed by Altay
	r-TCAGCTGTCGCAAACATTTC	

*f, forward primer; r, reverse primer

DNA sequencing was carried out with a Bigdye Kit (Biotech Center, University of Wisconsin). PCR mix prepared and the thermal cycler were given below.

Table 2.8 Sequencing PCR mix for MLST (Bigdye Kit - University of Wisconsin Biotechnology Center)

Reagent	**Amount**
DNA template	10 ng for each 100 bp amplicons
Primer	2 pmol
Bigdye buffer	2.5 µl
Bigdye	0.5 µl
Water	To a final volume of 12 µl
DMSO	0.25 µl

Thermal Cycler program for sequencing of MLST genes:

Initial denaturation: 98 ºC for 1 min
30 cycles: 98 ºC for 10 sec
50 ºC for 10 sec
60 ºC for 4 min
Final extension: 72 ºC for 1 min
Soak: 4 ºC for ∞

Sequencing PCR products were cleaned with magnetic beads (Beckman Coulter) according to producers manual and then sequences were determined at the Biotech Center, University of Wisconsin using ABI 3730xl DNA Analyzer.

2.3.7.7 MLST Data Analysis

Sequences of each locus were aligned and concatenated sequences of five MLST genes (*pur*K, *pnc*B, *pst*S, *pro*A and *tuf*) were formed and saved in fasta and MEGA files using MEGA software version 4.0 (Tamura 2007). For each gene different sequences were assigned as different alleles. For each isolate, combination of alleles obtained at five loci provided the isolate's allelic profile and sequence types (STs) were assigned using the allelic profiles. Sequences which have even only one single nucleotide polymorphism (snp) were defined as a distinct allele.

Phylogenetic tree based on the concatenated sequences was formed using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap (Felsenstein, 1985) test (1000 replicates) was used to estimate the confidence of branching in the tree using MEGA 4.0 (Tamura et al., 2007).

The software, DnaSP, (version 4.0) (Librado and Rozas, 2009) were used to calculate descriptive analysis parameters of each locus which were G+C content, number of variable sites, and number of synonymous and non synonymous sites.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Experimental Design

The experimental strategy followed during this study was given in three parts in Figure 3.1, Figure 3.2 and Figure 3.3. In the first part, isolates from traditionally produced Turkish yogurts were identified using biochemical techniques and putative *S. thermophilus* or *L. bulgaricus* isolates were selected for further research. Then, in the second part, technological properties of these isolates were determined and compared with commercial isolates. In the third part, it was focused on putative *S. thermophilus* isolates. Biochemical identification test results of 60 of these isolates were checked out using 16S rRNA gene sequencing and 56 isolates were genetically identified as *S. thermophilus* and selected for further genotypic characterization. Isolates differ from *S. thermophilus* (4 isolates) were eliminated from culture collection. CRISPR1 locus analysis of *S. thermophilus* isolates was performed and selected isolates according to this analysis were subjected to MLST to compare MLST with CRISPR analysis. Genotypic diversity (CRISPR1 locus analysis) and evolutionary history (CRISPR1 locus analysis and MLST) of the *S. thermophilus* isolates from traditional Turkish yogurts were also analyzed. Additionally, potential phage resistance pattern of the isolates was obtained after CRISPR1 locus analysis.

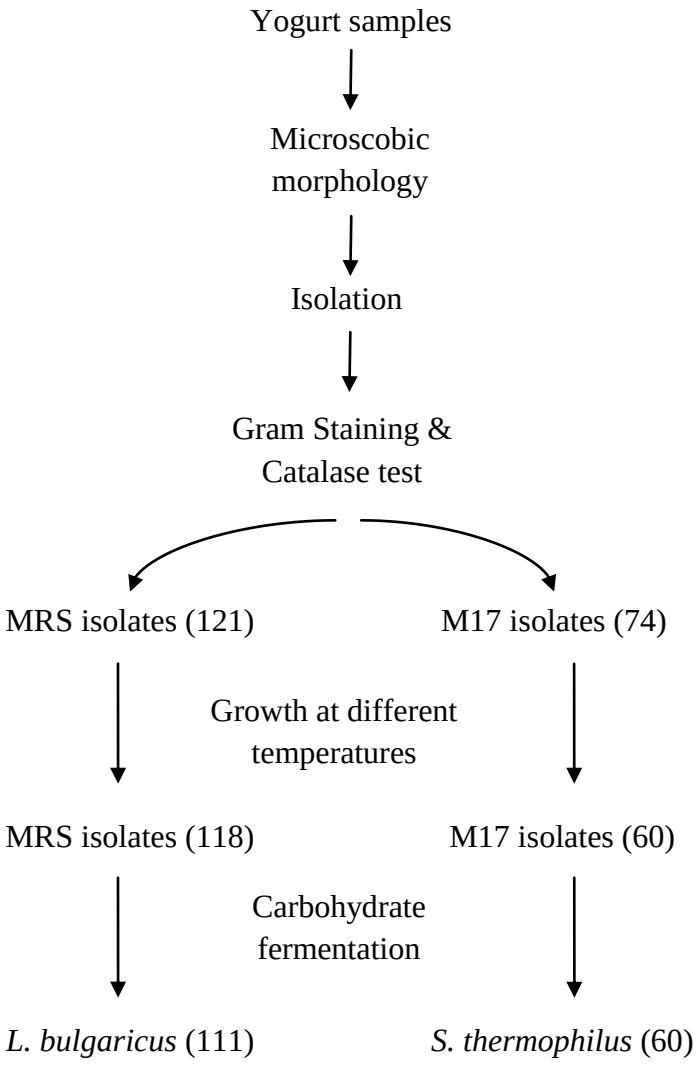


Figure 3.1 Experimental strategy (Part 1). Isolation and biochemical identification of isolates. Number of isolates was given in parenthesis which was studied in related analysis

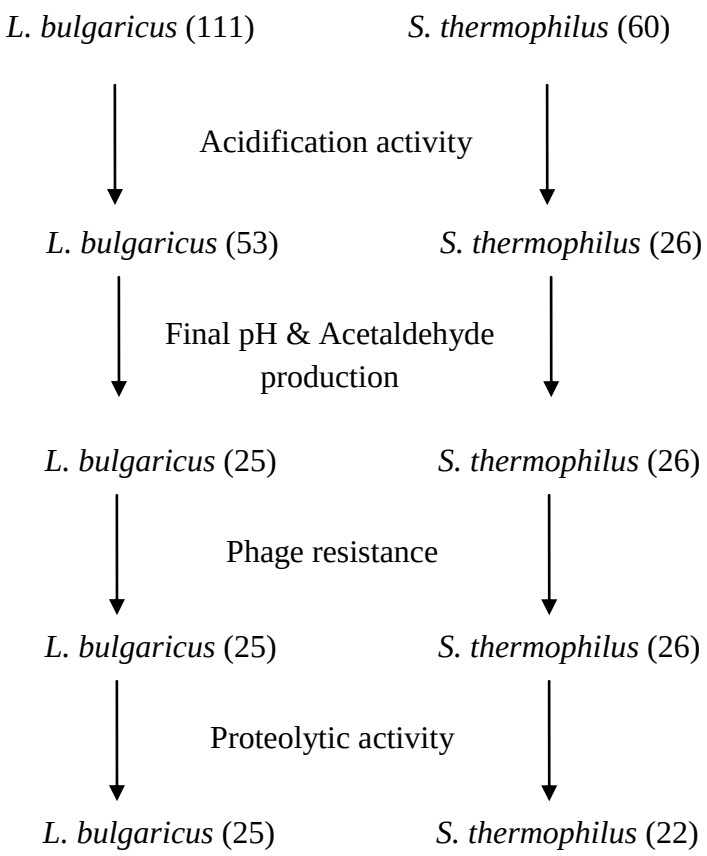


Figure 3.2 Experimental strategy (Part 2). Technological properties of isolates. Number of isolates was given in parenthesis which was studied and determined after related tests.

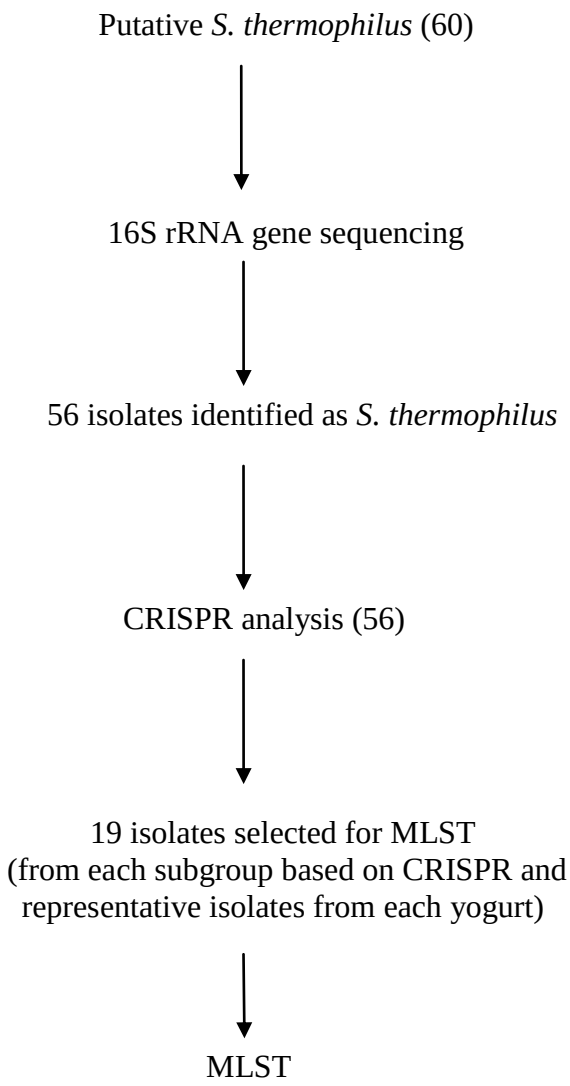


Figure 3.3 Experimental strategy (Part 3). Confirmation of *Streptococcus thermophilus* isolates and genotypic analysis. Number of isolates was given in parenthesis which was studied in related analysis

3.2 Isolation of Bacteria and Biochemical Identification

Since this study was focused on *S. thermophilus* and *L. bulgaricus* in traditionally produced Turkish yogurt bacteria, a very strict strategy was followed to eliminate the other bacteria possibly found in these non-commercial yogurts. Preliminary

experiments of the M17 medium with pH 7.2±0.2 (Merck) revealed growth deficiencies in *S. thermophilus.* However, M17 medium showed improved growth at pH 6.8 which is consistent with the works of other researchers (Terzaghi and Sandine, 1975). This could be because of the pH similarity to the fresh raw milk (pH 6.6-6.7 (Özer, 2010)). The pH of MRS medium was not changed because the growth of *L. bulgaricus* on MRS with its original pH (pH 5.7 ± 0.2) was as expected. After selecting the media and defining their properties, the first step for isolation was checking the colony morphology of bacteria on MRS or M17 agar. Colonies which were similar to colonies of *L. bulgaricus* and *S. thermophilus* were isolated. After obtaining pure cultures of these selected bacteria, they were tested in terms of their microscopic morphology. The organisms having typical microscopic morphologies of rod and cocci for *L. bulgaricus* and *S. thermophilus* respectively were subjected to Gram staining and catalase production tests. Gram positive and catalase negative isolates of 74 from M17 medium and 121 from MRS medium were selected for further studies. *Streptococcus thermophilus* isolates were differentiated from species of *Lactoccocci* and *Enterococci* based on their ability to growth at different temperatures i.e., 10 ºC and 45 ºC (Table 3.1). Additionally, *L. bulgaricus* does not grow at 10 ºC (Buchanan and Gibbons, 1974, Cogan, 2000). Therefore, 14 isolates from M17 and 3 isolates from MRS grown at 10ºC were eliminated from culture collection.

Table 3.1 Differentiation of *S. thermophilus* from the genera of *Lactococcus* spp. and *Enterecoccus* spp. (Holt et al., 1994)

	S. thermophilus	*Enterecoccus* spp.	*Lactococcus* spp.
Growth at 10ºC	-	+	+
Growth at 45ºC	+	+	-

All the selected isolates (178) and reference strains i.e. *S. thermophilus* LMG 18311 and *L. bulgaricus* DSM 20081 were tested for their fermentation ability of 17 carbohydrates. Carbohydrate fermentation profiles in literature were given in Table 3.2 and Table 3.4 for *S. thermophilus* and *L. bulgaricus*, respectively. The carbohydrate fermentation patterns observed in this study were given in Table 3.3 and Table 3.5 for *S. thermophilus* and *L. bulgaricus*, respectively. Carbohydrate fermentation results of the isolates were given in Appendix C.

The isolates from M17 had similar carbohydrate fermentation pattern with *S. thermophilus* LMG 18311. Seventeen slightly different carbohydrate fermentation patterns were observed within isolates form M17 (Table 3.3). All these isolates (60) display acidification on lactose and glucose and on saccharose with one exception (Appendix C) while different acidification profiles for some other carbohydrates were observed such as fructose, galactose, maltose and mannose. These results were similar with the carbohydrate fermentation profiles of *S. thermophilus* strains reported by van den Bogaard et al. (2004). In their study, almost all of *S. thermophilus* strains had utilized glucose, lactose and sucrose. They had additionally observed fructose utilization in some of the strains and also reported naturally occurring Galactose + (Gal+) isolates. In their study, Gal + strains were also able to utilize fructose. In this study, interestingly most of the isolates produced acid from galactose. However no tendency of Gal+ isolates to be fructose + was observed within our isolates. In the study of van den Bogaard et al. (2004), one of their strains had showed acidification of the API 50 CH indicator medium containing maltose, mannose or cellobiose without any growth on these sugars. They suggested the possible reason for this unusual observation as the presence of transport proteins and metabolic pathways with insufficient efficiency or specificity. In our study, this could be also the reason of weak, very weak or positive acidification for maltose and mannose.

Seven out of 118 isolates from MRS were eliminated from culture collection since having different carbohydrate patterns from *L. bulgaricus* (Appendix C). The rest of isolates from MRS (111) had similar carbohydrate fermentation pattern with *L. bulgaricus* (Table 3.4). Nineteen slightly different carbohydrate fermentation

patterns were observed within putative *L. bulgaricus* isolates (Table 3.5). Acidification on arabinose, cellobiose, melibiose, sorbitol and xylose was not observed any of these 19 profiles of *L. bulgaricus* isolates while all these isolates showed acidification on lactose and mannose and most of them also showed acidification on fructose and glucose.

After carbohydrate fermentation tests of the isolates, 60 isolates from M17 and 111 isolates from MRS were selected as putative *S. thermophilus* and putative *L. bulgaricus*, respectively and were further studied in terms of their technological properties.

Table 3.2 Carbohydrate fermentation profiles in literature for *S. thermophilus*

	Esculin	**L(+)Arabinose**	**D(+)Cellobiose**	**D-Fructose**	**D(+)Galactose**	**D(+)Glucose**	**lactose**	**Maltose**	**D-Mannitol**	**D(+)Mannose**	**Melibiose**	**D(-)Ribose**	**Saccharose**	**Salicin**	**D(-)Sorbitol**	**Trehalose**	**D(+)Xylose**	**References**
S. thermophilus	-						+		-			ND		-	-	ND		Holt et al.,1994
S. thermophilus	-	-		-	-	+	+			-	-		+		-		-	Badis et al., 2004
S. thermophilus			-				+	-	-		d	d			-	-		Gobbetti et al., 2000

ND: not determined; d: 11-89% of strains are positive

Table 3.3 Carbohydrate fermentation patterns observed within putative *S. thermophilus* isolates from Turkish yogurts

Patern number[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Number of isolate
Pattern 1	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	13
Pattern 2	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	7
Pattern 3	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	7
Pattern 4	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	6
Pattern 5	-	-	-	-	+	+	+	vw	-	-	-	-	+	-	-	-	-	2
Pattern 6	-	-	-	-	w	+	+	vw	-	-	-	-	+	-	-	-	-	4
Pattern 7	-	-	-	-	w	w	+	-	-	-	-	-	+	-	-	-	-	1
Pattern 8	-	-	-	-	-	+	+	vw	-	+	-	-	+	-	-	-	-	1
Pattern 9	-	-	-	vw	+	+	+	-	-	vw	-	-	+	-	-	-	-	3
Pattern 10	-	-	-	vw	w	+	+	-	-	-	-	-	+	-	-	-	-	2
Pattern 11	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	8
Pattern 12	-	-	-	-	w	+	+	-	-	vw	-	-	+	-	-	-	-	1
Pattern 13	-	-	-	-	+	+	+	-	-	vw	-	-	+	-	-	-	-	1
Pattern 14	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	1
Pattern 15	-	-	-	w	-	+	+	+	-	+	-	-	+	-	-	-	-	1
Pattern 16	-	- -	- -	w	w	+	+	- -	- -	- -	- -	- -	+	- -	- -	- -	- -	1
Pattern 17	-	- -	- -	vw	- -	+	+	vw	- -	vw	- -	- -	+	- -	- -	- -	- -	1

[a] After 16S rDNA sequencing of 60 isolates, one isolate from each of the patterns given bold were detected as not being *S. thermophilus*.

+: reaction; -: no reaction; vw: very weak reaction; w: weak reaction

Table 3.4 Carbohydrate fermentation profiles in literature for *L. bulgaricus*

	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	References
L. bulgaricus	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	Buchanan et al., 1974
L. bulgaricus	-	-		+	+	+	+			-	-		-		-		-	Badis et al., 2004
L. bulgaricus				+	-	+	+	-	-	+	-		-			-		Gomez-Zavaglia, 1999

+: positive reaction; -: no reaction

Table 3.5 Carbohydrate fermentation patterns observed within putative *L. bulgaricus* isolates from Turkish yogurts

Patern number	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Number of isolate
Pattern 1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	81
Pattern 2	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	2
Pattern 3	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-	2
Pattern 4	-	-	-	w	-	+	+	-	-	w	-	-	-	-	-	-	-	1
Pattern 5	-	-	-	+	-	w	+	-	-	w	-	-	-	-	-	-	-	1
Pattern 6	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	2
Pattern 7	-	-	-	+	-	+	+	-	-	+	-	w	-	-	-	w	-	1
Pattern 8	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	5
Pattern 9	-	-	-	+	-	w	+	-	-	w	-	-	-	-	-	w	-	2
Pattern 10	-	-	-	+	-	+	+	-	-	w	-	-	-	-	-	+	-	1
Pattern 11	-	-	-	+	-	+	+	-	-	+	-	w	-	-	-	+	-	2
Pattern 12	-	-	-	+	-	-	+	-	-	vw	-	-	-	-	-	+	-	1
Pattern 13	-	-	-	+	-	w	+	-	-	+	-	vw	-	-	-	+	-	1
Pattern 14	-	-	-	+	-	w	+	-	-	+	-	-	-	-	-	+	-	3
Pattern 15	-	-	-	+	-	vw	+	-	-	+	-	-	-	-	-	+	-	1
Pattern 16	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	2
Pattern 17	-	-	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	1

Table 3.5 Carbohydrate fermentation patterns observed within putative *L. bulgaricus* isolates from Turkish yogurts (cont'd)

Patern number	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Number of isolate
Pattern 18	-	-	-	+	-	+	+	+	-	+	-	w	+	-	-	+	-	1
Pattern19	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	1

+: reaction; -: no reaction; vw: very weak reaction; w: weak reaction

3.3 Growth of the Reference Bacteria

Growth of *S. thermophilus* LMG 18311 and *L. bulgaricus* DSM 20081^T were prepared and given on Figure 3.4 and Figure 3.5 for *S. thermophilus* LMG 18311 and *L. bulgaricus* DSM 20081^T, respectively. OD_{600} values were given in Appendix B. These curves were helpful to detect the starting point for standardization of inoculums in technological property experiments in preliminary experiments.

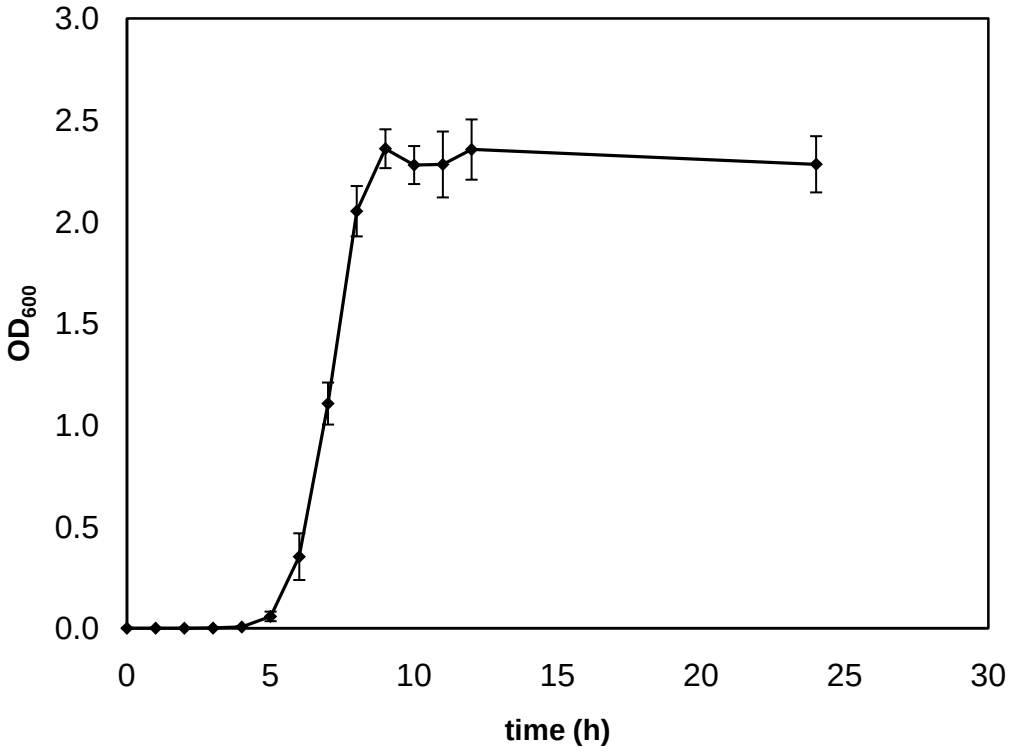


Figure 3.4 Growth of *Streptococcus thermophilus* LMG 18311 in M17 medium (pH 6.8) incubated at 42 ºC

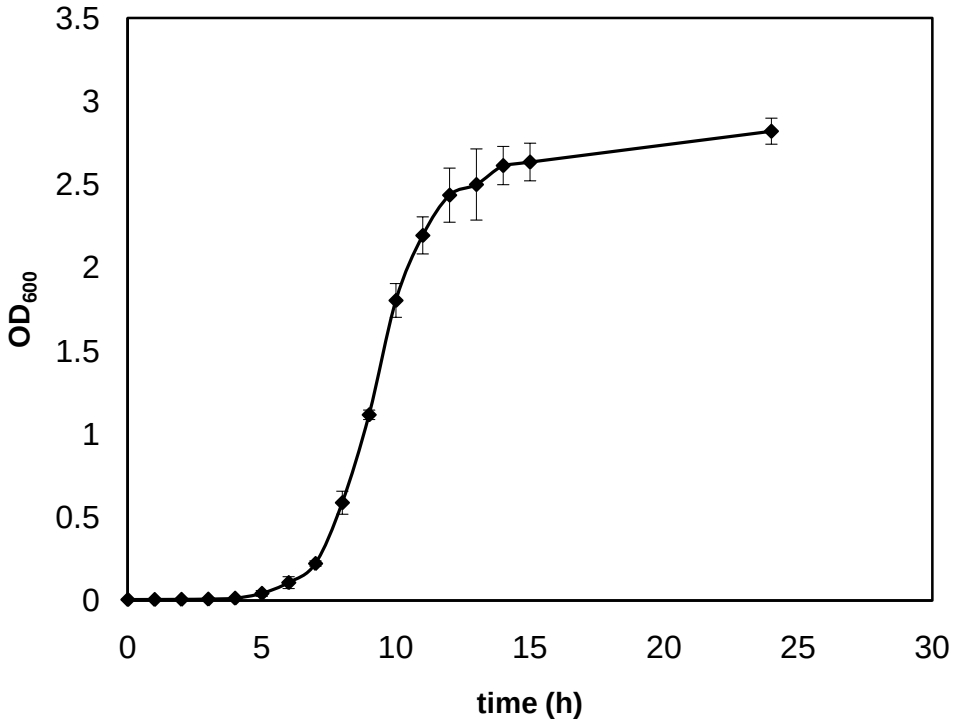


Figure 3.5 Growth of *Lactobacillus bulgaricus* DSM 20081 in MRS medium (pH 5.7) incubated at 42 ºC

3.4 Technological Characterization of Isolates

Acidification activity, acetaldehyde production ability, phage resistance and proteolytic activity of the isolates were studied to select cultures which have potential to be used as yogurt starter culture. Four cocci organisms identified biochemically as *S. thermophilus* were revealed as not being *S. thermophilus* after 16S rRNA gene sequencing performed in the third part of experimental strategy. Thus, the results of the technological property experiments belong to these four organisms were omitted.

3.4.1 Acidification Activity

Acidification activity is probably the most important technological property of yogurt cultures, since acidification causes coagulation of casein and hence production of yogurt (Tamime and Robinson, 2007). In this study, acidification activities of biochemically identified 55 *S. thermophilus* isolates and 110 putative *L. bulgaricus* isolates were measured and isolates were classified as good, medium and fair according to their acidification capability of reconstituted skim milk (RSM) via comparing ΔpH at 4 h and 6 h for *S. thermophilus* isolates and putative *L. bulgaricus* isolates, respectively. Acidification activity of 1 isolate from MRS and 5 isolates from M17 were not detected, because OD_{600} of these isolates was lower than 2. Since there could be slight differences between initial pH of RSM, ΔpH were used as comparing parameter instead of pH. While defining the range of ΔpH to classify the isolates, ΔpH of commercial isolates were used as reference.

pH changes of commercial *S. thermophilus* isolates (4) and one of our isolate were given in Figure 3.6. All the commercial isolates have similar pattern in decreasing the pH of RSM. At the beginning a very fast pH decrease was observed followed by a slower decrease after 4 h incubation.

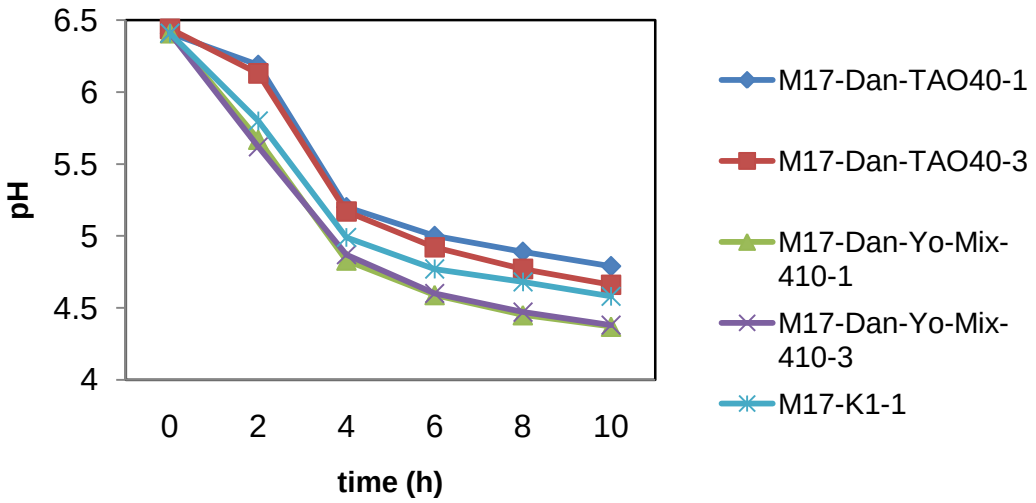


Figure 3.6 pH changes of some selected putative *Streptococcus thermophilus* isolates; all isolates except M17-K1-1 are commercial starter culture isolates.

pH changes of commercial *L. bulgaricus* isolates (5) and one of our isolate were given in Figure 3.7. All the commercial *L. bulgaricus* isolates, except MRS-Dan-Yo-Mix-410-2 decreased pH of RSM very fast during first 6 h incubation followed by a slower decrease. A slow decrease of pH was observed in MRS-Dan-Yo-Mix-410-2 case.

It was seen in the Figures 3.6 and 3.7 that *S. thermophilus* isolates decrease pH initially in a higher rate than *L. bulgaricus* (e.g. after 4 h incubation, pH was measured as 4.75-5.25 for *S. thermophilus* and 5.5-6 for *L. bulgaricus*). However, *L. bulgaricus* isolates decreased the pH to a lower level at the end of incubation (i.e. after 10h incubation). In yogurt production these properties of yogurt bacteria are also valid (Jay, et al., 2005). This observation is in agreement with the general characteristics of yogurt bacteria.

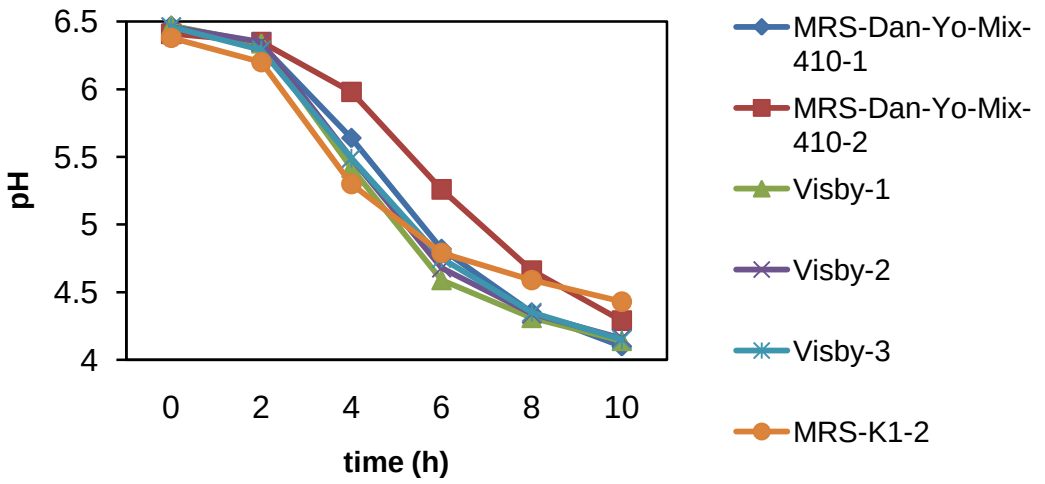


Figure 3.7 pH changes of some selected putative *Lactobacillus bulgaricus* isolates; all isolates except MRS-K1-2 are commercial starter culture isolates.

In Figure 3.8 and Figure 3.9, number of isolates was given for each acidification group for *S. thermophilus* isolates and putative *L. bulgaricus* isolates, respectively. Twenty-six *S. thermophilus* isolates and 53 putative *L. bulgaricus* isolates having medium or good acidification ability were selected to detect their acetaldehyde production ability.

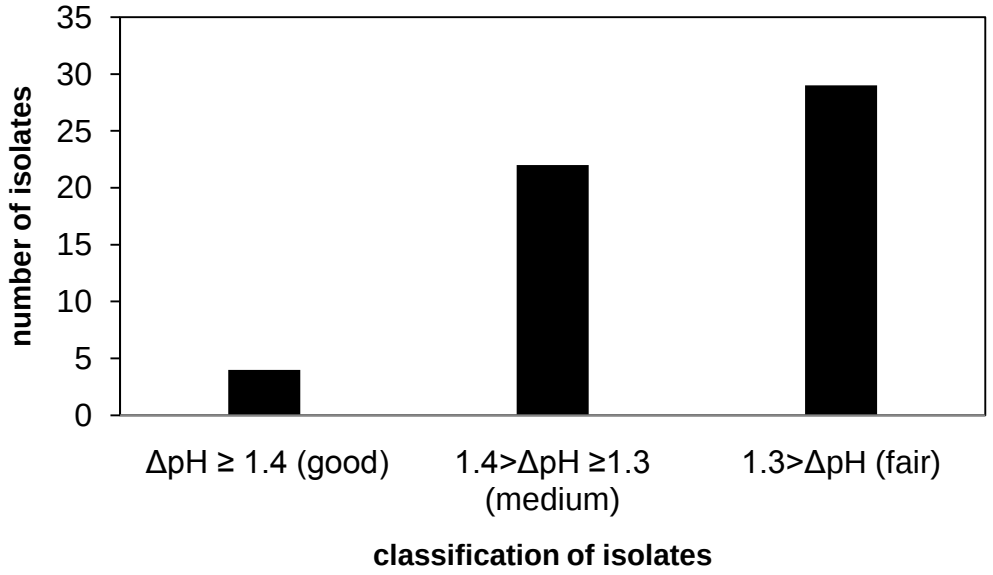


Figure 3.8 Classification of *Streptococcus thermophilus* isolates according to their acidification activities at 4 h

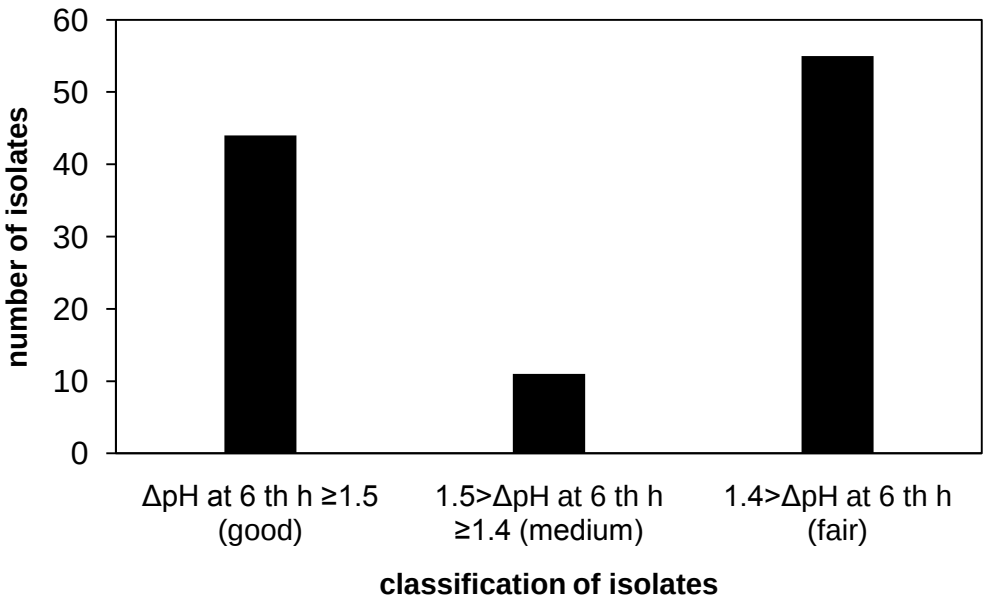


Figure 3.9 Classification of putative *Lactobacillus bulgaricus* isolates according to their acidification activities at 6 h

3.4.2 Acetaldehyde Production and Final pH

Acetaldehyde is claimed to be the major aroma compound in yogurt giving typical yogurt flavor (Tamime and Robinson, 2007). Therefore, acetaldehyde production ability of the selected isolates was examined to determine their capability of improving the characteristic aroma of yogurt. The acetaldehyde produced was determined in RSM after 24 h of fermentation at 42°C. Before the assay, the pH of the sample was also measured to determine final pH after 24 h incubation at 42 ºC. Acetaldehyde production ability of 53 *L. bulgaricus* isolates and 26 *S. thermophilus* isolates selected according to their acidification activities were measured. Acetaldehyde production of 25 *L. bulgaricus* isolates out of 53 was comparable with commercial isolates. Since *L. bulgaricus* is claimed as the major acetaldehyde producer, acetaldehyde production of these 25 isolates were measured in three parallels and given in Figure 3.10. Acetaldehyde production of all 53 isolates was given in Appendix F. Biochemical identification of 23 out of these 25 (except MRS-M2-13 and MRS-N2-2) *L. bulgaricus* isolates were confirmed using 16S rRNA gene sequencing by Cebeci Aydın (2008).

All *S. thermophilus* (26) and 25 out of 53 putative *L. bulgaricus* isolates produced comparable amounts of acetaldehyde with commercial isolates. Acetaldehyde produced by *S. thermophilus* isolates was given in Figure 3.12 and ranged between 3.96 μg/g (M17-K1-7) and 7.02 μg/g (M17-K1-29). Acetaldehyde produced by *L. bulgaricus* isolates was given in Figure 3.10 and ranged between 6.95 μg/g (MRS-N6-2) and 18.78 μg/g (MRS-M2-12). Especially within *L. bulgaricus* isolates, there were some isolates with greater acetaldehyde production compared to commercial isolates. This could be an expected result, since traditionally produced Turkish yogurts generally have intensive yogurt aroma, which may be not preferred for commercially produced yogurt. Nevertheless, acetaldehyde level produced by *S. thermophilus* and *L. bulgaricus* isolates are compatible with the observations of other researchers. Tamime and Robinson (2007) who summarized previous works has presented acetaldehyde production of *S. thermophilus*, *L. bulgaricus* and mixed culture as 1.0-13.5 μg/g, 1.4-77.5 μg/g and 2.0-41.0 μg/g,

respectively and emphasized that greater acetaldehyde production has been observed in mixed cultures due to associative growth.

The final pH by *S. thermophilus* measured after 24 h incubation in RSM at 42 ºC was higher than *L. bulgaricus* isolates which is consistent with general characteristics of these two bacteria. Most of the *S. thermophilus* isolates gave similar final pH with commercial isolates besides some of the isolates gave higher final pH values (Figure 3.13). Two groups were observed within commercial *L. bulgaricus* isolates according to their final pH results (Figure 3.11). At the first group including MRS-Dan-Yo-Mix-410-1, MRS-Dan-Yo-Mix-410-2 and Visby1, final pH was around 3.2 and at the second group formed by MRS-Visby-2 and MRS-Visby-3, final pH was much higher and recorded as 3.41 and 3.33, respectively.Four putative *L. bulgaricus* isolates, namely MRS-K1-43, MRS-N2-2, MRS-N2-4 and MRS-N2-5 gave comparably high final pH with the second group of commercial isolates. Undesirable postacidification formed during storage of yogurt is attributed to *L. bulgaricus* (Leroy and De Vuyst, 2004) since *S. thermophilus* tend to be inhibited at higher pH values than *L. bulgaricus* which can tolerate pH values of 3.5-3.8 (Jay et al., 2005). Therefore, these isolates could be important to get rid of postacidification.

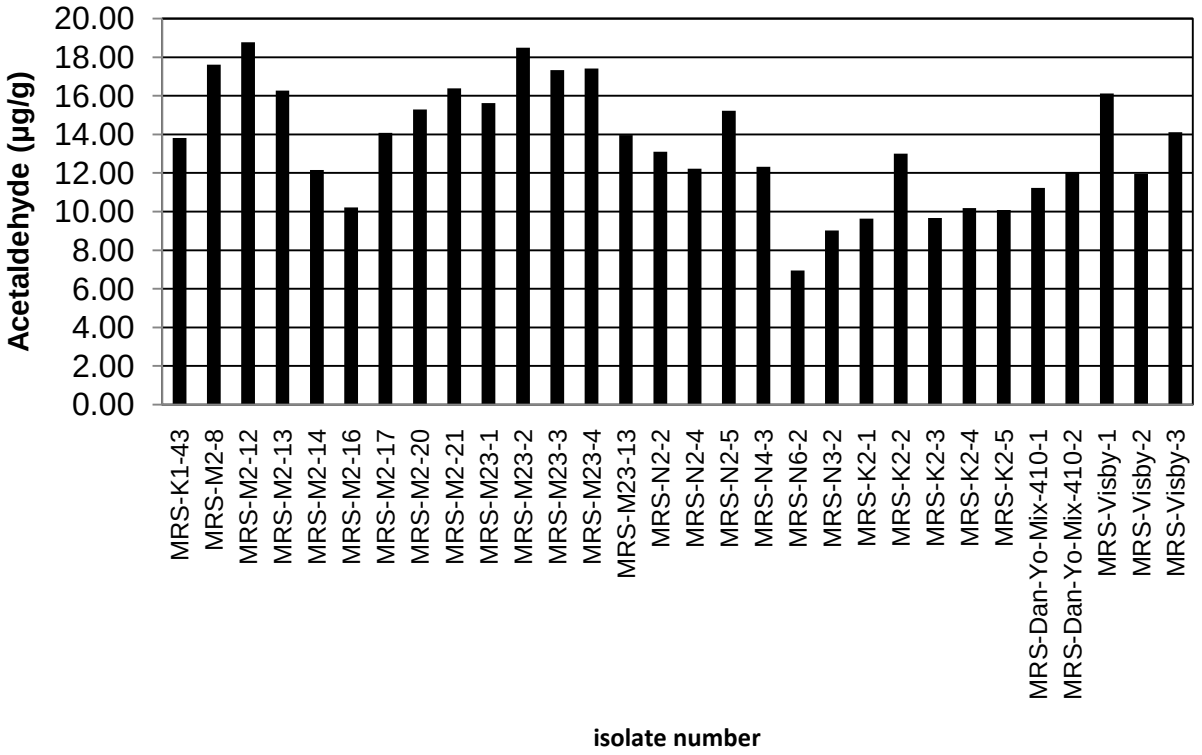


Figure 3.10 Screening of *Lactobacillus bulgaricus* isolates for acetaldehyde production in RSM

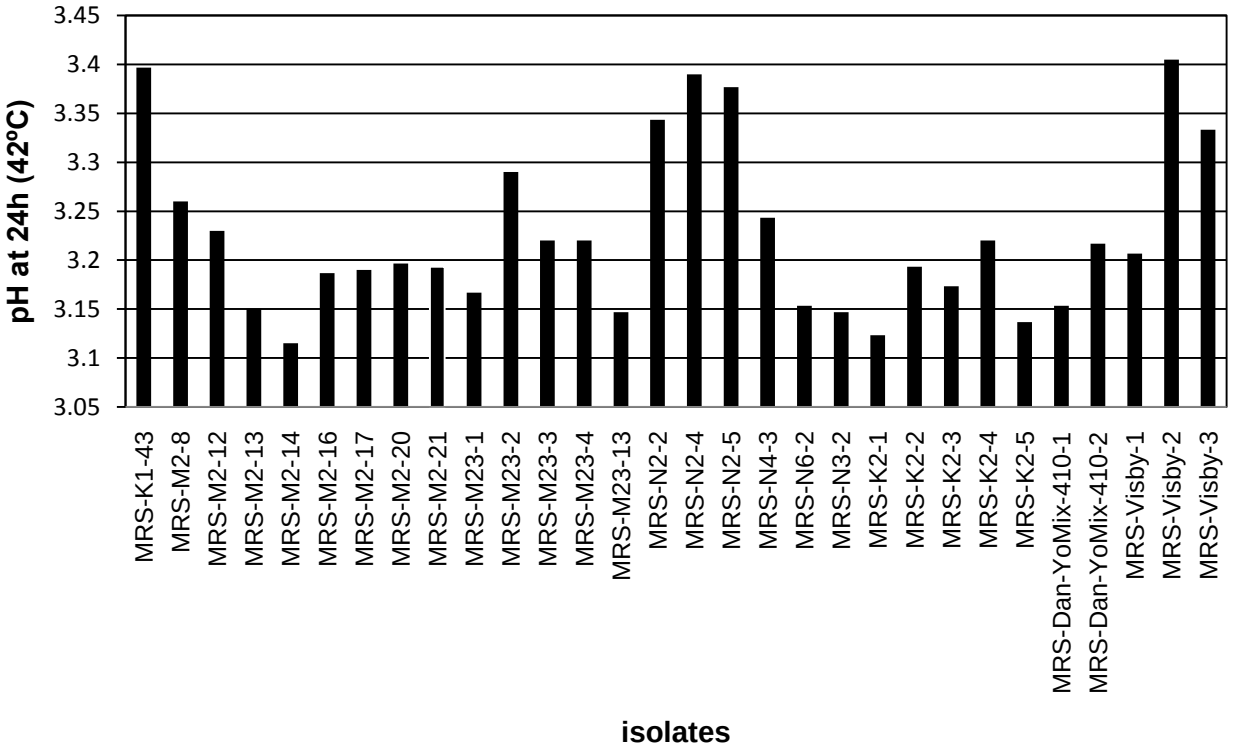


Figure 3.11 pH of *Lactobacillus bulgaricus* isolates in RSM after 24 h incubation at 42 ºC

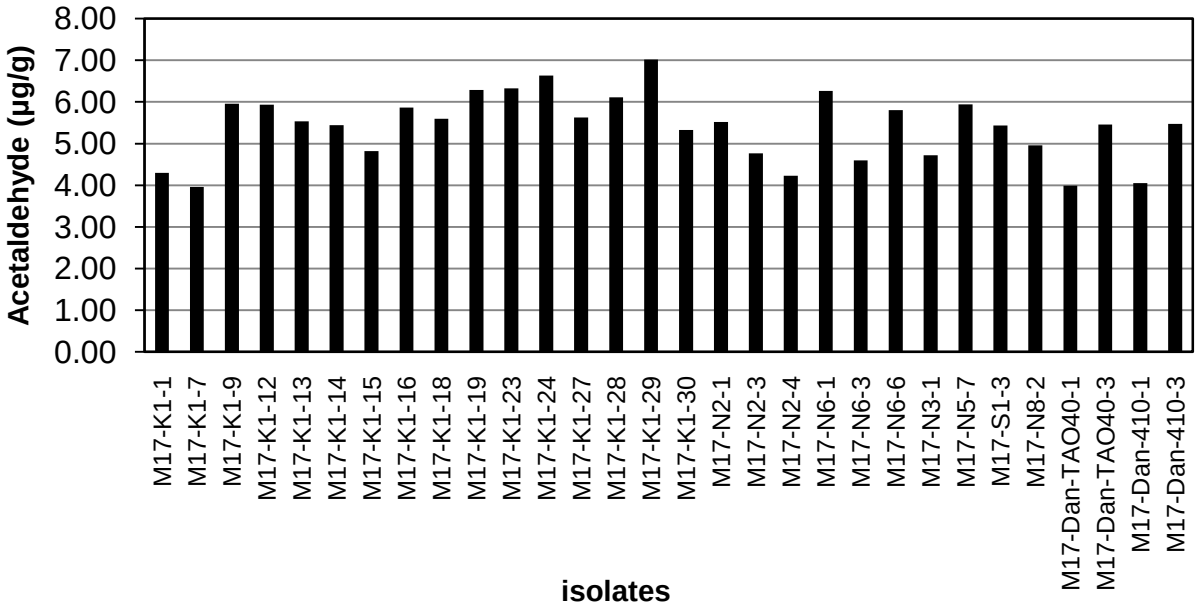


Figure 3.12 Screening of *Streptococcus thermophilus* isolates for acetaldehyde production in RSM

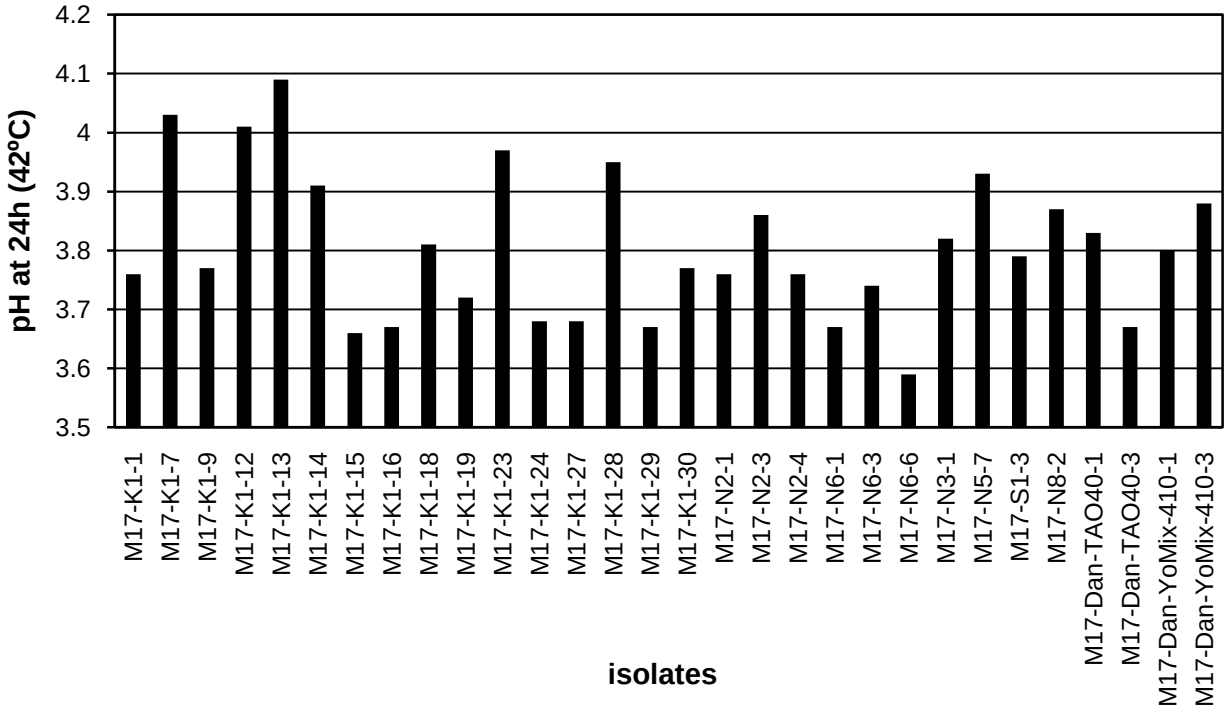


Figure 3.13 pH of *Streptococcus thermophilus* isolates in RSM after 24h incubation at 42 ºC

3.4.3 Phage Resistance

L. bulgaricus isolates (25 traditional and 4 commercial) were challenged with 15 *L. bulgaricus* phages and 9 *S. thermophilus* phages (Table 3.6-Table 3.7). *S. thermophilus* isolates (26 traditional and 4 commercial) were also challenged with 15 *L. bulgaricus* phages and 28 *S. thermophilus* phages (Table 3.8-Table 3.9). All *L. bulgaricus* isolates including commercial cultures were detected as resistant to *S. thermophilus* phages. However, they were sensitive to *L. bulgaricus* phages except two of traditional isolates (MRS-M2-16 and MRS-M23-2). One of the plates of *L. bulgaricus* isolates is shown in Figure 3.14. In general, *S. thermophilus* isolates were resistant to the phages. Nevertheless, *S. thermophilus* isolates which did not have a clear zone due to a complete lysis but a clear ring-like area having less dense bacterial growth inside were detected within K1 yogurt isolates. The reason of this uncommon response to phages might be the presence of phage resistant mutants in culture and increasing of these mutants in number during transferring the glycerol stocks. The phage resistance tendency of traditional *L. bulgaricus* and *S. thermophilus* isolates from yogurts determined in this study were compatible with the tendency previously observed (Özyurt, 2005; Acar-Soykut, 2007) as *S. thermophilus* isolates from Turkey were resistant to *S. thermophilus* phages isolated in Turkey while *L. bulgaricus* isolates from Turkey were in general sensitive to *L. bulgaricus* phages isolated in Turkey. Additionally, Kaleli (2001) were also studied phage resistance of *S. thermophilus* isolates from raw milks collected at different regions of Turkey and observed that all the isolates were resistant to both *S. thermophilus* and *L. bulgaricus* phages in the collection.

In this study, *S. thermophilus* and *L. bulgaricus* isolates were challenged with both *S. thermophilus* phages and *L. bulgaricus* phages since bacteriophages which infect more than one genus have been previously reported (Jensen et al., 1998; Özyurt, 2005; Acar-Soykut, 2007). However, the isolates tested in this study were infected only by their own phages even if some of the phages used in this study were previously shown as broad-host-range phages (Özyurt, 2005; Acar-Soykut, 2007).

In lactococci, lots of the phage resistance systems are related with plasmids (Josephsen and Neve, 1998) and loss of plasmid which encodes phage resistance can result phage sensitivity. Although a plasmid encoding a complete restriction modification system has been isolated from *S. thermophilus* (Solow and Somkuti, 2001), carrying plasmids is uncommon for both *S.thermophilus* and *L. bulgaricus* (Mercenier and Lemonie, 1989; Pridmore et al., 2000, Lee et al., 2007). Therefore it is difficult to relate extreme phage sensitivity observed among *L. bulgaricus* isolates from Turkey with plasmid loss. The phage sensitivity of *L bulgaricus* isolates from Turkey and the possible reasons of this property should be investigated.

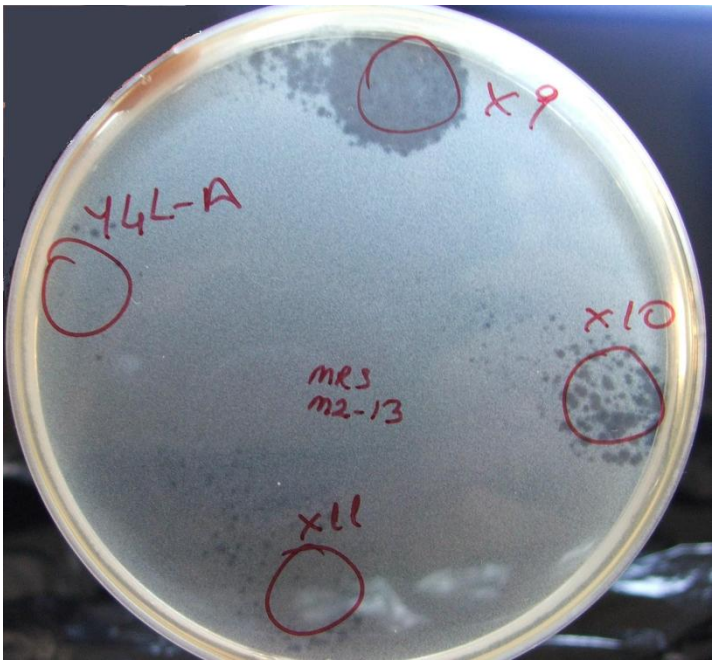


Figure 3.14 The plate of the isolate MRS-M2-13 challenged with four phages, ΦY4-X9, ΦY4-X10, ΦY4-X11 and ΦY4L-A

Table 3.6 Phage resistance profiles of putative *L. bulgaricus* isolates challenged with *L. bulgaricus* phages

	***L. bulgaricus* phages**														
Strain numbers	ΦY4-X1	ΦY4-X2	ΦY4-X3	ΦY4-X4	ΦY4-X5	ΦY4-X6	ΦY4-X7	ΦY4-X8	ΦY4-X9	ΦY4-X10	ΦY4-X11	ΦY4L-A	ΦV1-X20	ΦLbA-A	ΦLbA-Z
MRS-K1-43	+	+	+	+	+	+	(+)	+	+	+	+	+	+	-	+
MRS-M2-8	-	-	(+)	(+)	(+)	(+)	-	-	+	-	(+)	-	(+)	-	(+)
MRS-M2-12	(+)	(+)	-	-	-	-	(+)	(+)	(+)	-	(+)	-	-	+	+
MRS-M2-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-M2-14	(+)	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+
MRS-M2-16	-	-	-	-	(+)	-	-	-	-	-	-	-	-	(+)	-
MRS-M2-17	(+)	(+)	-	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
MRS-M2-20	(+)	-	-	(+)	(+)	-	(+)	-	(+)	(+)	-	-	-	(+)	(+)
MRS-M2-21	+	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-	-	-
MRS-M23-1	(+)	+	-	-	(+)	-	(+)	-	+	+	+	(+)	-	-	-
MRS-M23-2	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
MRS-M23-3	-	+	+	+	+	+	+	(+)	+	+	(+)	+	-	-	-
MRS-M23-4	+	+	+	+	-	-	-	(+)	+	-	-	+	-	-	-
MRS-M23-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-N2-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3.6 Phage resistance profiles of putative *L. bulgaricus* isolates challenged with *L. bulgaricus* phages (cont'd)

Strain numbers	*L. bulgaricus* phages														
	ΦY4-X1	ΦY4-X2	ΦY4-X3	ΦY4-X4	ΦY4-X5	ΦY4-X6	ΦY4-X7	ΦY4-X8	ΦY4-X9	ΦY4-X10	ΦY4-X11	ΦY4L-A	ΦV1-X20	ΦLbA-A	ΦLbA-Z
MRS-N2-4	(+)	-	-	(+)	(+)	-	(+)	(+)	(+)	-	-	-	-	-	-
MRS-N2-5	(+)	+	(+)	+	+	+	(+)	(+)	+	+	(+)	(+)	+	+	+
MRS-N4-3	(+)	(+)	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	(+)	-	(+)	-
MRS-N6-2	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	-	-
MRS-N3-2	(+)	(+)	-	-	(+)	(+)	-	-	(+)	(+)	(+)	-	(+)	-	-
MRS-K2-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-K2-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-K2-3	+	+	+	+	+	(+)	+	(+)	+	+	+	+	+	+	+
MRS-K2-4	+	+	(+)	+	+	+	+	+	+	+	+	(+)	+	+	+
MRS-K2-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-Dan-Yo-Mix-410-1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
MRS-Visby-1	+	+	+	+	(+)	(+)	(+)	(+)	+	+	+	(+)	(+)	(+)	-
MRS-Visby-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRS-Visby-3	+	+	+	+	+	+	+	+	+	+	+	+	+	(+)	(+)

+: sensitive, -: resistant, (+):1 or 2 plaque were detected

Table 3.7 Phage resistance profile of putative *L. bulgaricus* isolates challenged with *S. thermophilus* phages

	***S. thermophilus* phages**								
Strain numbers	ΦB3-X11	ΦB3-X19	ΦB3-X15	ΦB3-X20	Φ1B3A	Φ709-x1	Φ231-X23	Φ231S-A1MÖ	Φ231-X6
MRS-K1-43	-	-	-	-	-	-	-	-	-
MRS-M2-8	-	-	-	-	-	-	-	-	-
MRS-M2-12	-	-	-	-	-	-	-	-	-
MRS-M2-13	-	-	-	-	-	-	-	-	-
MRS-M2-14	-	-	-	-	-	-	-	-	-
MRS-M2-16	-	-	-	-	-	-	-	-	-
MRS-M2-17	-	-	-	-	-	-	-	-	-
MRS-M2-20	-	-	-	-	-	-	-	-	-
MRS-M2-21	-	-	-	-	-	-	-	-	-
MRS-M23-1	-	-	-	-	-	-	-	-	-
MRS-M23-2	-	-	-	-	-	-	-	-	-
MRS-M23-3	-	-	-	-	-	-	-	-	-
MRS-M23-4	-	-	-	-	-	-	-	-	-
MRS-M23-13	-	-	-	-	-	-	-	-	-
MRS-N2-2	-	-	-	-	-	-	-	-	-

-: resistant

Table 3.7 Phage resistance profile of putative *L. bulgaricus* isolates challenged with *S. thermophilus* phages

	***S. thermophilus* phages**								
Strain numbers	ΦB3-X11	ΦB3-X19	ΦB3-X15	ΦB3-X20	Φ1B3A	Φ709-x1	Φ231-X23	Φ231S-A1MÖ	Φ231-X6
MRS-N2-4	-	-	-	-	-	-	-	-	-
MRS-N2-5	-	-	-	-	-	-	-	-	-
MRS-N4-3	-	-	-	-	-	-	-	-	-
MRS-N6-2	-	-	-	-	-	-	-	-	-
MRS-N3-2	-	-	-	-	-	-	-	-	-
MRS-K2-1	-	-	-	-	-	-	-	-	-
MRS-K2-2	-	-	-	-	-	-	-	-	-
MRS-K2-3	-	-	-	-	-	-	-	-	-
MRS-K2-4	-	-	-	-	-	-	-	-	-
MRS-K2-5	-	-	-	-	-	-	-	-	-
MRS-Dan-Yo-Mix-410-1	-	-	-	-	-	-	-	-	-
MRS-Visby-1	-	-	-	-	-	-	-	-	-
MRS-Visby-2	-	-	-	-	-	-	-	-	-
MRS-Visby-3	-	-	-	-	-	-	-	-	-

-: resistant

Table 3.8 Phage resistance profile of putative *S. thermophilus* isolates challenged with *L. bulgaricus* phages

Strain numbers	*L. bulgaricus* phages														
	ΦY4-X1	ΦY4-X2	ΦY4-X3	ΦY4-X4	ΦY4-X5	ΦY4-X6	ΦY4-X7	ΦY4-X8	ΦY4-X9	ΦY4-X10	ΦY4-X11	ΦY4L-A	ΦV1-X20	ΦLbA-A	ΦLbA-Z
M17-K1-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.8 Phage resistance profiles of putative *S. thermophilus* isolates challenged with *L. bulgaricus* phages (cont'd)

Strain numbers	*L. bulgaricus* phages														
	ΦY4-X1	ΦY4-X2	ΦY4-X3	ΦY4-X4	ΦY4-X5	ΦY4-X6	ΦY4-X7	ΦY4-X8	ΦY4-X9	ΦY4-X10	ΦY4-X11	ΦY4L-A	ΦV1-X20	ΦLbA-A	ΦLbA-Z
M17-N2-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N2-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N2-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N3-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N5-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-S1-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N8-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: resistant

Table 3.9 Phage resistance profiles of putative *S. thermophilus* isolates challenged with *S. thermophilus* phages

Strain numbers	***S. thermophilus* phages**																
	Φ1B3-A	Φ2B3-A	Φ709S-B1	Φ709-X1	Φ709-X2	Φ709-X3	Φ709-X4	Φ709-X5	Φ231SAlmö	Φ231SB1	Φ231-X6	Φ231-X7	Φ231-X8	Φ231-X9	Φ231-X10	Φ231-X22	Φ231-X23
M17-K1-1	-	(+)	-	(+)	(+)	-	(+)	(+)	(+)	-	-	-	-	-	-	-	-
M17-K1-7	-	-	-	(+)	(+)	-	(+)	-	-	-	-	-	-	-	-	-	-
M17-K1-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-13	-	-	-	(+)	-	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-
M17-K1-14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-16	-	-	-	-	-	-	(+)	(+)	-	-	-	-	-	-	-	-	-
M17-K1-18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-19	-	-	-	-	-	-	(+)	(+)	-	-	-	-	-	-	-	-	-
M17-K1-23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-27	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-28	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-
M17-K1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-K1-30	-	-	(+)	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.9 Phage resistance profiles of putative *S. thermophilus* isolates challenged with *S. thermophilus* phages (cont'd)

	***S. thermophilus* phages**																
Strain numbers	Φ1B3-A	Φ2B3-A	Φ709S-B1	Φ709-X1	Φ709-X2	Φ709-X3	Φ709-X4	Φ709-X5	Φ231SAlmö	Φ231SB1	Φ231-X6	Φ231-X7	Φ231-X8	Φ231-X9	Φ231-X10	Φ231-X22	Φ231-X23
M17-N2-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N2-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N2-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N6-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N3-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N5-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-S1-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-N8-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: resistant, (+):a zone having a shape of a ring were detected with a low density of bacteria growth within it

Table 3.9 Phage resistance profiles of putative *S. thermophilus* isolates challenged with *S. thermophilus* phages (cont'd)

Strain numbers	***S. thermophilus* phages**										
	ΦB3S-B1	ΦB3-X11	ΦB3-X12	ΦB3-X13	ΦB3-X14	ΦB3-X15	ΦB3-X16	ΦB3-X17	ΦB3-X18	ΦB3-X19	ΦB3-X20
M17-K1-1	-	-	-	-	-	-	-	-	(+)	(+)	-
M17-K1-7	-	(+)	-	-	-	-	-	-	-	(+)	(+)
M17-K1-9	-	-	-	-	-	-	-	-	-	-	-
M17-K1-12	-	-	-	-	-	-	-	-	-	-	-
M17-K1-13	-	-	-	-	-	-	-	-	-	-	-
M17-K1-14	-	-	-	-	-	-	-	-	-	-	-
M17-K1-15	-	-	-	-	-	-	-	-	-	-	-
M17-K1-16	-	(+)	-	-	-	-	-	-	-	-	-
M17-K1-18	-	-	-	-	-	-	-	-	-	-	-
M17-K1-19	-	-	-	-	-	-	-	-	-	-	-
M17-K1-23	-	-	-	-	-	-	-	-	-	-	-
M17-K1-24	-	-	-	-	-	-	-	-	-	-	-
M17-K1-27	-	-	-	-	-	-	-	-	-	-	-
M17-K1-28	-	-	-	-	-	-	-	-	-	-	-
M17-K1-29	-	-	-	-	-	-	-	-	-	-	-
M17-K1-30	-	-	-	-	-	-	-	-	-	-	-

-: resistant, (+):a zone having a shape of a ring were detected with a low density of bacteria growth within it

Table 3.9 Phage resistance profiles of putative *S. thermophilus* isolates challenged with *S. thermophilus* phages (cont'd)

	***S. thermophilus* phages**										
Strain numbers	ΦB3S-B1	ΦB3-X11	ΦB3-X12	ΦB3-X13	ΦB3-X14	ΦB3-X15	ΦB3-X16	ΦB3-X17	ΦB3-X18	ΦB3-X19	ΦB3-X20
M17-N2-1	-	-	-	-	-	-	-	-	-	-	-
M17-N2-3	-	-	-	-	-	-	-	-	-	-	-
M17-N2-4	-	-	-	-	-	-	-	-	-	-	-
M17-N6-1	-	-	-	-	-	-	-	-	-	-	-
M17-N6-3	-	-	-	-	-	-	-	-	-	-	-
M17-N6-6	-	-	-	-	-	-	-	-	-	-	-
M17-N3-1	-	-	-	-	-	-	-	-	-	-	-
M17-N5-7	-	-	-	-	-	-	-	-	-	-	-
M17-S1-3	-	-	-	-	-	-	-	-	-	-	-
M17-N8-2	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-1	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-TA040-3	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-1	-	-	-	-	-	-	-	-	-	-	-
M17-Dan-Yo-Mix-410-3	-	-	-	-	-	-	-	-	-	-	-

-: resistant, (+):a zone having a shape of a ring were detected with a low density of bacteria growth within it

The phage resistance profiles of the isolates suggest that especially traditional *S. thermophilus* isolates have potential to be used as starter culture alone or in a rotation scheme with commercially available *S. thermophilus* cultures to overcome phage problem in yogurt plants in Turkey. For traditional *L. bulgaricus* isolates, although the majority of them are sensitive to *L. bulgaricus* phages, having two resistant isolates are promising to find phage resistant *L. bulgaricus* strains within traditional yogurt cultures. Additionally, sensitivity of commercial isolates to phages isolated in Turkish dairy plants emphasizes the necessity to have a national phage collection to serve dairy producer to control the starter cultures they purchase abroad, since phage diversity might change depending on location.

3.4.4 Proteolytic Activity

Proteolytic activity for yogurt production is not as important as in cheese production, but still has a secondary importance (Tamime and Robinson, 2007) and may have an important role while selecting best strain combination of *L. bulgaricus* and *S. thermophilus*. Proteolytic activities of 22 *S. thermophilus* and 25 *L. bulgaricus* selected according to their acidification activity and acetaldehyde production were measured. The proteolytic activity of the cultures was denoted as the absorbance of free amino groups at 340 nm. Proteolytic activities of 4 of *S. thermophilus* isolates could not be measured because of low OD_{600} (i.e. < 2) of these bacteria at the time of proteolytic activity experiments, even if their other technological properties were comparable with commercial isolates. These four isolates were eliminated due to losing their fast growing ability which is an important property for a yogurt starter culture. Proteolytic activities of the isolates were given in Figure 3.15 and Figure 3.16 for *S. thermophilus* (22) and *L. bulgaricus* (25), respectively.

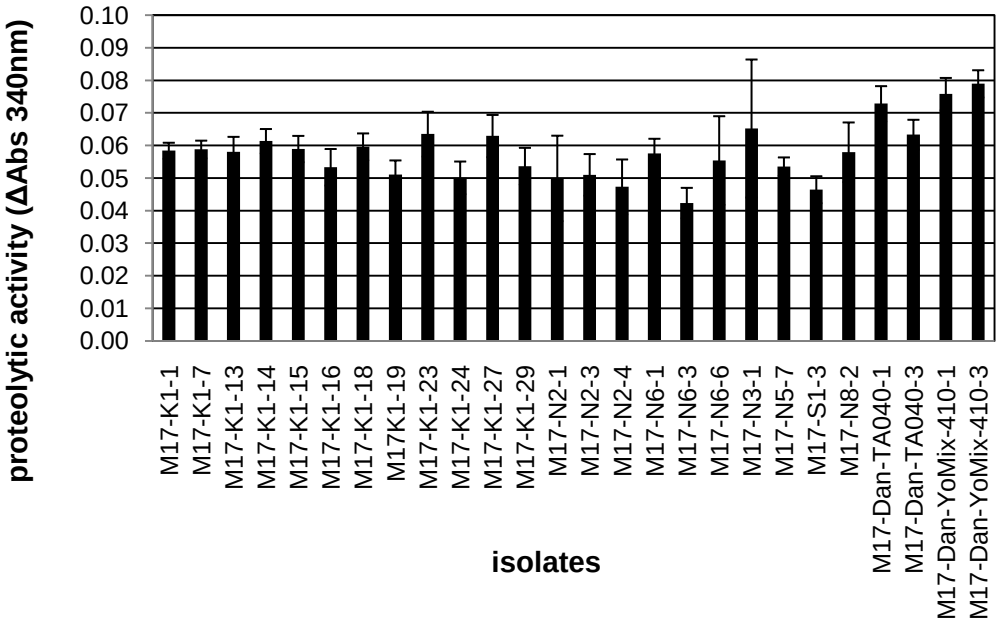


Figure 3.15 Proteolytic activities of *Streptococcus thermophilus* isolates in RSM after 6 h incubation at 42 ºC

Proteolytic activities of *L. bulgaricus* isolates were much higher than that of measured within *S. thermophilus* isolates and this is compatible with general properties of these species. *L. bulgaricus* has a greater proteolytic activity than *S. thermophilus* (Slocum et al., 1988a; Rajagopal and Sandine, 1990; Courtin and Rul, 2004), although the opposite case was also observed (Shihata and Shah, 2000). *S. thermophilus* isolates showed proteolytic activities (ΔAbs at 340 nm) very close to each other, including commercial isolates, and ranged between 0.04-0.08 (Figure 3.15). However a common bias within *L. bulgaricus* isolates could not be detected. Even commercial isolates grouped into two according to their proteolytic activities.

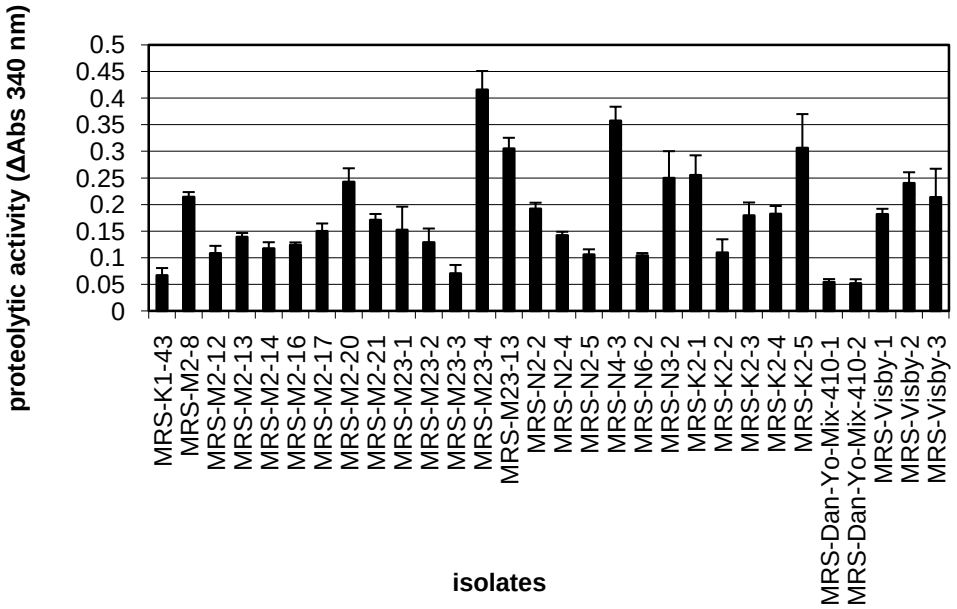


Figure 3.16 Proteolytic activities of *Lactobacillus bulgaricus* isolates in RSM after 6 h incubation at 42 ºC

Four of *L. bulgaricus* cultures (MRS-M23-4, MRS-M23-13, MRS-N4-3 and MRS-K2-5) exhibited higher proteolytic activity than commercial cultures (Figure 3.16). It is known that excessive proteolysis may cause bitter taste in yogurt (Slocum et al., 1988b). Rest of the tested *L. bulgaricus* isolates was comparable with commercial isolates in terms of their proteolytic activity. Nevertheless, having *L. bulgaricus* isolates with different proteolytic activities in a culture collection could be important to combine consistent *L. bulgaricus* and *S. thermophilus* isolates for yogurt production, since proteolytic activities of these species are important in their associative growth (Tamime and Robinson, 2007). *S. thermophilus* and *L. bulgaricus* isolates from Dan-Yo-Mix 410 could be example for this case. In this mix culture, *L. bulgaricus* with relatively low proteolytic activity combined with *S. thermophilus* having relatively high proteolytic activity. Proteolytic activities of

these strains might be one of the reasons of bringing them together within this commercially available mix yogurt culture.

As a conclusion, these technological property studies have demonstrated the significant phenotypic diversity within traditional yogurt cultures and revealed the high potential of traditionally produced Turkish yogurts for being a source of starter culture, which is compatible with the study of Çelik (2007). Raw milk could also have the potential for the isolates as starter culture for yogurt production in Turkey as it was shown by Ayhan et al., 2005.

3.5 16S rRNA Gene Sequencing of *S. thermophilus* Isolates

Strains of the same species can exhibit phenotypic variability and this may cause difficulties during identification using phenotypic methods (Drancourt et al., 2000). Therefore, genotypic confirmation of the results of phenotypic identification methods is necessary and 16S rRNA sequencing is a commonly used method for identification purposes.

S. thermophilus is closely related to the species *S. salivarius* and *S. vestibularis* (Poyart et al., 1998; Botina et al., 2007). Therefore, in this study, two parts within 16S rRNA gene were sequenced for 60 isolates from M17 instead of sequencing whole 16S rRNA gene and these two parts were selected in such a way to differentiate *S. thermophilus* from *S. salivarius* and *S. vestibularis*, although the origins of these species are distinct (dairy for *S. thermophilus* and human for *S. salivarius* and *S. vestibularis* (Facklam, 2002).).

Fifty-six out of 60 isolates were identified as *S. thermophilus* using 16S rRNA gene sequencing. *Streptococcus salivarius* ATCC 7073^T and *Streptococcus vestibularis* ATCC 49124^T strains were differentiated from *S. thermophilus* via 16S rRNA gene identification using the same primer and conditions used for *S. thermophilus*. The four isolates (M17-K1-25, M17-N5-4, M17-N7-1 and M17-N7-4) were not *S. thermophilus* according to their sequencing results. Blast Analysis

of a representative *S. thermophilus* isolate (M17-K1-11) was given below for both sequenced part 1 and part 2. Additionally, Blast Analysis of *S. thermophilus* LMG 18311 (Appendix G) *Streptococcus salivarius* ATCC 7073^{T}, *Streptococcus vestibularis* ATCC 49124^{T}, M17-K1-25, M17-N5-4, M17-N7-1 and M17-N7-4 (Appendix H) were also given in Appendices. Since the primers specifically design for identification of *S. thermophilus*, exact identification of the isolates M17-K1-25, M17-N5-4, M17-N7-1 and M17-N7-4 was not established.

3.5.1 Blast Analysis of Representative *S. thermophilus* Isolate (M17-K1-11)

Sequence-part1 for M17-K1-11

>gb|GU344730.1| *Streptococcus thermophilus* strain STKWT 16S ribosomal RNA gene, partial sequence

```
 Score =  817 bits (442),  Expect = 0.0
 Identities = 442/442 (100%), Gaps = 0/442 (0%)
 Strand=Plus/Plus

Query  1    TTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29   TTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGC  88

Query  61   GGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTCATT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89   GGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTCATT  148

Query  121  TATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  149  TATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGT  208

Query  181  GAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  GAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACAC  268

Query  241  TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG  328

Query  301  GGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329  GGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTC  388

Query  361  TGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  389  TGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCA  448

Query  421  GAAAGGGACGGCTAACTACGTG  442
            ||||||||||||||||||||||
Sbjct  449  GAAAGGGACGGCTAACTACGTG  470
```

Sequence-part2 for M17-K1-11

> gb|CP000419.1| *Streptococcus thermophilus* LMD-9, complete genome

```
 Score =  809 bits (438),  Expect = 0.0
 Identities = 438/438 (100%), Gaps = 0/438 (0%)
 Strand=Plus/Plus

Query  1      ATCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20281  ATCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATG  20340

Query  61     ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20341  ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACA  20400

Query  121    ACGAGTTGCGAGTCGGTGACGGCGAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20401  ACGAGTTGCGAGTCGGTGACGGCGAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGT  20460

Query  181    AGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20461  AGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCG  20520

Query  241    GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20521  GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC  20580

Query  301    CGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20581  CGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGT  20640

Query  361    GAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20641  GAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAA  20700

Query  421    AAACGGAATGTACTTGAG  438
              ||||||||||||||||||
Sbjct  20701  AAACGGAATGTACTTGAG  20718
```

Percent identities between the first part of sequenced 16S rRNA gene of *S. thermophilus* LMG 18311 and some other *Streptococcus* species were also given in Table 3.10. The identity of this part is between 89% and 99%. Although there is 99% identity between *S. thermophilus* and the closely related species (*S. salivarius* and *S. vestibularis*), *S. thermophilus* could be differentiated from *S. salivarius* and *S. vestibularis* by checking the snps. The alignments of 16S rRNA gene sequences of *S. thermophilus* LMG 18311 with *S. salivarius* ATCC 7073 and with *S. vestibularis* ATCC 49124 were given in Appendix G.

Table 3.10 Percent identities between sequenced 16S rRNAgene-part1 of some *Streptococcus* species and *S. thermophilus* LMG 18311.

***Streptococcus* species**	**percent identity**
S. agalactiae 2603V/R	89.367
S. equi subsp. *equi* 4047	89.14
S. equinus strain KLDS 3.0603	92.986
S. gallolyticus subsp. *gallolyticus* strain 904	91.855
S. gordonii str. Challis substr. CH1	92.308
S. pneumoniae 70585	93.197
S. pyogenes M1 GAS	90.724
S. salivarius strain ATCC 7073	99.548
S. sanguinis SK36	94.118
S. suis 05ZYH33	90.271
S. uberis 0140J	89.819
S. vestibularis strain ATCC 49124	99.093

3.6 Analyzing of CRISPR1 Locus

CRISPR1 locus of *S. thermophilus* isolates (56) confirmed using 16S rRNA gene sequencing was analyzed. Totally sixteen distinct CRISPR1 amplicons were defined based upon amplicon size. In Figure 3.17, amplicons belongs to isolates from K1 yogurt are presented. 3 distinct CRISPR1 amplicon sizes were observed. However amplicon size gives only limited information about diversity within the CRISPR1 locus, therefore these amplicons were sequenced to assess the level of CRISPR1 diversity within these isolates.

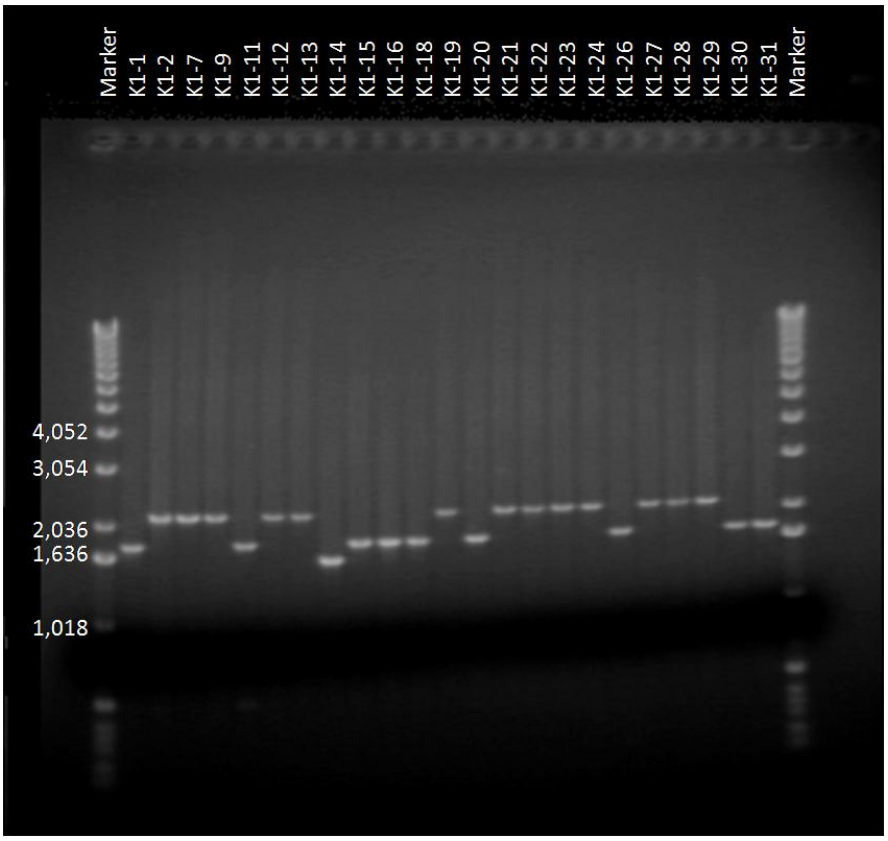


Figure 3.17 CRISPR1 amplicons of *Streptococcus thermophilus* isolates from K1 yogurt

The amplicons were sequenced and spacers were determined. Graphic representation of spacers was prepared as in the same way represented previously by Barrangou et al. (2007) and Horvath et al. (2008) (Figure 3.18). One representative sequence from each subgroup (1-15) was given in Appendix I. *S. thermophilus* isolates were grouped into 6 clusters with a total of 15 sub-clusters. Hypervariability defining the sub-clusters in a group was mostly located towards the leader end of the locus as in previous studies (Horvath et al., 2008) with the exception of subgroup 14 and 15 (Figure 3.18). Two separate deletions in the CRISPR1 spacers of a common ancestor might be the reason of this atypical case. In each group, deletions of distinct 2 spacers among 4 ancestoral spacers would result the other two spacers to appear in the locus.

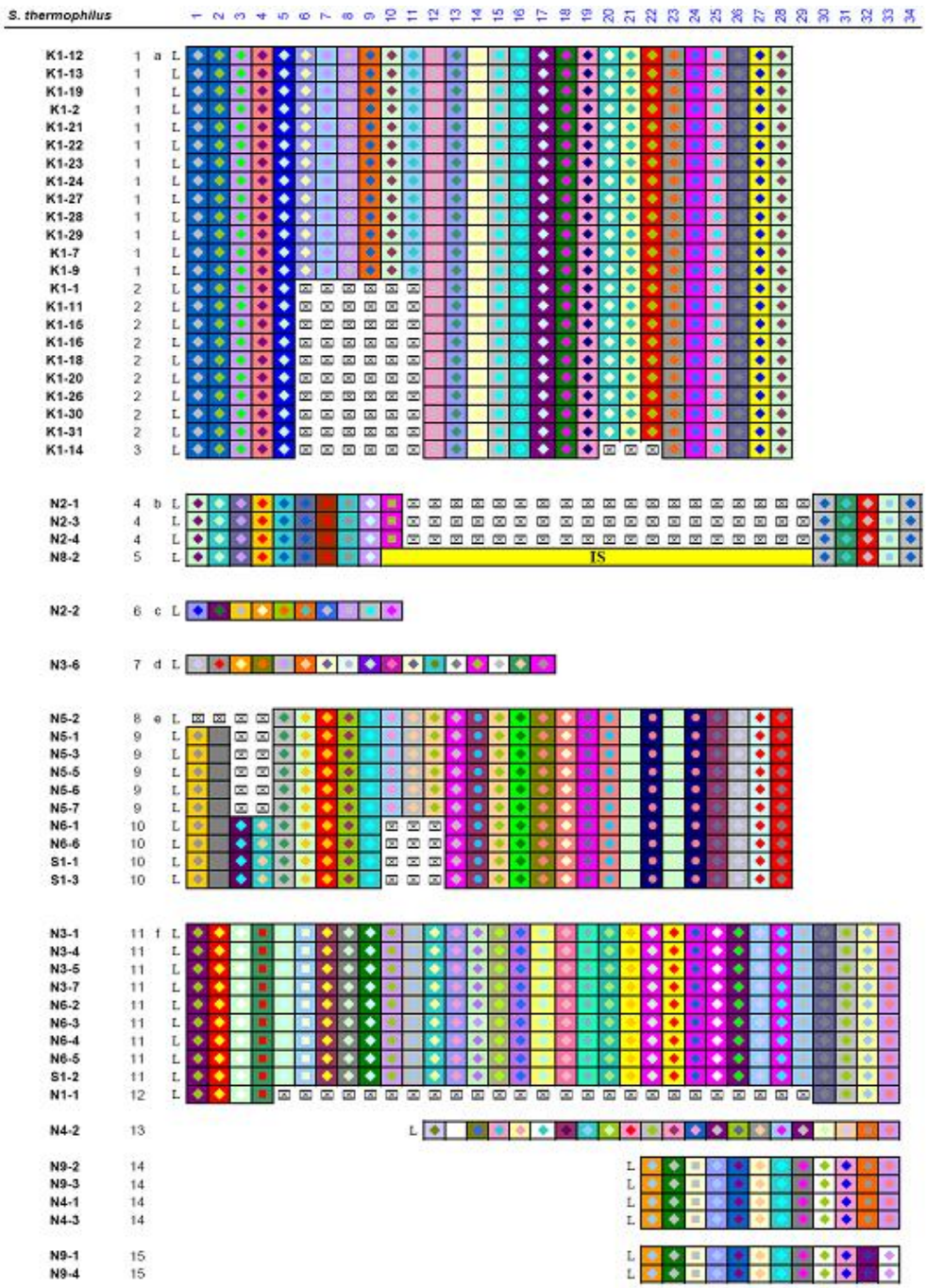


Figure 3.18 Graphic representation of CRISPR1 locus spacers of *Streptococcus thermophilus* isolates.

Direct repeats are eliminated; only spacers are represented. Each spacer is represented by a combination of one character with particular font color, on a particular background color. The color combination allows unique representation of a particular spacer. Crossed squares display lacking spacers. L; Leader sequence. IS; insertion sequence.

In Figure 3.18, the clusters and sub-clusters were shown as labeled with letters (a-f) and numbers (1-15), respectively. A total of 161 unique spacers were identified. Length of the spacers was 29 bp, 30 bp or 31 bp while about 90% of spacers were 30 bp long and this observation is in agreement with previous studies (Horvath et al., 2008; Deveau et al., 2008). Spacers detected in CRISPR1 locus of the isolates M17-N2-2, M17-N3-6 and M17-N4-2 were different from spacers in the remaining of the isolates. A transposone was detected within CRISPR1 locus of M17-N8-2. Spacers having homology with *S. thermophilus* phages (37), plasmids (13) and previously sequenced CRISPR spacers (6) were found out. It is possible that the remaining spacers were acquired from phages or plasmids whose genomes have not been sequenced yet. Additionally, very limited number of the spacers (6) had homology with previously sequenced CRISPR spacers. This low level of homology may point out the distinction of the isolates from Turkish yogurts than isolates from different locations in the world. Graphical representation of CRISPR1 locus of some representative *S. thermophilus* strains which was previously published by Barrangou et al. (2007) was given in Figure 3.19. The spacers (37) having homology to seven distinct *S. thermophilus* phages were given in Table 3.11. These spacers had homology with loci involved in lysogeny module (3), replication (3), tail morphogenesis (16), head morphogenesis (5), packaging (6) and host lysis (4).

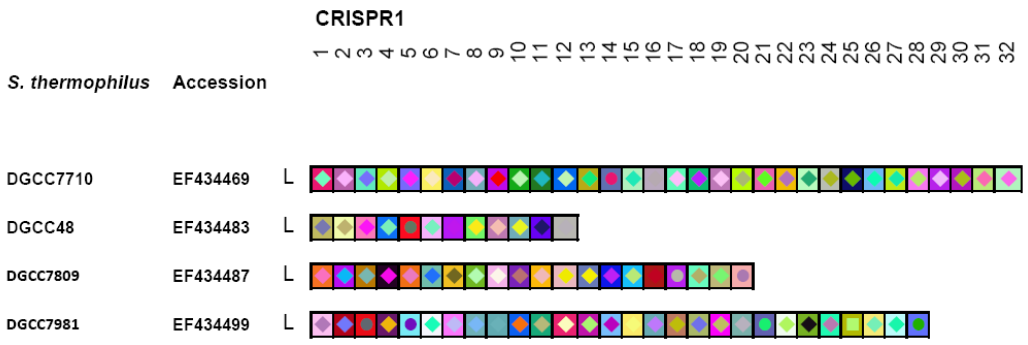


Figure 3.19 Graphic representation of CRISPR1 locus spacers of representative *Streptococcus thermophilus* isolates, which are previously published by Barrangou et al. (2007).
Direct repeats are eliminated; only spacers are represented. Each spacer is represented by a combination of one character with particular font color, on a particular background color. The color combination allows unique representation of a particular spacer. L; Leader sequence.

A CRISPR motif (AGAAW), located two nucleotide downstream of proto-spacers, was previously discovered in *S. thermophilus* and the importance of this motif for phage resistance phenotype was indicated (Deveau, 2008, Horvath, 2008). The CRISPR motif was also investigated in this study and addition to the motif previously detected (AGAAW), another possible motif, AAAA was commonly observed. It was previously showed that a mutation within AGAA motif sequence allowed the phage to escape CRISPR-mediated resistance (Deveau et al., 2008). Nevertheless, detection of AAAA in high level (9 of 37 phage proto-spacer) on the downstream of the proto-spacers related to non-commercial Turkish *S. thermophilus* isolates may indicate either a new CRISPR motif or different phage diversity in Turkey. The spacers (13) having homology to four distinct *S. thermophilus* plasmids were given in Table 3.12. Further analysis of proto-spacers related to phages and plasmids revealed that the spacer sequences were acquired from both strands of phages and plasmids, which is in agreement with previous study of Deveau et al. (2008). The majority of the phage proto-spacers (20) (i.e.

homologous regions on phage genome to spacers) were originated at positive strand (Table 3.11) and this observation is compatible with the study of Deveau et al. (2008) stated that new spacers from phages were originated often from coding strand. However, this bias was reverse in our plasmid proto-spacers since most of the plasmid proto-spacers (10) were originated at negative strand (Table 3.12).

Table 3.11 CRISPR1 spacers having homology with phages and characteristics of the related proto-spacers

Spacer	Spacer sequences[a]	3' flanking region	% Id[b]	Start	End	Sd[c]	Phage	Functional Unit[d]
K1-12_**7**	CCCTCTGTGTTAACTTGCCCAGATGTTATT	TT**AGAGA**TAC	96.67	12628	12605	-	7201	Packaging
K1-12_**9**	TAATCCAAAAGAATGGGATACACAAACGGT	CA**AGAAA**TCA	100	13674	13703	+	sfi21	Tail m.
K1-12_**11**	CTTGCTACACTAAACGATGGTAATGACAGC	CC**AGAAA**CAA	96.67	11309	11338	+	sfi21	Tail m.
K1-12_**20**	GAGAATGGCGATAACTGGATTCGTAAAGAT	AT**GGAAA**TAG	93.33	18367	18396	+	sfi11	Tail m.
K1-12_**21**	TGAGTTAGGACACGTCCAAGACGACAAACC	AA**AGAAA**AAG	100	7435	7464	+	sfi11	Head m.
K1-12_**25**	TACCGAGAGATGCTCGTCAATGCCATGCTC	GT**AGAAA**ACT	100	21079	21050	-	sfi21	Host lysis
K1-13_**16**	GTTTATTATGAAAATGAAACTTCTGTATAC	TT**AGAGC**AAA	100	21808	21837	+	sfi11	Tail m.
N2-1_**7**	ACCGAGAGATGCTCGTCAATGCCATGCTC	GT**AGAAA**ACT	100	21078	21050	-	sfi21	Host lysis
N2-1_**8**	CCAAATTTGCATTAAACAAAACGCTCCTTC	CA**ACTAA**TTT	96.67	14624	14595	-	Sfi21	Tail m.
N2-2_**1**	CAGGTCTTGATGAAGCGTTAGAGGGTTGGC	TT**AAAAC**GGT	100	17632	17661	+	7201	Head m.
N3-1_**17**	TTGGTTTTAACCACTACGACTTTCTTACTT	TG**AAAAA**GCG	100	17566	17595	+	7201	Head m.
N3-1_**10**	TGGTAAGCTATTACCAATAGACCACGAAAA	CT**AAAAA**AAT	96.67	26533	26562	+	858	Host lysis
N3-1_**11**	ATAATACCAACGTTTCTGACTATTTTTAT	GT**AAAAA**AGT	96.55	33118	33090	-	858	Replication
N3-1_**22**	ACGGTGACTATCAATCATGATTTCAACGGT	AA**AAAAA**CTT	100	22900	22929	+	sfi11	Tail m.
N3-1_**30**	CCAGTCTGCTACCAGCAATGCAAGACTAGA	GT**AAAAA**AAG	100	251	222	-	858	Packaging
N3-1_**33**	ATCCTAGATATTCTATTCCTGAAATCAAAG	GG**TAAAA**AAT	96.67	14252	14280	+	sfi21	Tail m.
N3-5_**15**	AGTCAACAGTCTAGCACGCTTATCGGACGT	TT**GAAGA**ATA	93.33	20391	20420	+	7201	Tail m.
N3-6_**2**	ACTAAAAGAGCTACTTGACGGCAAAGAATT	TG**GTGAA**ATA	100	8330	8359	+	sfi19	Head m.
N3-6_**5**	ATCAGATGGAAAAGGTGGATACGTCTATCA	AG**GTGAA**AAA	96.67	8989	9018	+	sfi19	Tail m.
N3-6_**7**	TAAATTCGACAAAAGCACTACATGAATACT	GA**GCAAA**AGT	96.67	31161	31132	-	7201	Tail m.

Table 3.11 CRISPR1 spacers having homology with phages and characteristics of the related proto-spacers (cont'd)

Spacer	Spacer sequences[a]	3' flanking region	% Id[b]	Start	End	Sd[c]	Phage	Functional Unit[d]
N3-6_**10**	ATTTAGAAGAAGTGTTTAAACCTGAAACGT	GG**GCAAA**GAG	96.43	12778	12807	+	7201	Packaging
N3-6_**11**	TAAACTCGACAAAAGCACTACAGGTATACT	GA**GCAAA**AGT	93.33	31161	31132	-	7201	Tail m.
N3-6_**16**	CGTTTTGCTACTCGTTCAGCATACTCTACA	TT**GTGAA**CAT	96.67	29391	29362	-	858	Replication
N4-2_**17**	TAAAATCATTTTCAACGAGTTGAGAAACAT	AA**AAAAC**GTG	100	14877	14848	-	sfi21	Tail m.
N4-2_**19**	CCACCTCCTTAGTTGCTAGATTTCTTTGCA	TT**AAATA**AAG	100	13830	13801	-	o1205	Packaging
N4-2_**26**	CTTCCTAAGTGCATGAAAATCGCAAACGGA	TA**AAAAA**TTA	100	25500	25471	-	sfi11	Host lysis
N5-2_**8**	CTACAATCTCGTCATAAGTAGTAGTACCGT	CT**ACAAT**GCT	100	25968	25997	+	sfi21	Lysogeny
N5-2_**7**	TTCTGGTAGTGGTTTTAGTCAAACAGATGT	CA**ATAAA**CCA	100	17084	17113	+	sfi21	Tail m.
N5-2_**11**	ATGAGTGGTTAAGAATCCGTATTATCAGCA	GA**ACAAC**GGG	100	11384	11413	+	7201	Packaging
N5-2_**12**	TATCAAATGCAGCACAAGTAACGTTGATGG	AT**ATCGT**TGA	96.67	20725	20754	+	sfi21	Tail m.
N5-2_**17**	CATTTCATAAGCTGTTCCTTCTTGAACATA	TC**ATAAT**AAG	100	28006	27977	-	sfi11	Lysogeny
N5-2_**21**	ATCTGTCCATCTGGTCTAAATCCAAACAGG	TC**ACAAA**AAC	100	33339	33310	-	858	Replication
N5-2_**26**	ATGGCATAATCTTCAAAAGCATACATACCA	TC**ATAGA**AAG	96.67	13639	13610	-	7201	Packaging
N5-2_**28**	TTTGAGGCAAGTTGACATTCTTAGACAGTC	GG**AAAAA**TTC	100	2418	2389	-	o1205	Lysogeny
N9-1_**32**	ACCCAGCGTTAAATAGTTGCGTTTTATCGC	TA**GTAAC**TTG	90	1068	1039	-	dt1	Tail m.
N9-2_**24**	TTAGCTGTCCAATCCACGAACGCTGATGGCA	GA**AAAAA**TGG	100	8078	8108	+	sfi19	Head m.
N9-2_**28**	CGTGTACAGCACGCAGTTGTTGATTTACAA	CA**AAAAA**ATC	93.33	9600	9629	+	sfi11	Tail m.

[a] The nucleotide(s) differs from proto-spacers is underlined; [b]% Identity; [c] Strand; [d]Tail m., Tail morphogenesis; Head m., Head morphogenesis; Lysogeny, Lysogeny module

Table 3.12 CRISPR1 spacers having homology with plasmids and characteristics of the related proto-spacers

Spacer	Spacer Sequence	3' flanking region	% Id[a]	Start	End	sd	Plasmid[b]
K1-12_**1**	GAGGTCTGTAATTTTATTCCCTCGTAATCT	TT**AGAAA**TTG	100.00	5258	5229	-	ST plasmid pSt08
K1-12_**4**	TCTAGCGCTTCATCAAGCATGGTAAAGCCT	GT**AGAAA**TAC	96.67	1547	1518	-	ST plasmid pSMQ-316
K1-12_**12**	TCGACACATAAAATTATAACACGAAACCTT	TC**AGAAA**TGA	100.00	155	126	-	ST plasmid pt39
N2-1_**11**	AATATCGTGAAATAGGCAACCGAAAAATAT	CA**AGAAT**TTC	100.00	131	102	-	ST plasmid pSt08
N2-2_**10**	TAGTCCGCCATATCCAAGTTGCCGTTTTCT	AT**ATAAA**CCA	100.00	1796	1767	-	ST plasmid pSt08
N5-2_**20**	TTCCAACAAGCCCGCGCCTAATTATTCCAG	TT**ACAAA**AAC	100.00	929	958	+	ST plasmid pSMQ-316
N5-2_**22**	GCCTAGCCCTACAGCTACCCCGCCTACTT	CC**ATAAT**GCT	100.00	2193	2165	-	ST plasmid pSMQ308
N4-2_**21**	TTTAACTGCCTTTCTTTCTTGCTAGGTCGT	GG**AAAAT**TTC	100.00	4253	4224	-	ST plasmid pSMQ-316
N4-2_**24**	GCACTCAGACAGTTTTTTAACTACTTAGCT	AT**AAACG**GAA	100.00	3312	3341	+	ST plasmid pSMQ-316
N4-2_**20**	CTTATTAGTAGCTGTACCGTTAAGCATAGG	GC**AAAAG**TTA	100.00	5768	5739	-	ST plasmid pSMQ-316
N9-2_**25**	AGGTAACACGTAGAACCATTTACAATTACA	TT**AAAAA**TGG	96.67	4305	4334	+	ST plasmid pSMQ-316
N9-2_**26**	GAACACTGATAACAGAAAGAGCTAAAAATG	TG**GTAAA**ATA	93.33	4300	4271	-	ST plasmid pSt08
N9-2_**27**	TAAGATTTATATCGCTGCTTACTTTAGAAC	GT**AAAAA**AAT	100.00	6392	6363	-	ST plasmid pSt08

[a] % Identity; [b] ST, *Streptococcus thermophilus*

3.7 Multilocus Sequence Typing

Five housekeeping genes widely distributed on genome of *S. thermophilus* LMG 18311 were analyzed among 19 *S. thermophilus* isolates from Turkish yogurts selected according to CRISPR analysis. A phylogenetic tree based on MLST data resolved 4 main clusters (Figure 3.20) with a significant discrimination within the *S. thermophilus* isolates. Comparison of whole genomes of *S. thermophilus* strains LMG 18311 and CNRZ 1066 was resulted in 3000 single nucleotide differences (Hols et al., 2005). Previously, MLST analysis was performed within the salivarius group by sequencing seven housekeeping genes and resulted with no significant clustering among *S. thermophilus* strains (Delorme C., Bolotin A., Ehrlich S.D., Renault P., unpublished data, Hols et al., 2005, Delorme, 2008). However in their study, the loci for MLST analysis were selected to detect the genetic diversity within salivarius group, not specifically within *S. thermophilus*. In our study, the housekeeping genes were selected to reveal the genetic diversity within *S. thermophilus* and resulted significant discrimination. Hence, the five loci analyzed in this study might provide MLST as a typing method for *S. thermophilus* isolates.

Clustering according to MLST analysis was highly similar to grouping based on CRISPR1 analysis. Isolates from K1 yogurt (Erzincan) formed a separate group as in CRISPR analysis. However, these isolates were not discriminated each other using MLST. The isolates in subgroup-f11 (CRISPR) were clustered together in phylogenetic tree (MLST). M17-N2-2 which was the isolate form the group 6 by itself after CRISPR analysis was seem related to M17-N2-3 and M17-N8-2 according to MLST analysis (Figure 3.20).

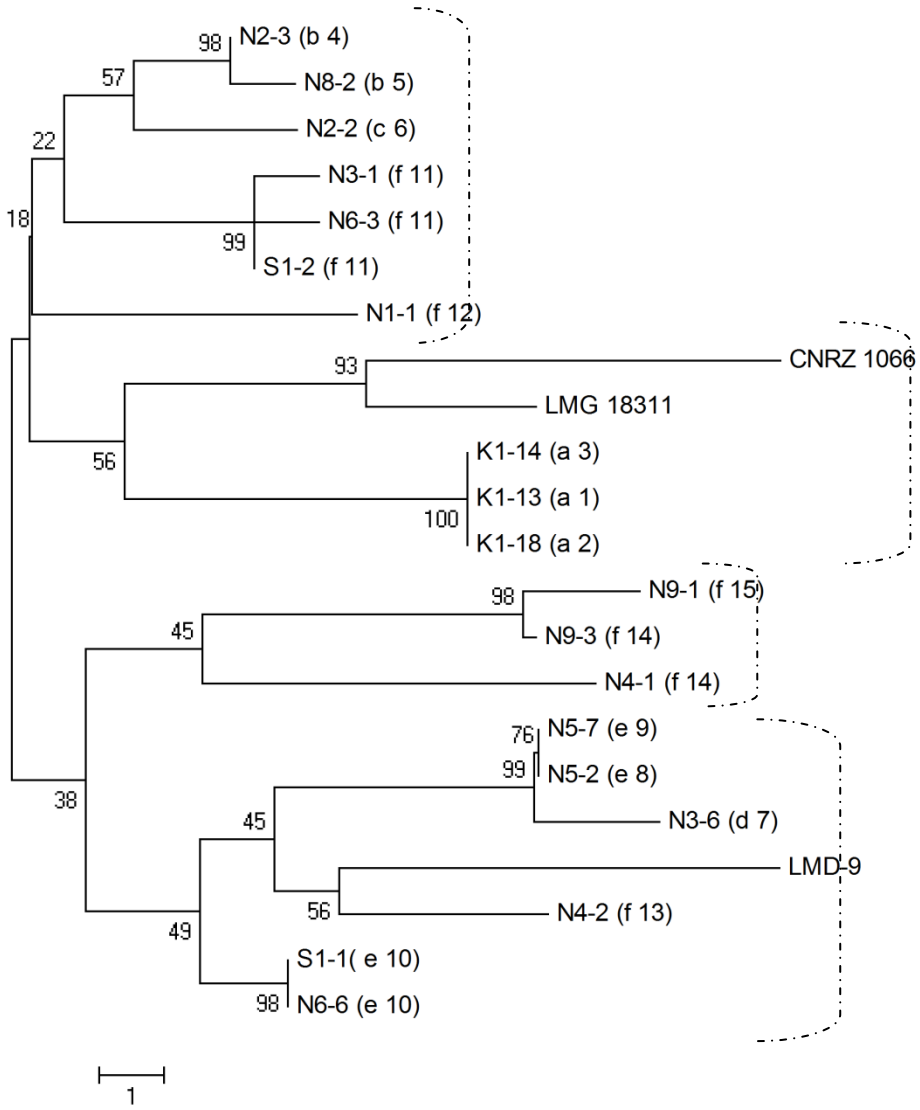


Figure 3.20 Phylogenetic tree of 19 *Streptococcus thermophilus* isolates and the *S. thermophilus* strains with known complete genome sequence (CNRZ 1066, LMD-9 and LMG 18311) based on alleles of 5 housekeeping genes.
The tree was constructed using the Neighbor-Joining method (Saitou et al., 1987). The cluster names according to CRISPR1 analysis are given in parenthesis.

According to CRISPR1 analysis M17-N2-3 and M17-N8-2 were in subgroup b which included isolates mostly from N2 yogurt and the isolate M17-N8-2. Pourcel et al. (2005), Barrangou et al. (2007) and Horvath et al. (2008) previously suggested CRISPR1 loci analysis as a typing method while CRISPR analysis as a

typing method was applied unsuccessfully for subtyping of seven *Lactobacillus casei* strains (Diancourt, 2007). Diancourt et al. (2007) were able to amplify a CRISPR locus from only 4 of these *L. casei* strains. However, analyzing the CRISPR sequences in these four strains did not discriminate the strains. In this study, CRISPR1 locus of *S. thermophilus* isolates was analyzed for typing and the clustering, after CRISPR1 analysis was confirmed by MLST. Therefore, analysis of CRISPR1 locus can be applied as a typing method for S. *thermophilus* isolates, besides providing information about phage resistance and phage exposure history of isolates

Allelic profiles of 19 Turkish isolates were also prepared according to analysis of five housekeeping genes (Table 3.12). All five genes showed identical alleles in multiple isolates. Additionally, three of sequence types i.e.ST1, ST2 and ST11 were observed in more than one isolate. The isolates in ST1 and ST2 were in different subgroups and hence discriminated via CRISPR1 analysis. However, M17-N6-6 (ST11) and M17-S1-1 (ST11) were also grouped in the same subgroup (e10) based on CRISPR1 analysis. Contrarily, there were isolates which were not discriminated by CRISPR analysis, but MLST analysis. M17-N3-1 (ST1), M17-N6-3 (ST10) and M17-S1-2 (ST15) isolates had the same CRISPR1 sequence and form the subgroup f11or M17-N9-3 (ST14) and M17-N4-1 (ST7) isolates had also the same CRISPR1 sequence and form the subgroup f14 (Figure 3.18 and Table 3.13). Nevertheless, these isolates grouped together at phylogenetic tree (Figure 3.20).

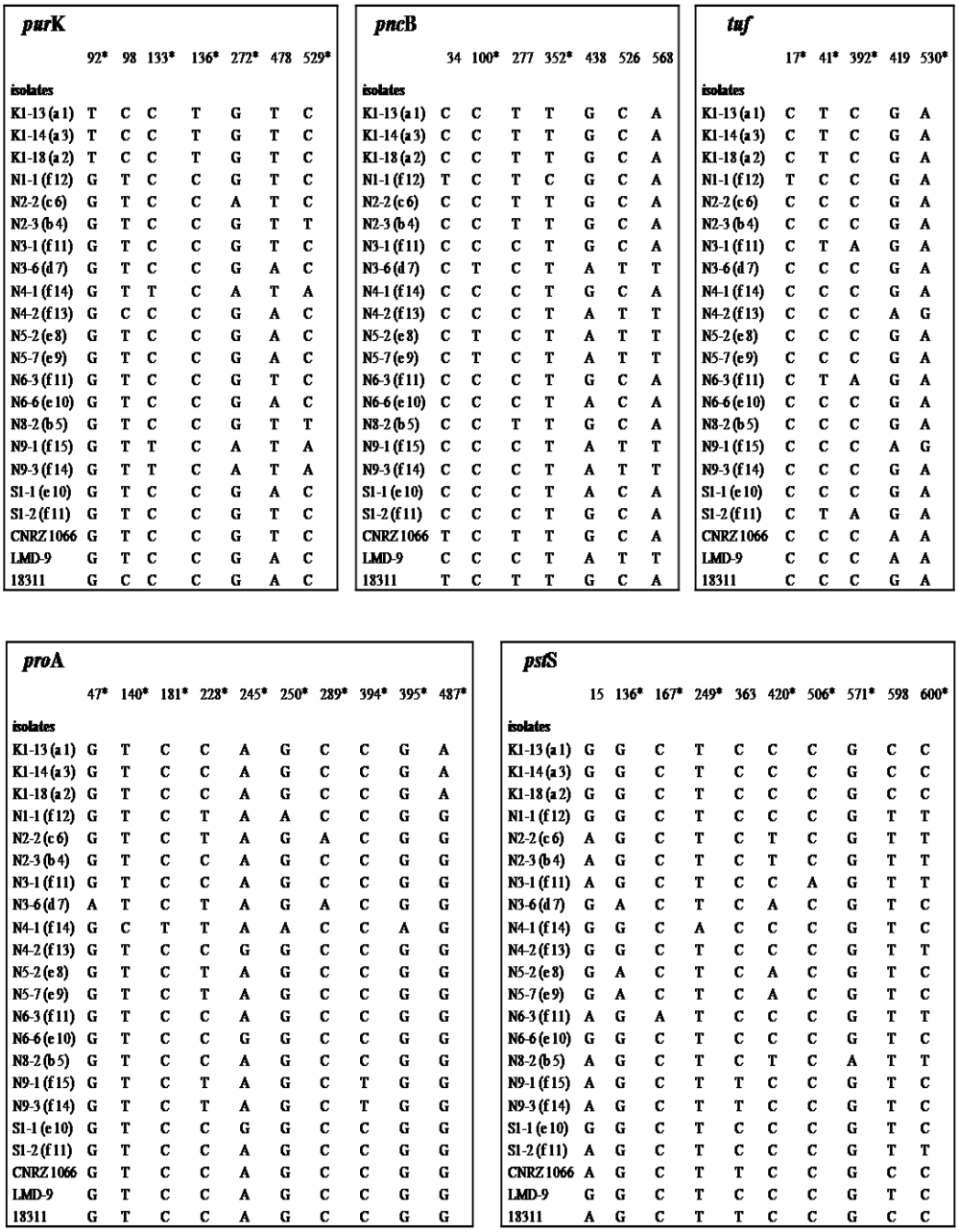
The polymorphic sites detected in the analyzed MLST genes within isolates from Turkish yogurts were compared to known complete genomes of *S. thermophilus* strains (Figure 3.21). Twenty-eight of 39 polymorphic sites detected among the MLST genes were not detected within the three completely genome sequenced *S. thermophilus* isolates (CNRZ 1066, LMD-9 and LMG 18311). This diversity observed between Turkish isolates and complete genome sequenced *S. thermophilus* strains may reveal that *S. thermophilus* isolates from Turkey might have potential to produce yogurts with distinct features than the ones currently available on the market.

Table 3.13 Allelic profiles of 19 isolates

Isolates[a]	ST[b]	Allele				
		*pur*K	*pnc*B	*tuf*	*pst*S	*pro*A
K1-13 (a 1)	1	1	1	1	1	1
K1-14 (a 3)	1	1	1	1	1	1
K1-18 (a 2)	1	1	1	1	1	1
N1-1 (f 12)	2	2	2	2	2	2
N2-2 (c 6)	3	3	1	3	3	3
N2-3 (b 4)	4	4	1	3	3	4
N3-1 (f 11)	5	2	3	4	4	4
N3-6 (d 7)	6	5	4	3	5	5
N4-1 (f 14)	7	6	3	3	6	6
N4-2 (f 13)	8	7	5	5	2	7
N5-2 (e 8)	9	5	4	3	5	8
N5-7 (e 9)	9	5	4	3	5	8
N6-3 (f 11)	10	2	3	4	7	4
N6-6 (e 10)	11	5	6	3	8	7
N8-2 (b 5)	12	4	1	3	9	4
N9-1 (f 15)	13	6	5	5	10	9
N9-3 (f 14)	14	6	5	3	10	9
S1-1 (e 10)	11	5	6	3	8	7
S1-2 (f 11)	15	2	3	4	11	4

[a]subgroup names according to CRISPR1 analysis were given in parenthesis

[b] ST: Sequence type



purK

isolates	92*	98	133*	136*	272*	478	529*
K1-13 (a 1)	T	C	C	T	G	T	C
K1-14 (a 3)	T	C	C	T	G	T	C
K1-18 (a 2)	T	C	C	T	G	T	C
N1-1 (f 12)	G	T	C	C	G	T	C
N2-2 (c 6)	G	T	C	C	A	T	C
N2-3 (b 4)	G	T	C	C	G	T	T
N3-1 (f 11)	G	T	C	C	G	T	C
N3-6 (d 7)	G	T	C	C	G	A	C
N4-1 (f 14)	G	T	T	C	A	T	A
N4-2 (f 13)	G	C	C	C	G	A	C
N5-2 (e 8)	G	T	C	C	G	A	C
N5-7 (e 9)	G	T	C	C	G	A	C
N6-3 (f 11)	G	T	C	C	G	T	C
N6-6 (e 10)	G	T	C	C	G	A	C
N8-2 (b 5)	G	T	C	C	G	T	T
N9-1 (f 15)	G	T	T	C	A	T	A
N9-3 (f 14)	G	T	T	C	A	T	A
S1-1 (e 10)	G	T	C	C	G	A	C
S1-2 (f 11)	G	T	C	C	G	T	C
CNRZ 1066	G	T	C	C	G	T	C
LMD-9	G	T	C	C	G	A	C
18311	G	C	C	C	G	A	C

pncB

isolates	34	100*	277	352*	438	526	568
K1-13 (a 1)	C	C	T	T	G	C	A
K1-14 (a 3)	C	C	T	T	G	C	A
K1-18 (a 2)	C	C	T	T	G	C	A
N1-1 (f 12)	T	C	T	C	G	C	A
N2-2 (c 6)	C	C	T	T	G	C	A
N2-3 (b 4)	C	C	T	T	G	C	A
N3-1 (f 11)	C	C	C	T	G	C	A
N3-6 (d 7)	C	T	C	T	A	T	T
N4-1 (f 14)	C	C	C	T	G	C	A
N4-2 (f 13)	C	C	C	T	A	T	T
N5-2 (e 8)	C	T	C	T	A	T	T
N5-7 (e 9)	C	T	C	T	A	T	T
N6-3 (f 11)	C	C	C	T	G	C	A
N6-6 (e 10)	C	C	C	T	A	C	A
N8-2 (b 5)	C	C	T	T	G	C	A
N9-1 (f 15)	C	C	C	T	A	T	T
N9-3 (f 14)	C	C	C	T	A	T	T
S1-1 (e 10)	C	C	C	T	A	C	A
S1-2 (f 11)	C	C	C	T	G	C	A
CNRZ 1066	T	C	T	T	G	C	A
LMD-9	C	C	C	T	A	T	T
18311	T	C	T	T	G	C	A

tuf

isolates	17*	41*	392*	419	530*
K1-13 (a 1)	C	T	C	G	A
K1-14 (a 3)	C	T	C	G	A
K1-18 (a 2)	C	T	C	G	A
N1-1 (f 12)	T	C	C	G	A
N2-2 (c 6)	C	C	C	G	A
N2-3 (b 4)	C	C	C	G	A
N3-1 (f 11)	C	T	A	G	A
N3-6 (d 7)	C	C	C	G	A
N4-1 (f 14)	C	C	C	G	A
N4-2 (f 13)	C	C	C	A	G
N5-2 (e 8)	C	C	C	G	A
N5-7 (e 9)	C	C	C	G	A
N6-3 (f 11)	C	T	A	G	A
N6-6 (e 10)	C	C	C	G	A
N8-2 (b 5)	C	C	C	G	A
N9-1 (f 15)	C	C	C	A	G
N9-3 (f 14)	C	C	C	G	A
S1-1 (e 10)	C	C	C	G	A
S1-2 (f 11)	C	T	A	G	A
CNRZ 1066	C	C	C	A	A
LMD-9	C	C	C	A	A
18311	C	C	C	G	A

proA

isolates	47*	140*	181*	228*	245*	250*	289*	394*	395*	487*
K1-13 (a 1)	G	T	C	C	A	G	C	C	G	A
K1-14 (a 3)	G	T	C	C	A	G	C	C	G	A
K1-18 (a 2)	G	T	C	C	A	G	C	C	G	A
N1-1 (f 12)	G	T	C	T	A	A	C	C	G	G
N2-2 (c 6)	G	T	C	T	A	G	A	C	G	G
N2-3 (b 4)	G	T	C	C	A	G	C	C	G	G
N3-1 (f 11)	G	T	C	C	A	G	C	C	G	G
N3-6 (d 7)	A	T	C	T	A	G	A	C	G	G
N4-1 (f 14)	G	C	T	T	A	A	C	C	A	G
N4-2 (f 13)	G	T	C	C	G	G	C	C	G	G
N5-2 (e 8)	G	T	C	T	A	G	C	C	G	G
N5-7 (e 9)	G	T	C	T	A	G	C	C	G	G
N6-3 (f 11)	G	T	C	C	A	G	C	C	G	G
N6-6 (e 10)	G	T	C	C	G	G	C	C	G	G
N8-2 (b 5)	G	T	C	C	A	G	C	C	G	G
N9-1 (f 15)	G	T	C	T	A	G	C	T	G	G
N9-3 (f 14)	G	T	C	T	A	G	C	T	G	G
S1-1 (e 10)	G	T	C	C	G	G	C	C	G	G
S1-2 (f 11)	G	T	C	C	A	G	C	C	G	G
CNRZ 1066	G	T	C	C	A	G	C	C	G	G
LMD-9	G	T	C	C	A	G	C	C	G	G
18311	G	T	C	C	A	G	C	C	G	G

pstS

isolates	15	136*	167*	249*	363	420*	506*	571*	598	600*
K1-13 (a 1)	G	G	C	T	C	C	C	G	C	C
K1-14 (a 3)	G	G	C	T	C	C	C	G	C	C
K1-18 (a 2)	G	G	C	T	C	C	C	G	C	C
N1-1 (f 12)	G	G	C	T	C	C	C	G	T	T
N2-2 (c 6)	A	G	C	T	C	T	C	G	T	T
N2-3 (b 4)	A	G	C	T	C	T	C	G	T	T
N3-1 (f 11)	A	G	C	T	C	C	A	G	T	T
N3-6 (d 7)	G	A	C	T	C	A	C	G	T	C
N4-1 (f 14)	G	G	C	A	C	C	C	G	T	C
N4-2 (f 13)	G	G	C	T	C	C	C	G	T	T
N5-2 (e 8)	G	A	C	T	C	A	C	G	T	C
N5-7 (e 9)	G	A	C	T	C	A	C	G	T	C
N6-3 (f 11)	A	G	A	T	C	C	C	G	T	T
N6-6 (e 10)	G	G	C	T	C	C	C	G	T	C
N8-2 (b 5)	A	G	C	T	C	T	C	A	T	T
N9-1 (f 15)	A	G	C	T	T	C	C	G	T	C
N9-3 (f 14)	A	G	C	T	T	C	C	G	T	C
S1-1 (e 10)	G	G	C	T	C	C	C	G	T	C
S1-2 (f 11)	A	G	C	T	C	C	C	G	T	T
CNRZ 1066	A	G	C	T	T	C	C	G	C	C
LMD-9	G	G	C	T	C	C	C	G	T	C
18311	A	G	C	T	T	C	C	G	C	C

Figure 3.21 Single nucleotide polymorphisms in five MLST genes among *Streptococcus thermophilus* isolates from Turkish yogurts and comparison of these sites with complete genome sequenced *S. thermophilus* strains.

Site numbering of nucleotides starting from the first nucleotide of aligned portion of each gene are displayed upper part of nucleotides. * The polymorphisms observed only within Turkish isolates, but not within complete genome sequenced *Streptococcus thermophilus.*

Sequence variations at the five MLST gene fragments were analyzed for 19 Turkish isolates and *S. thermophilus* LMD-9, CNRZ 1066 and LMG 18311 together (Table 3.13). The number of variable sites were range from 7 (*tuf*) to 15 (*pro*A). G+C contents of the five gene fragments were range from 40.6% and 46.1% and hence similar to G+C content of *S. thermophilus* genome (39%). Most of the polymorphism resulted in synonymous substitution, which is typical for housekeeping genes.

Table 3.13 Sequence variation at gene fragments[a]

Gene	G+C content (mol %)	Number of variable sites	Number of syn. sites	Number of nonsyn sites
proA	42,1	15	8	7
pstS	40,6	12	7	5
tuf	43,5	7	7	0
pncB	43,9	8	6	2
purK	46,1	11	8	3

[a] Analysis were performed using 19 Turkish isolates and *S. thermopilus* LMD-9, CNRZ 1066 and LMG18311.

CHAPTER 4

CONCLUSION

Yogurt is a very popular fermented dairy product in Turkey. Its popularity is also increasing all over the world. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are used together in yogurt production as starter culture and starter cultures are known as one of the most important factors determining the quality of the fermented foods and hence determining the acceptability of the products by consumers.

In this study, yogurt cultures were isolated from traditionally produced yogurts collected from different regions in Turkey. Isolates were identified using biochemical identification methods and the bacteria identified as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were studied for their technologically important properties as acidification ability, acetaldehyde production, phage resistance and proteolytic activity. Technological property studies showed that there is significant phenotypic diversity within traditional yogurt cultures. 25 *L. bulgaricus* and 22 *S. thermophilus* isolates were selected because of their good technological properties. Although the consumption of yogurt is very common and yogurt production is a great industry in Turkey, there is only one starter culture production company founded in Turkey. Results of this study demonstrate the high potential of traditionally produced yogurts for being a source of starter culture in Turkey. Additionally, this study could be a starting point of establishing a culture collection to provide starter cultures to yogurt producer and hence prevent these traditional cultures from disappearing.

In the second part of the study, traditional *S. thermophilus* isolates were analyzed for their genotypic diversity and evolutionary history. Analyses of CRISPR1 locus sequences and MLST within *S. thermophilus* isolates were studied. The results of

this part of the study reveal that significant genotypic diversity is also present within traditional *S. thermophilus* isolates. Additionally, sequencing CRISPR1 locus provides information about the potential phage resistance pattern of bacteria and spacers detected within traditional *S. thermophilus* isolates are mostly different from other known *S. thermophilus* CRISPR spacers, suggesting that traditional isolates would have different phage resistance profiles from that of strains with known CRISPR sequence.

Additionally, CRISPR analysis was previously suggested as a tool for typing (Pourcel et al., 2005; Barrangou et al., 2007; Horvath et al., 2008 and Horvath et al., 2010). In this study, CRISPR analysis (dynamic and rapid evolutionary changes) was compared with MLST (slowly generated evolutionary changes) in terms of their discrimination power for evolutionary diversity. Multilocus sequence typing results point out an evolutionary relationship within the strains compatible with that obtained from the CRISPR sequences. This reveals that CRISPR1 analysis can be used as a typing method for *S. thermophilus* isolates. Moreover, it could be preferred over MLST, since it provides additional information about potential phage resistance of the isolates. Furthermore, CRISPR study has reemphasized that *S. thermophilus* cultures could gain acquired resistance against phages if they are exposed to phages (Barrangouet al., 2007). This information could be crucial for the development of food grade phage resistant mutants.

CHAPTER 5

RECOMMENDATION

Lab scale yogurts can be produced using the isolates with good technological properties as starter cultures and best combinations of *L. bulg*aricus and *S. thermophilus* can be determined. Plant scale yogurt production can be also tested to see the problems faced in plants. As a subsequent stage, freeze dried starter cultures can be produced using selected cultures. Additionally, genotypic methods can be performed to confirm biochemical identification of rod isolates and reveal the genotypic diversity within these isolates. *S. thermophilus* isolates can be further studied for their genotypic diversity using pulsed-field gel electrophoresis (PFGE) which is a method known as its better discrimination power than MLST (Cai et al., 2007). The *S. thermophilus* isolates from K1 yogurt which could not be discriminated by CRISPR and MLST might be discriminated using PFGE. Additionally, CRISPR analysis can be compared with PFGE as a typing method and hence a better understanding of discrimination power of CRISPR analysis can be provided.

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APPENDIX A

A. GROWTH MEDIA

A.1 Media Used in Carbohydrate Fermentation Experiments

Table A. 1 Basal medium for carbohydrate fermentation experiments[a]

Compounds	Amonth
Peptone from casein	10 g
Yeast extract	4 g
Di-potassium hydrogen phosphate	2 g
Tween 80	1 ml
Di-ammonium hydrogen citrate	2 g
Sodium acetate trihydrate	8.3 g
Magnesium sulfate heptahydrate	0.41 g
Manganase sulfate monohydrate	0.038
Bromocresol purple	0.04 g
Distilled H_2O	1000 ml

[a] pH 6.5 ±0.2

A.2 Media Used in Phage Resistance Experiments

Table A. 2 Modified M17 broth (Krush et al., 1987; Acar, 2002)

Compounds	Amonth
Polypeptone	5 g
Phytone peptone	5 g
Yeast Extract	2.5 g
Beef Extract	5 g
Lactose	8 g
Ascorbic Acid	0.5 g
β-Disodium glycerophosphate	9.5 g
1 M $MgSO_4.7H_2O$	1 ml
1 M $CaCl_2$	1.2 ml
Distilled H_2O	1000 ml

Table A. 3 Modified M17 agar (Krusch et al., 1987)

Compounds	Amount
M17 broth	42.5 g
Agar	15 g
Lactose	8 g
Distilled H_2O	1000 ml

Table A. 4 Modified M17 soft agar

Compounds	Amonth
Polypeptone	2.4 g
Phytone peptone	2.4 g
Yeast Extract	3.68 g
Casein hydrolysate	2.4 g
Beef Extract	3.3 g
Ascorbic Acid	0.568 g
β-Disodium glycerophosphate	12.72 g
1 M $MgSO_4.7H_2O$	1.32 ml
Agar	6 g
Tryptone	6.68 g
Gelatine	0.84 g

Table A.4 Modified M17 soft agar (cont'd)

Compounds	Amonth
Dextrose	1.68 g
Lactose	1.68 g
Sucrose	1.68 g
NaCl	1.32 g
Na-acetate	0.5 g
Distilled H_2O	1000 ml

A.2.1 MRS Agar for phage resistance experiments

1% of $CaCl_2$ (1 M) was added to MRS agar (Merck) and sterilized at 118 ºC for 15 min (pH 5.7 ± 0.2).

A.2.2 MRS Soft Agar for phage resistance experiments

0.45 % agar (Lab M) was added to MRS broth (Merck) and dissolved homogenously by heating and dispensed 3 ml to tubes and then sterilized at 118 ºC for 15 min (pH 5.7 ± 0.2). Before using 100 µl $CaCl_2$ (1 M) was added to 3 ml soft MRS agar.

APPENDIX B

B. SOLUTIONS USED IN GENOMIC DNA ISOLATION

0.5M EDTA (pH=8.0) (Sambrook et al., 1989)

"Add 186.1 g disodium ethylenediaminetetraacetate.$2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by autoclaving."

1M Tris (pH=8.0) (Sambrook et al., 1989)

"Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl (for pH 8.0, add 42 ml HCl)." Allow the solution to cool to room temperature before the final pH adjustment. "Adjust the volume of the solution to 1l with H_2O. Dispense into aliquots and sterilize by autoclaving."

20% SDS (Sambrook et al., 1989)

Dissolve 200 g SDS in 900 ml of H_2O. "Heat to 68 ºC to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 l with H_2O."

TE (Tris-EDTA) (pH = 8.0) (Sambrook et al., 1989)

10 mM Tris.Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (Sambrook et al., 1989)

Mixture of equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1).

TES (Stahl et al., 1990)

50 mM NaCl, 100 mM Tris, 70 mM disodium EDTA, pH 8.0

RNase = Epicenter RNase A for master Pure Kit 5µl/ml cat number: MRNA092

APPENDIX C

C. CARBOHYDRATE FERMENTATION PROFILE OF THE ISOLATES

Table C. 1 Carbohydrate fermentation patterns of cocci isolates from M17[a].

Isolate numbers	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
M17-K1-1	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-K1-2	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	P2
M17-K1-7	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-K1-9	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-K1-11	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-12	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-13	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-14	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-15	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3

[a] Isolates whose isolate numbers were written in bold were not *S. thermophilus* according to 16S rDNA sequencing

Table C.1 Carbohydrate fermentation patterns of cocci isolates from M17**.** Isolates whose isolate numbers were written bold were not *S. thermophilus* according to 16S rRNA gene sequencing (cont'd)

Strain numbers	**Esculin**	**L(+)Arabinose**	**D(+)Cellobiose**	**D-Fructose**	**D(+)Galactose**	**D(+)Glucose**	**alpha-lactose**	**Maltose**	**D-Mannitol**	**D(+)Mannose**	**Melibiose**	**D(-)Ribose**	**Saccharose**	**Salicin**	**D(-)Sorbitol**	**Trehalose**	**D(+)Xylose**	**Pattern**
M17-K1-16	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-18	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	P3
M17-K1-19	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-K1-20	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-21	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-22	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-23	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-24	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-25	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	P4
M17-K1-26	-	-	-	-	+	+	+	vw	-	-	-	-	+	-	-	-	-	P5
M17-K1-27	-	-	-	-	+	+	+	vw	-	-	-	-	+	-	-	-	-	P5
M17-K1-28	-	-	-	-	w	+	+	vw	-	-	-	-	+	-	-	-	-	P6
M17-K1-29	-	-	-	-	w	+	+	vw	-	-	-	-	+	-	-	-	-	P6
M17-K1-30	-	-	-	-	w	+	+	vw	-	-	-	-	+	-	-	-	-	P6

Table C.1 Carbohydrate fermentation patterns of cocci isolates from M17**.** Isolates whose isolate numbers were written bold were not *S. thermophilus* according to 16S rRNA gene sequencing (cont'd)

Strain numbers	**Esculin**	**L(+)Arabinose**	**D(+)Cellobiose**	**D-Fructose**	**D(+)Galactose**	**D(+)Glucose**	**alpha-lactose**	**Maltose**	**D-Mannitol**	**D(+)Mannose**	**Melibiose**	**D(-)Ribose**	**Saccharose**	**Salicin**	**D(-)Sorbitol**	**Trehalose**	**D(+)Xylose**	**Pattern**
M17-K1-31	-	-	-	-	w	+	+	vw	-	-	-	-	+	-	-	-	-	P6
M17-N2-1	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1
M17-N2-2	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1
M17-N2-3	-	-	-	-	-	+	+	- -	-	-	-	-	+	-	-	-	-	P2
M17-N2-4	-	-	-	-	-	+	+	- -	-	-	-	-	+	-	-	-	-	P2
M17-N6-1	-	-	-	-	-	+	+	- -	-	-	-	-	+	-	-	-	-	P2
M17-N6-2	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1
M17-N6-3	-	-	-	-	-	+	+	- -	-	-	-	-	+	-	-	-	-	P2
M17-N6-4	-	-	-	-	w	w	+	- -	-	-	-	-	+	-	-	-	-	P7
M17-N6-5	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1
M17-N6-6	-	-	-	-	-	+	+	- -	-	-	-	-	+	-	-	-	-	P2
M17-N4-1	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1
M17-N4-2	-	-	-	w	w	+	+	- -	-	-	-	-	+	-	-	-	-	P16
M17-N4-3	-	-	-	-	w	+	+	- -	-	-	-	-	+	-	-	-	-	P1

Table C.1 Carbohydrate fermentation patterns of cocci isolates from M17**.** Isolates whose isolate numbers were written bold were not *S. thermophilus* according to 16S rRNA gene sequencing (cont'd)

Strain numbers	**Esculin**	**L(+)Arabinose**	**D(+)Cellobiose**	**D-Fructose**	**D(+)Galactose**	**D(+)Glucose**	**alpha-lactose**	**Maltose**	**D-Mannitol**	**D(+)Mannose**	**Melibiose**	**D(-)Ribose**	**Saccharose**	**Salicin**	**D(-)Sorbitol**	**Trehalose**	**D(+)Xylose**	**Pattern**
M17-N1-1	-	-	-	-	-	+	+	vw	-	+	-	-	+	-	-	-	-	P8
M17-N3-1	-	-	-	vw	+ +	+	+	-	-	vw	-	-	+	-	-	-	-	P9
M17-N3-4	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-N3-5	-	-	-	vw	w	+	+	-	-	-	-	-	+	-	-	-	-	P10
M17-N3-6	-	-	-	vw	w	+	+	-	-	-	-	-	+	-	-	-	-	P10
M17-N3-7	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N5-1	-	-	-	-	w	+	+	-	-	vw	-	-	+	-	-	-	-	P12
M17-N5-2	-	-	-	-	+	+	+	-	-	vw	-	-	+	-	-	-	-	P13
M17-N5-3	-	-	-	vw	+	+	+	-	-	vw	-	-	+	-	-	-	-	P9
M17-N5-4	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N5-5	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N5-6	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N5-7	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N7-1	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	P14

Table C.1 Carbohydrate fermentation patterns of cocci isolates from M17**.** Isolates whose isolate numbers were written bold were not *S. thermophilus* according to 16S rRNA gene sequencing (cont'd)

Strain numbers	**Esculin**	**L(+)Arabinose**	**D(+)Cellobiose**	**D-Fructose**	**D(+)Galactose**	**D(+)Glucose**	**alpha-lactose**	**Maltose**	**D-Mannitol**	**D(+)Mannose**	**Melibiose**	**D(-)Ribose**	**Saccharose**	**Salicin**	**D(-)Sorbitol**	**Trehalose**	**D(+)Xylose**	**Pattern**
M17-N7-4	-	-	-	w	-	+	+	+	-	+	-	-	+	-	-	-	-	P15
M17-N9-1	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-N9-2	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-N9-3	-	-	-	-	w	+	+	-	-	-	-	-	+	-	-	-	-	P1
M17-N9-4	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-S1-1	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	P2
M17-S1-2	-	-	-	vw	w	+	+	-	-	vw	-	-	+	-	-	-	-	P11
M17-S1-3	-	-	-	vw	+	+	+	-	-	vw	-	-	+	-	-	-	-	P9
M17-N8-2	-	-	-	vw	-	+	+	vw	-	vw	-	-	+	-	-	-	-	P17
LMG 18311	-	-	-	-	+	+	+	w	-	-	-	-	+	-	-	-	-	

+: reaction; -: no reaction; vw: very weak reaction; w: weak reaction

LMG 18311: *S. thermophilus* LMG 18311

Table C. 2 Carbohydrate fermentation patterns of rod isolates from MRS

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-K1-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-4	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-5	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-6	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-7	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-8	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-9	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-10	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-11	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-12	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-13	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-14	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-15	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	P2

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-K1-16	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	P2
MRS-K1-17	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-18	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-	P3
MRS-K1-19	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-20	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-22	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-23	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-24	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-25	-	-	-	w	-	+	+	-	-	w	-	-	-	-	-	-	-	P4
MRS-K1-26	-	-	-	+	-	w	+	-	-	w	-	-	-	-	-	-	-	P5
MRS-K1-27	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-29	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-30	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	P6
MRS-K1-31	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-32	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-K1-33	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-34	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-35	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-36	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-37	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-38	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-39	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-40	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-43	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-44	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-45	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K1-46	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M2-1	-	-	-	+	-	+	+	-	-	+	-	w	-	-	-	w	-	P7
MRS-M2-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	P8
MRS-M2-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	P8
MRS-M2-5	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	P8

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-M2-7	-	-	-	+	-	w	+	-	-	w	-	-	-	-	-	w	-	P9
MRS-M2-8	-	-	-	+	-	+	+	-	-	w	-	-	-	-	-	+	-	P10
MRS-M2-12	-	-	-	+	-	+	+	-	-	+	-	w	-	-	-	+	-	P11
MRS-M2-13	-	-	-	+	-	-	+	-	-	vw	-	-	-	-	-	+	-	P12
MRS-M2-14	-	-	-	+	-	w	+	-	-	w	-	-	-	-	-	w	-	P9
MRS-M2-15	-	-	-	+	-	w	+	-	-	+	-	vw	-	-	-	+	-	P13
MRS-M2-16	-	-	-	+	-	w	+	-	-	+	-	-	-	-	-	+	-	P14
MRS-M2-17	-	-	-	+	-	w	+	-	-	+	-	-	-	-	-	+	-	P14
MRS-M2-18	-	-	-	+	-	+	+	-	-	+	-	w	-	-	-	+	-	P11
MRS-M2-19	-	-	-	+	-	vw	+	-	-	+	-	-	-	-	-	+	-	P15
MRS-M2-20	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	P8
MRS-M2-21	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	-	P8
MRS-M2-23	-	-	-	+	-	w	+	-	-	+	-	-	-	-	-	+	-	P14
MRS-G3-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G3-5	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G3-7	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-G3-8	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G3-9	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G3-10	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-12	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-13	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-14	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-15	ND	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	-	
MRS-G1-16	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P16
MRS-G1-17	ND	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	
MRS-G1-18	-	-	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	P17
MRS-G1-19	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-20	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-21	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-22	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-G1-23	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-24	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-G1-25	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-4	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-5	ND	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	
MRS-M23-6	ND	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	
MRS-M23-7	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-M23-10	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P16
MRS-M23-13	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS Y1-1	ND	-	-	+	-	+	+	+	w	+	-	w	w	-	-	-	-	
MRS-Y1-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-Y1-4	ND	+	+	+	+	+	+	+	+	+	w	+	+	+	-	+	-	

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-Y1-5	ND	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	
MRS-Y1-6	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-Y1-7	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-Y1-8	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N2-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N2-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N2-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N2-4	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N2-5	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N4-1	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-	P3
MRS-N4-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N4-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N6-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N6-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N3-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N3-5	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1

Table C.2 Carbohydrate fermentation patterns of rod isolates from MRS (cont'd)

Strain numbers[a]	Esculin	L(+)Arabinose	D(+)Cellobiose	D-Fructose	D(+)Galactose	D(+)Glucose	alpha-lactose	Maltose	D-Mannitol	D(+)Mannose	Melibiose	D(-)Ribose	Saccharose	Salicin	D(-)Sorbitol	Trehalose	D(+)Xylose	Pattern
MRS-K2-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K2-2	-	-	-	+	-	+	+	+	-	+	-	w	+	-	-	+	-	P18
MRS-K2-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K2-4	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-K2-5	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	P6
MRS-S1-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-S1-2	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-S1-3	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N9-1	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	P1
MRS-N5-3	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	P19
DSM 20081	-	-	-	w	-	+	+	-	-	w	-	-	-	-	-	-	-	

DSM 20081: *L. bulgaricus* DSM 20081; ND: not determined

[a]Isolates written in bold were eliminated from collection because of having different carbohydrate profiles from *L. bulgaricus*

+: reaction; -: no reaction; vw: very weak reaction; w: weak reaction

APPENDIX D

D. OD_{600} DATA OF GROWTH

Table D. 1 OD_{600} values measured for *Streptococcus thermophilus* LMG 18311 in M17 (pH 6.8) incubated at 42ºC

	time (h)							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
OD600-1-1	0,001	0,001	0,001	0,001	0,008	0,041	0,244	1,140
OD600-1-2	0,001	0,001	0,001	0,002	0,004	0,042	0,238	1,044
OD600-2-1	0,002	0,002	0,001	0,001	0,004	0,040	0,253	1,020
OD600-2-2	0,001	0,001	0,000	0,001	0,003	0,037	0,242	1,000
OD600-3-1	0,001	0,002	0,001	0,001	0,003	0,037	0,253	1,012
OD600-3-2	0,001	0,001	0,001	0,001	0,005	0,039	0,241	1,018
OD600-1-1	-0,002	-0,002	-0,002	-0,002	0,004	0,064	0,438	1,080
OD600-1-2	-0,002	-0,001	-0,002	-0,001	0,005	0,064	0,428	1,100
OD600-2-1	-0,001	-0,001	0,000	0,000	0,007	0,069	0,446	1,115
OD600-2-2	-0,001	-0,001	-0,001	0,000	0,005	0,067	0,434	1,150
OD600-3-1	0,002	0,004	0,003	0,005	0,016	0,102	0,513	1,255
OD600-3-2	0,003	0,003	0,003	0,005	0,014	0,100	0,500	1,335
OD600 Ave	0,001	0,001	0,001	0,001	0,007	0,059	0,353	1,106
σ	0,002	0,002	0,002	0,002	0,004	0,024	0,115	0,103

Table D.1 OD_{600} values measured for *Streptococcus thermophilus* LMG 18311 in M17 (pH 6.8) incubated at 42 ºC (cont'd).

	time (h)					
	8	**9**	**10**	**11**	**12**	**24**
OD600-1-1	2,095	2,225	2,425	2,190	2,565	2,140
OD600-1-2	2,265	2,435	2,230	2,295	2,550	2,010
OD600-2-1	2,160	2,560	2,135	2,325	2,290	2,285
OD600-2-2	2,185	2,450	2,450	2,110	2,445	2,250
OD600-3-1	2,000	2,275	2,230	2,055	2,410	2,135
OD600-3-2	2,150	2,420	2,370	2,135	2,585	2,365
OD600-1-1	1,885	2,285	2,255	2,580	2,225	2,270
OD600-1-2	1,940	2,370	2,280	2,560	2,300	2,455
OD600-2-1	1,905	2,285	2,215	2,350	2,215	2,300
OD600-2-2	1,920	2,345	2,285	2,215	2,260	2,490
OD600-3-1	2,075	2,380	2,195	2,265	2,190	2,300
OD600-3-2	2,045	2,285	2,280	2,305	2,230	2,395
OD600 Ave	2,052	2,360	2,279	2,282	2,355	2,283
σ	0,124	0,096	0,093	0,162	0,148	0,138

OD_{600} Ave: Average values for OD_{600},σ : standart deviation

Table D. 2 OD values measured for *Lactobacillus bulgaricus* DSM 20081^{T} in MRS (pH 5.7) incubated at 42 ºC.

	time (h)								
	0	1	2	3	4	5	6	7	8
OD600-1-1	0,001	0,002	0,001	0,002	0,008	0,029	0,079	0,214	0,516
OD600-1-2	0,002	0,003	0,001	0,002	0,008	0,029	0,079	0,211	0,531
OD600-2-1	0,001	0,002	0,001	0,002	0,007	0,026	0,075	0,203	0,501
OD600-2-2	0,001	0,002	0,003	0,002	0,006	0,027	0,077	0,2	0,504
OD600-3-1	0,002	0,001	0,003	0,002	0,007	0,028	0,079	0,21	0,534
OD600-3-2	0,002	0,002	0,001	0,001	0,008	0,028	0,08	0,216	0,559
OD600-1-1	0,008	0,008	0,01	0,012	0,019	0,052	0,126	0,237	0,642
OD600-1-2	0,009	0,008	0,011	0,013	0,019	0,054	0,121	0,247	0,666
OD600-2-1	0,009	0,009	0,01	0,016	0,021	0,05	0,113	0,222	0,656
OD600-2-2	0,009	0,009	0,012	0,014	0,019	0,078	0,183	0,226	0,644
OD600-3-1	0,01	0,012	0,013	0,016	0,022	0,055	0,12	0,234	0,622
OD600-3-2	0,01	0,011	0,013	0,016	0,021	0,057	0,156	0,244	0,676
OD600Ave	0,005	0,006	0,007	0,008	0,014	0,043	0,107	0,222	0,588
σ	0,004	0,004	0,005	0,007	0,007	0,017	0,036	0,016	0,069

Table D.2 OD values measured for *Lactobacillus bulgaricus* DSM 20081^T in MRS (pH 5.7) incubated at 42 ºC (cont'd).

	time (h)							
	9	10	11	12	13	14	15	24
OD600-1-1	1,16	1,636	2,44	2,365	2,66	2,57	2,48	2,932
OD600-1-2	1,114	1,748	2,28	2,24	2,52	2,48	2,668	2,9
OD600-2-1	1,092	1,788	2,215	2,25	2,91	2,855	2,78	2,836
OD600-2-2	1,088	1,744	2,1	2,355	2,38	2,46	2,644	2,748
OD600-3-1	1,144	1,86	2,08	2,29	2,635	2,48	2,624	2,752
OD600-3-2	1,138	1,968	2,26	2,31	2,61	2,64	2,64	2,908
OD600-1-1	1,132	1,958	2,076	2,548	2,196	2,572	2,892	2,828
OD600-1-2	1,113	1,826	2,236	2,564	2,604	2,648	2,544	2,688
OD600-2-1	1,12	1,724	2,12	2,404	2,216	2,724	2,608	2,716
OD600-2-2	1,052	1,89	2,204	2,604	2,316	2,572	2,576	2,82
OD600-3-1	1,11	1,71	2,06	2,752	2,324	2,696	2,508	2,844
OD600-3-2	1,124	1,778	2,244	2,54	2,616	2,656	2,644	2,86
OD600Ave	1,116	1,803	2,193	2,435	2,499	2,613	2,634	2,819
σ	0,029	0,101	0,111	0,163	0,213	0,115	0,113	0,078

OD_{600}Ave: Average values for OD_{600}, σ : standart deviation

APPENDIX E

E. ACIDIFICATION ACTIVITIES OF THE ISOLATES

Table E. 1 Acidification activities of putative *S. thermophilus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$

isolate number		time (h)					
		0	2	4	6	8	10
M17-Dan-TA 040 -1	pH	6.41	6.19	5.2	5	4.89	4.79
	ΔpH	0	0.22	1.21	1.41	1.52	1.62
M17-Dan-TA 040 -3	pH	6.44	6.13	5.17	4.92	4.77	4.66
	ΔpH	0	0.31	1.27	1.52	1.67	1.78
M17-Dan-Yo-Mix-410-1	pH	6.41	5.67	4.83	4.59	4.45	4.37
	ΔpH	0	0.74	1.58	1.82	1.96	2.04
M17-Dan-Yo-Mix-410-2	pH	6.35	5.62	4.87	4.61	4.49	4.4
	ΔpH	0	0.73	1.48	1.74	1.86	1.95
M17-Dan-Yo-Mix-410-3	pH	6.41	5.62	4.87	4.6	4.47	4.38
	ΔpH	0	0.79	1.54	1.81	1.94	2.03
M17-K1-1	pH	6.41	5.8	4.99	4.77	4.68	4.58
	ΔpH	0	0.61	1.42	1.64	1.73	1.83
M17-K1-2	pH	6.43	6.26	5.21	4.9	4.76	4.67
	ΔpH	0	0.17	1.22	1.53	1.67	1.76
M17-K1-7	pH	6.43	5.96	5.1	4.83	4.73	4.6
	ΔpH	0	0.47	1.33	1.6	1.7	1.83
M17-K1-9	pH	6.45	6.11	5.13	4.83	4.69	4.54
	ΔpH	0	0.34	1.32	1.62	1.76	1.91
M17-K1-11	pH	6.45	6.42	5.41	4.96	4.72	4.56
	ΔpH	0	0.03	1.04	1.49	1.73	1.89
M17-K1-12	pH	6.48	6.09	5.15	4.83	4.67	4.49
	ΔpH	0	0.39	1.33	1.65	1.81	1.99
M17-K1-13	pH	6.44	6.02	5.07	4.77	4.62	4.49
	ΔpH	0	0.42	1.37	1.67	1.82	1.95
M17-K1-14	pH	6.44	6.19	5.14	4.86	4.72	4.61
	ΔpH	0	0.25	1.3	1.58	1.72	1.83

Table E.1 Acidification activities of putative *S. thermophilus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		time (h)					
		0	2	4	6	8	10
M17-K1-15	pH	6.41	5.91	5.03	4.78	4.65	4.51
	ΔpH	0	0.5	1.38	1.63	1.76	1.9
M17-K1-16	pH	6.43	6	5.12	4.81	4.65	4.5
	ΔpH	0	0.43	1.31	1.62	1.78	1.93
M17-K1-18	pH	6.42	5.93	5.09	4.78	4.63	4.5
	ΔpH	0	0.49	1.33	1.64	1.79	1.92
M17-K1-19	pH	6.41	6.05	5.11	4.79	4.64	4.53
	ΔpH	0	0.36	1.3	1.62	1.77	1.88
M17-K1-20	pH	6.44	6.39	5.51	4.93	4.73	4.64
	ΔpH	0	0.05	0.93	1.51	1.71	1.8
M17-K1-21	pH	6.47	6.43	5.37	4.95	4.77	4.61
	ΔpH	0	0.04	1.1	1.52	1.7	1.86
M17-K1-22	pH	6.42	6.41	5.86	5.03	4.78	4.67
	ΔpH	0	0.01	0.56	1.39	1.64	1.75
M17-K1-23	pH	6.43	6.08	5.08	4.74	4.6	4.48
	ΔpH	0	0.35	1.35	1.69	1.83	1.95
M17-K1-24	pH	6.41	5.99	5.09	4.78	4.66	4.55
	ΔpH	0	0.42	1.32	1.63	1.75	1.86
M17-K1-26	pH	6.45	6.44	6.11	5.13	4.85	4.67
	ΔpH	0	0.01	0.34	1.32	1.6	1.78
M17-K1-27	pH	6.42	5.94	5.04	4.77	4.63	4.51
	ΔpH	0	0.48	1.38	1.65	1.79	1.91
M17-K1-28	pH	6.42	5.79	5.02	4.8	4.69	4.58
	ΔpH	0	0.63	1.4	1.62	1.73	1.84
M17-K1-29	pH	6.42	5.93	4.99	4.69	4.56	4.46
	ΔpH	0	0.49	1.43	1.73	1.86	1.96
M17-K1-30	pH	6.41	6.03	5.11	4.83	4.68	4.54
	ΔpH	0	0.38	1.3	1.58	1.73	1.87
M17-K1-31	pH	6.45	6.2	5.18	4.85	4.66	4.46
	ΔpH	0	0.25	1.27	1.6	1.79	1.99
M17-N2-1	pH	6.45	6.02	5.09	4.82	4.63	4.45
	ΔpH	0	0.43	1.36	1.63	1.82	2
M17-N2-2	pH	6.46	6.29	5.28	4.97	4.74	4.55
	ΔpH	0	0.17	1.18	1.49	1.72	1.91

Table E.1 Acidification activities of putative *S. thermophilus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		**time (h)**					
		0	2	4	6	8	10
M17-N2-3	pH	6.4	5.98	5.01	4.74	4.58	4.44
	ΔpH	0	0.42	1.39	1.66	1.82	1.96
M17-N2-4	pH	6.44	6.05	5.08	4.79	4.62	4.48
	ΔpH	0	0.39	1.36	1.65	1.82	1.96
M17-N6-1	pH	6.44	6.1	5.08	4.8	4.65	4.47
	ΔpH	0	0.34	1.36	1.64	1.79	1.97
M17-N6-2	pH	6.42	6.06	5.14	4.85	4.68	4.57
	ΔpH	0	0.36	1.28	1.57	1.74	1.85
M17-N6-3	pH	6.42	5.99	5.08	4.79	4.62	4.51
	ΔpH	0	0.43	1.34	1.63	1.8	1.91
M17-N6-5	pH	6.41	6.18	5.21	4.87	4.68	4.55
	ΔpH	0	0.23	1.2	1.54	1.73	1.86
M17-N6-6	pH	6.41	5.96	5.02	4.76	4.6	4.46
	ΔpH	0	0.45	1.39	1.65	1.81	1.95
M17-N4-1	pH	6.41	6.15	5.14	4.85	4.68	4.55
	ΔpH	0	0.26	1.27	1.56	1.73	1.86
M17-N4-3	pH	6.46	6.01	5.18	4.85	4.66	4.54
	ΔpH	0	0.45	1.28	1.61	1.8	1.92
M17-N3-1	pH	6.45	6.01	5.15	4.88	4.72	4.58
	ΔpH	0	0.44	1.3	1.57	1.73	1.87
M17-N3-5	pH	6.43	6.15	5.28	4.96	4.76	4.62
	ΔpH	0	0.28	1.15	1.47	1.67	1.81
M17-N3-6	pH	6.36	6.26	5.4	4.97	4.73	4.59
	ΔpH	0	0.1	0.96	1.39	1.63	1.77
M17-N3-7	pH	6.43	5.93	5.14	4.83	4.66	4.52
	ΔpH	0	0.5	1.29	1.6	1.77	1.91
M17-N5-1	pH	6.47	5.99	5.24	5	4.87	4.72
	ΔpH	0	0.48	1.23	1.47	1.6	1.75
M17-N5-2	pH	6.38	6.04	5.16	4.87	4.72	4.59
	ΔpH	0	0.34	1.22	1.51	1.66	1.79
M17-N5-3	pH	6.46	6.03	5.27	4.94	4.73	4.58
	ΔpH	0	0.43	1.19	1.52	1.73	1.88

Table E.1 Acidification activities of putative *S. thermophilus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		**time (h)**					
		0	2	4	6	8	10
M17-N5-5	pH	6.45	6.24	5.17	4.83	4.67	4.52
	ΔpH	0	0.21	1.28	1.62	1.78	1.93
M17-N5-6	pH	6.46	6.05	5.24	4.92	4.74	4.59
	ΔpH	0	0.41	1.22	1.54	1.72	1.87
M17-N5-7	pH	6.44	6.16	5.02	4.7	4.55	4.42
	ΔpH	0	0.28	1.42	1.74	1.89	2.02
M17-N9-1	pH	6.45	5.97	5.16	4.87	4.75	4.62
	ΔpH	0	0.48	1.29	1.58	1.7	1.83
M17-N9-3	pH	6.34	6.25	5.08	4.67	4.51	4.36
	ΔpH	0	0.09	1.26	1.67	1.83	1.98
M17-N9-4	pH	6.45	5.88	5.24	4.99	4.83	4.7
	ΔpH	0	0.57	1.21	1.46	1.62	1.75
M17-S1-1	pH	6.35	6.19	5.2	4.87	4.69	4.55
	ΔpH	0	0.16	1.15	1.48	1.66	1.8
M17-S1-2	pH	6.31	6.15	5.21	4.89	4.72	4.58
	ΔpH	0	0.16	1.1	1.42	1.59	1.73
M17-S1-3	pH	6.34	5.93	5.04	4.81	4.64	4.51
	ΔpH	0	0.41	1.3	1.53	1.7	1.83
M17-N8-2	pH	6.44	5.99	5.06	4.79	4.62	4.47
	ΔpH	0	0.45	1.38	1.65	1.82	1.97

Table E. 2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$

isolate number		**time (h)**					
		0	2	4	6	8	10
MRS-Dan-Yo-Mix-410-1	pH	6.47	6.33	5.64	4.82	4.35	4.1
	ΔpH	0	0.14	0.83	1.65	2.12	2.37
MRS-Dan-Yo-Mix-410-2	pH	6.41	6.35	5.98	5.26	4.66	4.29
	ΔpH	0	0.06	0.43	1.15	1.75	2.12
MRS-Visby-1	pH	6.47	6.34	5.42	4.59	4.31	4.14
	ΔpH	0	0.13	1.05	1.88	2.16	2.33
MRS-Visby-2	pH	6.46	6.35	5.49	4.68	4.34	4.16
	ΔpH	0	0.11	0.97	1.78	2.12	2.3
MRS-Visby-3	pH	6.46	6.29	5.49	4.75	4.35	4.15
	ΔpH	0	0.17	0.97	1.71	2.11	2.31
MRS-K1-1	pH	6.37	6.23	5.99	5.77	5.56	5.4
	ΔpH	0	0.14	0.38	0.6	0.81	0.97
MRS-K1-2	pH	6.38	6.2	5.3	4.79	4.59	4.43
	ΔpH	0	0.18	1.08	1.59	1.79	1.95
MRS-K1-3	pH	6.38	6.25	5.66	5.51	5.38	5.3
	ΔpH	0	0.13	0.72	0.87	1	1.08
MRS-K1-4	pH	6.36	6.32	5.79	5.55	5.38	5.25
	ΔpH	0	0.04	0.57	0.81	0.98	1.11
MRS-K1-5	pH	6.37	6.26	5.68	5.34	5.20	5.14
	ΔpH	0	0.11	0.69	1.03	1.17	1.23
MRS-K1-6	pH	6.44	6.23	5.37	5.06	4.9	4.73
	ΔpH	0	0.21	1.07	1.38	1.54	1.71
MRS-K1-7	pH	6.36	6.23	5.61	5.24	5.08	4.94
	ΔpH	0	0.13	0.75	1.12	1.28	1.42
MRS-K1-8	pH	6.45	6.35	5.88	5.42	5.27	5.17
	ΔpH	0	0.1	0.57	1.03	1.18	1.28
MRS-K1-9	pH	6.46	6.32	5.73	5.31	5.14	4.96
	ΔpH	0	0.14	0.73	1.15	1.32	1.5
MRS-K1-10	pH	6.46	6.31	5.74	5.08	4.68	4.39
	ΔpH	0	0.15	0.72	1.38	1.78	2.07
MRS-K1-11	pH	6.5	6.37	5.7	5.29	5.1	4.99
	ΔpH	0	0.13	0.8	1.21	1.4	1.51
MRS-K1-12	pH	6.51	6.36	5.66	5.42	5.27	5.18
	ΔpH	0	0.15	0.85	1.09	1.24	1.33

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		time (h)					
		0	2	4	6	8	10
MRS-K1-13	pH	6.56	6.41	5.7	5.45	5.26	5.11
	ΔpH	0	0.15	0.86	1.11	1.3	1.45
MRS-K1-14	pH	6.55	6.42	5.86	5.27	4.93	4.7
	ΔpH	0	0.13	0.69	1.28	1.62	1.85
MRS-K1-15	pH	6.46	6.3	5.64	5.41	5.25	5.13
	ΔpH	0	0.16	0.82	1.05	1.21	1.33
MRS-K1-16	pH	6.45	6.22	5.35	4.65	4.3	4.14
	ΔpH	0	0.23	1.1	1.8	2.15	2.31
MRS-K1-17	pH	6.46	6.29	5.45	5.18	5.04	4.89
	ΔpH	0	0.17	1.01	1.28	1.42	1.57
MRS-K1-18	pH	6.44	6.36	5.86	5.64	5.47	5.37
	ΔpH	0	0.08	0.58	0.8	0.97	1.07
MRS-K1-19	pH	6.47	6.31	5.59	5.27	5.16	5.07
	ΔpH	0	0.16	0.88	1.2	1.31	1.4
MRS-K1-20	pH	6.54	6.32	5.9	5.68	5.53	5.36
	ΔpH	0	0.22	0.64	0.86	1.01	1.18
MRS-K1-22	pH	6.49	6.31	5.51	5.02	4.87	4.7
	ΔpH	0	0.18	0.98	1.47	1.62	1.79
MRS-K1-23	pH	6.45	6.35	5.26	4.73	4.45	4.27
	ΔpH	0	0.1	1.19	1.72	2	2.18
MRS-K1-24	pH	6.44	6.34	5.84	5.31	5.02	4.77
	ΔpH	0	0.1	0.6	1.13	1.42	1.67
MRS-K1-25	pH	6.42	6.39	6.06	5.72	5.48	5.11
	ΔpH	0	0.03	0.36	0.7	0.94	1.31
MRS-K1-26	pH	6.5	6.31	6.01	5.4	4.79	4.5
	ΔpH	0	0.19	0.49	1.1	1.71	2
MRS-K1-27	pH	6.49	6.32	5.83	5.22	4.73	4.38
	ΔpH	0	0.17	0.66	1.27	1.76	2.11
MRS-K1-29	pH	6.44	6.24	5.61	4.92	4.59	4.38
	ΔpH	0	0.2	0.83	1.52	1.85	2.06
MRS-K1-30	pH	6.49	6.34	5.79	5.07	4.68	4.48
	ΔpH	0	0.15	0.7	1.42	1.81	2.01
MRS-K1-31	pH	6.48	6.38	5.85	5.26	4.88	4.7
	ΔpH	0	0.1	0.63	1.22	1.6	1.78

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		time (h)					
		0	2	4	6	8	10
MRS-K1-32	pH	6.47	6.27	5.59	5.06	4.82	4.68
	ΔpH	0	0.2	0.88	1.41	1.65	1.79
MRS-K1-33	pH	6.47	6.32	5.59	4.82	4.42	4.28
	ΔpH	0	0.15	0.88	1.65	2.05	2.19
MRS-K1-34	pH	6.47	6.33	5.57	5.08	4.78	4.57
	ΔpH	0	0.14	0.9	1.39	1.69	1.9
MRS-K1-35	pH	6.5	6.42	6.17	5.77	5.52	5.4
	ΔpH	0	0.08	0.33	0.73	0.98	1.1
MRS-K1-36	pH	6.49	6.28	5.85	5.26	4.85	4.59
	ΔpH	0	0.21	0.64	1.23	1.64	1.9
MRS-K1-37	pH	6.48	6.33	5.73	5.3	5.11	4.99
	ΔpH	0	0.15	0.75	1.18	1.37	1.49
MRS-K1-38	pH	6.5	6.2	5.32	4.97	4.69	4.45
	ΔpH	0	0.3	1.18	1.53	1.81	2.05
MRS-K1-39	pH	6.5	6.17	5.34	5.04	4.83	4.7
	ΔpH	0	0.33	1.16	1.46	1.67	1.8
MRS-K1-40	pH	6.41	6.23	5.46	5.03	4.81	4.67
	ΔpH	0	0.18	0.95	1.38	1.6	1.74
MRS-K1-43	pH	6.5	6.4	5.62	4.69	4.22	4.05
	ΔpH	0	0.1	0.88	1.81	2.28	2.45
MRS-K1-44	pH	6.5	6.15	4.92	4.51	4.33	4.21
	ΔpH	0	0.35	1.58	1.99	2.17	2.29
MRS-K1-45	pH	6.5	6.25	5.59	5.36	5.22	5.1
	ΔpH	0	0.25	0.91	1.14	1.28	1.4
MRS-K1-46	pH	6.25	6.02	5.68	5.52	5.41	5.36
	ΔpH	0	0.23	0.57	0.73	0.84	0.89
MRS-G3-3	pH	6.26	6.01	4.87	4.32	4.11	4.04
	ΔpH	0	0.25	1.39	1.94	2.15	2.22
MRS-G3-5	pH	6.25	6.07	5.01	4.39	4.17	4
	ΔpH	0	0.18	1.24	1.86	2.08	2.25
MRS-G3-7	pH	6.31	6.17	5.44	4.74	4.34	4.13
	ΔpH	0	0.14	0.87	1.57	1.97	2.18
MRS-G3-8	pH	6.32	6.2	5.59	5.04	4.71	4.54
	ΔpH	0	0.12	0.73	1.28	1.61	1.78

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		**time (h)**					
		0	2	4	6	8	10
MRS-G3-9	pH	6.3	6.13	5.29	4.65	4.36	4.24
	ΔpH	0	0.19	1.03	1.67	1.96	2.08
MRS-G3-10	pH	6.32	6.15	5.36	4.64	4.3	4.12
	ΔpH	0	0.17	0.96	1.68	2.02	2.2
MRS-M2-1	pH	6.34	6.2	5.65	5.1	4.71	4.46
	ΔpH	0	0.12	0.67	1.22	1.61	1.86
MRS-M2-2	pH	6.32	6.25	5.77	5.12	4.71	4.42
	ΔpH	0	0.07	0.55	1.2	1.61	1.9
MRS-M2-3	pH	6.32	6.15	5.62	4.98	4.67	4.43
	ΔpH	0	0.17	0.7	1.34	1.65	1.89
MRS-M2-5	pH	6.36	6.2	5.63	5.3	5.19	5.1
	ΔpH	0	0.16	0.73	1.06	1.17	1.26
MRS-M2-7	pH	6.39	6.32	5.85	5.03	4.51	4.23
	ΔpH	0	0.07	0.54	1.36	1.88	2.16
MRS-M2-8	pH	6.38	6.28	5.73	4.96	4.52	4.26
	ΔpH	0	0.1	0.65	1.42	1.86	2.12
MRS-M2-12	pH	6.41	6.33	5.78	4.97	4.44	4.17
	ΔpH	0	0.08	0.63	1.44	1.97	2.24
MRS-M2-13	pH	6.42	6.15	5.22	4.54	4.3	4.14
	ΔpH	0	0.27	1.2	1.88	2.12	2.28
MRS-M2-14	pH	6.38	6.15	5.39	4.63	4.32	4.1
	ΔpH	0	0.23	0.99	1.75	2.06	2.28
MRS-M2-16	pH	6.36	6.13	5.36	4.57	4.21	4.05
	ΔpH	0	0.23	1	1.79	2.15	2.31
MRS-M2-17	pH	6.37	6.25	5.65	4.9	4.54	4.35
	ΔpH	0	0.12	0.72	1.47	1.83	2.02
MRS-M2-18	pH	6.37	6.26	5.85	5.31	4.93	4.64
	ΔpH	0	0.11	0.52	1.06	1.44	1.73
MRS-M2-19	pH	6.38	6.27	5.74	5.17	4.8	4.58
	ΔpH	0	0.11	0.64	1.21	1.58	1.8
MRS-M2-20	pH	6.42	6.28	5.59	4.83	4.37	4.16
	ΔpH	0	0.14	0.83	1.59	2.05	2.26
MRS-M2-21	pH	6.39	6.28	5.74	4.96	4.52	4.27
	ΔpH	0	0.11	0.65	1.43	1.87	2.12

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		**time (h)**					
		0	2	4	6	8	10
MRS-M2-23	pH	6.42	6.34	5.99	5.28	4.76	4.39
	ΔpH	0	0.08	0.43	1.14	1.66	2.03
MRS-G1-3	pH	6.42	6.33	5.94	5.32	4.88	4.69
	ΔpH	0	0.09	0.48	1.1	1.54	1.73
MRS-G1-12	pH	6.4	6.27	5.46	4.77	4.48	4.31
	ΔpH	0	0.13	0.94	1.63	1.92	2.09
MRS-G1-13	pH	6.4	6.34	6.19	6.06	6.03	5.99
	ΔpH	0	0.06	0.21	0.34	0.37	0.41
MRS-G1-14	pH	6.38	6.27	5.74	5.09	4.74	4.46
	ΔpH	0	0.11	0.64	1.29	1.64	1.92
MRS-G1-16	pH	6.41	6.18	5.61	4.82	4.37	4.16
	ΔpH	0	0.23	0.8	1.59	2.04	2.25
MRS-G1-18	pH	6.41	6.29	5.48	4.73	4.4	4.22
	ΔpH	0	0.12	0.93	1.68	2.01	2.19
MRS-G1-19	pH	6.35	6.22	5.54	4.78	4.49	4.27
	ΔpH	0	0.13	0.81	1.57	1.86	2.08
MRS-G1-20	pH	6.43	6.32	5.68	4.9	4.48	4.3
	ΔpH	0	0.11	0.75	1.53	1.95	2.13
MRS-G1-21	pH	6.41	6.2	5.4	4.86	4.63	4.47
	ΔpH	0	0.21	1.01	1.55	1.78	1.94
MRS-G1-22	pH	6.42	6.21	5.32	4.91	4.78	4.66
	ΔpH	0	0.21	1.1	1.51	1.64	1.76
MRS-G1-23	pH	6.43	6.24	5.38	4.81	4.46	4.27
	ΔpH	0	0.19	1.05	1.62	1.97	2.16
MRS-G1-24	pH	6.43	6.23	5.36	4.82	4.67	4.5
	ΔpH	0	0.2	1.07	1.61	1.76	1.93
MRS-G1-25	pH	6.42	6.13	5.2	4.74	4.47	4.28
	ΔpH	0	0.29	1.22	1.68	1.95	2.14
MRS-M23-1	pH	6.41	6.31	5.62	4.84	4.37	4.17
	ΔpH	0	0.1	0.79	1.57	2.04	2.24
MRS-M23-2	pH	6.42	6.32	5.64	4.81	4.37	4.19
	ΔpH	0	0.1	0.78	1.61	2.05	2.23
MRS-M23-3	pH	6.42	6.29	5.57	4.83	4.38	4.17
	ΔpH	0	0.13	0.85	1.59	2.04	2.25

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		time (h)					
		0	2	4	6	8	10
MRS-M23-4	pH	6.44	6.34	5.82	4.97	4.52	4.3
	ΔpH	0	0.1	0.62	1.47	1.92	2.14
MRS-M23-7	pH	6.43	6.38	6.29	6.14	6.16	6.16
	ΔpH	0	0.05	0.14	0.29	0.27	0.27
MRS-M23-10	pH	6.44	6.21	5.87	5.66	5.57	5.46
	ΔpH	0	0.23	0.57	0.78	0.87	0.98
MRS-M23-13	pH	6.43	5.88	5.02	4.6	4.38	4.24
	ΔpH	0	0.55	1.41	1.83	2.05	2.19
MRS-Y1-3	pH	6.39	6.35	6.02	5.34	4.76	4.4
	ΔpH	0	0.04	0.37	1.05	1.63	1.99
MRS-Y1-6	pH	6.42	6.36	6.02	5.1	4.58	4.31
	ΔpH	0	0.06	0.4	1.32	1.84	2.11
MRS-Y1-7	pH	6.41	6.37	5.81	4.82	4.42	4.21
	ΔpH	0	0.04	0.6	1.59	1.99	2.2
MRS-Y1-8	pH	6.42	6.3	5.52	4.72	4.38	4.19
	ΔpH	0	0.12	0.9	1.7	2.04	2.23
MRS-N2-1	pH	6.4	6.24	5.46	5.19	5.07	4.96
	ΔpH	0	0.16	0.94	1.21	1.33	1.44
MRS-N2-2	pH	6.44	6.27	5.44	4.76	4.42	4.22
	ΔpH	0	0.17	1	1.68	2.02	2.22
MRS-N2-3	pH	6.43	6.26	5.5	5.15	5.01	4.87
	ΔpH	0	0.17	0.93	1.28	1.42	1.56
MRS-N2-4	pH	6.43	6.17	5.08	4.65	4.44	4.25
	ΔpH	0	0.26	1.35	1.78	1.99	2.18
MRS-N2-5	pH	6.42	6.32	5.76	4.99	4.65	4.42
	ΔpH	0	0.1	0.66	1.43	1.77	2
MRS-N4-1	pH	6.44	6.33	5.93	5.27	4.79	4.49
	ΔpH	0	0.11	0.51	1.17	1.65	1.95
MRS-N4-2	pH	6.42	6.35	5.95	5.36	5.01	4.76
	ΔpH	0	0.07	0.47	1.06	1.41	1.66
MRS-N4-3	pH	6.46	6.3	5.4	4.8	4.47	4.28
	ΔpH	0	0.16	1.06	1.66	1.99	2.18
MRS-N6-1	pH	6.45	6.34	5.72	5.34	5.17	5.01
	ΔpH	0	0.11	0.73	1.11	1.28	1.44

Table E.2 Acidification activities of putative *L. bulgaricus* isolates.

$\Delta pH = pH_{at\ zero\ time} - pH_{at\ any\ time}$ (cont'd)

isolate number		**time (h)**					
		0	2	4	6	8	10
MRS-N6-2	pH	6.44	6.26	5.57	4.74	4.41	4.2
	ΔpH	0	0.18	0.87	1.7	2.03	2.24
MRS-N3-2	pH	6.42	6.26	5.29	4.46	4.2	4.03
	ΔpH	0	0.16	1.13	1.96	2.22	2.39
MRS-N3-5	pH	6.43	6.16	5.37	4.71	4.36	4.19
	ΔpH	0	0.27	1.06	1.72	2.07	2.24
MRS-K2-1	pH	6.42	6.32	5.58	4.69	4.34	4.15
	ΔpH	0	0.1	0.84	1.73	2.08	2.27
MRS-K2-2	pH	6.39	6.29	5.46	4.75	4.42	4.21
	ΔpH	0	0.1	0.93	1.64	1.97	2.18
MRS-K2-3	pH	6.37	6.13	5.01	4.49	4.24	4.06
	ΔpH	0	0.24	1.36	1.88	2.13	2.31
MRS-K2-4	pH	6.37	6.29	5.59	4.9	4.56	4.35
	ΔpH	0	0.08	0.78	1.47	1.81	2.02
MRS-K2-5	pH	6.36	6.11	5.02	4.36	4.16	3.99
	ΔpH	0	0.25	1.34	2	2.2	2.37
MRS-S1-1	pH	6.37	6.22	5.56	5.03	4.79	4.59
	ΔpH	0	0.15	0.81	1.34	1.58	1.78
MRS-S1-2	pH	6.38	6.15	5.12	4.56	4.36	4.17
	ΔpH	0	0.23	1.26	1.82	2.02	2.21
MRS-S1-3	pH	6.4	6.24	5.73	5.29	4.97	4.72
	ΔpH	0	0.16	0.67	1.11	1.43	1.68
MRS-N9-1	pH	6.4	6.33	5.91	5.17	4.96	4.4
	ΔpH	0	0.07	0.49	1.23	1.44	2
MRS-N5-3	pH	6.39	6.34	5.96	5.32	4.53	4.04
	ΔpH	0	0.05	0.43	1.07	1.86	2.35

APPENDIX F

F. ACETALDEHYDE PRODUCTION OF SELECTED *L. BULGARICUS* ISOLATES

Table F. 1 Acetaldehyde production ability of selected *L. bulgaricus* isolates according to their acidification abilities.

Strain numbers	ΔpH at 6th hour	pH at 24 h	Acetaldehyde (µg/g)
MRS-K1-16	1.8	3.56	6.74
MRS-K1-22	1.47	3.55	4.88
MRS-K1-23	1.72	3.81	1.21
MRS-K1-29	1.52	3.65	4.88
MRS-K1-30	1.42	3.54	5.88
MRS-K1-32	1.41	3.82	4.21
MRS-K1-33	1.65	3.63	6.61
MRS-K1-39	1.46	3.69	4.91
MRS-K1-43	1.81	3.41	15.20
MRS-K1-44	1.99	3.46	6.80
MRS-M2-8	1.42	3.44	23.93
MRS-M2-12	1.44	3.44	21.84
MRS-M2-13	1.88	3.36	27.02
MRS-M2-14	1.75	3.22	19.66
MRS-M2-16	1.79	3.38	11.12
MRS-M2-17	1.47	3.29	17.82
MRS-M2-20	1.59	3.3	15.07
MRS-M2-21	1.43	3.33	18.42
MRS-G3-3	1.94	3.21	8.01
MRS-G3-5	1.86	3.26	6.42
MRS-G3-7	1.57	3.34	4.52
MRS-G3-9	1.67	3.31	5.83
MRS-G3-10	1.68	3.34	3.39
MRS-G1-12	1.63	3.37	3.98
MRS-G1-16	1.59	3.2	4.21
MRS-G1-18	1.68	3.31	3.54
MRS-G1-19	1.57	3.28	2.57
MRS-G1-20	1.53	3.27	3.25

Table F.1 Acetaldehyde production ability of selected *L. bulgaricus* isolates according to their acidification abilities (cont'd)

Strain numbers	ΔpH at 6th hour	pH at 24 h	Acetaldehyde (µg/g)
MRS-G1-21	1.55	3.22	6.79
MRS-G1-22	1.51	3.2	4.56
MRS-G1-23	1.62	3.29	3.38
MRS-G1-24	1.61	3.23	6.47
MRS-G1-25	1.68	3.31	5.00
MRS-M23-1	1.57	3.22	11.04
MRS-M23-2	1.61	3.17	18.51
MRS-M23-3	1.59	3.16	16.67
MRS-M23-4	1.47	3.19	17.82
MRS-M23-13	1.83	3.17	16.03
MRS-Y1-7	1.59	3.21	3.62
MRS-Y1-8	1.7	3.35	0.56
MRS-N2-2	1.68	3.38	15.76
MRS-N2-4	1.78	3.26	15.89
MRS-N2-5	1.43	3.32	17.56
MRS-N4-3	1.66	3.28	13.05
MRS-N6-2	1.7	3.17	9.08
MRS-N3-2	1.96	3.14	11.20
MRS-N3-5	1.72	3.31	2.94
MRS-K2-1	1.73	3.13	11.54
MRS-K2-2	1.64	3.2	13.69
MRS-K2-3	1.88	3.17	9.48
MRS-K2-4	1.47	3.34	13.01
MRS-K2-5	2	3.22	13.41
MRS-S1-2	1.82	3.37	5.61

APPENDIX G

G. 16S rRNA GENE OF *S. THERMOPHILUS* LMG 18311 AND ALLIGNMENT WITH *S. VESTIBULARIS* AND *S. SALIVARIUS*

>gi|55820103:17819-19373 *Streptococcus thermophilus* LMG 18311, complete genome_16S rRNA gene region and ITS region (highlighted with grey) is given below. Primer pairs are highlighted with color (yellow and pink, each color indicates one pair). Sequence obtained using NCBI-Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi)

TTAATGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGT
AGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGT
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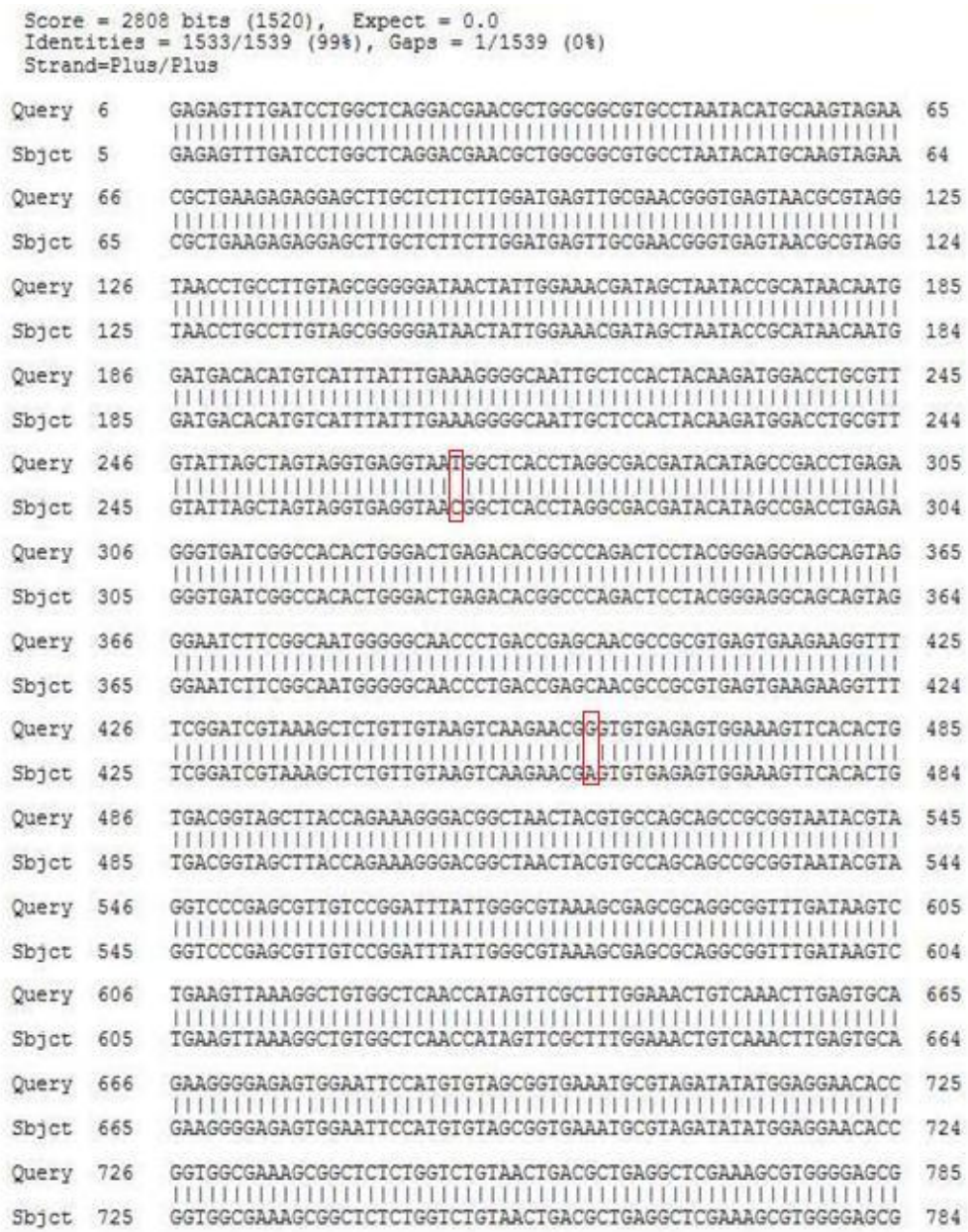
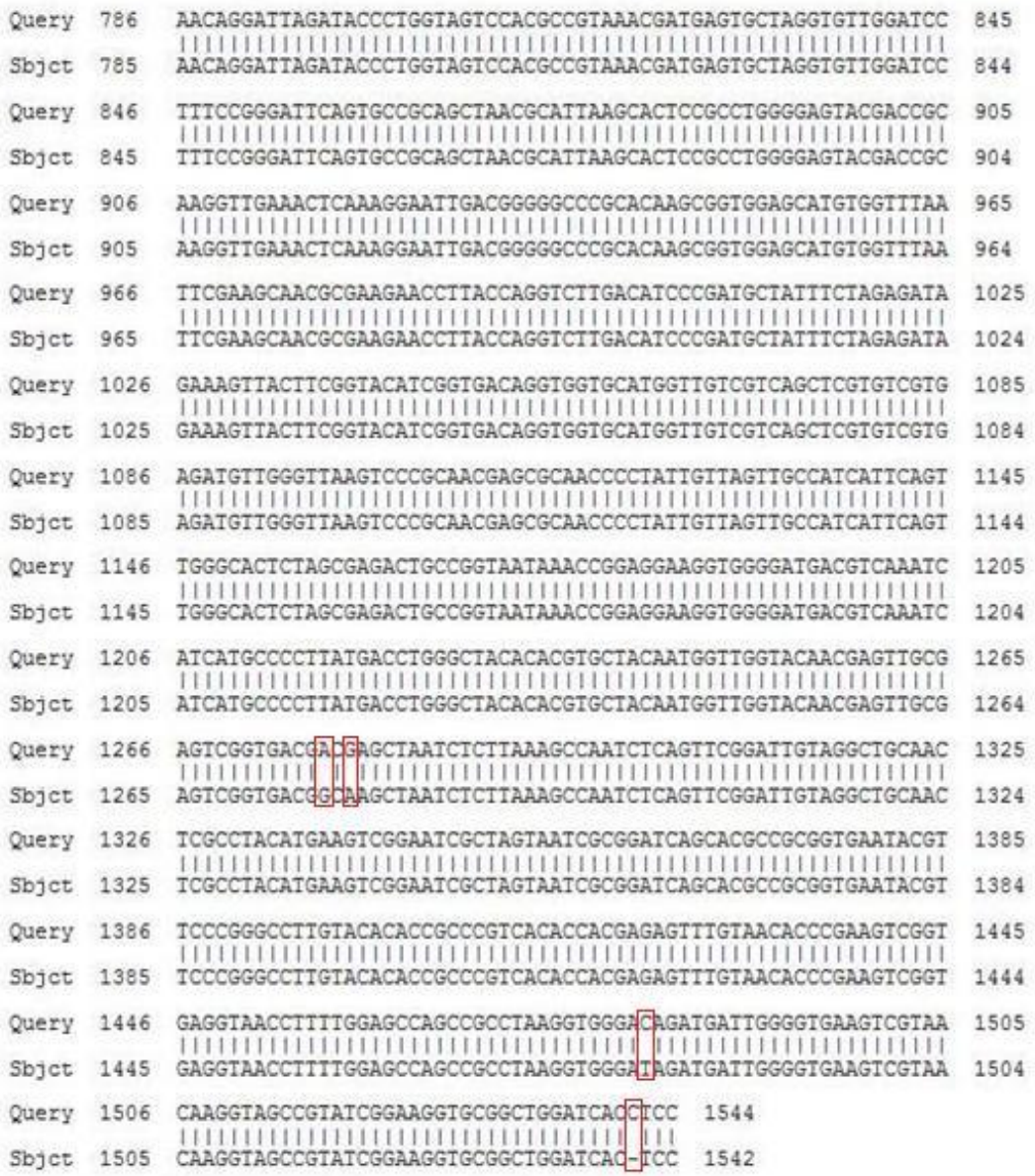
GTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCACACTGTGA
CGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTC
CCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAG
TTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGG
GAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGC
GAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGG
ATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGATAGAAAGTTACTT
CGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGA
GACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGACGAGC
TAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGA
ATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAG
CCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGG
TGCGGCTGGATCACCTCCTTTCTAAGGAAAAACGGAATGTACTTGAGTTTCTTATTTAGTTT
TGAGAGGTCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTC
AGCGGTTCGATCCCGCTAGGCTCCATTGAATCGAAAGATTCAAGTATTGTCCATTGAAAATT
GAATATCTATATCAAATTCCATATGTAAGTAATTACATATAGATAGTAACAAGAAAATAAA
CCGAAACGCTGTGAATATTTAATGAGTTAGGTCGAAAGGCCAAAAATAAGG

Alignment of rRNA gene sequences of *S. thermophilus* LMG 18311 and *S. salivarius* strain ATCC 7073 using BLAST. The snps were marked.

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Sbjct 785 AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCC 844
Query 846 TTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 905
Sbjct 845 TTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 904
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Sbjct 905 AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA 964
Query 966 TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGATA 1025
Sbjct 965 TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGATA 1024
Query 1026 GAAAGTTACTTCGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG 1085
Sbjct 1025 GAAAGTTACTTCGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG 1084
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Sbjct 1085 AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGT 1144
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Sbjct 1205 ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCG 1264
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Sbjct 1265 AGTCGGTGACGGCAAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAAC 1324
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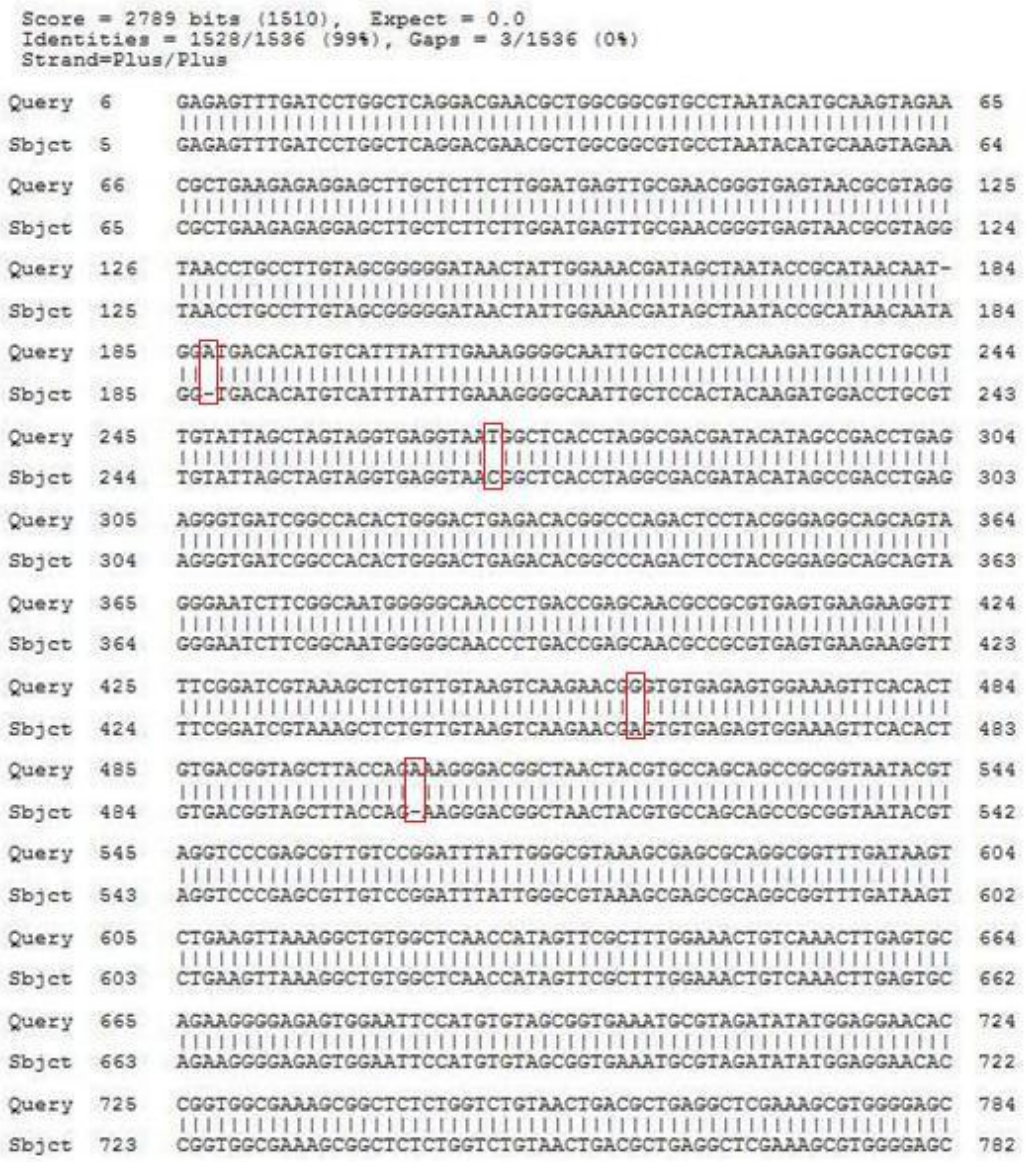
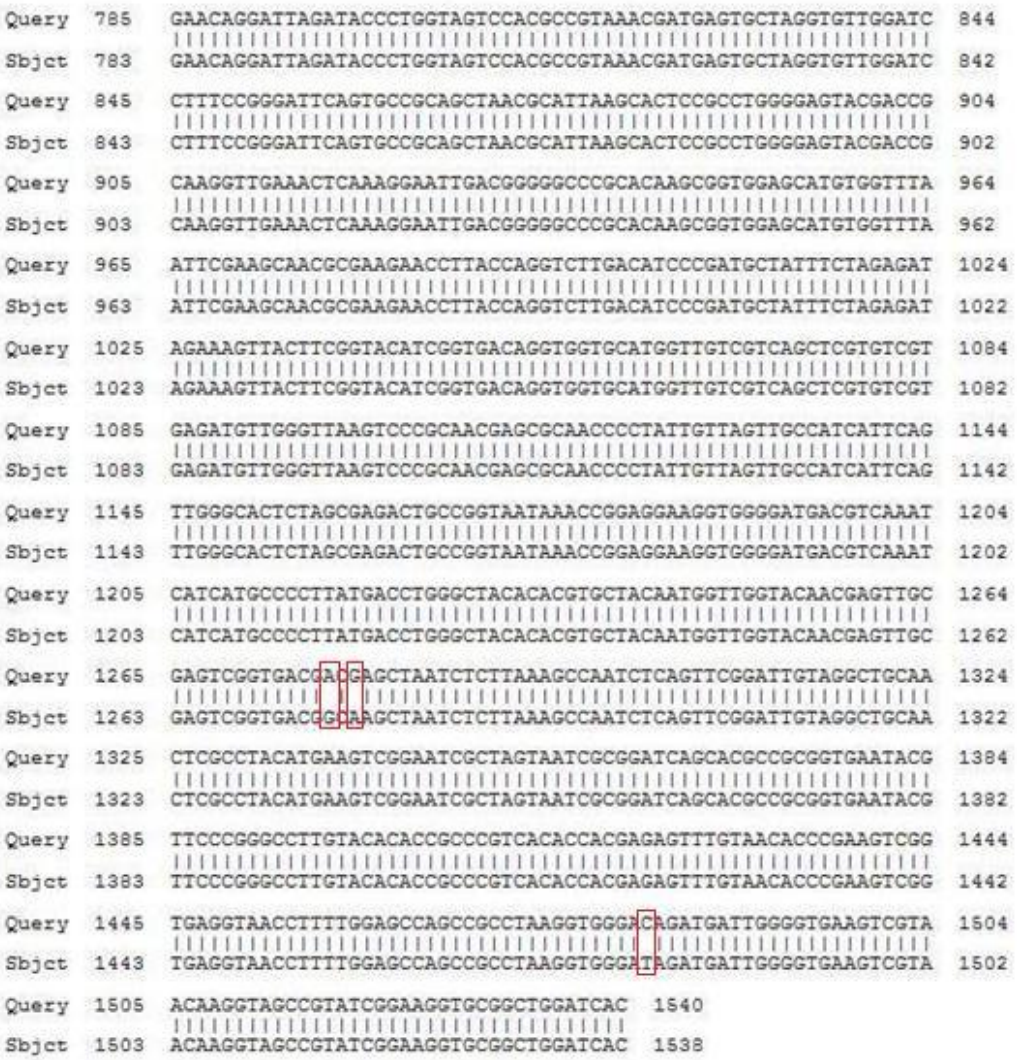
Sbjct 1325 TCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGT 1384
Query 1386 TCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGT 1445
Sbjct 1385 TCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGT 1444
Query 1446 GAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA 1505
Sbjct 1445 GAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAA 1504
Query 1506 CAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCC 1544
Sbjct 1505 CAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCAC-TCC 1542

Alignment of rRNA gene sequences of *S. thermophilus* LMG 18311 and *S. vestibularis* strain ATCC 49124 using BLAST. The snps were marked.

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Sbjct 783 GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATC 842
Query 845 CTTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCG 904
Sbjct 843 CTTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCG 902
Query 905 CAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA 964
Sbjct 903 CAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA 962
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Sbjct 963 ATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGAT 1022
Query 1025 AGAAAGTTACTTCGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT 1084
Sbjct 1023 AGAAAGTTACTTCGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT 1082
Query 1085 GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAG 1144
Sbjct 1083 GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAG 1142
Query 1145 TTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAAT 1204
Sbjct 1143 TTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAAT 1202
Query 1205 CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGC 1264
Sbjct 1203 CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGC 1262
Query 1265 GAGTCGGTGACGAACGAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAA 1324
Sbjct 1263 GAGTCGGTGACGCGAAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAA 1322
Query 1325 CTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACG 1384
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Sbjct 1383 TTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGG 1442
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Sbjct 1443 TGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTA 1502
Query 1505 ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCAC 1540
Sbjct 1503 ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCAC 1538

APPENDIX H

H. BLAST ANALYSIS OF PARTIAL 16S rRNA GENE OF REPRESENTATIVE ORGANISMS

The following BLAST analysis of 16S rRNA gene was performed for the two sequenced parts. Sequences by Primer Pro26 and Primer St2 were aligned and hence Sequence-part1was obtained. Sequences by Primer St3 and Primer St4 were also aligned and gave Sequence-part 2.

H.1 Blast analysis of *Streptococcus salivarius* ATCC 7073^{T}

H.1.1 Sequence-part1 for *Streptococcus salivarius* ATCC 7073^{T}

> gb|GU561396.1| *Streptococcus salivarius* strain H1_9 16S ribosomal RNA gene, partial sequence

```
 Score =  907 bits (491),  Expect = 0.0
 Identities = 491/491 (100%), Gaps = 0/491 (0%)
 Strand=Plus/Plus

Query  1    ATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTAT  60
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Sbjct  49   ATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTAT  108

Query  61   TGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  109  TGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGG  168

Query  121  CAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  169  CAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTC  228

Query  181  ACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  229  ACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA  288

Query  241  CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGA  300
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Sbjct  289  CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGA  348

Query  301  CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  349  CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAA  408

Query  361  GAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCT  420
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Sbjct  409  GAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCT  468

Query  421  AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  469  AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG  528

Query  481  CGTAAAGCGAG  491
            ||||||||||| 
Sbjct  529  CGTAAAGCGAG  539
```

H.1.2 Sequence-part2 for *Streptococcus salivarius* ATCC 7073^{T}

>gb|AF459433.1| *Streptococcus salivarius* 16S ribosomal RNA gene, partial sequence

```
 Score =  684 bits (370),  Expect = 0.0
 Identities = 370/370 (100%), Gaps = 0/370 (0%)
 Strand=Plus/Plus

Query  1     TAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1169  TAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA  1228

Query  61    CACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGGCAAGCTAATCTCTTA  120
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Sbjct  1229  CACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGGCAAGCTAATCTCTTA  1288

Query  121   AAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1289  AAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAG  1348

Query  181   TAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC  240
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Sbjct  1469  AAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGC  1528

Query  361   TGGATCACCT  370
             ||||||||||
Sbjct  1529  TGGATCACCT  1538
```

H.2 Blast analysis of *Streptococcus vestibularis* ATCC 49124^{T}

H.2.1 Sequence-part1 for *Streptococcus vestibularis* ATCC 49124^{T}

>gb|FJ154805.1| *Streptococcus vestibularis* strain CCRI 17387 16S ribosomal RNA gene, partial sequence

```
 Score =  815 bits (441),  Expect = 0.0
 Identities = 441/441 (100%), Gaps = 0/441 (0%)
 Strand=Plus/Plus

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Sbjct  136  GATAGCTAATACCGCATAACAATAGGTGACACATGTCATTTATTTGAAAGGGGCAATTGC  195

Query  121  TCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTCACCTAGG  180
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Sbjct  196  TCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTCACCTAGG  255

Query  181  CGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  CGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA  315

Query  241  GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCA  375

Query  301  ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGAG  360
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Sbjct  376  ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGAG  435

Query  361  TGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACG  420
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Sbjct  436  TGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACG  495

Query  421  TGCCAGCAGCCGCGGTAATAC  441
            |||||||||||||||||||||
Sbjct  496  TGCCAGCAGCCGCGGTAATAC  516
```

H.2.2 Sequence-part2 for *Streptococcus vestibularis* ATCC 49124^{T}

>gb|AY188353.1| *Streptococcus vestibularis* strain ATCC 49124 16S ribosomal RNA gene, complete sequence

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 Score =  604 bits (327),  Expect = 7e-170
 Identities = 327/327 (100%), Gaps = 0/327 (0%)
 Strand=Plus/Plus

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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  GCGAGTCGGTGACGGCAAGCTAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGC  1320

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Sbjct  1321  AACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATA  1380

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Sbjct  1381  CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTC  1440

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Sbjct  1441  GGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCG  1500

Query  301   TAACAAGGTAGCCGTATCGGAAGGTGC  327
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Sbjct  1501  TAACAAGGTAGCCGTATCGGAAGGTGC  1527
```

H.3 Blast analysis of isolate M17-K1-25

H.3.1 Sequence-part1 forM17-K1-25

> gb|GU460416.1| *Enterococcus faecium* strain UPAA88 16S ribosomal RNA gene, partial sequence

```
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 Identities = 508/509 (99%), Gaps = 0/509 (0%)
 Strand=Plus/Plus

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Sbjct  97   GGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGC  156

Query  61   TAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  157  TAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCG  216

Query  121  CTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  217  CTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCAC  276

Query  181  GATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  277  GATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACT  336

Query  241  CCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  337  CCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGC  396

Query  301  CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAANAAGGATG  360
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  397  CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATG  456

Query  361  AGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  457  AGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA  516

Query  421  GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  517  GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGC  576

Query  481  GCAGGCGGTTTCTTAAGTCTGATGTGAAA  509
            |||||||||||||||||||||||||||||
Sbjct  577  GCAGGCGGTTTCTTAAGTCTGATGTGAAA  605
```

H.3.2 Sequence-part2 forM17-K1-25

> emb|AJ301830.1| *Enterococcus faecium* 16S rRNA gene, strain LMG 11423

```
 Score =  752 bits (407),  Expect = 0.0
 Identities = 422/429 (98%), Gaps = 2/429 (0%)
 Strand=Plus/Plus

Query  1     CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1171  CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG  1230

Query  61    GGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1231  GGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAA  1290

Query  121   TCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1291  TCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAA  1350

Query  181   TCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC  240
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1351  TCGCTAGTAATCGCGGATCAGCACGCCGCG-TGAATACGTTCCCGGGCCTTGTACACACC  1409

Query  241   GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCC  300
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  1410  GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTT-GGAGCC  1468

Query  301   AGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAA  360
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1469  AGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCTGAA  1528

Query  361   GGTGCGGCTGGATCACCTCCTTTCTAAGGAATATTACGGAGACTACACAATTTGTTTTTA  420
             |||||||||||||||||||||||||||||||||||||||||| |||||||| ||| ||||||
Sbjct  1529  GGTGCGGCTGGATCACCTCCTTTCTAAGGAATATTACGGATACTACACACTTTTTTTTTA  1588

Query  421   CTTTGTTCA  429
             |||| ||||
Sbjct  1589  CTTTTTTCA  1597
```

H.4 Blast analysis of isolate M17-N5-4

H.4.1 Sequence-part1 forM17- N5-4

> emb|FN552257.1| *Streptococcus equinus* partial 16S rRNA gene, strain CBN292-08

```
 Score =  806 bits (436),  Expect = 0.0
 Identities = 436/436 (100%), Gaps = 0/436 (0%)
 Strand=Plus/Plus

Query  1    GGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCATGTTAGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  GGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCATGTTAGA  161

Query  61   TGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCTAGTTGGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162  TGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCTAGTTGGT  221

Query  121  GAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  GAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACAC  281

Query  181  TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG  341

Query  241  GGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  GGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTC  401

Query  301  TGTTGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAACTTACCA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  TGTTGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAACTTACCA  461

Query  361  GAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  GAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTC  521

Query  421  CGGATTTATTGGGCGT  436
            ||||||||||||||||
Sbjct  522  CGGATTTATTGGGCGT  537
```

H.4.2 Sequence-part2 forM17- N5-4

> emb|FN597254.1| *Streptococcus gallolyticus* UCN34 complete genome

```
 Score =  771 bits (417),  Expect = 0.0
 Identities = 424/427 (99%), Gaps = 1/427 (0%)
 Strand=Plus/Plus

Query  1      GGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19559  GGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCA  19618

Query  61     TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCGA  120
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  19619  TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAA  19678

Query  121    GTCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19679  GTCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACT  19738

Query  181    CGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19739  CGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTT  19798

Query  241    CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19799  CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG  19858

Query  301    AGGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19859  AGGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAA  19918

Query  361    CAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGATAAA-CGGAAG  419
              |||||||||||||||||||||||||||||||||||||||||||||||||| ||| ||||||
Sbjct  19919  CAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAAACGGAAG  19978

Query  420    CACGTTT  426
              |||||||
Sbjct  19979  CACGTTT  19985
```

H.5 Blast analysis of isolate M17-N7-1

H.5.1 Sequence-part1 for M17- N7-1

> emb|FN552257.1| *Streptococcus equinus* partial 16S rRNA gene, strain CBN292-08

```
 Score =  573 bits (310),  Expect = 2e-160
 Identities = 310/310 (100%), Gaps = 0/310 (0%)
 Strand=Plus/Plus

Query  1    CTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCATG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97   CTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCATG  156

Query  61   TTAGATGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCTAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  157  TTAGATGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCTAG  216

Query  121  TTGGTGAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  217  TTGGTGAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGC  276

Query  181  CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  277  CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG  336

Query  241  CAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  337  CAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAA  396

Query  301  AGCTCTGTTG  310
            ||||||||||
Sbjct  397  AGCTCTGTTG  406
```

H.5.2 Sequence-part2 for M17- N7-1

> gb|AY442813.1| *Streptococcus bovis* 16S ribosomal RNA gene, partial sequence

```
 Score =  730 bits (395),  Expect = 0.0
 Identities = 395/395 (100%), Gaps = 0/395 (0%)
 Strand=Plus/Plus

Query  1     TCATTAAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1094  TCATTAAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGA  1153

Query  61    CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1154  CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAA  1213

Query  121   CGAGTCGCGAGTCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1214  CGAGTCGCGAGTCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTA  1273

Query  181   GGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1274  GGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGG  1333

Query  241   TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1334  TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC  1393

Query  301   GAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1394  GAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGT  1453

Query  361   GAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCG  395
             |||||||||||||||||||||||||||||||||||
Sbjct  1454  GAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCG  1488
```

H.6 Blast analysis of isolate M17-N7-4

H.6.1 Sequence-part1 for M17- N7-4

> emb|FN552257.1| *Streptococcus equinus* partial 16S rRNA gene, strain CBN292-08

```
 Score =  776 bits (420),  Expect = 0.0
 Identities = 420/420 (100%), Gaps = 0/420 (0%)
 Strand=Plus/Plus

Query  1    TACTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   TACTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACCCA  154

Query  61   TGTTAGATGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  155  TGTTAGATGCTTGAAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCT  214

Query  121  AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215  AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCG  274

Query  181  GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275  GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  334

Query  241  GGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  GGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  394

Query  301  AAAGCTCTGTTGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395  AAAGCTCTGTTGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAA  454

Query  361  CTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455  CTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG  514
```

H.6.2 Sequence-part2 for M17- N7-4

> emb|FN597254.1| *Streptococcus gallolyticus* UCN34 complete genome

```
 Score =  774 bits (419),  Expect = 0.0
 Identities = 426/429 (99%), Gaps = 1/429 (0%)
 Strand=Plus/Plus

Query  1      GGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19560  GGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT  19619

Query  61     CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCGAG  120
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  19620  CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAAG  19679

Query  121    TCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19680  TCGGTGACGGCAAGCAAATCTCTTAAAGCCAATCTCAGTTCGGATTGTAGGCTGCAACTC  19739

Query  181    GCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19740  GCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTC  19799

Query  241    CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGA  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19800  CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGA  19859

Query  301    GGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAAC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19860  GGTAACCTTTTAGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAAC  19919

Query  361    AAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGATAAA-CGGAAGC  419
              ||||||||||||||||||||||||||||||||||||||||||||||||||| ||| |||||||
Sbjct  19920  AAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAAACGGAAGC  19979

Query  420    ACGTTTGGG  428
              |||||||||
Sbjct  19980  ACGTTTGGG  19988
```

APPENDIX I

I. SEQUENCES OF CRISPR1 LOCUS OF THE ISOLATES

One representative sequence from each subgroup obtained after CRISPR1 analysis was given below. Group and subgroup of the isolate according to CRISPR1 analysis were noted within brackets. In the sequence of M17-K1-13, direct repeats were highlighted and degenerate repeat were also underlined.

I.1 CRISPR1 Sequence of M17-K1-13 (a 1)

TATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGTAAG
GATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAA
TTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGAGGTCTGTAAT
TTTATTCCCTCGTAATCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGCTATC
ATCGTCTTACCTTGTGAACGAGCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CAAGACCGCTGTATTGGTCGGTATTCGTACCGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACTCTAGCGCTTCATCAAGCATGGTAAAGCCTGTTTTTGTACTCTCAAGATTT
AAGTAACTGTACAACGCTGAGTTAATGATTAAGTTTTCACCGCCAGTTTTTGTACTCTC
AAGATTTAAGTAACTGTACAACAGCTACCTACTACGTTAAGTTAAGACAAGCGTTTTT
GTACTCTCAAGATTTAAGTAACTGTACAACCCCTCTGTGTTAACTTGCCCAGATGTTAT
TGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAATCCTACTTCTCAAAGGATG
ATCCCAGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAATCCAAAAGAATG
GGATACACAAACGGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATAATTTT
GTAAATAAATTAGTACACCATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACC
TTGCTACACTAAACGATGGTAATGACAGCGTTTTTGTACTCTCAAGATTTAAGTAACTG
TACAACTCGACACATAAAATTATAACACGAAACCTTGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACAGCTTCCAAGTTGTTCCCACAGGGGCCCATGTTTTTGTACTCTCAA
GATTTAAGTAACTGTACAACTCGTGTTGAAAAAGATATTATTAACCCTGGTTTTTGTAC
TCTCAAGATTTAAGTAACTGTACAACACCCACACTTATATAGATATTGAACTAACTGTT
TTTGTACTCTCAAGATTTAAGTAACTGTACAACGTTTATTATGAAAATGAAACTTCTGT
ATACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCAAATGCTTCAATGGATTC
TTCCCATCCTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGAAAAAGTTCGT
GAGTATTTGCGAAATGCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGCT
ACTATTGGACGGAAAGCAAAACCTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTAC
AACGAGAATGGCGATAACTGGATTCGTAAAGATGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACTGAGTTAGGACACGTCCAAGACGACAAACCGTTTTTGTACTCTCAAG
ATTTAAGTAACTGTACAACTATTAGCAGGCACACCGTTATAGAAGTCCTGTTTTTGTAC
TCTCAAGATTTAAGTAACTGTACAACACCCTCTTAAAATTTTTACCCTTCAGCAACGTT
TTTGTACTCTCAAGATTTAAGTAACTGTACAACTGTAGGTCTTTTTTTGTTGTCATTATT
ATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTACCGAGAGATGCTCGTCAA
TGCCATGCTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTTCACTGGAAAA
TAAAGACCTTATCTTTGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAATTT
AGGAGGTAAGCAATGAGTGTATCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CAGGACTTCAGCCTTGAAGCTGAATACATTAGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAATACA
CGATAAACATAAGGATGT

I.2 CRISPR1 Sequence of M17-K1-18 (a 2)

CATTTTAGTTACCGGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAAT
TTTTAGAAAGTAAGGATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTAT
AAAAACGTCAAAATTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CGAGGTCTGTAATTTTATTCCCTCGTAATCTGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACGCTATCATCGTCTTACCTTGTGAACGAGCAGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACAAGACCGCTGTATTGGTCGGTATTCGTACCGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTCTAGCGCTTCATCAAGCATGGTAAAGCCTGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACGCTGAGTTAATGATTAAGTTTTCACCGCCAG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCGACACATAAAATTATAACACGA
AACCTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAGCTTCCAAGTTGTTCC
CACAGGGGCCCATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCGTGTTGAA
AAAGATATTATTAACCCTGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACACCC
ACACTTATATAGATATTGAACTAACTGTTTTTGTACTCTCAAGATTTAAGTAACTGTAC
AACGTTTATTATGAAAATGAAACTTCTGTATACGTTTTTGTACTCTCAAGATTTAAGTA
ACTGTACAACCAAATGCTTCAATGGATTCTTCCCATCCTTGTTTTTGTACTCTCAAGATT
TAAGTAACTGTACAACGAAAAAGTTCGTGAGTATTTGCGAAATGCTGTTTTTGTACTCT
CAAGATTTAAGTAACTGTACAACTTGCTACTATTGGACGGAAAGCAAAACCTAGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACGAGAATGGCGATAACTGGATTCGTAAA
GATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTGAGTTAGGACACGTCCAAG
ACGACAAACCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTATTAGCAGGCAC
ACCGTTATAGAAGTCCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACACCCTC
TTAAAATTTTTACCCTTCAGCAACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CTGTAGGTCTTTTTTTGTTGTCATTATTATAGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACTACCGAGAGATGCTCGTCAATGCCATGCTCGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACCTTCACTGGAAAATAAAGACCTTATCTTTGGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTAATTTAGGAGGTAAGCAATGAGTGTATCTGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACAGGACTTCAGCCTTGAAGCTGAATACATTA
GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTTTGATTCAACATAAAAAGCCAGT
TCAATTGAACTTGGCTTTTTAAAATACACGATAAACATAAGGATGT

I.3 CRISPR1 Sequence of M17-K1-14 (a 3)

GTTTTCATTTTAGTTACCGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACAT
AAATTTTTAGAAAGTAAGGATTGACAAGGACAGTTATTGATTTTATAATCACTATGTG
GGTATAAAAACGTCAAAATTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTG
TACAACGAGGTCTGTAATTTTATTCCCTCGTAATCTGTTTTTGTACTCTCAAGATTTAAG
TAACTGTACAACGCTATCATCGTCTTACCTTGTGAACGAGCAGTTTTTGTACTCTCAAG
ATTTAAGTAACTGTACAACAAGACCGCTGTATTGGTCGGTATTCGTACCGTTTTTGTAC
TCTCAAGATTTAAGTAACTGTACAACTCTAGCGCTTCATCAAGCATGGTAAAGCCTGTT
TTTGTACTCTCAAGATTTAAGTAACTGTACAACGCTGAGTTAATGATTAAGTTTTCACC
GCCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCGACACATAAAATTATAA
CACGAAACCTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAGCTTCCAAGTT
GTTCCCACAGGGGCCCATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCGTG
TTGAAAAAGATATTATTAACCCTGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CACCCACACTTATATAGATATTGAACTAACTGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACGTTTATTATGAAAATGAAACTTCTGTATACGTTTTTGTACTCTCAAGATTT
AAGTAACTGTACAACCAAATGCTTCAATGGATTCTTCCCATCCTTGTTTTTGTACTCTC
AAGATTTAAGTAACTGTACAACGAAAAAGTTCGTGAGTATTTGCGAAATGCTGTTTTT
GTACTCTCAAGATTTAAGTAACTGTACAACTTGCTACTATTGGACGGAAAGCAAAACC
TAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACACCCTCTTAAAATTTTTACCCT
TCAGCAACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTGTAGGTCTTTTTTTG
TTGTCATTATTATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTACCGAGAG
ATGCTCGTCAATGCCATGCTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCT
TCACTGGAAAATAAAGACCTTATCTTTGGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACTAATTTAGGAGGTAAGCAATGAGTGTATCTGTTTTTGTACTCTCAAGATTTAAG
TAACTGTACAACAGGACTTCAGCCTTGAAGCTGAATACATTAGTTTTTGTACTCTCAAG
ATTTAAGTAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTT
TTTAAAATACACGATAAACA

I.4 CRISPR1 Sequence of M17-N2-3 (b 4)

ATAAGGAACTATTACATAAATTTTTAGAAAGTAAGGATTGACAAGGACAGTTATTGAT
TTTATAATCACTATGTGGGTATGAAAATCTCAAAAATCATTTGAGGTTTTTGTACTCTC
AAGATTTAAGTAACTGTACAACAATAATTTTGCCCCTTCTTTGCCCCCTCGAGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACGTCTAACTAAAGACCCAGAATTAAAACATA
GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAATGACGAGGAGCTATTGGCAC
AACTTACAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTATAGATAATGGCGT
TATATGGGAGCGATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCATTATCA
GAAGATGGCAGACAGATATTAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
CTGCCCGTCAACGACGTCACCGTAAACTCCGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACACCGAGAGATGCTCGTCAATGCCATGCTCGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACCCAAATTTGCATTAAACAAAACGCTCCTTCGTTTTTGTACTCTCAA
GATTTAAGTAACTGTACAACATCACCTGGTTTGTAATCTCTAGGCTTAATGTTTTTGTA
CTCTCAAGATTTAAGTAACTGTACAACTTAAGGGGGTTATTCCCCTTTTTTAGTAGGTG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAATATCGTGAAATAGGCAACCGA
AAAATATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGTTCAAGATGCTATTG
AAAATGATGAAGACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGCGTGA
AGATGAATTAAACGACAGAGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACC
TAGGTCATTACGACCATATAAGTGTATTGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACAATATCGTGAAATAGGCAACCGAAAAATATGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAA
ATACACGAT

I.5 CRISPR1 Sequence of M17-N8-2 (b 5)

AGGTTACCGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAG
AAAGTAAGGATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATGAAAA
TCTCAAAAATCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAATAA
TTTTGCCCCTTCTTTGCCCCCTCGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CGTCTAACTAAAGACCCAGAATTAAAACATAGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACAATGACGAGGAGCTATTGGCACAACTTACAGTTTTTGTACTCTCAAGATTT
AAGTAACTGTACAACTATAGATAATGGCGTTATATGGGAGCGATAGTTTTTGTACTCTC
AAGATTTAAGTAACTGTACAACCATTATCAGAAGATGGCAGACAGATATTAGGTTTTT
GTACTCTCAAGATTTAAGTAACTGTACAACCTGCCCGTCAACGACGTCACCGTAAACT
CCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACACCGAGAGATGCTCGTCAATG
CCATGCTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCCAAATTTGCATTAA
ACAAAACGCTCCTTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATCACCTG
GTTTGTAATCTCTAGGCTTAATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTT
AAGGGGGTTATTCCCCTTTTGGCTCTTTGTCAACTGTAGTGGGTGACGAAAAGCTAACA
TCTAGAGAGGACCGGATAGGTTTTCTCTTTTTGTGGCTCTTTGTCAACTGTAGTGGGTA
GATGAAAAGCTAAATTCTTGAGAGGACCAGTTTGGTCTTCTCTTTTTTAATATTTACTG
CCATCAAAATCCGTTTCTTAAAGTTTTCAAAGTTCCTGAAGCCAAAGGCATTTCGCTTA
ATGACCTTGATGAGATTGTTCGTTGCTTCCAGTTTTGCGTTTGAATACGGCAGTTCGAG
GGCATTGATAATCTTGTCTTTATCTTTCAAAAAAGTTCTAAAAACTGTCTGAAAGATAG
GGTGTACGCTGGAACTCGTCTCTTCAATGAGGTCAAAGAAATGGTCCGAGTTCTTCTCT
TGGAAATGGAAAAGTAAAAGTTGGTAGAGTTCATAATGTTCTCTCAGTTCTTGAGAGT
ATGACAGTAGCTTTTCTAGGATTTCCTTGTTGGTTAAATGCGCACGAAAAGTCGGACG
ATAAAAGCGTTTATCGTTGAGTTTGCGACTATCTTGTTGGATCAGTTTCCAGTAGCGTT
TCAGGGCACGATATTCCTGTGACTGACGGTCCAAATGGTTCATGATTTGAATGCGGAC
GCGGTTCATAGCACGACTAAGGTGTTGCACAATGTGAAAGCGATCAAGGACAATCTTA
GCGTTAGGGAATAATTTTCTGGCGATGTCGTAATAAGGGCTAAACATGTCCATAGTGA
TGACTTTAACGCGATTTCTATCCTGTCTGGAATAGCGTAGGAAGTGGTTTCTAATCGTT
GCTTGCGTGCGTCCGTCGAGGATTGTGATGACTTTTAATGAGTCGAAATCCTGAGCGAT
GAAACTCATTTTTCCCTTCTTGAAGCCGTACTCATCCCAGCTCATATTCTCAGGCAGCC
AATTCCAGTCGGTTTTAAATTGGAACTCATTTAGCTTGCGACTGACGGTTGAGGTAGAG
ACGGCAAGGCGCTTGGCAATGTCGGTCATAGACCGTTTTTCAATGAGCAATTGAGCGA
TTTTCTGATAGACGACGGTTGCGATTTGGTGATTCTTCTTGACCAGAGAAGTTTCCGCG
ACAGCCATTTTTTCCGCACTCCTTACACTTGAAACGGCGCTTTTTCAGGCGAATGAGAGT
TCGGTAGCCAGCACACTCCAGATACGGGATTTTAGAGGCTTTCTGGAAGTCGTATTTGC
CCATCTGTCCCTTGCAGGCAGGACATTTAGGAGACTCGTAATCCAAGTAACCGTGAAG
TTCTTTGTGCGTTCCCATATCGTATTCATTAGTGATGATAATATTTTTGTCTTTTATTCC
AAGAAAATTTGTGATAAGATTTAGTTGTTCCATATGAGTCTTTCTAAAATGATGGTTTA
GTCGCTTTTCATTATAGGTCATATGGGACTTTTTTTCTACAATCAAAAAGGCTCCATAA
TCTCCATAGAGGATTTACCCACTACAGAAATTATAGAGCCCCCCTTTTTTAGTAGGTGT
TTTTGTACTCTCAAGATTTAAGTAACTGTACAACAATATCGTGAAATAGGCAACCGAA
AAATATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGTTCAAGATGCTATTGA
AAATGATGAAGACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGCGTGAA
GATGAATTAAACGACAGAGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCT
AGGTCATTACGACCATATAAGTGTATTGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACAATATCGTGAAATAGGCAACCGAAAAATATGTTTTTGTACTCTCAAGATTTAAG
TAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAA
TACACGATAAAC

I.6 CRISPR1 Sequence of M17-N2-2 (c 6)

TTTCATTTTAGTTACCGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAA
ATTTTTAGAAAGTAATGATTGACAAGAACAGTTATTGATTTTATAATCACTATGTGGGT
ATGAAAATCTCAAAAATCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACA
ACCAGGTCTTGATGAAGCGTTAGAGGGTTGGCGTTTTTGTACTCTCAAGATTTAAGTAA
CTGTACAACCAAAAGCAACAGTTGGTGAACCAGGGCCAAGTTTTTGTACTCTCAAGAT
TTAAGTAACTGTACAACTTAATATAAAGGAGGTGGTAAAAGTACCAAGTTTTTGTACT
CTCAAGATTTAAGTAACTGTACAACTTTGAACAATGCCCATCAGTTTATTATCTTGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACATTCAAGGCGGTATGTTCCCCCTATGC
TTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAAGACTCTTGCTACCGTCTT
GTTTACCGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCATAAGTATCTCCA
GAAGTCAAAGATGACGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATTGTCT
GTGTCTTTTGAAGATTTAAATGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACA
TATTAATATAGAGTGTTCGTTTGATATCGGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACTAGTCCGCCATATCCAAGTTGCCGTTTTCTGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAAT
ACACGATAAACATAAGG

I.7 CRISPR1 Sequence of M17-N3-6 (d 7)

TTTTAGTTACCGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTT
TAGAAAGTAAGGATTGACAAAGACAGTTATTGATTTTATAATCACTATGTGGGTATAA
AAACGTCAAAATTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACT
GGACACTTGAAACTTTACCAAGCTTTTTTGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACACTAAAAGAGCTACTTGACGGCAAAGAATTGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACTGGTAACCTTGATTATAGCCTTATTCGTCAGTTTTTGTACTCTCAA
GATTTAAGTAACTGTACAACCATAACAAAAGTCATTCAAGCTCAAGGCAGGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACATCAGATGGAAAAGGTGGATACGTCTATCA
GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCGTCCCTATGATTTTATCGATG
AAAATACGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAAATTCGACAAAAG
CACTACATGAATACTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTGTTTTTA
AATCCTTACCAAATTTATCCTGNTTTTGTACTCTCAAGATTTAAGTAACTGNACAACCT
AAAGGTGATGACTATCGTTTCAAACACAGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACATTTAGAAGAAGTGTTTAAACCTGAAACGTGTTTTTGTACTCTCAAGATTTAAG
TAACTGTACAACTAAACTCGACAAAAGCACTACAGGTATACTGNTTTTGTACTCTCAA
GATTTAAGTAACTGNACAACGAATTATTTAACTTGCTACCCTTATGAAAGTTTTTGTAC
TCTCAAGATTTAAGTAACTGTACAACTTTACTTAATTTATTCATTCTTCAACCTCTGTTT
TTGTACTCTCGAGATTTAAGTAACTGTACAACTCATTGATACTATCAACGCTTTCTTGG
TCTGNTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGTTTTGCTGTCTCACGAA
TTTCAAAAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCGTTTTGCTACTCG
TTCAGCATACTCTACAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAAGGA
GATTTTGACAATGAACAGACAAGGTTTTTGTATTCTCAATATTTAAGTAGCTGTACAGT
TTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAATACACGATAAA
CATAAGGA

I.8 CRISPR1 Sequence of M17-N5-2 (e 8)

TATAAGATATTCTCAGACACCTGGATAAGGAACTATTACATAAATTTTTAGAAAGTAA
GGATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAA
ATTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCAAAAATCGTA
AACGGTAAGCTACACGATGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAGA
TATTGAACTCACTGAAGAAATTGAAGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACTTCTGGTAGTGGTTTTAGTCAAACAGATGTGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACCTACAATCTCGTCATAAGTAGTAGTACCGTGTTTTTGTACTCTCAAGA
TTTAAGTAACTGTACAACGATGTAATGGATGATGGGGCTATCTATATGGTTTTTGTACT
CTCAAGATTTAAGTAACTGTACAACCATCATCGACTGATCTAATGAGCAAACCTCGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACATGAGTGGTTAAGAATCCGTATTATCA
GCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTATCAAATGCAGCACAAGTA
ACGTTGATGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAAAGTGTTTAC
AAACTATCATGTATGATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAAGC
AAATCGCGAGTATAAAGGATATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CTTGTCATAATAATTAAATCCAATAGGACTTGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACGGTATTCTTCCCAGTGTTTTCAGATGGTATGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACCATTTCATAAGCTGTTCCTTCTTGAACATAGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTTTGTCGATTAGCGATTATTTCATTAAATTGTTTTTGTA
CTCTCAAGATTTAAGTAACTGTACAACTGGCAGAGATTACACAGCAACGGAAACAGCG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTCCAACAAGCCCGCGCCTAATTA
TTCCAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATCTGTCCATCTGGTCT
AAATCCAAACAGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGCCTAGCCCT
ACAGCTACCCCGCCTACTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATCT
GTCCATCTGGTCTAAATCCAAACAGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTAC
AACGCCTAGCCCTACAGCTACCCCGCCTACTTGTTTTTGTACTCTCAAGATTTAAGTAA
CTGTACAACATCTACGTGTCAATACCTATCATAAAACAGGTTTTTGTACTCTCAAGATT
TAAGTAACTGTACAACATGGCATAATCTTCAAAAGCATACATACCAGTTTTTGTACTCT
CAAGATTTAAGTAACTGTACAACAAGACTACGTTGAATTACTAGAAAGGCAGTGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACTTTGAGGCAAGTTGACATTCTTAGACAG
TCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTTTGATTCAACATAAAAAGCCA
GTTCAATTGAACTTGGCTTTTTAAAATACACGATAAACATAA

I.9 CRISPR1 Sequence of M17-N5-7 (e 9)

CCGTATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGT
AAGGATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCA
AAATTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTTGAACA
AGCGAACAAAGACCATAATCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACA
TAGCCACATCTTTTCTGAAACCTTCACGTGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACCAAAAATCGTAAACGGTAAGCTACACGATGGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACAGATATTGAACTCACTGAAGAAATTGAAGAGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTTCTGGTAGTGGTTTTAGTCAAACAGATGTGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACCTACAATCTCGTCATAAGTAGTAGTACCGTG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGATGTAATGGATGATGGGGCTATC
TATATGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCATCATCGACTGATCTA
ATGAGCAAACCTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATGAGTGGTT
AAGAATCCGTATTATCAGCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAT
CAAATGCAGCACAAGTAACGTTGATGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACAAAAAGTGTTTACAAACTATCATGTATGATGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACAAAAGCAAATCGCGAGTATAAAGGATATAGTTTTTGTACTCTCAAGA
TTTAAGTAACTGTACAACTTGTCATAATAATTAAATCCAATAGGACTTGTTTTTGTACT
CTCAAGATTTAAGTAACTGTACAACGGTATTCTTCCCAGTGTTTTCAGATGGTATGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACCATTTCATAAGCTGTTCCTTCTTGAAC
ATAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGTCGATTAGCGATTATTT
CATTAAATNGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTGGCAGAGATTACA
CAGCAACGGAAACAGCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTCCAA
CAAGCCCGCGCCTAATTATTCCAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAA
CATCTGTCCATCTGGTCTAAATCCAAACAGGGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACGCCTAGCCCTACAGCTACCCCGCCTACTTGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACATCTGTCCATCTGGTCTAAATCCAAACAGGGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACGCCTAGCCCTACAGCTACCCCGCCTACTTGTTTTTGTA
CTCTCAAGATTTAAGTAACTGTACAACATCTACGTGTCAATACCTATCATAAAACAGGT
TTTTGTACTCTCAAGATTTAAGTAACTGTACAACATGGCATAATCTTCAAAAGCATACA
TACCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAGACTACGTTGAATTAC
TAGAAAGGCAGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGAGGCAA
GTTGACATTCTTAGACAGTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTTTG
ATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAATACCACGATAAACC
ATA

I.10 CRISPR1 Sequence of M17-N6-6 (e 10)

ATAAGATATTCTCAGACACCTGGATAAGGAACTATTACATAAATTTTTAGAAAGTAAG
GATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAA
TTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTTGAACAAGC
GAACAAAGACCATAATCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATAG
CCACATCTTTTCTGAAACCTTCACGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACA
ACCCTAAGTCTTCATCAGTCAATACTTTTCTCGTTTTTGTACTCTCAAGATTTAAGTAAC
TGTACAACGTCACAACCGATGACTATCCAAAATACATTGTTTTTGTACTCTCAAGATTT
AAGTAACTGTACAACCAAAAATCGTAAACGGTAAGCTACACGATGGTTTTTGTACTCT
CAAGATTTAAGTAACTGTACAACAGATATTGAACTCACTGAAGAAATTGAAGAGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACTTCTGGTAGTGGTTTTAGTCAAACAGAT
GTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTACAATCTCGTCATAAGTAG
TAGTACCGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGATGTAATGGATGA
TGGGGCTATCTATATGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAAAGT
GTTTACAAACTATCATGTATGATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
AAAAGCAAATCGCGAGTATAAAGGATATAGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACTTGTCATAATAATTAAATCCAATAGGACTTGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACGGTATTCTTCCCAGTGTTTTCAGATGGTATGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACCATTTCATAAGCTGTTCCTTCTTGAACATAGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACTTTGTCGATTAGCGATTATTTCATTAAATTG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTGGCAGAGATTACACAGCAACGG
AAACAGCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTCCAACAAGCCCGCG
CCTAATTATTCCAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATCTGTCCA
TCTGGTCTAAATCCAAACAGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGC
CTAGCCCTACAGCTACCCCGCCTACTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACATCTGTCCATCTGGTCTAAATCCAAACAGGGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACGCCTAGCCCTACAGCTACCCCGCCTACTTGTTTTTGTACTCTCAAGAT
TTAAGTAACTGTACAACATCTACGTGTCAATACCTATCATAAAACAGGTTTTTGTACTC
TCAAGATTTAAGTAACTGTACAACATGGCATAATCTTCAAAAGCATACATACCAGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACAAGACTACGTTGAATTACTAGAAAGGC
AGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTGAGGCAAGTTGACATTC
TTAGACAGTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTTTGATTCAACATA
AAAAGCCAGTTCAATTGAACTTGGCTTTTTAAAA

I.11 CRISPR1 Sequence of M17-N3-1 (f 11)

GATTTTATAATCACTATGTGGGTATAAAAACGTCAAAATTTCATTTGAGGTTTTTGTAC
TCTCAAGATTTAAGTAACTGTACAACCTTGCGATAGCAAACCGATATAAGAGAATGGT
TTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGATGATTGGAGGATAACATGACC
GATTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAGATAATCATTTATTT
ACTTATATACATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCCACGTGGAAC
GATTTGATAGCTATGTGCCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATT
TATTTGTACGGGAACCGTGACTTATTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACCTATGTTTTTTCTTTTTATGTACCCATTTTAGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACTAGTAGTCAGCAATATGAACTTTTTGCTCGGTTTTTGTACTCTCAAGA
TTTAAGTAACTGTACAACCTTAAATACTCACGAACTTTTTCAGATACTGTTTTTGTACTC
TCAAGATTTAAGTAACTGTACAACGCCATAATCTGTATAAGTTTTTCGCTCGTAGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACTGGTAAGCTATTACCAATAGACCACGA
AAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATAATACCAACGTTTCTGAC
TATTTTTATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGACATAGCAGAAAT
TTATTCTAACGAGCTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTTGTCA
GCATAGGCTCTACCAAGTTGCATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
ATTGGTAACCAAGTAAATATCACCATTGATGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACAGTCAACAGTCTAGCACGCTTATCGGACGTGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACATTAGCATACTGGCTGAGAACAATGTTCCAGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTTGGTTTTAACCACTACGACTTTCTTACTTGTTTTTGTA
CTCTCAAGATTTAAGTAACTGTACAACTAAAACATTTAGACCTAAACAAGTAACCATG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGTGGATTGAGAACAACTTGGAAA
ATATTCGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGTACCATCTGACCTAA
GAAATGTTCCATTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAAAATTGA
TGTGACTATCAATAAAGGCGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAC
GGTGACTATCAATCATGATTTCAACGGTGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACTTGTTTAGGAGAACATGAATGAAAAGAATAGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACACAATGGTGTTATATGGGAGCGATAAAATGGTTTTTGTACTCTCA
AGATTTAAGTAACTGTACAACTATAAGTTATATATCTCTTTTTATTTGTTGGTTTTTGTA
CTCTCAAGATTTAAGTAACTGTACAACCTTCTGAAATTAATTGTTATTTACCGAATAGT
TTTTGTACTCTCAAGATTTAAGTAACTGTACAACCGTTGATGGATATTATTGATAAACT
TTACGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGTAGTCAATGCTAGCG
CTTCTACTGCCTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCATCAATTATTT
TCGACCATCGATGATGTCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCCAGT
CTGCTACCAGCAATGCAAGACTAGAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACA
ACACGAAATTGGATCGCACGGTTACAAGTCGGGTTTTTGTACTCTCAAGATTTAAGTA
ACTGTACAACTGTAGTTTTAAGTTTAGTAAAAAAGTCAATGTTTTTGTACTCTCAAGAT
TTAAGTAACTGTACAACATCCTAGATATTCTATTCCTGAAATCAAAGGTTTTTGTACTC
TCAAGATTTAAGTAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTT
GGCTTTTTAAAATACACGATAAACATAAGGA

I.12 CRISPR1 Sequence of M17-N1-1 (f 12)

TAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGTAAGAA
TTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAATTT
CATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTTGCGATAGCAAAC
CGATATAAGAGAATGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGATGAT
TGGAGGATAACATGACCGATTAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACA
AAGATAATCATTTATTTACTTATATACATGTTTTTGTACTCTCAAGATTTAAGTAACTGT
ACAACCCACGTGGAACGATTTGATAGCTATGTGCCTGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAACCCAGTCTGCTACCAGCAATGCAAGACTAGAGTTTTTGTACTCTCAA
GATTTAAGTAACTGTACAACACGAAATTGGATCGCACGGTTACAAGTCGGGTTTTTGT
ACTCTCAAGATTTAAGTAACTGTACAACTGTAGTTTTAAGTTTAGTAAAAAAGTCAATG
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACATCCTAGATATTCTATTCCTGAAA
TCAAAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTTTGATTCAACATAAAAA
GCCAGTTCAATTGAACTTGGCTTTTTAAAATACACGATAAAC

I.13 CRISPR1 Sequence of M17-N4-2 (f 13)

AGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGTAAGGATT
GACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAATTTC
ATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCAATATCAATTACAA
AGTCCATGTGTTCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTATCTTCTT
TAAATTGTGGTTTGGTAAATGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCAG
TCCGATGGTTTACGCACCACTGTCAGCGTTTTTGTACTCTCAAGATTTAAGTAACTGTA
CAACTTAATAGGTTTTCTTCCTATTATATACTCAGTTTTTGTACTCTCAAGATTTAAGTA
ACTGTACAACTCTAGTTAGGCATTCTAAAACATCTATCACGTTTTTGTACTCTCAAGAT
TTAAGTAACTGTACAACTAAAATCATTTTCAACGAGTTGAGAAACATGTTTTTGTACTC
TCAAGATTTAAGTAACTGTACAACAATTTGTATCATCTGCATCCGATAGCAAGTGTTTT
TGTACTCTCAAGATTTAAGTAACTGTACAACCCACCTCCTTAGTTGCTAGATTTCTTTG
CAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTTATTAGTAGCTGTACCGTT
AAGCATAGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTTAACTGCCTTTC
TTTCTTGCTAGGTCGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAATTGTG
GTCACCACCATACTAATAGACGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
TAGTACCCTTAACGGTAGAGGTAGTCATCTGTTTTTGTACTCTCAAGATTTAAGTAACT
GTACAACGCACTCAGACAGTTTTTTAACTACTTAGCTGTTTTTGTACTCTCAAGATTTA
AGTAACTGTACAACACTTGGAAAGAGTTTCTATGAAGGAATGGAGTTTTTGTACTCTC
AAGATTTAAGTAACTGTACAACCTTCCTAAGTGCATGAAAATCGCAAACGGAGTTTTT
GTACTCTCAAGATTTAAGTAACTGTACAACAAGCTAGTGACAATCTAACGATTAACTC
TCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCCTGGTCACTTAATCTATTCGC
AAGACAAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAAAATGGCATAGAG
AATCTAAAGCTTGTGGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTATTATC
TTGATAGTAAACCTTATCCATAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
TAATTATGTTCAACATCTTGAACTTTATATGTTTTTGTACTCTCAAGATTTAAGTAACTG
TACAACTGACACAACCGATTTCAACTAGTAAAATAGTTTTTGTACTCTCAAGATTTAAG
TAACTGTACAACATCCTAGATATTCTATTCCTGAAATCAAAGGTTTTTGTACTCTCAAG
ATTTAAGTAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTT
TTTAAAATACACGATAAACATAAGG

I.14 CRISPR1 Sequence of M17-N9-3 (f 14)

TATAAGATATTCTCAGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGTAAG
AATTGACAAGGACAGTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAA
TTTCATTTGAGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTCTCAGCCAGT
ATGCTAATTGTGGTATGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGACCT
ATGCTGGTCAACTAACAATTATGTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACA
ACTTAGCTGTCCAATCCACGAACGCTGATGGCAGTTTTTGTACTCTCAAGATTTAAGTA
ACTGTACAACAGGTAACACGTAGAACCATTTACAATTACAGTTTTTGTACTCTCAAGAT
TTAAGTAACTGTACAACGAACACTGATAACAGAAAGAGCTAAAAATGGTTTTTGTACT
CTCAAGATTTAAGTAACTGTACAACTAAGATTTATATCGCTGCTTACTTTAGAACGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACCGTGTACAGCACGCAGTTGTTGATTTA
CAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACCTATCCGTTTCAACTTGGGC
GGGTTTAATCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTCTTCTAATATCTT
GCCAAGTTTTAGACTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGACCT
TAGAACCTGATGAGTATCTAAAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC
TGACACAACCGATTTCAACTAGTAAAATAGTTTTTGTACTCTCAAGATTTAAGTAACTG
TACAACATCCTAGATATTCTATTCCTGAAATCAAAGGTTTTTGTACTCTCAAGATTTAA
GTAACTGTACAGTTTGATTCAACATAAAAAGCCAGTTCAATTGAACTTGGCTTTTTAAA
ATACACGATAAAC

I.15 CRISPR1 Sequence of M17-N9-1 (f 15)

AGACACCTGATAAGGAACTATTACATAAATTTTTAGAAAGTAAGAATTGACAAGGACA
GTTATTGATTTTATAATCACTATGTGGGTATAAAAACGTCAAAATTTCATTTGAGGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACTTCTCAGCCAGTATGCTAATTGTGGTA
TGTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACGACCTATGCTGGTCAACTAA
CAATTATGTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTAGCTGTCCAAT
CCACGAACGCTGATGGCAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACAGGT
AACACGTAGAACCATTTACAATTACAGTTTTTGTACTCTCAAGATTTAAGTAACTGTAC
AACGAACACTGATAACAGAAAGAGCTAAAAATGGTTTTTGTACTCTCAAGATTTAAGT
AACTGTACAACTAAGATTTATATCGCTGCTTACTTTAGAACGTTTTTGTACTCTCAAGA
TTTAAGTAACTGTACAACCGTGTACAGCACGCAGTTGTTGATTTACAAGTTTTTGTACT
CTCAAGATTTAAGTAACTGTACAACCTATCCGTTTCAACTTGGGCGGGTTTAATCGTTT
TTGTACTCTCAAGATTTAAGTAACTGTACAACTCTTCTAATATCTTGCCAAGTTTTAGA
CTTGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTTGACCTTAGAACCTGATGA
GTATCTAAAAGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACACCCAGCGTTAAA
TAGTTGCGTTTTATCGCGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAACTAACTTC
TTATGTCTATTGTCTGCCTTACGGTTTTTGTACTCTCAAGATTTAAGTAACTGTACAGTT
TGATTCAA

APPENDIX J

J. SEQUENCES OF HOUSEKEEPING GENES USED IN MLST ANALYSIS

The following sequences of the housekeeping genes obtained from complete genome of *S. thermophilus* LMG 18311 (Bolotin et al., 2004) by NCBI-Genome Project/Microbial genomes (Internet: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi)

The primers designed for the related gene were highlighted on the sequences.

J.1 Sequence of *pro*A from *S. thermophilus* LMG 18311

In this gene primer were selected slightly outside of the gene to be able to sequence the polymorphic part close to the end of the gene. The sequence not belong to *pro*A were underlined.

>gi|55820103:c1516584-1515120 *Streptococcus thermophilus* LMG 18311, complete genome

ATGACATACATTGATACATTGGGCCAGCAGGCCAAAGTGGCGAGTCGTCAGATTGCTA
AATTGTCAACAGCAGCTAAAAACGACCTTTTAAATCAGGTAGCTAAAGCTCTAGTAGC
TGAGAGTGACTATATTATCACTGAAAATGCTAAGGATATGGCCAATGCCAGCGAAAAT
GGTATTTCAAAGATTATGCAAGACCGCTTGCTCTTAACGGAAGACCGTATCGCAGGGA
TTGCTGAAGGTGTTCGTCAAGTCGCAGATCTCCAAGATCCCATCGGCCAAGTAGTTCGT
GGTTATACAAATCTAGATGGTCTTAAAATTGTTCAAAAGCGTGTTCCCATGGGTGTTAT
CGCTATGATTTTTGAGAGCCGTCCTAATGTTTCAATCGATGCTTTTAGCCTTGCCTTTAA
GACTAATAATGCCATTATTCTTCGTGGAGGACGTGATGCTATCAATTCCAATAAGGCTT
TGGTTACGGTAGCTCGTAAAGCTTTGAAAAATGCAGGAATTACAGCAGATGCCGTTCA
GTTTGTTGAAGATACCTCTCATGAGGTAGCCGAGGAACTCATGGTGGCGACCAAATAT
GTTGACTTGCTCATCCCTCGTGGCGGAGCCCGTCTCATCCAAACTGTGAAAGAAAAAG
CCAAGGTTCCAGTCATCGAAACGGGTGTTGGGAACTGTCATATTTATGTGGATAAATA
TGCTAACTTAGATATGGCGACACAGATTGTCATCAATGCCAAGACCCAACGACCAAGT
GTGTGTAATGCTGCAGAATCTCTCGTTGTTCATGCTGATATTGTAGAAGAATTCTTGCC
TAACTTGGAAAAAGCTATTTTAAAAATTCAGTCTGTTGAGTTCCGCGCGGATGAAAGG
GCTTTGAAACTTATGGAAAAAGCTGTACCCGCTTCACCAGAGGATTTTGCGACAGAGT
TTCTTGACTACATTATGTCTGTTAAAGTAGTAGACAGCCTTGATGAGGCGATTAATTGG
ATTAATACTTACACGACATCACATTCAGAAGCTATCGTGACTCAGGACATCAGTCGTG
CTGAGCAATTCCAAGACGATGTTGACGCTGCAGCTGTCTATGTCAATGCCTCGACTCGT
TTCACAGACGGTTTCGTCTTTGGACTGGGTGCTGAAATCGGAATCTCAACTCAAAAAA
TGCACGCCCGTGGACCAATGGGACTTGAGGCCCTAACTTCAACCAAGTTCTATATTAA
TGGTCAAGGTCAAATTAGAGAATAACCCATCAAGCTCCATGAGGGGCTTTTTGGTCAG
TTTGCTAGAGTAAGACGCTTCTGATCTAGAGTAAGACGCTTCTGATATATTGGGGGCAT
CAAAAATGAGGAGCTTTGGTGATGTTTAAAGACCAATAAAAAGCTTCGTGTATACAAA
GCTAAAAAATGAGGTGATTTCATACGTCAGGTTATACTTGGCGTTTTTTATTATATTTA
AATT

J.2 Sequence of *pst*S from *S. thermophilus* LMG 18311

>gi|55736088:898589-899488 *Streptococcus thermophilus* LMG 18311, complete genome

GTGACAACATATCGGAGGATAGAAATGAAAAAAACAAAAGGCCTTGCAGTGATTTTA
TCACTGGCAATGGCCATGCTAGTCTTAACTGGTTGTGCCTCTTGGATTGACCGTGGTCA
GTCCATTACAGCAGTTGGTTCTACGGCTCTTCAACCCTTAGTAGAAGCAGCATCATATG
GTTTTGCTGAAAAAAATCCTGAAATTGTGGTTAACGTCCAAGGGGGCGGTTCTGGTAC
TGGACTTTCCCAAGTGCAATCAGGTGCTGTTGAAATTGGGAATAGTGACCTTTTCGCTG
AGGAAAAATCTGGAATCGATGCCAGTAAGCTCGTAGACTTTCAAGTTGCAGTTGCAGG
TATTGCAGTTATCACTAATCAGAAAGTATCCGTTGATAATTTGACAACAGAACAACTTC
GTAAAATCTTCACTGGAAAAATCACAAACTGGAAACAGCTTGGTGGACAGGATTTGGA
AATTACTATCGTTAACCGCGCAGCTAGCTCAGGAACACGCGTAACTTTCGATGCTGTG
ATTATGGATGGTAAATCACCAATCCGTACCCAGGAGCAAGATTCTAACGGAATGGTTA
AGTCAATTGTCGCTCAGACACCAGGTGCTATTTCATACCTATCATTTGCTTACCTTGAT
GATTCAGTTAAAACATTGAAACTAAATGGATTTGAACCTAATGCGAAAAATGTTGCGA
CTAACGATTGGCCTATTTGGTCCTACGAGCATATGTATACTAAAGGGAAACCTAATAG
CTACACCAAACAACTCTTAGACTACATGATTAGTGACGAGGTTCAAGAAAATATCGTT
AAAAAAATGGGATACATTCCAATTCATACTATGAAAGTTACTAAGGATGCTGACGGCA
AGGTTACAAAGAAGAGTGAGGAGTAA

J.3 Sequence of *tuf* from *S. thermophilus* LMG 18311

>gi|55736088:467189-468385 *Streptococcus thermophilus* LMG 18311, complete genome

ATGGCAAAAGAAAAATACGATCGTAGTAAACCACACGTTAACATTGGTACAATCGGA
CACGTTGACCACGGTAAAACTACTTTGACAGCTGCAATCACAACTGTATTGGCTCGTC
GTCTTCCTAGCGCAGTTAACACACCAAAAGACTACGCTTCAATCGACGCTGCTCCAGA
AGAACGTGAACGCGGTATCACAATCAACACTGCACACGTTGAATACGAAACTGAAAA
ACGTCACTACGCTCACATCGATGCGCCAGGACACGCGGACTACGTTAAAAACATGATC
ACTGGTGCCGCTCAAATGGACGGTGCGATCCTTGTAGTTGCATCTACTGACGGACCAA
TGCCACAAACTCGTGAGCACATCCTTCTTTCACGTCAGGTTGGTGTTAAACACCTTATC
GTCTTCATGAACAAAGTTGACTTGGTTGACGATGAAGAATTGCTTGAATTAGTTGAAA
TGGAAATCCGTGACCTTCTTTCAGAATACGATTTCCCAGGTGATGACATTCCAGTTATC
CAAGGTTCAGCTCTTAAAGCTCTTGAAGGTGATTCTAAATATGAGGACATCATCATGG
ACTTGATGAATACTGTTGACGAATACATTCCAGAACCAGAACGCGACACTGACAAACC
ATTGTTGCTTCCGGTCGAAGATGTATTCTCAATCACTGGTCGTGGTACTGTTGCGTCAG
GACGTATTGACCGTGGTGTTGTTCGTGTTAATGACGAAGTTGAAATTGTTGGTCTTAAA
GAAGAAAGCCAAAAAGCAGTTGTTACTGGTGTAGAAATGTTCCGTAAACAACTTGATG
AAGGTATTGCCGGTGATAACGTCGGTGTCCTTCTTCGTGGTATCCAACGTGATGAAATC
GAACGTGGTCAAGTATTGGCTGCGCCTGGTTCAATCAAGCCACACACTAAATTCAAAG
GTGAAGTTTACATCCTTACTAAAGAAGAAGGTGGACGTCACACTCCATTCTTCAATAA
CTACCGTCCACAGTTCTACTTCCGTACAACTGACGTAACAGGTTCAATCGAACTTCCTG
CAGGTACTGAAATGGTTATGCCTGGTGATAACGTGACTATCGACGTTGAGTTGATCCA
CCCAATTGCCGTTGAAAAAGGTACAACATTCTCTATCCGTGAAGGTGGACGTACTGTT
GGTTCAGGTATCGTAACTGAAATCGAAGCTTAA

J.4 Sequence of *pnc*B from *S. thermophilus* LMG 18311

>gi|55736088:229074-230609 *Streptococcus thermophilus* LMG 18311, complete genome

TTGCTCAAAAATGCTATAATAGTCAGCATAAACAAGCTATTAATAACAAAAAATAAGT
ACCTATTCGATTGGAGGAAATCCTTGTATAAAGATGATAGTTTAACCTTGCACACGGA
CTTGTATCAAATCAATATGATGCAGGTCTACTTCAACCAAGGTATTCACAATAAAAAG
GCCGTTTTTGAAGTTTATTTCCGTCAACTTCCGTTTAAAAATGGCTTTGCTGTGTTCGCA
GGTCTGGAGCATATTGTCAACTATCTTGAAAATCTGACTTTTTCAGAAACTGATATTGC
TTATCTGAAGGATTTAGGCTATCCGAAGGATTTTCTGGACTATCTGGCCAATCTAAAAC
TCGAGTTGACTATTAATTCAGCCCTTGAGGGTGATTTGGTATTTGCTAATGAACCGATT
TTTCAAGTGGAAGGTCCCTTGGCTCAGTGTCAGTTAGTAGAGACTGCCCTACTGAATAT
CCTTAATTACCAGATTCTTATTGCGACTAAGGCAGCTCGTATTCGTTCTGTTATTGAGG
ATGCTCCTCTGTTGGAATTTGGGACACGTCGTGCCCAAGAGATGGATGCAGCAATATG
GGGGACGCGTGCAGCCGTGATTGGTGGTGCTGACGCAACTTCAAATGTACGTGCAGGT
AAGATTTTCGGTATTCCTGTTTCAGGTACTCATGCCCATGCTCTTGTTCAAGCTTATGG
AAACGATTATGATGCCTTTAAAGCCTATGCATCTACTCATAAAGACTGCATATTTCTTG
TGGATACCTATGATACCCTTAAGATTGGTGTTCCAAATGCTATCCGTGTGGCTAAAGAG
CTAGGTGATAAGATCAACTTCTTGGGTGTTCGTCTTGATTCAGGTGACTTGGCTTATCT
GTCTAAGCAGGTCCGTAAGCAACTAGATGCGGCTGGTTTCCCTGATGCTAAGATTTAC
GCTTCAAATGACCTTGATGAAAATACCATTCTTAACTTGAAAATGCAGAAGGCCAAGA
TTGATGTTTGGGGTGTTGGTACTAATCTTATCACAGCCTATGATCAACCAGCCTTGGGT
GCGGTCTACAAAATTGTCTCAATCGAGAATGATCGGGGAGTCATGCAGGATACCATCA
AGTTGTCCAACAACGCTGAGAAGGTTTCGACACCAGGTAAGAAGCAAGTGTGGCGTAT
TACGAGCCGTGCTAAGGGGAAATCAGAAGGTGACTATATCACCTTCGCAGACACGGAT
GTTAATGCTTTAGAAGAAATTAACATGTTCCACCCGACTTACACCTACATTAACAAGA
CTGTCCGCGATTTTGATGCGGTGCCACTTTTGGTCCCAATCTACGACAAGGGGCAACTA
ATCTATGATTTGCCAAGTCTTGATGAAATCAAGAACTATGCGACTAAGAAATTGGATG
AGCTTTGGAATGAGTACAAGCGCGTTCTTAACCCCCAAGATTATCCAGTTGACTTGGCC
AAAGATGTCTGGGATCACAAGATGACCTTGATTGATAATATGCGTAAGAAAGCCCATG
ACTTGTCAGAGTAA

J.5 Sequence of *pur*K from *S. thermophilus* LMG 18311

>gi|55736088:47051-48142 *Streptococcus thermophilus* LMG 18311, complete genome

ATGAGCTCAACTAAAACCATTGGTATCATCGGTGGTGGCCAGCTTGGTCAGATGATGG
CCATTTCTGCTATCTATATGGGCCACAAGGTTATCACCCTTGATCCTGCATCAGATTGT
CCATCTTCTCGTGTGTCTGAGGTTATCGCGGCACCCTACGATGACGTAGATGCTCTTCG
TCAGTTGGCGGACCGCTGTGATGTTCTCACTTATGAATTTGAGAATGTCGACGCTGACG
GTCTTGACGCTGTCATCAAGGATGGACAACTTCCACAAGGAACAGAACTGCTTCGCAT
TTCACAAAACCGTATCTTTGAGAAGGACTTCCTTTCAAACAAGGCTCAAGTAACGGTG
GCACCTTACAAGGTCGTGACCTCTAGCCTTGATTTGGAAGATATTGATCTTTCTAAAAA
TTACGTCCTCAAGACTGCGACAGGTGGTTACGATGGCCACGGTCAAAAAGTCATCACA
TCAGCCGAAGATTTGGAAGAGGCAAATGCACTTGCTAACTCAGCTGAGTGTGTCTTGG
AAGAGTTCGTCAACTTCGACCTTGAAATTTCGGTTATCGTGTCAGGTAACGGCAAGGA
TGTGACGGTTTTCCCAGTTCAGGAAAATATCCACCGCAACAACATCCTCTCTAAGACTA
TCGTTCCAGCTCGTATTTCTGATAGACTAGCAGACAGAGCTAAAGCTATTGCTGTGAA
GATTGCTGAGCAACTTAACCTCTCTGGTACCCTTTGTGTAGAAATGTTTGCGACAGCTG
ATGACATCATTGTCAACGAAATTGCGCCACGCCCACACAATTCAGGGCACTACTCAAT
CGAAGCCTGCGACTTTTCACAATTTGACACACATATCTTGGGCGTTCTCGGAGCACCAC
TTCCAGCAATCAACCTCCATGAACCTGCTGTTATGCTCAACGTCCTCGGCCAACACGTC
GAAGCAGCTGAGCGTTATGTCACAGAAAATCCAAGCGCCCACCTCCACATGTATGGTA
AACTAGAAGCGAAGCACAACCGAAAGATGGGTCATGTGACTTTGTTTAGTAATGAGCC
AGATAATGTGGTTGAGTTTGGGAAAGGAATTGATTTTTAG

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2002-Present	METU Dept.of Food Engineering	Research Assistant

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

Chapter in book

Gurakan, G. C. and Altay, N. 2010. Yogurt Microbiology and Biochemistry. In *Development and Manufacture of Yogurt and Other Functional Dairy Products*. Ed. Yildiz, F. pp.97-121.CRC Press, Taylor and Francis Group, Fl, USA.

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HOBBIES

Literature, Travel, Photography