MOLECULAR IDENTIFICATION AND PROBIOTIC PROPERTIES OF '*LACTOBACILLUS ACIDOPHILUS* GROUP' ISOLATES FROM TURKISH KEFIR

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ABSTRACT

MOLECULAR IDENTIFICATION AND PROBIOTIC PROPERTIES OF '*LACTOBACILLUS ACIDOPHILUS* GROUP' ISOLATES FROM TURKISH KEFIR

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Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, rods, cocci or coccobacilli that ferment glucose primarily to lactic acid, or lactic acid, carbon dioxide and ethanol. These bacteria are among the most important group of microorganisms used in food and beverage fermentations. *Lactobacillus* species comprise the major part of LAB and a number of them are used as probiotic strains in order to benefit human health.

In this study, four '*Lactobacillus acidophilus* group' isolates from Turkish kefir were identified by using molecular identification techniques and also investigated for their probiotic characteristics. Their molecular identification was done by sequence comparison of PCR (polymerase chain reaction) amplified V1 region of the 16S rRNA gene, isolated from the strains, with sequences of *Lactobacillus acidophilus* group type strains. The comparison identified all four kefir isolates of *Lactobacillus acidophilus* group as *Lactobacillus amylovorus*. The identified strains were further investigated for their probiotic properties, including acid tolerance, bile salt tolerance and cell surface hydrophobicity. A commercial probiotic strain, LA05, was also

investigated in order to use its data as a reference for kefir isolates. All of these strains were also tested for antibiotic susceptibility to determine the existing antibiotic resistant genes that might be involve in horizontal gene transfer. Antibiotics belonging to the groups of inhibitors of cell wall synthesis, inhibitors of protein synthesis, inhibitors of nucleic acid synthesis and inhibitors of cytoplasmic membrane were used. All kefir isolates showed high tolerance against both acid and bile salts, but exhibited low hydrophobicity. They showed high resistance to nucleic acid synthesis inhibitors and cytoplasmic membrane inhibitors, but low resistance to the inhibitors of cell wall synthesis and to the majority of protein synthesis inhibitors. The results were the same for the commercial probiotic, the strain LA05. Since *in vitro* analyses do not necessarily correlate *in vivo* analyses, these results are sufficient to consider these '*Lactobacillus acidophilus* group' isolates from Turkish kefir as promising candidates for use as commercial probiotic strains.

Keywords: Probiotics, *Lactobacillus acidophilus* group, kefir, 16S rRNA gene, *L. amylovorus*.

ÖZ

TÜRK KEFİRİNDEN İZOLE EDİLEN '*LACTOBACILLUS ACIDOPHILUS* GRUP' İZOLATLARININ MOLEKÜLER TANIMLANMASI VE PROBİYOTİK ÖZELLİKLERİ

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Laktik Asit Bakterileri (LAB) glukozu öncelikli olarak laktik asite, veya laktik asit, karbondioksit ve etanole fermente eden Gram pozitif, spor oluşturmayan, yuvarlak, kokobasil ya da çubuklardır. Bu bakteriler yiyecek ve içecek fermantasyonlarında kullanılan mikroorganizma gruplarının en önemlileri arasında yer alır. *Lactobacillus* suşları LAB grubunun büyük bir kısmını oluşturur ve birçoğu insan sağlığına fayda sağlamak için probiyotik suş olarak kullanılır.

Bu çalışmada, Türk kefirinden izole edilen dört *Lactobacillus acidophilus* grubu izolatı moleküler teknikler kullanılarak tanımlandı ve ayrıca bu suşların probiyotik karakterleri araştırıldı. Moleküler tanımlama, suşlardan izole edilmiş 16S rRNA geninin PZR (polimeraz zincir reaksiyonu) ile çoğaltılmış V1 bölgesinin *Lactobacillus acidophilus* grubunun suşlarının sekansları ile karşılaştırılmasıyla yapıldı. Karşılaştırma tüm dört kefir izolatını *Lactobacillus amylovorus* olarak tanımladı. Tanımlanan suşlar asit tolerans, safra tuzu tolerans ve hücre yüzeyinin hidrofobikliğini de içeren probiyotik karakterleri için ilaveten araştırıldı. Ticari bir probiyotik suş, LA05, da verilerini kefir izolatları için referans olarak kullanabilmek

amacıyla araştırıldı. Yatay gen transferinde yer alabilecek var olan antibiyotik direnç genlerini belirlemek için tüm bu suşların ayrıca antibiyotik duyarlılıkları test edildi. Hücre duvarı sentezi inhibitörü, protein sentezi inibitörü, nükleik asit sentezi inhibitörü ve sitoplazmik membran inhibitörü gruplarına ait antibiyotikler kullanıldı. Tüm kefir izolatları hem aside hem de safra tuzlarına yüksek tolerans, fakat düşük hidrofobisite gösterdi. Nükleik asit sentezi inhibitörlerine ve sitoplazmik membran inhibitörüne yüksek direnç, fakat hücre duvarı inhibitörlerine ve protein sentezi inhibitörlerinin çoğuna düşük direnç gösterdiler. Ticari probiyotik, LA05 suşu, için de sonuçlar aynıydı. *İn vitro* analizler, *in vivo* analizler ile muhakkak bağdaşmadığından, bu sonuçlar Türk kefirinden izole edilen bu *Lactobacillus acidophilus* grup izolatlarını gelecek vaadeden ticari probiyotik suş adayları olarak değerlendirmek için yeterlidir.

Anahtar kelimeler: Probiyotikler, *Lactobacillus acidophilus* grup, kefir, 16S rRNA geni, *L. amylovorus*.

To My Parents,

ŞULE GÜLEL & ABDULLAH GÜLEL

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LIST OF ABBREVIATIONS

A: Absorbance

AFLP: Amplified fragment length polymorphism

bp : base pair

C: Cytosine

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleoside triphosphate

G: Guanine plus cytosine

GI: Gastrointestinal

GRAS: Generally recognized as safe

ITS: Intergenic transcribed spacer

LAB: Lactic acid bacteria

PCR: Polymerase chain reaction

RAPD: Randomly amplified polymorphic DNA

RFLP: Restriction fragment length polymorphism

rpm: Revolutions per minute

rRNA: Ribosomal ribonucleic acid

SDS-PAGE: Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

T: Type strain

w/v: Weight per volume

CHAPTER 1

INTRODUCTION

1.1. Probiotics

Probiotic is actually composed of two words which are pro, meaning for, and biotic, meaning life. Basically, it means “for life”. In 2001, probiotics are defined by World Health Organization as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host".

1.1.1. History of Probiotics

Henry Tissier, a French pediatrician, detected a Y-shaped bacterium in the intestines of breast-fed infants and named “Bacillus Bifidus Communis” in 1899. He reported that infants whose intestinal flora was rich with the bacteria had fewer gastrointestinal (GI) problems. This bacterium was used in diarrhea treatments of the infants and later renamed as *Bifidobacterium*.

The history of probiotics date back to thousands of years, but the modern concept of probiotics was first introduced in 1907 by Elie Metchnikoff, a Russian scientist and Nobel laureate. According to him, rates of atrophy and aging processes were affected by the activity of putrefactive (proteolytic) intestinal microbes. In order to prevent this intestinal auto-intoxication, Metchnikoff suggested modifying the gut flora by replacing harmful microbes with beneficial ones.

To prove his theory about aging, Metchnikoff observed Bulgarian people who had an average lifespan of 86 years despite the extreme poverty and harsh climate. He found out that they were consuming fermented milk in high amounts. Since lactic acid

bacteria decrease the pH as a result of lactose fermentation, he concluded that milk fermented with lactic acid bacteria inhibits the growth of proteolytic bacteria in the intestine. He called the bacteria "Bulgarian Bacillus" and introduced it in his diet.

In 1917, Alfred Nissle isolated a strain of *Eschericia coli* from the feces of a soldier who was not affected by the outbreak of shigellosis. He named the strain as "*Eschericia coli* Nissle 1917" and used it in the treatment of salmonellosis and shigellosis.

In 1920, Reetger and Cheplin reported that "Bulgarian Bacillus" could not live in human intestine and disproved Metchnikoff's theory. However, they observed changes in the composition of fecal microbiota when fed with *Lactobacillus acidophilus* and found that these strains were active in the human intestine.

In 1930, a *Lactobacillus* isolate from human feces was reported to survive passage through the gastrointestinal tract. The strain was called as "*Lactobacillus casei* strain Shirota" and used in the production of "Yakult", a fermented milk product.

1.1.2. Health Benefits

Probiotics exert many beneficial effects on human health. The studies show that they can be used effectively in the treatments of childhood diarrhea, ulcerative colitis, necrotizing enterocolitis, irritable bowel syndrome, vaginitis, diarrhea caused by *Clostridium difficile* bacteria, and Crohn's disease. They can also be used for preventing antibiotic-associated diarrhea, infectious diarrhea and pouchitis. They also propose health benefits by promoting lactose digestion in lactose-intolerant individuals, reducing toxic impact of small-bowel bacterial overgrowth, modulating immune system, reducing activity of ulcerative *Helicobacter pylori*, limiting urogenital infections, preventing vaginal infections and alternating gut microbiota, and also exhibits anticarcinogenic and antihypertensive effects. They can be used for their properties like lowering cholesterol, preventing heart disease, reducing gingivitis and preventing tooth decay. They might manufacture vitamins B-12 and K, and support immune system of the host. Some of these health benefits are summarized with proposed mechanisms in Table 1.1.

Table 1.1 Health benefits associated with probiotics (Leroy et al., 2008)

Health Benefit	Proposed Mechanism(s)
Cancer prevention	Inhibition of the transformation of pro-carcinogens into active carcinogens, binding/inactivation of mutagenic compounds, suppression of growth of pro-carcinogenic bacteria, reduction of the absorption of carcinogens, enhancement of immune function, influence on bile salt concentrations
Control of irritable bowel syndrome	Modulation of gut microbiota, reduction of intestinal gas production
Management and prevention of atopic disease	Modulation of immune response
Management of inflammatory bowel diseases (Crohn's disease, ulcerative colitis, pouchitis)	Modulation of immune response, modulation of gut microbiota
Prevention of heart diseases/influence on blood cholesterol levels	Assimilation of cholesterol by bacterial cells, deconjugation of bile acids by bacterial acid hydrolases, cholesterol-binding to bacteria cell walls, reduction of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to liver through influence of the bacterial production of short-chain fatty acids

Table 1.1 Health benefits associated with probiotics (Leroy et al., 2008) (cont'd)

Health Benefit	Proposed Mechanism(s)
Prevention of urogenital tract disorders	Production of antimicrobial substances, competition for adhesion sites, competitive exclusion of pathogens
Prevention/alleviation of diarrhea caused by bacteria/viruses	Modulation of gut microbiota, production of antimicrobial substances, competition for adhesion sites, stimulation of mucus secretion, modulation of immune response
Prevention/treatment of *Helicobacter pylori* infections	Production of antimicrobial substances, stimulation of the mucus secretion, competition for adhesion sites, stimulation of specific and non-specific immune responses
Relief of lactose indigestion	Action of bacterial β-galactosidases on lactose
Shortening of colonic transit time	Influence on peristalsis through bacterial metabolite production

1.1.3. Probiotic Strains

Probiotics are associated with so many health benefits. However, it should be noted that results can vary with the used strain.

"Different probiotic species and even different strains within a species exhibit distinctive properties that can markedly affect their survival in foods, fermentation characteristics, and other probiotic performance." (Klaenhammer, 2001, p.807).

To provide therapeutic effects, probiotic products should have a minimum concentration of 10^6 cfu/ml or cfu/g and daily consumption of probiotics strains should be 10^8-10^9 cfu/ml or cfu/g.

1.1.3.1. Selection Criteria for Probiotic Strains

In order to be used as a probiotic strain, a microorganism should meet certain criteria. These criteria can be grouped under four main criteria which are safety criteria, production and manufacturing criteria, functional criteria and performance criteria. These selection criteria are given in Table 1.2.

Table 1.2 Selection criteria for probiotic strains

Criteria	Property
Safety	Identification Origin Pathogenicity
Production and manufacturing	Mass production Storage Viability and stability Desired quality
Functionality	Survival and proliferation Resistance to acid and bile Adherence and colonization
Performance	Health benefits Production of antimicrobial substances Production of bioactive compounds

First of all, these strains should be well-defined. Their taxonomic identification must be done with phylogenetic analysis and the 16S rRNA sequencing. They should be originated from the normal inhabitant of the species targeted and isolated from healthy individuals. They should also meet safety requirements by being nontoxic and nonpathogenic.

Secondly, they should be suitable for the mass production and storage, having properties like adequate growth, recovery and concentration. They should be viable at high concentrations and stable during culture preparation, storage and delivery. They should also provide desirable qualities when introduced in foods or fermentation processes.

Third main criterion is about their capability of survival, proliferation, and metabolic activity at the target site. They should be resistant to acid (to survive in gastric juice) and bile (to colonize in the intestine). They should also have the ability to adhere to mucus and/or human epithelial cells and colonize *in vivo*. They should also be able to compete with the normal microflora to exert their therapeutic effects.

Finally, they should exert at least one clinically documented health benefit and produce antimicrobial substances like bacteriocins against potentially pathogenic bacteria. In addition, the production of bioactive compounds like enzymes, vaccines and peptides are also desired.

1.1.3.1.1. Tolerance to Acid and Bile Salt

Probiotic strains should have the ability to tolerate acid and bile salts in order to survive through gastrointestinal tract and reach intestine where they would provide their therapeutic effect for the host. In order to reach their target, strains should be resistance to low acidic conditions (pH 2) and digestive enzymes in the stomach. They should also be able to survive and colonize when bile salt concentration is 0.3% (w/v).

1.1.3.1.2. Adhesion to Intestinal Cells

In order to generate health benefit to the host, probiotic strains should be able to adhere to the intestinal epithelium. They should also have ability to colonize in the intestine until they present their beneficial effects.

1.1.3.2. Known and Used Probiotic Strains

Probiotic strains can be derived from both human and animal sources. Human sources are human large and small intestine, feces, and breast milk, whereas animal sources are raw milk and fermented foods.

Species of *Lactobacillus* and *Bifidobacterium* are most commonly used probiotics. In addition, *Streptococcus thermophilus*, the yeast *Saccharomyces cerevisiae* and some *Escherichia coli*, *Enterococcus* and *Bacillus* species are also used as probiotics. (Table 1.3)

Table 1.3 Probiotic strains (Holzapfel et al., 1998; Robinson, 2005)

Lactobacilli strains		Bifidobacteria	Other strains
L. acidophilus	*L. johnsonii*	*B. adolescentis*	*Bacillus cereus*
L. amylovorus	*L. paracasei*	*B. animalis*	*Bacillus coagulans*
L. brevis	*L. plantarum*	*B. bifidum*	*Enterococcus facealis*
L. bulgaricus	*L. reuteri*	*B. breve*	*Enterococcus faecium*
L. casei	*L. rhamnosus*	*B. infantis*	*Escherichia coli* Nissle 1917
L. crispatus	*L. salivarius*	*B. lactis*	*Lactococcus lactis*
L. fermentum		*B. longum*	*Saccharomyces boulardii*
L. gallinarum			*Saccharomyces cerevisiae*
L. gasseri			*Streptococcus thermophilus*

Most of these probiotic strains are used in commercial products for their therapeutic effects on human health. They are generally used in fermented dairy products, but tablets, capsules and powders containing probiotic strains are also available in the market as probiotic supplements. These commercial probiotic strains are listed with their producers in Table 1.4.

Table 1.4. Probiotic strains used commercially

Probiotic Strain	Producer
Bifidobacterium animalis DN 173 010	Danone/Dannon
Bifidobacterium breve Yakult	Yakult
Bifidobacterium infantis 35624	Procter & Gamble
Bifidobacterium lactis Bb-12	Chr. Hansen
Bifidobacterium lactis HN019 (DR10)	Danisco, France
Bifidobacterium longum BB536	Morinaga Milk Industry, Japan
Enterococcus faecium LAB SF 68	Cerbios Pharma, Switzerland
Escherichia coli Nissle 1917	Ardeypharm, Germany
Lactobacillus acidophilus LA-1	Chr. Hansen, USA
Lactobacillus acidophilus LA-5	Chr. Hansen, USA
Lactobacillus acidophilus LA10 (NCC 90)	Nestle, Switzerland
Lactobacillus acidophilus NCFM	Danisco, France
Lactobacillus acidophilus NCFB 1748	Rhodia, USA
Lactobacillus bulgaricus LBY27	Chr. Hansen, USA
Lactobacillus casei DN-114 001	Danone, France
Lactobacillus casei CRL431	Chr. Hansen, USA
Lactobacillus casei F19	Arla Foods, Denmark
Lactobacillus casei Shirota	Yakult, Japan
Lactobacillus crispatus CTV05	Gynelogix, Sweden

Table 1.4. Probiotic strains used commercially (cont'd)

Probiotic Strain	Producer
Lactobacillus fermentum RC-14	Urex, Canada
Lactobacillus johnsonii LA1 (NCC 533)	Nestle, Switzerland
Lactobacillus plantarum 299V	Probi AB, Sweden
Lactobacillus reuteri MM53	BioGaia, Sweden
Lactobacillus rhamnosus GG	Valio, Finland
Lactobacillus rhamnosus GR-1	Urex, Canada
Lactobacillus rhamnosus 271	Probi AB, Sweden
Streptococcus thermophilus STY-31	Chr. Hansen, USA

1.2. Kefir

Kefir is a traditional fermented dairy product made with kefir grains. Kefir grains are cauliflower-shaped clusters of lactic acid bacteria, acetic acid bacteria and yeasts. These microorganisms are held together in a symbiotic matrix consisting proteins, lipids and carbohydrates. Kefir grains also comprise kefiran, which is a water soluble polysaccharide containing glucose and galactose. Kefiran can be used as a food-grade functional additive for fermented dairy products due to its rheological properties (Rimada and Abraham, 2006).

1.2.1. History of Kefir

Kefir originates from the north of Caucasus Mountains in Russia, but the time when the kefir grains first originated or used is not known.

In old times, kefir was made in water-tight bags made of goat or sheep skin by inoculating milk with kefir grains. The inoculated bags were suspended in the sun until it went down, then the bags were brought inside and hung near the door. The idea was ensure the well mixing of milk and kefir grains during fermentation. The fermentation was continuous since the fresh milk was added as fermented milk was removed.

1.2.2. Health Benefits

Consisting of probiotic strains, kefir has many beneficial effects on health. It may be used in the treatment of atherosclerosis, allergic diseases, metabolic and digestive disorders, tuberculosis, cancer and gastrointestinal disorders. Kefir can also stimulate the immune system and enhance lactose digestion. It also has the ability to inhibit growth of tumors, fungi and pathogens including *Helicobacter pylori*. It has been also reported to have antimicrobial effect against other microorganisms including *Staphylococcus aureus*, *Staphylococcus epidermis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klesbsiella pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella*, *Shigella flexneri* and *Shiegella sonnei* (Cevikbas et al., 1994; Garrote et al., 2000).

1.2.3. Nutritional Content of Kefir

In addition to its probiotic strain content, kefir also contains minerals, essential amino acids and vitamins. This high nutritional content of kefir increases its beneficial effects on human health.

Kefir is abundant in calcium, magnesium and phosphorus. Calcium and magnesium are mainly important for nervous system whereas phosphorus has a role in the utilization of carbohydrates, fats and proteins for growth, maintenance and energy.

One of the essential amino acid found in high amounts in kefir is tryptophan, which is known for its relaxing effect on nervous system.

Kefir is also rich in vitamins and contains vitamins B1, B12 and K. It also comprises biotin, which is a B vitamin that helps with the assimilation of other B vitamins by the human body.

1.2.4. Production

Kefir production is classified as yeast-lactic fermentation since it consists of both lactic acid bacteria and yeasts. Milk from cow, sheep or goat can be used for the kefir production. Since each of these has varying organoleptic and nutritional qualities, taste and aroma of produced kefir will be depend on the type of milk used. Kefir grains can also ferment milk substitutes such as soy milk and rice milk. However, these media should contain all the required growth factors for the growth of kefir grains.

The traditional way of producing kefir is the 2-10% inoculation of milk with kefir grains. Fermentation process should be done in the dark, since light may degrade vitamins. The fermentation takes approximately 24 hours at 20-25°C. During the process, new kefir grains grow from the preexisting ones. At the end of the fermentation, the kefir grains are recovered and reserved as the starter for the new batch.

In commercial production of kefir, mostly lyophilized starter cultures are used. First, these starter cultures are activated in homogenized and pasteurized milk containing 2-5% milk fat. The fermentation is done with inoculation of 2-5% kefir grains at 25°C for 20-24 h. After fermentation, kefir grains are removed from the liquid media and stored for the reinoculation process.

1.2.5. Kefir Chemistry

Kefir contains lactic acid, ethanol, carbon dioxide, acetaldehyde, diacetyl and acetoin as the end products of fermentation by lactic acid bacteria and yeasts. Any change that would be made in the lactic acid bacteria or yeasts concentration will vary the concentrations of these metabolites. As a result, flavor of kefir would also be altered.

The quality of kefir depends on many factors. Some of these factors include microbial quality of kefir grains, the grain to milk ratio, type of milk, incubation time and temperature.

Different kefir grain to milk ratios resulted in altered pH, viscosity, microflora and carbon dioxide content in kefir (Garrote et al., 1998). When soy milk was used for kefir production, the content of kefir grains were altered from that of cow's milk. In soy milk, water and protein concentrations were higher whereas kefiran content was higher in cow's milk.

1.2.6. Kefir Microbiology

Kefir is mainly composed of lactic acid bacteria and yeasts. The strains in kefir grains and their concentrations show variability depending on the origin of the kefir.

Identified species in kefir grains are *Lactobacillus brevis*, *Lactobacillus viridescens*, *Lactobacillus gasseri*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Lactobacillus kefir*, *Lactobacillus acidophilus*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus parakefir* and *Leuconostoc*.

In Argentinean kefir, *Lactobacillus plantarum*, *Lactobacillus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, *Saccharomyces*, *Acetobacter*, *Lactobacillus parakefir*, *Kluyveromyces marxianus*, and *Lactobacillus lactis* subsp. *lactis* biovar. *diacetylactis* were identified (Garrote et al., 2001).

From Taiwanese kefir grains, *Lactobacillus helveticus* and *Leuconostoc mesenteroides*, and yeasts identified as *Kluyveromyces marxianus* and *Pichia fermentans* were isolated (ChinWen et al.,1999).

In Turkish kefir, *Lactococcus cremoris*, *Lactococcus lactis*, *Streptococcus thermophilus* and *Streptococcus durans* were identified (Yüksekdağ et al., 2004).

The yeasts isolated from kefir are *Saccharomyces cerevisiae*, *Candida kefir*, *Kluyveromyces lactis*, *Saccharomyces delbruecki*, *Torulopsis holmii*, *Candida holmii*, *Saccharomyces unisporus*, *Kluyveromyces marxianus*, *Torulaspora*

delbrueckii, *Candida friedricchi* and *Pichia fermentans* (ChinWen et al.,1999; Simova et al., 2002).

1.3. Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, catalase negative, non-spore-forming, rods, cocci or coccobacilli, aerotolerant microorganisms. They are named as lactic acid bacteria since they produce lactic acid as one of the main end products of carbohydrate fermentation process. Taxonomically, they belong to the phylum *Firmictes*, class *Bacilli*, and order *Lactobacillales*. The families and genera of LAB are given in Table 1.5.

Table 1.5 Families and genera of lactic acid bacteria

Family	Genus
Aerococcaceae	*Aerococcus*
Carnobacteriaceae	*Carnobacterium*
Enterococcaceae	*Enterococcus* *Tetrageonococcus* *Vagococcus*
Lactobacillaceae	*Lactobacillus* *Pediococcus*
Leuconostocaecae	*Leuconostoc* *Oenococcus* *Weissella*
Streptococcaceae	*Lactococcus* *Streptococcus*

Lactic acid bacteria can be categorized as homofermentative and heterofermentative according to their metabolic pathway of fermentation. The homofermentative pathway is based on glycolysis (Embden-Meyerhof-Parnas pathway) and lactic acid is produced as the sole fermentation product. However, in heterofermentative pathway (6-phosphogluconate pathway) in addition to lactic acid, carbon dioxide and ethanol or acetate are produced.

The LAB are crucial for food fermentation. They enhance the flavor and texture of fermented foods. They also inhibit food spoilage bacteria by producing lactic acid. They are mainly used in fermentation processes of yogurt, cheese, butter, sour cream, sausage, pickles, olives and sauerkraut. LAB strains are mostly used as probiotics and most of these probiotic strains belong to the genus *Lactobacillus*.

1.3.1. The Genus *Lactobacillus*

Lactobacilli are Gram-positive, catalase-negative, and rod-shaped bacteria. They are often found in pairs or chains of varying length and size (0.5-1.2 x 1-10 μm). Lactobacilli constitute a major group of lactic acid bacteria, and they are considered as generally recognized as safe (GRAS). The genus consists of both homofermentative and heterofermentative species. Their optimum growth temperature is 30-40 °C and pH is 5.5-6.2.

They play significant role in food fermentation and prevention of food spoilage. They are used as starter cultures and also as probiotics. Although they are naturally present in raw milk and fermented dairy products, sometimes they are intentionally added for their health promoting properties. Lactobacilli are also normal inhabitants of the human gastrointestinal tract and they play a major role in the maintenance of the colonic microbial ecosystem.

The genus *Lactobacillus* includes a wide variety of organisms. It contains a diverse assemblage of over 180 species. Their guanine plus cytosine (G+C) content ranges from 32% to 54%. This range is beyond the threshold limit for a well-defined genus. For accurate identification of *Lactabacillus* species at the strain level, genotypic identification techniques are required.

1.3.1.1. *Lactobacillus acidophilus* Group

In 1980, microorganisms previously identified as *Lactobacillus acidophilus* were reported to be highly heterogeneous (Lauer et al., 1980). Johnson et al. (1980) investigated these *Lactobacillus acidophilus* strains for their physiological properties, type of lactic acid produced, cell wall's sugar pattern, G+C content of their DNA and DNA homology values.

The strains were distributed among six distinct homology groups A and B, and it was concluded that *Lactobacillus acidophilus* group consists of *Lactobacillus acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii*. In Lauer et. al, these homology groups were designated as I and II (Table 1.6).

Table 1.6 *Lactobacillus acidophilus* group

Species	Homology groups	
	Johnson et al. (1980)	Lauer et al. (1980)
Lactobacillus acidophilus	A1	Ia
Lactobacillus amylovorus	A3	Ib
Lactobacillus crispatus	A2	Ic
Lactobacillus gallinarum	A4	Id
Lactobacillus gasseri	B1	IIa
Lactobacillus johnsonii	B2	IIb

Lactobacillus acidophilus is a rod-shaped microorganism with rounded ends, 0.6-0.9 x 1.5-6 μm in size, and occurs as single cell, in pairs and in short chains. The mol% G+C of its DNA is 32-37. It is obligate homofermentative. Since it is microaerophilic, it can also grow aerobically but it grows better under anaerobic conditions containing 5% carbon dioxide (CO_2), 10% water (H_2O) and 85% nitrogen (N) (Robinson, 2005).

Lactobacillus amylovorus is a rod-shaped, 1 x 3-5 μm in size, microorganism occurring as single cell and in short chains. It has the ability to grow at 45 °C, although this is beyond the optimum growth temperature of *Lactobacillus* species. They are obligate homofermentative. The mol% G+C of their DNA is 40-41. They actively ferment starch and display extracellular amylolytic enzyme activity.

Lactobacillus crispatus is straight to slightly curved rods with rounded ends, 0.8-1.6 x 2.3-11 μm in size, and occurs as single cell and in short chains. They are obligate homofermentative and they present good growth at 45 °C. The mol% G+C of its DNA is 35-38. They can be isolated from human feces, vagina and buccal cavities.

Lactobacillus gallinarum is a rod-shaped microorganism with varying lenghts, 0.5-1.5 x 1.5-10 μm in size, and occurs as single cell, in pairs and in short chains. They are obligate homofermentative. The mol% G+C of their DNA is 33-36. They differ from *L. acidophilus*, *L. amylovorus* and *L. crispatus* by being tolerant to 4.0% sodium chloride (NaCl).

Lactobacillus gasseri is rod with rounded ends, 0.6-0.8 x 3.0-5.0 μm in size, and occurs as single cell and in short chains. They are obligate heterofermentative. They do not hydrolyze arginine. The mol% G+C of their DNA is 33-35. They can be isolated from human mouth and vagina.

Lactobacillus johnsonii is a rod-shaped microorganism with varying lenghts, 0.5-1.5 x 1.5-10 μm in size, and occurs as single cell, in pairs and in short chains. They are obligate homofermentative. They are tolerant to 4.0% sodium chloride (NaCl). The mol% G+C of their DNA is 32-38. They can be isolated from human blood.

1.3.1.1.1. Genotypic Identification

Since *Lactobacillus acidophilus* group contains a genetically heterogeneous species, it is difficult to differentiate them by simple physiological and biochemical tests. In addition, most of the time these tests misidentify *Lactobacillus acidophilus* group species since they are closely related. In order to be able to make precise identification for these species, genotypic identification methods should be applied.

Throughout the years, many different methods have been suggested for identification of lactobacilli species. Nonetheless, most of these techniques failed to differentiate all strains belonging to the *Lactobacillus acidophilus* group. Precise identification has been achieved by applying more than one molecular characterization method and combining their results.

Gancheva et al. (1999) used SDS-PAGE method followed with RAPD-PCR and AFLP fingerprinting, Teanpaisan and Dahlen (2006) used PCR-RFLP and SDS-PAGE for the identification of these closely related species.

It is reported that species belonging to the *Lactobacillus acidophilus* group can be differentiated by the application of SDS-PAGE of whole-cell proteins (Pot et al. 1993; Klein et al.,1998), RAPD-PCR (Du Plessis et al., 1995; Klein et al., 1998). In addition, several species-specific probes and specific primers for PCR identification have been developed according to the sequencing data of the 16S and 23S rRNA (Collins et al., 1991; Schleifer and Ludwig, 1995) or the intergenic spacer regions (Hensiek et al., 1992; Hertel et al., 1993; Pot et al., 1993; Tilsala-Timisjarvi and Alatossava, 1997; Nour, 1998).

Rapid and accurate identification of species belonging to *Lactobacillus acidophilus* group achieved by Song et al. (2000) and Kullen et al. (2000). Song et al. (2000) attained good discrimination among all species of *Lactobacillus acidophilus* group by applying multiplex PCR with species-specific primers targeting the 16S-23S ITS and the flanking 23S rRNA gene region. Kullen et al. (2000) found that the V1 region alone was sufficient as well as the V1-V2 regions for discrimination among all type strains of *Lactobacillus acidophilus* group.

To summarize, all of these aforementioned methods emphasize the necessity of genotypic identification techniques for differentiation of closely related species within *Lactobacillus acidophilus* group. In addition, the importance of the 16S and 23S rRNA gene sequencing including the intergenic spacer region is important for the precise identification of these species.

1.4. Human Digestive System

The digestive system is a group of organs working together to convert food into energy. Digestion process starts in the mouth by breaking down of the food into smaller components with the help of teeth and saliva. The secreted saliva helps digestion by providing amylase enzyme. It also helps chewed food travel through esophagus to the stomach.

In the stomach, food is further broken down by peristalsis, rhythmic contraction of muscles in the wall of stomach, and gastric juice. Gastric juice composed of 0.1 M hydrochloric acid (HCl), potassium chloride (KCl) and sodium chloride (NaCl). The acid plays an important role in the digestion of proteins by activating digestive enzymes. The food turns into chyme and expelled by the stomach into the small intestine.

In the small intestine, the food is fully broken down and absorbed into the blood stream. The bile stored in the gallbladder is released when the food came into the duodenum, first section of the small intestine. The bile produced by liver is made up mostly water (85%), bile salts, mucus, bilirubin and other pigments, 1% fats and inorganic salts. Bile acts as a surfactant and helps to emulsify the fats in the chyme. After absorption of almost all the useful nutrients in the small intestine, the left of it reaches the large intestine where water and some minerals are absorbed. Then the waste products of digestion are excreted from anus via rectum.

1.4.1. Human Gastrointestinal (GI) Tract

The human gastrointestinal tract is an organ system responsible for the digestion. The tract is divided into the upper and lower GI tracts. The upper GI tract includes

the esophagus, stomach, and duodenum. The lower GI tract consists of the second and third parts of the small intestine which are jejunum and ileum, respectively, and the large intestine which is subdivided into the cecum, colon, rectum and anal canal (Figure 1.1).

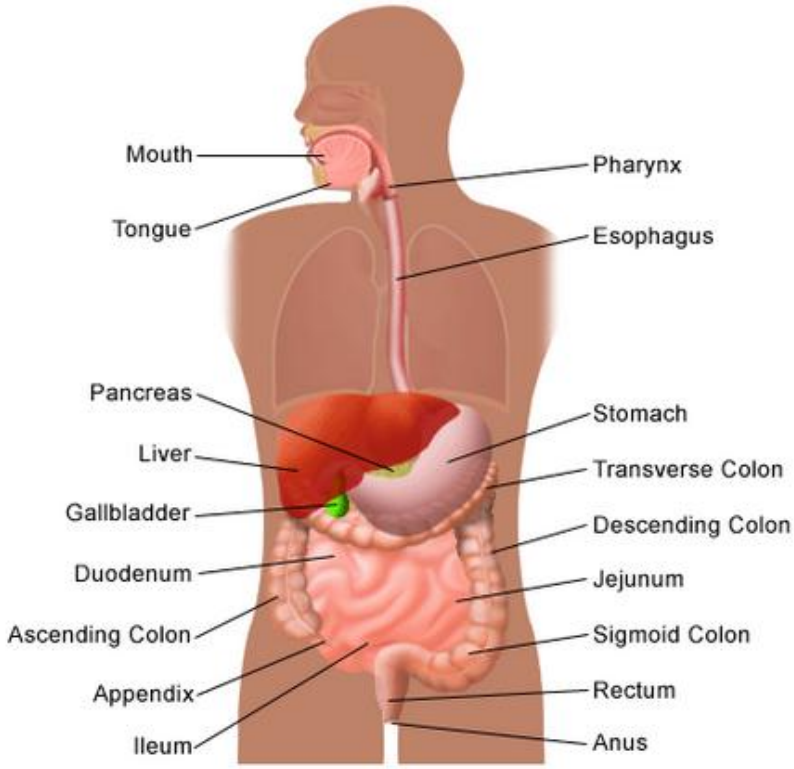

Figure 1.1 Human digestive system and components of the human GI tract. Retrieved from http://www.stanfordchildrens.org/en/topic/default?id=digestive-system-an-overview-85-P00380

1.4.1.1. Microbiology of the Human GI Tract

The human GI tract contains extensive microbiota since it serves as a home for about 10^{14} microbial cells. Majority of these microbial cells are bacteria. However, at the level of species and strains humans show significant microbial diversity. Every individual have their own distinctive pattern of bacterial composition since it is determined by both genotype and initial colonization at birth via vertical transmission.

The resident bacterial strains of human GI tract are shown with their approximate cell numbers in Figure 1.2.

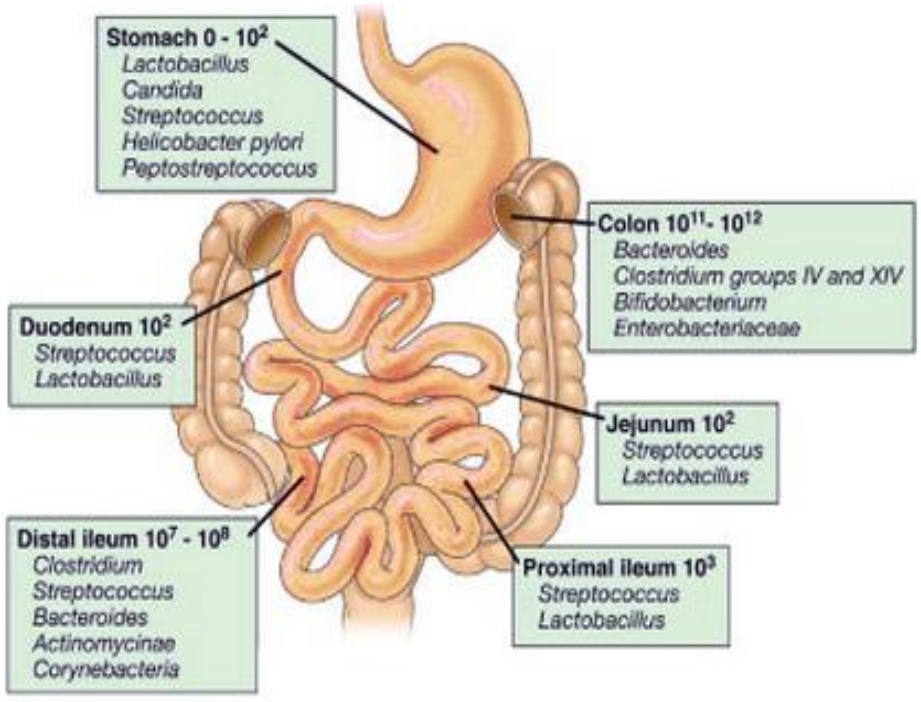

Figure 1.2 Distribution and abundance of bacteria in the human GI tract. Retrieved from http://www.wright.edu/~oleg.paliy/research.html

1.5. Scope of The Study

Probiotic strains are the microorganisms that provide health benefits to the host when consumed in adequate amount. Although they are naturally abundant in many fermented foods, they are also used as dietary adjuncts by intentional addition into the foods.

The interest in probiotic food and strains is increasing day by day, since people become more aware of their health. They do not want to consume just food, instead they demand food with health promoting effects. This increasing demand for probiotics leads not only the food industry but also research scientists to find new strains with probiotic characteristics that are suitable for industrial applications.

The aim of the study was to identify and characterize probiotic properties of novel strains of *Lactobacillus acidophilus* group that can be used as dietary adjuncts. For this purpose, *Lactobacillus acidophilus* group isolates from Turkish kefir were targeted since kefir is a known probiotic fermented dairy product which includes lactic acid bacteria along with other microorganisms. Turkish kefir was chosen for novelty of the strains, since the microorganisms found in kefir depend on the origin of kefir grains and vary from one geographic region to another.

In addition, these strains of interest were previously examined for their survival during storage and viability in yogurt comparing with a known probiotic, *Lactobacillus acidophilus* LA05 (Köse, 2011). Since the previous study showed that these strains are viable in yogurt and their number stays above the minimum suggested level for a probiotic during shelf life, these *L. acidophilus* group isolates were concluded as potential probiotics strains that need to be investigated further for probiotic properties.

In this study, four isolates of *Lactobacillus acidophilus* group from Turkish kefir were identified using molecular techniques including the16S rRNA gene sequencing. Then, the strains of interest were investigated for their probiotic properties including acid tolerance, bile salt tolerance and cell surface hydrophobicity to determine their

survival and colonization ability through the human GI tract. The antibiotic susceptibility of the strains was also tested.

The studied Turkish kefir isolates were putative *Lactobacillus amylovorus* strains. (Köse, 2011). Since *Lactobacillus amylovorus* is mainly associated with cholesterol-lowering and body adiposity decreasing properties (Grill et al., 2000; Omar et al., 2013), it makes them outstanding probiotics and superior to common commercially used probiotics for their digestive health properties, such as *Lactobacillus acidophilus* and *Bifidus bifidobacteria*.

In conclusion, the main idea behind the study was to discover new probiotic strains with novel properties that can be used as dietary adjuncts and in fermented dairy products to produce probiotic kefir, yogurt and milk.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

A total of 10 bacterial strains were used (Table 2.1). The reference strains were from American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), the commercial probiotic strain was from Chr. Hansen, the yogurt isolate was provided by Izzet Baysal University and kefir isolates were from Middle East Technical University (METU) Collection.

Table 2.1 The bacterial strains used in this study

Strain	Strain Number	Source
L. acidophilus (T)	ATCC 4356	ATCC
L. acidophilus	KPB4B	Izzet Baysal University
L. acidophilus	LA05	Chr. Hansen
L. acidophilus (T)	DSM 20079	DSMZ
L. amylovorus (T)	DSM 20531	DSMZ
L. crispatus (T)	DSM 20584	DSMZ
Kefir isolate[2]	A7	METU Collection
Kefir isolate[2]	A11-H[1]	METU Collection
Kefir isolate[2]	A11-P[1]	METU Collection
Kefir isolate[2]	A11-T[1]	METU Collection

[1] H,P,T abbrevations indicates morphological differences.
[2] Isolated from kefir grains provided by Ankara University (Köse, 2011).

2.1.2. Chemicals and Enzymes

The chemicals and enzymes used were listed with their suppliers in Appendix A.

2.1.3. Microbiological Growth Media and Buffers

Preparation of microbiological growth media and buffers were given in Appendix B.

2.2. Methods

2.2.1. Morphological Identification

Two different methods, colony morphology and microscopic examination, were used for the morphological identification of the bacterial strains of interest.

2.2.1.1. Colony Morphology

Colony morphology is a method to describe the characteristics of an individual colony of a bacterium growing on an agar medium in a Petri dish. The defined characteristics of colonies may be used to distinguish between types of microorganisms at a superficial level and it can also be an important tool in the description and identification of microorganisms.

Although dissecting microscopes can also be used, colony morphology observation is often made with the naked eye as it was done in this study. Colonies were described according to Smibert and Krieg (1994) by the examination of form, size, surface, texture, color, opacity, elevation and margin of the colony.

2.2.1.2. Microscopic Examination

Microscopic examination consists of analysis done by staining of cells to make them easily visible under microscopy. Three types of staining procedure were applied in the study, including simple staining, Gram staining and endospore staining.

2.2.1.2.1. Simple Stain

Simple staining is a method to make bacterial cells visible under microscope by the use of dyes. It allows determination of shape, size and arrangement of the organisms.

A loopful of an overnight culture grown in MRS broth was transferred onto a microscope slide and allowed to air dry. The smear was heat fixed and covered with methylene blue for 30 seconds. Then, the slide was rinsed with distilled water and dried by placing blotting paper on it. The morphology of bacterial cells was observed under a brightfield microscope at 100X magnification with immersion oil.

2.2.1.2.2. Gram Stain

Gram staining is a technique to differentiate bacterial species based on the chemical and physical properties of their cell walls. It requires four basic steps that include the application of a primary stain to a heat-fixed smear, followed by the addition of a mordant, then rapid decolorization and lastly the counterstaining. Bacteria with thick layers of peptidoglycan in their cell walls result in a purple-blue color and classified as Gram-positive, whereas the ones with thin layers of peptidoglycan in their cell walls are pink to red and classified as Gram-negative bacteria.

The protocol was adapted from *Manual of Methods for General Bacteriology* (Gerhardt et al., 1981). Cells from an overnight culture in MRS broth were transferred onto a microscope slide using a sterile loop and allowed to air dry. After heat fixation, the cells were flooded with crystal violet as the primary stain for 1 minute. The slide was washed gently with indirect stream of distilled water for 2 seconds. Gram's iodine solution was applied for 1 minute to serve as a mordant. After the repetition of washing step, ethanol solution as decolorizing agent was introduced for 15 seconds. Lastly, the slide was flooded with safranin as counterstain for 1 minute and washed as described above. After blot drying with adsorbent paper, the results were observed under a brightfield microscope at 100X magnification with immersion oil.

2.2.1.2.3. Endospore Stain

An endospore is a dormant and non-reproductive form of certain bacteria that demonstrate resistance to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants.

Endospore staining is one of the differential staining techniques used for the identification and the classifications of bacteria by confirming the presence or absence of the endospores. Since endospores are resistant to extreme conditions, special techniques are required for staining process. The Schaeffer-Fulton stain method is one of these special techniques. In this staining, the endospores result in a bright green color whereas the vegetative cells are brownish red to pink.

The staining was done according to Schaeffer and Fulton (1933). A loopful of an overnight culture grown in MRS broth was applied to a microscope slide and air-dried. The sample is heat fixed and covered with a blotting paper. The blotting paper was saturated with malachite green solution. Then, the slide was placed above a container of boiling water in order to steam the stain into the cells and the spores. It was steamed for 5 minutes by adding more dye to keep the paper moist. When the heating process was over, the slide was washed gently with distilled water. The water acts as a decolorizing agent for the vegetative cells, so the stain is not released by the endospores. Lastly, the slide was flooded with safranin for 30 seconds. The safranin is used as counterstain to give color to the vegetative cells. After washing and blot drying, the results were observed under a brightfield microscope at 100X magnification with immersion oil.

2.2.1.3. Motility Test

Motility is defined as the ability of an organism to move by itself and can be determined by the use of motility test medium. Addition of a triphenyltetrazolium chloride (TTC) as an indicator to the medium makes easier to read the results, since the colorless TTC will be absorbed by the inoculated bacteria and reduced to triphenylformazan (TPF) which is red-colored. The colored area shows where the bacterial growth occurs and the existence of the bacterial growth not only along the line of inoculation indicates that the bacterium is motile.

A well-isolated single colony is picked from MRS agar surface using a sterile needle and stabbed vertically to the motility test medium in the tube. Extra care was taken not to disrupt the straight inoculation line during the stabbing and removing process

of the needle. The inoculated tubes were incubated for 48 hours at 37ºC before examined.

2.2.2. Carbohydrate Fermentation Tests

Carbohydrate fermentation tests are done with different kinds of carbohydrates in order to determine which ones are utilized by the target bacteria. The results provide fermentation patterns that can be used for the differentiation of bacterial groups or species.

Positive results of fermentation reactions are detected by the color change of a pH indicator when acid forms as an end product of the fermentation process. It is safe to say that the pH change occurs as a result of carbohydrate fermentation, since the color will change if only the produced acid exceeds the alkaline by-products derived from the utilization of the peptones by the bacteria.

2.2.2.1. Starch Hydrolysis

Starch hydrolysis is defined as the breaking of the glycosidic linkage between glucose subunits of starch. Starch agar is used to determine the existence of extracellular α-amylase and oligo-1,6-glucosidase enzymes, which cause starch hydrolysis, in the bacteria.

A single colony from a 16-18 hour culture was inoculated onto the starch agar by single streaking. The plates were incubated for 48 hours at 37 ºC before flooding the agar surface with Gram's iodine solution. The zone around the bacterial growth was examined for clarity. A clear zone occurs when the starch gets hydrolyzed and it means that bacteria have these extracellular enzymes. If a clear zone does not occur after flooding, it is considered as a negative result.

2.2.2.2. Gas Production from Glucose

Lactic acid bacteria are categorized as homofermentative and heterofermentative according to their metabolic pathways of carbohydrate fermentation. "The homofermentative species produce lactic acid (<85%) as the sole end product, while

the heterofermentative species produce lactic acid, CO_2 and ethanol/acetate." (König et al., 2009, p.4). In order to differentiate homofermentative LAB and heterofermentative LAB, gas production from glucose is tested with Durham tubes.

Durham tubes were inserted upside down in regular test tubes and the test tubes were filled with modified MRS broth which lacks diammonium hydrogen citrate as recommended in Holzapfel and Gerber (1983). These tubes were autoclaved at 121°C for 15 minutes and the initial air gaps in Durham tubes were lost during this sterilization process. The medium was inoculated with a loopful of an overnight culture without disturbing the Durham tubes and incubated for 24 hours at 37ºC. After incubation, the Durham tubes were observed for the presence of gas that indicates the glucose utilization by the heterofermentative pathway.

2.2.2.3. Tube Fermentation Test

Tube fermentation test was performed for 10 different carbohydrates, which are fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, saccharose (sucrose) and trehalose. These carbohydrates were chosen to differentiate *Lactobacillus* species based on their carbohydrate metabolism.

The test was conducted in modified MRS broth which was simply the basal MRS broth containing bromocresol purple as a pH indicator. The basal MRS broth was prepared without meat extract, diammonium citrate and glucose, as recommended in Dworkin et al. (2006). After the distribution of this modified MRS broth to the test tubes, a single carbohydrate to be tested was added. The sugar solutions were prepared according to Gürakan (1991). The resulting carbohydrate fermentation patterns of the cultures were compared with the table for *Lactobacillus* genus from *Bergey's Manual of Systematic Bacteriology* (2005, vol. 2, p. 1222).

The cultures were grown in 9 ml MRS broth for 24 hours at 37ºC and they were centrifuged at 6,000 rpm for 10 minutes. The supernatant was removed and the pellet was dissolved in 9 ml modified MRS broth. After second centrifugation, the pellet was resuspended in 9 ml modified MRS broth.

The 10% (w/v) sugar solutions were prepared and 1 ml of a given solution was introduced to culture containing modified MRS broth, filtered through Minisart™ 0.22 μm filters (Sartorius, Germany) to give final sugar concentration of 1% (w/v). After 24 hours of incubation at 37ºC, the tubes were checked for the color change from purple to yellow that indicates the positive result for the carbohydrate fermentation.

2.2.2.4. API® 50 CH/CHL Test

API® 50 CH/CHL (BioMeriéux, France) is a system used for the determination of carbohydrate metabolism of lactic acid bacteria. The API® 50 CH is a strip that consists of 50 microtubes, 1 control (no substrate) and 49 substrates. These substrates belong to carbohydrate family and its derivatives such as heterosides, polyalcohols and uronic acids. The composition of the API® 50 CH strip is given in Table 2.2. API® 50 CHL is the medium used for the identification of *Lactobacillus* and related genera.

Table 2.2 The composition of the API® 50 CH strip

Microtube	Test	Active Ingredient	Quantity (mg/cup.)
0	-	Control	-
1	GLY	Glycerol	1.640
2	ERY	Erythritol	1.440
3	DARA	D-Arabinose	1.400
4	LARA	L-Arabinose	1.400
5	RIB	D-Ribose	1.400
6	DXYL	D-Xylose	1.400
7	LXYL	L-Xylose	1.400

Table 2.2 The composition of the API® 50 CH strip (cont'd)

Microtube	Test	Active Ingredient	Quantity (mg/cup.)
8	ADO	D-Adonitol	1.360
9	MDX	Methyl-βD-Xylopyranoside	1.260
10	GAL	D-Galactose	1.400
11	GLU	D-Glucose	1.560
12	FRU	D-Fructose	1.400
13	MNE	D-Mannose	1.400
14	SBE	L-Sorbose	1.400
15	RHA	L-Rhamnose	1.360
16	DUL	Dulcitol	1.360
17	INO	Inositol	1.400
18	MAN	D-Manitol	1.360
19	SOR	D-Sorbitol	1.360
20	MDM	Methyl-αD-Mannopyranoside	1.280
21	MDG	Methyl-αD-Glucopyranoside	1.280
22	NAG	N-Acetylglucosamine	1.280
23	AMY	Amygdalin	1.080
24	ARB	Arbutin	1.080
25	ESC	Esculin	1.160
		Ferric citrate	0.152
26	SAL	Salicin	1.040
27	CEL	D-Cellobiose	1.320
28	MAL	D-Maltose	1.400
29	LAC	D-Lactose (bovine origin)	1.400
30	MEL	D-Melibiose	1.320
31	SAC	D-Saccharose (sucrose)	1.320
32	TRE	D-Trehalose	1.320
33	INU	Inulin	1.280

Table 2.2 The composition of the API® 50 CH strip (cont'd)

Microtube	Test	Active Ingredient	Quantity (mg/cup.)
34	MLZ	D-Melezitose	1.320
35	RAF	D-Raffinose	1.560
36	AMD	Amidon (starch)	1.280
37	GLYG	Glycogen	1.280
38	XLT	Xylitol	1.400
39	GEN	Gentiobiose	0.500
40	TUR	D-Turanose	1.320
41	LYX	D-Lyxose	1.400
42	TAG	D-Tagatose	1.400
43	DFUC	D-Fucose	1.280
44	LFUC	L-Fucose	1.280
45	DARL	D-Arabitol	1.400
46	LARL	L-Arabitol	1.400
47	GNT	Potassium gluconate	1.840
48	2KG	Potassium 2-ketogluconate	2.120
49	5KG	Potassium 5-ketogluconate	1.800

First, the wells of the incubation tray were filled with demineralized water in order to create a humid atmosphere. Then, individual strips (5) were placed onto these wells. Pure culture of a single organism was used in a given test. The bacteria were grown on MRS agar and all of the resulting growth was harvested using a swab and transferred into 5 ml of sterile demineralized water to prepare a heavy suspension. This heavy suspension was used to prepare a second suspension with a turbidity

equivalent to 2-McFarland by transferring it into 2 ml of sterile demineralized water drop by drop. Two times of the drop number needed to reach 2-McFarland turbidity was transferred into the API® 50 CHL medium. The microtubes on the strips were filled with this inoculated API® 50 CHL medium and all of them were covered with sterile mineral oil in order to create anaerobic conditions. Incubation for 48 hours at 37ºC was carried out.

After the 48-hour incubation period, the results were evaluated by observing the color change in the microtubes. Occurrence of yellow color is derived from the acidification of bromocresol purple indicator and considered as a positive result. For esculin, color change to black is considered as positive result. If there is no color change in microtubes, it means the result is negative for that substrate.

2.2.3. Enzyme Tests

Enzyme tests are done in order to determine the ability of target bacteria to produce a certain enzyme. These tests reveal important information for classification, identification and differentiation of microorganisms. Catalase test, oxidase test, urease test, indole test, deoxyribonuclease (DNase) test, gelatinase test, and hemolytic activity test were conducted for these purposes.

2.2.3.1. Catalase Test

Catalase is an enzyme that breaks down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) in order to protect the cells from oxidative damage. The catalase test is applied to detect the presence of the catalase enzyme in bacteria. It also provides valuable information for the differentiation of aerobic and obligate anaerobic bacteria since anaerobes generally do not have the enzyme.

Catalase enzyme's presence is shown by the formation of bubbles due to the release of oxygen gas that forms as a result of the decomposition reaction of hydrogen peroxide.

$$2\ H_2O_{2\ (l)} \rightarrow 2\ H_2O_{\ (l)} + O_{2\ (g)}$$

A single colony was picked from an overnight culture grown on MRS agar surface and transferred onto a microscope slide placed inside a Petri dish. 1 drop of 3% H_2O_2 was dripped onto the bacteria on the microscope slide by using Pasteur pipette and the Petri dish was covered with its lid to prevent the escape of catalase aerosols. After the introduction of H_2O_2 to the culture, it was immediately observed for the bubble formation to detect the presence of catalase.

2.2.3.2. Oxidase Test

Oxidase is an enzyme that catalyzes the reduction of molecular oxygen (O_2) to water (H_2O) or hydrogen peroxide (H_2O_2).

Oxidase test is done to determine whether bacteria produce cytochrome c oxidase or not. Cythochrome c oxidase (Complex IV) is the last enzyme in the electron transport chain and plays a key role in the utilization of oxygen in the generation of energy.

Oxidase test was conducted by applying filter paper spot method according to Gerhardt et. al (1981). A well isolated colony from MRS agar surface was picked and rubbed onto a filter paper. Then, 1-2 drops of 1% Kovac's oxidase reagent was applied on the smear and it was monitored for 2 minutes for the color change.

Oxidase positive bacteria change the color to dark purple within 5-10 seconds, or 60-90 seconds for the delayed ones. However, if a color change takes longer than 2 minutes or it does not occur at all, it is considered as oxidase negative.

2.2.3.3. Urease Test

Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide (CO_2) and ammonia (NH_3) by the following reaction:

$$(NH_2)_2CO_{(s)} + H_2O_{(l)} \rightarrow CO_{2(g)} + 2NH_{3(l)}$$

Urease test is done in urea broth containing phenol red as an indicator. Produced ammonia as a result of urease activity increases the pH of the medium since it is basic molecule, and causes color change of the medium from yellow to pink. This

color change is considered as positive result. If the organism is urease negative, the medium remains yellow.

The tubes containing urea broth were heavily inoculated with overnight cultures of the bacteria to be tested and incubated for 48 hours at 37ºC. After incubation period, tubes were checked for color change in order to determine the presence of urease enzyme in the bacteria.

2.2.3.4. Indole Test

Indole is an aromatic heterocyclic organic compound generated by deamination of tryptophan by trytophanase enzyme. Indole test is performed to determine the ability of bacteria to convert tryptophan into indole in the presence of trytophanase as catalyst.

Tryptone broth is used as a test medium for indole test, since it contains high amount of trytophan. Production of indole is determined by the color change that occurs with the addition of Kovac's reagent.

The tubes including tryptone broth were inoculated with overnight culture of bacteria to be tested and incubated for 48 hours at 37ºC. Then, 5 drops of Kovac's reagent was introduced directly into the tubes and they were observed for the color change.

2.2.3.5. Deoxyribonuclease (DNase) Test

Deoxyribonuclease is a class of enzymes that catalyzes the hydrolysis of phosphodiester bond in the backbone of deoxyribonucleic acid (DNA). The ability of the microorganisms to produce DNase is called DNase activity and determined by DNase test agar plate method. DNase test agar is a culture medium that includes tryptose, DNA and sodium chloride. Peptides derived from tryptose serve as carbon and nitrogen sources, DNA as the substrate and sodium chloride ensures osmotic balance.

A pure culture of the bacteria to be tested was inoculated onto the surface of the test agar by streaking. The streaks were approximately 2 cm in length and 5 mm in width.

The inoculated plates were incubated for 48 hours at 37ºC and then flooded with 1N HCl.

If the DNA in DNAse test agar is not hydrolyzed by the target microorganism, it precipitates with the addition of the acid and forms opaque zones. On the other hand, if the DNA is hydrolyzed, the acid solves the oligonucleotides and clear zones appear around the colonies. In other words, if well-defined clear zones are observed, the organism is DNase-positive. If there is no clear zone, it is DNase-negative.

2.2.3.6. Gelatinase Test

Gelatinase is a proteolytic enzyme that hydrolyses gelatin, which is a protein derived from collagen. According to Leboffe and Pierce (2010), gelatin hydrolysis starts with the degradation of gelatin to polypeptides and followed by their further conversion into amino acids that can be used by the bacteria in their metabolic processes.

$$\text{Gelatin} + H_2O \rightarrow \text{Polypeptides}$$

$$\text{Polypeptides} + H_2O \rightarrow \text{Amino acids}$$

Gelatinase activity is detected by the use of a nutrient gelatin medium whether using a stab method or a plate method. The gelatin in the medium functions not only as a substrate but also as a solidifying agent. When gelatinase-positive bacteria is inoculated, the medium liquefies and remain in liquid phase even if it is placed on an ice bath, since the gelatin is degraded and cannot serve as a solidifying agent any more. If the inoculated bacteria are gelatinase negative, no reaction takes place and the medium remain solid since the gelatin in the medium continues to serve as a solidifying agent.

Nutrient gelatin medium is prepared according to the *Manual of Microbiological Culture Media* (Difco & BBL Manual, 2009). An overnight culture of the bacteria to be tested was stab-inoculated into the tubes containing the medium and incubated at 37ºC for 1 week by checking every day for the liquefaction. In order to be sure that the observed liquefaction was due to gelatin hydrolysis, the tubes were placed in an ice bath for 15-30 minutes before making any conclusion, since the gelatin is solid

under 20ºC and liquid above 28ºC. After the cooling process, the tubes were tilted to observe the gelatinase activity results.

2.2.3.7. Hemolytic Activity Test

Hemolysis is the destruction of erythrocytes, which are the red blood cells responsible for the oxygen delivery to the tissues, and the release of hemoglobin into the surrounding medium. There are three types of hemolysis defined in microbiology. These are alpha hemolysis (α-hemolysis), beta hemolysis (β-hemolysis) and gamma hemolysis (γ-hemolysis). Alpha hemolysis is the oxidation of the hemoglobin to methemoglobin by hydrogen peroxide produced by the bacteria. Since the cell membrane of the red blood cells stays intact, it doesn't considered as true lysis. Beta hemolysis is defined as complete lysis of erythrocytes caused by streptolysin enzyme, and the species with this ability are called as beta-hemolytic. Gamma hemolysis means no hemolytic reaction.

The ability of the bacterial colonies to cause hemolysis is called hemolytic activity and blood agar is used in order to determine their hemolytic properties.

A single colony grown on MRS agar plate was picked and transferred onto the blood agar which was tryptic soy agar base containing 5% unfibrinated sheep blood. The blood agar plates were incubated for 48 hours at 37ºC. After the incubation, the plates were held up to a light source and observed with transmitted light to be able to read the hemolytic reaction results easily.

The interpretation of hemolysis on blood agar plates was done according to Gerhardt (1994). The color changes on the blood agar plates where the bacterial colonies growth takes place play the key role for this interpretation. A green or brown discoloration on the blood agar is associated with the alpha hemolysis since the color is derived from the produced methemoglobin. Clear zones and the transparency of the base medium indicate complete lysis of red blood cells which is beta hemolysis. No color change on the media means lack of hemolysis, in other words gamma hemolysis.

2.2.4. Genotypic Identification

Genotypic identification of the isolates was done in order to confirm the findings of biochemical tests and also to discriminate among closely related species of *Lactobacillus acidophilus* group indisputably. This was achieved with 16S rRNA gene sequencing.

2.2.4.1. Genomic DNA Isolation

Isolation of genomic DNA was performed with NucleoSpin® Tissue Genomic DNA Purification Kit (Macherey-Nagel, Germany) according to the protocol for bacteria from the user manual of the kit with slight modifications and as follows: Overnight culture (2 ml) was centrifuged at 12,000 rpm for 5 minutes. After the removal of the supernatant, a preincubation with a lytic enzyme was applied, since Gram-positive bacteria are more difficult to lyse. The pelleted cells were resuspended in 180 µl of 20 mM TrisHCl, 2 mM EDTA, and 1% Triton X-100 solution supplemented with 20 mg/ml lysozyme and incubated for 1 hour at 37ºC. After the incubation, 25 µl proteinase K was added and the mixture was vortexed vigorously and incubated at 56ºC for 3 hours until complete lysis was obtained. During this incubation period, the cells were occasionally vortexed. Then, 200 µl buffer B3 was added and the samples were incubated at 70ºC for 20 minutes. Ethanol (210 µl) was added after brief vortex and then the samples were vortexed vigorously. Afterwards, the samples were transferred to Nucleospin® Tissue Columns which were placed into collection tubes. The samples were centrifuged at 12,000 rpm for 1 minute and the flow-through was discarded since the DNA bound to the silica membrane of the column. Then, the columns were washed first with 500 µl buffer BW and then with 600 µl buffer B5 and flow-through were discarded again. The centrifugations were done at 12,000 rpm for 1 minute for both of these washing steps. In order to remove the residual ethanol, the columns were centrifuged at 12,000 rpm for 1 minute. Lastly, they were placed into a 1.5 ml microfuge tubes and DNA was eluted with 100 µl buffer BE prewarmed to 70ºC. After incubation at room temperature for 1 minute, they were centrifuged again at 12,000 rpm for 1 minute. The purification column was discarded and the highly pure DNA was stored at -20ºC.

2.2.4.2. Determining DNA Concentration

DNA concentration should be sufficient to be able to proceed with further investigations. In addition, absorbance at 260 nm and at 280 nm should be determined and used for the calculation of their ratio as a measure for purity of the isolated nucleic acid. The accepted range of A_{260}/A_{280} ratio is 1.8-2.0. The ratio 1.8 represents purity for DNA and 2.0 for RNA. If the ratio is lower than 1.8, it means there is protein contamination whereas the ratio being higher than 2.0 means RNA contamination.

The concentrations of isolated DNA samples were determined (as ng/µl) at METU Central Laboratory with AlphaSpec µl Spectrophotometer (Alpha Innotech, Germany). The A_{260}/A_{280} ratio was used to determine their purity.

2.2.4.3. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is used to amplify a segment of DNA and produce millions of copies of it.

The PCR of the genomic DNA from strains of interest was performed according to Kullen et al. (2000). The plb16 (forward) and mlb16 (reverse) primers were used. These primers were purchased from Sacem Life Technologies (Ankara, Turkey). Their sequences are shown in Table 2.3.

Table 2.3 Primers used in this study

Primer	Target Gene	Primer Sequence (5' → 3')	Product Length	Reference
plb16 (forward)	16S rRNA	AGAGTTTGATCCTGGCTCAG	500 bp	Kullen et al., 2000
mlb16 (reverse)	16S rRNA	GGCTGCTGGCACGTAGTTAG	500 bp	Kullen et al., 2000

Thermo Scientific PCR Master Mix (2X) (Thermo Scientific, USA) containing PCR reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP), Taq polymerase and magnesium chloride ($MgCl_2$) was used. The reaction medium was prepared by adding forward and reverse primers and DNA samples into the master mix and diluting it with nuclease-free water. The volume of each reagent used in the reaction mixture is shown in Table 2.4.

Table 2.4 The composition of the PCR reaction mixture

Reagent	Volume	Percent Composition by Volume
Master Mix	12.5 µl	50.0 %
PCR reaction buffer	2X	
dNTPs	0.40 mM	
Taq polymerase	0.05 U/µl	
$MgCl_2$	4 mM	
plb16 primer (forward)	1.25 µl	5.0 %
mlb16 primer (reverse)	1.25 µl	5.0 %
Nuclease free water	9 µl	3.6 %
DNA	1 µl	4.0 %

All of the reagents except the DNA are pipetted into a 1.5 ml microfuge tube and then 24 µl aliquots were distributed into 11 PCR tubes, 10 for the bacterial strains of interest and 1 for PCR control (negative). Afterwards, 1 µl of genomic DNA extract

of each strain was added into these 10 tubes and 1 µl of nuclease-free water was added into the last tube to adjust the final volume to 25 µl for all PCR tubes.

Amplification of DNA was performed in Thermo Scientific Arktic™ thermal cycler (Thermo Scientific, USA). In order to amplify the desired region, it was programmed as shown in Table 2.5.

Table 2.5 Amplification conditions for the 16S rRNA gene

	Time	Temperature	Cycles
Initial denaturation	5 minutes	94 ºC	
Denaturation	15 seconds	94 ºC	35
Annealing	15 seconds	55 ºC	
Extension	1 minute	72 ºC	
Final extension	10 minutes	72 ºC	

2.2.4.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate DNA by size (length in base pairs) for visualization and purification. It moves the negatively charged DNA toward a positive electrode through an agarose gel by applying an electric field.

1.0% (w/v) agarose gel was prepared in TAE (Tris-Acetate-EDTA) buffer by microwaving until the agarose is completely dissolved. Then, agarose solution was cooled down to 45 ºC before pouring into the gel block. The gel was solidified in the gel block with placed comb inside the gel. After solidification, the comb was

removed and the gel was placed into electrophoresis tank. The tank was filled with TAE buffer and the gel was covered. Afterwards, 6X DNA Loading Dye (Thermo Scientific, USA) was added into the samples. The wells created by the comb were used for the sample introduction. The 1 kb DNA Ladder (New England Biolabs, UK) and PCR products were run for 1.5 hours at 90 V and post-stained with GelRed™ (Olerup SSP, Sweden), which is a fluorescent nucleic acid dye designed to replace the toxic ethidium bromide.

The PCR products were resolved by using Owl™ EasyCast™ B2 Gel Electrophoresis System (Thermo Scientific, USA) and visualized with Quantum ST4 1100/26 MX Gel Visualization and Analysis System (Vilber Lourmat, USA).

2.2.4.5. The 16S rRNA Gene Sequencing

The 16S rRNA gene sequencing was performed for the PCR products of 500 bp region of the genomic DNA from target bacterial strains belonging to *Lactobacillus acidophilus* group by RefGen Gene Research and Biotechnology (Ankara, Turkey). The primers used are shown with their sequences in Table 2.3.

Multiple sequence alignments were done with Clustal Omega program (1.2.1) provided by The European Bioinformatics Institute at the following link: http://www.ebi.ac.uk/Tools/msa/clustalo/.

2.2.4.6. BLAST Analysis

BLAST stands for Basic Local Alignment Search Tool, which is an algorithm provided by the National Center for Biotechnology Information (NCBI) for aligning query sequences against those present in a selected target database. It can be accessed from the homepage of NCBI BLAST (http://blast.ncbi.nlm.nih.gov).

The BLAST analyses were done for aligned sequences of each kefir isolates.

2.2.4.7. Comparison of the V1 Region

The aligned sequences of the highly variable V1 region, which is approximately 50 bp, of the 16S rRNA were compared with the sequences of reference strains, both

with the ones sequenced in this study and the ones given in Kullen et al. (2000) in order to be able to differentiate and identify the *Lactobacillus* isolates from Turkish kefir.

2.2.4.8. Phylogenetic Analysis

Phylogenetic analysis estimates the evolutionary relationships among species. It uses sequencing data and morphological data matrices. "The evolutionary history inferred from phylogenetic analysis is usually depicted as branching, treelike diagrams that represent an estimated pedigree of the inherited relationships among molecules (''gene trees''), organisms, or both." (Brinkman and Leipe, 2001, p.323)

Multiple sequence alignments were done with tools provided at www.megasoftware.net. The alignments of the 16S rRNA gene sequences, generated in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 provided at www.megasoftware.net, were used to compute pairwise distances and to construct phylogenetic trees with both maximum likelihood and UPMGA (Unweighted Pair Group Method with Arithmetic Mean) methods.

2.2.5. Probiotic Properties Tests

For a strain to be regarded as a probiotic, it should meet some required characteristics. First of all, it should survive through the human GI tract and reach its final destination where it will exhibit its beneficial health effects. To be able to do this, a probiotic bacterium should survive and at least temporarily colonize in GI tract. This long journey requires microorganisms to be tolerant to acid of gastric juice and bile salts of bile from liver. The microorganism should also have ability to adhere to the intestinal epithelium. In addition, antibiotic resistance/sensitivity of a probiotic strain should be determined.

2.2.5.1. Acid Tolerance

Acid tolerance is an important feature for probiotic bacteria since they should survive in gastric juice to move on through the GI tract.

Acid tolerance of *Lactobacillus* strains were examined according to Charteris et al. (1998a) with slight modifications. The 18-24 hours grown cultures were inoculated into 10 ml fresh MRS broth. Their growth was monitored at 600 nm until optical density value of 0.6, which indicates the existence of approximately 10^9 cells per ml, was reached. Then, cells were harvested by centrifugation at 6,000 rpm for 10 minutes and resuspended in 10 ml of previously pH adjusted MRS broth media. The pH adjustments of MRS broth media were done with 2N HCl (hydrochloric acid) to pH values of 1.5, 2.0 and 2.5. At time 0 h, 1 h, 1.5 h, 2.0 h and 3.0 h of incubation, total viable cell count was determined by spreading serial dilutions of MRS broth cultures, prepared in peptone water, on MRS agar. The plates were incubated anaerobically at 37ºC for 48 hours prior to counting colonies.

2.2.5.2. Bile Salt Tolerance

Probiotic bacteria should be tolerant to bile from liver and bile salts, since they are the main component of bile.

Bile tolerance experiment was done according to Fernandez et al. (2002) with some modifications. The 18-24 hours grown cultures were inoculated into 10 ml fresh MRS broth. Their growth was monitored at 600 nm until optical density value of 0.5, which indicates the existence of approximately 10^7 cells per ml, was reached. Then, cells were harvested by centrifugation at 6,000 rpm for 10 minutes and resuspended in 10 ml of previously bile salt added MRS broths. DIFCO® oxgall (BD, USA) was added at following concentrations: 0.1%, 0.3% and 0.5% (w/v). At time 0 h, 1 h, 1.5 h, 2.0 h and 3.0 h of incubation, total viable cell count was determined by spreading serial dilutions of MRS broth cultures, prepared in peptone water, on MRS agar. The plates were incubated anaerobically at 37ºC for 48 hours prior to counting colonies.

2.2.5.3. Cell Surface Hydrophobicity

Cell surface hydrophobicity of *Lactobacillus* strains of interest were determined in order to correlate the data with their ability to adhere to the intestinal epithelium.

The *in vitro* cell surface hydrophobicity was determined according to Rosenberg et al. (1980) by using microbial adhesion to hydrocarbons (MATH) method. Test bacteria were grown for 18-24 hours in 10 ml MRS broth before being harvested by centrifugation at 6,000 rpm for 10 minutes. The cells were washed twice and resuspended in 50 mM potassium phosphate buffer (pH 6.6). The absorbance at 560 nm was measured to adjust optical density to 0.8-1.0 and the data was recorded as A_0 to be used in calculation of percentage hydrophobicity. An aliquot (1.2 ml) of n-hexadecane ($C_{16}H_{34}$) was added to 6 ml of bacterial suspension. The mixture was vortexed for 120 seconds to homogenize. Then, tubes were incubated for 30 minutes at 37 ºC and the resulting two phases, organic and aqueous, were separated during this time. The absorbance at 560 nm was measured for aqueous phase and recorded as A value.

The percentage of cell surface hydrophobicity (% H) of the strains was calculated by the following formula:

$$\% \, \mathrm{H} = \frac{(\mathrm{A}_0 - \mathrm{A})}{\mathrm{A}_0} \times 100$$

2.2.5.4. Antibiotic Susceptibility

The determination of susceptibility of potentially probiotic bacterial strains to antibiotics is important due to horizontal gene transfer. A horizontal gene transfer of antibiotic resistance genes to intestinal bacteria or to epithelial cells of the gut of humans is not desired.

Antibiotic susceptibility was determined by disc diffusion method according to Charteris et al. (1998b) with slight modifications. 25 antibiotics were used in this study and their list is provided in Table 2.6.

Table 2.6 The studied antibiotics with disc concentrations

Group		Antibiotics	Conc. (μg)
1	Inhibitors of cell wall synthesis		
	Penicilins	Amoxicillin	30
		Ampicillin	10
	Cephalosporins	Cefoxitin	30
		Cephalothin	30
		Cephradine	30
	Monobactams	Aztreonam	30
	Glycopeptides	Vancomycin	30
	Single antibiotics	Bacitracin	10
2	Inhibitors of protein synthesis		
	Aminoglycosides	Amikacin	30
		Gentamicin	10
		Kanamycin	30
		Streptomycin	10
	Tetracyclines	Tetracycline	30
	Single antibiotics	Chloramphenicol	30
		Fusidic acid	10
	Macrolids	Erythromycin	15
	Lincosamides	Clindamycin	2
3	Inhibitors of nucleic acid synthesis		
	Sulphonamides	Sulphamethoxozole	100
	Trimethoprim	Trimethoprin	5
	Quinolones	Ciprofloxacin	5
	Rifampicins	Rifampicin	5
	Nitroimidazoles	Metronidazole	5

Table 2.6 The studied antibiotics with disc concentrations (cont'd)

Group		Antibiotics	Conc. (μg)
4	Inhibitors of cytoplasmic membrane function		
	Polymyxins	Polymyxin B	300
5	Other urinary tract antispectics		
	Single antibiotics	Nitrofurantoin	300
6	Other antimicrobials		
	Single antibiotics	Furazolidone	15

MRS agar plates were heavily inoculated, using sterile swabs, with overnight grown culture. Antibiotic discs (Bioanalyse, Turkey) were dispensed onto the inoculated agar surface. The plates were incubated at 37ºC for 24 hours. Then, the diameters of the growth inhibition zones were measured and susceptibility results of *Lactobacillus* strains were expressed as resistant (R), moderately susceptible (MS) and susceptible (S), by comparing the diameter values with the ones provided in Charteris et al. (1998b).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Experimental Strategy

This study consists of mainly two experimental parts: Identification and determination of probiotic properties of *Lactobacillus acidophilus* group isolates from Turkish kefir.

In the first part, four isolates from Turkish kefir were identified using both biochemical and molecular techniques. The flow chart of the experimental strategy of this part is shown in Figure 3.1. The diagram of biochemical tests used in this study is shown separately, in Figure 3.2. The 16S rRNA gene sequencing and BLAST analysis were done. Besides, the sequences of the V1 region of kefir isolates were compared with the type strains of *Lactobacillus acidophilus* group in order to identify and differentiate these closely related strains indisputably.

In the second part, probiotic properties of four *Lactobacillus amylovorus* strains, previously identified using microbiological, biochemical, and molecular methods, were investigated. Acid tolerance, bile salt tolerance, cell surface hydrophobicity and antibiotic susceptibility tests were done for the probiotic characterization of these isolates. A known commercial probiotic, the strain LA05 (*L. acidophilus*), was also included in these tests as a control. The flow chart of the experimental strategy of this part is shown in Figure 3.3.

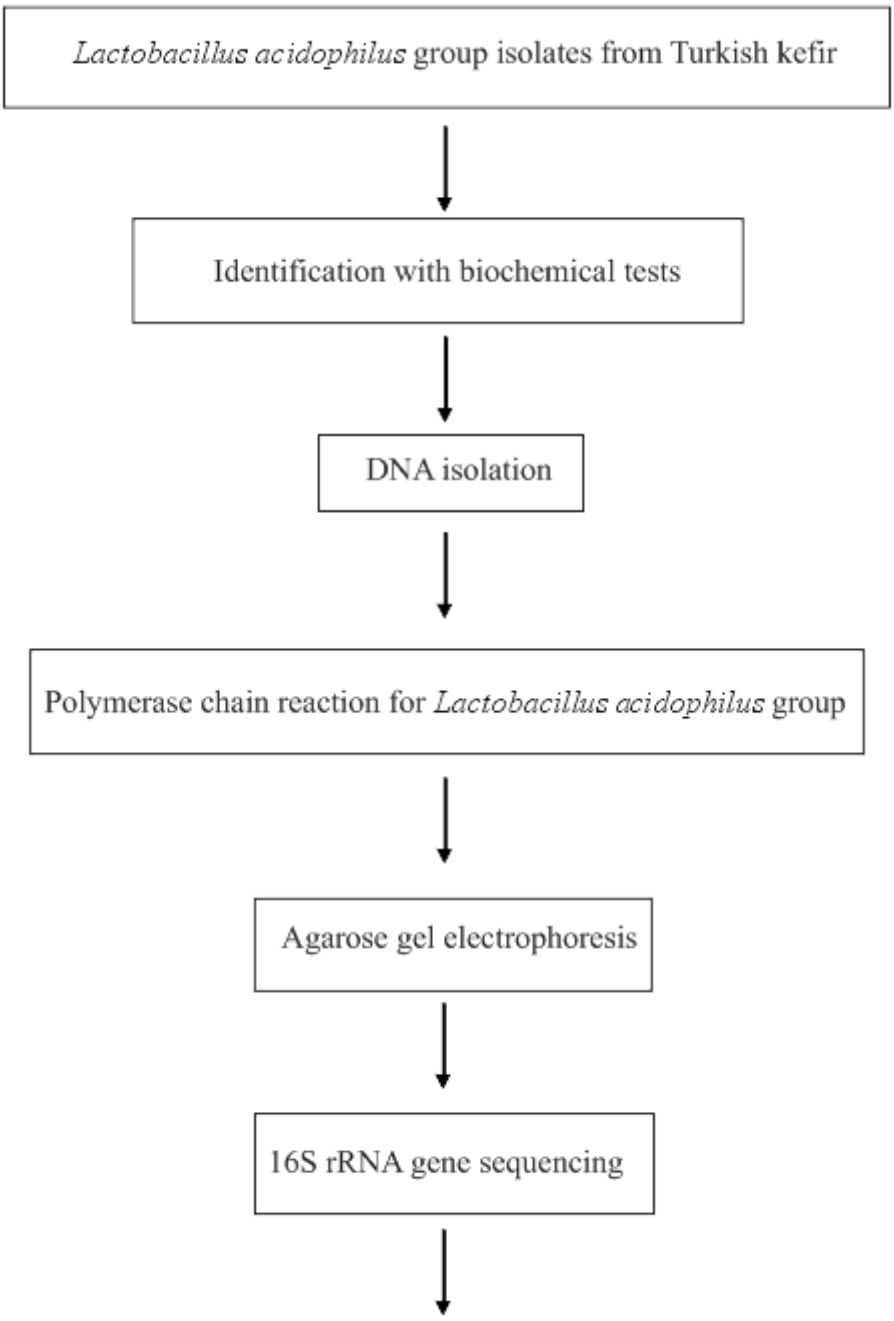

Figure 3.1 Flow chart of the experimental strategy (Part 1). Identification of *Lactobacillus acidophilus* group isolates from Turkish kefir.

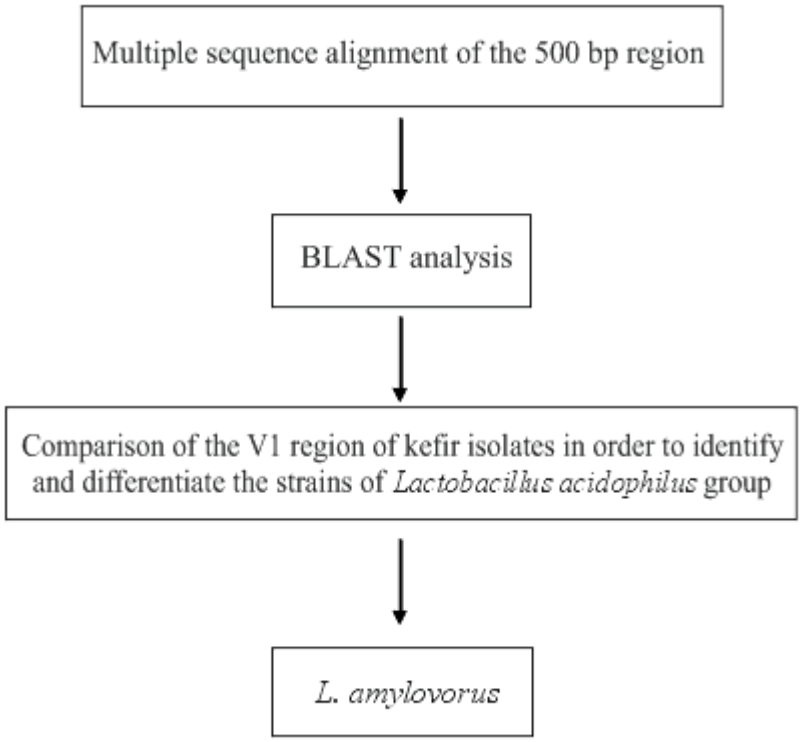

Figure 3.1 Flow chart of the experimental strategy (Part 1). Identification of *Lactobacillus acidophilus* group isolates from Turkish kefir. (cont'd)

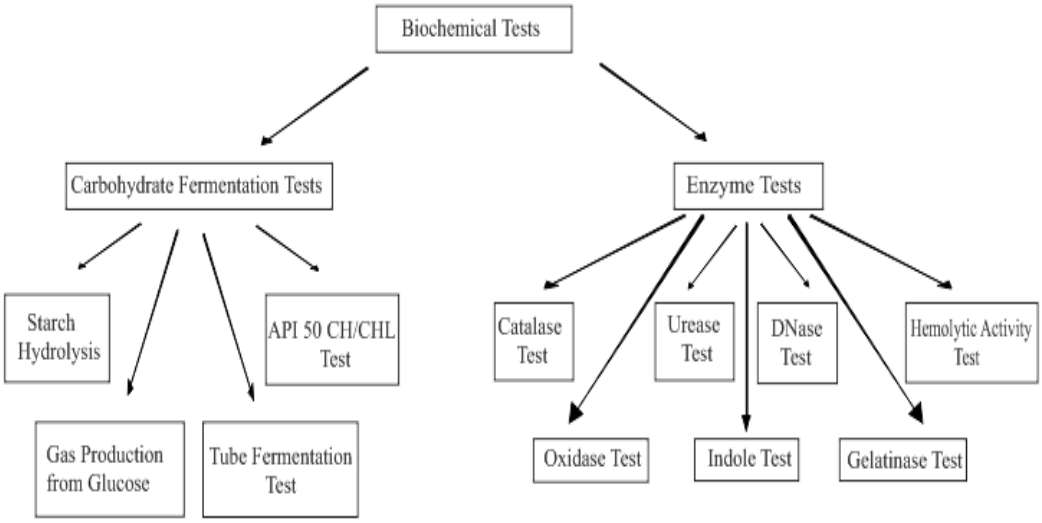

Figure 3.2 Diagram of biochemical tests applied in the study

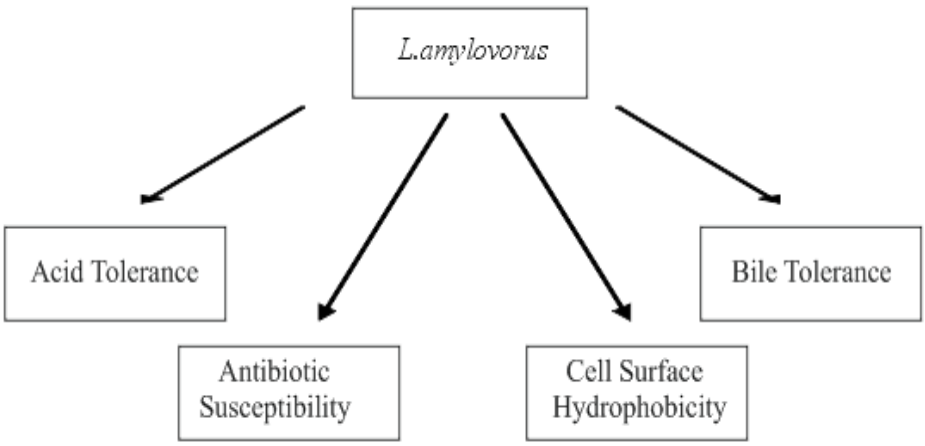

Figure 3.3 Flow chart of the experimental strategy (Part 2). Determination of probiotic properties of *L. amylovorus* strains isolated from Turkish kefir.

3.2. Colony Morphology

Colony morphology of *Lactobacillus* strains were examined and their properties including form, size, surface, texture, color, opacity, elevation and margin of the colony are summarized in Table 3.1 for *Lactobacillus* strains.

Table 3.1 Colony morphology of *Lactobacillus* strains used

	L. acidophilus			
	ATCC 4356	KPB4B	LA05	DSM 20079
Form	Circular	Circular	Circular	Circular
Size[1]	Tiny	Small	Tiny	Tiny
Surface	Moist	Moist	Moist	Moist
Texture	Smooth	Smooth	Smooth	Smooth
Color	Cream	Cream	Cream	Cream
Opacity	Opaque	Opaque	Opaque	Opaque
Elevation	Raised	Raised	Raised	Raised
Margin	Entire	Undulate	Undulate	Entire

[1]Results are expressed as tiny for colony diameter less than 2 mm and small for colony diameter 2-4 mm.

Table 3.1 Colony morphology of *Lactobacillus* strains used (cont'd)

	L. amylovorus	Kefir isolates			
	DSM 20531	A7	A11-H	A11-P	A11-T
Form	Circular	Circular	Circular	Circular	Circular
Size[1]	Small	Small	Small	Small	Tiny
Surface	Moist	Moist	Moist	Moist	Moist
Texture	Smooth	Smooth	Concentric rings	Smooth	Smooth
Color	Cream	Cream	Cream	Cream	Cream
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Raised	Raised	Raised	Raised	Raised
Margin	Entire	Erose	Undulate	Undulate	Entire

[1]Results are expressed as tiny for the ones with colony diameter less than 2 mm and small for colony diameter 2-4 mm.

All colonies were observed in circular form with moist surface and smooth texture except that of the strain A11-H, which has concentric rings on it. In addition, they were all cream, opaque and raised. They only differed from each other in size and some for some in margin characteristics. In the end, these findings were consistent with the morphology of *Lactobacillus* strains (Kandler and Weiss, 2005).

3.3. Microscopic Examination

Microscopic examination was done with three different types of staining methods, which are simple staining, Gram staining and endospore staining.

Simple staining was done to determine cell morphology of the bacterial strains of interest, including their shape, size and arrangements. All cells examined appeared to be single rods with rounded ends and in variable lengths from 1.5 μm to 5.0 μm. Gram staining was applied to differentiate the bacteria according to their cell wall characteristics. In order to determine the presence or absence of endospore, endospore staining was done. The results obtained from all these three staining procedures are summarized in Table 3.2.

Table 3.2 Microscopic examination results[1]

	Cell morphology	Cell wall characterization	Endospore forming
ATCC 4356	Rod	Gram (+)	-
KPB4B	Rod	Gram (+)	-
LA05	Rod	Gram (+)	-
DSM 20079	Rod	Gram (+)	-
DSM 20531	Rod	Gram (+)	-
A7	Rod	Gram (+)	-
A11-H	Rod	Gram (+)	-
A11-P	Rod	Gram (+)	-
A11-T	Rod	Gram (+)	-

[1] Results are expressed as + (positive) and - (negative).

3.4. Motility Test

Motility test medium was used to determine the ability of the strains to move by themselves during growth. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 6538) were used as positive and negative control, respectively. All the studied strains were determined as non-motile, as *Lactobacillus* strains are expected to be.

3.5. Carbohydrate Fermentation Tests

Carbohydrate fermentation profiles of the strains of interest were used to identify and differentiate them. The DSM 20584 strain, type strain of *L. crispatus*, was also included in these tests since it had not been considered in the study conducted by Köse (2011).

First, starch hydrolysis test was done to biochemically confirm *Lactobacillus* strains, since it is known that all strains of *L. amylovorus* hydrolyze starch actively. All kefir isolates showed positive results for starch hydrolysis test.

Second test done was to observe gas production from glucose fermentation. This test aims to classify the strains of interest as homofermentative or heterofermentative, according to their metabolic pathway of carbohydrate fermentation. None of the Durham tubes had gas accumulation, although the medium in all tubes had changed from purple to yellow as a result of the fermentation of glucose. Hence, all strains tested were classified as homofermentative.

Third experiment was tube fermentation test with 10 different carbohydrates. The tested carbohydrates were fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, saccharose (sucrose), and trehalose. The results were compared with the table, showing the carbohydrate fermentation profiles of the *Lactobacillus* genus, from *Bergey's Manual of Systematic Bacteriology* (1986, vol. 2, p. 1222). The carbohydrate fermentation results of *L. acidophilus* strains matched with the profile of *L. acidophilus* strain given in the table and all four kefir isolates showed the same profile as *L. amylovorus* strain. The obtained profiles are shown in Table 3.3.

Table 3.3 Tube fermentation test results[1]

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-
Saccharose (sucrose)	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	-	+	+	+	+

[1] Results are expressed as + (positive) and - (negative).

Lastly, API® 50 CH/CHL (BioMeriéux, France) system was used to generate more detailed carbohydrate fermentation profile of these strains. The system tests fermentation of 49 substrates preplaced in microtubes of the strips. The list of these substrates was provided in Table 2.2 with their quantities in each cupule. The profiles were determined by checking the color change of the medium after 48-hour of incubation and evaluating each microtube as a positive or a negative result. These results are shown in Table 3.4. The Apiweb™ software (BioMeriéux, France) provided in the link https://apiweb.biomerieux.com/ was used for the identification of the strains. The carbohydrate profiles were submitted to the software to get identification percentages. The identification results are shown in Table 3.5.

The strains ATCC 4356, KPB4B, LA05 and DSM 20079 were identified as *L. acidophilus* as expected whereas the strain DSM 20531 was identified as 98.9 % *L. crispatus* although being the type strain of *L. amylovorus*. The kefir isolates were also identified as *L. crispatus*, although they had presented different fermentation profiles than that of the strain DSM 20584, the type stain of *L. crispatus*, for lactose and trehalose, which are the two carbohydrates that can be used to differentiate *L. amylovorus* from *L. crispatus* according to *Bergey's Manual of Systematic Bacteriology* (1986). Even though the minimum identification percentage was 98.0 % for these species, the test results were considered as doubtful.

When contacted BioMeriéux to reach their database in Apiweb™ for API® 50 CH/CHL, it was revealed that there are three different profiles for *L. acidophilus*, one for *L. crispatus* but none for *L. amylovorus*. The database also lacks the profiles of *L. gallinarium*, *L. gasseri* and *L. johnsonii*, which are the other members of *Lactobacillus acidophilus* group. Therefore, the strain DSM 20531 and all four kefir isolates were identified as *L. crispatus* due to the species *L. crispatus* being the most closely related species present in the database of Apiweb™.

Table 3.4 API® 50CH/CHL results[1]

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Control	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-	-	-	-	-	-

[1] Results are expressed as + (positive) and - (negative).

Table 3.4 API® 50 CH/CHL results[1] (cont'd)

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
D-Galactose	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
D-Manitol	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-

[1] Results are expressed as + (positive) and - (negative).

Table 3.4 API® 50 CH/CHL results[1] (cont'd)

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Methyl-αD-mannopyranoside	-	-	-	-	-	-	-	-	-	-
Methyl-αD-glucopyranoside	-	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	-	+	-	-	+	+	+	+	+	+
Amygdalin	-	+	-	-	+	+	+	-	+	+
Arbutin	+	-	+	+	+	+	+	+	+	+
Esculin / Ferric citrate	+	+	+	+	+	+	+	+	+	+

[1] Results are expressed as + (positive) and - (negative).

Table 3.4 API® 50 CH/CHL results[1] (cont'd)

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Salicin	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	-	+	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-	-	-	-
D-Saccharose (sucrose)	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-

[1] Results are expressed as + (positive) and - (negative).

Table 3.4 API® 50 CH/CHL results[1] (cont'd)

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Amidon (starch)	+	-	-	+	+	+	+	+	+	+
Glycogen	+	-	-	+	+	+	+	+	+	+
Xylitol	-	-	-	-	-	-	-	-	-	-
Gentibiose	+	+	+	+	+	+	+	+	+	+
D-Turanose	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-
D-Tagatose	+	-	-	+	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-

[1] Results are expressed as + (positive) and - (negative).

Table 3.4 API® 50 CH/CHL results[1] (cont'd)

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
D-Arabitol	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-
Potassium gluconate	-	-	-	-	-	-	-	-	-	-
Potassium 2-ketogluconate	-	-	-	-	-	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-	-	-	-	-	-

[1] Results are expressed as + (positive) and - (negative).

Table 3.5 Apiweb™ identification results with percentages

Strain	Strain Number	Identification percentage	Identified strain
L. acidophilus	ATCC 4356	98.9 %	*L. acidophilus*
L. acidophilus	KPB4B	90.1 %	*L. acidophilus*
L. acidophilus	LA05	90.1 %	*L. acidophilus*
L. acidophilus	DSM 20079	98.9 %	*L. acidophilus*
L. amylovorus	DSM 20531	98.9 %	*L. crispatus*
L. crispatus	DSM 20584	99.4 %	*L. crispatus*
Kefir isolate	A7	98.9 %	*L. crispatus*
Kefir isolate	A11-H	98.0 %	*L. crispatus*
Kefir isolate	A11-P	98.9 %	*L. crispatus*
Kefir isolate	A11-T	98.9 %	*L. crispatus*

3.6. Enzyme Tests

Enzyme tests were done to classify the strains according to their ability to produce certain enzymes. They were investigated for catalase, oxidase and urease enzymes. Also, indole test was done to determine the presence of trytophanase. In addition, the strains were examined for DNase, gelatinase and hemolytic activity. These properties are important not only for identification but also their potential to be used as probiotic strains. If a strain is positive either one or more of these three enzymes, it cannot be used as probiotic since it can cause harm in human body instead of being beneficial for human health.

Staphylococcus aureus (ATCC 6538) was used as the positive control for catalase, DNase and gelatinase tests. *Escherichia coli* (ATCC 25922) was used as the negative control for oxidase, urease, DNase and gelatinase tests and as the positive control for indole test. *Pseudomonas aeruginosa* (ATCC 10145) was used as the positive control for oxidase test whereas *Proteus mirabilis* (ATCC 29906) was used as the positive control for urease test. *Streptococcus pyogenes* (ATCC 12344) was used as the negative control for catalase and indole tests and as the control for β hemolysis. S*treptococcus mutans* (ATCC 25175) was used as the control for α hemolysis whereas *Enterococcus faecalis* (ATCC 19433) was used as the control for γ hemolysis. These control strains are given in Table 3.6 and the test results are summarized in Table 3.7.

Table 3.6 The strains used as controls in enzyme tests

Test	Control	Strain	Strain Number
Catalase test	+	*Staphylococcus aureus*	ATCC 6536
	-	*Streptococcus pyogenes*	ATCC 12344
Oxidase test	+	*Pseudomonas aeruginosa*	ATCC 10145
	-	*Escherichia coli*	ATCC 25922
Urease test	+	*Proteus mirabilis*	ATCC 29906
	-	*Escherichia coli*	ATCC 25922
Indole test	+	*Escherichia coli*	ATCC 25922
	-	*Streptococcus pyogenes*	ATCC 12344
DNase	+	*Staphylococcus aureus*	ATCC 6536
	-	*Escherichia coli*	ATCC 25922
Gelatinase	+	*Staphylococcus aureus*	ATCC 6536
	-	*Escherichia coli*	ATCC 25922
Hemolytic Activity	α	*Streptococcus mutans*	ATCC 25175
	β	*Streptococcus pyogenes*	ATCC 12344
	γ	*Enterococcus faecalis*	ATCC 19433

Table 3.7 Enzyme test results[1]

	L. acidophilus				*L. amylovorus*	*L. crispatus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	DSM 20584	A7	A11-H	A11-P	A11-T
Catalase	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Trytophanase	-	-	-	-	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-
Hemolytic activity	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ

[1] Results are expressed as - (negative) and γ (gamma).

3.7. DNA isolation

Biochemical tests are not sufficient alone to identify the bacterial strains, especially the ones which are closely related. "*Lactobacillus acidophilus* cannot be differentiated reliably from *Lactobacillus gasseri*, *Lactobacillus crispatus*, and *Lactobacillus amylovorus* by any simple phenotypic test; electrophoretic analysis of soluble cellular proteins or lactate dehydrogenases, detailed cell wall studies or, preferentially, genotypic identification methods are necessary." (Bergey's Manual of Systematic Bacteriology, 2009, vol. 3, pp. 491-492).

For molecular characterization of the *Lactobacillus* isolates of interest, genomic DNA extraction and isolation were performed and the concentrations of resulting DNA samples were determined. The value of A_{260}/A_{280} was also determined, since the value should be in the range of 1.8 – 2.0 to confirm the purity of the isolated DNA. The A_{260}/A_{280} values and DNA concentrations are included, with their average values, in Table C.1 in Appendix C.

All four kefir isolates presented *L. amylovorus* profile in tube fermentation test but they were identified as *L. crispatus* according to API® 50 CH/CHL system. Although these kinds of biochemical tests are not reliable without support of genotypic identification methods, this situation raised some questions. In order to overcome this ambiguity, type strain of *Lactobacillus crispatus* (DSM 20584) was also included in further molecular characterization analysis.

3.8. Polymerase Chain Reaction

The PCR was performed with plb16 (5'-AGAGTTTGATCCTGGCTCAG-3') and mlb16 (5'-GGCTGCTGGCACGTAGTTAG-3') primers in order to amplify an approximately 500 bp region of the 16S rRNA gene according to Kullen et al. (2000). This region was chosen particularly, since it contained the variable V1 region (approximately 50 bp) and V2 region (approximately 50 bp). These V1 and V2 regions were used to differentiate the strains of interest belonging to *Lactobacillus acidophilus* group.

3.9. Agarose Gel Electrophoresis

Agarose gel electrophoresis was applied to PCR products to determine the size of the amplified DNA fragment. They were resolved in 1.0% (w/v) agarose gel by electrophoresis and visualized by staining with GelRed™ (Olerup SSP, Sweden). Figure 3.4 shows clearly that 500 bp region was amplified and resolved successfully for all the studied strains.

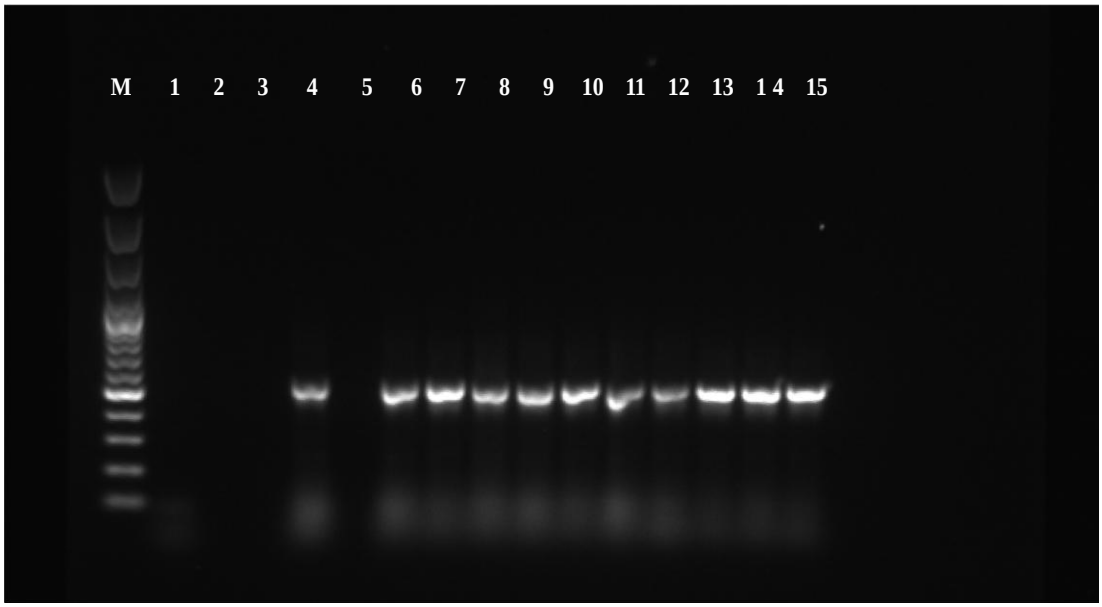

Figure 3.4 Agarose gel electrophoresis of 500 bp fragments of PCR amplified genomic DNA samples isolated from *Lactobacillus* strains. M: 1 kb DNA ladder, lane 1: Negative PCR control (no DNA), lane 2: Negative control for DNA ladder, lane 3: No sample, lane 4: A11-T (kefir isolate), lane 5: Negative control for loading dye, lane 6: A11-H (kefir isolate), lane 7: A11-P (kefir isolate), lanes 8-9: KPB4B (*L. acidophilus*), lane 10: DSM 20531 (*L. amylovorus*), lane 11: DSM 20584 (*L. crispatus*), lane 12: DSM 20079 (*L. acidophilus*), lane 13: ATCC 4356 (*L. acidophilus*), lane 14: LA05 (*L. acidophilus*), lane 15: A7 (kefir isolate).

3.10. 16S rRNA Sequencing

After the confirmation of amplification of approximately 500 bp region of the 16S rRNA gene with agarose gel electrophoresis, these PCR products were subjected to sequencing and sequence analysis.

The sequencing was done by RefGen Gene Research and Biotechnology (Ankara, Turkey), with plb16 and mlb16 primers which are used as forward and reverse primers, respectively. By doing so, all the bases of these regions were revealed and compared with each other in order to differentiate the strains.

3.11. Multiple Sequence Alignment and BLAST Analysis

Multiple sequence alignments were done with Clustal Omega program (1.2.1). In order to determine the differences in DNA base sequences, all the following strains were aligned: DSM 20079 (*L. acidophilus*), ATCC 4356 (*L. acidophilus*), KPB4B (*L. acidophilus*), LA05 (*L. acidophilus*), DSM 20531 (*L. amylovorus*), A7 (kefir isolate), A11-H (kefir isolate), A11-P (kefir isolate), A11-T (kefir isolate) and DSM 20584 (*L. crispatus*). The output data is shown in Figure D.1 in Appendix D.

Since the identical nucleotides for a given position were marked with * (asterisk), the variable bases were detected easily among *L. acidophilus*, *L. amylovorus* and *L. crispatus* strains. Although it is hard to differentiate *Lactobacillus acidophilus* group strains since they are closely related, their differences were revealed at the molecular level by the alignment.

In addition to comparison with reference strains, the 16S rRNA gene sequences of the kefir isolates of interest were compared with all the strains located in the gene bank database of NCBI by BLAST analysis.

The BLAST analyses of all kefir isolates (the strains A7, A11-H, A11-P, A11-T) with the type strains of *L. acidophilus*, *L. amylovorus* and *L. crispatus* are shown in Appendix E. The results are also summarized and shown with identification percentages in Table 3.8.

Table 3.8 BLAST analysis results

Query strain	Subject strain	Subject strain number	Identification percentage
A7	*L. acidophilus*	ATCC 4356	96 %
	L. amylovorus	DSM 20531	99 %
	L. crispatus	ATCC 33820	97 %
A11-H	*L. acidophilus*	ATCC 4356	96 %
	L. amylovorus	DSM 20531	99 %
	L. crispatus	ATCC 33820	97 %
A11-P	*L. acidophilus*	ATCC 4356	96 %
	L. amylovorus	DSM 20531	99 %
	L. crispatus	ATCC 33820	98 %
A11-T	*L. acidophilus*	ATCC 4356	96 %
	L. amylovorus	DSM 20531	99 %
	L. crispatus	ATCC 33820	98 %

According to Stackebrandt and Goebel (1994), strains showing homology of at least 97% might be considered as the same species. However, BLAST results of the kefir isolates indicated 97% or higher percent identity with both *L. amylovorus* and *L. crispatus*. Nonetheless, all of the isolates had the highest percent identity (99%) with *L. amylovorus*.

3.12. Comparison of the V1 region

According to Kullen et. al (2000), rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* group can be done by using DNA sequence of variable regions of the 16S rRNA gene. “Just downstream of the 5’ end of the 16S rRNA lies the highly variable ~50 bp V1 region, followed by ~80 bp of well-conserved sequence and finally, by the moderately divergent ~50 bp V2 region.” (Kullen et al., 2000, p.512). They concluded that the V1 region alone was sufficient for the differentiation of each of the type strains of *Lactobacillus acidophilus* group. Their alignment of the V1 region is shown in Figure 3.5.

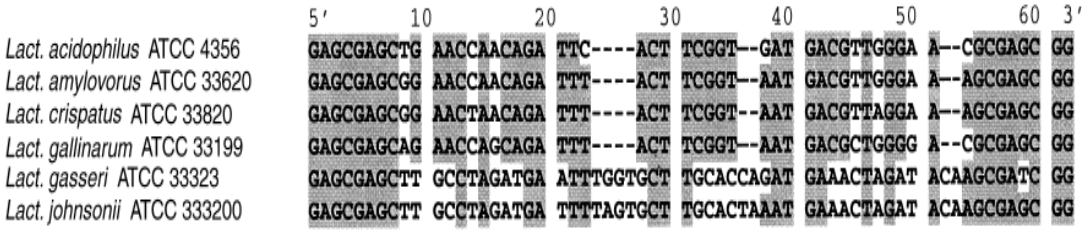

Figure 3.5 Alignment of the V1 region of the 16S rRNA gene sequences of type strains of the *Lactobacillus acidophilus* group. Shading denotes nucleotides that are identical for that position. Dashes (-) represent gaps in the sequences introduced during alignment. Adapted from “Use of DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex” by M.J. Kullen, R.B. Sanozky-Dawes, D.C. Crowell and T.R. Klaenhammer, 2000, *Journal of Applied Microbiology, 89,* p. 514. Copyright 2000 by Society for Applied Microbiology.

The V1 region of the 16S rRNA gene of *L. acidophilus* DSM 20079 (type strain), *L. acidophilus* ATCC 4356 (type strain), *L. acidophilus* KPB4B, *L. acidophilus* LA05, *L. amylovorus* DSM 20531 (type strain), kefir isolate A7, kefir isolate A11-H, kefir isolate A1-P, kefir isolate A11-T and *L. crispatus* DSM 20584 (type strain) were aligned with the sequences adapted from Kullen et al. (2000) by using Clustal Omega program (1.2.1.), and shown in Figure D.2 in Appendix D. It is also shown in Figure 3.6 where bases that differ for *L. acidophilus, L. amylovorus* and *L. crispatus* strains were highlighted.

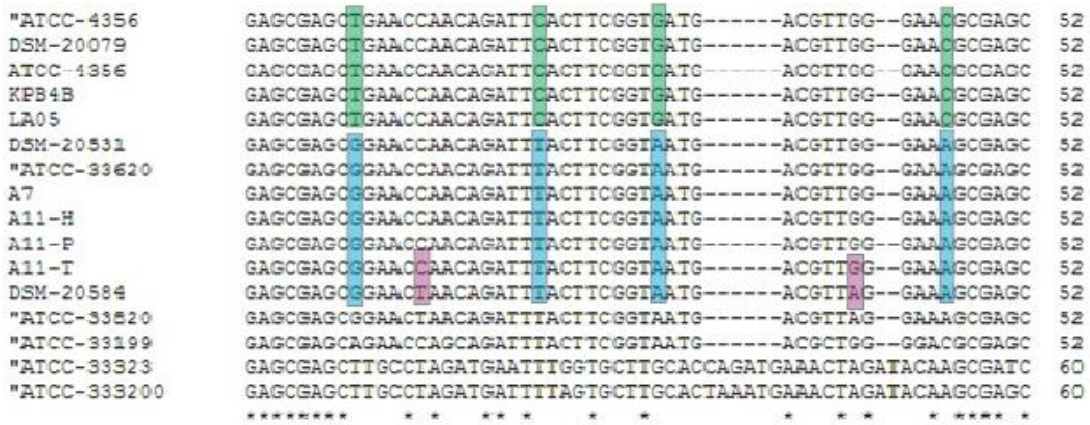

Figure 3.6 MSA of the V1 region of the 16S rRNA gene of *Lactobacillus* strains with highlighted bases. ATCC 4356: *L. acidophilus*, DSM 20079: *L. acidophilus*, KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, DSM 20531: *L. amylovorus*, ATCC 33620: *L. amylovorus*, A7: Kefir isolate, A11-H: Kefir isolate, A11-P: Kefir isolate, A11-T: Kefir isolate, DSM 20584: *L. crispatus*, ATCC 33820: *L. crispatus*, ATCC 33199: *L. gallinarium*, ATCC 33323: *L. gasseri* and ATCC33200: *L. johnsonii*. The sequences adapted from Kullen et al. (2000) are denoted with " (quotation mark). Fully conserved bases are denoted with * (asterisk). Green and blue color show where *L. acidophilus* and *L. amylovorus* strains differ whereas pink color highlights the bases where *L. amylovorus* and *L. crispatus* strains differ from each other.

The examination of the base differences in the V1 region and their comparison with the sequences of type strains identified all four kefir isolates as *Lactobacillus amylovorus*. The BLAST analysis of the V1 region of the 16S rRNA gene of the kefir isolates indicated 100% identity with all *L. amylovorus* strains, including the type strain DSM 20531, located in the gene bank database of NCBI. The results are shown in Figure E.13 in Appendix E.

3.13. Phylogenetic Analysis

Phylogenetic analysis of the strains of interest was done with MEGA6 program.

Before analyzing phylogenetic relationships of the strains of interest, DSM 20079: *L. acidophilus* (T), ATCC 4356: *L. acidophilus* (T), KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, DSM 20531: *L. amylovorus* (T), A7: *L. amylovorus* (kefir isolate), A11-H: *L. amylovorus* (kefir isolate), A1-P: *L. amylovorus* (kefir isolate), A11-T: *L. amylovorus* (kefir isolate) and DSM 20584: *L. crispatus* (T), multiple alignment sequences were obtained using MEGA6. Then, pairwise distances method was computed to measure the distance between each pair of the strains of interest and the obtained data is shown in Table 3.9.

Table 3.9 Evolutionary divergence among sequences of studied strains

	1	2	3	4	5	6	7	8	9	10
1. DSM 20079		0.002	0.007	0.007	0.009	0.010	0.008	0.008	0.008	0.007
2. ATCC 4356	0.002		0.007	0.006	0.008	0.010	0.008	0.008	0.008	0.007
3. KPB4B	0.026	0.023		0.007	0.012	0.009	0.011	0.011	0.011	0.010
4. LA05	0.023	0.021	0.023		0.011	0.010	0.011	0.010	0.010	0.010
5. DSM 20531	0.037	0.034	0.059	0.057		0.006	0.003	0.003	0.003	0.008
6. A7	0.048	0.045	0.041	0.045	0.019		0.006	0.005	0.005	0.009
7. A11-H	0.034	0.032	0.057	0.054	0.006	0.017		0.002	0.002	0.007
8. A11-P	0.032	0.030	0.054	0.052	0.004	0.015	0.002		0.000	0.007
9. A11-T	0.032	0.030	0.054	0.052	0.004	0.015	0.002	0.000		0.007
10. DSM 20584	0.023	0.021	0.045	0.043	0.026	0.037	0.023	0.021	0.021	

In Table 3.9, standard error estimates are shown above the diagonal. Analyses were conducted using the maximum composite likelihood model. All positions containing gaps and missing data were eliminated.

Two different methods were used to construct phylogenetic trees of these species. First, maximum likelihood method was used and the output is shown in Figure 3.7.

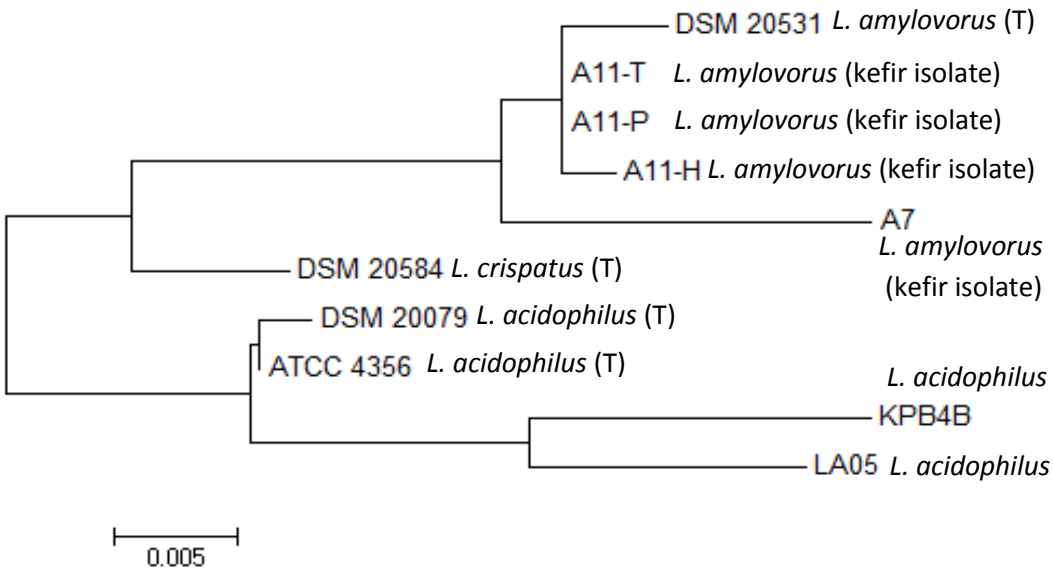

Figure 3.7 Phylogenetic analysis by maximum likelihood method. The tree with the highest log likelihood (-945.6389) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 477 positions in the final dataset.

Second applied method for phylogenetic analysis was UPMGA (Unweighted Pair Group Method with Arithmetic Mean). The constructed phylogenetic tree was shown in Figure 3.8.

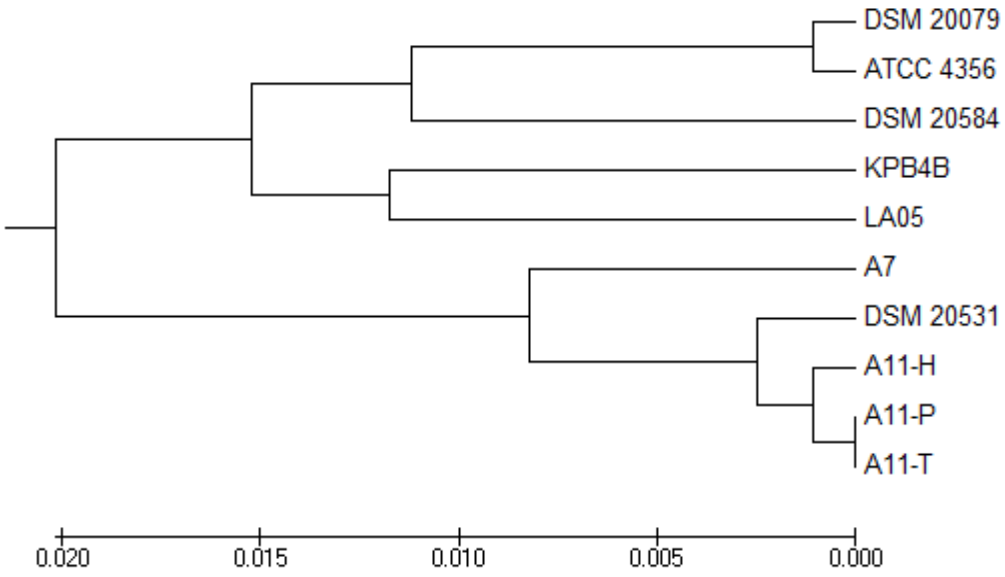

Figure 3.8 Evolutionary relationships of taxa**.** DSM 20079: *L. acidophilus* (T), ATCC 4356: *L. acidophilus* (T), DSM 20584: *L. crispatus* (T), KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, A7: *L. amylovorus* (kefir isolate), DSM 20531: *L. amylovorus* (T), A11-H: *L. amylovorus* (kefir isolate), A1-P: *L. amylovorus* (kefir isolate) and A11-T: *L. amylovorus* (kefir isolate). The optimal tree with the sum of branch length = 0.09124296 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 477 positions in the final dataset.

3.14. Acid Tolerance

Acid tolerance of four kefir isolates and the strain LA05, commercial probiotic, was determined. The data obtained from this known probiotic was used as a reference in the determination of probiotic characteristics of the kefir isolates.

Acid tolerance was examined at pH 1.5, 2.0 and 2.5 and at time 0 h, 1 h, 1.5 h, 2.0 h and 3.0 h. Both absorbance representing growth and total viable cell count were determined for the strains.

The absorbance measurement was performed at 600 nm. A bacterial cell suspension of 10^9 cells/ml was used since it is the generally recommended value for daily intake if therapeutic effects are desired from a probiotic microorganism.

Total viable cell count was performed three times for each strain. For each time, two sets of dilution in peptone water were prepared and duplicate Petri dishes were inoculated from each of these dilution tubes. After enumeration of the plates, $\log_{10}$ colony forming unit (cfu) per ml data were determined. These data are shown in Appendix F for pH 1.5, pH 2.0 and pH 2.5 in Table F.1, Table F.2 and Table F.3, respectively.

The average values obtained from these three experiments were used to plot $\log_{10}$ cfu/ml versus time (min.) graphs in order to exhibit acid tolerance of the strains. The graphs of the strains A7, A11-H, A11-P, A11-T and LA05 are shown in Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.12 and Figure 3.13, respectively.

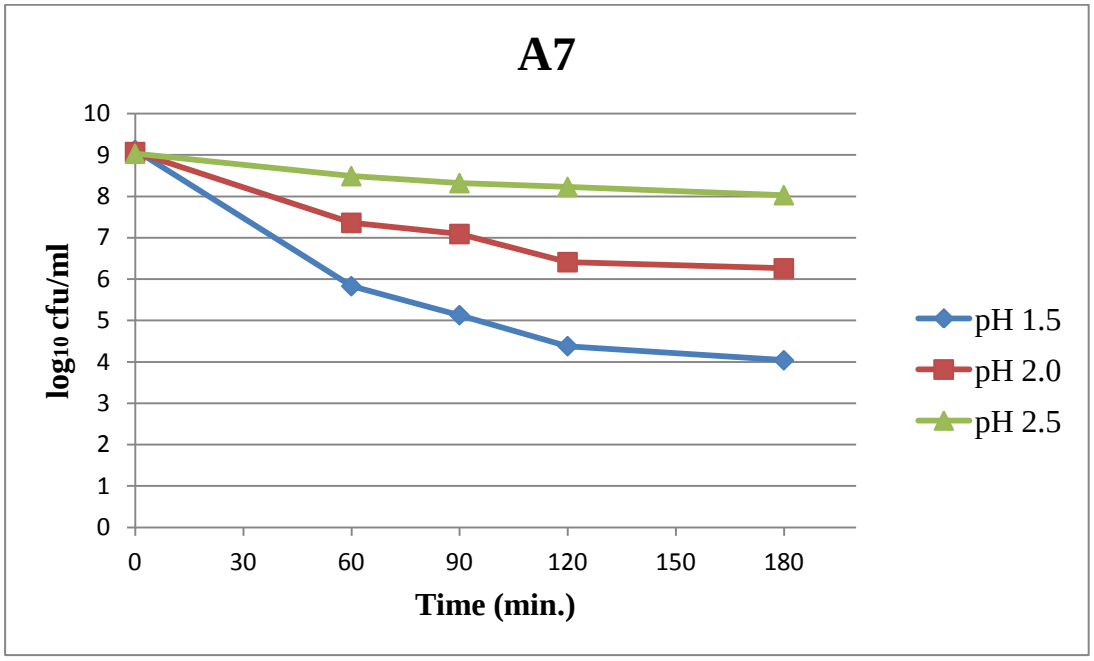

Figure 3.9 Acid tolerance of the strain A7 at different pH values

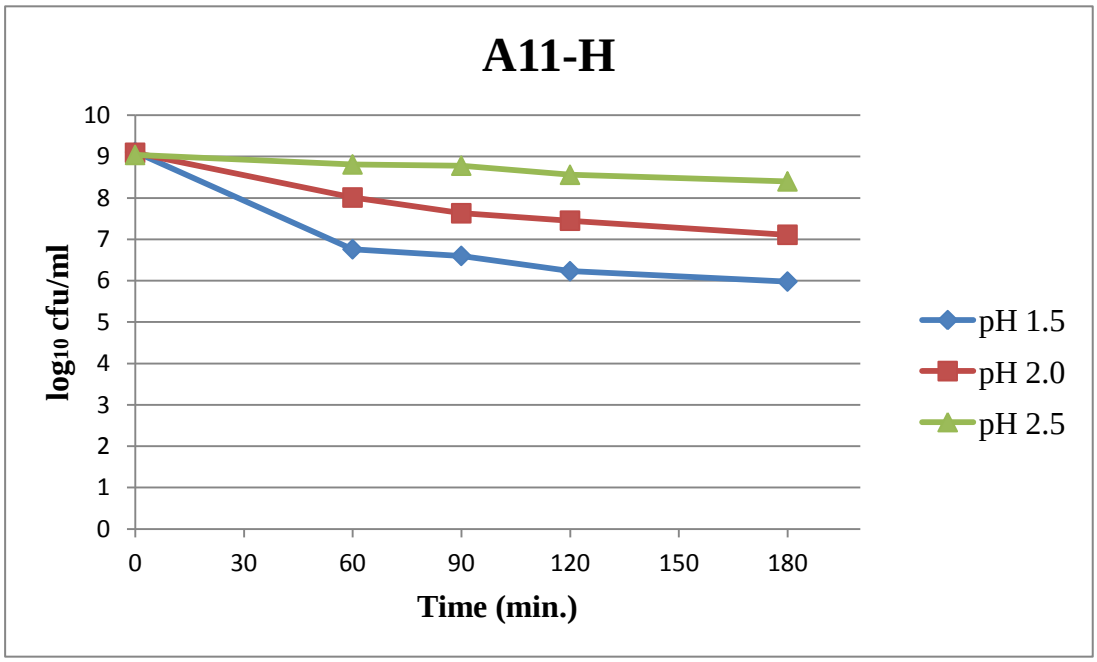

Figure 3.10 Acid tolerance of the strain A11-H at different pH values

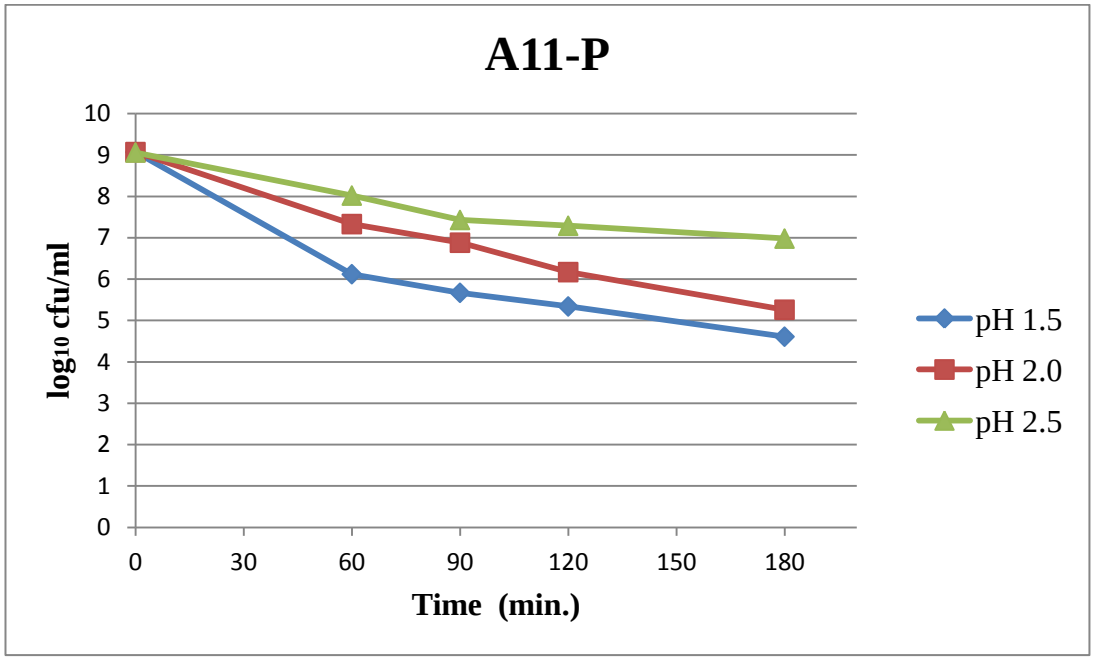

Figure 3.11 Acid tolerance of the strain A11-P at different pH values

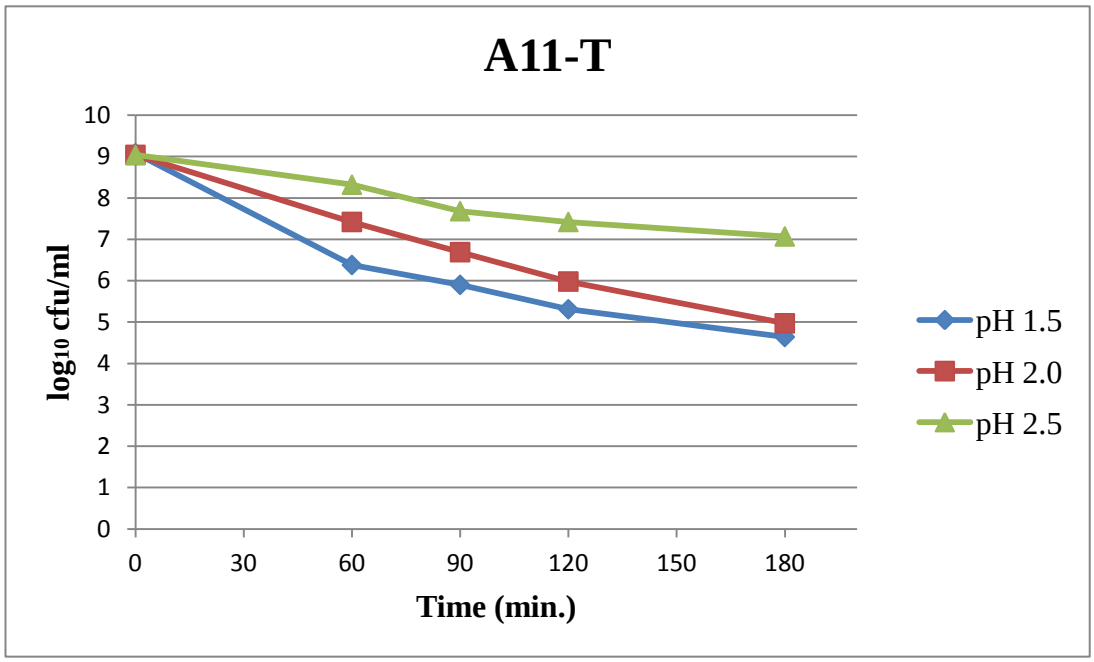

Figure 3.12 Acid tolerance of the strain A11-T at different pH values

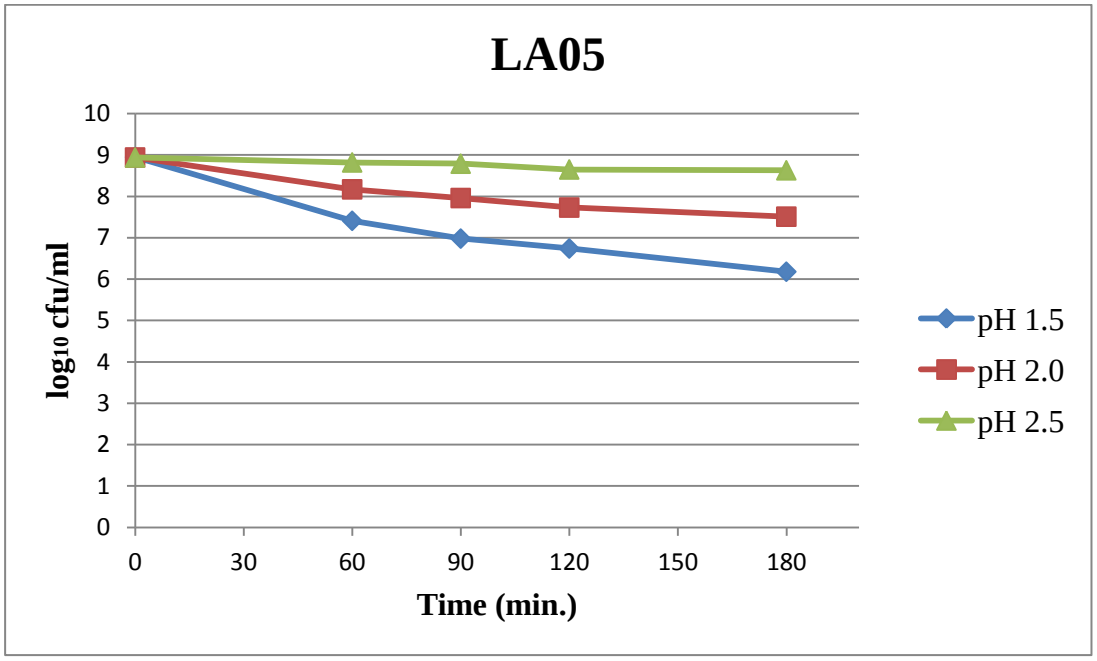

Figure 3.13 Acid tolerance of the strain LA05 at different pH values

Then, percent survivals of the strains were calculated for each pH values at 60 min., 90 min., 120 min. and 180 min., applying the following formula:

$$\% \text{ Survival} = \frac{(\log_{10} \text{cfu/ml})_{\text{t=60 min. } or \text{ t=90 min. } or \text{ t=120min. } or \text{ } t\text{=180 min.}}}{(\log_{10} \text{cfu /ml})_{\text{t=0}}} \text{ x } 100$$

The results are shown in Table 3.10 for pH 1.5, in Table 3.11 for pH 2.0 and in Table 3.12 for pH 2.5 with the relevant $\log_{10}$ cfu/ml data.

Table 3.10 Percent survival at pH 1.5

	t = 0	t = 60		t = 90		t = 120		t = 180	
	$\log_{10}$ cfu/ml	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival
A7	9.12	5.83	63.93	5.13	56.25	4.38	48.03	4.04	44.30
A11-H	9.11	6.76	74.20	6.60	72.45	6.23	68.39	5.98	65.64
A11-P	9.07	6.12	67.48	5.67	62.51	5.34	58.88	4.61	50.82
A11-T	9.08	6.38	70.26	5.90	64.98	5.31	58.48	4.64	51.10
LA05	8.95	7.41	82.79	6.98	74.99	6.74	75.31	6.18	69.05

Table 3.11 Percent survival at pH 2.0

	t = 0	t = 60		t = 90		t = 120		t = 180	
	log_{10} cfu/ml	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival
A7	9.07	7.36	81.15	7.09	78.17	6.41	70.67	6.26	69.02
A11-H	9.09	8.01	88.12	7.63	83.93	7.45	81.96	7.11	78.21
A11-P	9.07	7.33	80.82	6.88	75.85	6.17	68.03	5.26	57.99
A11-T	9.04	7.42	82.08	6.69	74.00	5.98	66.15	4.97	54.98
LA05	8.94	8.17	91.39	7.96	89.04	7.73	86.47	7.51	84.00

Table 3.12 Percent survival at pH 2.5

	t = 0	t = 60		t = 90		t = 120		t = 180	
	log_{10} cfu/ml	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival
A7	9.03	8.49	94.02	8.32	92.14	8.23	91.14	8.03	88.93
A11-H	9.04	8.81	97.46	8.78	97.12	8.56	94.69	8.40	92.92
A11-P	9.06	8.02	88.52	7.43	82.01	7.29	80.46	6.98	77.04
A11-T	9.04	8.32	92.04	7.68	84.96	7.42	82.08	7.07	78.21
LA05	8.94	8.82	98.66	8.79	98.32	8.65	96.76	8.63	96.53

According to Charteris et al. (1998a), acid tolerance test should be performed at pH 2.0 for three hours with intervals of 90 minutes to simulate the gastric conditions and bacterial residency in the stomach. In this study, pH values of 1.5 and 2.5 were used to examine the effects of pH on the strains, and time 60 min. and 120 min. were also used to increase the precision.

Total viable cell count was required to be able to determine the survival rate of the strains. After 3 hours of incubation, 69.05% of the strain LA05 survived at pH 1.5, 84.00% at pH 2.0 and 96.53% at pH 2.5. The maximum survival rate, after 3 h, among the kefir isolates was obtained for the strain A11-H as follows: 65.64% at pH 1.5, 78.21% at pH 2.0 and 92.92% at pH 2.5. The calculated minimum survival percentage was 44.30% and the data was obtained after 3 h incubation of A7 at pH 1.5. Except this data, all survival rates were higher than 50% which means that they all survived. Even though the strain A7 showed low survival rate at pH 1.5, its 69.02% survived at pH 2.0 after 3 h incubation. This survival rate was higher than the percent survival of the strains A11-P and A11-T which are 57.99% and 54.98%, respectively.

Evaluation of the data obtained by 90-minutes incubation at pH 2 was sufficient alone for probiotic characterization, since the pH of gastric juice is around 2 and it takes 90 minutes to release from stomach after entrance. None of the strains had percent survival less than 70% at that condition representing the gastric juice.

In the light of these findings, all the kefir isolates were defined as “acid tolerant” according to Santos et al. (2003) and thus potential probiotics.

3.15 Bile Salt Tolerance

Bile salt tolerance of four kefir isolates and the strain LA05, commercial probiotic, was determined. The data obtained from this known probiotic was used as a reference in the determination of probiotic characteristics of the kefir isolates.

Bile salt tolerance was examined in MRS broth containing: 0.1%, 0.3% and 0.5% (w/v) oxgall, at time 0 h, 1 h, 1.5 h, 2.0 h and 3.0 h. Both absorbance representing growth and total viable cell count were determined for the strains of interest.

The absorbance measurement was performed at 600 nm. A bacterial cell suspension of 10^7 cells/ml was used considering the loss (~ 2 log) of probiotic cells (recommended daily intake is 10^9 cells/ml) in the gastric environment and prior to their arrival to intestine.

Total viable cell count was performed three times for each strain. For each time, two sets of dilution in peptone water were prepared and duplicate Petri dishes were inoculated from each of these dilution tubes. After enumeration of the plates, $\log_{10}$ colony forming unit (cfu) per ml data were determined. These data are shown in Appendix F for bile salt concentration 0.1% (w/v), 0.3% (w/v) and 0.5% (w/v) in Table F.4, Table F.5 and Table F.6, respectively.

The average values calculated from these three experiments were used to plot $\log_{10}$ cfu/ml versus time (min.) graphs in order to exhibit bile tolerance of the strains. The graphs of the strains A7, A11-H, A11-P, A11-T and LA05 are shown in Figure 3.14, Figure 3.15, Figure 3.16, Figure 3.17 and Figure 3.18, respectively.

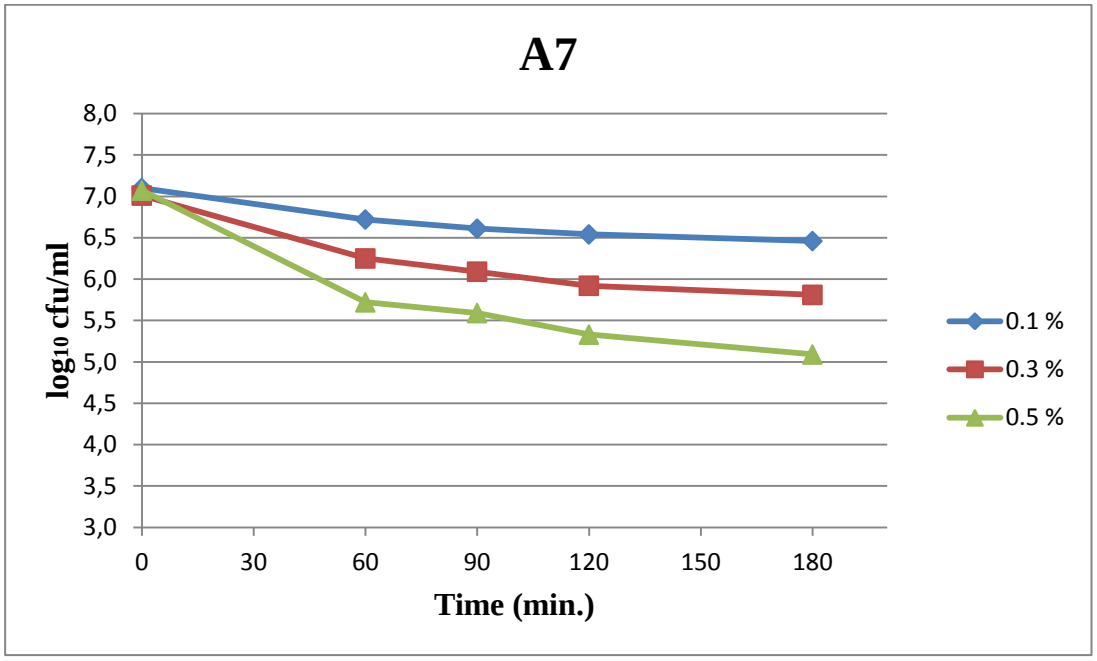

Figure 3.14 Bile tolerance of the strain A7 at different oxgall concentrations

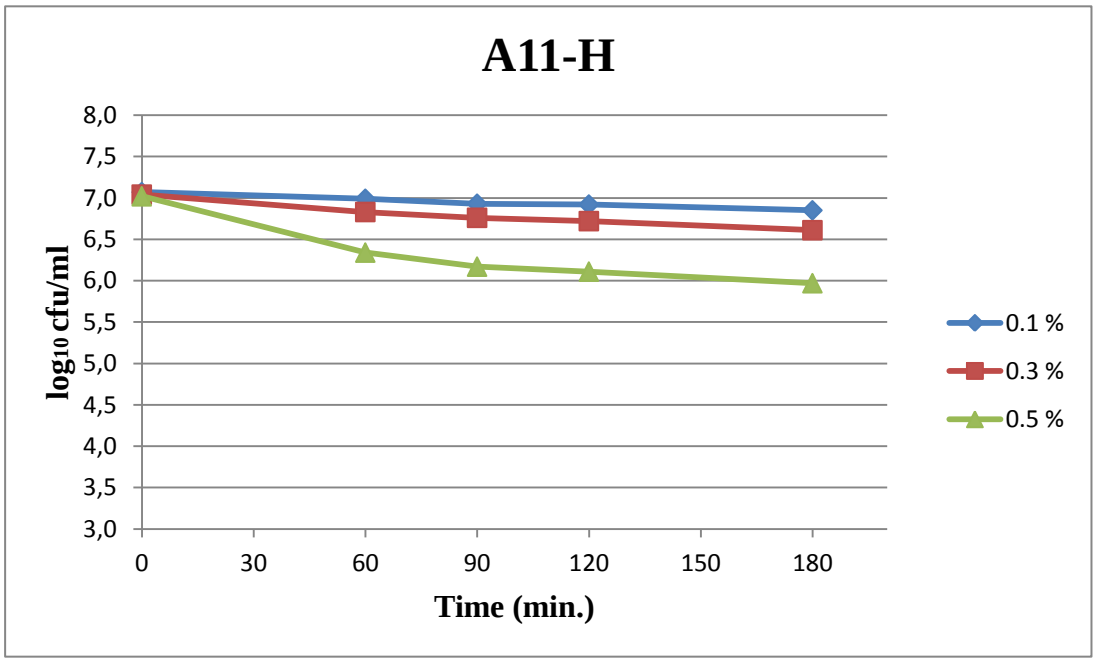

Figure 3.15 Bile tolerance of the strain A11-H at different oxgall concentrations

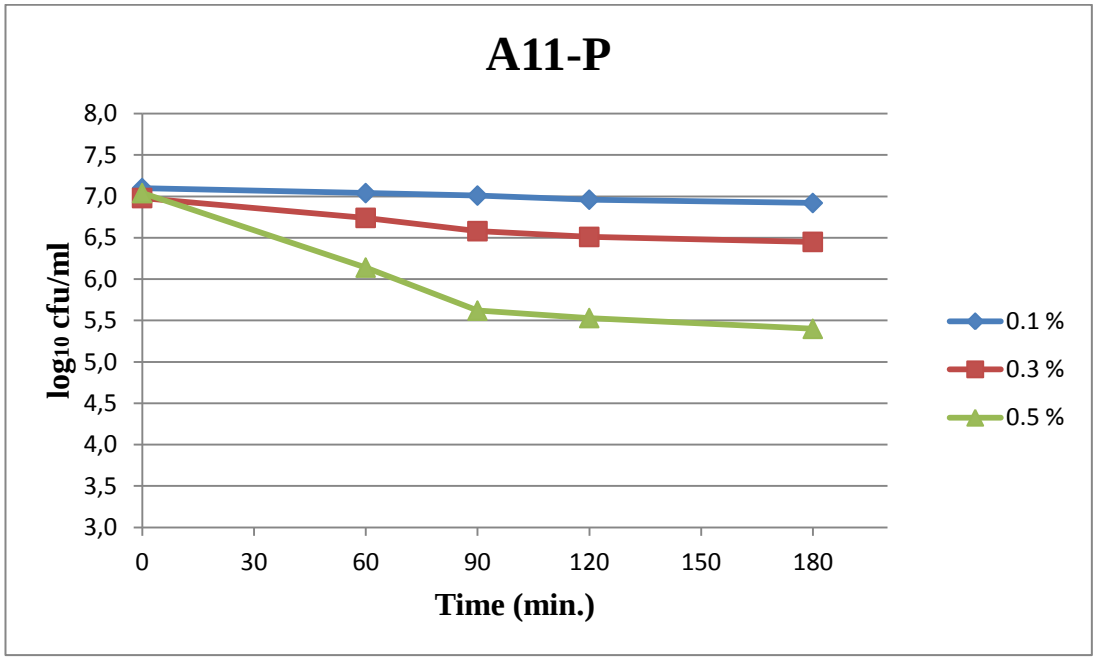

Figure 3.16 Bile tolerance of the strain A11-P at different oxgall concentrations

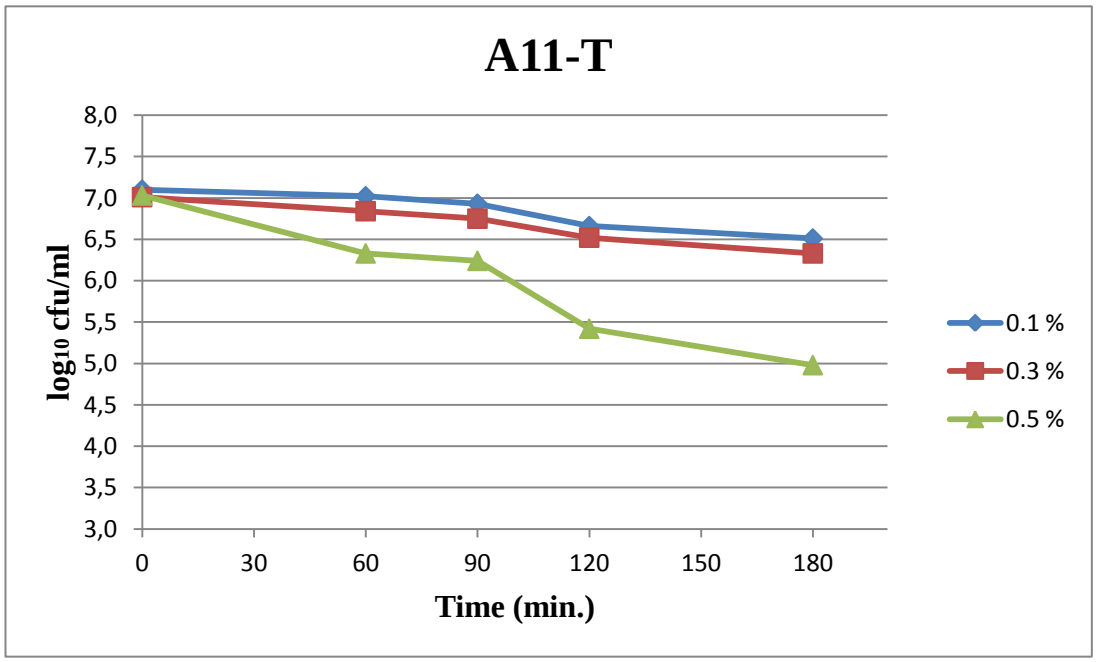

Figure 3.17 Bile tolerance of the strain A11-T at different oxgall concentrations

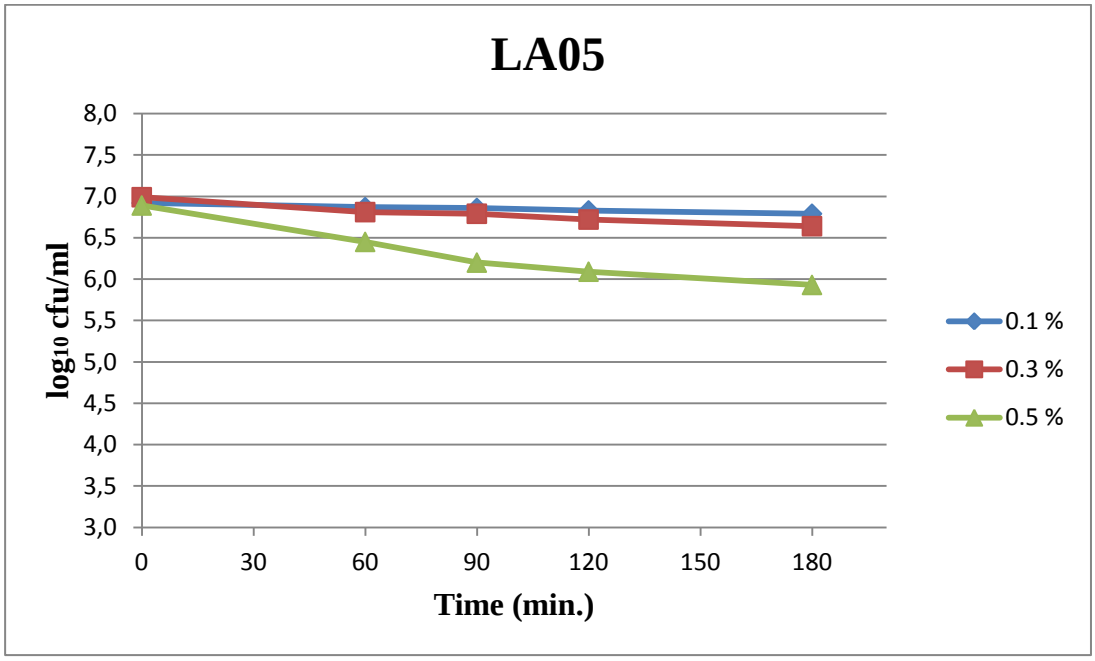

Figure 3.18 Bile tolerance of the strain LA05 at different oxgall concentrations

In addition, percent survivals of the strains were calculated for each bile concentration at 60 min., 90 min., 120 min. and 180 min., applying the following formula:

$$\% \text{ Survival} = \frac{(\log_{10} \text{cfu/ml})_{\text{t=60 min. } or \text{ t=90 min. } or \text{ t=120min. } or \text{ } t\text{=180 min.}}}{(\log_{10} \text{cfu /ml})_{\text{t=0}}} \text{ x } 100$$

The survival percentages of the strains are given in Table 3.13 for bile concentration 0.1% (w/v), in Table 3.14 for 0.3% (w/v), and in Table 3.15 for 0.5% (w/v) with the relevant $\log_{10}$ cfu/ml data.

Table 3.13 Percent survival at bile concentration 0.1% (w/v)

	t = 0	t = 60		t = 90		t = 120		t = 180	
	log_{10} cfu/ml	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival
A7	7.10	6.72	94.65	6.61	93.10	6.54	92.11	6.46	90.99
A11-H	7.07	6.99	98.87	6.93	98.02	6.82	97.88	6.85	96.89
A11-P	7.10	7.04	99.15	7.01	98.73	6.96	98.03	6.92	97.46
A11-T	7.10	7.02	98.88	6.93	97.61	6.66	93.80	6.51	91.69
LA05	6.92	6.87	99.28	6.86	99.13	6.83	98.70	6.79	98.12

Table 3.14 Percent survival at bile concentration 0.3% (w/v)

	t = 0	t = 60		t = 90		t = 120		t = 180	
	$\log_{10}$ cfu/ml	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival	$\log_{10}$ cfu/ml	% Survival
A7	7.01	6.25	89.16	6.09	86.88	5.92	84.45	5.81	82.88
A11-H	7.04	6.83	97.02	6.76	96.02	6.72	95.45	6.61	93.89
A11-P	6.98	6.74	96.56	6.58	94.27	6.51	93.27	6.45	92.41
A11-T	7.01	6.84	97.57	6.75	96.29	6.52	93.01	6.33	90.30
LA05	6.99	6.81	97.42	6.79	97.14	6.72	96.14	6.64	94.99

Table 3.15 Percent survival at bile concentration 0.5% (w/v)

	t = 0	t = 60		t = 90		t = 120		t = 180	
	log_{10} cfu/ml	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival	log_{10} cfu/ml	% Survival
A7	7.07	5.72	80.91	5.59	79.06	5.33	75.39	5.09	71.99
A11-H	7.02	6.34	90.31	6.17	87.89	6.11	87.04	5.97	85.04
A11-P	7.04	6.14	87.22	5.62	79.83	5.53	78.55	5.40	76.70
A11-T	7.03	6.33	90.04	6.24	88.76	5.42	77.10	4.98	70.84
LA05	6.89	6.45	93.61	6.20	89.99	6.09	88.39	5.93	86.06

Bile tolerance of probiotic bacteria should be determined in the presence of 0.3% (w/v) concentration of bile salts for at least 90 minutes to simulate the conditions in the intestine. In this study, 0.1% (w/v) and 0.5% (w/v) bile salt concentrations were also used to determine the effects of bile salt concentration on survival and colonization ability of the strains.

Total viable cell count was done to determine the survival rate of the strains. After 3 hours of incubation, 98.12% of the strain LA05 survived in 0.1% (w/v), 94.99% in 0.3% (w/v) and 86.06% in 0.5% (w/v) bile salt containing MRS broth. The maximum survival rate, after 3 h, among the kefir isolates was obtained for the strain A11-H as follows: 97.46% in 0.1% (w/v), 93.89% in 0.3% (w/v) and 85.04% in 0.5% (w/v). The calculated minimum percent survival was 70.84% and the data was obtained after 3 hour-incubation of the strain A11-T at bile salt concentration of 0.5% (w/v). All survival rates were higher than 70% which means that they all survived bile and thus protected their ability to colonize.

Evaluation of the data obtained by 90-minutes incubation in medium containing bile salt concentration of 0.3% (w/v) was sufficient alone for probiotic characterization. None of the strains survived at a rate less than 85% at that condition representing the transit through the intestine.

In the light of these findings, all the kefir isolates were defined as “bile tolerant” according to Santos et al. (2003) and thus potential probiotics.

3.16. Cell Surface Hydrophobicity

Cell surface hydrophobicity of the kefir isolates were determined to predict their ability to adhere to the intestinal epithelium. The strain LA05 was used as reference since it is a commercially used probiotic strain.

The experiment was performed three times for each strain by determining absorbance values in triplicate for duplicate samples. The change in absorbance after the introduction of n-hexadecane to bacterial suspension was used to calculate the percent cell surface hydrophobicity (%H).

Average values from these three experiments and the calculated percent cell surface hydrophobicity are shown in Table 3.16.

Table 3.16 Cell surface hydrophobicity percentages

	A_0		A		%H	
	Measured	Avg.	Measured	Avg.	Calculated	Avg.
A7	0.941	0.965	0.867	0.893	7.86	7.47
	0.983		0.916		6.82	
	0.972		0.897		7.72	
A11-H	0.954	0.932	0.836	0.798	12.37	14.35
	0.898		0.752		16.26	
	0.943		0.807		14.42	
A11-P	0.970	0.923	0.832	0.802	14.23	12.99
	0.889		0.765		13.95	
	0.909		0.811		10.78	
A11-T	0.981	0.931	0.918	0.881	6.71	5.43
	0.924		0.876		5.19	
	0.888		0.849		4.39	
LA05	0.984	0.935	0.822	0.745	16.46	20.48
	0.947		0.754		20.38	
	0.874		0.659		24.60	

The percent cell surface hydrophobicity for the strain LA05 was calculated to be 20.48%. Although this figure was the highest among all the tested strains, it is actually considered low for a probiotic. The highest percentage value was obtained for the strain A11-H among all kefir isolates. It was 14.35% and this value is not significantly low when compared to that of the strain LA05. The strain A11-P ranked second with 12.99%. However, the outcome was not as great for the strains A7 and A11-T with their percent cell surface hydrophobicity data of 7.47% and 5.43%, respectively.

Although acid tolerance and bile salt tolerance tests were concluded that all these kefir isolates survive in gastric and intestinal conditions, their low percent cell surface hydrophobicity indicate their low adhesion ability to the intestinal epithelium. However, these low levels of *in vitro* percent cell surface hydrophobicity values might be sufficient for *in vivo* adhesion, since *in vitro* analyses do not necessarily correlate *in vivo* analyses.

Since the strain LA05 is used commercially with 20.48% adhesion ability, the strains A11-H and A11-P can also be used with 14.35% and 12.99% adhesion ability, respectively. For potential probiotic usage of the strains A7 and A11-T, other ingredients may be used in probiotic preparations to increase their hydrophobicity. In addition, adhesion assay should also be done with Caco-2 intestinal epithelial cells to have a better understanding of these potential probiotics' ability to adhere *in vivo*.

3.17. Antibiotic Susceptibility

Antibiotic susceptibility of all *Lactobacillus acidophilus* and *Lactobacillus amylovorus* strains of interest was examined. It was done to determine the existing antibiotic resistant genes that might be involve in horizontal gene transfer.

In the evaluation of the inhibition zones observed, the antibiotic susceptibility table for *Lactobacillus* strains provided in Charteris et al. (1998b) was used. The results of the test, done with 25 antibiotics, were shown in Table 3.17.

Table 3.17 Antibiotic susceptibility of *Lactobacillus* strains[1]

	L. acidophilus				*L. amylovorus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	A7	A11-H	A11-P	A11-T
Amikacin	R	R	R	R	R	R	R	R	R
Amoxicillin	S	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	S	S	S	S	S	S
Aztreonam	R	R	R	R	R	R	R	R	R
Bacitracin	S	S	S	S	S	S	S	S	S
Cefoxitin	S	S	S	S	S	S	S	S	S
Cephalothin	S	S	S	S	S	S	S	S	S
Cephradine	S	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S
Ciprofloxacin	R	R	R	R	R	R	R	R	R

[1] Results are expressed as R (resistant), MS (moderately susceptible), S (susceptible).

Table 3.17 Antibiotic susceptibility of *Lactobacillus* strains[1] (cont'd)

	L. acidophilus				*L. amylovorus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	A7	A11-H	A11-P	A11-T
Clindamycin	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S
Furazolidone	S	S	S	S	S	S	S	S	S
Fusidic acid	R	R	R	R	R	R	R	R	R
Gentamicin	R	R	R	R	R	R	R	R	R
Kanamycin	R	R	R	R	R	R	R	R	R
Metronidazole	R	R	R	R	R	R	R	R	R
Nitrofurantoin	S	S	S	S	S	S	S	S	S
Polymyxin B	R	R	R	R	R	R	R	R	R
Rifampicin	MS	MS	MS	MS	MS	MS	MS	MS	MS

[1] Results are expressed as R (resistant), MS (moderately susceptible), S (susceptible).

Table 3.17 Antibiotic susceptibility of *Lactobacillus* strains[1] (cont'd)

	L. acidophilus				*L. amylovorus*	Kefir isolates			
	ATCC 4356	KPB4B	LA05	DSM 20079	DSM 20531	A7	A11-H	A11-P	A11-T
Streptomycin	R	R	R	R	R	R	R	R	R
Sulphamethoxozole	R	R	R	R	R	R	R	R	R
Tetracycline	S	S	S	S	S	S	S	S	S
Trimethoprin	R	R	R	R	R	R	R	R	R
Vancomycin	S	S	S	S	S	S	S	S	S

[1] Results are expressed as R (resistant), MS (moderately susceptible), S (susceptible).

All the studied strains showed similar susceptibility to the antibiotics used. No difference was observed between *L. acidophilus* and *L. amylovorus* strains.

The strains of interest showed resistance against only aztreonam from the antibiotic group of inhibitors of cell wall synthesis. They were susceptible to other members of this group which are amoxicillin, ampicillin, cefoxitin, cephalothin, cephradine, vancomycin and bacitracin. They were susceptible to tetracycline, chloramphenicol, erythromycin and clindamycin from the antibiotic group of inhibitors of protein synthesis. However, they showed resistance against aminoglycosides and fusidic acid from this group. The studied aminoglycosides were amikacin, gentamicin, kanamycin and streptomycin. They were all resistant to the examined antibiotics from the group of inhibitors of nucleic acid synthesis including sulphamethoxozole, trimethoprin, ciprofloxacin and metronidazole, but they were only moderately susceptible to rifampicin from this group. They were also showed resistance against polymyxin B which is a member of the group of inhibitor of cytoplasmic membrane. All of the strains were susceptible to nitrofurantoin, an urinary tract antiseptic, and furazolidone, an antimicrobial. All of these findings were in accordance with the results of Charteris et al. (1998b) for *Lactobacillus* strains.

CHAPTER 4

CONCLUSION

Kefir is a probiotic fermented dairy product produced by kefir grains, which contain lactic acid bacteria, acetic acid bacteria and yeasts. The ratios of these microorganisms may change from one geographic region to another and this variation gives regional kefirs their unique characteristics. Kefir helps to balance the ecosystem of gastrointestinal tract by providing probiotic strains.

Select members of the genus *Lactobacillus* is known for their health promoting properties. Most of the researches conducted on lactobacilli involve the selection, identification and development of new strains with novel and functional properties.

In this study, four *Lactobacillus acidophilus* group isolates from Turkish kefir were investigated for their probiotic characteristics after the strain identifications were made by molecular techniques. The idea was to identify novel strains with probiotic properties that can be used as dietary adjuncts and in fermented food products. For identification and differentiation of these *Lactobacillus acidophilus* group strains, PCR was used to amplify approximately the 500 bp region of the 16S rRNA gene, which contained the variable V1 region. The sequences of the V1 region of all the studied strains (*L. acidophilus* ATCC 4356, *L. acidophilus* KPB4B, *L. acidophilus* LA05, *L. acidophilus* DSM 20079, *L. amylovorus* DSM 20531, *L. crispatus* DSM 20584, kefir isolate A7, kefir isolate A11-H, kefir isolate A11-P and kefir isolate A11-T) were aligned with the type strains of *Lactobacillus acidophilus* group, which are *L. acidophilus* ATCC 4356, *L. amylovorus* ATCC 33620, *L. crispatus* ATCC 33820, *L. gallinarium* ATCC 33199, *L. gasseri* ATCC 33323 and *L. johnsonii* ATCC 333200 as per Kullen et al. (2000) to differentiate the strains of interest, indisputably.

All four of the Turkish kefir isolates were identified successfully as *Lactobacillus amylovorus*. Then, they were further investigated for their probiotic characteristics and susceptibility to antibiotics in order to determine their functional properties and decide on whether they are eligible to be used as dietary adjuncts and in fermented dairy products. A known probiotic, the strain LA05, was also included in these tests as a reference. The strains of interest were tested for acid tolerance, bile salt tolerance and cell surface hydrophobicity to determine their ability to survive in gastric juice, colonize in the intestine and adhere to intestinal epithelium, respectively. All these four Turkish kefir isolates showed resistance to acid and bile, but they exhibited low hydrophobicity. Since *in vitro* test results differ from those obtained *in vivo*, and the percent hydrophobicity for a commercial probiotic was also low, adhesion assay with Caco-2 intestinal epithelial cells is suggested for further investigation of their adherence ability.

In the light of all these test results, these four strains of *Lactobacillus acidophilus* group members isolated from Turkish kefir, which were identified as *L. amylovorus*, presented significant properties for survival in and colonization of the human gastrointestinal tract and concluded as promising probiotic strains that can be used commercially as dietary adjuncts and in fermented dairy products. However, prior to their commercial usage, the delivery medium should also be taken into account. Their viability in yogurt has already been investigated by Köse (2011) and the study concluded that they have good survival rates during yogurt shelf life. Even so, these strains should be investigated for their survival in any other fermented dairy product considered to contain these probiotic strains commercially. They should also be examined for their antimicrobial activity since kefir grains comprises not only lactic acid bacteria but also acetic acid bacteria and yeasts. The proteolytic activity of these strains should be determined and also sensory analysis should be done since it may alter the flavor, texture and quality of the kefir. Furthermore, these potential probiotic strains should be investigated for their health benefits especially for cholesterol-lowering and body adiposity decreasing properties that mainly associated with *Lactobacillus amylovorus* strains, before used as commercial probiotic strains.

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APPENDIX A

CHEMICALS AND ENZYMES

Table A.1 Chemicals and ezymes with their suppliers

Chemicals	Suppliers
Agar	Merck, Germany
Bromocresol purple	Sigma, USA
Diammonium hydrogen citrate	Applichem, Germany
Dihydrogen potassium phosphate	Applichem, Germany
Dipotassium hydrogen phosphate	Applichem, Germany
DNA loading dye	Fermentas, Lithuania
EDTA	Merck, Germany
Ethanol	Merck, Germany
Fructose	Merck, Germany
Galactose	Merck, Germany
GelRed™	Olerup SSP, Sweden
Glacial acetic acid	Merck, Germany
Glucose	Merck, Germany
Hydrochloric acid	Merck, Germany
Hydrogen Peroxide	Merck, Germany
Lactose	Sigma,USA
Lysozyme	Fermentas, Lithuania

Table A.1. Chemicals and ezymes with their suppliers (cont'd)

Chemicals	Suppliers
Magnesium sulphate	Applichem, Germany
Maltose	Merck, Germany
Manganese sulphate	Applichem, Germany
Mannose	Merck, Germany
Mastermix	Fermentas, Lithuania
Melibiose	Sigma, USA
Mineral Oil	Sigma, USA
MRS Agar	Merck, Germany
MRS Broth	Merck, Germany
n-hexadecane	Merck, Germany
Oxgall	BD, USA
Peptone water	Merck, Germany
Proteinase K	Fermentas, Lithuania
Raffinose	Sigma, USA
Saccharose	Merck, Germany
Sodium acetate	Sigma, USA
Trehalose	Applichem, Germany
Tris(hydroxymethyl)aminomethane	Sigma, USA
Tris-HCI	Sigma, USA
Triton X-100	Sigma, USA
Tryptone	Merck, Germany
Tween 80	Merck, Germany
Yeast Extract	Merck, Germany
1000 bp DNA ladder	Fermentas, Lithuania

APPENDIX B

MICROBIOLOGICAL GROWTH MEDIA AND BUFFERS

B.1. MRS Agar (per liter)

68.2 g MRS agar is dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 minutes.

B.2. MRS Broth (per liter)

52.2 g MRS broth is dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 minutes.

B.3. Basal MRS Broth (per liter)

Basal MRS broth is prepared without meat extract and glucose as in Table B.1 and autoclaved at 121°C for 15 minutes.

Table B.1 Basal MRS broth

Ingredients	Amount (g/L)
Tryptone	10
Yeast extract	5
Tween 80	1
Dipotassium hydrogen phosphate	2.6
Sodium acetate	5
Magnesium sulphate	0.2
Manganese sulphate	0.05

B.4. Modified MRS Broth (per liter)

Modified MRS broth is prepared by adding bromocresol purple to basal MRS broth.

Table B.2 Modified MRS broth

Ingredients	Amount (g/L)
Tryptone	10
Yeast extract	5
Tween 80	1
Dipotassium hydrogen phosphate	2.6
Sodium acetate	5
Magnesium sulphate	0.2
Manganese sulphate	0.05
Bromocresol purple	0.04

B.5. Peptone Water (per liter)

25.5 g peptone water is dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 minutes.

B.6. Potassium Phosphate Buffer (pH 6.6)

38.1 ml of 1 M dipotassium hydrogen phosphate (K_2HPO_4) solution is mixed ith 61.9 ml of 1 M dihydrogen potassium phosphate (KH_2PO_4) solution to get 100 ml of 1 M potassium phosphate buffer. Then, it is diluted to 50 mM.

B.7. TAE (Tris-Acetate-EDTA) Buffer (pH 8.0)

10x TAE buffer is prepared from 48.5 g Tris, 11.4 ml glacial acetic acid and 20 ml 0.5 M EDTA solution. It is diluted 10:1 to make 1x working solution.

APPENDIX C

QUANTITATION OF DNA

Table C.1 Absorbance ratio and concentration of isolated DNA samples

Strains	A_{260}/A_{280}				Concentration (ng/µl)			
	Measurements			Avg.	Measurements			Avg.
ATCC 4356	2.06	2.00	2.04	2.04	553.03	551.74	571.47	558.75
KPB4B	2.03	1.95	1.98	1.99	636.47	617.00	638.45	630.64
LA05	2.02	2.04	2.02	2.03	530.82	545.76	537.98	538.19
DSM 20079	1.86	1.82	1.86	1.85	716.73	712.27	735.73	721.58
DSM 20531	1.76	1.76	1.87	1.80	694.67	702.08	680.47	692.41
DSM 20584	1.80	1.88	1.88	1.85	742.31	734.92	738.53	738.59
A7	1.95	1.76	1.72	1.81	632.03	581.77	605.22	606.34
A11-H	1.94	1.91	1.89	1.91	728.50	743.40	736.51	736.14
A11-P	1.99	1.97	1.96	1.97	665.46	672.18	674.82	670.82
A11-T	1.84	1.83	1.80	1.82	632.89	633.76	619.25	628.63

APPENDIX D

MULTIPLE SEQUENCE ALIGNMENTS (MSAs)

MSAs were done with Clustal Omega program (1.2.1). Nucleotides which are identical for a particular position were aligned with * (asterisk).

D.1. MSA of the 500 bp Region of the 16S rRNA Gene

Figure D.1 MSA of the 500 bp region of the 16S rRNA gene of *Lactobacillus* strains. DSM 20079: *L. acidophilus* (T), ATCC 4356: *L. acidophilus* (T), KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, DSM 20531: *L. amylovorus* (T), A7: Kefir isolate, A11-H: Kefir isolate, A1-P: Kefir isolate, A11-T: Kefir isolate and DSM 20584: *L. crispatus* (T).

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DSM-20079       TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAA     180
ATCC-4356       TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAA     165
KPB4B           TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAA     155
LA05            TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAA     157
DSM-20531       TGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAA     178
A7              TGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAA     156
A11-H           TGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAA     165
A11-P           TGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAA     180
A11-T           TGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAA     168
DSM-20584       TGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAA     179
                ************************** ************** ******************

DSM-20079       TACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTA     240
ATCC-4356       TACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTA     225
KPB4B           TACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTA     215
LA05            TACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTA     217
DSM-20531       TACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA     238
A7              TACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA     216
A11-H           TACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA     225
A11-P           TACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA     240
A11-T           TACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA     228
DSM-20584       TACCGGATAAGAAAGCAGATCGCATGATCAGCTTTTAAAAGGCGGCGTAAGCTGTCGCTA     239
                ********** ********************** * ***********************

DSM-20079       TGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGAT     300
ATCC-4356       TGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGAT     285
KPB4B           TGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGAT     275
LA05            TGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGAT     277
DSM-20531       AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGAT     298
A7              AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGAT     276
A11-H           AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGAT     285
A11-P           AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGAT     300
A11-T           AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGAT     288
DSM-20584       TGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAAAGGCTTACCAAGGCGATGAT     299
                 ******************************** ***** *** ********* * ***

DSM-20079       GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     360
ATCC-4356       GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     345
KPB4B           GCATAGCCGAGTTGAGAGACTGAC-GGCCACATTGGGACTGAGACACGGCCCAAACTCCT     334
LA05            GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     337
DSM-20531       GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     358
A7              GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     336
A11-H           GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     345
A11-P           GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     360
A11-T           GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     348
DSM-20584       GCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT     359
                ********************** ***********************************
```

Figure D.1 MSA of the 500 bp region of the 16S rRNA gene of *Lactobacillus* strains. DSM 20079: *L. acidophilus* (T), ATCC 4356: *L. acidophilus* (T), KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, DSM 20531: *L. amylovorus* (T), A7: Kefir isolate, A11-H: Kefir isolate, A1-P: Kefir isolate, A11-T: Kefir isolate and DSM 20584: *L. crispatus* (T). (cont'd)

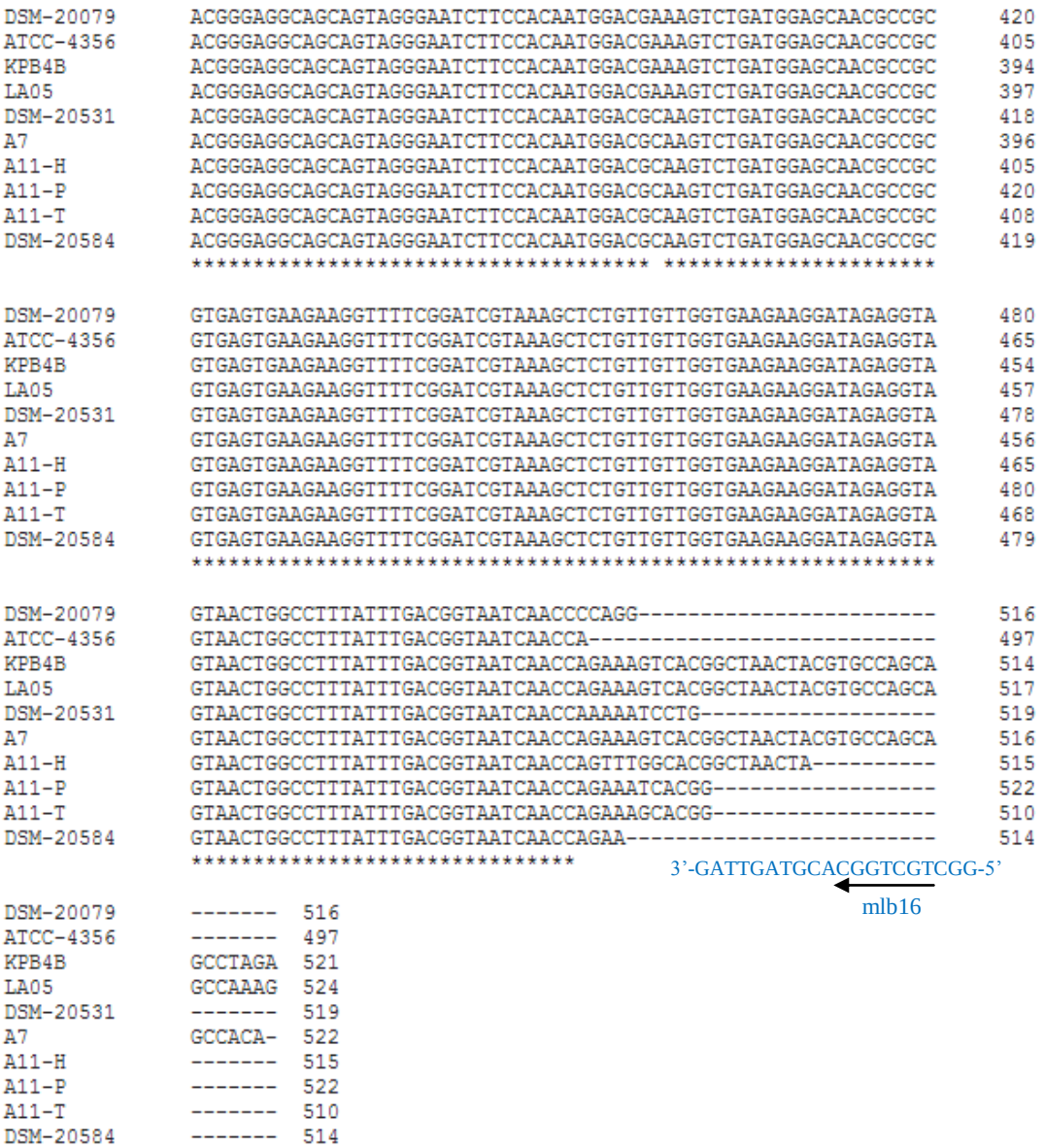

Figure D.1 MSA of the 500 bp region of the 16S rRNA gene of *Lactobacillus* strains. DSM 20079: *L. acidophilus* (T), ATCC 4356: *L. acidophilus* (T), KPB4B: *L. acidophilus*, LA05: *L. acidophilus*, DSM 20531: *L. amylovorus* (T), A7: Kefir isolate, A11-H: Kefir isolate, A1-P: Kefir isolate, A11-T: Kefir isolate and DSM 20584: *L. crispatus* (T). (cont'd)

D.2. MSA of the V1 Region of the 16S rRNA Gene

Clustal Omega program (1.2.1) output of multiple sequence alignment of the V1 region of the 16S rRNA gene of: *L. acidophilus* DSM 20079 (type strain), *L. acidophilus* ATCC 4356, *L. acidophilus* KPB4B, *L. acidophilus* LA05, *L. amylovorus* DSM 20531 (type strain), kefir isolate A7, kefir isolate A11-H, kefir isolate A1-P, kefir isolate A11-T and *L. crispatus* DSM 20584 (type strain).

The sequences denoted with " (quotation mark) are adapted from Kullen et al. (2000): *L. acidophilus* ATCC 4356, *L. amylovorus* ATCC 33620, *L. crispatus* ATCC 33820, *L. gallinarum* ATCC 33199, *L. gasseri* ATCC 33323 and *L. johnsonii* ATCC 333200.

```
"ATCC-4356        GAGCGAGCTGAACCAACAGATTCACTTCGGTGATG------ACGTTGG--GAACGCGAGC  52
DSM-20079         GAGCGAGCTGAACCAACAGATTCACTTCGGTGATG------ACGTTGG--GAACGCGAGC  52
ATCC-4356         GAGCGAGCTGAACCAACAGATTCACTTCGGTGATG------ACGTTGG--GAACGCGAGC  52
KPB4B             GAGCGAGCTGAACCAACAGATTCACTTCGGTGATG------ACGTTGG--GAACGCGAGC  52
LA05              GAGCGAGCTGAACCAACAGATTCACTTCGGTGATG------ACGTTGG--GAACGCGAGC  52
DSM-20531         GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
"ATCC-33620       GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
A7                GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
A11-H             GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
A11-P             GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
A11-T             GAGCGAGCGGAACCAACAGATTTACTTCGGTAATG------ACGTTGG--GAAAGCGAGC  52
DSM-20584         GAGCGAGCGGAACTAACAGATTTACTTCGGTAATG------ACGTTAG--GAAAGCGAGC  52
"ATCC-33820       GAGCGAGCGGAACTAACAGATTTACTTCGGTAATG------ACGTTAG--GAAAGCGAGC  52
"ATCC-33199       GAGCGAGCAGAACCAGCAGATTTACTTCGGTAATG------ACGCTGG--GGACGCGAGC  52
"ATCC-33323       GAGCGAGCTTGCCTAGATGAATTTGGTGCTTGCACCAGATGAAACTAGATACAAGCGATC  60
"ATCC-333200      GAGCGAGCTTGCCTAGATGATTTTAGTGCTTGCACTAAATGAAACTAGATACAAGCGAGC  60
                  ********    * *   ** *    *   *          *   * *    * **** *
```

Figure D.2 MSA of the V1 region of the 16S rRNA gene of *Lactobacillus* strains

APPENDIX E

BLAST ANALYSES OF KEFIR ISOLATES

E.1. BLAST Analysis of the Strain A7

Lactobacillus acidophilus ATCC 4356 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AF429493.1| Length: 504 Number of Matches: 1

Range 1: 10 to 500 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
804 bits(435)	0.0	475/494(96%)	3/494(0%)	Plus/Plus

```
Query  11   ggCGGTGCCTAATACATGGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATG  70
            ||| ||||||||||||| ||||||||||||||| ||||||||||||| |||||||| |||
Sbjct  10   GGC-GTGCCTAATACAT-GCAAGTCGAGCGAGCTGAACCAACAGATTCACTTCGGTGATG  67

Query  71   ACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGCAACCTGCCCCTAAGTC  130
            |||||||||| ||||||||||||||||||||||||||||||| |||||||||  ||||
Sbjct  68   ACGTTGGGAACGCGAGCGGCGGATGGGTGAGTAACACGTGGGG-AACCTGCCCCATAGTC  126

Query  131  TGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGC  190
            |||||||||| |||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  127  TGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGC  186

Query  191  TTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  250
            || | ||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  187  TTATAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  246

Query  251  TAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  310
            || ||||||||| ||||||||| | ||||||||||||||||||||||||||||||||||
Sbjct  247  TAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  306

Query  311  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  366

Query  371  GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  430
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  GGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  426

Query  431  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  486

Query  491  GAAAGTCACGGCTA  504
            ||||||||||||||
Sbjct  487  GAAAGTCACGGCTA  500
```

Figure E.1 BLAST analysis of the strain A7 with *L. acidophilus* (T)

Lactobacillus amylovorus partial 16S rRNA gene, type strain DSM 20531T

Sequence ID: emb|FR683089.1| Length: 1516 Number of Matches: 1

Range 1: 6 to 504 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
894 bits(484)	0.0	497/502(99%)	5/502(0%)	Plus/Plus

```
Query  11   ggCGGTGCCTAATACATGGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATG  70
            ||| ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    GGC-GTGCCTAATACAT-GCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATG  63

Query  71   ACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGCAACCTGCCCCTAAGTC  130
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  64   ACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGG-AACCTGCCCCTAAGTC  122

Query  131  TGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGC  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  TGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGC  182

Query  191  TTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  TTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  242

Query  251  TAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  TAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  302

Query  311  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  362

Query  371  GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  422

Query  431  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  482

Query  491  GAAAGTCACGGCTA-CT-CGTG  510
            |||||||||||||| || ||||
Sbjct  483  GAAAGTCACGGCTAACTACGTG  504
```

Figure E.2 BLAST analysis of the strain A7 with *L. amylovorus* (T)

Lactobacillus crispatus strain ATCC 33820 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_041800.1| Length: 1518 Number of Matches: 1

Range 1: 14 to 512 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
839 bits(454)	0.0	487/502(97%)	5/502(0%)	Plus/Plus

```
Query  11   ggCGGTGCCTAATACATGGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTAATG  70
            ||| ||||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  14   GGC-GTGCCTAATACAT-GCAAGTCGAGCGAGCGGAACTAACAGATTTACTTCGGTAATG  71

Query  71   ACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGCAACCTGCCCCTAAGTC  130
            ||||| ||||||||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct  72   ACGTTAGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGG-AACCTGCCCCATAGTC  130

Query  131  TGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGC  190
            |||||||||| |||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  131  TGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGC  190

Query  191  TTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  250
            |||| |||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  191  TTTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGG  250

Query  251  TAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  310
            |||||||| ||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  251  TAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACA  310

Query  311  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT  370

Query  371  GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT  430

Query  431  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  CTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCA  490

Query  491  GAAAGTCACGGCTA-CT-CGTG  510
            ||||||||||||||| || ||||
Sbjct  491  GAAAGTCACGGCTAACTACGTG  512
```

Figure E.3 BLAST analysis of the strain A7 with *L. crispatus* (T)

E.2. BLAST Analysis of the Strain A11-H

Lactobacillus acidophilus ATCC 4356 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AF429493.1| Length: 504 Number of Matches: 1

Range 1: 1 to 482 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
782 bits(423)	0.0	463/482(96%)	3/482(0%)	Plus/Plus

```
Query  12   AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTC  71
            ||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |||||
Sbjct  1    AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAACAGATTCACTTC  60

Query  72   GGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  131
            ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGTGATGACGTTGGGAACGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  120

Query  132  TAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATG  191
              ||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||
Sbjct  121  ATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATG  180

Query  192  ATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCT  251
            |||||||| | ||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  181  ATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCT  240

Query  252  AGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCG  311
            |||||||| ||||||||| ||||||||| | ||||||||||||||||||||||||||||||
Sbjct  241  AGTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCG  300

Query  312  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  371
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  360

Query  372  CACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  431
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  420

Query  432  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTT-AT--GACGGTAAT  488
            |||||||||||||||||||||||||||||||||||||||||||||| ||  |||||||||
Sbjct  421  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAAT  480

Query  489  CA  490
            ||
Sbjct  481  CA  482
```

Figure E.4 BLAST analysis of the strain A11-H with *L. acidophilus* (T)

Lactobacillus amylovorus partial 16S rRNA gene, type strain DSM 20531T

Sequence ID: emb|FR683089.1| **Length:** 1516 **Number of Matches:** 1

Range 1: 1 to 478 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
863 bits(467)	0.0	475/478(99%)	3/478(0%)	Plus/Plus

```
Query  16   CTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTA  75
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGGTA  60

Query  76   ATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAG  135
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAG  120

Query  136  TCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCA  195
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCA  180

Query  196  GCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTT  255
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCTAGTT  240

Query  256  GGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCA  315
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCGGCCA  300

Query  316  CATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA  375
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA  360

Query  376  ATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAG  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAG  420

Query  436  CTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTT-AT--GACGGTAATCA  490
            ||||||||||||||||||||||||||||||||||||||||||| ||  ||||||||||
Sbjct  421  CTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCA  478
```

Figure E.5 BLAST analysis of the strain A11-H with *L. amylovorus* (T)

Lactobacillus crispatus strain ATCC 33820 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_041800.1| Length: 1518 Number of Matches: 1

Range 1: 1 to 486 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
822 bits(445)	0.0	473/486(97%)	3/486(0%)	Plus/Plus

```
Query  8    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTA  67
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACTAACAGATTTA  60

Query  68   CTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  127
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTTCGGTAATGACGTTAGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  120

Query  128  CCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCG  187
            ||||  |||||||||||||| ||||||||||||||||||||||||||| ||||||||||||
Sbjct  121  CCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCG  180

Query  188  CATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATT  247
            |||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  181  CATGATCAGCTTTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATT  240

Query  248  AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTG  307
            |||||||||||||||||| ||||||||||||| |||||||||||||||||||||||||||
Sbjct  241  AGCTAGTTGGTAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTG  300

Query  308  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  360

Query  368  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  420

Query  428  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTT-AT--GACGG  484
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||  |||||
Sbjct  421  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGG  480

Query  485  TAATCA  490
            ||||||
Sbjct  481  TAATCA  486
```

Figure E.6 BLAST analysis of the strain A11-H with *L. crispatus* (T)

E.3. BLAST Analysis of the Strain A1-P

Lactobacillus acidophilus ATCC 4356 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AF429493.1| Length: 504 Number of Matches: 1

Range 1: 1 to 497 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
819 bits(443)	0.0	480/498(96%)	2/498(0%)	Plus/Plus

```
Query  27   AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTC  86
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||| |||||
Sbjct  1    AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAACAGATTCACTTC  60

Query  87   GGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  146
            ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGTGATGACGTTGGGAACGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  120

Query  147  TAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATG  206
               ||||||||||||||| |||||||||||||||||||||| ||||||||||||||||
Sbjct  121  ATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATG  180

Query  207  ATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCT  266
            |||||||| | |||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  181  ATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCT  240

Query  267  AGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCG  326
            |||||||| ||||||||| ||||||||| | ||||||||||||||||||||||||||||||
Sbjct  241  AGTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCG  300

Query  327  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  386
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  360

Query  387  CACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  446
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  420

Query  447  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTA-TTGACGGTAAT  505
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  421  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAAT  480

Query  506  CAACCAAGAAAGTCACGG  523
            |||||| ||||||||||
Sbjct  481  CAACCA-GAAAGTCACGG  497
```

Figure E.7 BLAST analysis of the strain A11-P with *L. acidophilus* (T)

Lactobacillus amylovorus strain DSM 20531 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_043287.1| Length: 1504 Number of Matches: 1

Range 1: 17 to 521 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
922 bits(499)	0.0	504/506(99%)	2/506(0%)	Plus/Plus

```
Query  19   TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGA  78
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17   TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGA  76

Query  79   TTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAA  138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAA  136

Query  139  CCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAG  198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  CCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAG  196

Query  199  ATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTG  258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  ATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTG  256

Query  259  CATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAG  318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  CATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAG  316

Query  319  ACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG  378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  ACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG  376

Query  379  GAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTT  438
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  GAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTT  436

Query  439  CGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTA-TTG  497
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||| |||
Sbjct  437  CGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTG  496

Query  498  ACGGTAATCAACCAAGAAAGTCACGG  523
            |||||||||||||| ||||||||||
Sbjct  497  ACGGTAATCAACCA-GAAAGTCACGG  521
```

Figure E.8 BLAST analysis of the strain A11-P with *L. amylovorus* (T)

Lactobacillus crispatus strain ATCC 33820 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_041800.1| **Length:** 1518 **Number of Matches:** 1

Lactobacillus crispatus strain ATCC33820 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AF257097.1|

Range 1: 1 to 501 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
859 bits(465)	0.0	490/502(98%)	2/502(0%)	Plus/Plus

```
Query  23   GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTA  82
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACTAACAGATTTA  60

Query  83   CTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  142
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTTCGGTAATGACGTTAGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  120

Query  143  CCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCG  202
            ||||  |||||||||||||| |||||||||||||||||||||||| ||||||||||||
Sbjct  121  CCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCG  180

Query  203  CATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATT  262
            ||||||||||||||| |||||||||||||||||||| |||||||||||||||||||||||
Sbjct  181  CATGATCAGCTTTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATT  240

Query  263  AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTG  322
            |||||||||||||||||| |||||||||||||| |||||||||||||||||||||||||||
Sbjct  241  AGCTAGTTGGTAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTG  300

Query  323  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  382
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  360

Query  383  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  442
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  420

Query  443  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTA-TTGACGG  501
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  421  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGG  480

Query  502  TAATCAACCAAGAAAGTCACGG  523
            |||||||||| ||||||||||
Sbjct  481  TAATCAACCA-GAAAGTCACGG  501
```

Figure E.9 BLAST analysis of the strain A11-P with *L. crispatus* (T)

E.4. BLAST Analysis of the Strain A11-T

Lactobacillus acidophilus ATCC 4356 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AF429493.1| Length: 504 Number of Matches: 1

Range 1: 1 to 495 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
813 bits(440)	0.0	477/495(96%)	2/495(0%)	Plus/Plus

```
Query  14   AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTC  73
            ||||||||||||||||||||||||||||||||||||||||| ||||||||||||| |||||
Sbjct  1    AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAACAGATTCACTTC  60

Query  74   GGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  133
            ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGTGATGACGTTGGGAACGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCC  120

Query  134  TAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATG  193
              ||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||
Sbjct  121  ATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATG  180

Query  194  ATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATTAGCT  253
            |||||||| | ||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  181  ATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCT  240

Query  254  AGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTGATCG  313
            |||||||| |||||||| ||||||||| | ||||||||||||||||||||||||||||||
Sbjct  241  AGTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCG  300

Query  314  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC  360

Query  374  CACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  433
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT  420

Query  434  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGT-AT  492
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  421  AAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAAT  480

Query  493  CAACCAGAAAG-CAC  506
            ||||||||||| |||
Sbjct  481  CAACCAGAAAGTCAC  495
```

Figure E.10 BLAST analysis of the strain A11-T with *L. acidophilus* (T)

Lactobacillus amylovorus strain DSM 20531 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_043287.1| Length: 1504 Number of Matches: 1

Range 1: 17 to 519 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	501/503(99%)	2/503(0%)	Plus/Plus

```
Query  6    TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGA  65
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17   TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGA  76

Query  66   TTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAA  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TTTACTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAA  136

Query  126  CCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAG  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  CCTGCCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAG  196

Query  186  ATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTG  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  ATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTG  256

Query  246  CATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAG  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  CATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAG  316

Query  306  ACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  ACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG  376

Query  366  GAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTT  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  GAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTT  436

Query  426  CGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTG  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  CGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTG  496

Query  486  ACGGT-ATCAACCAGAAAG-CAC  506
            ||||| |||||||||||||| |||
Sbjct  497  ACGGTAATCAACCAGAAAGTCAC  519
```

Figure E.11 BLAST analysis of the strain A11-T with *L. amylovorus* (T)

Lactobacillus crispatus strain ATCC 33820 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_041800.1| Length: 1518 Number of Matches: 1

Range 1: 1 to 499 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
854 bits(462)	0.0	487/499(98%)	2/499(0%)	Plus/Plus

```
Query  10   GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTA  69
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACTAACAGATTTA  60

Query  70   CTTCGGTAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  129
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTTCGGTAATGACGTTAGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTG  120

Query  130  CCCCTAAGTCTGGGATACCATTTGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCG  189
            ||||  |||||||||||||| |||||||||||||||||||||||| ||||||||||||
Sbjct  121  CCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCG  180

Query  190  CATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTAAGGGATGGCCCCGCGGTGCATT  249
            ||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  181  CATGATCAGCTTTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATT  240

Query  250  AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGAGACTG  309
            |||||||||||||||||| ||||||||||||||| ||||||||||||||||||||||||||
Sbjct  241  AGCTAGTTGGTAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTG  300

Query  310  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT  360

Query  370  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA  420

Query  430  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGG  489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGG  480

Query  490  T-ATCAACCAGAAAG-CAC  506
            | |||||||||||| ||| 
Sbjct  481  TAATCAACCAGAAAGTCAC  499
```

Figure E.12 BLAST analysis of the strain A11-T with *L. crispatus* (T)

E.5 BLAST Analysis of the V1 Region of the 16S rRNA Gene of the Turkish Kefir Isolates

The BLAST analysis was done for the V1 region of the 16S rRNA gene of the kefir isolates by using the gene bank database of NCBI. The results shown in Figure E.13 applies for all four kefir isolates, the strains A7, A11-H, A11-P and A11-T, since they had the same sequences for the V1 region.

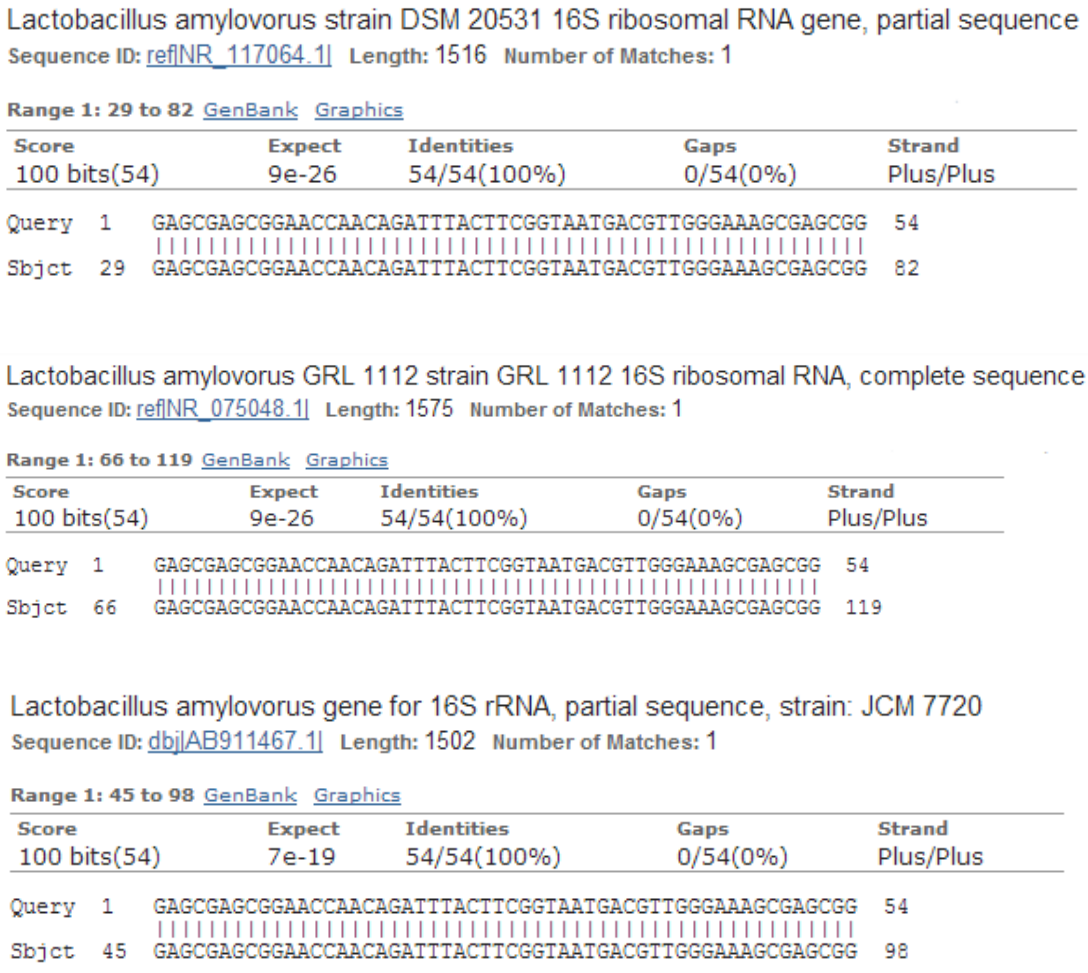

Figure E.13 BLAST analyses of the V1 region of the 16S rRNA gene of the Turkish kefir isolates

Lactobacillus amylovorus gene for 16S rRNA, partial sequence, strain: JCM 7722
Sequence ID: dbj|AB911468.1| Length: 1503 Number of Matches: 1

Range 1: 46 to 99 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
100 bits(54)	7e-19	54/54(100%)	0/54(0%)	Plus/Plus

```
Query  1   GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  54
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  46  GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  99
```

Lactobacillus amylovorus strain FQ103 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|KF418830.1| Length: 1433 Number of Matches: 1

Range 1: 12 to 65 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
100 bits(54)	7e-19	54/54(100%)	0/54(0%)	Plus/Plus

```
Query  1   GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  54
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12  GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  65
```

Lactobacillus amylovorus strain TCP071 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|KF312687.1| Length: 1437 Number of Matches: 1

Range 1: 12 to 65 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
100 bits(54)	7e-19	54/54(100%)	0/54(0%)	Plus/Plus

```
Query  1   GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  54
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12  GAGCGAGCGGAACCAACAGATTTACTTCGGTAATGACGTTGGGAAAGCGAGCGG  65
```

Figure E.13 BLAST analyses of the V1 region of the 16S rRNA gene of the Turkish kefir isolates (cont'd)

APPENDIX F

TOTAL VIABLE CELL COUNT DATA OF ACID TOLERANCE AND BILE SALT TOLERANCE TESTS

F.1. Total Viable Cell Count Data of Acid Tolerance Test

Table F.1 Total viable cell count at pH 1.5

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
A7	9.07	5.72	5.06	4.28	3.98
	9.16	6.03	5.17	4.49	4.12
	9.14	5.74	5.15	4.37	4.02
A11-H	9.16	6.82	6.66	6.26	6.06
	9.07	6.73	6.53	7.67	8.82
	9.10	6.74	6.62	6.15	5.88
A11-P	9.03	6.11	5.59	5.31	4.57
	9.09	6.12	5.68	5.35	4.63
	9.10	6.14	5.74	5.36	4.64
A11-T	9.06	6.34	5.86	5.27	4.60
	9.11	6.42	5.94	5.34	4.67
	9.08	6.38	5.91	5.32	4.66
LA05	8.93	7.39	6.96	6.72	6.18
	8.96	7.43	7.01	6.76	6.21
	8.95	7.42	6.98	6.74	6.16

Table F.2 Total viable cell count at pH 2.0

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
	9.12	7.48	7.12	6.34	6.30
A7	9.07	7.97	7.09	6.45	6.34
	9.02	7.24	7.07	6.44	6.14
	9.09	7.98	7.57	7.38	7.03
A11-H	9.14	8.06	7.67	7.49	7.18
	9.04	8.04	7.64	7.47	7.14
	9.04	7.29	6.82	6.12	5.20
A11-P	9.08	7.31	6.89	6.19	5.29
	9.08	7.38	6.94	6.21	5.30
	9.02	7.39	6.66	5.94	4.93
A11-T	9.03	7.41	6.68	5.98	4.96
	9.06	7.45	6.72	6.02	5.01
	8.96	8.20	7.99	7.76	7.54
LA05	8.94	8.18	7.95	7.74	7.52
	8.92	8.14	7.94	7.70	7.48

Table F.3 Total viable cell count at pH 2.5

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
	9.04	8.46	8.24	8.21	7.96
A7	9.01	8.53	8.39	8.26	8.03
	9.03	8.47	8.33	8.23	8.09
	9.01	8.78	8.74	8.47	8.31
A11-H	9.09	8.87	8.82	8.67	8.49
	9.02	8.79	8.77	8.53	8.41
	9.04	7.96	7.39	7.26	6.96
A11-P	9.05	8.04	7.44	7.28	6.98
	9.08	8.06	7.46	7.32	7.01
	9.07	8.38	7.71	7.44	7.09
A11-T	9.04	8.33	7.68	7.42	7.08
	9.01	8.26	7.65	7.41	7.05
	8.97	8.85	8.81	8.67	8.64
LA05	8.94	8.18	7.95	7.74	7.52
	8.92	8.79	8.77	8.63	8.61

F.2. Total Viable Cell Count Data of Bile Salt Tolerance Test

Table F.4 Total viable cell count at bile concentration 0.1% (w/v)

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
	7.06	6.68	6.55	6.48	6.39
A7	7.09	6.72	6.62	6.52	6.47
	7.14	6.75	6.67	6.62	6.51
	7.03	6.94	6.89	6.88	6.81
A11-H	7.08	7.01	6.95	6.93	6.86
	7.10	7.03	6.96	6.94	6.89
	7.08	7.02	6.98	6.95	6.90
A11-P	7.10	7.05	7.00	6.96	6.93
	7.11	7.06	7.04	6.96	6.92
	7.08	7.00	6.90	6.62	6.48
A11-T	7.09	7.03	6.94	6.68	6.53
	7.12	7.04	6.96	6.69	6.53
	6.90	6.86	6.84	6.80	6.77
LA05	6.92	6.88	6.86	6.84	6.78
	6.93	6.88	6.87	6.85	6.81

Table F.5 Total viable cell count at bile concentration 0.3% (w/v)

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
	7.02	6.25	6.08	5.92	5.81
A7	7.04	6.27	6.12	5.95	5.84
	6.98	6.24	6.07	5.90	5.79
	7.00	6.80	6.73	6.69	6.56
A11-H	7.05	6.83	6.77	6.71	6.64
	7.08	6.86	6.79	6.76	6.64
	6.96	6.70	6.54	6.47	6.41
A11-P	7.00	6.78	6.62	6.54	6.51
	7.06	6.17	5.64	5.55	5.42
	7.00	6.80	6.72	6.48	6.30
A11-T	7.01	6.83	6.76	6.54	6.34
	7.03	6.87	6.77	6.56	6.35
	6.97	6.79	6.77	6.70	6.63
LA05	7.00	6.82	6.80	6.72	6.64
	7.01	6.83	6.80	6.73	6.65

Table F.6 Total viable cell count at bile concentration 0.5% (w/v)

	$\log_{10}$ cfu/ml				
	t = 0	t = 60	t = 90	t = 120	t = 180
A7	7.04	5.66	5.55	5.31	5.04
	7.08	5.72	5.59	5.35	5.10
	7.10	5.78	5.64	5.38	5.14
A11-H	6.99	6.31	6.13	6.09	5.91
	7.03	6.35	6.19	6.12	5.96
	7.05	6.37	6.20	6.13	6.03
A11-P	7.03	6.11	5.59	5.51	5.38
	7.04	6.15	5.64	5.54	5.41
	7.06	6.17	5.64	5.55	5.42
A11-T	7.05	6.37	6.26	5.44	5.01
	7.03	6.34	6.23	5.41	4.98
	7.02	6.30	6.23	5.40	4.96
LA05	6.92	6.48	6.24	6.12	5.97
	6.87	6.39	6.17	6.09	5.91
	6.89	6.47	6.20	6.11	5.92