ISOLATION, CHARACTERIZATION, DETERMINATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA FROM HUMAN MILK

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ABSTRACT

ISOLATION, CHARACTERIZATION, DETERMINATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA FROM HUMAN MILK

Probiotics mean live microorganisms that have beneficial effects on their host's health. Although probiotic strains can be isolated from many sources; for human applications the main criteria is being human origin.

Breast milk is an important nutrient source for neonates. Lots of studies showed that this fluid has beneficial effects on the health of neonates. One reason of being beneficial is explaining by the microflora of human breast milk including beneficial lactic acid bacteria.

In this study, isolates were identified by biochemical and molecular characterization and also probiotic properties of lactic acid bacteria, isolated from human milk were investigated.

Three of the isolates were observed as potential probiotic. Two of them are bacilli and the other is cocci. These isolates showed resistance to stomach pH (pH 3,0), tolerance against 0,3% bile concentration and antimicrobial activity against *Salmonella thyphimurium* CCM 5445, *Escherichia coli* O157:H7 NCTC 129000 and *Escherichia coli* NRRL B-3008. After investigation the probiotic properties of these isolates, they were identified by biochemical characterization techniques and molecular identification by using amplification and restriction fragment length polymorphism (RFLP) of 16S ribosomal DNA (rDNA) and 16S sequencing. Two lactobacilli were identified as *Lactobacillus oris* and *Lactobacillus fermentum*. In the light of this study, it is observed that, human milk is a source of potential probiotic strains.

ÖZET

ANNE SÜTÜNDEKİ LAKTİK ASİT BAKTERİLERİNİN İZOLASYONU, KARAKTERİZASYONU VE PROBİYOTİK ÖZELLİKLERİNİN BELİRLENMESİ

Konakçı sağlığı üzerinde olumlu etkiler gösteren canlı mikroorganizmalar Probiyotik olarak adlandırılırlar. Probiyotik suşlar birçok kaynaktan izole edilebilmesine rağmen insan beslenmesinde kullanılacak olan suşların insan kaynaklı olması gerekmektedir.

Anne sütü yeni doğmuş bebekler için önemli bir gıda kaynağıdır. Birçok çalışma bu sıvının yeni doğan bebeklerin sağlığı üzerinde birçok olumlu etki yarattığını göstermektedir. Bu olumlu etkilere neden olan etkenlerden birisi de anne sütünün içeriğinde bulunan sağlığa yararlı laktik asit bakterileridir.

Bu çalışmada anne sütünden izole edilen laktik asit bakterilerinin probiyotik özellikleri taranmış ve aynı zamanda bu izolatların biyokimyasal ve moleküler düzeyde tanımlaması yapılmıştır.

İzole edilen bakterilerden üç tanesi probiyotik özellik göstermektedir. Bunlardan iki tanesi basil diğeri ise kok olarak gözlemlenmiştir. Bu izolatlar, mide pH sına (pH 3,0) direnç, bağırsak içerisindeki safra tuzuna (0,3%) tolerans ve aynı zamanda *Salmonella thyphimurium* CCM 5445, *Escherichia coli* O157:H7 NCTC 129000 and *Escherichia coli* NRRL B-3008 indikatör mikroorganizmalara karşı da antimikrobiyel aktivite göstermişlerdir. Probiyotik özellik gösteren suşlar hem biyokimyasal olarak hem de ribosomal RNA genlerinin 16S bölümünün amplifikasyonu ve RFLP'lerinin (Restriction Fragment Length Polymorphism) karşılaştırılmasına dayalı olarak moleküler düzeyde tanımlanmışlardır. Ayrıca 16S dizi anlaizi ile de bu sonuçlar doğrulanmıştır. Çalışma sonunda izole edilen iki laktobasil suşunun potensiyel probiyotik kültürü olarak kullanılabileceği gözlenmiş ve bu iki suş *Lactobacillus oris* ve *Lactobacillus fermentum* olarak tanımlanmıştır.

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spp., subsp.	: Subspecies
FAO/WHO	: Food and Agriculture Organization/World Health Organization
LAB	: Lactic Acid Bacteria
TD	: Traveller's diarrhea
ADD	: Antibiotic associated diarrhea
L. Lb.	: Lactobacillus
B.	: Bifidobacterium
E.	: Enterococcus
PCR	: Polymerase Chain Reaction
RFLP	: Restriction Fragment Length Polymorphism
ARDRA	: Amplified Ribosomal Restriction Analysis
RAPD	: Randomly Amplified Polymorphic DNA
DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
rDNA	: Ribosomal DNA
rRNA	: Ribosomal RNA
ITS	: Internal Transcribed Spacer
MRS	: de Man, Rogosa and Sharpe Medium
TPY	: Typticase Phytone Yeast
v/v	: volume/volume
w/v	: weight/volume
rpm	: Round per minute
cfu	: Colony forming unit
OD	: Optical Density
Bp	: base pair
TE	: Tris-EDTA
SDS	: Sodium Dodecyl Sulfate
CTAB	: Cethyl Trimethyl Ammonium Bromide
EDTA	: Ethylene Diamide Tetra Acetic Acid
TAE	: Tris Acetate EDTA

CHAPTER 1

INTRODUCTION

1.1. The History and the Definition of Probiotics

The word ‘probiotic’ comes from Greek language ‘pro bios’ which means ‘for life’ opposed to ‘antibiotics’ which means ‘against life’. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much (Gismondo, et al. 1999, Guarner, et al. 2005). In 1908 a Russian researcher Ellie Metchnikoff, who has a nobel prize, firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (*Lactobacillus* spp.). Therefore, these bacteria affect the gut microflora positively and decrease the microbial toxic activity (Gismondo, et al. 1999, Çakır 2003, Chuayana, et al. 2003).

The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word ‘probiotic’ was used in different meaning according to its mechanism and the affects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined ‘probiotic’ as ‘substances and organisms which contribute to intestinal microbial balance’. In 1989, the meaning use today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host’s health positively by improving its intestinal microbial balance. Then this definition was broadened by Havenaar and Huis in’t Veld in 1992 including mono or mixed culture of live microorganisms which applied for animal and man (Çakır 2003, Guarner, et al. 2005, Sanders 2003).

In the following years lots of researchers studied on probiotics and made so much definition. They are listed below.

1- ‘Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition’ by Shaafasma, 1996.

2- ‘A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract’ by Naidu et al., 1999.

3- ‘A live microbial food ingredient that is beneficial to health’ by Salminen et al. 1998.

4- ‘A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host’ by Schrezenmeir and de Vrese, 2001.

5- ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ is accepted by FAO/WHO (report in October 2001) (Guarner, et al. 2005, Sanders 2003, Klaenhammer 2000).

Probiotics are also challinging for the industrial applications. The probiotic concept is open to lots of different applications in a large variety of fields relevant for human and animal health. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have beneficial effects on the health of host. They can contain one or several species of probiotic bacteria. Most of products which destine human consumption are produced in fermented milk or given in powders or tablets. These capsules and tablets do not used for medicial applications. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. Lots of studies suggest that probiotics have beneficial effects on microbial disorders of the gut, but it is really difficult to show the clinical effects of such products. The probiotic preperations use for traveller’s diarrhoea, antibiotic associated diarrhoea and acute diarrhoea which is showned that they have positive therapeutic effect (Gismondo, et al. 1999, Çakır 2003, Quwehand 1999).

More than 400 bacterial species exit in human intestinal tract. It is an enormously complex ecosystem that includes both facultatively anaerobic and anaerobic microorganisms (Naidu, et al. 1999). The numbers of genera is nearly steady, because they each have their own growth niches (Fooks, et al.1999). The composition of the gut microflora is constant but can be affected by some factors such as; age, diet, environment, stress and medication (Albertcllasic 2007). To have a healthy intestine the balance of the bacteria must be maintained but this is difficult as the lifestyles change. Lots of factors may change the balance away from potenially beneficial or health

promoting bacteria like lactobacilli and bifidobacteria to potentially harmfull or pathogenic microorganisms like clostridia, sulphate reducers and *Bacteroides* species. It makes the host more susceptible to the illnesses. In this case the prevalence of the beneficial bacteria must be supported. Using of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks, et al. 1999). Probiotics are suggested as food to provide for the balance of intestinal flora (Holzapfel, et al. 1998).

Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects. Also probiotics are acceptable because of being naturaly in intestinal tract of healthy human and in foods (Çakır 2003, Albertcllasic 2007). The probiotics which are use to feed both man and animals are shown in the Table 1.1.

Table 1.1. Microorganisms applied in probiotic products

***Lactobacillus* species**	***Bifidobacterium* species**	**Others**
L. acidophilus	*B. bifidum*	*Enterococcus faecalis*
L. rhamnosus	*B. animalis*	*Enterococcus faecium*
L. gasseri	*B. breve*	*Streptococcus salivarus* subsp.
L. casei	*B. infantis*	*thermophilus*
L. reuteri	*B. longum*	*Lactococcus lactis* subsp. *lactis*
L. delbrueckii subsp. *bulgaricus*	*B. lactis*	*Lactococcus lactis* subsp. *cremoris*
L. crispatus	*B. adolascentis*	*Propionibacterium freudenreichii*
L. plantarum		*Pediococcus acidilactici*
L. salivarus		*Saccharomyces boulardii*
L. johnsonii		*Leuoconostoc mesenteroides*
L. gallinarum		
L.plantarum		
L. fermentum		
L. helveticus		

1.2. The Effects of Probiotics on Health

There are lots of studies on searching the health benefits of fermented foods and probiotics. However, in most of these studies researchers did not use sufficient test subjects or they use microorganisms were not identified definitely (Çakır 2003). So, while a number of reported effects have been only partially established, some can be regarded as well established and clinically well documented for specific strains. These health-related effects can be considered as in the below (Çakır 2003, Scherezenmeir and De Vrese 2001, Dunne, et al. 2001, Dugas, et al. 1999).

- Managing lactose intolerance.
- Improving immune system.
- Prevention of colon cancer.
- Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence).
- Lowering blood pressure.
- Reducing inflammation.
- Reduction of allergic symptoms.
- Beneficial effects on mineral metabolism, particularly bone density and stability.
- Reduction of *Helicobacter pylori* infection.
- Suppression of pathogenic microorganisms (antimicrobial effect).
- Prevention of osteoporosis.
- Prevention of urogenital infections.

1.2.1. Lactose Intolerance

Most of human commonly non-Caucasians become lactose intolerant after weaning. These lactose intolerant people can not metabolize lactose due to the lack of essential enzyme β-galactosidase. When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence, cramping and diarrhoea ensue. If lactose passes through from the small intestine, it is converted to gas and acid in the large intestine by the colonic microflora. Also the presence of breath hydrogen is a signal for lactose maldigestion. The studies provide that the addition of

certain starter cultures to milk products, allows the lactose intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms (Fooks, et al. 1999, Scheinbach 1998, Quewand and Salminen 1998, Lin, et al. 1991).

The beneficial effects of probiotics on lactose intolerance are explained by two ways. One of them is lower lactose concentration in the fermented foods due to the high lactase activity of bacterial preparations used in the production. The other one is; increased lactase active lactase enzyme enters the small intestine with the fermented product or with the viable probiotic bacteria (Salminen, et al. 2004).

When the yogurt is compared with milk, cause the lactose is converted to lactic acid and the yogurt consist of bacterial β-galactosidase enzyme; it is suitable end beneficial to consume by lactose intolerants. Furthermore, the LAB which is used to produce yogurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, are not resistant to gastric acidity. Hence, the products with probiotic bacteria are more efficient for lactose intolerant human.

It is thought that the major factor improves the digestibility by the hydrolyses of lactose is the bacterial enzyme β-galactosidase. Another factor is the slower gastric emptying of semi-solid milk products such as yogurt. So the β-galactosidase activity of probiotic strains and other lactic acid bacteria used in dairy products is really important. β-galactosidase activity within probiotics varies in a huge range. It has to be considered both the enzyme activity of probiotic strain and the activity left in the final product for their use in lactose intolerant subjects (Salminen, et al. 2004).

1.2.2. Immune System and Probiotics

The effects of immune system are promising. However, the mechanism is not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts (Mombelli and Gismondo 2000).

Several reserchers have studied on the effects of probiotics on immune system stimulation. Some in vitro and in vivo searchs have been carried out in mice and some with human. Data indicate that oral bacteriotherapy and living bacteria feeding in fermented milks supported the immune system against some pathogens (Scheinbach 1998, Dugas, et al. 1999). Probiotics affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA

concentrations (Çakır 2003, Scheinbach 1998, Dugas, et al. 1999). Some of these effects are related to adhesion while some of them are not (Quwehand, et al. 1999).

Link-Amster et al. (1994) examined whether eating fermented milk containing *Lactobacillus acidophilus* La1 and bifidobacteria could modulate the immune response in human. They give volunteers the test fermented milk over a period of three weeks during which attenuated *Salmonella typhi* Ty21a was administered to mimic an enteropathogenic infection. After three weeks, the specific serum IgA titre rise to *S. typhi* Ty21a in the test group was >4-fold and significantly higher (p=0.04) than in the control group which did not ate fermented foods but received *S. typhi* Ty21a. The total serum IgA increased. These results showed that LAB which Cn survive in the gastrointestinal tract can act as adjuvants to the humoral immune response (Lime-Amster, et al. 1994, Quwehand, et al. 1999).

Perdigon et al. (1986) feed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations (Scheinbach 1998). Also in a human trial Halpern et al. (1991) feed human with 450 g of yogurt per day for 4 months and at the end a significant increase is observed in the production of γ-interferon (Fooks, et al. 1999).

Mattilla-Sandholm and Kauppila (1998) showed that *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 derived extracts suppress lymphocyte proliferation in vitro. Further evidence for immunomodulation by these two strains a children trial with severe atopic eczema resulting from food allergy. Children fed with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 showed improvement in clinical symptoms compared to the placebo group (Saarela, et al. 2000).

1.2.3. Diarrhea

Diarrhea is many causes and many types so it is difficult to evaluate the eefects of probiotics on diarrhea. But there are lots of searchs and evidence that probiotics have beneficial effects on some types of dierrhea. Diarrhea is a severe reason of children death in the worldwide and rotavirus is its common cause (Scheinbach 1998). In the treatment of rotavirus dierrhea, *Lactobacillus* GG is reported really effective. The best documented probiotic effect is shortened duration of rotavirus diarrhea using *Lactobacillus* GG. It has been given proof in several studies around the world by some

researchers like Guandalini *et al.* (2000), Pant et al. (1996). Also *Lactobacillus acidophilus* LB1, *Bifidobacterium lactis* and *Lactobacillus reuterii* are reported to have beneficial effects on shortening the diarrhea (Salminen, et al. 2004).

One of types of dierrhea is traveller's diarrhea (TD) which affects the healthy travellers not only in developing countries but also in Europe. Probiotics have beneficial effects in preventing some forms of TD. Oksanen et al. (1990) evaluated the efficacy of *Lactobacillus* GG in preventing diarrhea in 820 people travelling from Finland to Turkey. In a double-blind study by Black et al. (1989) lyophylised bacteria (*L.acidophilus, B.bifidum, L.bulgaricus, S.thermophilus*) were given to 56 Danish tourists on a 2-week trip to Egypt. The occurence of diarrhea in the group receiving the lactic acid bacteria was 43% while it was 71% in the placebo group (Gismondo, et al. 1999).

Antibiotic therapy causes mild and severe outbreaks of diarrhea. The normal microflora may be suppresed during the microbial therapy and resulting with filling with pathogenic strains. The changes of microflora may also ancourage the resistant strains at least *Clostridium difficile* which is the reasan of antibiotic associated diarrhea (ADD). Several clinical trails (Surewiez, et al., Adam, et al., Mcfarland, et al., etc.) have used *Saccharomyces boulardii, Lactobacillus* spp. and *Bifidobacterium* spp. in ADD. Probiotics which are able to restore and replace the normal flora should be used. Also they shoukd be used in high risk patients such as old, hospitalised or immuno-compromised. Studies with *Saccharomyces boulardii* proved that *Clostridium difficile* concentraiton is decreased in the presence of *Saccharomyces boulardii* (Gismondo, et al. 1999).

1.2.4. Cancer

Epidemiological studies point out that if the consumption of saturated fats increases in the diet, the occurrence of colon cancer increases in Western World. Bacterial enzymes (β-glucornidase, nitroreductase and azoreductase) convert precarcinogens to active carcinogens in the colon. It is thought that probiotics could reduce the risk of cancer by decreasing the bacterial enzymes activity. Although the exact mechanism for the anti tumor action is not known, some suggestions have been proposed by McIntosh as follows (Fooks, et al. 1999, Scheinbach 1998):

1. Carcinogen/procarcinogen are suppressed by binding, blocking or removal.

2. Suppressing the growth of bacteria with enzyme activities that may convert the procarcinogens to carcinogens.

3. Changing the intestinal pH thus altering microflora activity and bile solubility.

4. Altering colonic transit time to remove fecal mutagens more efficiently.

5. Stimulating the immune system.

There are in vitro and in vivo evidences not only from animal studies but also from human studies that probiotics have beneficial effects on suppression of cancer. Oral administration of lactic acid bacteria has been shown to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa in rats. The consumption of lactobacilli by healthy volunteers has been demonstrated to reduce the mutagenicity of urine and feaces associated with the ingestion of carcinogens in cooked meat. When it comes to epidemiological studies, they show an association between fermented dairy products and colorectal cancer. The consumption of a large quantity of dairy products especially fermented foods like yogurt and fermented milk with containing *Lactobacillus* or *Bifidobacterium* may be related to a lower occurrence of colon cancer (Rafter 2003, Hirayama and Rafter 2000). A number of studies have shown that predisposing factors (increases in enzyme activity that activate carcinogens, increase procarcinogenic chemicals within the colon or alter population of certain bacterial genera and species) are altered positively by consumption of certain probiotics (Brady, et al. 2000).

1.2.5. Cholesterol Reduction

Lots of researchers proposed that probiotics have cholesterol reduction effects. However, the mechanism of this effect could not been explained definitely. There are two hypotheses trying to explain the mechanism. One of them is that bacteria may bind or incorporate cholesterol directly into the cell membrane. The other one is, bile salt hydrolysis enzymes deconjugate the bile salts which are more likely to be exerted resulting in increased cholesterol breakdown (Çakır 2003, Scheinbach 1998, Prakash and Jones 2004).

A study on the reduction of cholesterol was showed that *Lactobacillus reuteri* CRL 1098 decreased total cholesterol by 38% when it is given to mice for 7 days in the

rate of 10^4 cells/day. This dose of *Lactobacillus reuteri* caused a 40% reduction in triglycerides and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen and liver (Kaur, et al. 2002).

1.3. Mechanism of Probiotics

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel, et al. 1998). There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly (Rolfe 2000, Çakır 2003, Salminen, et al. 1999, Castagliuola, et al. 1999).

1. Production of inhibitory substances: Production of some organic acids , hydrogen peroxide and bacteriocins which are inhibitory to both gram-positive and gram-negative bacteria.

2. Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.

3. Competition for nutrients: Despite of the lack of studies in vivo, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.

4. Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvants and increase humoral immune response.

5. Degradation of toxin receptor: Because of the degredation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* protects the host against *C. difficile* intestinal disease.

Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks, et al. 1999).

1.4. Selection Criteria for Probiotics

In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a number of desirable properties. The selection criteria are listed in Table 1.2 briefly. Some of them will be disscussed in more details. A potential probiotic strains does not need to fulfill all such selection criteria (Quwehand, et al. 1999).

Table 1.2. Selection criteria for probiotics.
(Source: Quwehand, et al. 1999, Çakır 2003)

Probiotic Strain Properties	**Remarks**
Human origin for human usage	Although the human probiotic *Saccharomyces boulardii* is not human origin, this criteria is important for species-dependent health effects.
Acid and bile tolerance	Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surface	Imortant to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization.
Safe for food and clinical use	Identification and characterization of strains accuratly, documented safety. No invasion and no degradation of intestinal mucus.
Clinically validated and documented health effects	Minumum effective dosage has to be known for each particular strain and in different products. Placebo-controlled, double-blinded and randomized studies have to be run.
Good technological properties	Survival in products if viable organisms are required, phage resistance, strain stability, culturable in large scales, oxygen resistance, have no negative effects on product flavour.

The selection criteria can be categorized in four basic groups. Appropriatness, technological suitibility, competitiveness, performance and functionality (Klaenhammer and Kullen 1999). Strains which have these criteria should be used in order to get effective on health and functional probiotic strains. Probiotics are chosed by using the criteria in Table 1.2. Saarela et al. (2000) proposed the properties of probiotics in three basic groups as; safety aspects, aspects of functionality and technological aspects.

Some major selection criteria will be discussed in details below.

1.4.1. Acid and Bile Tolerance

Bacteria used as probiotic strains are joined in the food system with a journey to the lower intestinal tract via the mouth. In this food system, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. Then the journey will be going on in the stomach and enter the upper intestinal tract which contain bile.ın this stage strains should have the ability to resist the digestion processes. It is reported that time at the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile (Chou and Weimer 1999, Çakır 2003).

To show probiotic sufficiencies, they should reach to the lower intestinal tract and maintain themselves overthere. Because of desirable point the first criteria is looking for probiotic strains is being resistant to acid and bile. Bile acids are synthesized in the liver from cholesterol and sent to the gall –bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli* subspecies, *Klebsiella* spp., and *Enterococcus* spp. in vitro. The deconjugated acid forms are more effective on gram positive bacteria (Dunne, et al. 1999, Çakır 2003).

Lactobacillus acidophilus is the most used probiotic strain in the products like dairy products or capsules. Chou and Weimer (1999), tried to isolate acid and bile resistant variants of *L. acidophilus*. Probiotic strains were taken from American Type Culture Collection had been isolated from different sources. Some of these strains were

found resistant to acid at pH 3.5 for 90 min. at 37 °C. Also these strains were capable of growth in medium at pH 3.5 containing 0.2% mixed bile salts (Chou and Weimer 1999).

An investigation of probiotic potential of 47 selected strains of *Lactobacillus* spp. were examined for resistance to pH 2.5 and 0.3% oxgall. They showed high resistance to bile salts and growth was delayed from 1h to more than 4 h for 16 of these strains examined and except one all of these strains survived for in such conditions mentioned above. The results obtained in vitro experiments; five strains (*L.rhamnosus* 19070-2, *L.reuteri* DSM 12246, *L.rhamnosus* LGG, *L.delbrueckii* subsp. *lactis* CHCC2329 and *L. casei* subsp. *alactus* CHCC3137) were selected for in vivo studies (Jacobsen, et al. 1999).

For selection of acid and bile resistant bifidobacteria, human fecal samples were screened and isolated strains from these samples were examined for growth (pH 4.5 and 7.0) and oxgall (0.006% and 0.15%). Then conditions were updated and isolated strains were examined for survival in pH 2.0, 3.0 and 7.0, a final concenrations of 0, 0.05% and 1% of oxgall. According to the results for survival rate of the isolated bifidobacterium strains were very similar at bile concentrations of 0.5% and 1% for 12 hs exposure. Among two of selected bifidobacterium strains, HJ 30 and SI 31, showed higher rates of survival (Chung, et al. 1998).

In another study a large culture collection of lactic acid bacteria of NZDRI was screened to select strains to use as probiotics. For this, over 200 strains of *Lactobacillus* and *Bifidobacterium* were examined according to their ability of resistant to bile and acid and four of them selected. Three of them were from dairy origins and the last one was from human origin. They were compared with the two commercial probiotic strains namely *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* LA-1. The isolated strains were analyzed for a series of pH between 1 and 3 and also for tolerance against bile at final concentrations of 0, 0.5 and 1% w/v. They were tolerant for the conditions mentioned above. While the general survival patterns are similar, the strain from human orgin showed higher tolerance. These strains were identified as *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN067, *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* HN019 (Prasad, et al. 1998).

In another research, twenty nine Lactobacillus strains of dairy origin were tested in vitro for their probiotic potential. The resistance of bacteria was examined in pH 1 between pH 3. Tolerance to bile salt was tested against to 0.3% oxgall. All of the examined strains were resistant to pH 3 during 3h, but most of them lost their viability

in 1h in pH 1. Also all of them were tolerated 0.3% bile salts concentration in 4 h. For in vivo testing the most suitable strains were chosen, *L.casei* Shirota ACA-DC 6002, *L.plantarum* ACA-DC 146, *L.paracasei* subsp. *tolerans* ACA-DC 4037 (Maragkoudakis, et al. 2005). Also an experiment was performed on three *Lactobacillus* species isolated from human milk whether they may use potential probiotic strains. They were identified as *Lactobacillus gasseri* and one of them *Lactobacillus fermentum*. Survival in low pH and in gastrointestinal environment were examined for an comparison with commercial probiotic strains, *L.rhamnosus* GG, *L. casei* imunitass and *L. johnsanii* La1. The strains especially *L.gasseri* showed that it can be used as a potential probiotic strain (Martín, et al. 2004).

1.4.2. Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer Kullen 1999). Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Quwehand and Vesterlund 2004, Çakır 2003). Till today there are some researchs on showing that different species produce different antimicrobial substances. Here are some examples of these substances: *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuterin; subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocin acidophilucin A (Quwehand and Vesterlund 2004). Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time. Nisin, produced by L. lactis subsp. lactis is the well known bacteriocin and it is allowed to use in food preparations (Çakır 2003).

Lactobacilli and Bifidobacteria isolated from human ileum were assayed if they have antimicrobial activity against a range of indicator microorganisms, *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli,*

Lactobacillus, Streptococcus, Bifidobacterium and *Lactococcus.* Antimicrobial activity of *Lactobacillus salivarus* UCC118 was counted against to these bacteria listed above. The study showed that *Lactobacillus salivarus* UCC118 is significantly capable of inhibiting in vitro growth of both some gram positive and some gram negative bacteria such as, *L. fermentum* KLD, *B. longum, B. bifidum, Bacillus subtilus, B. cereus, B.thuringiensis, E. faecalis, E. faecium* etc. although it is not effective against some of *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* etc. species (Dunne, et al. 1999).

Some milk products were used to isolate potential probiotic bacteria and determination of their possible antimicrobial activities. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Serratia marcescens* and *Candida albicans* were used as indicator microorganisms. After the study, the results showed that, Yakult and Ski D' Lite probiotics inhibited all of the test indicator microorganisms, Nestle yogurt probiotics were bactericidal for *S.aureus* and *P. aeruginosa* but inhibitory for *S. typhi* , Neslac probiotics killed E. coli and *S. typhi* while they were only inhibitory for *S.aureus* and *C. albicans,* Gain probiotics inhibited *C. albicans* (Chuayana, et al. 2003).

In another study eight lactic acid bacteria strains producing bacteriocins were isolated from Burkina Faso fermented milk and they were examined for the antimicrobial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP. The lactic acid bacteria strains were identified as *Lactobacillus fermentum*, *Pediococcus* spp., *Lactococcus* spp., *Leuconostoc mesenteroides* subsp. *mesenteroides.* The diameters of inhibition zones were obtained between 8 mm and 12 mm. *Lactobacillus fermentum* (S1) gave the biggest zone around 12 mm on *Enterococcus faecalis* while the smallest one is obtained from *Leuconostoc mesenteroides* subsp. *mesenteroides* (S5) on the same strain *Enterococcus faecalis* (Savadogo, et al. 2004).

In a research which was aimed to test the production of bacteriocin in vaginal lactobacilli flora and characterization of this flora was also made. First antimicrobial activity was assayed for 100 vaginal lactobacilli isolates. Six of them were determined for the production of bacteriocin. In this study, common human pathogens *Gardneralla vaginalis*, *Pseudomonos aeroginosa*, *Proteus vulgaris*, *Escherichia coli*, *Enterobacter cloacae*, *Streptococcus milleri*, *Staphylococcus aureus* and *Candida albicans* were used as indicator microorganisms. Six of the strains had bacteriocin activity against eight of

ten different *Lactobacillus* species an also *S.milleri*, *P. vulgaris*, *P. aeroginosa*, *E. coli*, *E. cloacae* and *G. vaginalis.* But none of isolated strains showed efficiency on test organisms *S. aureus* and *C. albicans.* Also some characteristics of bacteriocins were obtained from the research (Karaoğlu, et al. 2002).

In another research, potential probiotic lactobacilli strains (*L.reuteri, L. plantarum, L.mucosae, L. rossiae* strains) (from pig feces), used as additives in pelleted feeding, were examined according to their antibacterial activity against to *Salmonella typhimurium* ATCC 27164, *E. coli*, *C. perfringens* 22G, *S. aureus* ATCC 25923, *B. megaterium* F_6, *L. innocua* DSM 20649 and *B. hyodysenteriae* ATCC 27164. Generally the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *B. hyodysenteriae* ATCC 27164. The study showed that, neutralization and treatment with catalase affect the antibacterial activity a little (De Angelis, et al. 2006). A similar study was conducted and in that study four *Lactobacillus* strains (*L. salivarus* CECT5713, *L. gasseri* CECT5714, *L.gasseri* CECT 5715 and *L.fermentum* CECT5716) isolated from human milk were investigated whether they have antimicrobial potential and for comparison *L. coryniformis* CECT5711 was used. All of the strains showed antibacterial properties against pathogenic bacteria (*Salmonella choleraesuis* CECT4155, CECT409 and CECT443, *Esherichia coli* CECT439 and *E. coli* O157:H7 serover CECT4076, *Staphylococcus aureus* CECT4013 and CECT9776, *Listeria monocytogenes* Scott A and the spoilage strain *Clostridium tyrobutyricum* CECT4011). However, the antimicrobial properties of lactobacilli strains varied and *L. salivarus* CECT5713 revealed not only the best in vitro antibacterial activity, but also the highest protective effect against a *Salmonella* strain in the murine infection model (Olivares, et al. 2005).

1.4.3. Safety Aspects of Probiotics

Today, there are evidences that probiotic strains used as commercial bacteria are safe to use in applications. The safety of the probiotic products is appraised with the phenotypic and genotypic characteristics and the statistics of used microorganisms (Çakır 2003). Safety aspects of probiotic bacteria include the following requirements.

1. Strains for human use are preferred to be human origin.
2. They are isolated from healthy human gastrointestinal tract.

3. They have to be non-pathogenic.

4. They have to no history of relationship with diseases like, infective endocarditis or gastrointestinal tract disorders.

5. They do not deconjugate bile salts.

6. They should not carry transmissible antibiotic resistance genes (Saarela, et al. 2000).

1.5. Molecular Identification of Probiotic Strains

Methods used for detection of probiotics in human gastrointestinal tract are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations they are used for identification. With the developing technology about the molecular typing it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for selection, enumeration and biochemical characterization (fermentation profiles, salt-pH-temperature tolerances) but it is not efficient to classify a culture taxonomically. Molecular characterization methods are powerful even between closely related species. There are number of alternative taxonomic classification methods well known including hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers (Klaenhammer and Kullen 1999). Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools (Bulut 2003). Comparison between these methods, the most powerful and accurate one is sequencing (Coeuret, et al. 2003).

Characterization of microorganisms according to their 16S rDNA regions sequencing was firstly proposed by Woese in 1987. The application of 16S or 23S rRNA-targeted oligonucleotide probes is the best and most reliable approach to identify bacteria on a phylogenetic basis. The 16S rRNA gene is nearly 1540 bases long and includes variable regions while the general structure is highly conserved. Because the probes have the broadest specificity ranging from universal to species specificity, it is possible to use 16S rRNA gene to study phylogenetic relationships between microorganisms and identify them more accurately (Çakır 2003, Holzapfel, et al. 1998, Charteris, et al. 1997).

In one study, the PCR-ARDRA technique was used to identify potential probiotic *Lactobacillus* species isolated from bovine vagina. 16S rRNA gene was amplified by PCR and products were digested with four restriction enzymes (*Sau* 3AI, *Hinf* I, *Hinc* II and *Dra* I). Most of the digestion profiles obtained from the amplified 16S rDNA gene of these strains agreed with the theoretical profile matching with *Lactobacillus fermentum*. Among all strains, four homofermentative lactobacilli showed a restriction profile that matched with *Lactobacillus gasseri* and a facultative heterofermentative strain was identified as *Lactobacillus rhamnosus* (Otero, et al. 2006).

Restriction enzyme analysis were done by using pulsed with gel electrophoresis (REA-PFGE) and intergenic transcribed spacers (ITS)-PCR restriction fragment length polymorphism (RFLP) techniques for identification of probiotic potential strains (by sequencing of the 16S rRNA gene) isolated from koko and koko sour water (African spontaneously fermented millet porridge and drink). *Taq* I and *Hae* III restriction enzymes were used for digestion. From the result of ITS-PCR RFLP, four groups were obtained including group 1 *Weisella confuse*, group 2 *Lactobacillus fermentum*, group 3 *Lactobacillus salivarus* and group 4 *Pediococcus* spp. At the end it was showed using for identification of these strains the ITS-PCR RFLP technique, 16S rRNA gene sequencing is very reliable (Lei and Jakobsen 2004).

To identify lactobacilli used as starter and probiotic cultures, amplified ribosomal DNA restriction analysis (ARDRA) was applied. Firstly group-specific and species-specific 16S rDNA primers were used to amplification. *Cfo* I, *Hinf* I, *Tru* 91 and *Scr*FI restriction enzymes were selected for digestion. The results revealed three groups: A, B and C. It is suggested that ARDRA by using *Cfo* I was reliable method for differentiation of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* (Roy, et al. 2001).

Some researchers aimed to develop a novel multiplex PCR primer set to identify seven probiotic Lactobacillus species (*L.acidophilus, L. delbrueckii, L.casei, L.gasseri, L. plantarum, L. reuteri* and *L. rhamnosus*). The primer set containing seven specific and two conserved primers, was obtained from the integrated sequences of 16S and 23S rRNA genes and their rRNA intergenic spacer region of each species. 93.6% accuracy was obtained to identify the seven target species. The study showed that the multiplex primer set is really efficient tool for simple, rapid and reliable identification of *Lactobacillus* species (Kwon, et al. 2004).

In another study potential probiotic *Lactobacillus* strains isolated from human, animal and food were identified by 16S-23S rRNA restriction profiling at species level. Firstly PCR amplification of 16S-23S rRNA intergenic spacers was done by using universal primers. It is followed by digestion of PCR products by 11 restriction enzymes with 6bp specificities. Some of the enzymes were *Sfu* I, *Hind* III, *Dra* I, *Eco*RI, *Eco*RV etc. the study was concluded that identification could be done by DNA fingerprints generated by restriction endonucleases. The amplified ribosomal DNA restriction analysis (ARDRA) was an easier, faster and more accurate method (Moreire, et al. 2005).

1.6. Human Milk – A Source of Potential Probiotic Strain

After birth, breast milk is the best food for infants because it fullfills all the nutritional requirements for them during months. Also breast milk protects the newborn against infectious diseases. This effect seems a result of the action of some breast milk components, like different antimicrobial compounds, immunoglobulins, immunocomponent cells (Martin, et al. 2003) and also breast milk contains prebiotic substances which stimulate the growth of the benefical bacteria neonate gut (Martin, et al. 2004, Martin, et al. 2003). In a general view human milk contains fat, protein, carbohydrate, minerals and bacteria.

Table 1.3. Contents of human milk
(Source: Prentice 1996)

fat	Fatty acids, polyunsaturated fatty acids,
protein	Casein, α-lactalbumin, lactoferrin, IgA, IgG, lysozyme, serum albumin, ß-lactoglobulin
carbohydrate	Oligosaccharides, lactose
minerals	Calcium, phosphorus, sodium, potassium, chlorine

When it comes to the microbiological point of breast milk, human milk is really an important factor in the initiation and development and of course composition of the neonatal gut microflora since it constitutes source of microorganisms to the infant gut for several weeks after birth (Martín, et al. 2005). It is estimated that an infant ingests

$1x10^5 – 1x10^7$ commensal bacteria while suckling if the infant comsumes approximately 800 ml breast milk per day (Martín, et al. 2004, Martín, et al. 2005, Heikilla and Saris 2003). From the studies the fact is that, the bacterial composition of the infant fecal flora seems to reflect the bacterial composition of breast milk (Heikillä and Saris 2003).

The composition of the gut microflora is thoroughly influenced by the diet of the infant. Thus, the presence of a few predominant Gram-positive species in breast milk may be a reason explaining why microbiota of breast-fed infants is composed of a narrow spectrum of species, and a more diverse microbiota develops after weaning (Martín, et al. 2004, Favier, et al. 2002).

The studies on the microbiology of human milk are restricted to the identification of potential pathogenic bacteria in clinical cases of mastitis or infant infections. However, it is clear that the prevention of infant from infectious diseases owing to the natural flora of human milk (Martín, et al. 2004). Although there are limited knowledge about the commensal or probiotic bacteria that breast milk contain, bacteria commonly isolated from this biological fluid include staphylococci, streptococci, micrococci, lactobacilli and enterococci (Martín, et al. 2004, Martín, et al. 2003, Martín, et al. 2005, Heikillä and Saris 2003). Bacteria from these genera can be easily isolated from fresh milk of healthy women. So, these groups of bacteria should be considered the natural microbiota of human milk rather than mere contaminant bacteria (Martín, et al. 2004, Martín, et al. 2005).

There are surprisingly not so much studies on the isolation and analysis of commensal or potential probiotic bacteria from breast milk (Martin, et al., 2003). However, if the bacteria with the ability to provide health benefits such as protection the host from pathogenic bacteria were isolated from human milk, they would be considered attractive probiotic organisms (Martín, et al. 2004). These isolated bacteria would fulfill some of the main criteria like being human origin, adaptation to dairy substrates and a histoy of long duration and safeintake by infants (Martín, et al. 2004, Klaenhammer and Kullen 1999).

From the bacteria isolated from breast milk, *Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus fermentum, or Enterococcus feacium* are founded and they can be regarded as potential probiotic bacteria (Martín, et al. 2004, Holzapfel, et al. 1998). Hence, breast milk, a natural source of potentially probiotic or biotherapeutic LAB, protects mother and infants against infectios diseases (Martín, et al. 2004).

There are lots of studies on the effect of human milk on the health of infants and the infant diseases but surprisingly lack of studies on the microbiology of breast milk. From the few studies, it is found that human milk is an attractive source for potential probiotic strains. As, the bacteria implement some of the main criteria for being probiotic strains such as, human origin, survival in the gastrointestinal conditions and particularly low pH and bile, production antimicrobial compounds, adhesion to the intestinal mucosa (Martín, et al. 2005, Olivares 2005).

Martin et al. (2003) aimed to investigate whether human breast milk contains potentially probiotic lactic acid bacteria, and therefore, whether it can be considered a synbiotic food. For this purpose; they isolated lactic acid bacteria from milk, mammary areola, and breast skin of eight healthy mothers and oral swabs and feces of their respective breast-fed infants. They identified the lactic acid bacteria by RAPD-PCR analysis and 16S rDNA sequencing. From the results they identified the bacteria isolated from human milk as *Lactobacillus gasseri, Lactobacillus fermentum* and *Enterococcus faecium*. These species are considered among the probiotic bacteria (Holzapfel, et al. 1998, Collins, et al. 1998) and contain strains that are used in commercial probiotic products. They concluded as their work indicates that breast milk contains lactic acid bacteria is a natural source of LAB for the newborns and may be considered a synbiotic food.

Martin et al. (2004) studied on three lactobacilli strains isolated from breast milk whether they were potential probiotic bacteria. They performed some assays to investigate some criteria need to be used as probiotic bacteria such as; survival to conditions simulating in the gastrointestinal tract, production of antimicrobial compounds, adherence to intestinal cells, production of biogenic amines, degradation of mucin, enzymatic profile and pattern of antibiotic resistance. 2 *Lactobacillus gasseri* and 1 *Lactobacillus fermentum* strains were evaluated and the results showed that the probiotic potential of lactobacilli isolated from human milk is similar to strains commonly used in commercial probiotic products.

Heikkilä and Saris (2003) were focused on the antimicrobial activity against *Staphylococcus aureus* of bacteria isolated from human milk. They identified the bacteria by different molecular characterization techniques and named the bacteria as staphylococci, streptococci, and LAB as *Lactobacillus crispatus, Lactobacillus rhamnosus, Lactococcus lactis* and *Leuonostoc mesenteroides* and also *Enterococcus feacalis.* Then they examined the antimicrobial activity of these bacteria against

Staphylococcus aureus. They concluded that the commensal bacteria in human milk may have a role in protecting the infant and mother against *Staphylococcus aureus.* Also the results supported that the commensal staphylococci and streptococci are predominant bacterial species in breast milk. The other isolated bacteria *Lactobacillus rhamnosus* had RAPD profile identical to the commercial strain *Lactobacillus rhamnosus* GG, which is a commonly used probiotic strain in milk products in Finland.

Olivares et al. (2006) aimed to evaluate the antimicrobial activity against some pathogenic bacteria of four lactobacilli (*Lactobacillus salivarius* CECT5713, *Lactobacillus gasseri* CECT5714, *Lactobacillus gasseri* CECT5715, *Lactobacillus fermentum* CECT5716) isolated from human breast milk. In the conclusion; the four lactobacilli and particularly *Lactobacillus salivarius* CECT5713 showed antibacterial activity. These results suggest that these lactobacilli strains from human breast milk could play a part of anti-infective protection in neonates and would be good strains to develop probiotic products for infant.

Human milk is an important food for neonates during some months to grow them up and protect the infants against some infectious. The high concentration of LAB in milk from healthy mother may play an important biological role during the first months of life. Studies on this biological fluid indicate that human milk is a challenging source for potential probiotic bacteria.

CHAPTER 2

MATERIALS AND METHODS

2.1. Isolation of Lactic Acid Bacteria from Human Breast Milk

The isolation material was human milk obtained from 15 healthy mother volunteers in Tepecik SSK Hospital. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the organisms. Samples were used directly and also diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS (Man, Rogosa and Sharpe) agar (pH 6.2 and pH5.5), TPY (Trypticase Phytone Yeast) agar (pH 6.5) and MRS-cystein agar (pH 5.5).

The plates were incubated at 37 °C for 3 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). The using of these mediums aimed to isolation and enumeration of lactobacilli, streptococci and enterococii. After incubation, individual colonies were selected and transferred into sterile broth mediums. The following step is purifying the selected colonies with streak plate technique. The isolates were examined according to their colony morphology, catalase reaction and gram reaction. Gram positive and catalase negative cocci and bacilli colonies were taken to the glycerol stocks as lactic acid bacteria.

2.1.1. Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. LAB are known to be gram positive. It means that they give blue-purple color by gram staining.

Cultures were grown in appropriate mediums at 37 °C for 24 h under anaerobic conditions. Cells from fresh cultures were used for gram staining. After incubation cultures were transferred aseptically into 1.5 ml eppendorf tubes and centrifuged for 5 min at 6000 rpm. Then, supernatant was removed and cells were resuspended in sterile

water. Gram staining procedure was applied. Then, under light microscopy gram positives and purified isolates were determined.

2.1.2. Catalase Test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.

$$2H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, two methods can be applied. Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were choosed. Since, LAB are known as catalase negative.

2.1.3. Long Term Preservation of Isolates

Gram positive and catalase negative isolates were preserved in MRS broth medium containing 20% (v/v) glycerol as frozen stocks at -80 °C. The glycerol stocks of samples were prepared by mixing 0.5 ml of active cultures and 0.5 ml MRS medium including 40% sterile glycerol.

2.2. Probiotic Properties of Isolates

For the determination of probiotic properties of isolates these major selection criteria were choused: resistance to low pH, tolerance against bile salt and the antimicrobial activity.

2.2.1. Resistance to Low pH

Resistance to pH 3 is often used in vitro assays to determine the resistance to stomach pH. Because the foods are staying during 3 hs, this time limit was taken into account (Prasad, et al. 1998). For this purpose, active cultures (incubated for 16-18 h) were used. Cells were harvested by centrifugation for 10 min at 5000 rpm and 4 °C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2). Then cell pellets were resuspended in PBS (pH 3) and incubated at 37 °C. Viable microorganisms were enumerated at the 0., 1., 2. and 3. hours with pour plate techniques. Appropriate dilutions were done and plates were incubated at 37 °C under anaerobic conditions for 48 h. Also growth was monitored at OD_{620} (Thermo Multiskan EX).

2.2.2. Tolerance against Bile

Because the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h (Prasad, et al. 1998). The experiment was applied at this concentration of bile for 4 h. MRS medium containing 0.3% bile (Oxoid) was inoculated with active cultures (incubated for 16-18 h). During the incubation for 4 h, viable colonies were enumerated for every hour with pour plate technique and also growth was monitored at OD_{620} (Thermo Multiskan EX).

2.2.3. Antimicrobial Activity

For the antimicrobial activity test, spot on lawn method was used. After 18 h incubation active cultures were spotted on the surface of MRS agar plates (2 strains for each plate). Then MRS plates were incubated to grow cultures for 24 h at 37 °C under anaerobic conditions. The next step is preparing the indicator microorganisms (Table 2.1). Overnight indicator pathogens inoculated (1%) to soft agar containing 0.7% agar and this inoculated agar were overlaid on MRS plates. These plates were incubated according to the appropriate conditions for indicator microorganisms. At the end of the incubation, inhibition zone diameters (surrounding the spotted isolates) were measured. Lots of researches were observed to give the results. Accordingly; isolates, which gave an inhibition zone bigger than 1 mm, were determined to have antimicrobial activity.

Table 2.1. Indicator microorganisms

Indicator microorganism	**Incubation conditions**
Salmonella thyphimurium CCM 5445	37 °C in TSB medium
Escherichia coli O157:H7 NCTC 129000	37 °C in TSB medium
Escherichia coli NRRL B-3008	37 °C in Nutrient broth medium

2.3. Physiological and Biochemical Characterization

Biochemical tests were run according to methods offered by Bulut, 2003.

2.3.1. Gas Production from Glucose

In order to determine the homonfermentative and heterofermentative characterization of isolates, CO_2 production from glucose test was applied. Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 37 °C for 5 days. Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose.

2.3.2. Growth at Different Temperatures

Temperature test media, MRS containing bromecresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 7 days at 10 °C, 15 °C, 45 °C. During these incubation time cells growth at any temperatures was observed by the change of the cultures, from purple to yellow.

2.3.3. Growth at Different NaCl Concentrations

Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 4% and 6.5% NaCl concentrations were selected. Test mediums containing bromecresol purple indicator were prepared according to the appropriate concentrations

and transferred into tubes in 5 ml. these tubes were inoculated with 1% overnight cultures and then incubated at 37 °C for 7 days. The change of the color from purple to yellow was proofed the cell growth.

2.3.4. Arginine Hydrolysis Test

Arginine MRS medium and Nessler's reagent were used in order to see ammonia production from arginine. MRS containing 0.3% L-arginine hydrocloride was transferred into tubes as 5 ml and inoculated with 1% overnight cultures. Tubes were incubated at 37 °C for 24 h. After incubation, 100 µl of cultures transferred onto a white background. The same amount of Nessler's reagent was pipetted on the cultures. The change in the color was observed. Bright orange color indicated a positive reaction while yellow indicated the negative reaction. A negative control, which did not contain arginine, was also used as negative control.

2.3.5. Carbohydrate Fermentations

Isolates were characterized according to their fermentation profiles of ability to ferment 17 different carbohydrates. All reactions were performed by using 96-well microtitre plates. Active cells and sugar solutions were prepared separately. For preparation of active cells; isolates were activated in 10 ml MRS medium and incubated at 37 °C for 24 h. Then, they were centrifuged 10 min at 10000 rpm. Pellets were washed twice and resuspended in MRS without glucose and containing pH indicator bromecresol purple. Each sugar solutions were prepared at a final concentration of 10% (w/v), only salicin was prepared at concentration of 5%. Then the solutions were filter sterilized with filters (0.22 µm pore diameter). After preparation steps the procedure was applied. Forty µl of sugar solutions were pipetted into each well and 160 µl of suspended cells were added onto the sugar solutions. Thus, 2% final sugar concentration was obtained. All the reactions were performed twice. Also positive and negative controls were used to indicate any contamination. 160 µl of suspended cells + 40 µl of glucose solution were used as positive control while 200 µl of suspended cells was used as negative one. After overnight incubation at 37°C, the turbidity and the color change from purple to yellow was recorded as positive fermentation results compared with the

positive and negative controls. Also results were compared with the absorbance of samples read at 620 nm in an automated microplate reader (Thermo Multiskan EX).

2.4. Molecular Identification

2.4.1. Genomic DNA Isolation

For genomic DNA of isolation of strains were done by using the following procedure (Cardinal, et al. 1997).

- Overnight activation of 10ml MRS cultures.
- Harvesting cells at 6.000 rpm for 5 min.
- Removing the liquid phase, washing pellet with 500µl 1xTE buffer (pH 8) and then centrifugation at 6.000 rpm for 5 min.
- Suspending cells in 200µl 1xTE buffer (pH 8) containing 25% sucrose and 30mg/ml lysozyme and mixing gently.
- Incubation for 1 h at 37°C.
- Addition of 370µl 1xTE buffer (pH 8) containing 1mg/ml Proteinase K.
- Addition of 30 µl SDS
- Incubation for 1 h at 37°C.
- Addition of 100µl 5M NaCl solution and 80µl CTAB/NaCl solution (10% cetytrimethylammonium bromide, 0.7M NaCl).
- Incubation for 10 min at 65°C.
- Adding 750 µl chloroform/isoamyl alcohol (24/1, v/v) and mixing effectively.
- Centrifugation at 6.000 rpm for 5 min.
- Transferring the upper aqueous phase into a new eppendorf tube.
- 2^{nd} Chloroform/isoamyl alcohol extraction and centrifugation at 6000 rpm for 5 min.
- Transferring the upper aqueous phase into a new eppendorf tube.
- DNA precipitation by the addition of an equal volume (750µl) of isopropanol.
- If DNA wool is observed, taking the wool with a yellow tip and transferring into a new eppendorf tube containing 500 µl 70% ethanol.

- If DNA wool is not observed, centrifugation at 6.000 rpm for 10 min to pellet DNA and washing with 500 µl 70% ethanol.
- Pelleting DNA with centrifugation at 6.000 rpm for 10 min after washing.
- Removing the ethanol and drying the pellet at 37°C for 10 min.
- Dissolving pellet in 100µl 1xTE buffer (pH 8) containing 100µg/ml RNase.
- Incubation for 1 h at 37°C.
- Adjusting the volume to 400µl with 1xTE buffer (pH 8)
- Dissolving DNA with alternating cold-heat shock twice (80 °C for 10 minutes, and -20 °C for 20 min).
- Addition of one volume (400µl) of phenol, mixing efficiently.
- Centrifugation at 6.000 rpm for 5 min.
- Transferring the upper aqueous phase into new eppendorf tube.
- Addition of one volume (400µl) of chloroform/isoamyl alcohol (24/1, v/v), mixing efficiently.
- Centrifugation at 6.000 rpm for 5 min.
- DNA precipitation with the addition of 1/10 volume (40 µl) of 5M NaCl and 2 volumes (800µl) of 99% ethanol.
- Centrifugation at the maximum spin for 10 min.
- Washing pellet with 500µl 70% ethanol.
- Centrifugation at 6.000 rpm for 5 min.
- Removing all the ethanol and drying DNA for 10 min at 37°C.
- Dissolving DNA in 50µl, 100µl or 150µl according to pellet size.
- Dissolving DNA with alternating cold-heat shock (80°C for 10 min, and -20° C for 20 min).
- Preservation of dissolved genomic DNA samples at -20°C.

At the end of the procedure all of the samples were checked whether genomic DNAs were isolated. It is visualized by agarose gel electrophoresis (in Section 2.4.3).

2.4.2. Amplification of 16S rDNA Region by Polymerase Chain Reaction

2 µl of genomic DNA was mixed with 48 µl of PCR mixture given in the Appendix E. the final reaction mixture was taken to the PCR steps in the final volume 50 µl.

For the amplification of 16S rDNA region of isolates and reference strains EGE1 forward primer and EGE2 reverse primer were used. Forward primer is complementary to the 5'end of 16S rDNA, and the reverse primer is complementary to the 3' end of 16S rDNA region.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACGA-3'

The PCR Conditions:

Step 1: 94°C for 5 min

Step 2: 94°C for 1 min (denaturation)
Step 3: 56°C for 1 min (annealing)
Step 4: 72°C for 1 min (elongation)
} 40 cycles

Step 5: 72°C for 10 min

2.4.3. Seperation of Amplified PCR Products

Preparation of Agarose Gel:

Agarose gel was prepared in the concentrations of 0.8%. 0.8 g agarose was dissolved in 100 µl of boiling TAE buffer (Appendix D). Then it was cooled nearly to 45 °C and 15 µl ethidium bromide solution (10mg/ml) was added. The prepared agarose gel was poured into the gel casting stand and the combs were placed. After having a rigid gel combs were taken to have wells for loading.

Loading of Agarose Gel:

5 µl of PCR products and 2 µl of loading dye (Appendix D) were mixed and loaded into wells. A DNA size-marker (1 kb, Fermentas) was loaded into the fist well to see if the right region was amplified.

Electrophoresis of the Products:

PCR products were electrophoresed at 80 mA for 45 min. Amplification products were visualized in a gel documentation system (Vilber-Lormat). The DNA fragments with the size of 1500-2000 bp indicates the right amplification.

Purification of PCR Products

PCR Products were purified before digestion with restriction enzymes. Following procedure was used for purification:

- Adjusting the volume of PCR products to 100µl by adding 50µl 1xTE buffer
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 5.000 rpm for 10 min
- Transferring the upper aqueous phase into a new eppendorf tube
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 5.000 rpm for 10 min
- Transferring the upper aqueous phase into a new eppendorf tube containing 0.l volume of 3M sodium acetate (pH 5.2) and mixing effectively
- Addition of 2 volumes (220µl) of 99% ethanol
- Centrifugation at 8.000 rpm for 10 min and removing the liquid phase
- Washing the pelleted DNA with the addition of 500µl of 70% ethanol
- Centrifugation at 5.000 rpm for 10 min
- Removing the ethanol and drying pellet at 37°C for 10 min
- Dissolving DNA in 50µl 1xTE solution
- Storing DNA solution at -20°C.

2.4.4. Restriction Fragment Length Polymorphism (RFLP)

Ten µl of purified amplification PCR products were used for each of the restriction enzyme digesiton. Three different enzymes were used; *Taq* I, *Hae* III, and *EcoR* I. 50 µl final reaction volume and given tempratures were used for digesiton; for *Taq* I, 65°C, for *Hae* III and *EcoR* I, 37°C. All of the reactions were performed overnight and additionally, *Taq* I restriction reactions were overlaid with mineral oil to avoid evaporation.

2.4.4.1. Purification of Restriction Products

- Adjusting the volume of restriction products to 100µl with the addition of 90µl 1xTE buffer
- Addition of 2 volumes (200µl) of chloroform/isoamyl alcohol solution (Appendix D) and mixing effectively
- Centrifugation at 8.000 rpm for 5 min
- Transferring the upper aqueous phase into the new eppendorf tubes containing 0.1 volume of 3 M sodium acetate (pH 5.2) and mixing effectively
- Addition of 250µl of 99% ethanol
- Centrifugation at 8.000 rpm for 15 min and removing the all liquid phase
- Washing pellet with 300µl of 70% ethanol
- Centrifugation at 8.000 rpm for 5 min
- Removing the ethanol and drying pellet at 37°C for 10 min
- Dissolving the pellet in 10-15µl 1xTE solution
- Storing DNA solution at -20°C

2.44.2. Electrophoresis of Restriction Fragments

Restricted fragments were separated in 1.6 % agarose gel.

Preparation of Agarose Gel:

2.4g of agarose were dissolved in 150ml 1x TAE buffer by boiling. After boiling, it was cooled to almost 45°C. 22.5 µl ethidium bromide solution (10mg/ml) was added and stirred. The agarose gel was poured into the gel casting stand and the combs were placed. The combs were removed when the gel was solidified.

Loading of Agarose Gel:

The solidified agarose gel was placed into the electrophoresis tank and 1.5lt TAE buffer containing 300µl of ethidium bromide was poured into the tank. 10–12µl of the digestion products were mixed with 2µl of gel loading dye. The samples were loaded into wells, starting from the second well on the gel. The fist well on the gel was loaded with the 2µl (500ng) of DNA molecular weight marker for 100 bp., and the last

well on the gel was loaded with the 2µl (500ng) of DNA molecular weight marker for 1kb.

Electrophoresis of the Products

The samples were electrophoresed at 60mA for 30 min and at 80mA for 4 h. Amplification products were visualized in a gel documentation system (Vilber-Lormat).

2.4.4.3. Interpretation of Results

RFLP paterns were visualized in gel documentation system. The images were modified in Adobe Photoshop 7.0 and analyzed by using BIO-ID++ software (Vilber-Lourmat). The similarities between strains were determined automatically by specifying the formula of Jaccard. Strain clustering was performed by the un-weighed pair group method with arithmetic averages, UPGMA, BIO-ID++. The dendrogram was prepared using 13% homology coefficient.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation of Lactic Acid Bacteria

15 samples of human milk taken from healthy mother volunteers were used as isolation source. Lactic acid bacteria were isolated from various mediums at 37 °C under anaerobic conditions. Some gram negative bacteria were detected on TPY agar but no one on MRS agar. Also lots of catalase positive bacteria and yeast were observed. The reason could be the contamination from mother's breast skin. From approximately 200 isolates, 60 isolates remained at the end of the isolation, purification after the loss of unstable isolates during purification and subculturing steps. All of the isolates were gram positive catalase negative rods and cocci. It was understood that the isolates from human milk were so sensitive to the subculturing. Also some of them were lost because of being intolerant to -80°C and also -20°C cryopreservation.

3.2. Probiotic Properties

3.2.1. Resistance to Low pH

Being resistant to low pH is one of the major selection criteria for probiotic strains (Quwehand, et al. 1999, Çakır 2003). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach (Chou and Weimer 1999, Çakır 2003). Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad, et al. 1998).

For selection the strains resistant to low pH, PBS pH-adjusted to 3.0 was used. The time that takes during the digestion in the stomach is 3 hours. So all the isolates were detected whether they were resistant to pH 3.0 during 3 hours. After the examination of all the isolates, the isolates that survive in pH 3.0 were taken to the next step. According to this experiment only three isolates were resistant to low pH. Two of

them are bacilli and the other is cocci. Experiments were run twice. Results, both cfu (colony forming units) values and OD_{620}, were shown as graphics (Figure 3.1 and Figure 3.2).

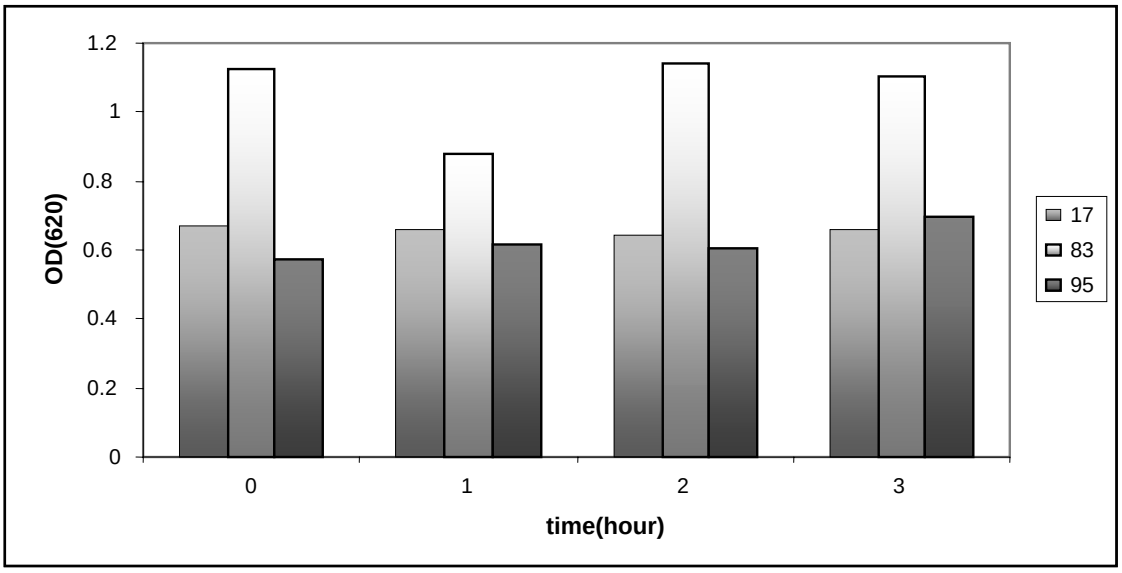


Figure 3.1. Survival in pH 3.0 – OD_{620} values

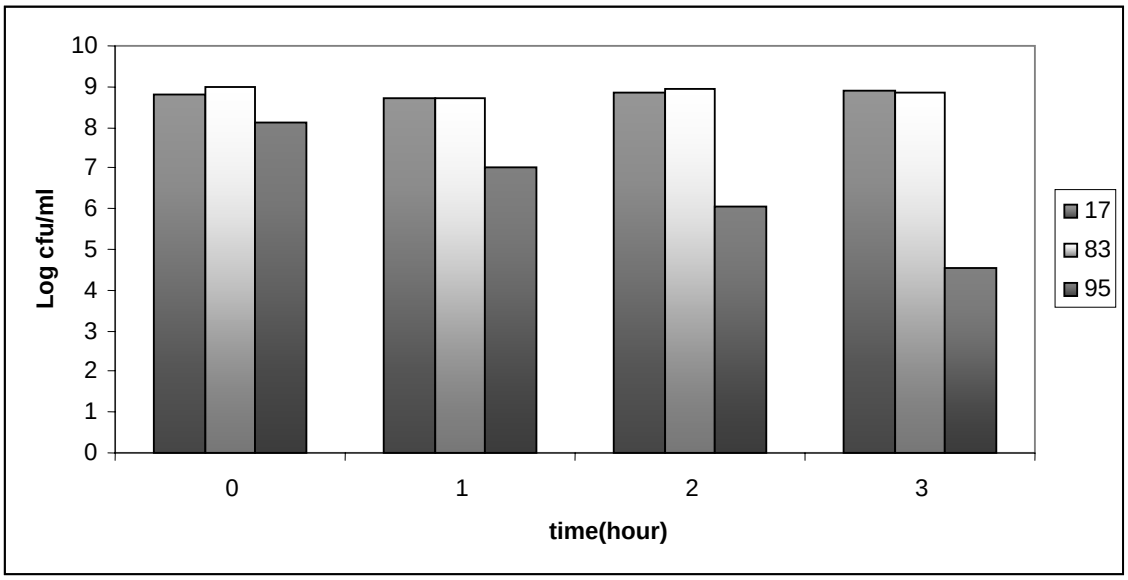


Figure 3.2. Survival in pH 3.0 – cfu values

As it is seen from the graphics AS17 bacilli is very stable in pH 3.0 which means that this isolate is able to survive in this pH value. AS83 bacilli is also stable during 3

hours. AS95 is able to tolerate pH 3.0 but it is more sensitive to low pH than AS17 and AS83.

When the two methods were compared, both of them gave the same results for AS17, nearly the same for AS83, but to decide the survival in pH 3.0 cfu values seem more reliable for AS95.

3.2.2. Tolerance against Bile

The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad, et al. 1998).

Strains were detected in 0.3% during 4 hours. The cfu values and OD_{620} were observed. According to the results all of the isolates are resistant to 0.3% bile salt. AS17 and AS83 are more tolerant than AS95. All of the isolates are also able to grow in 0.3% bile salt as they survive.

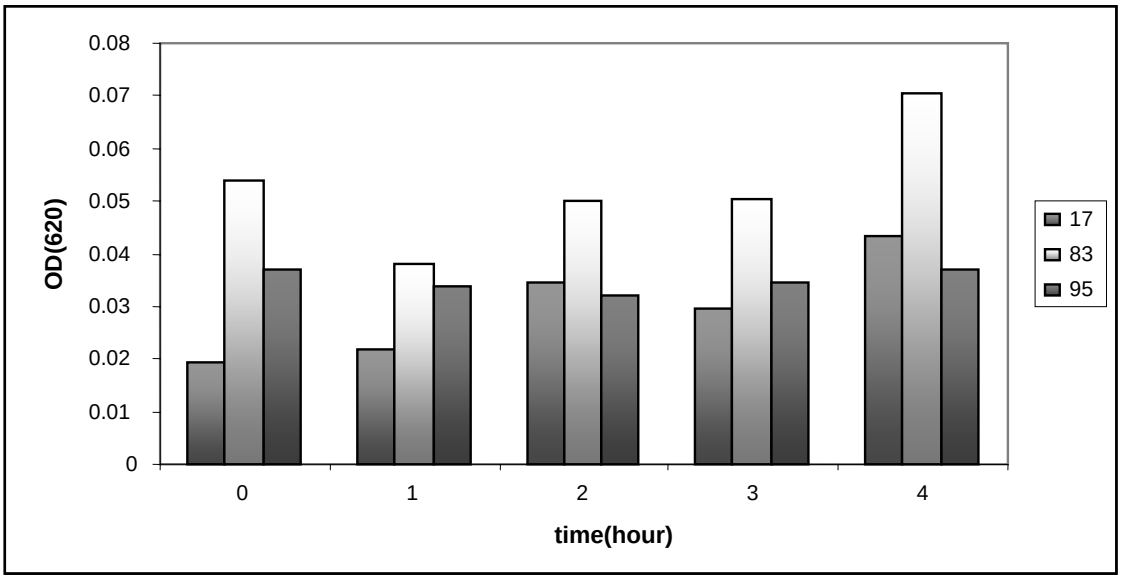


Figure 3.3. Tolerance against 0.3% bile – OD_{620} values

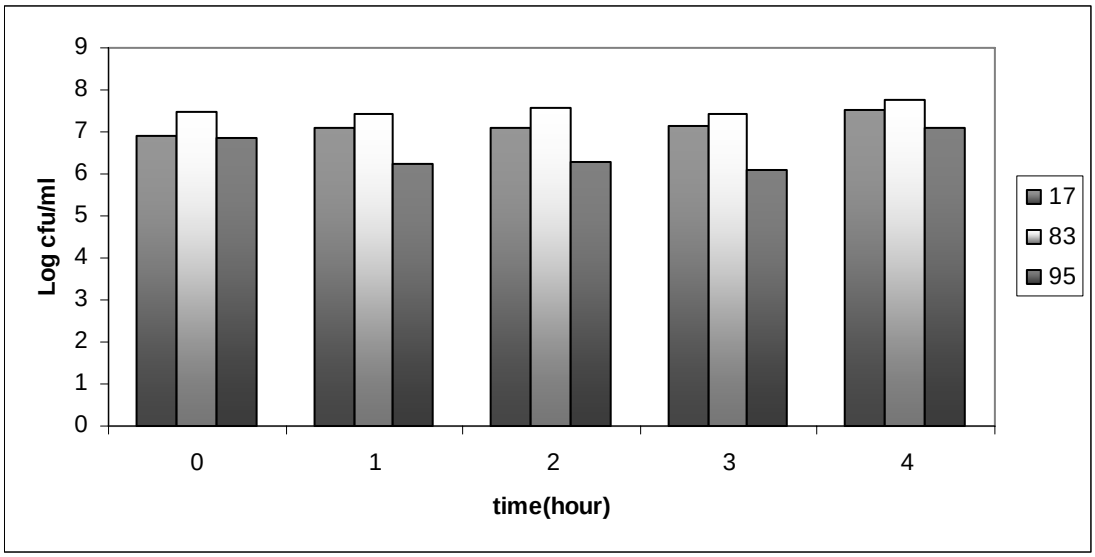


Figure 3.4. Tolerance against 0.3% bile – cfu values

3.2.3. Antimicrobial Activity

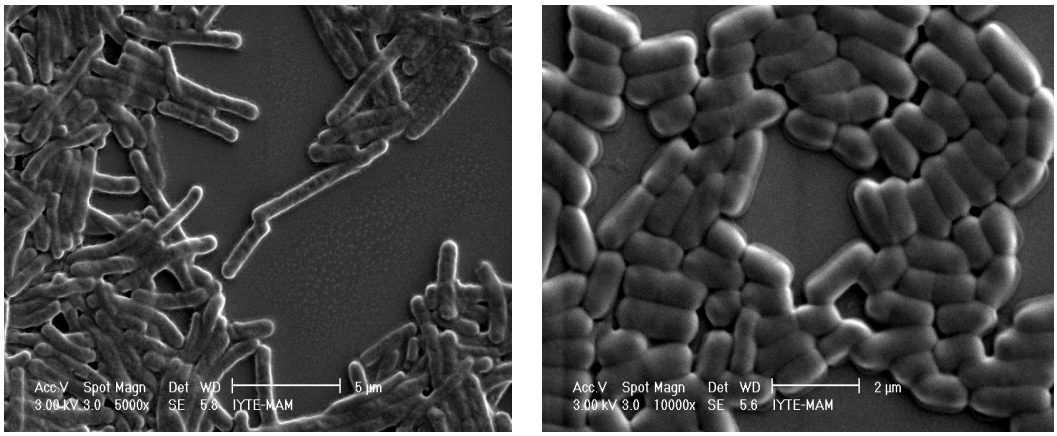
The selected strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganisms *Salmonella thyphimurium* CCM 5445, *Escherichia coli* O157:H7 NCTC 129000 and *Escherichia coli* NRRL B-3008. The diameter of inhibition zones (Table 3.1) showed that all of the isolates have antibacterial effect on the indicator microorganisms. The tests were applied two times and the averages of diameters of zones were given.

Table 3.1. Diameter of inhibition zones

Isolates	**Indicator Microorganisms**		
	Diameter of inhibition zones(mm)		
No	***Salmonella thyphimurium***	***Escherichia coli***	***Escherichia coli* O157:H7**
17	20	41	22
83	18	43	25
95	15	36	15

3.3. Physiological and Biochemical Characterization

All of the isolates were subjected to Gram staining and they were examined under light microscope. All the strains gave blue- purple color with staining; hence they all were Gram positive bacteria. AS17 was bacilli with long and rounded end (Figure 3.5.a). AS83 was cocobacilli morphology (Figure 3.5.b) while AS95 was cocci with spherical morphology.



(a) (b)

Figure 3.5. Scanning Electron Microscopic images **(a)** AS17 **(b)** AS83

Isolates were tested for catalase activity. They were all catalase negative (do not show catalase activity).

To test the gas production from glucose test tubes were observed for 5 days. AS95 showed no gas production while gas production was observed from AS17 and AS83. This indicates that, AS17 and AS83 are heterofermentative cultures whereas AS95 is homofermentative.

Another criterion for the identification the isolates was the ability of growth at different temperatures. From the results of 7 days observation, all of the isolates can grow at 45 °C however they can not grow at 10 °C and 15 °C.

Growth at different NaCl concentrations was observed. All of the isolates have the ability to grow at 2% NaCl concentration. AS17 and AS83 do not show the ability to grow at 6.5% NaCl concentration however AS95 can grow at this concentration.

Arginine hydrolysis test was another step to follow the identification procedure. The isolates which gave the bright orange were accepted that they can produce ammonia from arginine. The yellow color indicated negative arginine hydrolysis.

According to this test, AS17 can not hydrolyze arginine while both AS83 and AS95 can produce ammonia from arginine.

The most useful test for the determination of strain differences is carbohydrate fermentation. Eighteen (with glucose) different carbohydrates were used for identification. They give different fermentation patterns when they are compared. The patterns are showed in Table 3.2.

Table 3.2. Biochemical Test Results of Isolates

No	Shape	Catalase	gas from glucose	Ammonia from Arginine	2% NaCl	6.5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Glucose	Xylose	Ribose	Melezitose	Arabinose	Mannitol	Trehalose	Melibiose	Raffinose	Galactose	Salicin	Maltose	Sucrose	Mannose	Fructose	Lactose	Rhamnose	Sorbitol
AS17	bacilli	-	+	-	+	-	-	-	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-	+	+	-	-
AS83	bacilli	-	+	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	-	+	+	+-	+	+	-	-
AS95	cocci	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-

According to the biochemical test results AS17 produced gas from glucose while did not produce ammonia from arginine. It tolerated only %2 NaCl concentrations and only grew at 45 °C. This isolate gave positive results with the carbohydrates, glucose, xylose, ribose, arabinose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose. AS83 produced both gas from glucose and ammonia from arginine. It was resistant to %2 salt concentrations and grew at 45 °C. This isolate gave positive test results with sugars, glucose, ribose, arabinose, trehalose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose. When these biochemical test results are compared with the literature information (Table 3.3), it seems that AS17 is like to be *Lactobacillus oris*, AS83 is like to be *Lactobacillus fermentum* and AS 95 is like to be *Streptococcus* ssp. For the future experiments AS17 and AS83 were chosen.

Table 3.3. Literature Information of Biochemical Test Results source: (Roos, et al. 2005, Hammes and Hertel 1995)

Strains	Shape	Catalase	gas from glucose	Ammonia from Arginine	Growth at 15°C	Growth at 45°C	Glucose	Xylose	Ribose	Melezitose	Arabinose	Mannitol	Trehalose	Melibiose	Raffinose	Galactose	Salicin	Maltose	Sucrose	Mannose	Fructose	Lactose	Rhamnose	Sorbitol
Lactobacillus oris	bacilli	-	+	-	-	+	+	+	+	-	+	ND	d	+	+	+	-	+	+	d	+	+	-	ND
Lactobacillus fermentum	bacilli	-	+	+	-	+	+	d	+	-	d	-	d	+	+	+	-	+	+	w	+	+	-	ND

Symbols: +: 90% or more strains are positive, -: 90%or more are negative, d: 11-89% of strains are positive, w: weak positive reaction, ND: no data available

3.4. Molecular Identification

3.4.1. Genomic DNA Isolation

Genomic DNAs of isolates were isolated using the method offered by Cardinal et al. 1997 then isolated DNAs were visulized by agarose gel electrophoresis under UV light. Then they were taken to the PCR step.

3.4.2. Amplification of 16S rDNA Region

After DNA isolation the 16S rDNA region was amplified by PCR protocole. Then 50 µl of PCR products were visulized by agarose gel electrophoresis under UV light. The length of amplification products varied from 1500 to 2000 bp (Figure 3.6).

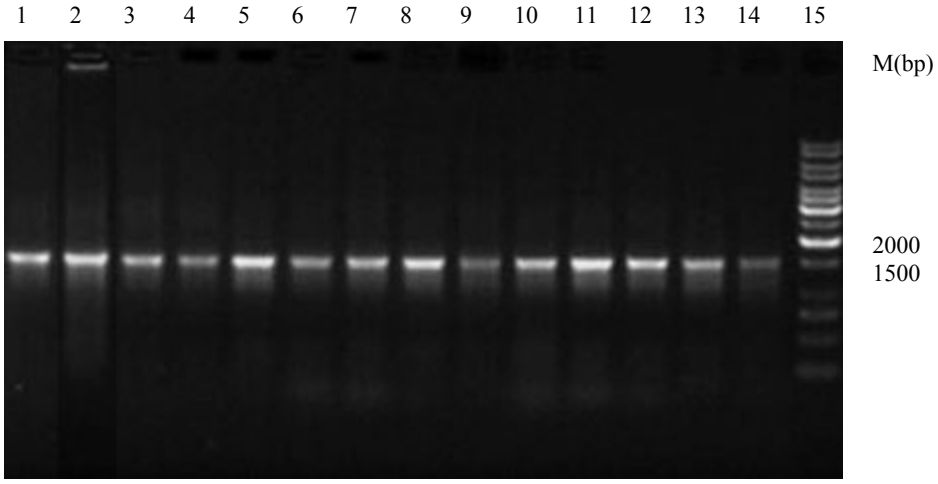


Figure 3.6. 16S Amplification Products of Isolates and Reference Strains
1. AS17 **2.** AS83 **3.** NRRL 14170 *Lb. reuteri* **4.** NRRL 4524 *Lb. fermentum* **5.** CCM 4833 *Lb. acidophilus* **6.** CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* **7.** CCM 2772 *Lb. delbrueckii* ssp *lactis* **8.** CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus* **9.** CH1 *Lb. casei* **10.** NRRL 2178 *Lb. johnsonii* **11.** NRRL 1954 *Lb. plantarum* **12.** NRRL 4526 *Lb. helveticus* **13.** NRRL 4527 *Lb. brevis* **14.** NRRL 442 *Lb. rhamnosus* **15.** 1kb DNA ladder Gene Ruler™

3.4.3. Digestion of Amplified 16S rDNA region

3.4.3.1. *Hae* III digestion

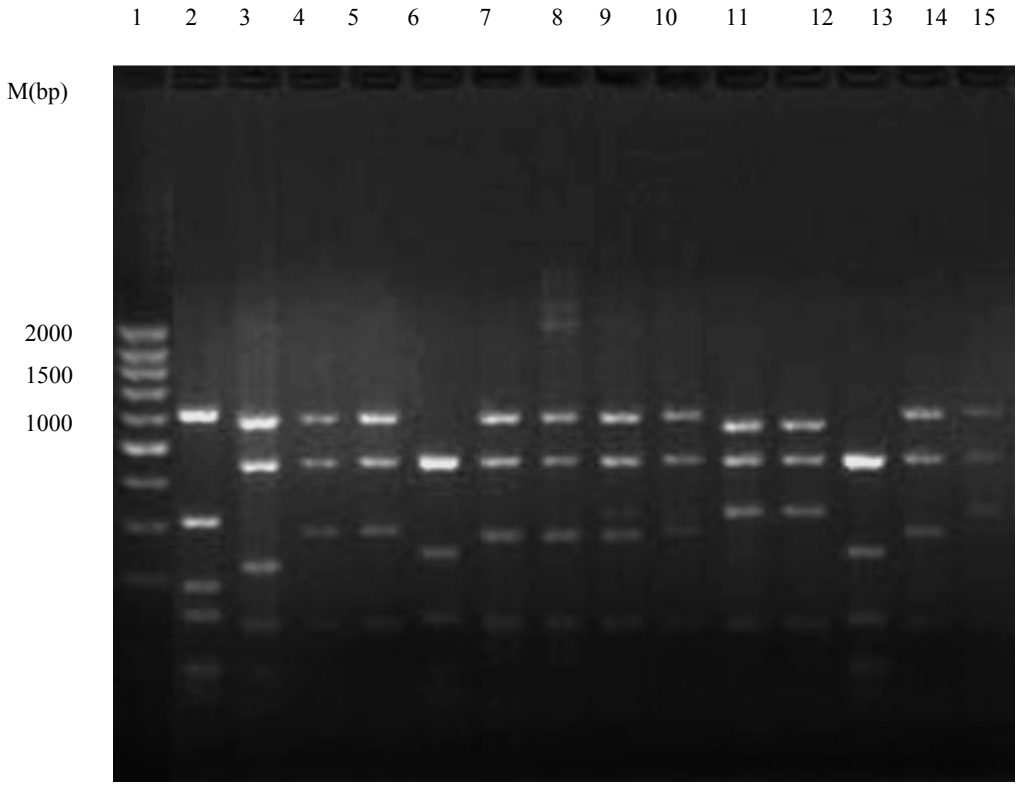


Figure 3.7. *Hae* III digests of Isolates and Reference Strains
1. 100bp DNA ladder Gene Ruler^{TM} **2.** AS17 **3.** AS83 **4.** NRRL 14170 *Lb. reuteri* **5.** NRRL 4524 *Lb. fermentum* **6.** CCM 4833 *Lb. acidophilus* **7.** CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* **8.** CCM 2772 *Lb. delbrueckii* ssp *lactis* **9.** CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus* **10.** CH1 *Lb. casei* **11.** NRRL 2178 *Lb. johnsonii* **12.** NRRL 1954 *Lb. plantarum* **13.** NRRL 4526 *Lb. helveticus* **14.** NRRL 4527 *Lb. brevis* **15.** NRRL 442 *Lb. rhamnosus*

Ten reference strains and two isolates were digested by *Hae* III enzyme to get a restriction profile (Figure 3.7). A dendrogram was created according to this profile (Figure 3.8). From the dendrogram, seven different groups were obtained. AS17 shows 88% homology with the pattern of *Lb. helveticus* while AS83 shows 100% homology with the group consist of *Lb. reuteri* and *Lb. fermentum*.

It could be concluded that PCR-RFLP method by using *Hae* III enzyme revealed good correlation with the phenotypic methods for AS83. Because AS83 had been identified as *Lb. fermentum* by analysing the phenotypic methods.

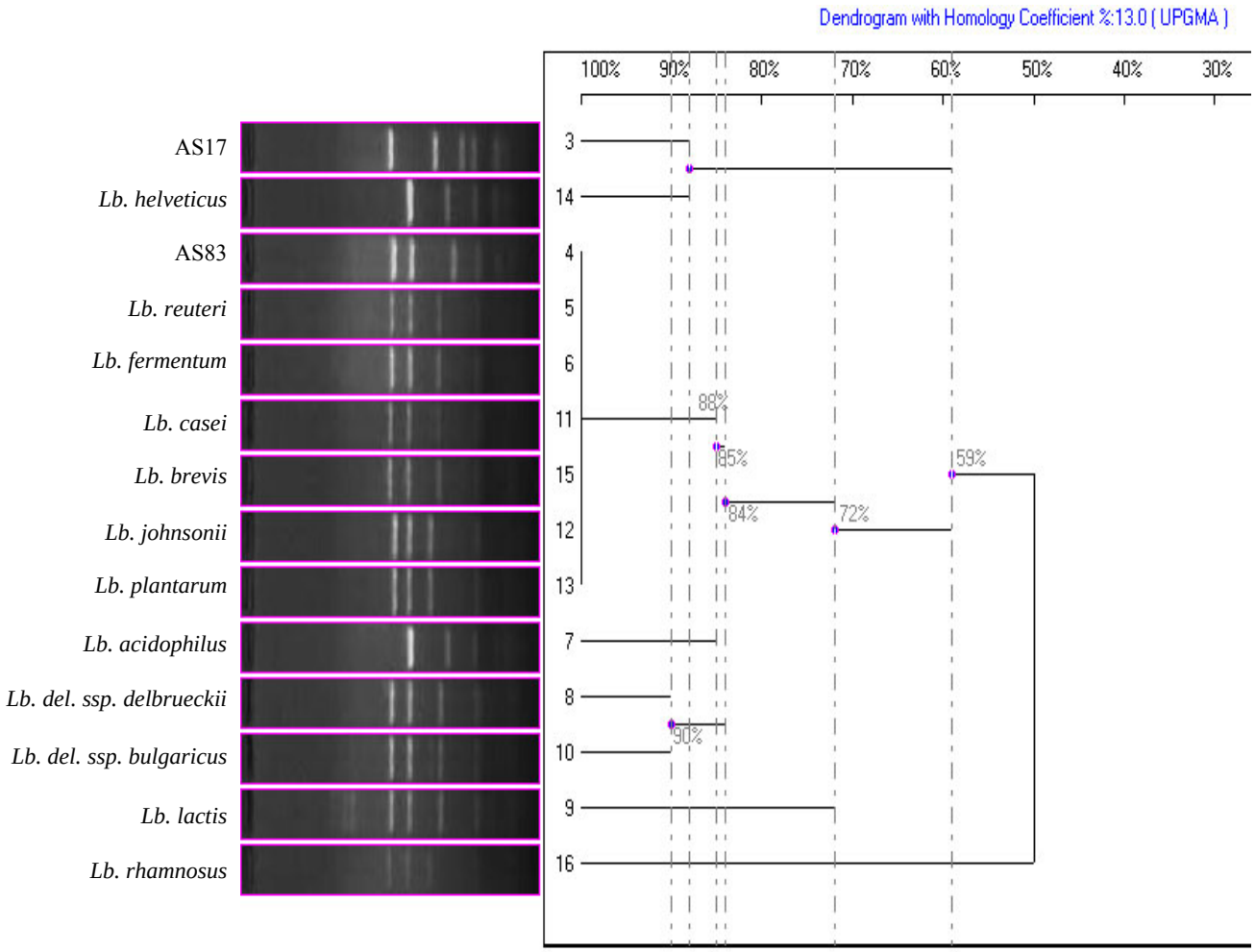


Figure 3.8. Dendrogram of *Hae* III digests of isolates and reference strains

3.4.3.2. *Taq* I digestion

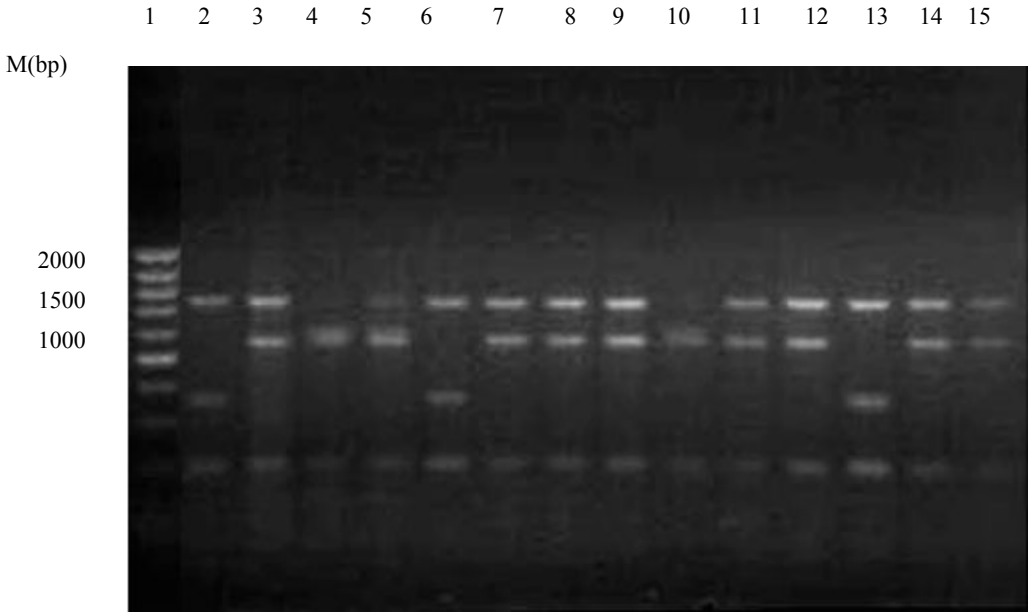


Figure 3.9. *Taq* I digests of Isolates and Reference Strains

1. 100bp DNA ladder Gene Ruler^{TM} **2.** AS17 **3.** AS83 **4.** NRRL 14170 *Lb. reuteri* **5.** NRRL 4524 *Lb. fermentum* **6.** CCM 4833 *Lb. acidophilus* **7.** CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii* **8.** CCM 2772 *Lb. delbrueckii* ssp *lactis* **9.** CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus* **10.** CH1 *Lb. casei* **11.** NRRL 2178 *Lb. johnsonii* **12.** NRRL 1954 *Lb. plantarum* **13.** NRRL 4526 *Lb. helveticus* **14.** NRRL 4527 *Lb. brevis* **15.** NRRL 442 *Lb. rhamnosus*

All reference strains and isolates were digested by *Taq* I enzyme (Figure 3.9). From the dendrogram(Figure 3.10) of *Taq* I digests of reference strains and isolates, it was obtained that AS17 showed 100% homology with *Lb. helveticus* and *Lb. acidophilus* while AS83 showed 100% homology with the group which consist of *Lb. fermentum*.

Additionally to these two enzymes also strains were digested by EcoR I enzyme. But it was observed that *EcoR* I was not suitable for these strains.

In the conclusion of PCR-RFLP studies *Hae* III and *Taq* I enzyme digesitons showed good correlation with phenotypic methods for AS83 isolates. The number of reference strains was not sufficient to identify the isolate AS17, so phenotypic and molecular methods gave different results. Also different enzymes could be used to differentiate the reference strains more sufficiently.

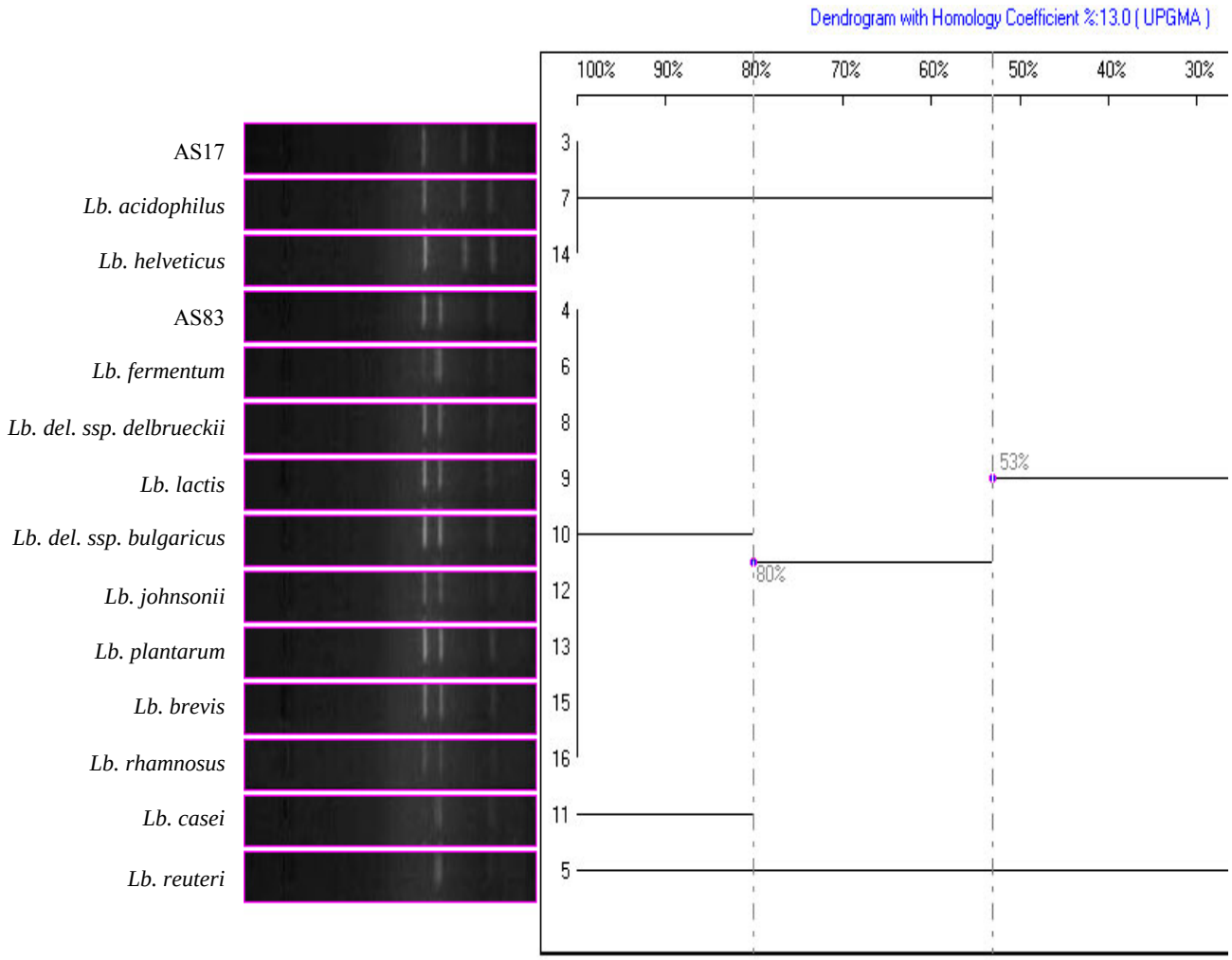


Figure 3.10. Dendrogram of *Taq* I digests of isolates and reference strains

3.4.4. Sequencing of Isolates

Because of the absence of some reference strains, isolates were identified by 16S DNA seguencing. Isolates were sequenced at REFGEN(Gen Araştırma ve Biyoteknoloji Ltd. Şti). Acccording to seguencing results AS17 showed 100% homology with *Lactobacillus oris* and AS83 showed 100% homology with *Lactobacillus fermentum* (Figure 3.11). AS17 also showed genetically similarities with *Lb. antri*, *Lb. panis*, *Lb. vaginalis* and *Lb. reuteri*. The sequences of isolates are given in Appendix G.

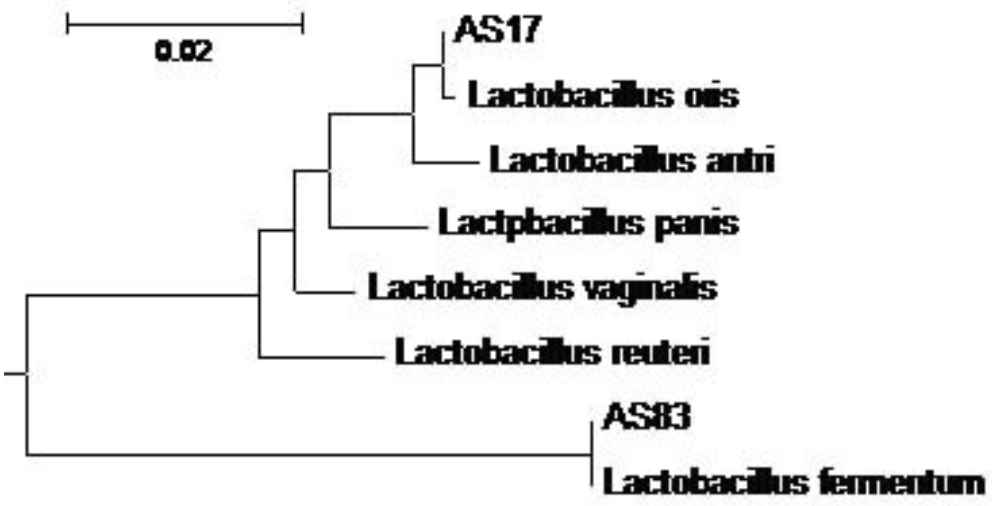


Figure 3.11. Philogenetic tree of isolates

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVE

Characterization and determination of probiotic properties of Lactic Acid Bacteria isolated from human milk was the aim of this study. To determine the probiotic properties different tests were applied such as resistance to low pH and bile salt and antimicrobial activity tests. After the determination of potential probiotic isolates, these isolates were characterized by phenotypic and genotypic methods. For the phenotypic characterization, morphologic examination, resistance to different temperatures and salt concentrations, gas production from glucose, ammonia production from arginine, determination of sugar fermentaiton profiles were applied. For molecular identification, ARDRA based on 16S rRNA gene was performed and 16S DNA sequencing was applied to support the ARDRA method. Finally the following results were obtained;

1. Lactic Acid Bacteria were isolated from human milk.
2. Probiotic properties of isolated bacteria were determined. Only 3 of them showed resistance to low pH, tolerance to bile salt, antimicrobial activity against some indicator microorganisms.
3. Phenotyic and genotypic identifications were effectively diferentiate the isolates especially sugar fermentation patterns support the genotypic characterization results. Two of them was determined that they could be potential probiotic strains even if some forward tests were applied.

In this study the first step was taken to use the isolates as cultures for probiotic products. The main criteria of being probiotic strains were determined and the selected isolates were identified. Therefore some future studies should be performed to use these isolates reliably. It will be beneficial to test the following characteristics;

1. Adhesion to mucosal surface.
2. Clinical studies for human health.
3. Technological properties (strain stability, viability in products, bacteriophage resistance).
4. Antibiotic resistance.

REFERENCES

Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A., Ragni, A., and Gobbetti, M. 2006. Selection of potential probiotic Lactobacilli from pig feces to be used as additives in pelleted feding. *Research in Microbiology.* 157:792-801.

Brady, L.J., Gallaher, D.D., Busta, F.F. 2000. The role of probiotic cultures in the prevention of colon cancer. *The Journal of Nutrition.* 130:410S-414S.

Bulut, Ç. 2003. Isolation and molecular characterization of lactic acid bacteria from cheese. *IYTE Thesis of Ms.*

Çakır, İ. 2003. Determination of some probiotic properties on Lactobacilli and Bifidobacteria. *Ankara University Thesis of Ph.D.*

Cardinal, M.J., Meghrous, J., Lacroix, C., Simard, R.E., 1997. Isolation of *Lactococcus lactis* strain producing inhibitory activity against *Listeria*. *Food Biotechnology* 11:129.

Castagliuoluolo, I., Rıegler, M.F., Valenick, L., Lamont, J.T., and Pothoulakis, C. 1999. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins a and b in human colonic mucosa. *American Society for Microbiology* 67(1):302-307.

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J.K. 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *International Journal of Food Microbiology* 35:1-27.

Chou, L.S and Weimer, B. 1999. Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. *Journal of Dairy Science* 82:23-31.

Chuayana, Jr. E.L., V. Ponce, C., Rivera, M.R.B., Cabrera, E.C. 2003. Antimicrobial activity of probiotics from milk products. *Phil J. Microbiol. Infect. Dis.* 32(2):71-74.

Chung, H.S., Kim, Y.B., Chun, S.L., and Ji, G.E. 1999. Screening and selection of acid and bile resistant Bifidobacteria. *International Journal of Food Microbiology* 47:25-32.

Coeuret, V., Dubernet, S., Bernardeau, M., Gueguen, M., Vernoux, J.P., 2003. Isolation, characterization and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait* 83:269.

Collins, J.K., Thornton, G., Sullivan, G.O. 1998. Selection of probiotic strains for human applications. *International Dairy Journal* 8:487-490.

Dugas, B., Mercenier, A., Lenoir-Wijnkoop, I., Arnaud, C., Dugas, N., and Postaire, E. 1999. Immunity and probiotics. *Trends Immunology Today* 20(9):387-390.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O'Sullivan, G.C., Shanahan, F., and Collins, J.K. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie van Leeuwenhoek* 76:279-292.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., C O'Sullivan, G., Shanahan, F., and Collins J.K., 2001. In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. *Am. J. Clin. Nutr.* 73:386S-392S.

Favier, C.F., Vaughan, E.E., De Vos, W.M., Akkermans, A.D.L. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ Microbiol*ogy 68:219-226.

Fooks, L.J., Fuller, R., Gibson, G.R. 1999. Prebiotics, probiotics and human gut microbiology. *International Dairy Journal* 9:53-61.

Fuller, Roy. 2007. History and development of probiotics, http://www.albertcllasic.net/probiotics.php (accessed June 3, 2007).

Fuller, Roy. 2007. Probiotics for farm animals, http://www.albertcllasic.net/probiotics.php (accessed June 3, 2007).

Gismondo, M.R., Drago, L., Lombardi, A. 1999. review of probiotics available to modify gastrointestinal flora. *International Journal of Antimicrobial Agents.* 12:287-292.

Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B. and Morelli, L. 2005. Should yoghurt cultures be considered probiotic?. *British Journal of Nutrition.* 93:783-786.

Hammes, W.P., Vogel, R.F., 1995. The genus *Lactobacillus*. *The Genera of Lactic Acid Bacteria.* United Kingdom: B.J.B. Wood Blackie Academic and Professional.

Heikkila, M.P. and Saris, P.E.J. 2003. Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *Journal of Applied Microbiology* 95:471-478.

Hirayama, K., and Rafter, J. 2000. The role of probiotic bacteria in cancer prevention. *Microbes and Infection* 2:681-686.

Holzapfel, W.H., Haberer, P., Snell, J., Schillinger, U., Huis in't Veld, J. 1998. Overview of gut flora and probiotics. *International Journal of Food Microbiology* 41:85-101.

Jacobsen, C.N., Nielsen, V.R., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandström, B., Tvede, M., and Jacobsen, M. 1999. Screening of probiotic activities of forty seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology* 65(11):4949-4956.

Karaoğlu, Ş. A., Aydın, F., Kılıç, S. S., Kılıç, A.O. 2003. antimicrobial activity and characteristics of bacteriocins produced by vaginal Lactobacilli. *Turk J Med. Sci.* 33:7–13.

Kaur, I.P., Chopra, K., Saini, A. 2002. Probiotics: Potential pharmaceutical applications. *European Journal of Pharmaceutical Sciences* 15:1-9.

Klaenhammer, T.R. 2000. Probiotic Bacteria: today and tomorrow. *J. Nutr.* 130:415S-416S.

Klaenhammer, T.R. and Kullen, M.J. 1999. Selection and design probiotics. *International Journal of Food Microbiolog* 50(1):45-57.

Kwon, H.S., Yang, E.H., Yeon, S.W., Kang, B.H., Kim, T.Y. 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex pcr using species-specific primers based on the region extending from 16s rRNA through 23s rRNA. *FEMS Microbiology Letters* 239:267-275.

Lei, V., and Jacobsen, M. 2004. Microbiological characterization and probiotic potential af koko and koko sour water african spontaneously fermented millet porridge and drink. *Journal of Applied Microbiolog* 96:384-397.

Lin, M., Savaiano, D. and Harlender, S. 1991. Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. *Journal of Dairy Science* 74: 87-95.

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E. 2005. Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal* 16:189-199.

Martin, R., Langa, S., Reviriego, C., Jimenez, E., Marin, L.M., Olivares, M., Boza, J., Jimenez, J., Fernandez, L., Xaus, J. and Rodriguez, J.M. 2004. the commensal microflora of human milk: New perspectives for food bacteriotherapy and probiotics. *Trends in Food Science&Technology* 15 :121-127.

Martin, R., Langa, S., Reviriego, C., Jimenez, E., Marin, L.M., Xaus, J., Fernandez, L. and Rodriguez, J.M. 2003. Human milk is a source of lactic acid bacteria for infant gut. *The Journal of Pediatrics* 143:754-758.

Martin, R., Olivares, M., Marin, M.L., Fernandez, L., Xaus, J., Rodriguez, J.M. 2005. Probiotic potential of 3 Lactobacilli strains isolated from breast milk. *J Hum Lact***.** 21(1):8-17.

Mombelli, B., Gismondo, M.R. 2000. The use of probiotics in medical practice. *International Journal of Antimicrobial Agents* 16:531-536.

Moreira, J. L. S., Mota, R. M., Horta, M.F., Teixeira, S. MR., Neuman E., Nicoli J. R., Nunes A.C. 2005. Identification to the species level of *lactobacillus* isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. *BMC Microbiology* 5(15):1-9.

Naidu, A. S., Bidlack, W. R. and Clemens, R. A. 1999. Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition* 39(1):13-126.

Olivares, M., Diaz-Ropero, M.P., Martin, R., Rodriguez, J.M. and Xaus, J. 2006. Antimicrobial potential of four *Lactobacillus* strains isolated from breast milk. *Journal of Applied Microbiology* 101:72-79.

Otero, M.C., Morelli, L., Nader-Macias, M.E. 2006. Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. *Letters in Applied Microbiology* 43:91-97.

Quwehand, A.C., Kirjavainen, P.V., Shortt, C., Salminen, S. 1999. Probiotics: Mechanisms and established effects. *International Dairy Journal* 9:43-52.

Prakash, S. and Jones, M. L., 2005. Artificial cell therapy: New strategies for the therapeutic delivery of live bacteri. *Journal of Biomedicine and Biotechnology* 2005:1 :44-56.

Prasad, J., Gill, H., Smart, J., and Gopal, P.K. 1998. Selection and Characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. *International Dairy Journal* 8:993-1002.

Prentice A. 1996. Constituents of human milk. *Food and Nutrition Bulletin* 17, No.4. http://www.unu.edu/ (accessed June 7, 2007).

Quewand, A.C. and Salminen, S.J. 1998. The health effects of cultured milk products with viable and non-viable bacteria. *International Dairy Journal* 8:749-758.

Quwehand, A.C. and Vesterlund, S.. eds. 2004. Antimicrobial components from lactic acid bacteria. *Lactic Acid Bacteria Microbiological and Functional Aspects*. New York: Marcel Dekker Inc.

Rafter, J. 2003. Probiotics and colon cancer. *Best Practice and Research Clinical Gastroenterology* 17(5):849-859.

Rolfe, R.D. 2000. The Role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 130:396S-402S.

Roos, S., Engstrand L., Jonsson H. 2005. *Lactobacillus gastricus* sp. nov., *Lactobacillus antri* sp. nov., *Lactobacillus kalixensis* sp. nov. and *Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa. *International Journal of Systematic and Evolutionary Microbiology* 55:77-82.

Roy, D., Sirios, S., and Vincent, D. 2001. Molecular discrimination of Lactobacilli used as starter and probiotic cultures by amplified ribosomal DNA restriction analysis. *Current Microbiology an International Journal* 42:282-289.

Salminen, S. 1999. Probiotics: scientific support for use. *Food Technology* 53(11):66.

Salminen, S., Gorbach, S., Lee, Y.K., Benno, Y. 2004. Human studies on probiotics: What is scientifically proven today?. *Lactic Acid Bacteria Microbiological and Functional Aspects*, New York: Marcel Dekker Inc.

Salminen, S., Ouwehand, A., Benno, Y., and Lee, Y.K. 1999. Probiotics: How should they be defined?. *Trends in Food Science&Technology* 10:107-110.

Sanders, M.E. 2003. Probiotics: Considerations for human health. *Nutr. Rev.* 61:91-99.

Scheinbach, S. 1998. Probiotics: Functionality and commercial status. *Biotechnology Advances* 16(3):581-608.

Schrezenmeir, J. and Vrese, M. 2001. Probiotics, prebiotics, and synbiotics – approaching a definition. *Am. J. Clin. Nutr.* 73:361S-364S.

APPENDIX A

CHEMICALS FOR MICROBIOLOGICAL EXPERIMENTS AND MOLECULAR CHARACTERIZATION

Table A.1. Chemicals Used in Microbiological Experiments

No	Chemical	Code
1	MRS Broth	Merck 1.10661
2	M17 Broth	Merck 1.15029
3	Bacteriological Pepton	Oxoid LP037
4	Yeast extract	Merck A 1.03753
5	Lablemco Meat Extract	Oxoid LP029
6	Sodium Acetate	Sigma S2889
7	Agar	AppliChem A0949
8	D(-) Mannitol	AppliChem A1903
9	D(+) Sucrose	AppliChem A2211
10	Fructose-	AppliChem A3688
11	D(-) Salicin	Fluka 84150
12	Esculin	AppliChem A1537
13	Mannose	Aldrich 11,258-5
14	(D+) Raffinose	AppliChem A6882
15	Arabinose	Aldrich A,9190-6
16	Trehalose	Merck 1.08216
17	(D-) Ribose	Merck 1.07605
18	L-Arginine hydrochloride	AppliChem A3709
19	D(+) Glucose	AppliChem A3666
20	D(+) Lactose	Sigma L3750
21	D(+) Maltose Monohydrate	AppliChem A3891
22	D(+) Galactose	Aldrich 11259-3

(cont. on next page)

Table A.1. Chemicals Used in Microbiological Experiments (cont.)

No	Chemical	Code
23	D(+) Xylose	Merck 1.08689
24	D(+)Melesitose	Sigma M5375
25	L Rhamnose	AppliChem A4336
26	Melibiose	Sigma M5500
27	Triammonium citrate	Sigma A1332
28	Sodium Citrate trisodiumsalt	Sigma S4641
29	$MgSO_4.7H_2O$	Merck 1.05886
30	$MgCl_2$	Merck 1.4733
31	$MnSO_4.4H_2O$	Merck 1.02786
32	NaCl	AppliChem A2942
33	K_2HPO_4	Sigma P8281
34	Sodium hydroxide	Merck 1.06498
35	Glyserol-2-phosphate disodium salt	Sigma G6376
36	Tween 80	AppliChem A1390
37	Glycerol	AppliChem A2926
38	Anaerogen	Oxoid AN0025A
39	Safranine	Merck 1.15948
40	Crystal Violet	Sigma C3886
41	Potasium iodide	Sigma C6757
42	Methylene blue	AppliChem A1402
43	Bromcresol purple	Merck 1.03025
44	Mineral oil	Sigma M5904
46	Hyrogen peroxide (%30)	Merck 1.07209
1	Sorbitol	Merck 1.07759
2	L-Cysteine hydrochloride	Fluka 30120
3	Trypticase peptone	BD/BBL 211921
4	Phytone peptone	BD/BLL 211906
5	Nessler's reagent	Merck 1.09028
6	Bile salt	Oxoid LP0055

APPENDIX B

RECIPES FOR CULTURE MEDIA AND BIOCHEMICAL TESTS

B.1. MRS Broth

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.2. MRS Agar

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml

K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15.0
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

B.3. TPY Broth

Ingredients	**g/l**
Trypticase peptone	10.0
Phytone peptone	5.0
Glucose	15.0
Yeast extract	2.5
Tween 80	1 ml
Cysteine HCL	0.5
K_2HPO_4	2.0
$MgCl_26H_2O$	0.5

All the ingredients were suspended into deionized water, and pH was adjusted to 6.5. Then solution was dispensed to the test tubes and autoclaved at 110 °C for 30 min.

B.4. TPY Agar

Ingredients	**g/l**
Trypticase peptone	10.0
Phytone peptone	5.0
Glucose	15.0
Yeast extract	2.5

Tween 80	1 ml
Cysteine HCL	0.5
K_2HPO_4	2.0
$MgCl_26H_2O$	0.5
Agar-agar	0.5%

All the ingredients were suspended into deionized water, and pH was adjusted to6.5. Then solution was autoclaved at 110°C for 30 min.

B.5. Modified MRS Broth for Testing the Growth at Different Temperatures

Ingredients	**g/l**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.6. Modified MRS Broth for Testing the Growth at Different NaCl Concentrations

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
NaCl	20,40,65 for the concentration of 2%, 4% and 6.5%
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

B.7. Modified MRS Broth for Gas Production from Glucose

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0

$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and inverted durham tubes were distributed to each test tube, and lastly sterilized by autoclaving at 121°C for 15 min.

B.8. Modified MRS for Carbohydrate Fermentations

Ingredients	**g/l**
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
Tween 80	1ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromcresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

B.9. Arginine MRS

Ingredients	**g/l**
Peptone	10.0
Yeast extract	5.0
Tween 80	1 ml
K_2HPO_4	2

Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Arginine	1,5
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

APPENDIX C

CARBOHYDRATES USED FOR CARBOHYDRATE FERMENTATION TESTS

Sugar solutions prepared at concentration 10%

1. D(+) Xylose
2. D(-) Ribose
3. Melezitose
4. L(+) Arabinose
5. Mannitol
6. D(+) Trehalose
7. Melibiose
8. Raffinose
9. D(+) Galactose
10. Maltose
11. Sucrose
12. D(+) Mannose
13. Fructose
14. Lactose
15. Rhamnose
16. Sorbitol
17. Glucose

Sugar solution prepared at concentration 5%

18. D(-) Salicin

APPENDIX D

BUFFERS AND STOCK SOLUTIONS FOR MOLECULAR CHARACTERIZATION

D.1. 1M Tris-HCl pH 7.2 and pH 8.0

121.1 g of Tris base was dissolved in 800 ml of deionized H_2O. pH was adjusted to the desired value by adding concentrated HCl. The approximate values of the amount of HCl required for the desired pH values are given below.

pH	*HCl*
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool to room temperature before making final adjustments to the pH, and the volume of the solution was adjusted to 1 L with H_2O. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. It was dispensed into aliquots and sterilized by autoclaving. If the 1 M solution had a yellow color, it was discarded and obtained Tris of better quality.

D.2. 0.5M EDTA pH 8.0

186.1 g of disodium EDTA•$2H_2O$ was added to 800 ml of deionized H_2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with 10N of NaOH (or approx. 20 g of NaOH pellets). Volume was adjusted to 1 L with deionized water. It was dispensed into aliquots and sterilized by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

D.3. 50X TAE

242 g of Tris base was dissolved in deionized H_2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Lastly volume was adjusted to1 L with deionized water.

D.4. 1X TAE

20ml of 50 X TAE buffer was taken and the volume was adjusted to 1 L with deionized water. The 1x working solution was 40 mM Tris-acetate/1 mM EDTA.

D.5. 3M NaCl

175.32g NaCl was dissolved in deionized water and the volume was adjusted to 1 L.

D.6. 5M NaCl

292.2g NaCl was dissolved in deionized water and the volume was adjusted to 1 L.

D.7. Ethidium Bromide Stock Solution (10mg/ml)

0.5g ethidium bromide was dissolved in 50ml deionized water and the solution was storred in dark bottle at room temperature.

D.8. 3M Sodium Acetate pH 5.2

408.3 g of sodium acetate•$3H_2O$ was dissolved in 800 ml of deionized H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 L with deionized H_2O.

D.9. Chloroform-Isoamyl Alcohol Solution

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

D.10. Phenol

Most batches of commercial liquefied phenol are clear and colorless and can be used in molecular techniques without redistillation, however some batches of liquefied phenol are pink or yellow, and these should be rejected. Crystalline phenol was preferred in experiments. First, it was allowed to warm at room temperature and then it was melted at 68°C. Before use, phenol must be equilibrated to a pH of >7.8 because the DNA partitions into the organic phase at acid pH. Gloves, full face protection, and a lab coat should be weared when carrying out this procedure.

To the melted phenol, an equal volume of 0.5 M Tris-Cl (pH 8.0) buffer was added at room temperature. The mixture was stirred on a magnetic stirrer for 15 minutes. When the two phases have separated, the aqueous phase (upper phase) was removed with separation funnel. Then an equal volume of 0.1 M Tris-Cl (pH 8.0) was added to the phenol. The mixture was stirred on a magnetic stirrer for 15 min. The upper aqueous phase was removed as described before. The extractions were repeated until the pH of the phenolic phase is >7.8 (as measured with pH paper).

After the phenol was equilibrated and the final aqueous phase has been removed, phenol was divided to aliquots and 0.1 volume of 0.1 M Tris-Cl (pH 8.0) was added on top of each aliquot. The phenol solution should be stored in this form under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at -20°C. When needed, phenol was melted at room temperature. Hydroxyquinoline (to a final concentration of 0.1%), and ß-mercaptoethanol (to a final concentration of 0.2%) were added before use. Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.

D.11. 1 X TE BUFFER

100mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) was mixed and the buffer was stored at room temperature.

D.12. CTAB/NaCl Solution

4.1g NaCl was dissolved in 80ml deionized water. 10g CTAB was added slowly while heating and stirring. The solution can be heated to 65°C to increase the dissolution. Lastly, the final volume was adjusted to 100ml.

D.13. 10% Sodium Dodecyl Sulfate (SDS)

100g of SDS was dissolved in 900ml of deionized water. Solution was heated to 68°C to dissolve. The pH was adjusted to 7.2 with the addition of a few drops of concentrated HCl. The volume was adjusted to 1L with deionized water.

D.14. Gel Loading Dye

2ml of 10XTBE and 6ml of glycerol was mixed in a falcon and the volume was adjusted to 20ml with sterile deionized water. Bromohenol blue was added until the adequate color was obtained.

APPENDIX E

PCR RECIPES

E.1. PCR Mixture

Mg free Taq DNA polymerase buffer	5µl
$MgCl_2$ (25Mm)	3µl
Sterile deionized water	32µl
Oligo forward 10 picomole/µl	1µl
Oligo reverse 10 picomole/µl	1µl
dNTP (2 mM each)	5µl

E.2. dNTP (10X)

Twenty microliters of each 100mM dATP, dCTP, dGTP, and dTTP are taken and mixed in an eppendorf tube. 920µl of sterile deionized water was added to a final concentration of 2mM. Solution was mixed gently and stored at -20°C.

E.3. PRIMER OF EGE1

590µg primer EGE1 was dissolved in 295 µl of sterile deionized water to obtain 2µg/µl stock solution. 5µl of stock solution were then taken and mixed with 95µl sterile deionized water. The resulting solution had 100µl, 10 picomole /µl concentration. Stock and working solutions were stored at -20°C.

APPENDIX F

RESTRICTION ENZYMES AND OTHER ENZYMES USED FOR MOLECULAR CHARACTERIZATON

F.1 Restriction Enzyme Reaction Mixture

Restriction enzyme buffer	5µl
Sterile deionized water	34.5µl
Restriction Enzyme	0.5µl (from 5U)
DNA	10µl

F.2 Restriction Enzymes Used for Molecular Characterization

Taq I

Hae III

*Eco*R I

APPENDIX G

SEQUENCES OF ISOLATES

Sequence of AS17

GCGGGGGATTACATTTGGAAACAGGTGCTAATACCGCATAACTTGGAAAA
CCACATGGTTTTCCAATAAAAGATGGTTTCGGCTATCACTTTGGGATGGG
CCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAATGA
TGCATAGCCGAGTGAGAGACTGATCGGCCACAATGGAACTGAGACACGGT
CCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAA
GCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAA
CTCTGTTGTTGGAGAAGAACGTGCGTAAGAGTAACTGTTTACGCAGTGAC
GGTATCCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCA
GGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGAAGTG
CATCGGAAACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATG
TGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAAC
AGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGT
TGGAGGGTTTCCGCCCTTCAGTGCCGAAGCTAACGCAGAAAGCACTCCGC
CTGGGGAGTACGACCGCAAGTGG

Sequence of AS83

ACTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTCCGGCACT
GAAGGGCGGAAACCCTCCAACACCTAGCACTCATCGTTTACGGCATGGACT
ACCAGGGTATCTAATCCTGTTCGCTACCCATGCTTTCGAGTCTCAGCGTCAG
TTGCAGACCAGGTAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGC
ATTCCACCGCTACACATGGAGTTCCACTACCCTCTTCTGCACTCAAGTTATC
CAGTTTCCGATGCACTTCTCCGGTTAAGCCGAAGGCTTTCACATCAGACTTA
GAAAACCGCCTGCACTCTCTTTACGCCCAATAAATCCGGATAACGCTTGCC
ACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTT
AAATACCGTCAACGTATGAACAGTTACTCTCATACGTGTTCTTCTTTAACAA
CAGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGTGTTGCTCCATCA
GGCTTGCGCCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTATG
GGCCGTGTCTCAGTCCCATTGTGGCCGATCAGTCTCTCAACTCGGCTATGCA
TCATCGCCTTGGTAGGCCGTTACCCCACCAACAAGCTAATGCACCGCAGGT
CCATCCAGAAGTGATAGCGAGAAGCCATCTTTTAAGCGTTGTTCATGCGAA
CAACGCTGTTATGCGGTATTAGCATCTGTTTCCAAATG