SURVIVAL OF PROBIOTIC MICROORGANISMS DURING STORAGE AFTER MARKETING

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ABSTRACT

SURVIVAL OF PROBIOTIC MICROORGANISMS DURING STORAGE AFTER MARKETING

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Probiotics are viable microorganisms that show beneficial effects on the health of the host by improving their intestinal microflora. The microorganisms applied as probiotics mainly include *Lactobacillus* and *Bifidobacterium* species. Probiotics can inhibit the bacterial pathogens, reduce serum cholesterol levels, improve lactose tolerance and stimulate the immune response. They also have other properties such as; tolerance to acid and bile salts, adherence to gastrointestinal cells for colonization, resistance to antibiotics and β-galactosidase acitivity.

The properties of probiotic products are determined by the characteristics of the microorganisms they contain. For that reason, isolation and characterization of new strains having probiotic properties is an important issue. New strains are generally isolated from their natural habitats which are fermented dairy products such as kefir.

In order to exert beneficial health affects in the digestive system, commercial probiotic products should contain adequate numbers of viable cells. Probiotic

microorganisms should protect their viability during their shelf storage. Therefore, the viability of probiotics is especially important for food manufacturers that search for new probiotic strains with good survival and stability properties upon storage.

In this study, probiotic microorganisms were isolated from traditional kefir grains known as a 'complex probiotic'. The isolates were firstly identified using biochemical tests, then the putative species belonging to '*Lactobacillus acidophilus* group' were identified with 16S rRNA gene sequencing. Analysis of sequencing resulted in differentiation of "*L. acidophilus* group" organisms, namely *L. amylovorus* and *L. acidophilus*. Moreover, typing of commercial and traditional *L. acidophilus* strains and *L. amylovorus* strains were performed with RAPD-PCR by using primer M13. While several *L. acidophilus* strains showed different RAPD fingerprints most of the *L. acidophilus* and *L. amylovorus* strains could not be differentiated due to high similarity of their RAPD fingerprints. Following identification, survival of these isolates in probiotic yogurt preparations were investigated and compared to the survival of commercial probiotics.

Consequently, although the survival of kefir grain isolates were less than commercial probiotics, they sustained the minimum recommended level for probiotics (10^6 cfu/ml) during cold storage. Such level of survival makes them considerably good candidates to be used as commercial probiotic cultures.

Keywords: Probiotics, survival, kefir grains, *L. acidophilus*, shelf life, 16S rRNA gene.

ÖZ

PROBİYOTİK MİKROORGANİZMALARIN PAZARLAMA SONRASI, DEPOLAMA SÜRESİNDEKİ CANLILIKLARI

Köse, Işkın

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Probiyotik mikroorganizmalar bağırsak florasını düzenleyerek konağa fayda sağlayan mikroorganizmalardır. Probiyotik olarak kullanılan mikroorganizmalar genellikle *Lactobacillus* ve *Bifidobacterium* cinslerine aittir. Probiyotikler patojenleri inhibe etmekte, serum kolesterol seviyesini azaltmakta, laktoza karşı toleransı arttırmakta ve bağışıklık sistemini düzenlemektedir. Bunların dışında safra ve mide asitlerine karşı dayanıklı olma, mide ve bağırsak hücrelerine tutunarak koloni oluşturabilme, antibiyotiklere karşı dirençli olma ve β-galaktosidaz aktivitesi gösterebilme gibi özellikleri vardır.

Probiyotik ürünlerin özellikleri içerdikleri mikroorganizmaların karakteristikleri tarafından belirlenir. Bu nedenle probiyotik özelliklere sahip yeni suşların izolasyonu ve karakterizasyonu önemlidir. Yeni suşlar genellikle doğal habitatları olan fermente süt ürünlerinden izole edilmektedir. Fermente süt ürünü olan kefir bunlardan bir tanesidir.

Probiyotik ürünlerin sindirim sistemine faydalı etkilerini gösterebilmeleri için ticari probiyotik ürünler yeterli sayıda canlı hücre içermelidirler. Ayrıca probiyotik mikroorganizmalar probiyotik ürünlerin raf ömürleri süresince, canlılıklarını korumalıdırlar. Bu nedenle probiyotiklerin canlılığı depolama süresince stabilite ve canlı kalma özelliği gösteren yeni suş arayışları içerisinde olan gıda üreticileri için önem taşımaktadır.

Bu çalışmada, 'kompleks probiyotik' olarak bilinen gelenkesel kefir mayasından probiyotik mikroorganizmalar izole edilmiştir. İlk olarak biyokimyasal testler ile tanımlamaları yapılmış ve ardından '*L. acidophilus* grup' una ait olabilecek olan olası türlerin tanımlanması 16S rRNA geni dizilemesi ile yapılmıştır. Sekans analizi '*L. acidophilus* grup'organizmalardan *L. acidophilus* ve *L. amylovorus* 'u ayırt etmiştir. Ayrıca, ticari ve geleneksel *L. acidophilus* türleri ile kefirden izole edilen *L. amylovorus* suşlarının RAPD-PZR ile tiplendirilmesi M13 primeri kullanılarak yapılmıştır. Birkaç *L. acidophilus* türü farklı parmakizi gösterirken, çoğu *L. acidophilus* ve *L. amylovorus* türü RAPD parmakizlerinin yüksek oranda benzemesinden ötürü ayırt edilememiştir. Tanımlamayı takiben, izolatların probiyotik yoğurt içerisindeki canlılığı araştırılmış ve ticari probiyotiklerin canlılığı ile kıyaslanmıştır.

Sonuç olarak kefir izolatları, ticari probiyotiklerden daha düşük bir canlılık göstermelerine rağmen probiyotik mikroorganizmalar için önerilen en düşük değer olan 10^6 kob/ml değerini soğuk depolama süresince korumuşlardır. Bu gibi iyi bir canlılık göstergesi probiyotik olarak kullanabilmeleri için onları dikkate değer kılmıştır.

Anahtar sözcükler: Probiyotikler, canlılık, kefir mayası, *L. acidophilus*, raf ömrü, 16S rRNA gen.

To my family;

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LIST OF ABBREVIATIONS

bp: Base Pair

CD: Crohn's Disease

CT: Cholera Toxin

DGGE: Denaturing Gradient Gel Electrophoresis

dNTP: Deoxyribonucleoside Triphosphate

DNA : Deoxyribonucleic Acid

FAO/WHO: Food and Agricultural Organization and World Health Organization

GC: Guanine-Cytosine

IgA: Immunoglobin A

IgE: Immunoglobin E

ILSI: International Life Science Institute

NCBI: National Center for Biotechnology Information

OD: Optical Density

PCR: Polymerase Chain Reaction

RAPD: Randomly Amplified Polymorphic DNA

rRNA: ribosomal RNA

rpm: Revolution Per Minute

SEM: Scanning Electron Microscope

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SSU: Small subunits

TBE: Tris Borate EDTA

TH: T helper cells

UC: Ulserative Colitis

UHT: Ultra High Temperature

UPGMA: Unweighted Pair Group Method of Aritmetic Mean

UV: Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Functional Foods

Consumers increasingly become conscious for their health and they have started to search for ways to improve their life quality. They expect the food that they consume to be healthy and also capable of prevent illness. Besides, the development of chemical and biological sciences and the high cost of treating chronic diseases changed the focus of medicine from the treatment of disease to prevent the disease. This tendency for health care and prevention of diseases accelerated the research and development activites in functional foods (Vasilijevic and Shah, 2008).

The functional food concept first came up, a long time ago in Far Eastern countries as a thought that food can act as a drug (Kwak et al., 2001). The idea started with the dictum of Hippocrates; ‘ Let food be your medicine’. Nowadays, it is known that, diet is a crucial factor for the regulation of physiological functions in the body (Vasilijevic and Shah, 2008).

Functional foods are defined as; foods that provide not only nutrients but also provide some components to the organism that prevent or cure diseases. Beside their nutritional value, these foods, also have physiological affects on the consumer. As reported by International Life Science Institute (ILSI), in order to regard a food as functional, it should have beneficial affects on the specific function or functions of the body, besides its nutritional value (Kvak and Jukes 2001, Roberfroid 2002, Prado et al., 2008).

Fermented dairy products can be included in functional foods because of their health effects on the intestinal flora of humans. Nowadays, fermented dairy products are curiously researched because of their potential health benefits. One of their use is being vehicles for probiotics. Lactic acid bacteria include an important group of microorganisms that are used in the production of dairy products and most of them are considered as probiotics (Robinson et al., 2000, Ljung and Wadstrom 2006, Prado et al., 2008).

There are numerous functional foods available, among them probiotics are the most important. Because of their well-studied and established health benefits, they symbolise a strong and wide area in the concept of functional foods and an area for dairy products investigations (Vasilijevic and Shah, 2008, Prado et al., 2008).

1.1.1. History of Probiotics

From the second half of 19th century, scientists realized that traditional milk compounds have lots of benefical effects beside prolonging shelf-life. From that time, they studied the interactions between human host and microorganisms (Goktepe et al., 2005). As a consequence in numerous studies, considerable developments were achieved on the knowledge of the microbiology of human body (Vasilijevic and Shah, 2008).

As early as 1885, Escherich described the microbiota by discovering the bacteria in the intestine and faeces. He found out the physiology of digestion and the therapy for the intestinal diseases were originated from intestinal microorganisms. Döderlein, (1892) was the first who suggested that the bacteria in the vagina prevent or inhibit the growth of pathogenic bacteria by producing lactic acid from sugars. Even at those days, lactic acid producing bacteria were named as 'lactic acid bacteria' and the association between lactic acid bacteria and fermented milk products was known. In 1900, Tissier and Moro isolated some microorganisms from breast-fed infants. Tissier found an anaerobically cultured organism whose morphology and staining results were similar to lactobacilli, however, whose appearance was bifurcated. So he termed them as *Bacillus bifidus.* Moro named the

isolate as *Bacillus acidophilus* since it had acid tolerance and told that the origin of the bacteria was mother's breast (Vasilijevic and Shah, 2008).

At the same time, Elie Metchnikoff, Russian bacteriologist with a Nobel Laureate, noticed that Bulgarian people have an average life of 86 years which was extraordinary for that time. The main difference in their nutrition was high consumption of fermented milk. Metchnikoff suggested that the long life of Bulgarians resulted from high consumption of milk products. In his theory 'longevity-without aging', he stated that lactic acid bacteria prevents the toxin production by pathogenic bacteria and as a result, life span increased (Vasilijevic and Shah, 2008).

Metchnikoff s theory was based on a bacteria which was isolated by Grigorrof (1905). Grigorrof used this bacteria for the production of a Bulgarian yogurt which is called 'kiselo mleko' and named the bacteria *Lactobacillus bulgaricus*. During his study he found another organism; *Streptococcus thermophilus*. Until that time *S. thermophilus* was considered as a pathogenic organism. As a result of Metchnikoff's studies, Grigoroff believed that *L. bulgaricus* could colonize in the intestine and prevent the colonization of harmful bacteria. On the other hand, Herter and Kendall (1908) revealed that *L. bulgaricus* can not colonize in the gut. Such findings conflicted with Metchnikoff's approach. However, scientists carried on studying health benefits of bacteria for human health and found *L. acidophilus* strains that could be able to colonize digestive tract. Rettger and Horton (1914), found that feeding the rats or human with milk, cause a change in the intestinal microflora which ends with high numbers of *Lactobacillus bifidus* and *Lactobacillus acidophilus*. Such studies increased curiosity of scientists about *L. acidophilus* and products of its fermentation. In 1930, a strain of *Lactobacillus* that was able to survive in the gastrointestinal tract was isolated. This strain was identified as *Lactobacillus casei* strain Shirota. This strain was then used for the manufacturing of fermented dairy product called 'Yakult'.

Such studies performed resulted with the start of probiotic concept (Vasilijevic and Shah, 2008, Hattingh and Viljoen, 2001).

1.1.2. Definitons of probiotics

'Probiotic' word arised from Greek words and means 'for life'. The name was first mentioned by Kollath in 1953 to describe the organic and inorganic food complexes contrary to antibiotics. Then, Vergin (1954) suggested that the disrupted balance caused by the usage of antibiotics in human body, could be restored with a probiotic diet. In 1965, Lilly and Stilwell defined probiotics as microorganims that promote the growth of other microorganisms. Sperti (1971) and Cook (1973) expressed probiotics as compounds which provoke microbial growth and promote immune response. In 1974, Parker described the probiotics as microorganisms that affect the intestinal microbial balance. Fuller (1989) defined probiotics as 'live microbial supplements which benefically affects the host animal by improving its intestinal microbial balance' (Lee and Salminen, 2009). The latest definition was stated by Food and Agricultural Organization and World Health Organization (FAO/WHO) in 2002; 'live microorganisms that when administered in adequate amounts confer a health benefit on the host'.

1.1.3. Human gastrointestinal tract as an ecosystem

The gastrointestinal system and its surface in human body, contributes an environment for microbial colonization (Holzapfel et al., 2002). The area of our gastrointestinal system (150-200m^2) is much larger than the skin surface (2m^2). Such a huge area, with its circular folds and intestinal villi provides an area for the interactions during the digestive process and for the adhession to the mucosal wall (Holzapfel et al., 1998).

Intestinal epithelium and its microbial flora act as a barrier in order to prevent the colonization of pathogenic bacteria and also prevent the uptake of harmful compounds and antigens to the gut lumen. In a healthy individual, this barrier is

stable and protects the host. In addition to host protection, it provides immunological resistance and makes intestine function normally (Holzapfel et al., 1998).

The flora of the intestine start to change from the first day of a newborn to the adulthood period. The intestinal flora of a newborn infant does not have any bacterial colonization but just after birth, colonization starts. In a few days, coliforms, clostiridia, enterococci and lactobacilli start to colonize. Bifidobacteria colonize the gut afterwards (Holzapfel et al., 2001, Benno et al., 1984).

The bacterial population of a human adult is approximately 10^{14} viable cells. This number is 10 times more than all of the human cells in body (Luckey and Floch, 1972). The numbers of microbial populations through the gastrointestinal tract varies in number; 10^{1} to 10^{3}cfu/g in the stomach, 10^{9} cfu/g in terminal ileum and 10^{7}cfu/g in the jejenum where the main population is generated by lactobacilli, enterobacteriaceae and streptococci. The large intestine is mainly colonized by *Bacteriodes* and the Gram positive *Eubacterium* and *Bifidobacterium.* (Figure 1.1.). Clostridia, streptococci and lactobacilli also play an important role like sustaining the stability of gut mucosa. In the small intestine the main role belongs to lactobacilli. A healthy human adult has 10^{3} to 10^{4} cfu/g lactobacilli in his/her oral cavity, 10^{3} to 10^{7} cfu/g in the ileum and 10^{4} to 10^{8} cfu/g in the colon.

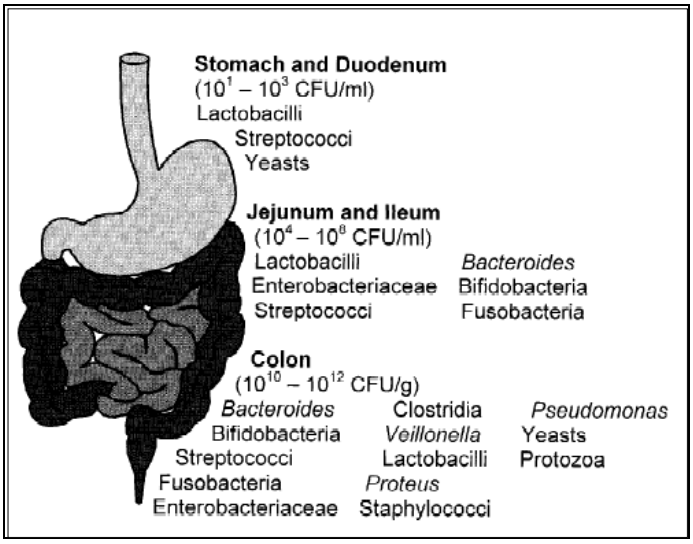

Figure 1.1. Microbial colonization of human gastrointestinal tract (Holzapfel et al.,1998).

This dense population help the digestion process and in addition, toxification and detoxification processes, which have important roles in human immune system (Goktepe et al., 2005).

A healthy intestinal epithelium with its stable microbial population, is a main barrier for the prevention of the colonization of pathogenic microorganisms. Moreover, the intestinal mucosa assimilates antigens and this triggers specific immune response mechanisms in the villus epithelium and Peyer's patches. The major physiological functions of the gut microflora can be listed as follows (Holzapfel et al., 2002, Goktepe et al., 2005);

- Restoration and maintenance of the barrier function,
- Immune system stimulation,
- Maintenance of mucosa nutrition by degrading certain components,
- Improvement of the bioavailibility of nutrients,
- Bowel motility stimulation,

1.1.4. Probiotic Microorganisms

A number of microbial species and genera with their functional properties are supplied in the market as yogurt type fermented products, in lyophilized form, as food supplements or as pharmaceutical forms (Goktepe et al., 2005; Vasilijevic and Shah 2008,).

A variety of different human probiotic preparations are commercially available. It has been estimated that approximately 70 different probiotic containing products are marketed worldwide. Organisms that are generally used as probiotics include species of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium* and *Enterococcus* (Table 1.1.). Among them, the main species are *Lactobacillus acidophilus* and *Bifidobacterium* spp. because of their long and safe history in dairy products (Shah 2007).

Table 1.1. Microorganisms used as probiotics (Holzapfel et al., 1998; Robinson 2002)

Lactobacilli	Bifidobacteria	Other lactic acid bacteria	Non-Lactics
L. acidophilus	*B. adolescentis*	*Ent. facealis*	*B. cereus*('toyoi')[a,d]
L. amylovorus	*B. adolescentis*	*Ent. faecium*	*E. coli* (Nissle 1917)[d]
L. crispatus	*B. bifidum*	*Lacto. lactis*[c]	*P. freudenreichii*[a,d]
L. gallinarum[a]	*B. breve*	*Leuc. mesenteroides*[c]	*S. cerevisiae ('boulardii')*[c]
L. gasseri	*B. infantis*		
L. johnsonii	*B. lactis*		
L. paracasei	*B. longum*		
L. plantarum			
L. reuteri			
L. rhamnosus			

[a] Mainly used for animals

[b] Probably synonymus with *B. animalis*

[c] Little known about probiotic properties

[d] Mainly as pharmaceutical preparations

The consumption of probiotic bacteria in food products is a way for preserving the microbial balance in human digestive tract. Lots of bioproducts like probiotic yogurt, fresh milk, cottage cheese, health food and ice cream have been produced as delivery agents of probiotics into human gastrointestinal system (Hattingh et al., 2001). These products mainly include different commercial strains of lactic acid bacteria (Table 1.2.).

Table 1.2. Worldwide marketed probiotic bacteria (Prado et al., 2008)

Strains	Company
L. casei F19	Arla Foods, Denmark /Sweden
L. casei	ShirotaYakult, Japan
L. reuteri MM53	BioGaia, Sweden
L. crispatus CTV05	Gynelogix, Sweden
B. lactis HN019	Danisco, France
L. rhamnosus GG	Valio, Finland
P. freudenreichii ssp. shermanii	JSValio, Finland
L. acidophilus NCFB 1748	Rhoida, USA
L. acidophilus NCFM	Rhodia, USA
L. casei DN-114001	Danone, France
L. plantarum 299v	Probi AB, Sweden
L. rhamnosus 271	Probi AB, Sweden
L. acidophilus LA10 (NCC 90)	Nestle-, Switzerland
L. johnsonii LA1 (NCC 533)	Nestle-, Switzerland
L. casei CRL 431	Chr. Hansen, USA
B. lactis BB-12	Chr. Hansen, USA
L. acidophilus LA-5	Chr. Hansen, USA
L. acidophilus LA-1	Chr- Hansen, USA
L. bulgaricus LBY27	Chr. Hansen, USA
S. thermophilus STY-31	Chr. Hansen, USA
L.fermentum RC-14	Urex, Canada
L. rhamnosus GR-1	Urex,Canada
B. animalis DN-173010	Danone, France

Table 1.2. Worldwide marketed probiotic bacteria (Prado et al., 2008)(cont'd).

Strains	Origin
L. acidophilus DDS-1	Nebraska cultures
L. acidophilus SBT2062	Snow brand milk products

1.1.5. General properties of lactic acid bacteria

Lactic acid bacteria are Gram-positive non-spore forming, non-pigmented, catalase negative microorganisms. They grow anaerobically but are aerotolerant microorganisms. They are strictly fermentative and lactic acid is produced as a major product from sugar fermentation. In order to grow they require specific amino acids, B vitamins and other growth factors (Marth and Steele, 2001).

The lactic acid bacteria group consist of ; *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc, Pedicococcus, Carnobacterium, Aerococcus, Tetragenococcus*. The GC (guanine-cytosine) content of the genera in lactic acid bacteria is lower than 55 % mol DNA **(**Tran et al., 2004).

Lactic acid bacteria are divided into two main groups according to their sugar fermentation pattern; homofermentative and heterofermentative. Homofermentative lactic acid bacteria, apply Embden-Meyerhof-Parnas pathway for fermentation and as a result of their fermentation they convert their carbon source into lactic acid (Homolactic metabolism). Heterofermentative lactic acid bacteria apply 6-phosphogluconate/ phosphoketolase pathway and as a result of this metabolism (heterolactic metabolism) beside producing lactic acid they also produce CO_2, lactate, ethanol or acetate as end product (Tran et al., 2004; Vasilijevic and Shah, 2008).

Lactic acid bacteria are the major group used in fermentation of dairy products. Besides dairy products, they are also found in meat, beverages and vegetables. Among lactic acid bacteria, lactobacilli is the one of the most interested since they

are found in raw milk and other fermented milk products (Couret, 2003; Tran et al., 2004).

1.1.5.1. The Genus Lactobacilli

Lactobacilli are Gram-positive, non spore forming, non-flagellated rod shape bacteria (0,5-1,2 x 1-10μm). They are the largest group in lactic acid bacteria and include a variety of different species according to their biochemical, phenotypicand physiological properties. This diversity arises from their GC content, which is between 32-55%. They are strictly fermentative and microareophilic or anaerobic. Some of them are homofermentative and some are heterofermentative. Some examples of homofermentative and heterofermentative members are given in Table 1.3 (Tran et al., 2004). Lactobacilli can grow between 5°C and 53°C, but their optimum growth temperature is between 30-40°C (Gomes and Malcata, 1999, Breed et al., 1957, Robinson et al., 2000). They are acidophilic and the optimum pH is 5.5-6.2 but they can also grow at a pH of 5.0 or less (Breed et al., 1957).

Table 1.3. The Genus *Lactobacillus* (Tran et al., 2004; Stiles and Holzapfel, 1996).

Character	Group I, obligate Homofermenter	Group II, Facultative Heterofermenter	Group III, Obligate Heterofermenter
Pentose fermentation	_	+	+
CO_2 from glucose	_	_	+
CO_2 from gluconate	_	+	+
FDP Aldolase	+	+	_
Phoshpoketolase	_	+	+
	L. acidophilus	*L. casei*	*L. brevis*
	L. delbrueckii	*L. curvatus*	*L. buchneri*
	L. salivarius	*L. plantarum*	*L. fermentum*
	L. helveticus	*L. sakei*	*L. reuteri*
	L. amylovorus		
	L. gasseri		

1.1.5.1.1. *Lactobacillus acidophilus as a member of 'L. acidophilus group'*

Lactobacillus acidophilus, which means 'acid-loving' bacteria was first isolated from infant faeces by Moro in 1900 and named as '*Bacillus acidophilus*'. In 1929, the species was placed in the genus '*Lactobacillus*'. Afterwards it was further investigated in terms of phenotypic properties like sugar fermentation patterns, serological properties, G+C content and DNA-DNA hybridization.

In 1980, strains of *Lactobacillus* that were originally identified as *L. acidophilus* according to sugar fermentation patterns and lactic acid isomers, were divided into several groups at species level according to their DNA-DNA hybridization (Fujisawa et al., 1992).

DNA-DNA hybridization studies performed by Johnson et al. (1980) revealed the heterogenity of *L. acidophilus* and persistence of six different homology groups. Strains in the homology group that exhibited high degree of DNA relatedness with *L. acidophilus* were included in this species. On the other hand, members of other homology groups were classified as; *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus johnsonii*, *Lactobacillus gasseri* and *Lactobacillus crispatus* (Holzapfel et al., 2001).

Today, six homology group have been suggested in the '*L. acidophilus* group' as valid species. Lauer et al. (1980) divided the group into two main groups; A and B. Johnsonn et al. (1980) divided the groups into group I and group II (Table 1.4., Göktepe et al., 2006).

Table 1.4. Species of the '*Lactobacillus acidophilus'* group (Holzapfel et al., 1998, Goktepe et al., 2006)

		DNA homology groups	
Species	G+C %	Lauer et al.	Johnson et al.
L. acidophilus	32-37	Ia	A-1
L. amylovorus	40	Ib	A-3
L. crispatus	35-38	Ic	A-2
L. gallinarum	33-36	Id	A-4
L. gasseri	33-35	IIa	B-1
L. johnsonii	32-38	IIb	B-2

Despite the fact that they are different, the species of '*L. acidophilus* group' are closely related so that they can not be differentiated from each other by simple phenotypic tests. The reason is that biohemical and physiological tests are limited when discriminating closely related species (Kwon et al., 2004). In order to detect their phylogenetic relationships, comparison of the genes encoding rRNA regions is perhaps the most common genetic tool. By using 16S rRNA gene sequences of '*L. acidophilus* group' organisms, their dendograms have been created. According to the dendograms obtained *L. acidophilus* group organisms were shown to belong to the same phylogenetic group (Figure 1.2).

According to 16S rRNA gene sequence analysis, *L. acidophilus* is most closely related to *L. helveticus*, *L. crispatus* and *L. amylovorus. L. gasseri* and *L. johnsonii* are close to each other in the group but they are more distant to *L. acidophilus* (Robinson et al., 2000).

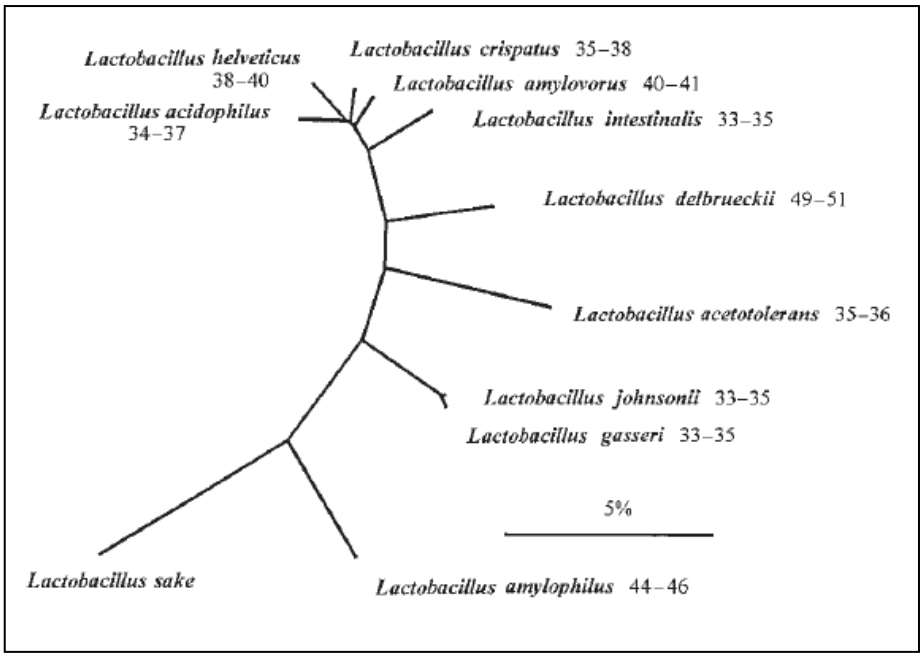

Figure 1.2. Phylogenetic relationships within the '*L. acidophilus* group' based on 16S rRNA gene sequencing (Holzapfel et al., 2001).

L. acidophilus is a rod shaped bacteria with rounded ends (0,5-1 x 2-10 μm). The colonies occur single, in pairs or in short chains (Figure 1.3.)

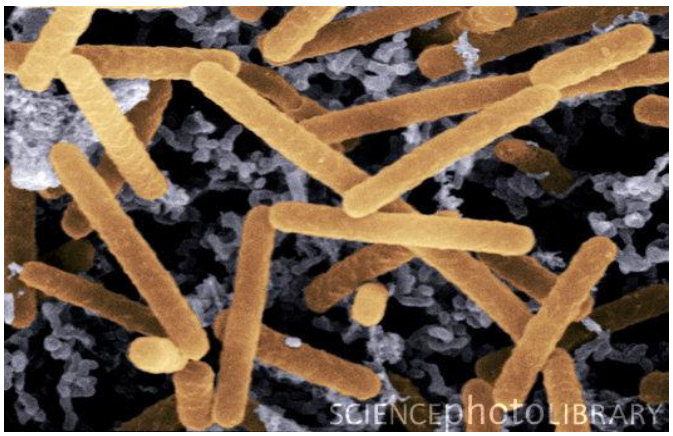

Figure 1.3. SEM of *L. acidophilus* (http://www.sciencephoto.com)

L. acidophilus is a microaerophilic bacteria and can also grow aerobically in static cultures without shaking. Beside this, it grows better in an anaerobic gas mixture consisting of 5% CO_2, 10% H_20 and 85 % N (Robinson et al., 2000). In order to grow, it requires calcium pentothenate, folic acid, niacin and riboflavin (Breed et al., 1957). Amygladin, cellobiose, fructose, galactose, lactose, maltose, glucose, mannose, salicin, sucrose, aesculin and trehalose are fermented by most strains of *L. acidophilus*. The optimum growth temperature is between 35°C and 40° but can also grow at 45°C. The optimum pH for *L. acidophilus* is between 5.5-6.0 (Gomes and Malcata, 1999).

L. acidophilus is one of the mostly used species in the group as a probiotic and has the ability to show beneficial effects on the microflora of gastrointestinal system. It has been widely used for the production of dairy products such as probiotic yogurts or dietary supplements.

L. amylovorus have been firstly isolated and identifed in a study of Nakamura (1981). In their study, several *Lactobacillus* species from cattle waste corn fermentations that have ability to hydrolyze starch were evaluated. The species studied exhibited different morphology, fermentation characteristics, DNA base compositions and amylolytic activities than *L. acidophilus*. This species was named as '*L. amylovorus'* (Gr. n. amylum: starch, L. v. Voro: to devour).

L. amylovorus is a rod shaped bacterium (1 x 3-5 μm). It has the ability to grow at 45°C but not at 15°C. This species can ferment cellobiose, esculin, fructose, galactose, glucose, maltose, mannose, salicin, sorbitol, sucrose and trehalose. In order to grow, it requires some growth factors like; niacin, pathothenic acid, folic acid and riboflavin (Breed et al., 1957).

In further studies, *L. amylovorus* has been isolated from the intestine of piglets where it is found in large numbers. Several studies dealing with this species showed that it has some probiotic properties including antimicrobial effect against several pathogens. Kant et al., (2011) reported the genome sequence of *L. amylovorus* GRL1118 isolated from porcine ileum. *L. amylovorus* GRL1118 possesses S-layer

proteins and adhere strongly to the pig intestine. This feature makes them notable as probiotics.

S-layer proteins are responsible for the protection of the cell from environmental hazards and maintain cellular integrity. S-layer proteins of *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lactobacillus crispatus* also have the ability to adhere to the host ephitelial cells (Kant et al., 2011).

In a study of Petsuriyawong and Khunajakr (2011), the probiotic properties of lactic acid bacteria isolated from piglet faeces have been studied. The isolates were primarily evaluated in terms of their antimicrobial acitivity against pathogenic bacteria. The isolates that had antimicrobial activity were then evaluated for their acid and bile salt tolerance. The species that exhibited high survival rate under gastric and intestinal conditions were characterized as *L. amylovorus* and *L. reuteri.* In addition, these species could adhere to the ephitelial cells. According to the results they were considered as candidates probiotics.

1.1.6. Necessary characteristics for probiotic cultures

The cultures used as probiotics should have some technological and physiological characteristics in order to provide their health benefits. One of the most important criteria is the number of probiotic bacteria in the products, since in order to show their therapeutical health effects, the suggested concentrations of probiotics in products should be at least 10^6 cfu/ml (Donkor et al., 2007). There are other researchers suggest that the concentration should be above 10^{7}-10^8 cfu/ml. (Hattingh and Viljoen, 2001; Vinderola and Reinheimer 2000). The recommended levels are suggested in order to compensate the decrease in numbers of probiotics during storage and in the intestinal conditions (Vasilijevic and Shah, 2008).

Numbers of criteria have been suggested for the selection of probiotics. They are listed as follows (Morelli 2007, Shah 2006, Sandholm et al., 2007);

- Acid tolerance and tolerance to human gastric bile,

- Adhesion to the mucosal surface of gastrointestinal tract,
- Immunomodulation,
- Antogonistic acitivity against pathogens like *Helicobacter pylori*, *Salmonella* spp., *Clostiridium difficile*
- Antimutagenic and anticarcinogenic effects,
- Phage resistance,
- Good sensory propeties,
- Viability and activity during processing and storage.

Viability and activity of probiotics during storage is an important issue, since the bacteria should survive during the shelf life of the product and should also survive in the acidic conditions of the stomach.

The dairy industry searches to produce products that contain enough number of probiotics in order to guarantee their health benefits. For this reason, there is a need for reliable enumeration of probiotics in the fermented milk products (Robinson et al., 2000).

1.1.7. Selective media for the enumeration of *L. acidophilus*

In order to evaluate the quality of a probiotic product, the important parameter is the ability to count the probiotic bacteria differentially. Because of the presence of similar microbes in a product, it is difficult to enumerate probiotic bacteria differentially.

Different media have been suggested for the selective enumeration of *L. acidophilus* from mixed bacterial populations. One of them is Bile-MRS; suggested for the selective enumeration of *L. acidophilus* in the presence of yogurt bacteria and *Bifidobacterium*. Bile-MRS agar (0,15 % w/v) with aerobic incubation, was found to eliminate the growth of yogurt bacteria and aerobic incubation suppressed the growth of anaerobic *Bifidobacterium* (Vinderola and Reinhemier, 1999; Mortazavian et al., 2007; Antunes et al.,2005).

Another suggested medium is MRS-maltose; recommended for the the selective enumeration of *L. acidophilus* in the presence of yogurt bacteria, if the product does not contain *Bifidobacterium* (Shah, 2000). On the other hand, Dave and Shah (1996) suggested the usage of MRS-maltose for the total counts of *L. acidophilus* and *Bifidobacteria.*

In another study, MRS-salicin and MRS-sorbitol was found to be selective for the enumeration of *L. acidophilus*, but sorbitol was favoured because of enumeration with salicin was costly compared to sorbitol. MRS-sorbitol was found to be selective for the enumeration of *L. acidophilus*, since *L. delbureckii* ssp. *bulgaricus*, *S. thermophilus* and *Bifidobacterium* did not grow on this medium. (Shah, 1999) (Table 1.5.).

Table 1.5. Sugar fermentation patterns of yogurt bacteria, *L. acidophilus* and *Bifidobacterium* (Shah, 1999).

Strains	**Salicin**	**Cellobiose**	**Fructose**	**Mannitol**	**Sorbitol**	**Glucose**
S. thermophilus						
2000	-	-	++	-	-	++
2002	-	-	++	-	-	++
2008	-	-	++	-	-	++
2010	-	-	++	-	-	++
2013	-	-	++	-	-	++
2014	-	-	++	-	-	++
L. delbrueckii* ssp. *bulgaricus						
2501	-	-	++	-	-	++
2505	-	-	++	-	-	++
2515	-	-	++	-	-	++
2517	-	-	++	-	-	++
2519	-	-	++	-	-	++
L. acidophilus						
2400	+++	++	++	+	+	++
2401	+++	++	++	+	+	++
2404	+++	++	++	+	+	++
2405	+++	+++	++	+	+	++
2409	+++	+++	++	+	+	++
2415	+++	+++	++	+	+	++
***Bifidobacterium* spp.**						
B. bifidum 1900	-	+	++	-	-	++
B. bifidum 1901	-	-	++	-	-	++
B. infantis 1912	-	-	++	-	-	++
B. adolescentis 1920	-	-	++	-	-	++
B. breve 1930	-	-	++	-	-	++
B. longum 1941	-	+	++	±	±	++
B. longum 20097	-	-	++	-	-	++
B. pseudolongum 20099	-	++	++	+	+	++
B. thermophilum 20210	-	-	++	-	-	++
-, no growth; ± pin point colonies; +, colony size 0.1 to 0.5 mm; ++ colony size 0.6 to 1.5 mm; +++, colony size >1.5mm.						

1.1.8. Factors affecting viability of probiotics in dairy products

Some probiotic microorganisms are influenced easily from the environmental conditions such as high temperature, high osmotic pressure, presence of oxygen and high acidity level. thus viability and the stability of probiotics is a important issue for the industry (Hattingh and Viljoen 2001).

In addition, most of probiotic definions underline that these micoorganisms should be viable until consumption and also in the intestine. In order to exert their beneficial health effects, they should reach the intestine in active and viable form (Ouwehand et al.,1991).

The viability of probiotic microorganisms depends on various factors such as; the strains used, the interaction between species, dissolved oxygen, final acidity, and presence of hydrogen peroxide coming from bacterial metabolism. Moreover, availibility of nutrients, growth promoters and inhibitors, inoculation level and fermentation time may also affect their viability (Hattingh and Viljoen 2001; Shah 2000).

Oxygen in probiotic products affect the viability of probiotics since *L. acidophilus* and *Bifidobacterium* are microaerophilic and anaerobic, respectively. These bacteria lack a complete electron transport chain and do not have the ability to reduce oxygen completely to hydrogen peroxide. They also lack the enzyme 'catalase' which functions for the decomposition of hydrogen peroxide into water. As a result, due to the accumulation of hydrogen peroxide, cell death occurs (Vasilijevic and Shah, 2008).

The final pH of the probiotic products is another crucial factor affecting viability. When the pH of the product decreases under 4.4, probiotics usually start to decrease in number. The decrease of the pH usually occurs in yogurt production (post-acidification) and arise from the metabolic acitivity of starter culture *L. delbrueckii* ssp. *bulgaricus* (Donkor et al.,2006).

1.1.9. Improvement of the survival of probiotics

For the improvement of the viability of probiotics, different methods have been suggested including controlling and modifying the production process and storage conditions.

According to Marshall (1992), post acidification can be avoided by lowering the storage temperature to less than 3-4°C. Yogurt is normally fermented at 43°C, the optimal temperature for growth of starter cultures. However during the manufacturing process of a probiotic yogurt, lowering the incubation temperature to 37-40°C will favour the growth of probiotic cultures (Lourens and Hattingh, 2001).

Addition of growth promoting substances is another way for the improvement of the survival of probiotics. Adding casitone, casein hyrdolysate, fructose and whey protein concentrate trigger the growth of *L. acidophilus*. This occurs because of the availability of growth promoters for *L. acidophilus*: minerals and sugars, especially glucose and fructose (Lourens and Hattingh, 2001).

Another suggested method is microencapsulation; in which probiotics are encapsulated in a gelatine or vegetable gum. It is known to be an effective method for the protection of probiotics, especially for acid sensitive ones, to retain their viability (Vasilijevic and Shah, 2008).

1.1.10. Health Effects of Probiotics

From the first appearance of probiotics, numbers of health benefits have been attributed to the products that contain probiotic microorganisms. While some of these benefits are well studied and established, some of them need more research. Due to the strain specifity of health benefits, there is no universal strain that show all benefical health effects. The mostly investigated probiotic cultures are *L. rhamnosus* GG (Valio), *L. casei* Shirota (Yakult), *B. animalis* BB-12 (Chr-Hansen) which are effective against lactose malabsorbtion, rotaviral diarrhoea and *Clostridium difficile* diarrhoea (Vasilijevic and Shah, 2008). Some of the health benefits of probiotics are listed as follows;

1.1.10.1. Reducing Lactose Intolerance

Lactose, the principal carbohydrate of milk, is normally hydrolyzed with the *β*-galactosidase enzyme into its monosaccharides, glucose and galactose. Glucose and galactose are then absorbed in bloodstream (Vasilijevic and Shah, 2008). People who have lactose malabsorbtion, lack the β-galactosidase enzyme and lactose can not be digested into its monomers. These people also suffer from 'gastric distress' when they consume milk or milk products. Undigested lactose cause some microbial action and as a result hydrogen gas is produced (Shah 2007).

The starter cultures *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and probiotic bacteria *L. acidophilus* and *B. Bifidum* produces β-galactosidase enzyme. With the consumption of fermented milk products like probiotics, lactose can be hydrolyzed (Hattingh and Viljoen et al., 2001).

1.1.10.2. Effectivenes against diarrhoea symptoms

Diarrhoea symptoms are often caused by *C. difficile* as a result of microbial imbalance. *C. difficile* is a gastrointestinal microorganism which is normally found in low numbers. With usage of antibiotics, the intestinal microflora changes and the numbers of *C. difficile* increases. In order to reconstitute the intestinal flora, probiotics should be consumed. In clinical studies, it was found that the consumption of probiotics reduced the diarrhoea, arising from antibiotic treatment, by %52 (Vasilijevic and Shah, 2008).

Another diarrhea symptom is caused by Rotavirus infection. Rotavirus disrupt the intestinal balance by invading in the intestinal ephitelium and cause the loss of microvilli. With the loss of microvilli, the intestine looses its permeability. Studies showed that some probiotics like; *L. rhamnosus* GG, *L. reuteri*, *L. casei* Shirota, and *B. lactis* Bb-12 can reduce the duration of diarrhoea. Several mechanisms have been suggested about how probiotics reduce the duration of diarrhoea; one of them is the increase of a rotavirus spesific IgA with the consumption of probiotics (Ouwehand et al., 2002).

1.1.10.3. Antimutagenic and anticarcinogenic properties

Mutagens occur as a result of viral or bacterial infections, stress and are also obtained via foods. Probiotic bacteria bind mutagens to the cell surface and can reduce some enzyme activities like β-glucuronidase, nitroreductase and azoreductase which cause activation of mutagens (Shah 2007).

Lankaputhra and Shah (1998) studied the antimutagenic effects of some organic acids produced by probiotic bacteria. Among the organic acids they studied, butyric acid showed better antimutagenic activity against mutagens. In addition, they also found that live bacterial cells showed higher antimutagenic acitivity than dead cells, showing the significance of the viability of probiotic bacteria (Vasilijevic and Shah, 2008; Shah 2007).

Different food habits can change the microflora of the intestine. Making diet with the high consumption of meat products, especially give rise to increased numbers of *Bacteriodes* and *Clostiridium*. With these bacteria, some faceal enzymes (β-glucuronidase, azoreductase, urease, nitroreductase) that convert the procarcinogens to carcinogens start to increase. The increase of these enzymes constitute a risk for colorectal cancer (Ouwehand et al., 2002).

Studies performed with some species of *Lactobacillus* and *Bifidobacterium* showed that these bacteria are able to decrease the number of the enzymes which in turn result in a declined risk of tumor development (Vasilijevic and Shah,2008).

1.1.10.4. Inflammatory bowel disease

Inflammatory bowel disease consist of ulcerative colitis (UC) and Crohn's disease (CD). The inflammation of the intestinal mucosa uncontrollably cause inflammatory bowel disease and also effect the gastrointestinal tract (Stephen and Hanauer, 2006). People who have inflamatory bowel disease have low numbers of *Lactobacilli* and *Bifidobacteria*, and high numbers of coccoids and anaerobic bacteria. The treatment is done with corticosteroids and probiotics help the patient for maintaining the remission period (Shah 2007).

1.1.10.5. *Helicobacter pylori* infection

Helicobacter pylori is an opportunistic pathogenic microorganism that give rise to peptic ulcers, chronic gastritis and type B gastritis. Normally this infection is treated with antibiotics and proton pump inhibitors. But this treatment can also cause a change in the balance of the intestine which can result in diarrohea (Vasilijevic and Shah, 2008). *H. pylori* infection depends on the strain, cell density and prolong of inflammation (Ernst and Gold 2000). The level of infection with *H. pylori* affects the risk of the development of peptic ulcer (Vasilijevic and Shah, 2008).

Probiotics can not destroy all of the *Helicobacter* but can reduce the number of pathogens accumulated in the intestine. *L. casei* Shirota was found to reduce the numbers of *H. pylori* in body mucosa and as a result a decline of mucosal inflammation was observed (Sgouras et al., 2004).

Probiotics produce different types of compounds like; lactic acid, acetic acid, hydrogen peroxide, bacteriocins, antifungal peptides and proteins. Among these compounds lactic acid and acetic acid are the main compunds produced by lactic acid bacteria and these compunds lower the pH of the intestine. This low pH show bacteriocidal or bacteriostatic effect on pathogens (Vasilijevic and Shah,2008). It was also shown that *Lactobacillus salivarius* produces high amount of lactic acid which cause the decline in numbers of *H. pylori* cell count (Parvez et al., 2007).

1.1.10.6. Immune system modulation

The immune system comprises a complex mechanism that protect against infections and disorderly growing tumors. Probiotic bacteria can affect the immune system directly or indirectly by altering the intestinal microflora (Robinson K., 2002, Marteau et al., 1997).

In a study it was shown that *L. johnsonii* LJ-1 and *L. salivarius* UCC 118 provoked mucosal IgA response. *L. rhamnosus* and *Bifidobacterium lactis* Bb-12 extracts were shown to have an impact on the suppression of lymphocyte proliferation in vitro (Saarela et al., 2000). The immunomodulation properties of these two strains were

confirmed with a study that children have atopic eczema. These children were fed with *L. rhamnosus* and *Bifidobacterium lactis* Bb-12 and they demonstrated a better improvement in contrast to placebo group (Sandholm et al., 1999).

1.1.10.7. Prevention of allergic diseases

Another beneficial effect of probiotics is the prevention of allergy. A lack of early childhood exposure to infectious agents change the immune system and makes them more allergenic (hygiene hypothesis). Reduced exposure to bacterial and viral pathogens change the immune system cells; TH2 cells start to increase and TH1 cells start to decrease which cause allergy (Yazdanbakhsh et al., 2002). Expansion of TH2 cells stimulate the production of IgE antibodies against eosinophilia and different types of antigens which is a proof of allergy. Kalloimaki and Isaouri (2003) suggested that the late colonization of *Bifidobacterium ssp*. and *Lactobacillus* species can be a reason for the allergic reactions in children. Consumption of the products that contain *Lactobacillus* GG in early times of life may reduce the currency of allergic diseases (Guenimode et al., 2006). Although the mechanism of how probiotics affect prevention of allergic diseases is not well known, it is suggested that they induce the gut defence mechanism for immunologic reactions (Isolauri et al., 2005).

1.1.11. Safety of probiotic microorganisms

Most of the probiotics are taken into the body with foods or drugs. So their safety is an important issue. Probiotic bacteria like *Lactobacillus, Leuconostoc and Pediococcus* species have been used in the foods for a long time and the safety of such probiotics has not been asked. But especially in recent years some species of *Lactobacillus, Leuconostoc* and *Pediococcus, Enterococcus* and *Bifidobacterium* were isolated from infective lesions. Regarding that they can be isolated from various type on infective lesions, safety of probiotics should be taken into account, especially for the ones that are being used in industrial applications (Ishibashi and Yamazaki 2001).

For the examination of the safety of probiotics; pathogenicity, toxicity and infectivity should be evaluated. Araya et al. (2002) from FAO/WHO justified that probiotic products should be labeled with the strain that it contains, the number of the probiotics in the food at the end of the shelf life, storage conditions and with the manufacture's contact details. Also some tests should be performed in order to characterize them as safe (Lee and Salminen 2009);

- Antibiotic resistance patterns should be determined,
- Side effects to human should be evaluated,
- Metabolic activities should be evaluated,
- Postmarket survey should be performed for the consumers who were affected adversely.

Beside these tests, their survival, translocation, colonization in the gastroinestinal tract and the effect of products derived from probiotics should be taken into consideration for the assesment of their effects (Saarela et al., 2000).

1.2. Kefir

1.2.1. Historical background of kefir

The origins of fermented milk beverages go back to the domestication of certain mammals. The first fermented milk products were produced by chance with the milk stored at environment temperature. Different strains of microorganisms become dominant in these fermented milk products all around the world because of the differences in climatic and environmental conditions. As a result, specific types of fermentations occurred which are distinct for different regions (Tamime 2006).

Such an example of fermented dairy products is kefir, which is originated from the Northern Caucasus mountain region in Russia. The long lives of the Caucasian people is believed to be due to their high consumption of dairy products, especially kefir (Yıldız, 2010). Kefir production was first achieved by traditional methods in the bags made from animal hides. It was produced by continuous fermentation and

as it was finished in the bag, fresh milk was added. During this procedure, microorganisms started to produce a thin layer and a cluster on the surface of the containers. The warm conditions of Caucasus weather aided this microbial film formation (Tamime, 2006).

Kefir is a natural probiotic that contains live active cultures of normal intestinal flora. It is a sour, carbonated fermented milk product. The pH of kefir is 4.0 and has a pleasant taste, without any bitterness. It also has a yeasty taste because of the yeast content and ethanol content (Garbers et al., 2004). In manufacturing processes kefir is generally produced with a final ethanol level of 0.01-0.1 %. The ethanol and CO_2 content of kefir are affected by the manufacturing conditions. 0.85-1.05 g/L of CO_2 content have been documented for the kefir produced from kefir grains (Farnworth 2005, Simova et al., 2002). The specific taste comes from the different compounds produced during fermentation.

Kefir is produced by incubating milk with kefir grains. It is produced under different type of names (kephir, kiaphur, kefer, knapon) and at different countries (Argentina, Taiwan, Portugal, Turkey and France) (Farnworth, 2005).

1.2.2. Kefir grains

Kefir grains are irregularly shaped, white to yellow in colour, folded and have uneven surface. Their shape are like cauliflower florets (Figure 1.4.)

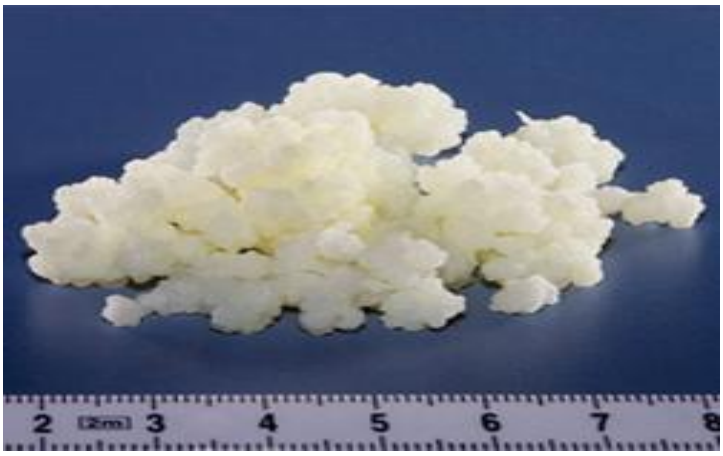

Figure 1.4. Kefir Grains (http://pinoygreenacademy.typepad.com)

The diameter of kefir grains depends on the agitation during its growth in milk but generally their diameter is between 0.5cm and 3.5cm (Koroleva 1991). In fresh milk, grains grow and transfer their properties to the newly formed kefir grains (Guzel-Seydim 2000b). In order to keep kefir grains viable, they should be transferred into fresh milk for fermentation. By this way new kefir grains will be formed (Farnworth 2005). The microbial profiles of grains fermented in milk and stored at room temperature or at 4°C differ from the fresh grains (Pintado et al., 1996).

Microbial population that constitutes the kefir grains seems to be almost constant over time, however seasonal differences can affect the flora of the grains and thus the product consistency. The microbial composition of the grains, the mother culture and the final product kefir are not the same (Table 1.6.)

Table 1.6. Microorganisms in kefir grains, mother culture and kefir drink (log cfu/g) (Farnworth 2005).

	Lactococci	Lactobacilli	Yeast
Kefir grains	7.37	8.94	8.30
Mother culture	8.43	7.65	5.58
Kefir drink	8.54	7.45	5.24

The microbial profile of the kefir drink is not much as the kefir grains. This is why kefir production should start with kefir grains instead of kefir (Farnworth, 2005).

1.2.3. Microbiology of kefir grains

Kefir grains consist of a complex symbiotic microbial population that comprises mostly lactic acid bacteria and yeasts included in a polysaccharide-protein matrix (Jianzhong et al., 2009). Yeasts in kefir grains are responsible for ethanol and carbondioxide production (Irigoyen et al., 2005). The diverse microbial population in kefir grains is an example of symbiosis. According to Koroleva (1991); when the bacteria and yeasts in the grains are divided, they can not grow well and can not carry out their biochemical acitivities properly.

The outer side of kefir grains mainly consist of lactic acid bacteria, yeasts are generally found in the core of the grain and the intermediate zone of the grains consist of yeasts and bacteria (Sarkar, 2008).

Different species have been found in kefir grains so far. Grains are generally consist of mostly lactic acid bacteria (10^8-10^9) CFU. And also contains yeasts (10^5-10^6) and acetic acid bacteria (10^5-10^6). This composition is mostly affected by fermentation. A list of different types of microorganisms isolated from kefir and kefir grains is given in table 1.7.

Table 1.7. The microflora of kefir and kefir grains (adapted from Sarkar,2008)

Bacteria	Yeasts
Lactobacillus kefir[a]	*Kluveromyces lactis*[k]
Lactobacillus kefiranofaciens[a]	*Kluveromyces marxianus*[k]
L. kefirangum[a]	*Saccharomyces cerevisiae*[k]
L. parakefir[a]	*Saccharomyces unisporus*[k]
L. brevis[b]	*Torulaspora delbrus*[k]
L. plantarum[b]	*Torulaspora delbrueckii*[k]
L. paraplantarum[c]	*Candida pseudotropicalis*[l]
L. gasseri[c]	*Candida kefir*[m]
L. helveticus[d]	*Candida holmii*[m]
L. acidophilus[b]	*Pichia fermentans*[e]
L. delbrueckii[b]	
L. rhamnosus[b]	
L. paracasei[b]	
L. fermentum[e]	
L. viridescens[e]	
L. bulgaricus[f]	
Lactococcus lactis subsp. *lactis*[g]	
Lactococcus lactis subsp. *cremoris*[h]	
Streptoccus thermophilus[d]	
Enterococcus faecium[f]	
Leuconostoc mesenteroides[h]	
Acetobacter aceti[h]	
Escerichia coli[e]	
Bacillus subtilis[j]	

[a] Takizawa et al., 1994, [b] Santos et al., 2003, [c] Anna et al., 2005, [d] Simova et al., 2002, [e] Angulo et al., 1993, [f] Wang et al., 2004, [g] Yoshima and Toyoshima 1994, [h] Koroleva 1991, [j] Yüksekdağ et al., 2004, [k] Loretan et al., 2003, [m] Engel et al., 1986.

1.2.3.1. Lactic acid bacteria in kefir grains

Lactococcus spp., *Lactobacillus* spp., *Streptococcus thermophilus* and *Leuconostoc* spp. generally mave up the main species of lactic acid bacteria in kefir grains. *Lactococcus* spp. species are located on the surface of the grains, lactobacilli and yeasts are generally found inside the kefir grains (Tamime 2006). Different studies have been performed for the isolation of bacteria from kefir grains;

Pintado et al., (1996) isolated *Lactobacillus kefir* and *Lactococcus lactis* from Portuguese kefir grains by using API 50^{TM} and SDS-PAGE analysis. Chen et al., (2008) identified *Lactobacillus kefiri*, *Leuconostoc mesenteroides* and *Lactococcus lactis* from the kefir grains occur in Taiwan.

In order to understand the behaviour of bacteria in kefir grains, Garrote et al. (2004) performed some tests and found that; *Lactobacillus kefir* and *Lactobacillus parakefir* strains include S-layer proteins and these proteins function in their auto-aggregation and hameagglutination properties. It is also found that these bacteria have the ability to adhere the Caco-2 cells.

Santos et al., (2003) studied antimicrobial properties of lactobacilli isolated from kefir. They isolated *Lactobacillus kefir*, *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lactobacillus kefiranofaciens* by using API50CH. According to their study, it was concluded that *L. acidophilus* and *L. kefiranofaciens* have good adhesion properties, have resistance to acid and bile conditions and have the ability for inhibiting pathogenic bacteria. *L. kefiranofaciens* is also known to produce kefiran, the matrix of kefir grains (Vuyst and Deegest, 1991).

Bosh et al., (2006), isolated heterofermentative *Lactobacillus kefir, Lactobacillus parakefir, Lactobacillus brevis* species and homofermentative *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei* species from kefir grains. Witthuhn et al., (2005) isolated different species of lactic acid bacteria from kefir grains by using microbiological tests and API50CH. They isolated species of *Lactococcus, Leuconostoc* and *Lactobacillus* species from kefir grains. It is indicated that, during the isolation process some species that grew on the media used

for the isolation did not grow during obtaining pure cultures.This was explained with that the cultures in the kefir grains can not be viable when they are outside the complex kefir grain environment.

Witthuhn et al. (2004) characterized the microbial population of different grains of South African region by using morphological and biochemical tests. They isolated species of *Lactobacillus, Leuconostoc* and *Lactococcus*.

1.2.3.2. Yeasts in kefir grains

In fermented dairy products, yeasts play an important role because they provide amino acids, nutrients, appropriate pH, ethanol and CO_2 production (Viljoen 2001). Yeasts provide an environment for the growth of the bacteria, and produce metabolites that give the taste and flavour of kefir (Simova et al., 2002).

Different species of yeasts have been found in kefir grains like *S. cerevisiae, Candida pseudotropicalis,* and *Pichia fermentans*. The properties of these and other species of yeasts in kefir grains differ from each other. For instance; some of them are inside the grains and are able to ferment lactose, while some can not. Some of the yeasts are located inside the grains, while some of them are located on the surface of the grains. This pattern affect their fermentation metabolism (Farnworth 2005).

Jianzhong et al (2009) studied the microbial community present in Tibetian kefir grains by using denaturing gradient gel electrophoresis (DGGE) of partially amplifed rRNA. They isolated different species of bacteria and different yeasts like; *Saccharomyces cerevisiae, Saccharomyes unisiporus, Kluveromyces marxianus*.

1.2.4. Production of kefir

The current methods available for kefir production are traditional and industrial method.

In traditional method, kefir grains are added into raw milk which is boiled and cooled down to incubation temperature with a ratio of 2-10%. Inoculated milk is fermented with grains for 24 hours at 25°C. After fermentation, the grains are

recovered from fermented product with a sterile sieve. Kefir can be stored at 4°C until consumption and the recovered grains can be used for next culture passage (Figure 1.5.)(Güzel-Seydim et al., 2010).

In industrial processes, kefir production differ from traditional method with the usage of lyophilized starter cultures that contain lactic acid bacteria and yeasts. In industrial processes, the use of kefir grains for inoculation is difficult because of the necessity of post-fermentation removal.

For producing kefir industrially, activated starter culture that contain lactic acid bacteria and yeasts is inoculated in homogenized and pasteurized milk that is composed of 2-5 % milk fat. Fermentation occurs at 25°C for 20-24 hours. Fermentation is accomplished when pH drops to 4.7 and following fermentation, the kefir is stored at refrigerator temperature (Simova et al., 2002).

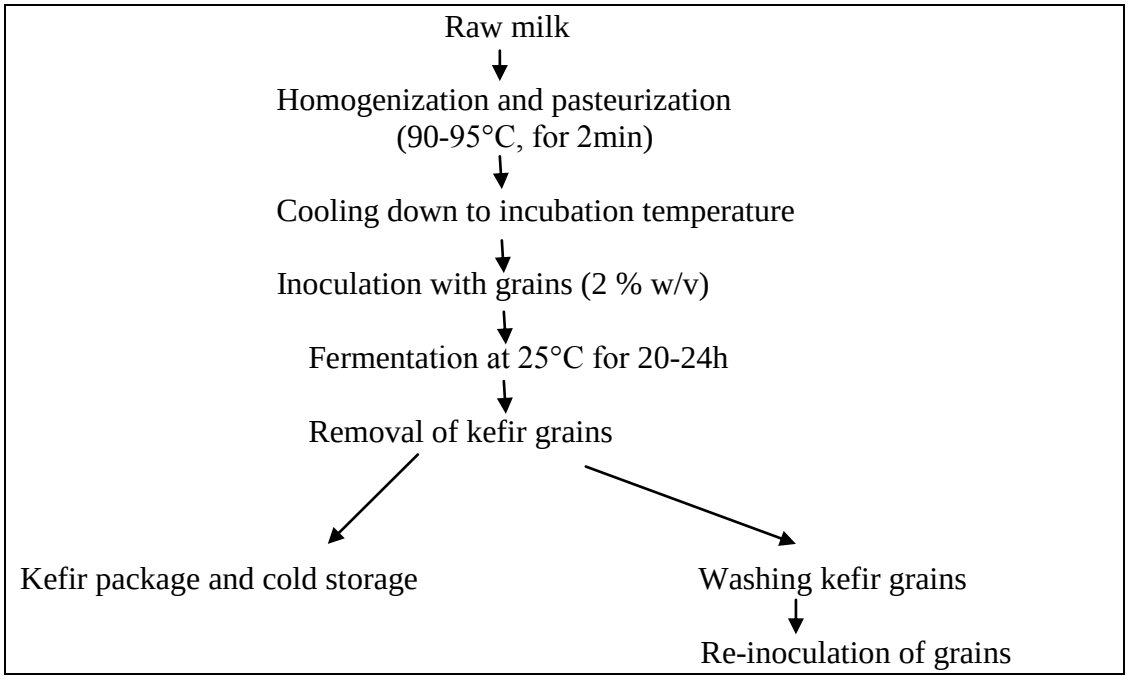

Figure 1.5. Traditional process of kefir production (Güzel-Seydim et al., 2010).

The quality of kefir is affected by different factors such as, the quantity of the grain inoculated, incubation time and temperature, stirring and storage conditions. These factors affect the microflora and thereby the fermentation.

Different grain-milk ratios have been suggested so far; 20-50g kefir grain/L , 20-100g/L, 1g/L (Marshall et al., 1984, Garotte et al., 1998). Garotte et al., (1998) studied different grain : milk ratios; 1g kefir/L, 10g kefir/L, 20g kefir/L, 50g kefir /L, 100g kefir/ L. With these ratios, they obtained fermented milks with different properties. Their results indicated that different ratios have a great impact on final pH, lactococci concentration, viscosity and carbon dioxide content. When the ratio of 100g/ L was used, it was observed that acidification occurs rapidly and accordingly, lactococci numbers decrease.

1.2.5. Nutritional Value of Kefir

The complex microbial flora of kefir provide different types of products from fermentations and so a product with a nutritional value. It was found that in 100 g of kefir, protein amount is 3.0-3.4 g, fat amount is 1.5g and lactose is 2.0-3.5 g after fermentation. Besides these values, the lactic acid content and alcohol content may be variable due to the use of kefir grains or starter culture as inoculum into the milk. Lactic acid content may differ between 0.6-1.0ml / 100mL, alcohol level may differ between 0.0-0,1g/ 100ml (Tamime, 2006).

Kefir is also rich in terms of vitamins, essential amino acids and minerals that help the body in the way of carry out its functions. Kefir is a good source of biotin, folic acid, panthotenic acid and B_{12}. These types of B vitamins are responsible of variable health benefits like regulation of the kidney, providing long life, alleviate skin problems. One important essential amino acid, tyrptophan is found in kefir and relaxes the nervous system. Calcium and magnesium that are important in nervous system is also found in kefir. Phosphorus, which is responsible for aiding in the utilization of carbohydrates, fats and proteins is also found in kefir. (Otes and Cağındı 2003, Salof Coste 1996).

1.2.6. Health benefits of kefir

1.2.6.1. Stimulation of immune system

One of the health benefits of probiotics is the stimulation of immune system. Kefir found to have an adjuvant effect on immune system modulation.

In a study conducted by Thoreux and Schmucker (2001); young and old rats were inoculated with cholera toxin (CT). The non spesific IgA titers did not differ in young and old rats, but the anti-CT IgA concentrations were higher in young rats because of the secretion of in vitro antibodies from the lymphocytes. Beside this, the non-spesific IgG concentrations were higher than anti-CT IgG concentrations in young and old rats. It was concluded that orally administered kefir stimulate intestinal immune response in young but not old rats.

1.2.6.2. Inhibition of tumour growth

The first information about the inhibition of tumour growth by kefir was given by Shiomi et al. (1982); water soluble polysaccharides isolated from kefir, were given to mice and it was observed that these polysaccharides were able to inhibit the growth of Ehrlich carcinoma or Sarcoma in contrast to the ones which were fed with other polysaccharides (Farnworth, 2005).

Güven et al. (2003), inoculated mice with a hepatoxin that cause oxidative damage, carbon tetrachloride, and feed them with kefir. They observed diminishing in the numbers of liver and kidney malondialdehyde, which is an oxidative stress marker. Their results also showed that kefir was more effective than vitamin E in defending cells from oxidative damage.

1.2.6.3. Improved lactose tolerance

A relative amount of the global population can not have the ability for fermenting lactose since they lack the enzyme β- galactosidase and thus can not tolerate yogurt. However, studies showed that when they consume yogurt which contain high number of live probiotic bacteria, they are able to tolerate yogurt. These

probiotic live bacteria protect their viability and their cell walls remained undamaged. By this way, their β-galactosidase enzyme is protected while passing through the stomach until the gastrointestinal tract (Farnworth, 2005).

De Vresse et al. (1992) found that β- galactosidase activity of kefir grains were active until kefir is consumed (Farnworth, 2005).

A commercial kefir, is produced with six starter cultures and one yeast was shown to be effective in reducing breath hydrogen in the people who have lactose intolerance (Hertzler and Clancy, 2003). Breath hydrogen test is used for the determination whether the milk sugar (lactose) is fermented or not. In this test, a solution that contains lactose or lactulose is given to the patient and the breath samples of the patient is evaluated. Large amount of hydrogen in the breath samples indicates lactose intolerance.

1.2.6.4. Antimicrobial activity of kefir

Kefir microorganisms have antimicrobial effects against other microorganisms. They are in a competition with other microorganisms in order to obtain polysaccharides, peptides, bacteriocins, organic acids and free fatty acids (Yıldız, 2010).

Garrote et al. (2000) studied the inhibitory effect of cow's milk fermented with kefir grains, against Gram positive and Gram negative bacteria. He observed that Gram positive bacteria were inhibited more than Gram negative bacteria. In addition, milk supplemented with some organic acids like lactic acid and acetic acid showed inhibitory effect against *E. coli* meaning that the organic acids produced from kefir have an inhibitory effect on pathogenic microorganisms (Farnworth, 2005).

Yüksekdağ et al. (2004a) studied metabolic acitivies of *Lactobacillus* species isolated from Turkish kefir and found that lactic acid isolates have the ability to produce the antimicrobial compound, hydrogen peroxide. In another study they found that lactococci isolates have also inhibitory effect on pathogens such as *S.*

aureus, E. coli NRRL B-704, and *P. aeruginosa* by producing hydrogen peroxide. Hydrogen peroxide was the most effective against *S. auerus.*

1.2.7. Preservation of kefir grains

In order to keep up the properties of kefir grains and for a successful marketing of kefir beverage , kefir grains should be stored in optimum conditions.

Liu et al., (1999) studied the preservation of kefir grains and found that kefir grains retain their stability when stored at -20°C.

Witthulm et al., (2005a) studied the effect of different storage temperatures on the metabolic activity of kefir grains. Kefir grains were stored at 4°C, -20°C and -80°C. It was observed that freezing is a better method for the preservation of the kefir grain properties. Grains which were stored at - 20°C and - 80°C increased their weight effectively when transferred into milk. The microflora of the milk which was fermented with grains stored at -80 °C and -20°C have almost similar microflora, acidity level, viscosity and carbon dioxide content with the milk fermented with control grains. For that reason, it was suggested to store kefir grains at -20 °C for maintaining its properties for production of kefir.

1.3. Identification and typing of '*L. acidophilus* group'organisms from kefir

'*L. acidophilus* group' organisms especially *L. acidophilus* is of considerable attraction because of its important role in human health and nutrition. For the isolation and characterization of this bacteria different methods have been suggested so far like hybridization with species specific probes (Pot et al., 1993) or primers (Tilsala-Timisjarvi and Alatossava,1997) and generation of randomly amplified polymorphic DNA markers (Kullen et al., 2000). Beside this methods, 16S rRNA gene amplification and sequence analysis have been used widely for the study of microbial diversity in different samples (Escalante et al., 2004). This method have been used for the identification of species that were isolated from vaginal flora (Vasquez et al., 2002), fermented mill product 'pulgue' (Escalante et al., 2004),

traditional cheese (Abriouel et al., 2008), faeces (Gu et al., 2008, Yun et al., 2008) and human stomach (Ryan et al., 2008).

1.3.1. Importance of 16S rRNA gene

Ribosomes are the site of protein sythesis and each ribosome is made up of two subunits. These subunits in prokaryotes consist of 30S and 50S. The 30S subunit consist of 16S rRNA and proteins, 50S subunit consist of 5S and 23S rRNA molecules.

Ribosomal RNAs have some properties that make them remarkable in the evolutionary relationships between organisms. Ribosomal genes are assumed to act like molecular clocks that can be used for the constitution of phylogenetic tree of living organisms. The features that make them important in evolution are; their universal distribution, containing highly conserved regions, functional stability, and ease of sequencing. Beside these features, the specificity of their primary and secondary structures also make them important (Tourova, 2002).

The first use of ribosomal small subunits (SSU) as phylogenetic tools was achieved by Carlos Woese in the early 1970s with the thought that setting up a phylogenetic tree based on molecular sequences could give an idea about the relationships between organisms. By using comparative analysis of 16S rRNA genes, he found out the three big domain; Eucarya, Bacteria and Archaea. The phylogenetic tree was constructed by aligning the organisms and then their differences were counted which were assumed as ‘evolutionary distances’ between them (Pace 1997).

Between the rRNAs of prokaryotes, the first study for determining the relationships between organisms was applied by using 5S rRNA. 5S rRNA molecules were extracted and separated phylogenetically due to different molecules that belong to different communities. After that, they were analysed phylogenetically. But the low nucleotide content of 5S rRNA- 120 base pair-, limited the use of this molecule in phylogenetic analysis. After that, the usage of larger rRNA molecules have been suggested; 16S rRNA gene which consist of 1500bp and 23S rRNA gene which

consist of 3000bp (Amman et al., 1995). Although 23S rRNA gene is a bigger molecule than 16S rRNA gene, its limited databases restricted its usage in phylogenetic studies (Bergey, 2005).

In prokaryotes the ribosomal operons may be present in several numbers but they are less than in eukaryotes. Almost half of the procaryotes carry one or two copies of rRNA. The organisms that contain several, have three to eight copies of rRNA. Analysing closely related species explained that they usually consist of similar numbers of ribosomal genes that enables to estimate the data from a known species to a related unknown species. Such an estimation is considerable in determining the number of organisms in environmental species using PCR (Tourova, 2003).

rRNA that contains highly conserved regions enable the synthesis of 'universal primers' that can anneal to the conserved regions of rRNA molecules (Pace, 1997). 16S rRNA gene sequence analysis have been widely used for the identification of *Lactobacillus* species. Sui et al. (2002), identified lactobacilli such as *L. acidophilus, L. rhamnosus, L. gasseri* and *L. fermentum* from faecal samples of humans by using 16S rRNA gene sequencing method. Kullen et al.(2000); identified species belong to the '*L. acidophilus'* complex by sequencing a 500bp region of 16S rRNA gene and compared the obtained sequences with type strains by using aligment of Clustal W. Furet et al. (2004) designed 16S rRNA gene targeted specific primers for the identification of lactobacillus species found in fermented milk products. Tsai et al. (2010) have designed specific primers for the detection and enumeration of *L. acidophilus* and *L. plantarum* species in food supplements and animal feeds.

1.3.2. Polymerase Chain Reaction

Polymerase chain reaction is an in vitro method that allows the amplification of deoxyribonucleic acid (DNA). PCR amplification of DNA is applied using oligonucleotide primers. Primers have the ability to bind to their complementary region on the DNA. DNA poylmerase –a thermostable enzyme that is isolated from *Thermus aquaticus*- extend the primers on single stranded DNA in the presence of deoxynucleoside triphosphates (dNTPs). With the extension of the primer, new

double -stranded DNA are being synthesized which are complementary to the template. This strand synthesis can be repeated again with the denaturation of double stranded DNA, annealing of the primers and primer extension by DNA polymerase, respectively. Each newly synthesized DNA act as a template for further cycles of DNA amplification (Newton and Graham, 1994).

1.3.3. Sequencing

With the introduction of PCR, different methods have been suggested for the sequencing of PCR generated fragments. These methods are, Sanger chain-terminating dideoxynucleotide sequencing, Maxam and Gilbert chemical cleavage method, cycle sequecing and next generation sequencing.

Next generation sequencing which is also known as massively parallel sequencing, process millions of sequencing reactions to occur in parallel (Reis-Filho, 2009). This technique is important in the way of the data it produces. On the contrary to long reads generated from a PCR-amplified sample, it produces millions of shorter reads (~21 to ~400 base pairs). It gives qualitative and quantitative information about any type of nucleic acid. Next generation sequence does not require DNA amplification and can be performed from single DNA molecules (Pettersson et al., 2009).

In cycle sequencing method, which differs from normal PCR with the absence of any new template formation, fluorescently labeled dideoxynucleotides are introduced as chain terminators. In a thermal cycler the reaction is performed with dye terminators and each dideoxynucleotide triphosphates is labeled with different fluorescent dyes. After the reaction the products are purified and their length is determined with gel electrophoresis. With this method, target DNA sequence can be amplified and after the purification of the product sequence data can be obtained. This method has been used for sequencing a 500bp PCR fragment (Bartlett and Stirling 2003, Cebeci Aydın 2008).

The sequences that are obtained can be analyzed by using different programmes such as; FASTA programme or BLAST programme. FASTA programme is a

sequence database that contains short common patterns between the query and target sequence. Another sequence database programme BLAST (Basic Local Alignment Search Tool) is a programme of NCBI. In this programme the identities between the query and the sequences in the database are researched (http://blast.ncbi.nlm.nih.gov/Blast

1.3.4. Randomly amplifed polymorphic DNA (RAPD)

Randomly amplifed polymorphic DNA (RAPD) is a method that depends on the creation of amplification products for a given nucleic acid with the usage of arbitrary priming nucleotides (Bartlet and Stirrling, 2003).

In RAPD technique, by using small oligonucleotide primers- generally 10 bp- genomic DNA is amplified. RAPD differs from normal PCR with the use of a single random oligonucleotide primer and no need for any brief information for the organism to be analyzed. In the presence of short primers, it is highly possible that the genome consist of several priming sites which are close to one another. The resulted profile of amplification products are reproducible only under strictly controlled conditions and depends on the combination of primer and template (Hadrys et al., 1992).

During thermal cycling at convenient annealing temperature, primers with random sequences bind to several priming site that have complementary sequences in template DNA (Figure 1.6). In case priming sites are in a amplifiable distance to each other, the binding of primers create several discrete products (Bardakcı, 2001).

The amplifed region generally include disorganized, variable and noncoding sequences. These differences change from one species to another. The arbitrary priming is affected by the complimentary regions in the DNA template, so variations at these regions provoke the generation of characteristic fingerprinting patterns (Bartlet and Stirrling, 2003).

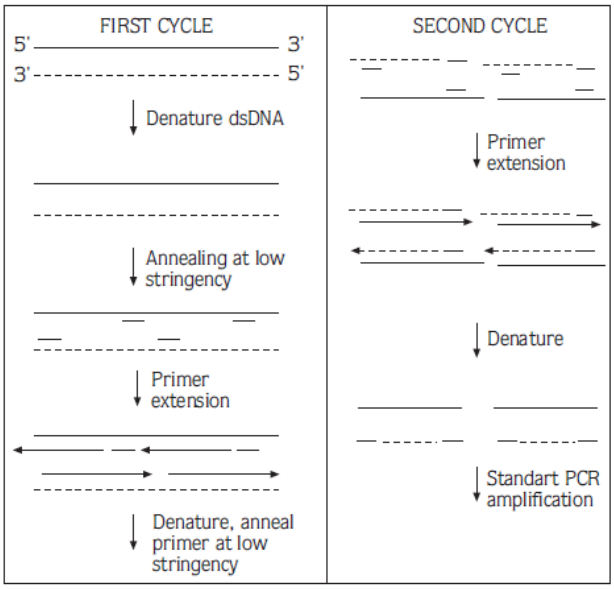

Figure 1.6. Schematic diagram of RAPD

The distance between priming regions should not be more than 3 to 4 Kb for amplification of the target region. The short primers desire stringent conditions for a reproducible PCR . The purity, quality and quantity of template DNA may cause differences between PCR products or absence of PCR products. (Bartlett and Stirling, 2003). In addition, primer-template concentration, differences in primer annealing temperatures, concentration of magnesium ions in the reaction mix can affect the reproducibility of PCR (Hadyrs et al., 1992).

RAPD technique has been widely used for typing lactic acid bacteria in different studies; Svec et al., (2010) studied typing of lactobacilli in dental carries with RAPD-PCR method by using M13 primer. M13 primer also was used for the differentiation of *Lactobacillus* strains isolated from probiotic yogurts (Schillinger et al., 2003).

Delferedico et al., (2005) studied typing of lactobacilli isolated from kefir grains. RAPD technique have also been used for typing of lactobacilli isolated from probiotic yogurts (Shillinger et al., 2003). Du Plesis and Dicks (1995) used RAPD

method to differentiate '*L.acidophilus* group' microorganisms. Roy et al., (2000) evaluated the similarities between the strains of '*L. acidophilus*' group by using RAPD technique.

1.4. Aim of the study

Consumers are increasingly becoming more sensitive of their health and try to consume foods that are healthy and able to prevent illness. The demand for the consumption of 'healthy' foods evoked the production of new products in food industry. It was established that by using probiotics, functional food products that having beneficial health effects can be produced.

Probiotic cultures can be added into food products. But their viability and stability is a problem for food industry. Because, in order to exert their health benefits, probiotics should remain viable at adequate numbers during shelf life of the products.

L. acidophilus is the most commonly suggested organism for the dairy products. It has been widely used in the production of probiotic yogurts with probiotic *Bifidobacterium* species.

The aim of this study was to isolate new possible probiotic strains that belong to '*L. acidophilus* group' from traditional kefir grains, examine their viability during the shelf life of a probiotic yogurt and compare their viability with commercial probiotics. By this way, new species that have probiotic properties could be added to commercial probiotic collection to be used in industrial probiotic yogurts. In addition, typing of *Lactobacillus acidophilus* and *Lactobacillus amylovorus* strains from commercial probiotics, from kefir grains and from traditional dairy products was aimed.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and Enzymes

A detailed list of chemicals and enzymes are listed in Appendix A.

2.1.2. Buffers and Reagents

A detailed list of the buffers, reagents, media and their preparation are given in Appendix B.

2.2. Methods

2.1.1. Kefir Grains

Kefir grains used in this study were obtained from three different area; one is obtained from Ankara University Agricultural Faculty Department of Dairy Technology and the two other grains were kindly provided by traditional home-made kefir producers from Ankara and Mersin.

2.2.1.1. Activation and Propagation of Kefir Grains

In order to activate and propagate kefir grains, 10 g of kefir grains were transferred in 500 ml UHT milk and incubated at 25°C for 24h. After fermentation, propagated grains were recovered from milk through filtration with a sterile sieve and were washed with sterile distilled water for the next propagation. This activation and

propagation procedure was repeated for three times in one week (Simova et al., 2001).

2.2.1.2. Homogenization of Kefir Grains

10 grams of activated and washed kefir grains were homogenized in 90 mL of sterile saline solution (8.5 g/L NaCI) in a stomacher for 15 min (Hsia-Chia Chen et al., 2008).

2.2.1.3. Isolation of lactic acid bacteria from Kefir Grains

For the isolation of lactic acid bacteria from kefir grains, several different media have been suggested including MRS agar (Santos et al., 2003, Mainville et al., 2005) and MRS agar that contain 200 ppm cycloheximide in order to eliminate yeast growth.

In order to determine the appropriate selective medium for lactic acid bacteria, both MRS agar and MRS agar + 200ppm (200 μg/mL) cyloheximide were evaluated. In order to provide a more specific environment to lactobacilli pH of MRS was adjusted to 5.5 (Robinson et al., 2000). In addition MRS with 0,15% Bile-salt was tested. Bile-salt was added in order to select cultures that have the ability to grow in the presence of bile.

For the isolation; homogenized kefir grains were subjected to serial dilutions up to 10^{-7} with sterile saline solution (8.5 g/L NaCI). MRS agar, previously prepared and cooled down to 50-55 °C, were poured on the plates. The plates were incubated aerobically at 37°C for 72-96 hours.

After the incubation, the colonies with different morphologies were selected and streaked on MRS Agar for obtaining pure cultures. Purified cultures were maintained in 20 % glycerol stocks at -80°C. The glycerol stocks were prepared as follows ;

- 1200 μl of overnight culture were transferred into eppendorf tubes,
- Centrifuged for 1 min at 8000rpm,

- The supernatant was removed,
- 900 µl medium were added on the pellet and dissolved by mixing with a pipette
- 600 µl glycerol (50%) was added.

2.2.1.4. Basic Physological and Biochemical Tests for Lactic Acid Bacteria

2.2.1.4.1. Gram staining Procedure

From an overnight fresh culture a loopful was taken and transferred on a microscope slide. It was air dried and then fixed with flame. After fixation it was stained with cristal violet for one minute and then washed with distilled water. After washing, it was subjected to iodine solution for 30-40 seconds. Iodine was then removed by washing with alcohol. After alcohol treatment it was stained with safranine for one minute and washed with distilled water. Then the slides were air dried and visualized under microscope with immersion oil (100X magnification).

After staining, gram positive bacteria were seen as purple, gram negative bacteria were seen as pink under microscope.

2.2.1.4.2. Catalase Test

Catalase enzyme is responsible for the decomposition of hydrogen peroxide into water and oxygen.

$H_20_2 \longrightarrow H_20 + 0_2$

In the presence of catalase enzyme, adding a small amount of hydrogen peroxide will result in bubbles of oxygen.

1 ml of 3 % H_2O_2 solution was added to 1 ml of an overnight culture grown on MRS broth to test bubble formation. Observed bubbles indicated catalase positive isolates.

2.2.1.4.3. Gas Production From Glucose

For the detection of homofermentative and heterofermentative metabolism gas production from glucose was tested.

Gas production from glucose was tested in MRS broth from which meat extract and citrate have been extracted and replaced by 2% glucose and durham tubes where included. Overnight grown cultures were transferred into these brothes and incubated at 37°C for 24 h (Altay 2010, Gürakan 1991).

After 24 hours of incubation, the bubbles observed in the durham tubes indicated gas production from glucose which belongs to a heterofermentative metabolism and no bubbles in durham tubes indicated homofermentative metabolism.

2.2.1.4.4. Endospore Forming

A loopful of culture was taken from an overnight fresh culture and transferred onto a microscope slide. It was air dried and then fixed with flame. Then it was stained with malachite green over a flame letting the dye steaming. As the dye steamed, it was stained again with malachite green. This procedure was repeated for 3 times. Then the slide was washed with distilled water and stained with safranine for one minute. After air drying, it was visualized under microscope with 100X magnification. The cells that do not contain endospore were appeared red, the cells that contain endospores were seen as green endospores inside the red cells.

2.2.1.4.5. Growth on Bile-MRS

Overnight grown cultures in MRS broth were streaked on Bile-MRS agar (0.15 %) and incubated in anaerobic jars (Anaerocult C) for 24h at 37°C.

2.2.1.4.6. Carbohydrate Fermentation Test

Carbohdyrate fermentation tests were performed in 96-well microplates with 14 different carbohydrates; arabinose, cellobiose, fructose, galactose, glucose, lactose,

maltose, mannitol, melibiose, saccharose, salicin, sorbitol, trehalose and xylose (Bulut et al. 2003).

For the carbohydrate fermentation test, cells free from sugar residues and sugar solutions were prepared. Then they were combined in microplates (Gurakan, 1991).

For the preparation of cell cultures; the cells were grown in 10 ml MRS broth for 24 h at 37°C. These active cells were centrifuged for 10 min at 10.000 rpm. The supernatant was removed and the pellet was dissolved in 5ml of modified MRS that contains 0.04 g/l bromocresol purple (Appendix B). After that, the cells were centrifuged again for 10 min at 10.000 rpm. The supernatant was removed again and the pellet was dissolved in 10ml modified MRS.

The sugar solutions were prepared by dissolving 1g of sugar in 10 ml of distilled water (1% w/v). Prepared sugar solutions were sterilized with 0,22 um filters (Minisart/Sartorius)

The wells were inoculated with 160 µl of modified MRS that contain cell culture and with 40 µl of sugar solutions. The first column of the wells were inoculated with 40 µl sterile distilled water instead of sugar solution (control for the cells). The last row of the wells were inoculated with 40ul sugar solution + 160ul sterile modified MRS (control for the sugars). At the last step all the wells were inoculated with 2-3 drops of mineral oil for providing anaerobic conditions and for preventing the vaporization of the liquid in the wells. All the sugar tests were performed twice (Dede Altay, 2010).

After the inoculation the plates were incubated at 37°C for 48h. The color change from purple to yellow indicated that the carbohydrate was fermented. *L. acidophilus* ATCC 4356 type strain was used as a positive control organism during the test.

Esculin test was performed differently from the carbohydrate test mentioned above; it was performed in modified MRS that does not contain meat extract and contain 5g/l esculin instead of glucose (Tjandraatmadja et al., 1990). This medium was prepared and autoclaved for 15min at 115°C. The tubes that contain this medium

were inoculated with overnight grown cultures. Then the tubes were incubated at 37°C for 48h. *L. casei* subsp. *casei* NRRL- B1922 was used as a positive control organism. After incubation the tubes were observed under UV light. The tubes that were fluorescent under UV indicated esculin negative isolates and the tubes that have lost their fluorescence indicated esculin positive isolates.

2.2.1.5. Molecular Characterization

In order to confirm putative '*L. acidophilus* group' organisms isolated from kefir grains, 16S rDNA sequencing was performed. Following identification, RAPD-PCR for typing different commercial and traditional *L. acidophilus* and *L. amylovorus* strains was performed.

2.2.1.5.1. Genomic DNA Isolation

Genomic DNA isolation was performed with the GENEJet Genomic DNA Purification Kit (Fermentas). The isolation procedure is indicated as follows; 2 ml of culture grown overnight was centrifuged at 15000 rpm for 3 min (Andreas, Hettich Germany). After the removal of the supernatant, the pellet was dissolved in 180 µl of lysis buffer (Appendix B) and incubated at 37° C for 30 min. After the incubation, 200 µl lysis solution (Fermentas) for the lysis of cells and 20 µl of proteinase K (Fermentas) was added and mixed by vortexing. It was incubated at 56 ° C for 30 min with mixing occasionally (until the cells are completely lysed). Then 20 µl of Rnase (Fermentas) was added to the solution, mixed by vortexing and incubated for 10 min at room temperature. Afterwards, 400 µl of 50 % ethanol was added to the solution for precipitation and was transfered into a GeneJet Genomic DNA Purification Column (DNA binds to the silica membrane of the column). It was centrifuged at 15000 rpm for 1 min. Following centrifugation, the collection tube which contains the flow-through solution was discarded and the column was inserted into a new collection tube. For the removal of the impurities 500 µl wash buffer I was added and centrifuged at 15000 rpm for 1 min. The flow-through solution was discarded and the column was placed into the same collection tube. 500 µl wash buffer II was added and centrifuged at 15000 rpm for 3 min. The flow-

through solution was discarded again and the column was put into a sterile microcentrifuge tube. For eluting the DNA from the membrane, 50 µl of elution buffer was added into the microcentrifuge tube above the column and incubated for 2 min at room temperature. After incubation it was centrifuged for 15000 rpm for 1 min. The purification column was discarded and the purified DNA was stored at -20 ° C for following applications.

2.2.1.5.2. Determining DNA Concentration

The quality and quantity of isolated DNA concentrations were measured with Alphaspec µL spectrophotometer at Central Laboratory at the Biology Department. At this spectrophotometer direct ratio of the absorbance at 260nm and 280 nm were measured (OD_{260} / OD_{280}).

The ratio of absorbances OD_{260} / OD_{280} , were used as a degree of contamination. When the OD_{260} / OD_{280} was equal to 1.8, it represents purity, when the ratio is bigger than 2.0 it represents RNA contamination and when it is smaller than 1.8 it represents protein contamination. The samples that have OD_{260} / OD_{280} ratio between 1.8- 2.0 were used in this study.

2.2.1.5.3. Electrophoresis

For the visualization of DNA and PCR products electrophoresis was performed. In order to visualize genomic DNA or PCR products, agarose gel was prepared as follows; 1.5 % w/v agarose (Prona Agarose) was dissolved and boiled in TBE Buffer (Appendix B). After dissolved, it was cooled to 45 °C and poured into the gel block and the combs were placed.

Following the solidification of the gel, the combs were removed from the gel and the gel was placed into to the electrophoresis tank (Thermo EC330 Electrophoretic Gel System) containing TBE Buffer. The next step after preparation of the agarose gel, was loading the agarose gel;

- For DNA isolates; 3µl of isolated DNA was mixed with 2 µl of loading dye (Fermentas) and loaded into the wells of the gel.
- For PCR products; 10 µl of PCR products was mixed with 2 µl of loading dye and loaded into the wells of the gel.
- For all of the gels, the first well was loaded with 100bp marker (Fermentas).

The loaded agarose gel was run at 50 V for 2 hours. The staining was done in 1.0 µg/ml EtBr solution for 20 minutes. After staining, it was destained in distilled water for 10 minutes and the gel was visualized under UV in GelDocXR (BioRad, USA).

2.2.1.5.4. Polymerase Chain Reaction

PCR reaction was performed by preparing a PCR mix containing; 1X PCR buffer (Fermentas), $MgCI_2$ (Fermentas), dNTP (Fermentas), Taq Polymerase (Fermentas) and sterile ddH_20 (Table 2.2).

For all PCRs performed, the chemicals mentioned above were prepared in one eppendorf and then distributed into PCR tubes and the genomic DNA of each culture added afterward. The final volume of PCR mix for all tubes were 50 µl. For the control of contamination, in all PCR's done, one PCR tube contained the same amount of sterile distilled water instead of genomic DNA.

The PCR reactions were conducted in MJ Mini thermal cycler (BioRad, USA) machine.

2.2.1.5.5. 16S rRNA gene sequencing

16S rRNA gene sequence was performed for putative *L. amylovorus* species; A-7, A-11 isolates. The primers and their sequences are given in Table 2.1.

Table 2.1. 16S rRNA primers used for sequencing

Primer	**Target gene**	**Primer sequence (5'→3')**	**Product length**	**Reference**
A (forward)	16S rRNA	AGAGTTTGATCCTGGCTCAG		Mora et al., 1998
U926 (reverse)	16S rRNA	CCGTCAATTCCTTTRAGTTT	926bp	Baker et al., 2003

The reaction mix and pcr conditions is given in table 2.2 and 2.3, respectively;

Table 2.2. Reaction mix for 16S rRNA gene sequencing

Reaction components	Final concentration
ddH_20	-
$MgCI_2$	1.5mM
PCR Buffer	1X
dNTP	200mM
Forward Primer	1mM
Reverse Primer	1mM
Taq polymerase	0,5U
DNA	500ng

Table 2.3. PCR conditions for 16S rRNA gene sequencing

	Temperature-Time	
Initial denaturation	94˚C 2 min	
Denaturation Annealing Extension	94˚C 30 sec 54˚C 40 sec 72 ˚C 40 sec	45 cycle
Final Extension	72 ˚C 10 min	

PCR products were run on agarose gel for 2 hours at 50V, stained with EtBR for 20 min and destained for 10 min. Then, they were visualized under UV in GelDocXR (Biorad, USA). The PCR products were sent to Iontek (Istanbul) for sequencing and purifying procedures.

2.2.1.5.6. Evaluation of Sequencing Results

The sequences obtained from Iontek were visualized and analysed via Blast Programme of NCBI. According to the sequences obtained, their multiple alignments were compared by using ClustalW Programme.

2.2.1.5.7. Random Amplified Polymerase Chain Reaction (RAPD-PCR)

In order to type different *L. acidophilus* strains and *L. amylovorus* strains RAPD-PCR was performed. M13 primer was used for RAPD-PCR analysis (Cebeci Aydın 2008, Shillinger et al., 2003, Delfederico et al., 2006).

L. acidophilus and *L. amylovorus* that were used for differentiation in RAPD-PCR are listed in table 2.4.

Table 2.4. *L. acidophilus* and *L. amylovorus* strains used in RAPD

Name	Source
L. acidophilus ATCC 4356	American Type Culture Collection
L. acidophilus LA05	Chr-Hansen
L. acidophilus NCFM (HowaruDophilus LYO 40 DCU)	Danisco
L. acidophilus 74-2	Danisco
L. amylovorus A7	Kefir isolate (this study)
L. amylovorus A11	Kefir isolate (this study)
L. acidophilus LgER	İzzet Baysal Unv.
L. acidophilus KPB4B	Izzet Baysal Unv.

The cultures *L. acidophilus* 74-2 and *L. acidophilus* NCFM were kindly provided by Danisco via Sütaş/Bursa. *L. acidophilus* LgER (isolate from breast-fed baby faeces) and *L. acidophilus* KPB4B (yogurt isolate) cultures were kindly provided by İbrahim Çakır (Izzet Baysal University).

The sequence of primer M13 is given below in table 2.5.

Table 2.5. Sequence of M13 primer

Primer	**Target gene**	**Primer sequence (5'→3')**
M13	Whole genome	GAGGGTGGCGGTTCT

The reaction mix and PCR conditions are given in tables 2.6. and 2.7., respectively;

Table 2.6. Reaction mix for RAPD

Reaction components	Final concentration
ddH_20	-
$MgCI_2$	3.0 mM
PCR Buffer	1X
dNTP	200mM
Primer	1mM
Taq polymerase	0,5U
DNA	150ng

Table 2.7. PCR conditions for RAPD

	Temperature-Time
Initial denaturation	94˚C 2 min
Denaturation Annealing Extension	94˚C 1 min 42˚C 20 sec 72 ˚C 2 min } 40 cycle
Final Extension	72 ˚C 10 min

PCR products were run in agarose jel for 2 hours at 50V. After running they were stained with ethidium bromide for 20 min, destained for 10 min and visualized under UV in GelDocXR (Biorad, USA).

2.2.1.5.8. Evaluation of RAPD results

Upon electrophoresis, the gel photos of RAPD were taken by using GelDoc (BioRad). After taking the photos of the gel, the patterns were observed by using QuantityOne (BioRad). Dendograms were obtained by matching the bands which were detected. Cluster analysis was carried out by using Unweighted Pair Group Method Aritmetic Mean (UPGMA). A similarity of 80% was selected as a verge for the definiton of RAPD based clusters (Cebeci Aydin, 2008, Aquilanti 2007, Kenny et al., 2005).

2.2.2. Yogurt

2.2.2.1. Bacterial Strains Used

In order to evaluate different suggested media for the selective enumeration of *L. acidophilus* from a probiotic yogurt, different cultures were used. These cultures are listed in table 2.8.;

Table 2.8. Microorganisms used in this study

Species or subspecies	Strain number	Source
L.acidophilus	ATCC 4356	ATCC
L.acidophilus	LA05	Chr-Hansen
L. delbrueckii subsp. *bulgaricus*	DSM20081	DSMZ
L. delbrueckii subsp. *bulgaricus*	B1000-1	Visby
L. delbrueckii subsp. *bulgaricus*	B1000-2	Visby
L. bulgaricus	Yo-mix 410-1	Danisco
L. delbrueckii subsp. *bulgaricus*	YCX-11	Chr-Hansen
S. thermophilus	Ta 040-3	Danisco
S. thermophilus	Yo-mix 410-2	Danisco
S. thermophilus	Yo-mix 410-3	Danisco
S. thermophilus	YCX-11	Chr-Hansen
B. lactis	BB-12	Chr-Hansen
L. amylovorus	A-7	Kefir grains
L. amylovorus	A-11	Kefir grains
L. acidophilus	KPB4B	Izzet Baysal Unv.

[1] American Type Culture Collections

[2] German Resource Center for Biological Material

[3] National Institue of Agronomical Research, France

The cultures of *L. bulgaricus, L. acidophilus* and *Bifidobacterium* were taken from their glycerol stocks (20 %) and they were activated three times in MRS broth (pH ± 5,7) 37°C prior to usage. The frozen cultures of *S. thermophilus* were activated three times in M17 broth (pH 6.8) at 42°C prior to usage. After activation they were serially diluted with peptone water (0,1 %) and poured on the media used.

The culture YCX-11 (Table 2.8) was a freeze-dried yogurt culture and contained *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* together. In order to activate and

differentiate the bacteria from each other; one loop was taken from the culture under aseptic conditions and transferred into MRS broth (pH 5.7± 0,2) for the growth of *L. delbrueckii subsp. bulgaricus* and one loop was transferred into M17 broth (pH 6.8) for the growth of *S. thermophilus*. The tubes were incubated at 42°C for 24 hours. After incubation in order to have pure cultures of *S. thermophilus* and *L. delbrueckii subsp.bulgaricus* they were streaked on M17 (pH 6.8) and MRS agar, respectively. After purification they were Gram stained in order to observe their colony shapes. Finally, 20% glycerol stocks of differentiated purified *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* cultures were prepared and they were stored in -80°C.

2.2.2.2. Selective media for *L. acidophilus* and *L. amylovorus* in probiotic yogurt

For the selective enumeration of *L. acidophilus* in a probiotic yogurt, three different media were tested with the strains (Table 2.8) mentioned above. These media were; MRS-maltose (Chr-Hansen), MRS-sorbitol (Tharmaraj N. and Shah N.P., 2003) and Bile-MRS (Vinderola C.G. and Reinheimer J.A., 2000). During the experiment, pour-plate method was used and all the plates were incubated aerobically at 37°C for 72h.

For the preparation of MRS-maltose, MRS-sorbitol and MRS-Salicin; basal medium was prepared (Appendix B) and autoclaved at 121°C for 15 min. After being autoclaved they were cooled down to 50-55°C in a water bath. 20% of sugar solutions prepared and sterilized with 0,22 μm filters (Minisart, Sartorius). Prepared sugar solutions were added to the basal media that were cooled down to 50-55°C (final sugar concentration 2%) (Thamaraj and Shah, 2003).

Bile-MRS was prepared by adding % 0,15 (w/v) Bile Salt to MRS medium and autoclaved at 121°C for 15min (Vinderola and Reinheimer, 1999). The pH of the medium was measured as 5.7 ± 0.2.

For MRS-maltose the pH was measured as 6.9 ± 0.1. For MRS-sorbitol the pH was adjusted from 6.7 ± 0.2 to 5.5 ± 0.1.

Beyond the media tested, MRS-sorbitol was choosen as the selective medium for *L. acidophilus* in the presence of yogurt bacteria and *Bifidobacterium*. The selected medium was then tested for its selectivity on *L. amylovorus.* Before starting the enumeration of *L. acidophilus* from commercial probiotic yogurt, the starter cultures of this commercial probiotic yogurt (YC-X11) and the *Bifidobacterium* (BB-12) culture of this probiotic yogurt were tested on MRS-sorbitol in order to see whether they grow on MRS-sorbitol or not.

One loop were taken from each freeze-dried culture and they were serially diluted with peptone water. After diluting they were poured with MRS-sorbitol agar and incubated at 37°C for 72h.

2.2.2.3. Enumeration of *L. acidophilus* LA05 in commercial probiotic yogurt during its shelf life

The commercial probiotic yogurts were kindly provided from the manufacturer on the first day of it's production and were brought to the laboratory in cold chain. The yogurts were stored at 4°C during the experiment.

During the experiment pour plate method was applied and the yogurt samples were analyzed in duplicates. One ml of the yogurt was added to a 9 ml of sterile peptone water (0,1 % w/v) and dilutions were made from 10^{-1} to 10^{-8} in triplicates. MRS-sorbitol (Appendix B) was prepared and poured on the plates. After the plates were cooled they were turned upside down and incubated aerobically at 37°C for 72h.

After incubation, plates that contain 30-300 colonies were enumerated and the colonies were observed under microscope for the selectivity of growth medium used (100X magnification).

By starting from the 1^{st} day of the yogurt production, the experiment was repeated every 7 -day intervals during the shelf life of the product which was 34 days.

2.2.2.4. Production of probiotic yogurt with traditional *L. acidophilus* and *L. amylovorus* cultures

Probiotic yogurt was produced with some species belong to the '*L. acidophilus* group'; two of them were isolated from kefir grains in our laboratory which are characterized as *L. amylovorus* species and one of them, a traditional yogurt isolate *L. acidophilus* KPB4B (Table 2.9.).

Table 2.9. Traditional isolates used for probiotic yogurt production

Isolates	Source
L. amylovorus A-7	Kefir grains (Ank.Unv.)
L. amylovorus A-11	Kefir grains (Ank. Unv.)
L.acidophilus KPB4b	Traditional yogurt (Izzet Baysal Unv.)

In order to determine the viability of these cultures, three different sets of probiotic yogurts were produced with these cultures. They were produced with lyophilized commercial yogurt cultures, lyophilized commercial *Bifidobacterium lactis* culture and traditional '*L. acidophilus* complex' isolates; *L. amylovorus* A-7, *L. amylovorus* A-11 and *L. acidophilus* KPB4B .

2.2.2.4.1. Adjustment of the initial load of probiotics into milk

According to the commercial probiotic yogurt producers, the initial quantity of probiotics in probiotic yogurt should be approximately 10^7cfu/ml (Sutaş/Bursa). 1000 ml milk was used for yogurt production in this study. So the inital load of the cultures A-7, A-11 and KPB4B were adjusted to 10^{10}cfu/ml.

For the adjustment of the probiotics concentration to 10^{10}cfu/ml, the optical density of the cultures were determined at different cell concentrations at a wavelength of 600nm (Boukseim et al., 2000) using spectrophotometer (Analytikjena Specord 50).

For the measurement of the optical density the cells were diluted 1/10 (100ul cell culture + 900ul water). After different measurements, it was determined that the optical density of *L. amylovorus* A-7 and *L. amylovorus* A-11 should be 0.8 at 600 nm in order to have approximately 10^{10} cfu /ml cells. And the optical density of *L. acidophilus* KPB4B should be 0.9 at 600 nm in order to have approximately 10^{10} cfu/ml cells.(Appendix E).

2.2.2.4.2. Probiotic yogurt production

For the probiotic yogurt production, the recommended procedure was provided by Sütaş Bursa; the initial amount of *L. amylovorus* A-7, *L. amylovorus* A-11 and *L. acidophilus* KPB4B were adjusted to 10^7 cfu/ml for the inoculation into the milk.

Reconstituted and heat treated milk used in the production of probiotic yogurt which was kindly provided by Atatürk Orman Çiftliği. The milk had a pH of 6.5, fat content of 3 % and 13.3% of dry matter.

From the freeze dried yogurt cultures (YC-X11) that contain 10^{10} cfu/ml cells 1 g was weighed and 0.1 g was weighed from freeze dried *Bifidobacterium animalis* subsp. *lactis* culture (BB-12) that contain 10^{11} cfu/ml cells. In order to adjust initial load of A-7, A-11 and KPB4B to 10^{10} cfu/ml into milk, optical density was measured and adjusted to 0.8 at 600 nm for A-7 and A-11, and for *L. acidophilus* KPB4B the optical density was adjusted to 0.9 at 600 nm.

Right after the adjustment of optical densities of the cultures; the yogurt cultures, *Bifidobacterium* culture and the isolates were inoculated into 1000 ml milk and stirred subsequently. The 1000 ml inoculated milk were divided into 100 ml portions and these portions were incubated at 39°C for fermentation. During the incubation, pH was measured periodically and when pH was $4.5 \pm 0{,}1$ fermentation was ceased. At the end of fermentation, the yogurts were stored at 4°C for 34 days and viable counts of A-7, A-11 and KPB4B in these products were taken at 7 day intervals (Figure 2.1.). The counting was done as mentioned in section 2.2.2.3.

Briefly, three sets of probiotic yogurts which contain same yogurt cultures and *Bifidobacterium* culture but different *L. acidophilus* and *L. amylovorus* cultures were produced;

- P1 (Yogurt culture YC-X11 + *B. lactis* BB-12+ *L. amylovorus* A-7),
- P2 (Yogurt culture YC-X11 + *B. lactis* BB-12+ *L. amylovorus* A-11),
- P3 (Yogurt culture YC-X11 + *B. lactis* BB-12+ *L. acidophilus* KPB4B).

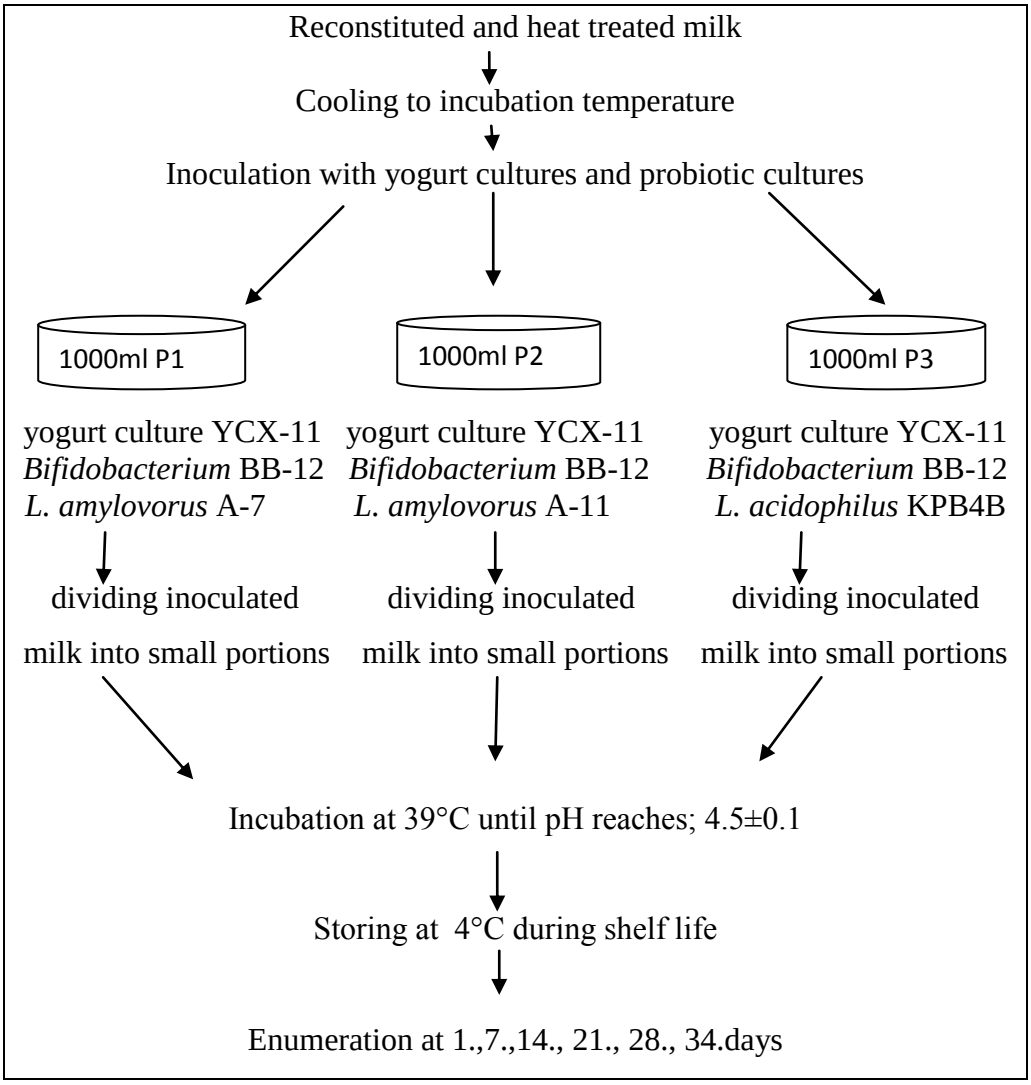

Figure 2.1. Flowchart of probiotic yogurt production

2.2.2.5. pH Measurement

During the enumerations pH of the yogurts were measured with a pH meter. Prior to pH measurement calibration was done with the standard buffers at pH 4 and 7.

2.2.2.6. Statistical Analysis

Enumeration results were presented in mean $\log_{10}$ ± standard deviation (n=12). Repeated Measures of One Way Anova was applied using General Linear Model of the SAS system in order to test differences between the viability of commercial probiotic and traditional probiotics where $p<0.05$ were considered as statistically different.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Experimental Strategy

Today, probiotics are widely spread both in scientific and industrial fields. For the increasing consumption of probiotic products, food industry should fulfil consumers' desires. Probiotic products should be safe for human health and beside this, in order to function properly in the digestive system, they should contain probiotics in adequate numbers. They should protect their numbers and activities during the shelf life of probiotic products. Nowadays, lots of industrial culture manufacturers search for ways in order to find new strains that contain such properties.

In this study, our aim was to isolate probiotic bacteria from traditional kefir grains, determine their viability during the shelf life of a probiotic yogurt and compare their survival with commercial *L. acidophilus* LA05 strain. Besides these, typing of *L. acidophilus* and *L. amylovorus* strains with RAPD-PCR method was also aimed. For this purpose, some isolations from kefir grains were performed. After the isolations in order to select potential lactic acid bacteria, biochemical tests were performed. Following biochemical tests, the potential species that could belong the '*L. acidophilus* group' were analyzed via 16S rRNA gene sequencing and multiple alignment. According to 16S rRNA gene sequencing and multiple alignment results, two isolates were identified as '*L. amylovorus*'. Moreover, RAPD-PCR was performed in order to observe diversity between *L. acidophilus* and *L. amylovorus* strains.

Following identification of kefir grains isolates as '*L. amylovorus'*, they were used for the production of probiotic yogurt and their viability were determined during the shelf life of the product. Besides the shelf life of *L. amylovorus* species, the shelf life of traditional yogurt isolate: *L. acidophilus* KPB4B strain and the shelf life of a commercial probiotic *L. acidophilus* LA05 was also determined. The experimental strategy of the isolation, identification of *L. amylovorus* and survivals are explained in Figure 3.1. The experimental strategy for typing of *L. acidophilus* and *L. amylovorus* strains is explained in Figure 3.2.

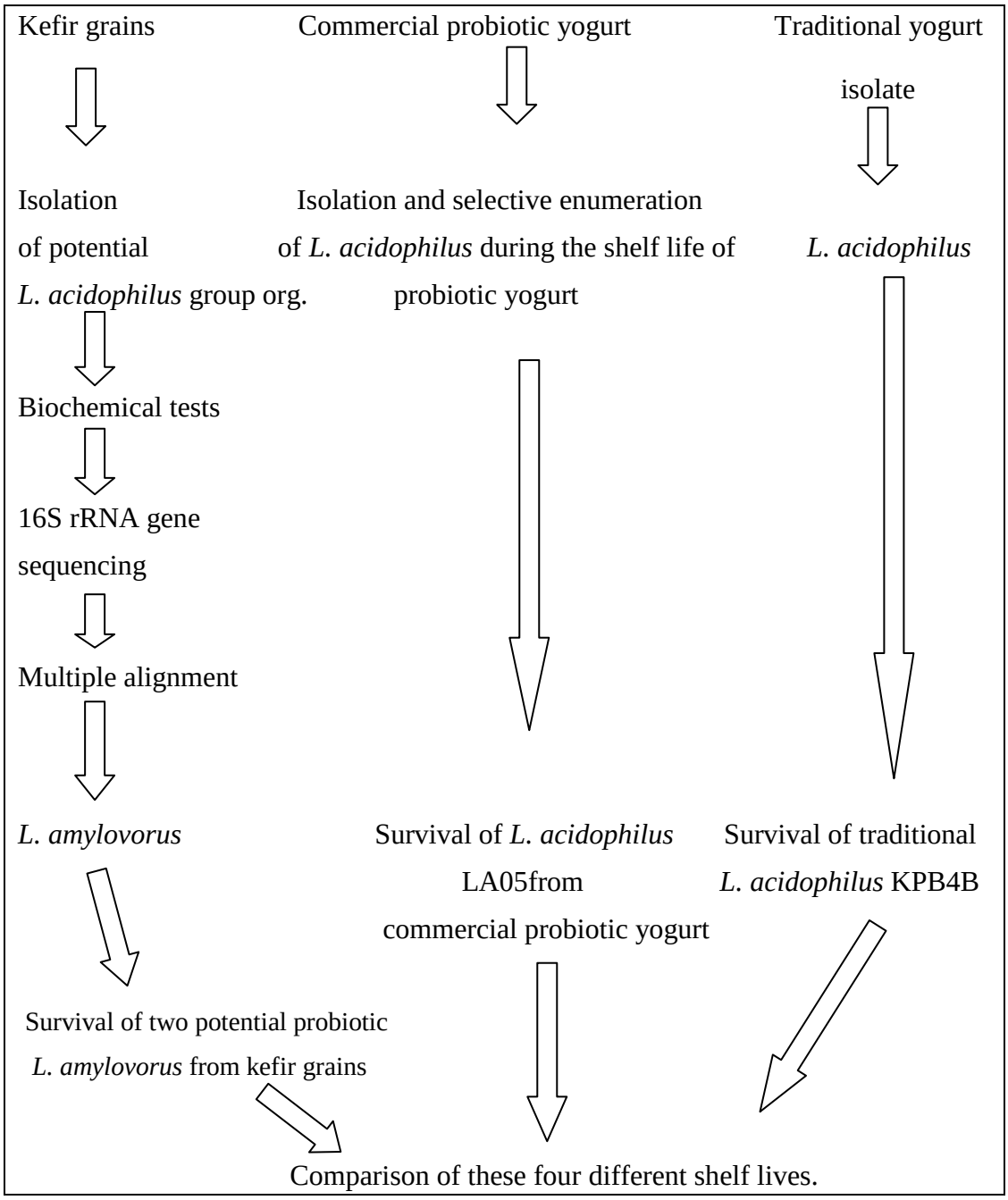

Figure 3.1. Flowchart of the experimental strategy for the survival of probiotic strains (Part I)

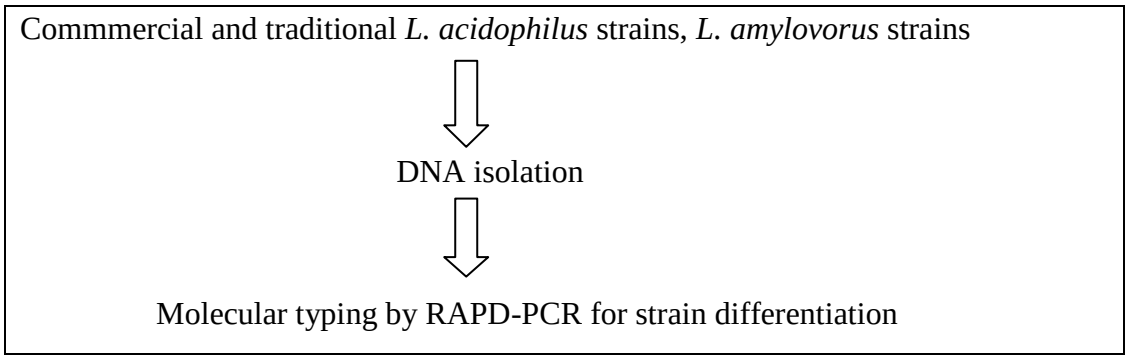

Figure 3.2. Flowchart of the experimental strategy for typing *L. acidophilus* and *L. amylovorus* strains (Part II).

3.2. Isolation of lactic acid bacteria from kefir grains

For the isolation of lactic acid bacteria three different kefir grains were evaluated. The grains that were obtained from Ankara University Agricultural Faculty Department of Dairy Technology were named as 'A' in this study. The other grains obtained from traditional home made kefir producer in Ankara were named as 'B' and the third one was from Mersin were named as 'C'.

For the isolation, MRS (pH:5.5 ±0.1) agar was used since it is the optimum pH for lactobacilli (Robinson 2000). After 72h- 96h of incubation, different types of colonies especially white or creamy smooth colonies which morphologically resemble species of lactic acid bacteria were selected and purified on MRS agar.

3.2.1. Biochemical tests for the identification of putative *Lactobacilli*

For all the isolates, Gram staining was performed in order to select Gram positive bacteria. Gram negative ones were eliminated and further studies continued with Gram positive ones. Some of Gram positive bacteria observed as single rods while some of them observed rods in chains. After Gram staining, catalase test was performed in order to select catalase negative isolates since all species of *Lactobacillus* genus are catalase negative organisms.

After that, gas production from glucose, growth on bile-MRS and endospore test was performed.

The isolates that did not produce gas from glucose were determined with the absence of bubbles in Durham tubes which indicated homofermentative metabolism. These species with homofermentative metabolism could belong to '*L. acidophilus* group' since organisms of this group are obligate homofermenters (Table 3.1.) Some of the isolates could grow on Bile-MRS while some of them not (Table 3.2.).Growth on Bile-MRS test was performed in order to select cultures that have the ability to grow in the presence of bile.

Table 3.1. Some biochemical properties of reference strains

	Gram staining	Gas production from glucose	Catalase rxn (% 3 H_2O_2)	Endospore Forming
L. acidophilus	Gr (+) rod	-	-	-
L. amylovorus	Gr (+) rod	-	-	-

Table 3.2. Biochemical test results of potential lactobacilli

	Gram staining	Gas production from glucose	Growth on bile-MRS	Catalase rxn (% 3 H_2O_2)	Endospore Forming
A-7	Gr (+) rod	-	+	-	-
A-10	Gr (+) rod	-	-	-	-
A-11	Gr (+) rod	-	+	-	-
A-15	Gr(+) rod	-	+	-	-
A-16	Gr (+) rod	-	-	-	-
A-19	Gr (+) rod	-	+	-	-
B-1	Gr (+) rod	+	+	-	-
B-2	Gr (+) rod	+	+	-	-
B-3	Gr (+) rod	-	+	-	-
B-4	Gr (+) rod	-	+	-	-
B-5	Gr (+) rod	-	+	-	-
B-6	Gr (+) rod	-	+	-	-
B-7	Gr (+) rod	-	+	-	-

Table 3.2. Biochemical test results of potential lactobacilli (cont'd).

	Gram staining	Gas production from glucose	Growth on bile-MRS	Catalase rxn (% 3 H_2O_2)	Endospore Forming
B-8	Gr (+) rod	+	+	-	-
B-9	Gr (+) rod	-	+	-	-
B-10	Gr (+) rod	+	+	-	-
B-11	Gr (+) rod	-	+	-	-
B-12	Gr (+) rod	-	+	-	-
B-14	Gr(+) rod	-	+	-	-
C-2	Gr(+) rod	-	+	-	-
C-3	Gr(+) rod	-	+	-	-
C-6	Gr(+) rod	-	+	-	-
C-7	Gr(+) rod	-	+	-	-
C-9	Gr(+) rod	-	+	-	-
C-10	Gr(+) rod	-	+	-	-
C-11	Gr(+) rod	-	+	-	-
C-12	Gr(+) rod	-	+	-	-
C-13	Gr(+) rod	-	+	-	-
C-14	Gr(+) rod	-	+	-	-

A: isolates from the grains of Ankara University

B: isolates from traditional grains of Mersin

C: isolates from traditional grains of Ankara

3.2.1.1. Carbohydrate Fermentation

Carbohydrate fermentation tests were performed with 15 different sugars and different sugar fermentation profiles were obtained (Table 3.4.). The results were interpreted with the colour change from purple to yellow in the wells. The colour change from purple to yellow in the wells were accepted as positive, due to the fermentation of the carbohydrate. With the usage of the carbohydrate the pH of the medium changed and the indicator in the medium, bromocresol purple, changes its colour. The wells with no colour change accepted as negative results which means that the carbohydrate was not fermented (Dede Altay, 2010). The results were

compared with the carbohydrate fermentation patterns of lactobacilli obtained from Bergey's Manual (1986) (Table 3.3.).

According to the carbohydrate fermentation results, the patterns of A-7 and A-11 were similar to *L. amylovorus* but A-7 was different from *L. amylovorus* by unusually fermenting mannitol. A-11 was different from *L. amylovorus* by not fermenting esculin. Besides these, they did not produce gas from glucose which means that they are homofermentative like *L. amylovorus* (Table 3.1.). The isolate A-16 was similar to a facultative heterofermentative organism *Lactobacillus curvatus* but A-16 differed from *L. curvatus* by not fermenting esculin. Also, A-16 differed from *L. curvatus* by fermenting mannitol. The carbohydrate profile of A-19 was similar to *L. curvatus* also, but differed from it by fermenting trehalose.

The patterns of the isolates B-1 and B-2 were similar to the carbohydrate profile of an obligate heterofermentative organism *Lactobacillus reuteri* except sucrose utilization since *Lactobacillus reuteri* ferments sucrose. But these strains did not ferment sucrose. B-1 and B-2 were heterofermentative organisms since they produced gas from glucose (Table 3.2.). The patterns of the isolate B-7, B-9, B-11 and B-12 were similar to a facultative heterofermentative organism *L. plantarum* except xylose and esculin utilization. B-8, B-10 and B-14 were more similar than the others to *L. plantarum* since only one carbohydrate utilization, of xylose, was different from *L. plantarum*.

The carbohydrate patterns of the isolates C-3, C-6, C-7, C-10, C-11 and C-12 were similar to a heterofermentative organism *Lactobacillus kefir*. C-9 was also similar to *L. kefir* but differed from it by fermenting sorbitol.

For further genotypic analysis, mannitol positive ones were eliminated since they are most probably *L. plantarum*. Sorbitol negative ones were eliminated since they are most probably *L. curvatus*. Instead mannitol negative and sorbitol positive ones were selected as they are most probably *L. acidophilus* or *L. amylovorus*.

Table 3.3. Carbohydrate fermentation patterns of representative lactobacilli (Bergey, 1986) and putative lactobacilli.

Name	**Arabinose**	**Xylose**	**Galactose**	**Glucose**	**Fructose**	**Mannitol**	**Sorbitol**	**Esculin**	**Salicin**	**Cellobiose**	**Maltose**	**Lactose**	**Melibiose**	**Sucrose**	**Trehalsoe**
L. acidophilus	-	-	+	+	+	-	+	+	+	+	+	+	d	+	+
L. amylovours	-	-	+	+	+	-	+	+	+	+	+	-	-	+	+
L. curvatus	-	-	+	+	+	-	-	+	+	+	+	d	-	-	-
L. kefir	d	-	-	+	+	-	-	-	-	-	+	+	+	-	-
L. plantarum	d	d	+	+	+	+	+	+	+	+	+	+	+	+	+
L. reuteri	+	-	+	+	+	-	-	-	-	-	+	+	+	+	-
Ank.Unv. A-7	-	-	+	+	+	w	+	+	+	+	+	-	-	+	+
Ank.Unv. A-10	w	-	w	-	-	-	w	-	w	-	w	-	w	-	-
Ank.Unv. A-11	-	-	+	+	+	-	+	-	+	+	+	-	-	+	+
Ank.Unv. A-15	-	-	+	+	+	w	-	+	+	+	+	-	w	w	w
Ank.Unv. A-16	-	-	+	+	+	w	-	-	+	+	+	+	-	-	-
Ank.Unv. A-19	-	-	+	+	+	-	-	+	+	+	+	+	-	-	+
Mersin B-1	+	-	+	+	+	-	-	-	-	-	+	+	+	-	-
Mersin B-2	+	-	+	+	+	-	-	-	-	-	+	+	+	-	-
Mersin B-3	+	-	+	+	+	-	w	-	w	-	+	+	-	-	-
Mersin B-4	+	-	+	+	+	-	w	-	w	-	+	+	w	-	-
Mersin B-5	+	-	+	+	-	-	w	-	-	-	+	+	w	-	w
Mersin B-6	+	-	+	+	-	-	w	-	-	-	+	+	-	-	-
Mersin B-7	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Mersin B-8	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Mersin B-9	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Mersin B-10	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3.3. Carbohydrate fermentation patterns of representative lactobacilli (Bergey, 1986) and putative lactobacilli (cont'd).

Name	**Arabinose**	**Xylose**	**Galactose**	**Glucose**	**Fructose**	**Mannitol**	**Sorbitol**	**Esculin**	**Salicin**	**Cellobiose**	**Maltose**	**Lactose**	**Melibiose**	**Sucrose**	**Trehalose**
Mersin B-11	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Mersin B-12	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Mersin B-14	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Ankara C-2	+	-	+	+	+	+	w	+	+	+	+	+	+	+	+
Ankara C-3	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-
Ankara C-6	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-
Ankara C-7	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-
Ankara C-9	+	-	-	+	+	-	+	-	-	-	+	+	+	-	-
Ankara C-10	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-
Ankara C-11	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-
Ankara C-12	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-

+ : positive reaction

- : negative reaction

w: weak reaction

d: 11-89 % strains positive (Bergey, 1986)

For most of the bacterial species it is difficult to identify them only with their biochemical properties since they can not give accurate results alone (Lan and Reeves, 1996). Biochemical tests should be supported with molecular genetic methods (Mainville et al., 2006). *Lactobacillus* species have similar nutritional requirements and grow under similar environmental conditions (Garbers et al., 2004). The species belong to '*L. acidophilus* complex'contain closely related species that can not be easily differentiated. In a study of Du Plesis and Dicks (1995); some species of '*L. acidophilus* complex'; *L. acidophilus*, *L. crispatus* and *L. gallinarum* could not be differentiated from each other with simple biochemical tests even by using lactic dehydrogenase profiles which is used for differentiating species (Garbers et al., 2004). On the contrary, genotypic methods can give more precise results (Lan and Reeves, 1996). 16S rRNA gene sequencing method is a widely accepted method used for the identification of *Lactobacillus* species (Mora and Amman, 2001).

3.3. 16S rRNA gene sequencing for potential '*L. acidophilus* group' organisms from kefir grains

To isolate and identify probiotic '*L. acidophilus* group' organisms mannitol negative and sorbitol positive isolates (A-7 and A-11) were selected as presumptive '*L. acidophilus* group' species. For the further characterization of the species A-7 and A-11, 16S rRNA gene sequencing was performed.

The 16S rRNA gene sequences of potential '*L. acidophilus* complex' species were analyzed in the NCBI database via BLAST programme. A region corresponding to a 600 bp of 16S rRNA from 5' downstream gene was analyzed via Blast Programme (Appendix C). According to blast analysis; A-7 species sequences showed %100 identity with *L. acidophilus* 30SC (RDP), but also with *L. amylovorus* DSM20531T (RDP). Similarly, A-11 species sequences showed %100 identitiy with *L. acidophilus* 30SC but also with *L. amylovorus* GRL1118.

Blast analysis of these two species A-7 and A-11 showed that they could belong to the species of either *L. acidophilus* or *L. amylovorus* which are both included in the

'*L. acidophilus* complex'. In order to determine the identity of the strains A-7 and A-11, their 16S rRNA gene sequences were aligned with type strains of *L. acidophilus* and *L. amylovorus* along with some other strains present in the Ribosomal Database Project (RDP). The alignment was performed via Clustal W2 Programme of MEGA 4. Differences between bases are highlighted as given in Figure 3.3. (The whole multiple alignment is given in Appendix D).

The strains used for alignment are given as follows;

L. acidophilus CECT4179 (acidophilus 1), *L. acidophilus* BCRC 10695(T) (acidophilus 2) , *L. acidophilus* NCFM (acidophilus 3), *L. amylovorus* DSM 20531(T) AY944408 (amylovorus 1), *L. amylovorus* GRL1112 (amylovorus 2), *L. amylovorus* DSM 20531 (T) FR683089 (amylovorus 3).

```
Acidophilus 1    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 96
Acidophilus 2    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 102
Acidophilus 3    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 117
Amylovorus  1    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 111
Amylovorus  2    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 120
Amylovorus  3    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 83
a-11             A-GTCGAGCGAGCGGAACCA-CAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 86
a-7              ----------------------ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 36
                                        *** ******** ************* *********

Acdiophilus 1    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAG 156
Acidophilus 2    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAG 162
Acidophilus 3    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAG 177
Amylovorus  1    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAG 171
Amylovorus  2    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAG 180
Amylovorus  3    GGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAG 143
a-11             GGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAG 146
a-7              GGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAG 96
                 ********************************  ************* **********
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Figure 3.3. Clustal W multiple sequence alignment for 16S rRNA gene of *L. amylovorus* A-7 , *L. amylovorus* A-11, Acidophilus 1,2,3 and Amylovorus 1,2,3(Different bases are highlighted);

```
Acidophilus 1   GTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCT 216
Acidophilus 2   GTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCT 222
Acidophilus 3   GTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCT 237
Amylovorus  1   GTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCT 231
Amylovorus  2   GTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCT 240
Amylovorus  3   GTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCT 203
a-11            GTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCT 206
a-7             GTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCT 156
                ********** ********** ********** * **********

Acidophilus 1   GTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGG 276
Acidophilus 2   GTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGG 282
Acdiophilus 3   GTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGG 297
Amylovorus  1   GTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG 291
Amylovorus  2   GTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG 300
Amylovorus  3   GTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG 263
a-11            GTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG 266
a-7             GTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG 216
                ******* ********************************* ********* ********

Acidophilus 1   CAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 336
Acidophilus 2   CAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 342
Acidophilus 3   CAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 357
Amylovorus  1   CGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 351
Amylovorus  2   CGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 360
Amylovorus  3   CGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 323
a-11            CGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 326
a-7             CGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA 276
                * * ******************************************************

Acidophilus 1   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCA 396
Acidophilus 2   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCA 402
Acidophilus 3   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCA 417
Amylovorus  1   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA 411
Amylovorus  2   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA 420
Amylovorus  3   AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA 383
a-11            AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA 386
a-7             AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA 336
                ****************************************** **************

Acidophilus 1   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 456
Acidophilus 2   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 462
Acidophilus 3   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 477
Amylovorus  1   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 471
Amylovorus  2   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 480
Amylovorus  3   ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 443
a-11            ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 446
a-7             ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT 396
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Figure 3.3. Clustal W multiple sequence alignment for 16S rRNA gene of *L. amylovorus* A-7 , *L. amylovorus* A-11, Acidophilus 1,2,3 and Amylovorus 1,2,3(cont'd).

```
Acidophilus 1   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 516
Acidophilus 2   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 522
Acidophilus 3   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 537
Amylovorus  1   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 531
Amylovorus  2   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 540
Amylovorus  3   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 503
a-11            AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 506
a-7             AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 456
                ************************************************************

Acidophilus 1   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 576
Acidophilus 2   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 582
Acidophilus 3   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 597
Amylovorus  1   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 591
Amylovorus  2   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 600
Amylovorus  3   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 563
a-11            GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 566
a-7             GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 516
                ************************************************************

Acidophilus 1   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 636
Acidophilus 2   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 642
Acidophilus 3   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 657
Amylovorus  1   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 651
Amylovorus  2   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 660
Amylovorus  3   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 623
a-11            GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 626
a-7             GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 576
                ************** ********* ***********************************

Acidophilus 1   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 696
Acidophilus 2   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 702
Acidophilus 3   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 717
Amylovorus  1   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 711
Amylovorus  2   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 720
Amylovorus  3   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 683
a-11            CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 686
a-7             CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 636
                ************************************************************

Acidophilus 1   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 756
Acidophilus 2   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 762
Acidophilus 3   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 777
Amylovorus  1   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 771
Amylovorus  2   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 780
Amylovorus  3   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 743
a-11            TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 746
a-7             TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 696
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Figure 3.3. Clustal W multiple sequence alignment for 16S rRNA gene of *L. amylovorus* A-7, *L. acidophilus* A-11, Acidophilus 1,2,3 and Amylovorus 1,2,3(cont'd).

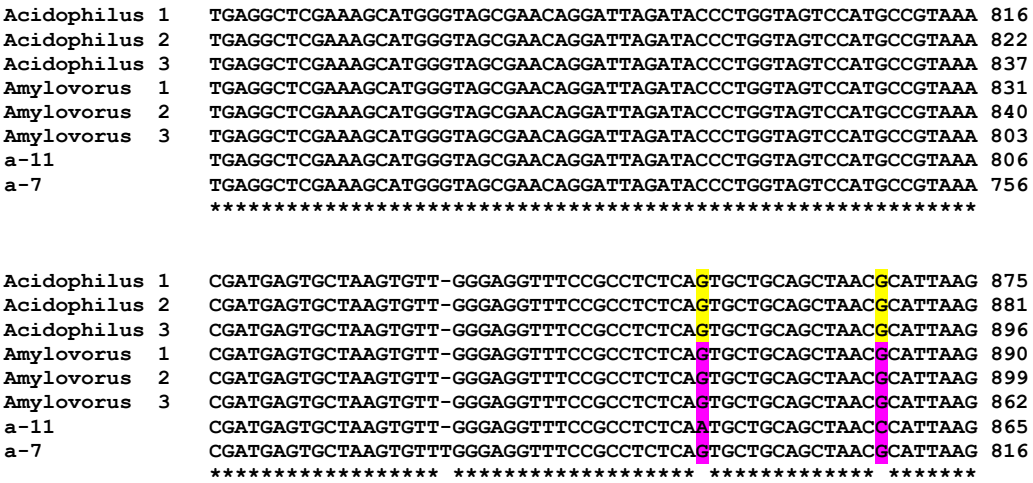

Figure 3.3. Clustal W multiple sequence alignment for 16S rRNA gene of *L. amylovorus* A-7, *L. acidophilus* A-11, Acidophilus 1,2,3 and Amylovorus 1,2,3 (cont'd).

According to the aligments of 16S rRNA gene sequences, comparison of the variable regions(V1-V3) (Baker et al., 2003) revealed that at the regions where *L. acidophilus* and *L. amylovorus* type cultures differ from each other, A-7 and A-11 sequences showed identity with *L. amylovorus* type cultures. This aligment results and biochemical tests performed in this study demonstrated that these species A-7 and A-11 belong to *L. amylovorus*. Following alignment, their phylogenetic tree were generated with MEGA 4.0. (Figure 3.4). *L. acidophilus* and *L. amylovorus* strains were grouped seperately from each other. *L. amylovorus* A-7 and A-11 were grouped within the same cluster with *L. amylovorus* strains that were selected from Ribosomal Database Project (RDP).

Since *L. amylovorus* is considered as a potential probiotic (Kant et al., 2011), survival studies were performed with the isolates A-7 and A-11.

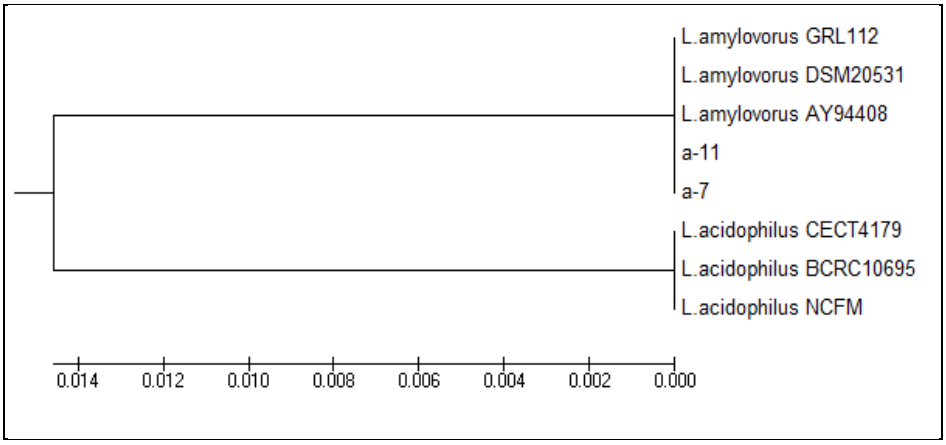

Figure 3.4. Phylogenetic tree generated with MEGA4 on the basis of 16S rRNA gene sequences of *L. acidophilus* and *L. amylovorus* from RDP and A-7 and A-11 from kefir grains.

Such an alignment approach is in agreement with the study of Kullen et al. (2000). In their study, a DNA sequence based identification system was developed in order to identify the unkown isolates that belong to '*L. acidophilus* group'. A 500 bp region of 16S rRNA gene was amplified from the unkown isolates by PCR and sequenced. After that the 16S rRNA sequences of the unkown isolates were aligned with the 16S rRNA regions of type strains. Following alignment, a comparison of the differences between 16S rRNA regions of unkown isolates and type strains enabled the identification of unkown isolates of '*L. acidophilus* group'. The approach applied in this study was stated as a successful method for the identification of species within the '*L. acidophilus* group' which is important for the development of new probiotic strains.

3.4. RAPD-PCR patterns of *L. acidophilus* and *L. amylovorus*

Following identification of the kefir isolates, RAPD-PCR was performed in order to observe the diversity between *L. acidophilus* and *L. amylovorus* strains. M13 primer was used for RAPD-PCR analysis.(Schilligner et al., 2003). The isolates were grouped into five clusters based on the 80% similarity level (Figure 3.5.).

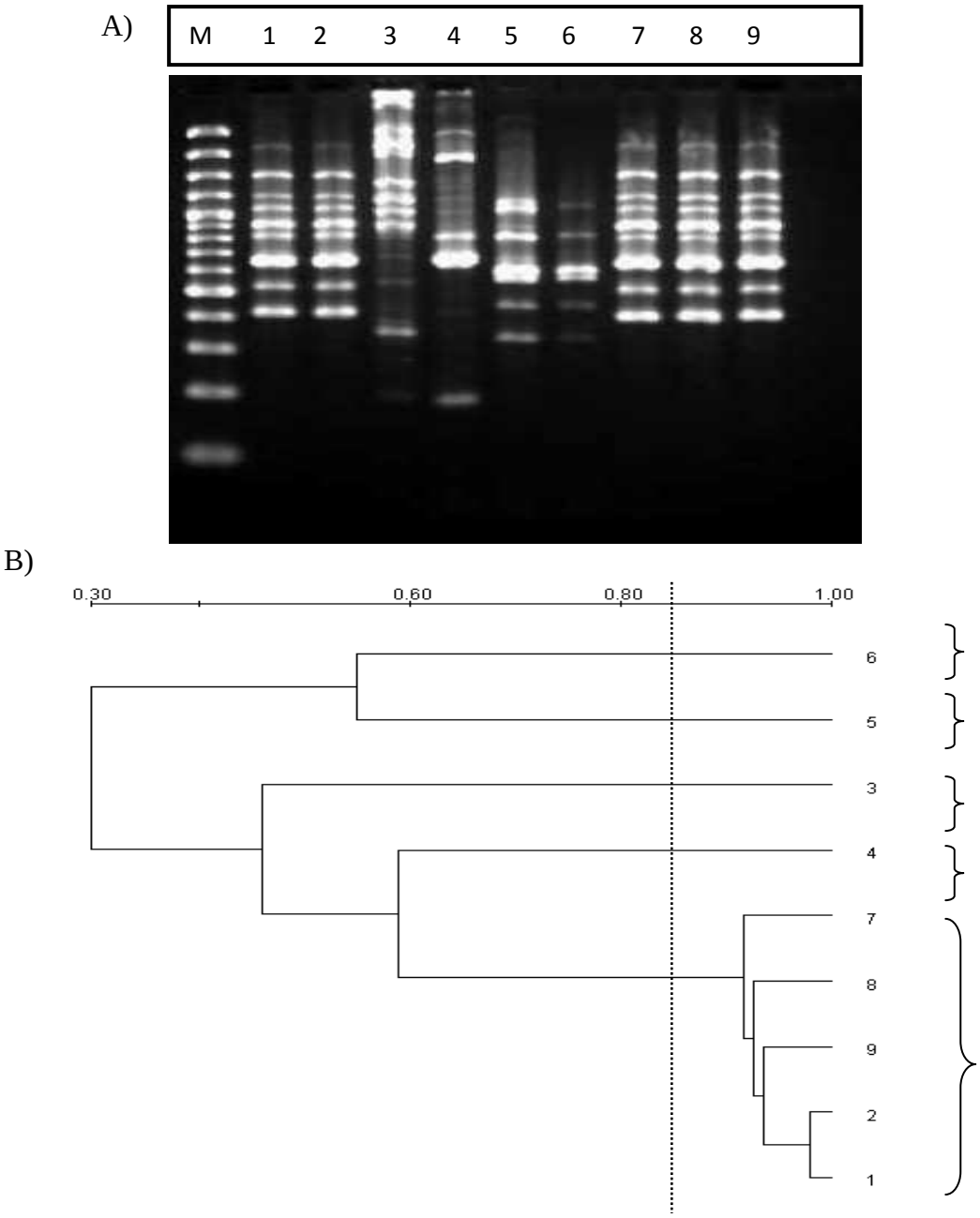

Figure 3.5. RAPD analysis of *L. acidophilus* and *L. amylovorus* A) Gel photograph of RAPD analysis of *L. acidophilus* and *L. amylovorus* species with primer M13. M: 100bp ladder, lane 1: *L. acidophilus* LA05, lane 2*: L. acidophilus* ATCC4356, lane:3 *L. acidophilus* LgER lane 4: *L. acidophilus* KPB4B lane 5: *L. acidophilus* Howarudophilus LYO40, lane 6: *L. acidophilus* 74-2, lane 7: *L. amylovorus* A-7, lane 8: *L. amylovorus* A-11, lane 9: *L. amylovorus* NRRL-B4540 B) UPGMA dendogram obtained by using RAPD profiles.

According to the results obtained, commercial probiotic *L. acidophilus* LA05, type strain *L. acidophilus* ATCC4356 kefir grain isolates *L. amylovorus* A-7, *L. amylovorus* A-11 and type strain *L. amylovorus* NRRRL-B4540 gave identical banding patterns and grouped in the same cluster with a similarity level above 80%. For *L. amylovorus* A-7 and *L. amylovorus* A-11 to be in same cluster with *L. amylovorus* 4540 was an expected result. Because 16S rRNA gene alignment results of A-7 and A-11 with *L. amylovorus* type strain were similar (Appendix D).

Traditional yogurt isolate *L. acidophilus* KPB4B, baby faeces isolate *L. acidophilus* LgER, commercial probiotic cultures *L. acidophilus* Howarudophilus LYO40 and *L. acidophilus* 74-2 were clustered separately from each other.

RAPD-PCR analysis with primer M13 have been previously used for the typing of '*L. acidophilus* group' organisms that are mostly used in probiotic yogurts. Schillinger et al. (2003) applied RAPD-PCR with primer M13 to the strains within the '*L. acidophilus* group'. According to the banding pattern results, a clear distinction obtained between the strains within the '*L. acidophilus* group'; *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* and they could be differentiated from each other. However, in a study of Delfederico et al. (2006) typing of lactobacilli isolated from kefir grains which was performed with primer M13 did not produce different banding patterns and could not be able to differentiate kefir grain isolates.

Similarily to the study of Delfederico et al. (2006), in this study M13 primer could not be able to differentiate isolates since the banding patterns of *L. acidophilus* and *L. amylovorus* were almost the same.

Taken as a whole, in this study putative '*L. acidophilus* group' organisms isolated from kefir grains were identified with 16S rRNA gene sequencing and with multiple alignment which was previously performed by Kullen et al., (2000) and considered as a successful and rapid method for the identification of unknown '*L. acidophilus* group' isolates. According to the multiple alignment results, the isolates were differentiated from *L. acidophilus* and identified as *L. amylovorus*. Additionally,

RAPD-PCR with M13, which was performed in order to observe the diversity between *L. amylovorus* and *L. acidophilus* strains could not be able to differentiate strains of *L. acidophilus* and *L. amylovorus*.

Multiple alignment of 16S rRNA gene sequences applied in this study is considered to be a sufficient method for the identification of the unkown isolates of '*L. acidophilus* group' organisms. On the other hand, it can be recommended to apply additional molecular methods for the identification and differentiation of '*L. acidophilus* group' organisms such as PCR-DGGE (Walter et al., 2000) which was previously applied for the identification of '*L.acidophilus* group' organisms.

3.5. Selective media for enumeration of *L. acidophilus* and *L. amylovorus* in probiotic yogurt

Three different media, Bile-MRS, MRS-Maltose and MRS-Sorbitol were preliminary tested for their selectivity to *L. acidophilus* in the presence of starter cultures and *Bifidobacterium*.

In this study different yogurt cultures and *L. acidophilus* were tested for the evaluation of Bile-MRS (Table 3.4.)

Table 3.4. Selectivity evaluation of Bile-MRS for *L. acidophilus*

Strains	Growth
S. thermophilus Yo-mix 410-3	+
S. thermophilus Ta 040-3	+
S. thermophilus YCX-11	+
L. delbrueckii ssp. *bulgaricus* B1000-1	-
L. delbrueckii ssp. *bulgaricus* Yo-mix 410-1	-
L. bulgaricus YCX-11	+
L. acidophilus LA05	+
L. acidophilus ATCC4356	+

Bile-MRS agar was used as a selective medium for the enumeration of *L. acidophilus* in the presence of yogurt bacteria by Vinderola and Reinheimer (2000). Also Mortazavian et al. (2007) enumerated *L. acidophilus* by using Bile-MRS agar. However in our study not only *L. acidophilus* grew well on Bile-MRS, but also some species of *S. thermophilus* and *L. bulgaricus* grew on this media. So, Bile-MRS was not chosen as selective medium for the enumeration of *L. acidophilus*.

For the evaluation of MRS-maltose whether it is selective for *L. acidophilus* or not, different yogurt cultures were tested. (Table 3.5.)

Table 3.5. Selectivity evaluation of MRS-Maltose for *L. acidophilus*

Strains	Growth
S. thermophilus Ta 040-3	-
S. thermophilus YCX-11	+
L. delbrueckii ssp. *bulgaricus* B1000-2	+
L. bulgaricus YCX-11	+
L. acidophilus LA05	+
L. acidophilus ATCC4356	+

According to Lankaputhra et al., MRS-Maltose was determined to be selective for the enumeration of *L. acidophilus* in the presence of yogurt bacteria if the product does not contain Bifidobacterium spp. (Cogan et al., 2006). However, in this study some yogurt cultures also grown on MRS-maltose in addition to *L. acidophilus*.

Another suggested medium for the selective enumeration of *L. acidophilus* was MRS-sorbitol. In this study yogurt cultures, *L. acidophilus* and *Bifidobacterium* Bb-12 which is used in probiotic yogurts were evaluated for their growth on MRS-Sorbitol (Table 3.6.).

Table 3.6. Selectivity evaluation of MRS-Sorbitol for *L. acidophilus* and *L. amylovorus*

Strains	Growth
Yogurt culture YCX-11	-
L. delbrueckii ssp. bulgaricus B1000-2	+
S. thermophilus Ta 040-3	+
L. acidophilus LA05	+
L. acidophilus ATCC4356	+
L. amylovorus NRRL-B4540	+
Bifidobacterium Bb-12	-

Experiments performed with MRS-sorbitol showed that only *L. acidophilus* cultures could grow on MRS-sorbitol. The yogurt cultures YCX-11 (*S. thermophilus*+ *L. bulgaricus*) and *Bifidobacterium* Bb-12 and *L. acidophilus* LA05 were the cultures that used for the production of probiotic yogurt. The inability of yogurt cultures and *Bifidobacterium* Bb-12 to grow on MRS-sorbitol was an important point for the selective enumeration of *L. acidophilus.*

This result was in agreement with the study of Donkor et al., (2006) who studied the survival of *L. acidophilus* L10 in yogurt (*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342) by using MRS-sorbitol as a selective medium. Dave and Shah (1996), Tharmaraj and Shah (2003) also suggested MRS-sorbitol for the selective enumeration of *L. acidophilus* in the presence of yogurt bacteria.

According to the results obtained from the evaluations of different selective media, MRS-sorbitol was selected for the enumeration of *L. acidophilus* and *L. amylovorus* in probiotic yogurts.

The *L. acidophilus* colonies grew on MRS-sorbitol after 72h, were observed as white-creamy disc type colonies(Figure 3.6). These colonies were also observed under microscope for their purity.

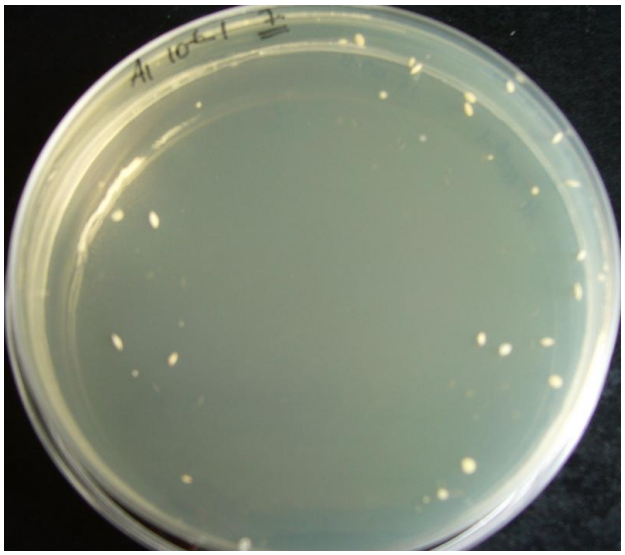

Figure 3.6. *L. acidophilus* colony morphology on MRS-sorbitol

3.6. Survival of commercial probiotics and putative probiotics

Enumeration of commercial *L. acidophilus*, traditional *L. acidophilus* KPB4B, kefir isolates *L. amylovorus* A-7 and *L. amylovorus* A-11 were performed with pour plate method by using MRS-sorbitol agar. Plates that contained 30-300 colonies were enumerated. During the enumeration the selectivity of the medium was confirmed with microscopic examination.

All the enumerations were carried out during the shelf life of commercial probiotic yogurt with 7 days of intervals. The results of the evaluation of the viability of

probiotic cultures are the averages of replicates. The raw data of evaluation results were given in Appendix F.

3.6.1. Enumeration of commercial *L. acidophilus* LA05 in commercial probiotic yogurt during its shelf life

For the enumeration of *L. acidophilus* LA05 in commercial probiotic yogurt A, MRS-sorbitol was used. The enumeration was performed at 1, 7, 14, 21, 28 and 34 days of cold storage at 4°C.

After an incubation of 72h at 37°C, colonies of *L. acidophilus* LA05 which were observed as disc type creamy colonies were counted.

The enumeration results and viability of commercial *L. acidophilus* LA05 were given in Table 3.7 and Figure 3.7., respectively.

Table 3.7. Viable counts of *L. acidophilus* LA05 in commercial probiotic yogurt A. Mean[a] (n=12)± standard deviation.

Storage at 4°C	**Viable counts[a] (log_{10} colony forming units (cfu) /mL)**
Day 1	7.43± 0.03
Day 7	7.39± 0.05
Day 14	7.32± 0.05
Day 21	7.28 ± 0.07
Day 28	7.25 ± 0.13
Day 34	7.22 ± 0.20

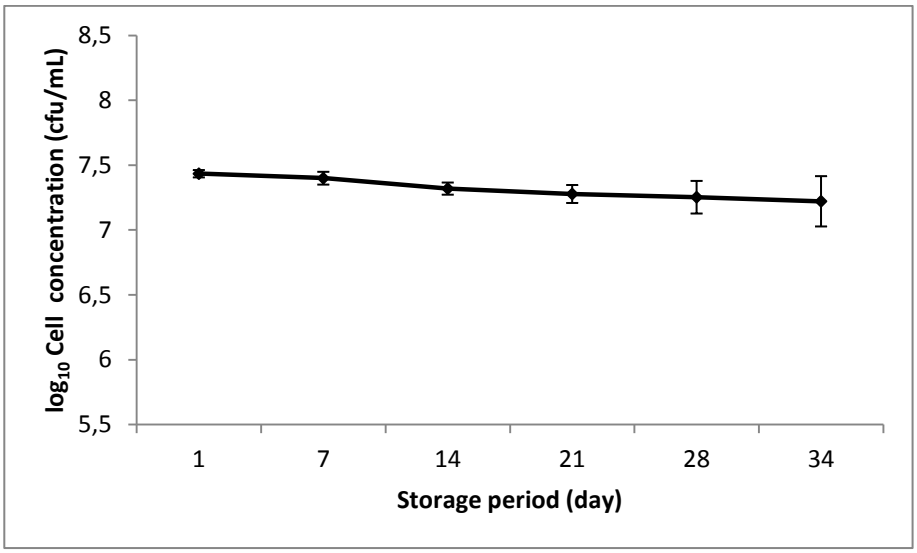

Figure 3.7. Viability of commercial *L. acidophilus* LA05 during the cold storage of probiotic yogurt A. Error bars represent standard deviation of means (n=12).

According to the enumeration results obtained during refrigerated storage of probiotic yogurt A, commercial *L. acidophilus* showed a steady survival and remained above 7 log cfu/mL during the shelf life of the product (Figure 3.6.). Throughout the refrigerated storage period the pH of probiotic yogurt A has a range of: 4.6-4.21.

A similar survival result for a commercial *L. acidophilus* NCFM was observed in the study of Gilliland et al. (2002); in their study the viability of *L. acidophilus* NCFM in probiotic yogurt exhibited a good stability during storage at 5°C for 35 days.

3.6.2. Enumeration of traditional *L. acidophilus* and *L. amylovorus* species in probiotic yogurt

Within the same storage conditions with commercial probiotic yogurt A, the three probiotic yogurts P1, P2 and P3 were stored. The enumeration of the species *L. acidophilus* KPB4B and *L. amylovorus* A-7 and *L. amylovorus* A-11 were applied in same conditions (+4°C) during 34 days with 7 days intervals. The statistical analysis results of the enumerations are given in Appendix G.

The viable counts of *L. amylovorus* A-7 demonstrated a significant decrease ($p<0.0001$) in comparison to commercial *L. acidophilus.* It decreased from 7.37 to 6.60 log cfu/mL over the storage at 4°C. However, it remained above the minimum recommended therapeutic level of 6 log cfu/mL at the end of the storage (Figure 3.6). During the refrigerated storage period the pH of the probiotic yogurt P1 has a range of: 4.6-4.12. The enumeration results and viability of *L. amylovorus* A-7 is given in table 3.8 and figure 3.8., respectively.

Table 3.8. Viable counts of *L.amylovorus* A-7 in probiotic yogurt P1. Mean[a] (n=12)± standard deviation.

Storage at 4°C	**Viable counts[a] (log_{10} colony forming units (cfu) /mL)**
Day 1	7.37± 0.05
Day 7	7.04± 0.40
Day 14	6.87±0.06
Day 21	6.85±0.11
Day 28	6.77±0.07
Day 34	6.60±0.09

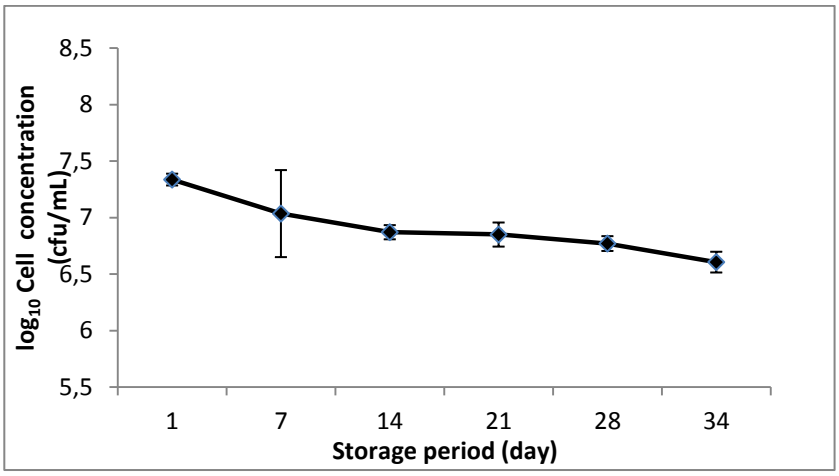

Figure 3.8. Viability of *L. amylovorus* A-7 during the storage of probiotic yogurt P1

During cold storage, the numbers of *L. amylovorus* A-11 in probiotic yogurt P2 decreased from 7.43 to 6.55 log cfu/mL (Table 3.9). In comparison to commercial *L. acidophilus* it exhibited a signifcant decrease ($p<0.0001$), however it remained above 6 log cfu/mL. (Figure 3.9). During the refrigerated storage period, the pH of the probiotic yogurt P2 has range of: 4.6-4.0.

Table 3.9. Viable counts of *L.amylovorus* A-11 in probiotic yogurt P2. Mean[a] (n=12)± standard deviation.

Storage at 4°C	**Viable counts[a] (log_{10} colony forming units (cfu) /mL)**
Day 1	7.43± 0.03
Day 7	7.00± 0.04
Day 14	6.90± 0.27
Day 21	6.73±0.08
Day 28	6.67±0.05
Day 34	6.55±0.06

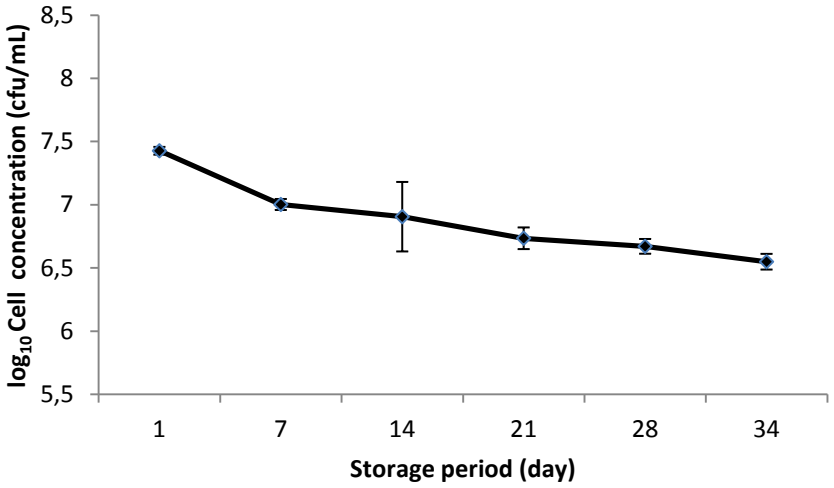

Figure 3.9.Viability of *L. amylovorus* A-11 during the storage of probiotic yogurt P2

Similarly to *L. amylovorus* A-7 and *L. amylovorus* A-11, *L. acidophilus* KPB4B demonstrated a significant decrease ($p<0.0001$) in comparison to commerical *L.*

acidophilus (Table 3.10.) Although it decreased from 7.34 to 6.83 log cfu/mL, it was above 6 log cfu/mL at the end of the storage (Figure 3.10.). The highest pH decrease was observed in this probiotic yogurt, since its pH decreased from 4.55 to 3.9 at the end of the storage.

Table 3.10. Viable counts of *L.acidophilus* KPB4B in probiotic yogurt P3. Mean[a] (n=12)± standard deviation.

Storage at 4°C	**Viable counts[a] (log_{10} colony forming units (cfu) /mL)**
Day 1	7.34± 0.04
Day 7	7.26± 0.02
Day 14	7.14± 0.07
Day 21	6.94± 0.05
Day 28	6.88± 0.03
Day 34	6.83±0.05

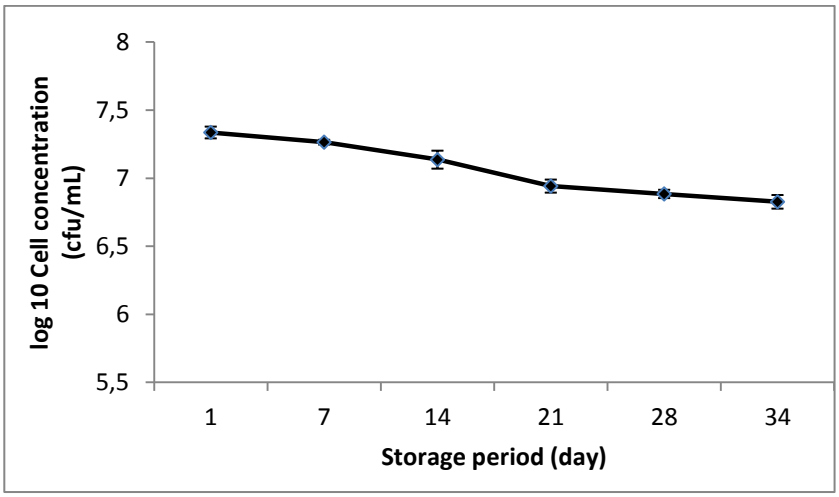

Figure 3.10. Viability of *L. acidophilus* KPB4B during the storage of probiotic yogurt P3.

Altough three of the traditional cultures *L. amylovorus* A-7, *L. amylovorus* A-11 and *L. acidophilus* KP4B demonstrated a significant decrease ($p<0.0001$), they showed maximum 1 log reduction and remained above 6 log cfu/mL throughout the refrigerated storage period.

Several studies have been reported for the factors that cause a loss in the viability of probiotics. The decrease of the pH and the accumulation of organic acids due to microbial metabolism are the main factors that affect the viability (Shah., 1999, Lourens-Hattingh ., and Viljoen., 2001). The increase of lactic acid content after fermentation during storage which is known as 'post-acidification' cause a decline in the pH of the product. Mainly *L. bulgaricus* strains are responsible from this post acidification process and the low pH of the environment cause a decline in the numbers of probiotics (Lourens-Hattingh A., and Viljoen B.C., 2001).

In a study of Hood and Zottola (1998), a strain of *L. acidophilus* showed better survival at a pH of 4.0 than at a pH of 2.0. This findings are in agreement with the study of Lankaputhra and Shah (1995) who studied the survival of six different *L. acidophilus* strains and observed that they survive well at pH of 3.0 or higher

(Lourens-Hattingh A., and Viljoen B.C., 2001). In this study, it was observed that the commercial *L. acidophilus* in probiotic yogurt A, maintained its number at a level of 7 log cfu/mL. A reason for remaining at the same level may be because of the acid tolerance of *L. acidophilus*. In addition, commercial food manufacturers minimize the interaction between yogurt cultures and probiotic strains in order to eliminate their antogonistic effect. Such an approach may be a reason for high level of viability and stability (Chr-Hansen).

In a study of Lorca and Valdez (2001), it was stated that several micoorganisms such as *L. acidophilus* that are inhabitants of the gastrointestinal tract have an acid tolerance response which is mediated by $F_1 F_0$ -ATPase. $F_1 F_0$ –ATPase of lactic acid bacteria have the most important role for sustaining the pH across the cell membrane. Such a pH homoestasis system is mediated by proton-translocating ATPase in order to increase the $pH_{i.}$ This mechanism may play a major role in protecting *L. acidophilus* cells from low pH environments (Lorca and Valdez, 2001). In addition, high cytoplasmic buffering capacity of *L. acidophilus* which was reported by Ruis et al. (2000) may be another factor for the stability and resistance to pH changes (Kailasapathy and Chin, 2000).

Although pH of the probiotic yogurts produced in this study decreased more than the commercial probiotic yogurt during cold storage especially for yogurt P3, the numbers of *L. acidophilus* KPB4B, *L. amylovorus* A-7 and *L. amylovorus* A-11 did not reduce under the lowest recommended level which is considered to be 6 log cfu/mL at the end of the shelf life of a probiotic product. They may have a similar acid tolerance response with commercial probiotic organisms that enabled them to remain above 6 log cfu/mL.

The presence of oxygen in probiotic yogurts might have caused a decline in the numbers of probiotics in our study. Oxygen have a detrimental affect on probiotic microorganisms. According to Dave and Shah (1997), oxygen may affect probiotics in two ways; one of is the direct effect of oxygen which cause an accumulation of hydrogen peroxide and finally cell death. The other, hydrogen peroxide production in the presence of oxygen by *L. delbrueckii* ssp. *bulgaricus* is the indirect effect of

oxygen that cause cell death. In our study, oxygen may have such an affect on the numbers of probiotics since the species evaluated in this study are micoaerophilic organisms. Besides the final numbers of commercial *L. acidophilus* was higher in comparison to three other species; *L. acidophilus* KPB4B, *L. amylovorus* A-7 and *L. amylovorus* A-11 in products. The packaging method may have caused such a better survival in commercial probiotic organism. Because during yogurt production oxygen can directly get in and dissolve in milk (Lourens Hattingh and Viljoen, 2001).

Differences between the survivals of commercial probiotic *L. acidophilus*, traditional *L. acidophilus* KPB4B, *L. amylovorus* A-7 and *L. amylovorus* A-11 cultures may be because of strain to strain variation (Nighswonger et al., 1995).

Besides the biochemical reasons that caused a difference between in numbers of commercial probiotic *L. acidophilus* LA05 and traditional isolates *L. acidophilus* KPB4B, *L. amylovorus* A-7 and *L. amylovorus* A-11, commercial probiotic yogurt may have include some stabilizers and prebiotics that caused a difference between the survival of commercial probiotic and traditional isolates. Because, prebiotics and stabilizers are generally added into commercial probiotic yogurts in order to maintain the stability of yogurt and enhance the growth and activity of probiotic bacteria (Chr-Hansen). However, the probiotic yogurt produced in this study did not contain any stabilizers and prebiotics. Additionally, using a different milk from commercial probiotic yogurt producers and differences between yogurt processing may have caused a difference between the survival of commercial probiotic and traditional isolates.

In our study, commercial *L. acidophilus* showed a better survival because of its stability. *L. amylovorus* A-7 and *L. amylovorus* A-11 almost showed a similar survival during refrigerated storage and protected their level above 6 log cfu/mL. Such a number is known to compensate the possible decline at the levels of probiotics during their way to stomach and intestine (Shah,2000). From this point of view, it can be concluded that our isolates *L. amylovorus* A-7 and *L. amylovorus* A-11 showed a good survival activity during cold storage.

CHAPTER 4

CONCLUSION

Day by day consumers become more concerned about their health and they start to search for ways in order to protect and improve their health. Diet is a main factor that affects the health of humans through their life. For that reason public health approaches try to sustain optimum health during the whole life, prevent gastrointestinal problems, cardiovascular diseases and cancer. Such a tendency for being healthy has accelerated the focus on 'healthy foods'. Probiotics are live microbial additives that have beneficial health affects to the consumers. In order to show their health effects the main issue is their viability and stability during the shelf life of probiotic yogurts.The main aim of this study was to isolate new probiotics that belongs to '*L. acidophilus* complex', determine their viability during the shelf life of probiotic yogurts and compare their viability with commercial probiotics. In addition, typing of the kefir grains isolates, commercial and traditional *L. acidophilus* species was aimed.

For the isolation of probiotics traditional kefir grains as a source of valuable probiotics were selected. The reason for selecting kefir grains was due to their complex microflora and well established health benefits. Isolations were performed from several kefir grains with different origins, isolates were purified and evaluated in terms of their colony morphology, cell morphology, gram stain and catalase test. The species that could belong to lactic acid bacteria were subjected to carbohydrate fermentation tests. Considering the carbohydrate test results, the putative isolates that resemble to the species of '*L. acidophilus* complex' were selected and their 16S

rRNA gene sequencing were performed. 16S rRNA gene sequences of the isolates were aligned at Clustal W2 with the 16S rRNA gene sequences of different strains obtained from Ribosomal Database Project (RDP). Multiple alignment results allowed identification of strains as *L. amylovorus.* Having similar carbohydrate fermentation patterns with *L. amylovorus* and analysis of 16S rRNA sequencing revealed that these isolates belong to *L. amylovorus* species.

Considering the 16S rRNA gene analysis of the strains *L. amylovorus* A-7 and *L. amylovorus* A-11 they belonged to the same strain. However, their esculin and mannitol fermentation patterns were different. Such a difference may be due to a strain level mutation. The mutation may resulted with the loss of the gene that is responsible for fermenting esculin and mannitol. Different carbohydrate fermentation patterns within the strains of *L. amylovorus* were observed in the literature (Fujisawa et al., 1992).

After identification, RAPD-PCR was performed with primer M13 in order to observe the diversity between *L. acidophilus* and *L. amylovorus* strains. However, *L. acidophilus* LA05 , *L. acidophilus* ATCC4356, *L. amylovorus* NRRL-B4540, *L. amylovorus* A-7 and *L. amylovorus* A-11 were grouped in the same cluster with a high similarity level.

Following the identification of two of the isolates as *L. amylovorus* (A-7 and A-11), two sets of probiotic yogurt (named as P1 and P2) were produced with each of these two cultures. For the probiotic yogurt production, commercial starter cultures and a commercial *Bifidobacterium* culture was used in addition to these *L. amylovorus* cultures. In addition, another probiotic yogurt was produced (named as P3) by using the same commercial starters and *Bifidobacterium* culture and a *L. acidophilus* KPB4B culture which was previously isolated from traditional yogurt. After the production of these probiotic yogurts P1, P2 and P3; the survival of the *L. amylovorus* and *L. acidophilus* species that they contain were determined during the shelf life of probiotic yogurts and compared with the survival of a commercial probiotic organism.

According to the survival results, the best survival was observed in the commercial probiotic organism since it maintained its level above 10^7 cfu/mL. Besides, kefir grain isolates *L. amylovorus* A-7 and *L. amylovorus* A-11 also showed good survival. They decreased from 10^7 cfu/mL to 10^6 cfu/mL but at the end of the shelf life of probiotic yogurt they were still at the minimum suggested level of 10^6 cfu/mL for probiotic products. The traditional yogurt isolate *L. acidophilus* KPB4B showed a similar survival with those *L. amylovorus* species.

Consequently, the good survival of *L. amylovorus* A-7 and *L. amylovorus* A-11 during the shelf life of probiotic yogurts could make them as potential probiotic cultures. Such a result may be considered for food manufacturers since they search for new probiotics that have good viability properties. Moreover these species can be investigated in terms of S-layer proteins since S-layer proteins are important in adhesion to intestinal cells and which were previously reported for some *L. amylovorus* species.

This is the first study for the isolation of potential probiotic *L. amylovorus* species from kefir grains. In addition this study is important for the study of the survival of these isolates for their use as commercial probiotics. As next, probiotic properties of these isolates should be studied and in order to investigate strain diversity, doing the PFGE of on these isolates is also recommended. Furthermore, in order to observe the total population of kefir grains DGGE is also recommended.

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APPENDIX A

CHEMICALS ENZYMES AND SUPPLIERS

Table A.1. Chemicals, enzymes and suppliers

Chemicals	Suppliers
MRS Agar	Merck, Germany
MRS Broth	Merck, Germany
M17 Agar	Merck, Germany
M17 Broth	Merck, Germany
Tryptone	Merck, Germany
Yeast Extract	Merck, Germany
Tween 80	Merck, Germany
di-potassium hydrogen phosphate	Applichem, Germany
di-amonium hydrogen citrate	Applichem, Germany
Sodium acetate	Sigma, USA
Magnesium sulphate	Applichem, Germany
Manganese sulphate	Applichem, Germany
Agar	Merck, Germany
L (+) Arabinose	Sigma, USA
D(+) Cellobiose	Applichem, Germany
Glucose	Merck, Germany
Galactose	Merck, Germany
Fructose	Merck, Germany
Lactose	Sigma,USA
Maltose	Merck, Germany
D-Mannitol	Applichem, Germany
Melibiose	Sigma, USA
Saccharose	Merck, Germany
Salicin	Sigma, USA
D(-)Sorbitol	Applichem, Germany
Trehalose	Applichem, Germany
D(+) Xylose	Sigma,USA

Table A.1. Chemicals, enzymes and suppliers (cont'd)

Esculin	Applichem, Germany
Ethanol	Merck, Germany
Ethididum Bromide	Sigma, USA
EDTA	Merck, Germany
Tris-HCI	Sigma, USA
Ribonuclease A	Fermentas, Lithuania
Proteinase K	Fermentas, Lithuania
$MgCI_2$	Fermentas, Lithuania
dNTP	Fermentas, Lithuania
PCR Buffer	Fermentas, Lithuania
Taq Polymerase	Fermentas, Lithuania
100 bp DNA ladder	Fermentas, Lithuania

APPENDIX B

PREPARATIONS OF BUFFER, GROWTH MEDIA AND SOLUTIONS

B. 1. MRS Agar (per liter)

68.2 g MRS agar is dissolved in 1000mL of distilled water and the pH is adjusted to 5.7±0.2 with HCI and autoclaved at 121°C for 15min.

B. 2. MRS Broth (per liter)

52.2 g MRS agar is dissolved in 1000mL of distilled water and the pH is adjusted to 5.7±0.2 with HCI and autoclaved at 121°C for 15min.

B. 3. M17 Broth (per liter)

42 .5g of M17 broth is dissolved in 1000mL of distilled water and the pH is adjusted to 6.8±0.2 with HCI and autoclaved at 121°C for 15min.

B. 4. M17 Agar (per liter)

42 g of M17 broth is dissolved in 1000mL of distilled water and the pH is adjusted to 6.8±0.2 with HCI and autoclaved at 121°C for 15min.

B. 5. Basal Medium for the preparation of MRS agar with different dextroses instead of glucose (per liter)

The pH was adjusted to 5.4 ±0.2 and autoclaved at 121°C for 15min.

Ingredients	Amount(g/lt)
Tryptone	10
yeast Extract	5
tween 80	1
di-potassium hydrogen phosphate	2.6
Sodium acetate	5
di-ammonium hydrogen citrate	2
magnesium sulphate	0.2
manganese sulphate	0.05
Agar	13

B. 6. Modified MRS (for carbohydrate fermentation tests)

For the carbohydrate tests modified MRS was prepared without meat extract and glucose and with bromocresol purple (0.04g/l).

Ingredients	Amount
Tryptone	10g
Yeast Extract	4g
Di-potassium hydrogen phosphate	2g
Sodium acetate	8.3g
di-ammonium hydrogen citrate	2g
magnesium sulphate	0.41g
manganese sulphate	0.038g

Bromocresol purple	0.04g

The compunds given above were dissolved in 1000mL of distilled water and autoclaved at 121˚C for 15 min. (pH: 6.5± 0.2).

B. 7. 10X TBE Buffer

108 g of Tris base, 55g of boric acid and 0.5M of EDTA (pH 8.0) was dissolved in 800 ml of distilled water. After dissolving the volume was adjusted to 1000ml with distilled water.

B. 8. 0.5M EDTA (pH:8.0)

186g of ethylenedinitrilotetraacetic acid disodium salt dihydrate was added to 800ml of distilled water and stirred vigorously on a magnetic stirrer. The volume of the solution was adjusted to 1L and the pH was adjusted to 8.0 with 10N of NaOH and dispensed into aliquots. The solution was sterilized by autoclaving.

B. 9. Lysis Buffer (TE Buffer)

20mM Tris-HCI (pH 8.0), 2mM EDTA and 1.2% Triton-X-100 was mixed and 20mg/ml lysozyme was added immediately prior to usage.

B. 10. NaOH (10N)

40g of NaoH was added to 80mL of dH_20. After dissolving NaOH pellets the volume was adjusted to 100mL.

B. 11. Lyozyme (20mg/ml)

3.6 mg of lysozyme was dissolved in 180µl of distilled water.

B. 12. Ethidium Bromide Solution

10mg/ml EtBr was prepared by dissolving in dH_20.

APPENDIX C

BLAST ANALYSIS OF PRESUMPTIVE '*L. acidophilus* COMPLEX' SPECIES

C.1. Blast analysis of strains A-7

C.1.1. gb|CP002559.1| *Lactobacillus acidophilus* 30SC, complete genome
Length=2078001

Features in this part of subject sequence:
rRNA-16S ribosomal RNA

Score = 1109 bits (600), Expect = 0.0
Identities = 600/600 (100%), Gaps = 0/600 (0%)
Strand=Plus/Plus

Query: strain A-7
Subject: *L. acidophilus* 30SC

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Query  1      ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57175  ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  57234

Query  61     ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57235  ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  57294

Query  121    GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57295  GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  57354

Query  181    GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57355  GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  57414

Query  241    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57415  GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  57474

Query  301    GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57475  GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  57534

Query  361    TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  57535  TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  57594

Query  421    GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57595  GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  57654

Query  481    GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57655  GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  57714

Query  541    AATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTGCA  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57715  AATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTGCA  57774
```

C.1.2. >emb|FR683089.1| *Lactobacillus amylovorus* partial 16S rRNA gene, type strain DSM 20531T Length=1516

Score = 1109 bits (600), Expect = 0.0
Identities = 600/600 (100%), Gaps = 0/600 (0%)
Strand=Plus/Plus

Query: strain A-7

Subject: *L. amylovorus* DSM 20531T

```
Query  1    ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48   ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  107

Query  61   ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  108  ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  167

Query  121  GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  227

Query  181  GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  287

Query  241  GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288  GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  347

Query  301  GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348  GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  407

Query  361  TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  408  TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  467

Query  421  GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  468  GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  527

Query  481  GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  528  GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  587

Query  541  AATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTGCA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  588  AATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTGCA  647
```

C.2. Blast analysis of strain A-11

C.2.1. >gb|CP002559.1| *Lactobacillus acidophilus* 30SC, complete genome Length=2078001

Features in this part of subject sequence: rRNA-16S ribosomal RNA

Score = 1016 bits (550), Expect = 0.0
Identities = 550/550 (100%), Gaps = 0/550 (0%)
Strand=Plus/Plus

Query: A-11
Subject: *L. acidophilus* 30SC

```
Query  1      ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57175  ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  57234

Query  61     ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57235  ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  57294

Query  121    GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57295  GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  57354

Query  181    GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57355  GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  57414

Query  241    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57415  GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  57474

Query  301    GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57475  GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  57534

Query  361    TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57535  TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  57594

Query  421    GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57595  GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  57654

Query  481    GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57655  GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  57714

Query  541    AATGTGAAAG  550
              ||||||||||
Sbjct  57715  AATGTGAAAG  57724
```

C.2.2. >gb|CP002609.1| *Lactobacillus amylovorus* GRL1118, complete genome Length=1894401

Features in this part of subject sequence:
rRNA-16S ribosomal RNA

Score = 1016 bits (550), Expect = 0.0
Identities = 550/550 (100%), Gaps = 0/550 (0%)
Strand=Plus/Plus

Query: strain A-11
Subject: *L. amlyovorus* GRL1118

```
Query  1      ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  55985  ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGA  56044

Query  61     ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56045  ACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCA  56104

Query  121    GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56105  GATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGT  56164

Query  181    GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56165  GCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA  56224

Query  241    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56225  GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG  56284

Query  301    GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56285  GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTT  56344

Query  361    TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56345  TCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTT  56404

Query  421    GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56405  GACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  56464

Query  481    GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56465  GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT  56524

Query  541    AATGTGAAAG  550
              ||||||||||
Sbjct  56525  AATGTGAAAG  56534
```

APPENDIX D

MULTIPLE SEQUENCE ALIGNMENT OF 16S rRNA GENE

CLUSTAL W multiple sequence alignment for 16S rRNA gene of; *L. acidophilus* CECT4179 (acidophilus 1), *L. acidophilus* BCRC 10695(T) (acidophilus 2) , *L. acidophilus* NCFM (acidophilus 3), *L. amylovorus* DSM 20531(T) AY944408 (amylovorus 1), *L. amylovorus* GRL1112 (amylovorus 2), *L. amylovorus* (T) DSM 20531 (T) FR683089 (amylovorus 3) and kefir grain isolates from this study A-7 and A-11 (different bases are highlighted).

```
Acidophilus 1    ------------------------CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 36
Acidophilus 2    ------------------TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 42
Acidophilus 3    ---AAAACGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 57
Amylovorus  1    ---------AGGGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 51
Amylovorus  2    TTTAAAATGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 60
Amylovorus  3    -------------------------------------CTGGCGGCGTGCCTAATACATGC 23
a-11             ------------------------CCGCGAGGGGGGACTGACGG--------GCACGTGC 28
a-7              ------------------------------------------------------------

Acidophilus 1    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 96
Acidophilus 2    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 102
Acidophilus 3    AAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGC 117
Amylovorus  1    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 111
Amylovorus  2    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 120
Amylovorus  3    AAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 83
a-11             A-GTCGAGCGAGCGGAACCA-CAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 86
a-7              ------------------------ATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGC 36
                                          *** ******** ************* *********
```

Figure D.1. Clustal W mulitple sequence alignment for 16S rRNA gene of *L. acidophilus* and *L. amylvorus* strains

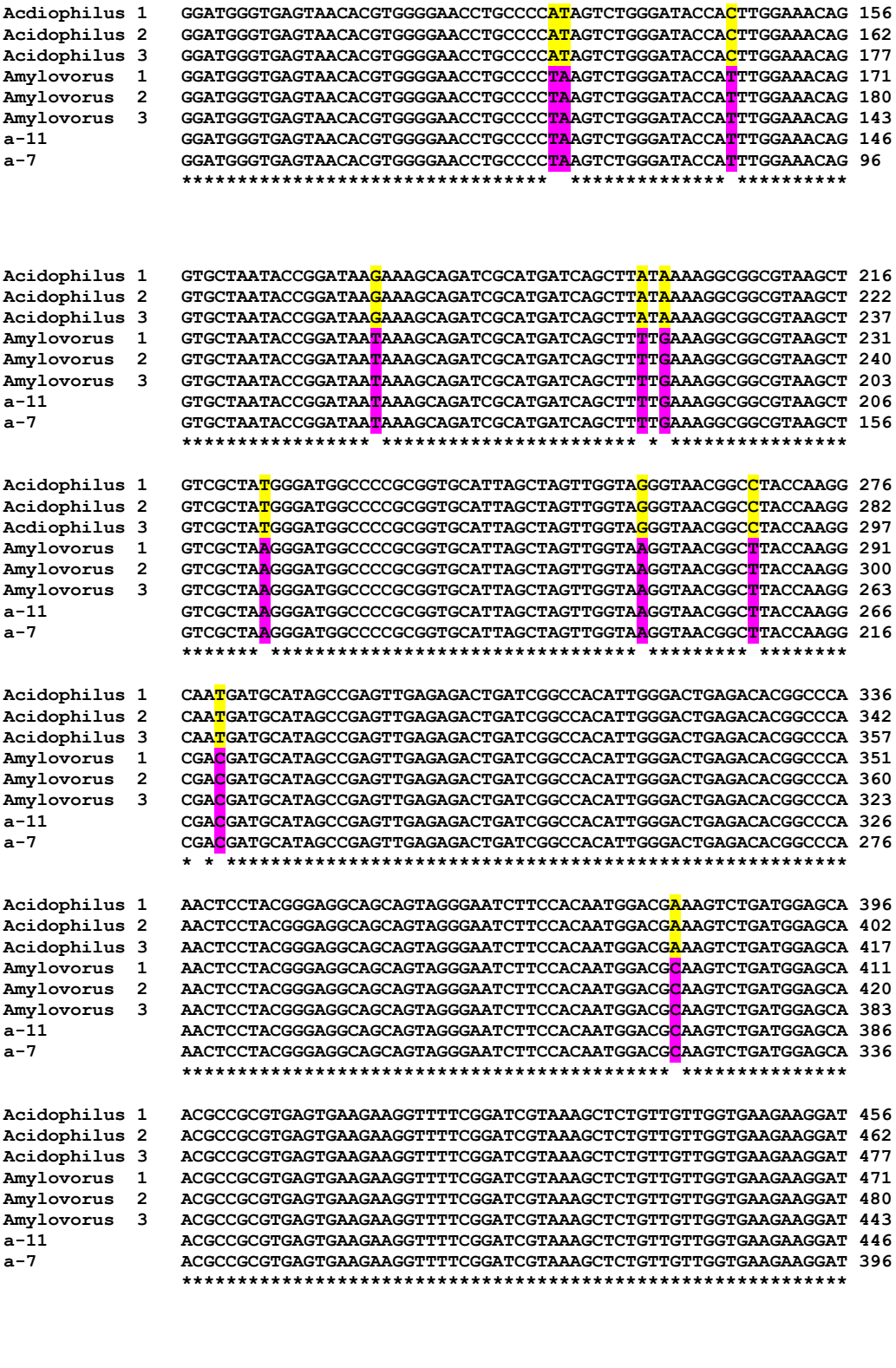

Figure D.1. Clustal W mulitple sequence alignment for 16S rRNA gene of *L. acidophilus* and *L. amylvorus* strains (cont'd).

```
Acidophilus 1   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 516
Acidophilus 2   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 522
Acidophilus 3   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 537
Amylovorus  1   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 531
Amylovorus  2   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 540
Amylovorus  3   AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 503
a-11            AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 506
a-7             AGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT 456
                ************************************************************

Acidophilus 1   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 576
Acidophilus 2   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 582
Acidophilus 3   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 597
Amylovorus  1   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 591
Amylovorus  2   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 600
Amylovorus  3   GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 563
a-11            GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 566
a-7             GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC 516
                ************************************************************

Acidophilus 1   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 636
Acidophilus 2   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 642
Acidophilus 3   GAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 657
Amylovorus  1   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 651
Amylovorus  2   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 660
Amylovorus  3   GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 623
a-11            GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 626
a-7             GAGCGCAGGCGGAAAAATAAGTCTAATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCAT 576
                ************** ********* ***********************************

Acidophilus 1   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 696
Acidophilus 2   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 702
Acidophilus 3   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 717
Amylovorus  1   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 711
Amylovorus  2   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 720
Amylovorus  3   CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 683
a-11            CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 686
a-7             CGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAA 636
                ************************************************************

Acidophilus 1   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 756
Acidophilus 2   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 762
Acidophilus 3   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 777
Amylovorus  1   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 771
Amylovorus  2   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 780
Amylovorus  3   TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 743
a-11            TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 746
a-7             TGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGC 696
                ************************************************************

Acidophilus 1   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 816
Acidophilus 2   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 822
Acidophilus 3   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 837
Amylovorus  1   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 831
Amylovorus  2   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 840
Amylovorus  3   TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 803
a-11            TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 806
a-7             TGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA 756
                ************************************************************
```

Figure D.1. Clustal W mulitple sequence alignment for 16S rRNA gene of *L. acidophilus* and *L. amylvorus* strains (cont'd).

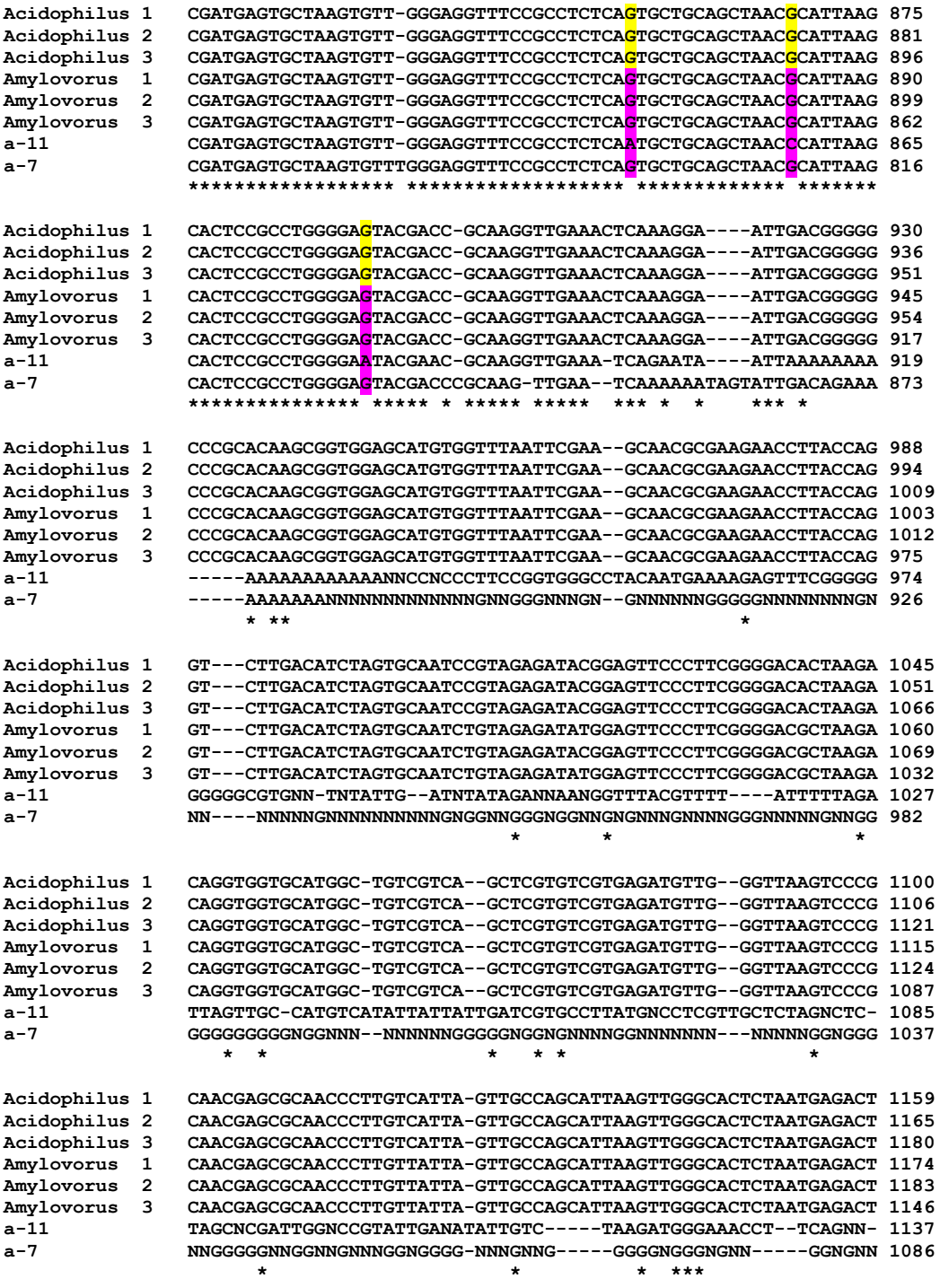

Figure D.1. Clustal W mulitple sequence alignment for 16S rRNA gene of *L. acidophilus* and *L. amylvorus* strains (cont'd).

```
Acidophilus 1   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1216
Acidophilus 2   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1222
Acidophilus 3   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1237
Amylovorus  1   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1231
Amylovorus  2   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1240
Amylovorus  3   GCCGGTGA-CAAACCGGAGGAA-GGTGGGGATGACGTCAAGTCATCATGCCCCT-TATGA 1203
a-11            -GCGGTATTCGCATTTGAACACTAGTGGGNTAAATGTTGTGT----GTATCCNTATATG- 1191
a-7             GGGGGNNNGGGGGNNGGGGNNNGGGNNGGGNGGGNG---------------------- 1122
                   **          *       *  **      *

Acidophilus 1   CCTGGGCTACACACGTGCTACAATGGACAGTA--CAACGAGGAGCAAGCCTGCGAAGGCA 1274
Acidophilus 2   CCTGGGCTACACACGTGCTACAATGGACAGTA--CAACGAGGAGCAAGCCTGCGAAGGCA 1280
Acidophilus 3   CCTGGGCTACACACGTGCTACAATGGACAGTA--CAACGAGGAGCAAGCCTGCGAAGGCA 1295
Amylovorus  1   CCTGGGCTACACACGTGCTACAATGGGCAGTA--CAACGAGAAGCAAGCCTGCGAAGGCA 1289
Amylovorus  2   CCTGGGCTACACACGTGCTACAATGGGCAGTA--CAACGAGAAGCAAGCCTGCGAAGGCA 1298
Amylovorus  3   CCTGGGCTACACACGTGCTACAATGGGCAGTA--CAACGAGAAGCAAGCCTGCGAAGGCA 1261
a-11            -TTAG--------TATAATATAGTTAGTAATAATTGATNAAATTCAANCATATGAATGGA 1242
a-7             ---GG--------NNNGGNNNGGGGGGNGNGNGGNGGNNNGNNGNNGGGNNNNGNNGGGN 1171
                    *                                                 *   *

Acidophilus 1   A----GCGAATCT----CTTAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1326
Acidophilus 2   A----GCGAATCT----CTTAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1332
Acidophilus 3   A----GCGAATCT----CTTAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1347
Amylovorus  1   A----GCGAATCT----CTGAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1341
Amylovorus  2   A----GCGAATCT----CTGAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1350
Amylovorus  3   A----GCGAATCT----CTGAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACT 1313
a-11            NCATGGCGATTTTGATTCTGNNA--TGTTTGTAGTTTNGTCCCTAAT-TGTGA---ANCT 1296
a-7             N----GNNNNGNG------NGNGNNNGNNNNNNG---GGNNNGGNGNGGGNGG---GGGG 1215
                     *                   *      *    *          *

Acidophilus 1   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1386
Acidophilus 2   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1392
Acidophilus 3   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1407
Amylovorus  1   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1401
Amylovorus  2   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1410
Amylovorus  3   GCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG 1373
a-11            CCTTATTGTT----TTGTTGN--ATNGTATGT-AGCATGT-GTTTCAGTTANNTTCNTTA 1348
a-7             GGNGGGGGGNGGGGNGGGNNG--NNNNNGGGNGNNNNNGG-GNNNNGGGNNGNNNNNNGG 1272
                       *        *                     *  *

Acidophilus 1   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1442
Acidophilus 2   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1448
Acidophilus 3   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1463
Amylovorus  1   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1457
Amylovorus  2   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1466
Amylovorus  3   GCCTTGTACACACCGCCCGTCACACCATGGGA-GTCTGCAATGCC--CAAAGCC-GGTGG 1429
a-11            GATTTAN---CGCTATNCGACCAGATGTGGAATANTTACTTTGCTATTGTNGTNTGATGA 1405
a-7             G-----------------GGGNNGGNNNGGGNGGGGNGNNNNG-----GGGGNGGGNNNG 1310
                *                 *         **            *        *   *

Acidophilus 1   CCTAACCTTCG---------------------------------------------------- 1453
Acidophilus 2   CCTAACCTTCGGGAAGGAGCCGTCTAAGGC------------------------------- 1478
Acidophilus 3   CCTAACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGTAACA 1523
Amylovorus  1   CCTAACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGG------------- 1504
Amylovorus  2   CCTAACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGTAACA 1526
Amylovorus  3   CCTAACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGTAACA 1489
a-11            TCNAATNNTNG---------TGTTTTNNGNGN---------------------------- 1428
a-7             --GNNNGNNNGA---------ANNNAANGNA---------------------------- 1330
                          *
```

Figure D.1. Clustal W mulitple sequence alignment for 16S rRNA gene of *L. acidophilus* and *L. amylvorus* strains (cont'd)

APPENDIX E

DETERMINATION OF THE INITAL LOAD OF PROBIOTIC MICROORGANISMS

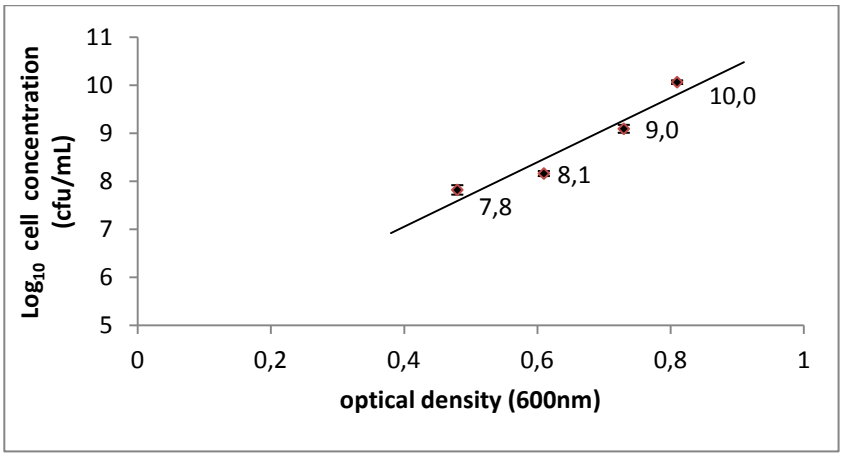

Figure E.1. Cell concentration of *L. amylovorus* at different optical density values. (O.D. values are averages of three different measurements).

Table E.1. Cell concentrations of *L. amylovorus* corresponding to different optical density values

O.D. values (600nm	Cell concentration (cfu/mL)
0,472	
0,464	10^7
0,468	
0,610	
0,607	10^8
0,613	

Table E.1. Cell concentrations of *L. amylovorus* corresponding to different optical density values (cont'd)

O.D. values (600nm	Cell concentration (cfu/mL)
0,793	
0,693	10^9
0,704	
0,807	
0,811	10^{10}
0,813	

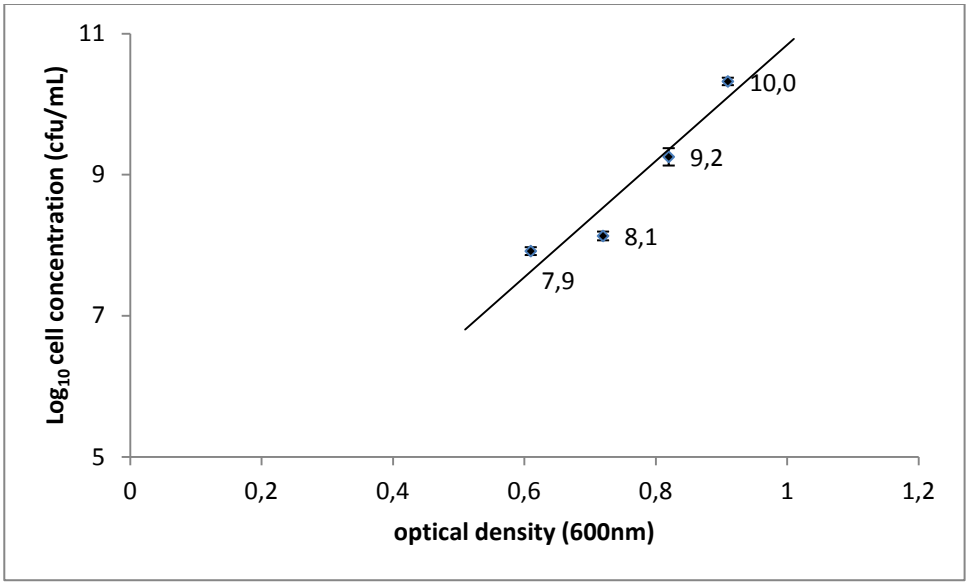

Figure E.2. Cell concentration of *L. acidophilus* at different optical density values. (O.D. values are averages of three different measurements).

Table E.2. Cell concentrations of *L. acidophilus* corresponding to different optical density values

O.D. values (600nm	Cell concentration (cfu/mL)
0,619 0,621 0,605	10^7
0,714 0,725 0,730	10^8
0,837 0,814 0,819	10^9
0,910 0,919 0,925	10^{10}

APPENDIX F

ENUMERATION RESULTS OF COMMERCIAL AND TRADITONAL CULTURES

Table F.1. Enumeration results of commercial *L. acidophilus* in commercial probiotic yogurt A (A_1 and A_2 are the duplicates of commercial probiotic yogurt A)(Six enumeration were performed from each duplicates)

10^{-5} dilution	**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 28**		**Day 34**	
A_1	270	278	210	220	190	197	170	165	188	171	300	239
A_1	289	280	230	245	220	225	200	189	310	235	68	82
A_1	256	235	245	280	256	245	235	247	130	145	164	153
A_2	279	276	265	276	202	198	185	190	120	119	211	171
A_2	256	276	219	262	205	212	198	200	222	220	195	121
A_2	298	287	290	287	178	189	165	150	174	174	245	218

Table F.2. Enumeration results of *L. amylovorus* A-7 in probiotic yogurt P1. ($P1_1$ and $P1_2$ are the duplicates of probiotic yogurt P1)(Six enumeration were performed from each duplicates)

10^{-5} dilution	**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 28**		**Day 34**	
$P1_1$	203	210	81	80	75	77	80	61	60	50	50	55
$P1_1$	180	179	74	70	72	53	64	49	62	48	34	30
$P1_1$	200	210	65	63	70	93	80	87	70	68	59	36
$P1_2$	220	239	82	80	70	73	42	71	50	55	32	38
$P1_2$	246	250	83	79	74	69	93	90	60	50	40	40
$P1_2$	235	252	65	70	89	85	80	75	70	72	45	37

Table F.3. Enumeration results of *L. amylovorus* A-11 in probiotic yogurt P2. **(**$P2_1$ and $P2_2$ are the duplicates of probiotic yogurt P2)(Six enumeration were performed from each duplicates).

10^{-5} dilution	**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 28**		**Day 34**	
$P2_1$	245	269	96	93	54	75	65	47	47	45	30	32
$P2_1$	275	270	110	97	79	72	48	40	63	55	41	45
$P2_1$	230	249	100	103	65	70	54	40	50	48	34	38
$P2_2$	279	283	99	98	72	63	65	50	40	43	40	43
$P2_2$	294	286	87	91	56	64	75	57	48	39	32	31
$P2_2$	276	260	117	121	58	74	62	60	47	42	34	30

Table F.4. Enumeration results of *L. acidophilus* KPB4B in probiotic yogurt P3.($P3_1$ and $P3_2$ are duplicates of probiotic yogurt P3)(Six enumeration were performed for each duplicates).

10^{-5} dilution	**Day 1**		**Day 7**		**Day 14**		**Day 21**		**Day 28**		**Day 34**	
$P3_1$	220	235	190	187	120	145	90	85	80	69	60	65
$P3_1$	229	210	195	191	130	120	92	85	75	73	80	63
$P3_1$	246	260	184	180	138	138	99	70	82	70	85	75
$P3_2$	215	220	181	178	125	160	97	100	85	75	54	74
$P3_2$	200	191	188	190	156	100	86	94	83	82	77	70
$P3_2$	197	190	170	176	164	164	84	74	76	72	62	65

APPENDIX G

STATISTICAL ANALYSIS OF ENUMERATION RESULTS (THE GLM PROCEDURE-REPEATED MEASURES ANALYSIS OF VARIANCE-TEST FOR HYPHOTHESIS FOR BETWEEN SUBJECTS EFFECTS)

G.1. Statistical analysis of the difference between the survival of commercial *L. acidophilus* and *L. amylovorus* A-7

Source	DF	Type III SS	Mean Square	F Value
type	1	564251.3611	564251.3611	1822.85
Error	22	6809.9444	309.5429	

Source	Pr > F
type	<.0001
Error	

G.2. Statistical analysis of the difference between the survival of commercial *L. acidophilus* and *L. amylovorus* A-11

Source	DF	Type III SS	Mean Square	F Value
type	1	516242.2500	516242.2500	3257.62
Error	22	3486.3889	158.4722	

Source	Pr > F
type	<.0001
Error	

G.3. Statistical analysis of the difference between the survival of commercial *L. acidophilus* and *L. acdiophilus* KPB4B

Source	DF	Type III SS	Mean Square	F Value
type	1	270486.6736	270486.6736	1210.00
Error	22	4917.9306	223.5423	

Source	Pr > F
type	<.0001
Error	