ISOLATION, CHARACTERIZATION, AND SCREENING PROBIOTIC PROPERTIES OF ARTISANAL YOGHURT STARTER STRAINS FROM URLA REGION

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ABSTRACT

ISOLATION, CHARACTERIZATION, AND SCREENING PROBIOTIC PROPERTIES OF ARTISANAL YOGHURT STARTER STRAINS FROM URLA REGION

Probiotics are bacteria that help to maintain the natural balance of microflora in the intestine. The largest group of probiotic bacteria in the intestine is lactic acid bacteria, found in yoghurt with live culture. In this study, 13 different artisanal yoghurt samples were collected from Urla region to isolate artisanal starter strains. These isolated strains were firstly identified and characterized by using basic biochemical, physiological and probiotic characterization methods. Among 453 LAB strains in total, 5 *Streptecoccus thermophilus* and 26 *Lactobacillus delbrueckii* ssp. *bulgaricus* isolates resulted as probiotic candidates. PCR-RFLP and PFGE-RFLP methods were used for the differentiation of probiotic candidates. Probiotic features of strains were screened by using bile salt tolerance, bile salt deconjugation, and cholesterol assimilation, transit tolerance to gastrointestinal tract, antibiotic resistances, autoaggregation, cell surface hydrophobicity, antimicrobial activities, adhesion abilities, and growth capacity with prebiotics. The results showed that, the isolated strains gave different properties against these tests; but all of them were acid and bile resistant, had ability to adhesion Caco-2 cell lines, and grown very well with prebiotic sources. They had also antimicrobial activity against *Escherichia coli* and *Listeria innocua*. Further studies were conducted; probiotic yoghurts were produced by using probiotic candidates. Sixteen starter combinations were made using the two cocci and eight bacilli isolates, and thus these yoghurt products were characterized by using physical, chemical, rheological, and organoleptic methods. Acetaldehyde content of yoghurt samples varied between 5.61 and 15.38 mg/L and apparent viscosity values ranged from 261 and 608 m Pa.s. Hardness value of samples ranged from 3.81 and 6.71 N. Yoghurt starter having probiotic features is an advantage for dairy industry to produce large amount of functional yoghurt. In this study, a good number of cocci and bacilli were paired as probiotic/starter strain combinations that could perfectly be used for the production of functional yoghurt for dairy industry.

ÖZET

URLA BÖLGESİ DOĞAL YOĞURT STARTER SUŞLARININ İZOLASYONU, KARAKTERİZASYONU VE PROBİYOTİK ÖZELLİKLERİNİN TARANMASI

Probiyotikler bağırsak mikroflorasının doğal dengesini korumaya yardımcı bakterilerdir. Bağırsak mikroflorasındaki en büyük probiyotik grubunu yoğurtta canlı olarak bulunan laktik asit bakterileri oluşturur. Bu çalışmada, doğal starterleri izole etmek için Urla Bölgesi'nden 13 faklı doğal yoğurt örneği toplanmıştır. İzole edilen suşlar ilk olarak biyokimyasal, fizyolojik metotlar ve temel probiyotik özellikler kullanılarak tanımlanıp, karakterize edilmiştir. Dört yüz yetmiş üç adet LAB suşu arasından, toplamda 5 kok ve 26 basil izolatı probiyotik adayı olarak belirlenmiştir. Probiyotik adayların ileri tanımlanması için PCR-RFLP ve PFGE-RFLP metotları kullanılmıştır. Suşların probiyotik özellikleri; safra tuzu toleransları, safra tuzunu dekonjuge etmeleri, kolesterolü düşürmeleri, gastrointestinal sistemden geçiş toleransları, antibiyotik dirençlilikleri, kendi kendine yığın oluşturmaları, hücre yüzey hidrofobikliği, antimikrobiyal aktiviteleri, yapışma kabiliyetleri ve prebiyotikle birlikte büyüme kapasitelerine göre belirlenmişitir. Sonuçlara göre, suşlar probiyotik özellikler açısından farklı özellikler göstermelerine rağmen hepsi düşük aside ve safra tuzlarına dayaklı olup Caco-2 hücrelerine yapışma özelliği göstermip, prebiyotikli ortamda oldukça iyi büyümektedirler. Ayrıca *Escherichia coli* ve *Listeria innocua* üzerinde antimikrobiyal etkiye sahip oldukları belirlenmiştir. Sonuç olarak probiyotik adaylar kullanılarak probiyotik yoğurtlar üretilmiştir. İki kok ve sekiz basil izolatı kullanılarak on altı yoğurt kombinasyonu oluşturulmuş, bunlar fiziksel, kimyasal, reolojik ve duyusal olarak karakterize edilmiştir. Yoğurt örnekleri arasında asetaldehit miktarı 5.61-15.38 mg/L arasında ve görünür viskozite sonuçları 261-608 m Pa.s. arasında bulunmuştur. Sertlik sonuçları ise 3.81-6.71N arasında değişmektedir. Probiyotik özelliklere sahip yoğurt starterleri büyük hacimde fonksiyonel yoğurt üretiminde süt endüstrisi için avantaj oluşturmaktadır. Bu çalışmada, kok ve basil probiyotik/stater suş kombinasyonları fonksiyonel yoğurt üretiminde kullanılmak üzere eşleştirilmiştir. Bu kombinasyonlar süt endüstrisi için probiyotik starter karışımları olarak önerilmektedir.

It is dedicated to my mother;

Nigar OKUKLU

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CHAPTER 1

INTRODUCTION

Lactic acid bacteria (LAB) have been used for the production, as starters, and for the improvement, as probiotics, of fermented foods. Probiotic bacteria have often been used as functional food for the reestablishment of beneficial microflora in the human gastrointestinal tract.

Probiotic lactic acid bacteria are expected to harbour the following biochemical characteristics: they should be safe to human health; tolerant to harsh physiological conditions of gastrointestinal system, such as extreme acid or salt environment, be able to adhere to the intestinal epithelium, and keep acceptable levels of viability while passing along the tract and an acceptably long shelf life. They are also expected to exert antimicrobial activity against pathogenic microorganisms (Lin, et al. 2006; Musikasang, et al. 2009).

The following guidelines are proposed for evaluating probiotics in food that could lead to the substantiation of health claims: Identification of the genus and species of the probiotic strain by using a combination of phenotypic and genotypic tests, in vitro testing to delineate the mechanism of the probiotic effect, substantiation of the clinical health benefit of probiotic strains with human trials (Narayanan 2013).

Large scale production of probiotics have recently been dramatically increased worldwide, and marketing have often been achieved in the form of industrial yoghurts, supplemented with a consortium of probiotic strains which belong to diverse genera and species of LAB.

Yoghurt, as food, is an excellent source of protein, calcium, and vitamins [riboflavin (vitamin B2), thiamin (vitamin B_1), and vitamin B_{12}]. It is also a valuable source of zinc, magnesium, and folate. Yoghurt fermentation involves the use of two of the LAB species, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophiles* in combination. There is a symbiotic relationship between these organisms: growth of *S. thermophilus* is promoted by amino acids that are produced by *Lb. delbrueckii* ssp. *bulgaricus* during the early stages of fermentation; and, in turn,

organic acids, produced by *S. thermophilus*, promote the growth of *Lb. delbrueckii* ssp. *bulgaricus.*

The traditional use of microorganisms as starters in food process has been subject to little or no regulation. However, probiotic microorganisms represent a novel application and may be consumed as self-medication by consumers. The safety of these microorganisms is of interest food industry. There are no established or validated testing criteria to determine the safety of microorganisms. This situation has created a requirement for regulation of probiotics (Wright 2005).

In Europe, traditional starters are classified as food ingredients and processing aids or additives. If classified as an ingredient, they should be listed within the additive list of end products. When the probiotic cultures are incorporated in food, they are classified as food supplements. To date, it is only in Danish Veterinary and Food Administration, Denmark, must be notified by the manufacturer prior to the use of new strains. The Danish statue on food additives lists the necessary documents, including microbiological and toxicological investigations but no specific tests are listed (Saarela et al. 2008)

In the U.S. the use of probiotics can result in following regulatory categories: food or food ingredients; medical food; dietary supplements; and drug or biological products. According to the Food and Drug Administration, a probiotic product intended for use as a drug is also a biological product (Narayanan 2013).

For a substance to be considered as Generally Recognized as Safe (GRAS), it must be well defined. For example, *Lb. bulgaricus* that had been traditionally used to fermented foods and has been consumed in food at levels of 1010-1011 live microorganisms/day would likely qualify a GRAS use (Narayanan 2013).

Reference system for functional foods in Japan is Foods for Specified Health Use (FOSHU). They are specific foods for which the manufacturers or sellers have provided sufficient scientific justification of specific functions can use approved health claims and the FOSHU logo on their labels.

According to the current review on regulation of the category, functional foods, probiotics still exist in grey area. International trade in these products continues to grow and they need to develop an appropriate regulatory framework to control these products and to prevent the consumer deception. The following scientific recommendations and regulatory recommendations need consideration. (i)Microorganisms must be able to confer defined health benefits on human, if the microorganisms are termed a probiotic.

(ii) In terms of quality control, good manufacturing practices must be applied. (iii) The regulatory status of probiotics as a component of foods should be established on an international level (Narayanan 2013).

1.1. Thesis Objectives

The aim of the study is production of yoghurt starter mixture having probiotic properties. Generally, probiotic yoghurts are made with starters and addition of probiotic bacteria such as *Lactobacillus acidophilus* or *Bifidobacterium bifidus*. The cost of the production and end product will increase if the plants use this type of process. Using starter with probiotic properties eliminates these problems. Viability of the probiotics during the storage is an important parameter for probiotic food. Using starters having probiotic characteristics eliminates the viability problem during storage.

The main goals of the thesis are threefold:

I. Isolation and identification of novel LAB strains,
II. Characterisation of most desired probiotic properties,
III. Use of the characterised strains in yoghurt production.

Biochemical methods included carbohydrate fermentation, the presence of proteinase, gelatinase, urease and β-galactosidase activities, and indole production. Physiological characterisation was performed using a range of NaCl concentration and temperature. Molecular methods included PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and PFGE (pulsed field gel electrophoresis) of genomic DNA.

Probiotic properties were screened by growing the isolated strains in simulated gastric and intestinal juice, and in the presence of antibiotics. Adhesion capacity, onto the epithelial cells and auto-aggregation, and surface hydrophobicity, were also determined. Selected combinations of isolated *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* strains were used for yoghurt production. The products were then characterised in terms of texture and aroma.

1.2. Lactic Acid Bacteria (LAB)

Fermentation, especially lactic acid fermentation, is an old invention and has traditionally been used worldwide. The aim was preservation, during storage and transportation, of perishable foods such as milk, meat, certain cereals, and vegetables. The microbial nature of fermentation was first revealed in 1857 by Louis Pasteur.

LAB produce lactic acid by fermenting carbohydrates. They are typically known as non-spore forming, non-motile, Gram-positive, catalase-negative, and acid tolerant bacteria.

The term LAB is used synonymously with "milk souring organisms." Important progress in the classification of these bacteria was made when the difference between milk-souring bacteria and other lactic acid producing bacteria of other habitats was recognized (Axelsson, 1993).

The name 'lactic acid bacteria' essentially specifies those microorganisms causing the coagulation of milk by fermentation of lactose to lactic acid. The family name *Lactobacteriaceae* was annotated by Orla-Jensen (1919) to a physiological group of bacteria producing lactic acid alone or acetic and lactic acids, alcohol, and carbon dioxide. Today, almost all LAB genera are confined within the family of Lactobacteriaceae (Breed et al., 1957).

Lactic acid bacteria consist of diverse genera and are grouped according to the fermentation end-products. Homofermenters use homolactic fermentation via Embden-Meyerhof-Parnas (EMP) pathway (Figure 1.1.). Lactic acid is the major end-product for homolactic fermentation. LAB producing carbondioxide, ethanol, and acetic acid besides lactic acid are called heterofermenters. These use pentose-monophosphate pathway (Figure1.2). Only the homofermentative LAB possess the key enzyme aldolase, which hydrolyses glucose to lactic acid.

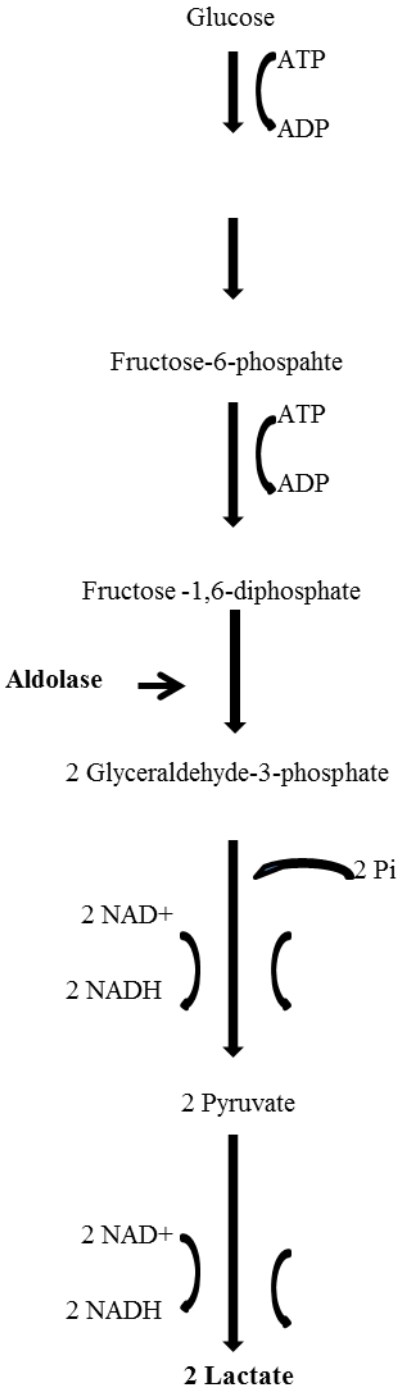

Figure 1.1. The pathway of homolactic fermentation.

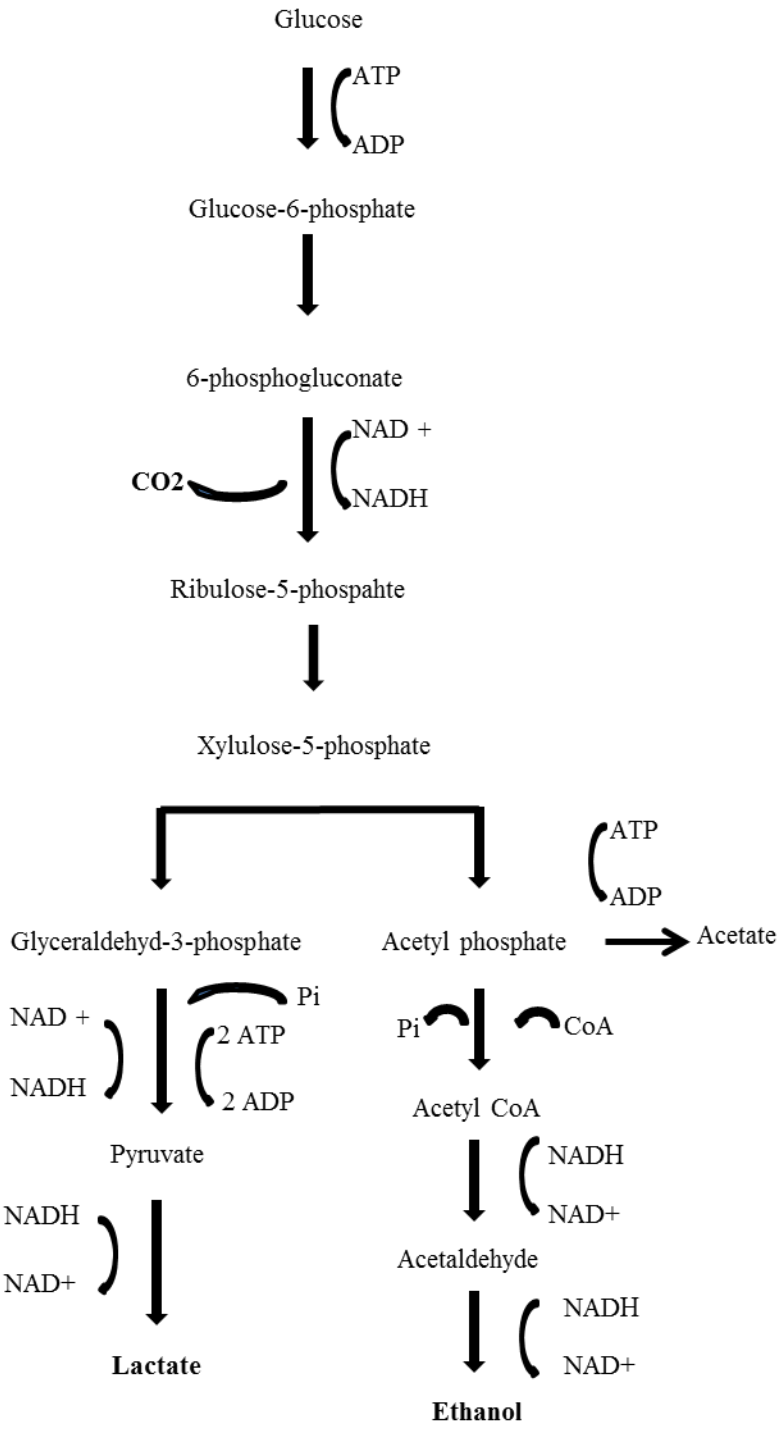

Figure 1.2. The pathway of heterolactic fermentation.

LAB are generally associated with rich nutrient habitats like milk, meat, or vegetables, but some are also members of the normal flora of mouth, intestine, and vagina of mammals. The known LAB genera, so far, are *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Some of the rod-shaped lactic acid

bacteria, previously included in *Lactobacillus*, have now been included in the genus *Carnobacterium* (Bergey's Manual, 1986; Collins et al., 1987).

1.3. Starter Lactic Acid Bacteria

Fermentation is basically a microbial process and all of the fermented foods are produced by microorganisms such as LAB, mould, or yeast. Among these, LAB are the most important and common bacterial group used for the production of fermented dairy products. They are normally inoculated into milk and are called as starter culture, reducing the pH of milk below 4.6 within an industrially accepted period of fermentation time (Beresford et al., 2001). Starter strains can be mesophilic, thermophilic (Mayra-Makinen and Bigret, 1998).

1.3.1 Mesophilic Starters Cultures

The growth temperatures of mesophilic starters range from10 to 40 ºC, and their optimum growth temperature is around 30 ºC. *Lactococcus lactis* and its three sspecies (*L. lactis, L. cremoris,* and *L. diactylactis*) are classified into this group, and they are known as acid producing microorganisms. Two of the *Leuconostoc* species (*lactis* and cremoris) are also recognized as mesophilic starters but they are used for flavour, not for coagulation.

Mesophilic starters can also be divided into four different subgroups by the type of flavour they produce: (1) O Type: Non-flavor producers, *Lactococcus lactis* ssp. *lactis* and *cremoris*; (2) D Type: Citrate fermeter *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*; (3) B or L Type: Citrate fermenter *Leuconostoc* species; and (4) BD or LD Type: Includes aroma forming species, both *Leuconostoc* species and *Lc. lactis* ssp. *lacti*s biovar. *diacetylactis* (Mayra-Makinen and Bigret, 1998). Mesophilic starters are generally used for the production of many types of cheese.

1.3.2. Thermophilic Starter Cultures

Some of the species of the *Lactobacillus* and *Streptecoccus* genera can be thermophilic, with an optimum growth temperature of 42 ºC.

The genus Lactobacillus includes both homofermentative and heterofermentative species. Homofermentative species are used for the initiation of fermentation, and heterofermentative species are used for flavour formation.

Streptococcus thermophilus is the only food associated streptococci. It is used for yoghurt production, in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus* (Robinson, 2002).

1.4. Yoghurt Starters

A starter culture can be simply a sample of fermented food. These types of starters are called artisanal or undefined cultures. They contain historically selected combinations of starter organisms. But the actual identities of the organisms present are not known, and the individual species are not characterized microbiologically or biochemically. The proportion of different organisms in a mixed culture may not be constant from one product to another. Thus the main disadvantage of artisanal cultures is that they may yield products of inconsistent quality. In addition, fermentation rates may vary from day to day, affecting production schedules. In large production facilities, where precise schedules are essential and consistent product quality is expected, artisanal starters cannot be used (Durso and Hutkins, 2003).

Defined starters are identified by physiological, biochemical, and genetically characterisation. Most of the defined strains have been isolated from wild or artisanal fermentation products (Hebert et al., 2000). Hence they are expected to maintain quality, and be flexible to be used for the modification of the product when needed.

1.4.1. *Lactobacillus delbrueckii* ssp. *bulgaricus*

Lactobacillus delbrueckii ssp. *bulgaricus* is a member of *Lb. delbrueckii* species. Previously, three separate species with similar phenotypes were included within this

species. These were *Lb. delbrueckii, Lb. lactis,* and *Lb. bulgaricus* (Limsowtin et al., 2002).

Lb. delbrueckii ssp. *bulgaricus* belongs to a group of lactobacilli related to *Lb. acidophilus, Lb. johnsonii,* and *Lb. gasseri.* A combination of these species are formed and named as the acidophilus complex, and used as a probiotic formulation. *Lb. delbrueckii* is considered to be unique within this group because of their atypical G-C content, which is 49.7% (Gutche et al., 2006).

Lb. delbrueckii ssp. *bulgaricus* can be found as single cell or as a chain of three or four cells. It is a facultative anaerobe and very sensitive to O_2 exposure. It is catalase negative because it lacks the cytochromes. Its cardinal temperatures are as follows: optimum growth at 45°C; minimum and maximum, 22°C and 53°C, respectively. It ferments lactose, fructose, and glucose. Its major end-product of fermentation is D (-) lactic acid. *Lb. delbrueckii* ssp. *bulgaricus* cannot utilise arginine.

1.4.2. *Streptococcus thermopilus*

Streptococcus thermophilus has been reclassified as *S. salivarius* ssp. *thermophilus.* It forms relatively longer chains, including 10 or 20 cells. It is facultative anaerobe, catalase negative, homofermentative, and it produces L (+) lactic acid as the major fermentation end-product. *Streptococcus thermophilus* has an optimum growth temperature around 45°C, and it survives at 60°C for 30 min.

The genome of *S. thermophilus* is approximately 1.8 Mb, making it among the smallest genomes of all lactic acid bacteria. The genome of *S. thermophilus* contains approximately1, 900 genes and 1,500 of them are known as orthologous. It means that *S. thermophilus* have common physical and cellular properties with pathogenic streptococci. The G-C content of *S. thermophilus* is low, 40 % (Guetche et al., 2006).

1.4.3. Synergistic Growth of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*

S. thermophilus and *Lb. delbrueckii* ssp. *bulgaricus* have been used together to ferment milk to yoghurt. The synergistic relationship is based on the metabolic

compatibility between the two species. Studies have shown that combined culture of these bacteria produces much higher acidity, >10g/L within 4 h. When alone, *S. thermophilus* produces 4g/L and *Lb. delbrueckii* ssp. *bulgaricus* produces 2g/L (Robinson, 2002).

S. thermophilus grows much faster than *L. delbrueckii* ssp. *bulgaricus*. *S. thermophilus* also releases CO_2 from the breakdown of urea and formic acid. It uses up oxygen in the medium, which leads the oxidation-reduction potential be much more favourable for the growth of *L. delbrueckii* ssp. *bulgaricus*. The increased acidity (around pH of 5.4), CO_2, and formic acid, stimulate the growth of *L. delbrueckii* ssp. *bulgaricus*. In turn, *Lb. delbrueckii* ssp. *bulgaricus* secretes extracellular proteinases which hydrolyse casein and other milk proteins, and produces the amino acids, including valine, essential for the growth of *S. thermophilus*. The optimum temperature for the symbiotic growth is 42°C (Shah, 2003).

1.5. Characterization of Starters

1.5.1 Biochemical Characterization

Carbohydrate fermentation profile of *S. thermophilus* is highly variable (Table 1.1.) (Vin et al., 2005).

Table 1.1. Main biochemical characteristics of *S. thermophiles* (Source: Erkus, 2007)

Peptidoglycan type	Lysine-Alanine	**Mannitol**	-
G-C Content (mol %)	37-40 %	**Mannose**	+/-
Growth (15-45 ºC)	-/+	**Mellibiose**	D
NH_3 from Arginine	-	**Raffinose**	D
Cellobiose	-	**Sucrose**	+
Galactose	D	**Fructose**	+
Lactose	+	**Glucose**	+
Maltose	-/+	**Sorbitol**	-

Symbols: (+): 90% or more strains are positive, (-): 90% or more strains are negative, (D):11-89% of strains are positive

Lactobacillus delbrueckii consists of three different sspecies; *Lb. delbrueckii, Lb. bulgaricus*, and *Lb. lactis*. The natural habitat of *Lb. delbrueckii* ssp. *bulagricus* and *lactis* is milk. *Lb. delbrueckii* ssp. *delbrueckii* can grow on vegetables. Their carbohydrate fermentation patterns also differ (Table 1.2). For example, lactose is not utilized by *Lb. delbrueckii* ssp. *delbrueckii*, when compared to *Lb. delbrueckii* ssp. *bulgaricus*, and to *Lb. lactis* (Germond et al., 2003).

Table 1.2. Biochemical classification characteristics of *Lb. delbrueckii*
(Source: Erkus, 2007)

Strain type	G-C Content	Growth at 15-45 °C	NH_3 from Arginine	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Mellibiose	Raffinose	Salicin	Sucrose	Trehalose	Fructose	Glucose	Sorbitol	Esculine
Lb. delb.* ssp. *bul.	49-51%	-/+	-	-	-/D	+	+/-	-	-/+	-	-	-	-	-	+	+	+	+
Lb. delb.* ssp. *delb.	49-51%	-/+	D	D	-	-	D	-	+	-	-	-	+	D	+	+	+	-
Lb. delb.* ssp. *lactis	49-51%	-/+	D	D	D	+	D	-	+	-	-	+	+	+	+	+	+	+

Symmbols; (+):90% or more strains are positive; (-):90% or more strains are negative; (D): 11-89% or strains positive

1.5.2. Molecular Characterization

Classical identification is generally performed by using basic biochemical and microbiological methods, such as morphology, carbohydrate fermentation profile, growth ability at differing temperature range, and nutritional requirements (Morata et al., 1999). These methods can produce phenotypic information that may not be stably expressed under certain environmental conditions; thus, this information cannot be sufficient for the differentiation of closely related microorganisms (Busch and Nitschko, 1999). Furthermore, artisanal cultures generally display atypical biochemical behaviours (Milliere et al., 1996).

Reproducibility, discriminatory power, and ease of interpretation of data obtained are the major criteria for strain characterization. Reproducibility is to be able to produce the same typing results on the same organism. Techniques having high discriminatory power can distinguish closely related strains, at species or sspecies level. Molecular methods generally display such high discriminatory powers (Farber, 1996).

There are a number of molecular classification methods which include hybridization with species-specific probes or the generation of specific DNA fragments by PCR (polymerase chain reaction) (Klaenhammer and Kullen, 1999). Such methods are easy to perform and highly versatile molecular typing tools (Bulut, 2003). Here, 16S ribosomal RNA (rRNA) is often used as the phylogenetic marker. Because ribosomes are the universal organelles in the biosphere, and the composition of them has remained almost unchanged during evolution.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has often been used for the molecular characterization of yoghurt starters. Here, 16S rRNA gene is first amplified using specifically designed primers, amplification products (amplicons) are then cut with suitable restriction enzymes to produce a specific finger print. Genomic DNA manipulations can also be used in the identification of environmental strains. These include techniques of high discriminatory power, such as PFGE (pulsed field gel electrophoresis) (Tanskanen et al., 1990; Tenover et al., 1995).

CHAPTER 2

PROBIOTICS

2.1. Definition and History of Probiotics

Probiotics are live microorganisms and are thought to be beneficial for the health of host organism. They are also called friendly bacteria or good bacteria.

The word “probiotic” comes from Latin preposition “pro” and the Greek adjective “biotic” derived from the noun “bios” which means life (Reid et al., 2003; Vouloumanou et al., 2009). Metchnikoff (1907) was the first who demonstrated the beneficial effects of probiotics on human health, and then developed the theory that the presence of lactic acid bacteria in the intestines could control infections, resulting from pathogenic microorganisms, and help control toxin-producing bacteria. Metchnikoff also proposed that acid-producing organisms in fermented dairy products could prevent what he called "fouling" in the large intestine and, if consumed regularly, lead to a longer, healthier life. Lilley and Stillwell (1965) used the term probiotic to describe the substances secreted by one microorganism which stimulated the growth of another. In 1971, Sperti used the term to describe tissue extracts which stimulated microbial growth. Parker (1974) defined the probiotics “organisms and substances which contribute to the microbial balances”. Fuller (1989) was defined the probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. The most common used definition, developed by the WHO (World Health Organization) and FAO (the Food and Agriculture Organization) of UN (the United Nations), is that “probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Reid et al., 2003).

2.2. Gut Microbiota

The human gastro intestinal tract (GIT) is a complex system and it contains many different types of bacterial species. The intestinal epithelium has a combined surface area of 400 m^2 and provides ecological niches for the three domains of life: prokarya, archaea and eukarya. Some of them are the members of endogenous flora which are expected to be beneficial, and the remaining is considered to be contaminants from the environment. The GIT is one of the densest microbial ecosystems on earth (Whitman et al., 1998), and its population number of up to 10^{14} cells exceeds that of the human cells by a factor of 10.

The microbial colonization of the GIT varies with age and is characterized by temporary changes. Gram-positive and non-spore forming rods of several genera constitute an important part of the flora in the GI tract. These include obligate anaerobe *Propionibacterium,* and *Eubacterium* and *Bifidobacterium* (*B. bifidum* and *B. infantis*), found in GI tract of breast-fed infants. The facultative anaerobe genus, *Lactobacillus* also has many species residing in the human GI tract. Several types of spore-forming rods and cocci are also normal inhabitants of the gut. Among these, the genus *Clostridium* is the most ubiquitous. *Vibrio* and *Campylobacter,* which are serious pathogens, are also present in GI tract. The significance of the presence of yeasts and moulds in the gut is uncertain. Some of the yeasts associate with the murine stomach wall but in general they are thought to be transient contaminants (Desai, 2008).

The human GI tract can be divided into upper and lower parts. Stomach, duodenum, jejunum, and upper ileum are the parts of upper intestinal tract, and they contain transient microorganisms which pass through with food (Zoetendal, 2008). The human stomach is almost sterile because of the low pH, although some microorganisms such as *Helicobacter pylori*, can reside on the stomach mucosal layer (Rathbone and Heatley, 1992). Small intestine can maintain bacterial numbers at below 10^6 / ml of contents. Large intestine accommodates the maximum microbial load, approximately 10^{14} cells of bacteria. Most of the microorganisms found in the GI tract have not been cultured, differentiated or characterized as yet (Hayashi et al., 2002).

The GI tract of foetus is supposed to be sterile, and it is established and re-established many times throughout life. Bacteria colonize GI tract of foetus from birth canal. After birth, the vaginal and faecal microbiota of the mother colonizes the

newborns gastrointestinal tract. Aerobes are the first colonizers (streptococci and enterobacteria), the late colonizers are anaerobes such as eubacteria and clostridia (Palmer et al., 2007). Bacterial species and colonization is host specific, and the members of genus *Bacteroides* are the dominant microflora in some babies (Mackie et al., 1999).

There is a symbiotic relationship with host and gut microbiota. The host provides the microflora a nutrition-rich and protective habitat, while the microorganisms ferment non-digestible dietary substrates and indigenous mucus produced by epithelial cells, resulting in the production of short chain fatty acids (SCFA) which are absorbed by the host (Shanahan, 2002). Additionally, the commensal flora provides a barrier against colonization of exogenous and pathogenic bacteria by competing them for nutrients and binding sites (Guarner et al., 2006) and by producing antimicrobials such as bacteriocins inhibiting growth of pathogens (Shanahan, 2002).

Intrinsic factors such as ageing and infectious diseases change microbial composition of the GI tract. In addition to these factors, external environment can also influence the composition and activity of the intestinal microbiota. One of these is diet, affecting the activity and composition of GI microbiota. Consumption of probiotics and/or prebiotics, stress conditions, or infectious diseases, also affects the composition of microbiota (Hawrelak and Myers, 2004).

2.3. Probiotics

As mentioned previously, the probiotic concept was first developed by Metchnikoff. Metchnikoff observed prolonged lifetime in Bulgarian people who consumed soured milk and refined the treatment by using pure cultures of what is now called *Lactobacillus delbruckeii* ssp. *bulgaricus*, which, with *Streptococcus salivarius* ssp. thermophilus, is used to ferment milk in the production of traditional yoghurt (Vouloumanou et al., 2009).

Research has been directed towards the use of intestinal isolates of bacteria as probiotics (Fernandes et al., 1987). Over the years many species of microorganisms have been used that many of which included well known LAB genera: *Bifidobacterium* (*Bifidobacterium animalis*, *B. breve*, *B. infantis*, *B. lactis*, and *B. longum*), *Enterococcus*, *Lactococcus*, *Lactobacillus* (*Lactobacillus acidophilus*, *Lb. casei*, *Lb.*

johnsonii, Lb. lactis, Lb. plantarum, Lb. reuteri, Lb. rhamnosus, and *Lb. salivarius*). Though not often, some *Bacillus, Aspergillus*, and *Saccharomyces* species have also been used (Table 2.1). Most probiotic foods contain lactobacilli or bifidobacteria. Enterococci are occasionally used in food. The yeasts are also used as a human probiotic, but they are delivered in capsules or powders rather than in food (Wright, 2005).

Table 2.1. Organisms used as probiotics in the food and agricultural industry (Source: Goldin and Gorbch, 1992)

Microorganisim	**Comment**
Saccharomyces boulardii	Treatment of diarrhoea
Lactobacillus acidophilus	Dairy products supplements and used for fermentations; numerous health claims
L. plantarum	In dairy products, pickled vegetables and silage
Lactobacillus GG	In yoghurt and whey drink; numerous health claims
L. casei ssp. rhamnosus	In dairy products and silage
L. brevis	In dairy products and silage
L. delbrueckii spp. bulgaricus	Production of yoghurt; health claims have been made
Streptococcus thermophilus	For the production of yoghurt
Bifidobacterium bifidum	Component of new dairy products and in preparation for new born; health claims
Bifidobacterium infantis	Similar to *B. bifidum*
Enterococcus faecium	Being introduced in certain health products; health claims
L. lactis ssp. lactis and cremoris	Used in production of buttermilk and certain cheeses

2.4. Selection of Probiotics

Probiotic effect can be direct or indirect, through the modulation of endogenous flora or of the immune system. Biological activity or the components of probiotic mechanism have been poorly understood. But it has been well documented that in order to exert this activity, a given probiotic population must contain a certain number of cells, upon reaching the target organ. Some of the probiotic species have a high survival capacity in the small intestine, and sometimes in the large intestine, the others are rapidly destroyed while they pass through the GI tract. Furthermore, the strength of adherence as well as adherence time to the epithelium of the intestine, show great variability among different LAB species (Marteau, 2001). These two factors play a key role in the reestablishment of GI microflora.

A given probiotic strain should possess and maintain the following characteristics (Marteau, 2001; Gorbach, 2002; Millette et al., 2008; Vasiljevic and Shah, 2008):

i. To have demonstrable beneficial effect(s) on the human health,
ii. to be non-pathogenic,
iii. to survive during the transit through the GI tract; and thus resistant to extremes of acids and bile salts,
iv. good adherence to human epithelial cells,
v. to colonize human intestine,
vi. and to produce an antimicrobial substance.

2.4.1. Resistance to Acid and Bile and Survival in the GI Tract

In vitro tests assessing the effect of exposure to different pH values, and to the presence of bile salts, typically mimicking the conditions during the GI transit, have been routinely used to screen for the search of potential probiotic strains (Jacobsen et al., 1999).

Tolerance to low pH levels and bile salts is vital for bacteria to survive and grow in the GI tract, making these the main requirements for bacteria to be considered probiotic. Bile is a steroid produced by the liver and secreted through the bile duct in the form of bile salts. These salts can occur as conjugates between cholic acid and the

amino acids glycine or taurine (forming glycholic or taurocholic acid), or as deconjugates such as sodium deoxycholate (Ramirez-Chavarin et al., 2013).

Acid and bile tolerance ability is strain dependent. Bacteria are generally sensitive to the stomach's low pH values (Conway *et al.*, 1987), however, some LAB can survive and grow at relatively low pH because they have a system that simultaneously transports lactic acid and protons to the cell's exterior.

Lankaputhra and Shah (1995) have studied with *Lb. acidophilus* and *Bifidobacterium* strains from dairy origin, they have demonstrated that only a few strains survived under acidic conditions. It cannot be generalized that all of the probiotics are acid and bile tolerant (Desai, 2008).

2.4.2. Adhesion to Host Tissue

Adhesion onto intestinal epithelium is an important criterion for the selection probiotic candidates. Studying bacterial adhesion *in vivo* is difficult; therefore, *in vitro* models with intestinal cell lines are widely adapted for this assessment.

Upon arriving in the intestine, a probiotic strain must fix itself to the tips of the microvilli and then adhere itself to the mucus to avoid being swept off by peristalsis (Fernández et al., 2003). The intestinal mucus is a classic model for testing *in vitro* adherence since different receptors can be located in the small and large intestine mucus using the specific adherence (Ramirez-Chavarin et al., 2013).

This ability enhances the survival of probiotics. Adhesion to epithelial cells is species specific. And this has very important health implications on the host, as the interactions between the host- and probiotic cells could modulate the development and/or functioning of the immune system of the host (Desai, 2008).

2.4.3. Antimicrobial Activities of Probiotics

Production of antimicrobial substances is another significant criterion for the selection of probiotics. Such substances can be as simple as organic acids, or proteins, for example, bacteriocins (Dave and Shah, 1997; Desai, 2008; Dobson et al., 2007; Kwak et al., 2001) (Table 2.2).

Table 2.2. Antimicrobial Substances

Probiotic Microorganisms	**Product**
***Lactobacillus* GG**	Antibiotic (wide range)
Lb. acidophilus	*Acidolin, Acidophilin, Latocidin*
Lb. delb.* ssp. *bulgaricus	*Bulgarican*
Lb. plantarum	*Lactolin*
Lb. reuteri	*Reuterin*
Lb. brevis	*Lactobacillin, Lactobrevin*

Co-aggregation with pathogenic bacteria is another important feature of a good probiotic strain. This interferes with the interaction between host epithelia and pathogens (Collado et al., 2007).

2.4.4 Anticarcinogenic Effects of Probiotics

Probiotics can also exert anti-carcinogenic activities. It has been suggested that this activity can be affected by one of the three mechanisms (Wollowski et al., 2001):

I. by eliminating procarcinogens,

II. by modulation the activity of a procarcinogenic enzyme,

III. or by tumour suppression.

Diet or antibiotic treatments can reduce the chemically induced tumours in the colon, and also prevent the generation of carcinogens (Goldin and Gorbach, 1984). Probiotic microorganisms within the colon microflora can mediate an anti-carcinogenic effect via inhibiting intestinal bacterial enzymes that could convert pro-carcinogenic substances to carcinogens. Experimental and epidemiological studies provide some evidence that fermented milk and bacterial cultures, routinely used to ferment the milk, can also reduce the risk of certain types of cancer by inhibiting the growth of certain tumours and tumourous cells (Gibson et al., 1995).

CHAPTER 3

PHYSICOCHEMICAL PROPERTIES OF YOGHURT

3.1. The Use of LAB Starters in Yoghurt Production

A standard yoghurt production process includes the following steps: pasteurization, homogenization, heat treatment, and fermentation of a batch of milk, cooling and storage of the fermentation product.

The selection step involves the determination of fat and protein content of milk to be used (Tamime and Robisonson, 1999). Many commercial yoghurt products have around 15% of total solid content. Codex regulations (2008) for yoghurt indicate that the minimum milk protein content should be 2.7%, except for concentrated yoghurts in which the minimum protein content is 5.6% after concentration, and the maximum fat content should be 1.5%. The total solids content of milk can be increased by concentration processes, such as evaporation under vacuum, or ultrafiltration. The use of stabilizers may also help provide a more uniform consistency and lessen batch variations (Vedamuthu, 2006).

Milk is heated prior to the addition of a starter culture. Heating influences the physical properties and microstructure of the fermentation product (Lucey et al., 1998). The combinations of temperature and time for the batch-heat treatments include 85°C for 30 min or 90-95°C for 5 min (Tamime and Robinson, 1999).

Homogenization of milk is an important processing step in yoghurt making. Milk is typically homogenized, using first 15 and then 5 MPa pressure at 60 °C. This step results in milk fat globules being disrupted into smaller fat globules, and therefore the fat surface area becomes greatly increased. It also prevents partitioning (creaming) of fat during fermentation or storage. It also increases whiteness, and enhances the consistency of the fermentation product by slowing the separation of whey (Vedamuthu, 2006).

After heat treatment, the milk base is then cooled to the incubation temperature suitable for growth of the starter cells. An optimum temperature of the thermophilic lactic acid bacteria, (*Streptococcus* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp.

bulgaricus), is around 40-45°C. Bacterial fermentation converts lactose into lactic acid, which reduces the pH of milk. During acidification of milk, the pH decreases from 6.7 to ≤4.6. After reaching the desired pH, the product is partially cooled at 20°C, and is then chilled to 5°C in a refrigerated store to minimise acid development (Tamime and Robinson, 1999).

3.2. Physico-Chemical Mechanisms Involved in the Formation of Yoghurt Gels

Gelation occurs at a pH range between 5.2 and 5.4 for a batch of milk after the heat treatment. Acidification of milk leads to the disruption of internal structure of casein micelles. As casein molecules approach their isoelectric point (pH 4.6), the net negative charge on the surface is reduced. This leads to decrease in the electrostatic repulsion between charged groups. Thus, protein-protein attraction starts to increase via enhanced hydrophobic interactions (Lucey, 2004).

Physicochemical mechanisms for the formation of acid milk gels can be discussed for three different pH regions (Lucey, 2004). When the pH of milk decreases from 6.6 to 6.0, the net negative charge on the casein micelles also decreases. This results in a decrease in electrostatic repulsion. As the pH of milk decreases further, from pH 6.0 to 5.0, the net negative charge on casein micelles greatly decreases and the charged "hairs" of κ-casein may shrink (or curl up). This results in a further decrease in electrostatic repulsion and steric stabilization. These are both responsible for the stability of casein micelles in the original milk. Dalgleish and Law (1988) have reported that the amounts and proportions of casein molecules dissociated from the micelles were both temperature- and pH-dependent. Dissociation becomes accelerated from the micelles into the serum when the temperature decreases from 30 to 4°C. Maximum dissociation occurs at a pH point around 5.5 (Dalgleishand and Law, 1988).

When the pH of milk becomes close to the isoelectric point of casein (pH 4.6), there is a decrease in the net negative charge on casein, which leads to a decrease in electrostatic repulsion between casein molecules. On the other hand, casein-casein attractions increase due to increased hydrophobic and plus-minus (electrostatic) charge interactions (Horne, 1998). The whole acidification process results in the formation of

three-dimensional network, consisting of clusters and chains of casein molecules (Mulvihill and Grufferty, 1995).

Yoghurt products can be classified as pseudoplastic materials that can exhibit Non-newtonian behavior, because their viscoelastic fluids. Thus, viscosity is an important parameter for the quality of yoghurt. Milk types, total solid content of milk, starter culture combinations, and cooling conditions can all influence the viscosity (Afonso et al. 2003). Among these, the type of starter strains used is the most important parameter in determining viscosity. For example, EPS (exo-polysaccharides) producing strains can dramatically increase the viscosity (Bouzar et al., 1996; Folkenberg et al., 2006).

3.3. Aroma Compounds of Yoghurts

Flavour is the most important characteristic of industrial food products as it determines the product acceptance by consumers. In fermented dairy products, flavour is essentially composed of volatile substances (Kalviainen et al., 2003). Several flavour compounds have been isolated. The most prominent of them is mainly lactic acid, imparting an acidic refreshing taste in the mouth. Carbonyl compounds, such as acetaldehyde, ethanol, acetone, diacetyl, and 2-butanone can also be named as other flavouring agents. Among these, acetaldehyde appears to be the major flavouring compound (Chaves et al., 2002; Ott et al., 1997). Both the ratio and balance between these flavouring agents must be taken into account when the final aroma of the product is decided (Gardini et al., 1999; Chaves et al., 2002). Yet, a greater number of volatile organic compounds identified in yoghurt originate from milk (Beshkova et al.,1998).

3.4. The Texture of Yoghurt

Appearance, flavour, texture, and nutritional capacity are the main quality factors of any type of industrially produced foods (Bourne, 2002).

The definition of texture, especially for food, is very complicated because of its multi-dimensional nature. Some of the terms, which have been used to describe the textural properties, were given below (Table 3.1).

Table 3.1. Relations between textural parameters and common nomenclatures (Source: Bourne, 2002)

Mechanical Characteristics		
Primary Parameters	**Secondary Parameters**	**Common Terms**
Hardness		Soft, Firm, Hard
Cohesiveness	Brittleness	Crumbly, Crunchy, Brittle
	Chewiness	Tender, Chewy, Tough
	Gumminess	Short, Mealy, Pasty, Gummy
Viscosity		Thin, Viscous
Elasticity		Plastic, Elastic
Adhesiveness		Sticky, Tacky, Gooey
Geometric Parameters		
Particle size and shape		Gritty, Grainy, Coarse
Particle shape and orientation		Fibrous, Cellular, Crystalline
Other characteristics		
Primary Parameters	**Secondary Parameters**	**Common Terms**
Moisture content		Dry, Moist, Wet, Watery
Fat content	Oiliness	Oily
	Greasiness	Greasy

Recently developed instrumental techniques, simulating both human perception and human jaw, have enabled the texture profile analysis (TPA) (Texture Analyzer, TA.XT2, Rawson and Marshall, 1997). The basic principle of TPA is the mechanical compression of a food sample. All TPA measurements can be carried out using two-cycle uniaxial compression instruments. A 'bite-size' food sample is placed on the base plate, compressed, and decompressed twice by the plate, and then attached to a drive system, with a high compression, to imitate the chewing action of the teeth (Bourne, 2002) (Fig.3.1).

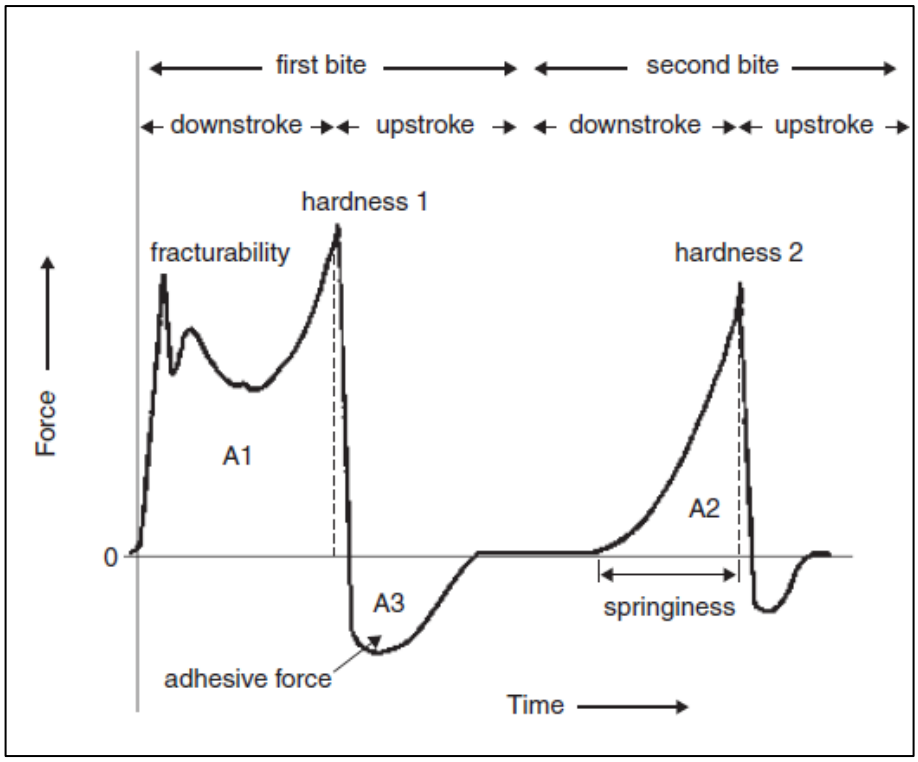

Figure 3.1. A TPA curve obtained with TA.XT2
(Source: Ozcan, 2003)

Hardness (N) = Maximum force of the first compression

Cohesiveness = Area under first compression (A1)/Area under second compression (A2)

Adhesiveness = Negative area in the graph (A3)

Springiness (mm) = Length 1/ Lenhgt2

Chewiness (N) = Hardness X Cohesiveness X Springiness

Hardness is the peak force during the first compression cycle. Cohesiveness is defined as the ratio of the positive force area (Area1/Area2). Adhesiveness is the negative force area of the first compression cycle (Area 3).

According Rawsan and Marshal (1997) the assessment of texture of yoghurt (adhesiveness and cohesiveness) is probably linked to exopolysaccharides produced by specific strains of yoghurt *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

Chemicals used in this study were given in Appendix A.

4.1.2. Yoghurt Samples

Thirteen different artisanal yoghurt samples were collected from Urla district of Izmir, Turkey, and were evaluated according to their organoleptic properties such as aroma, viscosity and acidity (Table 4.1).

Table 4.1. The Origin of the Yoghurt Samples

Yoghurt Sample Codes	**Location of Yoghurt Samples**	**Yoghurt Sample Codes**	**Location of Yoghurt Samples**
GM	Gulbahce, Urla	**UZ**	Zeytindi, Urla
GS	Gulbahce, Urla	**UIB**	Birgi, Urla
UF	Merkez, Urla	**UIIB**	Birgi, Urla
US	Merkez, Urla	**UN**	Merkez, Urla
GG	Gulbahce, Urla	**UIN**	Nohutalan, Urla
GA	Guzelbahce	**UIIN**	Nohutalan, Urla
DT	Merkaez, Urla		

4.1.3. Reference Strains

Lactobacillus delbrueckii ssp. *bulgaricus* CCM 7190, *Streptecoccus thermophilus* CCM 4757 strains from Czech Culture Collections were used as reference yoghurt starters.

Esherichia coli RSHM 4024 (ATTC 25922), *Staphylococcus aureus* RSSK 1009 and *Listeria innocua* NRRL-B 33314 were used as food-borne pathogens.

4.2. METHODS

4.2.1. Isolation of Bacteria

Ten milliliter aliquots of each of the samples were homogenized in 90 ml of sterile peptone water (0.1 % w/v). Dilutions up to 10^{-8} were then prepared and 1 ml aliquots from 10^{-6}, 10^{-7}, 10^{-8} dilutions were used for starter isolation by pour-plate method using duplicate plates for each of the samples (Bulut, 2003).

4.2.1.1. Selective Media and Growth Conditions

M17 agar (pH 6.9) was used for *Streptecocus thermophilus* and MRS agar (pH 6.2) was used *Lactobacillus delbrueckii* ssp. *bulgaricus* at 42 °C (De Man, 1960). Microaerophilic conditions were achieved using Anerogen kit (Oxoid) and plates were incubated for 3 days. After the incubation, plates with colony forming units (cfu) between 30 and 300 were counted and results were recorded as viability of yoghurt bacteria.

MRS or M17 broth medium was used for enrichment of bacteria. Streak-plate technique was used for the further purification of isolates. The isolated strains were then examined under the light microscope after Gram staining.

4.2.2. Long Term Preservation of the Isolates

MRS or M17 medium containing 40% (v/v) glycerol was prepared and was mixed with equal amounts of overnight bacterial cultures. Hence, the resulting suspensions included 20% glycerol (v/v). The samples were then stored at -80°C (Bulut, 2003).

4.2.3. Characterization of the Isolates

Isolates were characterized by using basic phenotypic, biochemical and molecular methods.

4.2.3.1. Phenotypic Characterization

Isolated strains were firstly identified using microscopic examination to determine both morphology and gram status.

4.2.3.1.1. Gram Staining

Gram status of the isolates was examined under light microscope. Fresh bacterial cultures were used for Gram staining. Blue-purple color indicates the Gram positive nature of *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*.

Isolated strains were cultured using MRS or M17 medium at 42°C for overnight. One ml fresh culture was transferred into the Eppendorf tube, then centrifuged at 6 000 rpm for 5 min, and supernatant was removed. The cells were re-suspended in 1 ml sterile water. Ten microliters of the suspension were streaked onto the glass slide and dried in open air approximately for 5 min. Cells were then fixed by heat (1s exposure to flame for 3 times). Gram staining was effected as follows: 1 min staining with crystal violet, removing the excess of the dye under running tap water (washing), second staining with iodine solution, and washing. The dyed cells were then fixed with ethanol (95 %) for 5s. The slides were then dried after washing. Final staining was performed for 30s by using safranine.

4.2.3.2. Catalase Reaction

The absence of the catalase is a very important characteristic of lactic acid bacteria. Hence, *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are catalase negative.

Catalase enzyme breaks downs hydrogen peroxide into water and oxygen. Released oxygen produces gas bubbles which indicate the presence of the catalase enzyme in the bacterium.

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

The isolated strains, after grown overnight in an appropriate agar medium at 42°C, were treated with hydrogen peroxide (H_2O_2). Onto the individual colonies, 3% H_2O_2 solutions were pipeted.

4.2.3.3. Biochemical Identification

4.2.3.3.1. Gas Production from Glucose

Lb. delbrueckii ssp. *bulgaricus and S. thermophilus* are both homofermentative bacteria. The production of CO_2 from glucose is the main criteria in order to differentiate the homofermentative and heterofermentative bacteria. Main fermentation product of homofermentative bacteria is lactic acid. Heterofementative bacteria can also produce acetic acid, ethanol and CO_2. This released carbon dioxide can be trapped into inverted Durham tubes, inserted into the culture media.

Isolated strains were incubated overnight in modified MRS broth; in which citrate was omitted because its presence can also cause the production of carbon dioxide. Fifty microliter aliquots of the overnight cultures were inoculated into fresh media and allowed for incubation for 5 days at 42 ºC.

4.2.3.3.2. Growth at Different Temperatures

For the isolation of lactobacilli, the growth temperatures 15 and 45°C are mostly used (Hammes and Vogel, 1995). However, *Lb. delbrueckii* ssp. *bulgaricus* can grow at 45 °C, but not at 15 °C. Lactobacilli can grow both 10°C and 45°C. Yet *S. thermophilus* can grow at 45 °C, but not at 10°C. Bromocresol purple was supplemented into the appropriate both media to determine the lactic acid production and also cell growth. It is a pH indicator, and acidic pH turns its purple color into yellow. Fifty microliters of active culture were inoculated into 10 ml fresh media and allowed for incubation for 7 d. After the incubation, the growth of the isolates and the change of the medium color were examined.

4.2.3.3.3. Growth at Different NaCl Concentrations

S. thermopihlus and *Lb. delbrueckkii* ssp. *bulgaricus* strains are not resistant to NaCl. For the identification of bacilli, 4 and 6.5 % NaCl (w/v) and for the cocci, 2 and 4 % NaCl (w/v) concentrations are used. In this study, 2, 4, 6.5, 8 and 10 % NaCl concentration were used. Fifty microliters of active culture were inoculated into the test media, MRS or M17, which were again supplemented with bromocresol purple, as indicator of the growth. Incubation time was 7 d. Yellow color indicated the NaCl tolerance and growth (Hoque et al., 2010).

4.2.3.3.4. Carbohydrate Fermentation Profiles

Carbohydrate fermentation profiles could differentiate between lactic acid bacteria. Nineteen different sugars were used. Fermentation profiles were obtained by reading the color change in the cultures using the 96- well plate method (Bulut et al., 2005). The isolated strains were first activated overnight in the appropriate 5 ml broth media at 42 °C. Cells were pelleted at 10 000 rpm. Supernatant was removed and the cells were re-suspended and washed in sugar-free MRS. The cells were then taken into 5 ml sugar free MRS medium, supplemented with bromocresol purple. Forty microliter aliquots of the carbohydrate solutions (10 % w/v), sterilized by microfiltration (0.22

µm), were then pipetted onto the 160µl cell suspensions in the 96 well plates, giving a final sugar concentration of 2 % (v/v). Incubation was allowed to proceed for 24h at 42°C. After the incubation, the turbidity was checked and color change purple to yellow was recorded as positive result.

4.2.3.3.5. Proteolytic Activity

A qualitative method, involving modified MRS agar supplemented with skimmed milk was used for the evaluation of proteolytic ability (Musikasang et al., 2009). Ten microliter of the overnight cultures were spread onto the agar plates and incubated for 24h at 37°C. The diameters of halo zone around the colonies were then measured. Those strains, around which a clear zone of >1 cm formed, were classified as positive.

4.2.3.3.6. ß-galactosidase Activity of the Isolates

ß-Galactosidase activity was performed by using the ONPG disc assay (Iyer et al., 2010). Isolates grown overnight at 37 °C in MRS were centrifuged for 5 min at 12 000 rpm at 4 °C. The pelleted cells were first washed twice with 0.85 % (w/v) sterile saline solution and then incubated in L-MRS broth medium for 24h (0.5% (w/v) lactose) at 37 °C. Incubation was terminated. A drop of chloroform and a drop of 0.1% SDS (v/v) were then added into the cultures and mixed by vortexing for 10s. The mixture was incubated for 1 min at 37 °C in a water-bath. OPNG discs soaked with 100 µl 0.85 % (w/v) sterile saline were then inserted into the cultures. The final samples were further incubated for 24h 37 °C. The development of any shade of yellow color indicated the ß-galactosidase activity.

4.2.3.3.7. Gelatinase Activity

The gelatinase activity of the isolates was screened according to the method as described by Sialedea et al. (2011) with some modifications. Bacterial cultures were grown overnight in MRS or M17 broth medium at 42 °C. Ten µicroliters of bacterial

suspension were then inoculated into the 10 ml nutrient gelatin medium (3g/L LabLemco; 5g/L peptone; 120g/L gelatine) and incubated overnight at 37 °C. After this incubation, isolates were kept for 10 min at 4 °C. Liquefaction of gelatin indicates the strong gelatinase activity.

4.2.3.3.8. Urease Activity

Urease activity of the isolated strains was evaluated using the method described Mora et al. (2002). A loopful of a fresh culture of each strain was re-suspended in a solution containing one volume of solution A (urea, 2 g dissolved in ethanol, 2 ml; sterilized water, 4 ml) and 19 volumes of solution B (KH_2PO_4, 1 g /1; K_2HPO_4, 1 g/l; NaCl, 5 g/ l; phenol red, 20 mg/ ml). The suspension was incubated for 1–2h at 37ºC and the development of red-violet color indicated the presence of urease activity.

4.2.3.3.9. Indole Production

This biochemical test is known as a tryptophanase system, involving a chain of intracellular enzymes, and has often been performed on bacterial species to determine the ability of the organism to split indole from the amino acid tryptophan.

A tryptone broth medium (tryptone 10 g/L, NaCl 5 g/L) was prepared and the isolates strains were inoculated into this medium. After overnight incubation at 37 ºC, 5 drops of Kovac's reagent were added directly into the tubes. Formation of a pink to red colour ("cherry-red ring") indicates the presence of an active tryptophanase system. A yellowish color indicates the opposite.

4.2.3.4. Genetic Characterization of Probiotics

4.2.3.4.1. Genomic DNA Isolation

Genomic DNA was isolated using the following procedure published by Bulut et al. (2005). Isolated strains were grown overnight in MRS or M17 broth medium at 37 °C. The cultures were centrifuged for 5 min at 6 000 rpm. Supernatants were removed,

and cells were then suspended in 200 µl 1x TE (pH 8.0) containing 25% (w/v) sucrose and 30 mg/ml lysozyme. The cells were lysed for 1h at 37 °C. After the lysis, 370µl 1x TE with 1 mg/ml proteinase K and 30 µl 10% SDS were added. The samples were then incubated further for 1h at 37 °C. After the deproteinization step, 100 µl 5M NaCl and 80 µl CTAB/NaCl solution were added and the samples were incubated for 10 min at 65 °C. An equal volume of chloroform (chloroform/isoamylalcohol 24:1) was added, mixed manually, and the samples were centrifuged for 5 min at 6 000 rpm. The chloroform extraction was repeated twice. The aqueus phase was gently transformed into a fresh Eppendorf tube. DNA was precipitated by the addition of an equal volume of isopropanol, and by manual mixing. DNA wool was pelleted, washed with 70 % (v/v) ethanol, and the DNA pellet was dried for 10 min at 37 °C. DNA was dissolved in 100 µl 1x TE, including 100µg/ml RNase, and the samples were incubated for 1h at 37 °C. After the RNase treatment, the sample volume was adjusted to 400 µl by the addition of 1xTE. Chromosomal DNA was dissolved by alternating heat shocks (20 min at 80 °C, and 10 min at -20 °C). DNA was purified using one equal volume of phenol. The samples were centrifuged for 5 min at 6 000 rpm. The aqueus phase was gently transferred into a fresh tube. Similarly, a chloroform extraction step was performed. The aqueous phase was then transferred into a new tube containing 1/10 volume of 5M NaCl and DNA was precipitated by adding two volumes of 99 % ethanol. Precipitated DNA was collected by centrifugation for 10 min at 10 000 rpm. DNA pellets were washed with 70 % ethanol. DNA was dried at room temperature, dissolved in 100 µl 1x TE, and was then stored at -20 °C. The quality of the isolated genomic DNA was checked by using NanoDrop 8000.

4.2.3.4.2. Amplification of 16S-ITS (Internally Transcribed Spacer) Region of Bacilli Strains by Polymerase Chain Reaction (PCR)

Internally Transcribed Spacer region (ITS) is situated between 16S and 23S ribosomal RNA (rRNA) genes. 16S region is highly conserved and served as genetic marker to study microbial diversity (Bulut et al., 2005).

16S rRNA gene sequences including the ITS region were amplified using the following primers:

Forward Primer: 5'-AGA GTT TTG ATC CTG GCT CAG-3'
Reverse Primer: 5'-CAA GGC ATC CAC CGT-3'

The forward primer is complementary to the 5' end of the 16S rRNA, and reverse is complementary to the 3' end of ITS.

PCR reaction was performed in a final volume of 50 µl. Genomic DNA (500 ng, 2µl) was aliquoted into the 0.2 ml PCR tubes and 48 µl of master mix solution (0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each of the DNA primers, 1x PCR buffer, and 1.25U Taq DNA polymerase) were then added.

PCR reactions were performed in a thermocycler, using the following program:

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

4.2.3.4.3. Electrophoresis of Amplified 16S-ITS Fragments

Amplified PCR products were resolved in 1 % (w/v) agarose gel, containing 15 µl of ethidium bromide. Five microliter of the amplified PCR products were mixed with 1 µl of gel loading dye and loaded into the agorose gel wells. Four microliters of DNA molecular weight marker (500 ng) were also loaded into the gel. The electrophoresis was performed for 1h at 80 mA. DNA was visualized under UV light in a gel documentation system (Vilbert Lourmat).

4.2.3.4.4. Characterization of Cocci Isolates by ARDRA

The results of ARDRA was also verified by species specific PCR. To increase detection sensitivity, a species-specific PCR amplification procedure has been

developed and primers have been used for the amplification of an intragenic fragment of 968bp within the lacZ gene sequence of *S. thermophilus* (Lick et al., 1995).

IYTE1 and IYTE2 primers were used.

Forward Primer: IYTE1: 5'-CAC TAT GCT CAG AAT ACA-3'

Reverse Primer: IYTE2: 5'-CGA ACA GCA TTG ATG TTA-3'

PCR reactions were performed in a thermocycler, using the following program:

Step 1: 94°C for 5 min

Step 2: 94°C for 1 min (denaturation)

Step 3: 46°C for 1 min (annealing) } 40 cycles

Step 4: 72°C for 1 min (elongation)

Step 5: 72°C for 10 min

4.2.3.4.5. Purification of Amplified PCR Products

Chloroform extraction method was used for purification of amplified PCR products (Bulut, 2003). The volume of the sample was adjusted to 150 µl by addition of 100 µl 1xTE buffer. To purification, 200 µl chloroform/isoamyl alcohol solutions (24:1 v/v) were added and mixed well. The samples were centrifuged for 5 min at 4 000 rpm and the aqueus phase was transferred into new eppendorf tube and second chloroform extraction was applied. After centrifugation, the aqueus phase was taken into new tubes containing 10 µl of 3 M sodium acetate (pH5.2) and mixed well. Then, 450 µl of 99% ethanol were added and mixed again. To obtain DNA pellet, the samples were centrifuged for 15 min at 7000 rpm. After that, ethanol was removed from the tube and DNA pellet was washed with 500 µl 70% ethanol. To remove ethanol, tubes centrifuged for 5 min at 6000 rpm after this step, pellets were dried for 10 min at 37 °C. Finally, pellets were dissolved in 55 µl 1xTE, and stored at -20°C.

4.2.3.4.6. Restriction Enzyme Digestion of Amplification Products

Ten µl of the purified amplification products were used for restriction enzyme digestions.

*Eco*RI endonuclease was used for the identification of bacilli isolates. *Hae*III was used for differentiation of cocci isolates. Digestions with two of the endonucleases were performed at 37°C in a water bath at overnight.

4.2.3.4.7. Purification of Digested DNA Products

The volumes of the samples were adjusted to 100 µl with 1x TE after restriction digestion. Chloroform extraction was performed twice using two volume of chloroform/isoamyl alcohol solution. After this step, aqueous phase was taken into a new eppendorf tube with 1/10 volume of sodium acetate solution (3 M, pH5.2) and mixed well. Two and half volume of 99% ethanol were added and mixed. After centrifugation for 15 min at 7 000 rpm, liquid phase was removed, and DNA pellet washed with 300 µl 70% ethanol. Ethanol was removed and sample was dried for 10 min at 37 °C. Finally, sample was dissolved in 15 µl 1xTE and 3 µl of 6x gel loading buffer were added.

4.2.3.4.8. Electrophoresis of Restriction Digestion of Products

The digested fragments were separated in a 2.0 % agarose gel. For this purpose, 2.0 g agarose was dissolved in 100 ml 1x TAE by boiling. After cooling, 20 µl of ethidium bromide (10 mg/ml) were added into gel and it was poured into the gel casting stand and comb was placed. When the gel solidified, the combs were removed. The tank was filled with 1x TAE buffer. Ten µl of the samples were loaded into each well and 500 ng of DNA molecular weight marker were loaded into the first well. The electrophoresis conditions; for the first 30 min, electrophoresis was performed at 60 mA, and the current was adjusted to 80 mA and the electrophoresis was allowed to last for about 2 h. After the electrophoresis, RFLP patterns were observed in gel documentation system.

4.2.3.4.9. Pulsed Field Gel Electrophoresis RFLP

4.2.3.4.9.1. Preparation of Agarose Blocks

A few specks of the frozen bacterial stock samples were inoculated in MRS or M17 and 5 ml overnight cultures were prepared at 42 ºC.

The cultures were harvested by centrifugation for 5 min at 10 000 rpm. Cell pellets were then resuspended in 500 µl of cell suspension buffer (10 mM Tris pH 7.0, 20 mM NaCl, 50 mM EDTA, pH 8.0) and were washed twice. The cell pellets were resuspended in the same buffer (50 µl) and mixed with 50 µ of 2% (w/v) low melting temperature agarose (prepared in sterile water, at 50 ºC). The mix samples were then pipetted into the wells of disposable 10-welled plug mould holder (Bio-Rad). The plugs were then allowed to solidify for 15 min at 4ºC. The cells embedded into the plugs were lysed in 1.5 ml Eppendorf tubes containing 1 ml lysozyme solution (30 mM Tris, pH 8.0, 50 mM NaCl, 5mM EDTA, pH 8.0, 10 mg/ml lysozyme), and were then incubated for 4h at 37 ºC without agitation in a water bath. The plugs were transferred into 50 ml Falcon tubes containing 5 ml of 1x wash buffer (20 mM Tris pH 8.0 and 50 mM EDTA pH 8.0), and were then incubated with gentle agitation for 45 min at 37 ºC in water bath. The plugs were then transferred into 1.5 ml Eppendorf tubes containing 1 ml proteinase K buffer (100 mM EDTA pH 8.0, 0.2% (w/v) sodium deoxycholate, 1% (w/v) sodium N-laurylsarcosinate, 1 mg/ml proteinase K), and were incubated overnight at 50 ºC without agitation. Before the restriction enzyme digestion, the plugs were washed 4 times in 5 ml washing buffer by gentle agitation for 45 min at 37 ºC on an orbital shaker. First and second washes were in 1× wash buffer (20 mM Tris pH 8.0 and 50 mM EDTA pH 8.0) plus 1 mM NaCl. Third washing was in 1× wash buffer plus 1 mM PMSF (phenyl methyl sulfonyl floride). Fourth wash was in 1× wash buffer and the final wash was in 0.1× wash buffer (Okuklu, 2005).

4.2.3.4.9.2. Restriction Enzyme Digestion of Agarose Plugs

For digestion of the genomic DNA, *Sma*I restriction endonuclease (5'-CCCGGG-3') (Fermentas) was used. The plugs were first equilibrated in 1ml of 1x

*Sma*I restriction enzyme buffer for 15 min at 37 ºC with gentle agitation. DNA was then digested overnight using 30 units of *Sma*I in 100 µl reaction volume at 30 ºC. Before the electrophoresis the plugs were equilibrated for 15 min in 1 ml of 0.5x TBE buffer at room temperature with gentle agitation on an orbital shaker.

4.2.3.4.9. 3. Pulsed Field Gel Electrophoresis

The electrophoresis was performed in 1% (w/v) PFGE grade agarose (Bio-Rad) gel. One gram of agarose was dissolved in 100 ml of 0.5x TBE buffer by boiling. After the gel was cooled, it was poured into the platform of casting stand provided by CHEF Mapper equipment (Bio-Rad). The 30-wells comb was placed into the comb holder and gel was then allowed to solidify at room temperature. The agarose plugs loaded into the wells by using a spatula.

Two liters of 0.5x TBE at 4 ºC was poured into the electrophoresis chamber. Electrophoresis was performed in a CHEF DRIII system with 5-40 pulse times, for 22 h at 4V/cm at 14 ºC.

4.2.3.4.9.4. Staining the PFGE Gels

When the electrophoresis was complete, the gel was removed and stained for 45 min in 200 ml dH_2O containing 200µl (10 mg/ml) ethidium bromide with gentle agitation. The gel was then de-stained with deionized water for 30 min with gentle agitation for 3 times. The image of the gel was analyzed in a gel documentation system (Vilber Lourmat, Torcy, France).

4.2.4. Screening of Isolates for Probiotic Criteria

4.2.4.1. Tolerance to Low pH

The low pH tolerance experiments were conducted according to the Iyer et al. (2010). MRS and M17 medium were prepared at pH 2.0, pH 3.0, pH 4.0, and at pH 7.0, as control. These media were then inoculated with 2 % active culture and incubated for

3h at 37°C. After the incubation, total viable cell counts were determined by pour-plate method, using MRS or M17 agar plates.

The experiment was repeated by using another protocol (Bao et al., 2010). A phosphate saline solution (PBS, 0.8 % (w/v) NaCl and 0.02 % (w/v) KH_2PO_4 and 0.115 % (w/v) Na_2HPO_4) was prepared and its pH was adjusted to 2.5 before sterilization. After the sterilization, 2 % active culture inoculated into the solution, and incubated for 3h at 37 °C. Total viable cell counts were determined by pour-plate method.

4.2.4.2. Tolerance to the Simulated Human Gastric Environment

4.2.4.2.1. Tolerance to the Simulated Human Gastric Juice

Simulated gastric juice was prepared according to the method described by Guo et al. (2009). The pH of the PBS medium was adjusted to 3.0 with 1N HCl, and sterilized for 15 min at 121 °C. Pepsin solution was prepared at a final concentration 3 g/L, sterilized by microfiltration (0.22 μm), and was then mixed with the PBS solution.

Activated test culture was centrifuged at 2 500 g at 4 °C for 10 min. Cells were resuspended into the 0.85 % (w/v) sterile saline solution. Cell suspensions (1 %) were inoculated into PBS (pH 3.0) supplemented with pepsin and incubated for 3h at 37 °C. After the incubation, total viable cell counts were determined by pour-plate assay.

The simulated gastric juice was also prepared according to the Musikasang et al. (2009). A sterile saline solution (0.85 % NaCl, w/v) was prepared and its pH was adjusted to 3.0 with 1.0 M HCl. A pepsin solution (3 mg/ml) was prepared in PBS and sterilized by microfiltration (0.22 μm). Activated test culture cells, washed twice with PBS, were resuspended in the simulated gastric juice, and were then incubated for 3h at 37 °C. Tolerance was assessed by pour-plate method and survival rates were calculated by using the following equation:

Survival rate were calculated by using the following Equation 4.1.

$$\text{Survival rate \%} = (\log \text{cfu } N_1 / \log \text{cfu } N_0) \times 100\% \quad (4.1.)$$

(N_1= the total viable count of strains after treatment by gastric juice, N_0= the total viable count of strains before treatment)

4.2.4.2.2. Tolerance to the Simulated Human Intestinal Juice

Simulated intestinal juice was prepared by supplementing PBS with trypsin (0.1g/L). One milliliter of the culture was transferred into the 9 ml of PBS (pH 8.0) with trypsin. The intestinal transit tolerance was evaluated by determining the total viable cell count after incubation for 0, 1, 2, 3, 4, and 24h at 37 °C.
Survival rates were calculated by using the following Equation 4.2.

$$\text{Survival rate } \% = (\log \text{cfu } N_1 / \log \text{cfu } N_0) \times 100\% \quad (4.2.)$$

(N_1= the total viable count of strains after treatment by gastric juice, N_0= the total viable count of strains before treatment)

4.2.4.3. The Bile Salts Tolerance

The maximum concentration of bile salts, tolerated by tested strains, was determined (Bao et al., 2010). MRS broth was prepared with a range of concentrations of bile salts: 0.3%, 0.4%, 0.6%, 0.8%, and 1.0% (w/v). The media were inoculated with 1% active culture and incubated for 24 h at 37°C. During incubation, absorbance was read at 600 nm with one hour intervals.

4.2.4.4. Bile Salt Deconjugation Assay

The ability of the strains to deconjugate bile salts was examined (Iyer et al., 2010). MRS agar, supplemented with 0.5% sodium salts of sodium taurocholic acid and sodium thioglycholate, was prepared. The cells were spread onto the agar plates and allowed incubation for 72h at 37°C. Tolerance was indicated by the presence of the precipitated bile acids around the colonies (white opaque halo).

Bile salt deconjugation assay was also performed using MRS agar supplemented with 0.5% (w/v) sodium taurodeoxcholic acid and 0.37 g/L $CaCl_2$.

4.2.4.5. Autoaggregation Assays

Autoaggregation assays were determined using the autoaggregation percentage described by Mathara et al. (2008) with some modifications. Active cultures were harvested by centrifugation for 15 min at 5 000 g. Cells were washed two times and re-suspended in PBS (pH 7.4). Two hundred microliter aliquots of the cell suspensions were incubated 24h at 37 ºC for. During the incubation time, every hour absorbance was measured at 600 nm with 1h intervals in spectrophotometer (Varioscan).

4.2.4.6. Screening for Antibiotic Resistance

Screening was performed by using the disk diffusion method, following NCCLS standard. A bacterial suspension was made by picking colonies from MRS or M17 agar plates using a sterile loop, and suspending these in PBS to reach a density corresponding to 0.5 McFarland. Using a sterile swap, cell suspension was then spread onto the agar plates in 2 directions and allowed to dry. Antibiotic disks were placed on the agar and plates were incubated for 48h at 37 °C. The inhibition zones were measured and results were expressed in terms of resistance and susceptibility (Mathara et al., 2008).

Concentrations of antibiotics used were as follows: azitromycin (15 µg), rifampicin (5 µg), cephalothin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), linomycin (2 µg), amoxycillin (25 µg), streptomycin (10 µg), kanamycin (30 µg), pefloxacin (5 µg), and tetracyline (30 µg).

4.2.4.7. Cholesterol Assimilation Assay

4.2.4.7.1. Preparation of Cholesterol Solution

The cholesterol levels of isolates were also studied (Mathara et al., 2008). Cell cultures were prepared in MRS for 24 h at 37°C. Cholesterol solution (10 mg/ml cholesterol in 96% ethanol), 70 µl, was mixed with 10 ml MRS broth, containing 0.2%

(w/v) bile salts. Cell culture, at 1% (v/v), was inoculated in the medium and incubated for 24h at 37 °C. Supernatant was used for the assimilation assay.

4.2.4.7.2. Quantitative Assay for Cholesterol

Cells were removed by centrifugation for 10 min at 10 000 g at 4 ºC. Three milliliters of supernatant were mixed with 2 ml, 10% KOH, and 3 ml ethanol, then mixed by vortexing for 20s. The mixture was incubated for 15 min at 60 °C in a water bath. After cooling, 5 ml hexane and 1ml of distilled water were added. The mixture was incubated at room temperature for phase separation. Hexane layer (3 ml) was then transferred into a new tube and evaporated under the flow of N_2 gas approximately for 15 min. Four milliliters of freshly prepared o-phytalaldehyde was added (0.5 mg o-pyhtalaldehyde / ml of acetic acid). After adding 2 ml H_2SO_4 the samples were allowed incubation for 10 min. Two hundred microliters of the sample were transferred into a microtiter plate and the absorbance was read at 550 nm to determine cholesterol content.

Cholesterol assimilation levels (in percentage) were calculated using the following Equation 4.3.

$$A = 100 - (B/C) \times 100 \qquad (4.3.)$$

(Where A=% of cholesterol removed, B=absorbance of the sample containing the cells and C=absorbance of the sample without cells)

4.2.4.8. Assessment of Cell Surface Hydrophobicity

The cell surface hydrophobicity assays was determined using the method described by Iyer et al. (2010). The actively grown cultures were centrifuged for 5 min at 12 000 g at 5°C. Cells were washed two times and then re-suspended into 1.2 ml phosphate-urea-magnesium sulphate buffer (pH 6.5). The initial absorbance was adjusted to 1.0 at 450 nm. Three milliliters of bacterial suspension were mixed with 0.6 ml n-hexadecane and xylene. The samples were then pre-incubated for 10 min at 37°C, and were mixed by vortexing for 2 min. After 15 min incubation, the hydrocarbon layer was removed. The final absorbance was measured, and the decrease in the absorbance was taken as a measure of cell surface hydrophobicity.

The following Equation 4.4. was used for the calculation of hydrophobicity;

$$\% \text{ Hydrophobicity} = [(OD_{initial} - OD_{final}) / (OD_{initial})] \times 100 \quad (4.4)$$

($OD_{initial}$= the absorbance before extraction of hydrocarbons, OD_{final}= the absorbance after extraction of hydrocarbons)

4.2.4.9. Growth in the Presence of Different Prebiotics

The experiment was performed by following the method adopted from Pennacchia et al. (2006). Active bacterial cultures were prepared in MRS or M17 broth for 24 h at 37°C. One milliliter of active culture was centrifuged for 10 min at 10 000 g. The cell pellet was re-suspended in 50 ml of fresh media, without glucose, supplemented with 1.5 % (w/v) lactulose and inulin prebiotic as prebiotic sources. The samples were incubated at 37°C. Absorbance, at 600 nm, was measured at 0, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 24^{th} and 30^{th} h with spectrophotometer (Varioscan).

4.2.4.10. Adhesion Assay

Adhesion properties of the bacterial cells were examined by following the procedure described by Maragkoudakis et al (2006). For the assay, a human colon carcinoma cell line (Caco-2) was used.

Caco-2 cells were grown in Dulbecco's modified Eagel's medium (DMEM) containing heat inactivated 10 % (v/v) foetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids, and 20 μg/mL streptomycin and penicillin. Cells were seeded onto six-well tissue culture plates and incubated at 37 °C in 10% CO_2. After reaching confluency, on the 3^{rd} day of incubation, the monolayer was scraped, and approximately 1.2 $x10^5$ cells were then seeded into the new wells. Incubation was then allowed to proceed for 15d.

Bacterial cell cultures were activated twice by incubating in 10 ml MRS or M17 for 18h at 42° C. The cells were then centrifuged for 10 min at 3 500 g at 4 ºC. Cell pellets were rinsed twice in 5 ml PBS (pH 7.2). Cells were then re-suspended in 5 ml of PBS. Cell densities were then adjusted in DMEM to 0.5 McFarland.

Fifteen days old Caco-2 monolayers were washed twice in PBS. Bacterial-suspensions in DMEM were placed onto the CaCo- 2 monolayers. The cell mixture was then incubated for 90 min at 37 ºC in 10% CO_2. The adsorbed bacterial cells were recovered by washing the plates with 0.004 % (v/v) Tween 80. Residual tween was then removed by rinsing the plates with PBS (pH7.2) twice. Recovered bacterial cells were enumerated by the pour-plate method. The adhesion of the bacterial cells to CaCo-2 cells was expressed as percentage of the viable bacteria compared to the initial population. The experiment was repeated twice.

4.2.5. Screening for Technological Properties

4.2.5.1. Production of Exopolysaccharides (EPS)

Two different media were used. (1) Skimmed milk (10%, w/w) agar (SMA) was prepared and autoclaved at 110 ºC for 10 min. SMA was supplemented with 0.05% (w/v) ruthenium red dye to obtain modified RSMA. Strains were streaked onto the plates and incubated overnight at 42 ºC or at 37 ºC. EPS producing strains were selected based on their ability to resist the penetration of ruthenium red, which appeared as white colonies on pink background of the agar plate (Elli et al., 2006). (2) A modified MRS medium was used to screen EPS producing *Lactobacillus* strains while modified M17 medium for *Streptococcus* strains. MRS and M17 media were supplemented with ruthenium red (0.05%). Colonies grown on the agar surface were pink in non-ropy strains, and white in ropy strains (Hongpattarakere et al., 2012).

4.2.5.2. Rate of Acidification

4.2.5.2.1. Milk Acidifying Activity

Active bacterial cultures were prepared in MRS or M17 broth by overnight incubation overnight at 42 ºC. Aliquots of the cultures were inoculated into 200 ml, 10% skimmed milk containing 1% yeast extract, and were allowed incubation for 6h at 42 ºC. pH was determined with 1h intervals. Isolated strains reaching a pH point within

4.60-4.70 by 5th h of incubation were selected as promising candidates for yoghurt fermentation.

4.2.5.2.2. Proteolytic Activity

The agar medium used for the determination of proteolytic activity was made up of 1.5 % agar, 19% sodium glycerophosphate, 5% skimmed milk powder, and 0.01 g/1000 ml bromocresol purple. The isolated strains were propagated overnight in MRS or M17 broth at 42 ºC. Aliquots of the cultures were spread onto the agar plates, and were then allowed incubation for 72h at 37 ºC. Strains producing relatively larger colonies with yellow color and opaque appearance were classified as protease positive and fast acidifiers. Strains yielding smaller, white and translucent colonies were disregarded (Dandoy et al., 2011).

4.2.5.3. Probiotic Yoghurt Production

4.2.5.3.1. Assessment of Coagulation Properties

Strains of *Lb. bulgaricus* and *S. thermophilus* were activated in MRS (pH 6.2) and M17 broth (pH 6.9), respectively. Active cultures were inoculated (1% final volume) into 10% skimmed milk solution, including 1% yeast extract and the samples were then incubated for 5h at 42 °C.

4.2.5.3.2. Yoghurt Production and Selection of Starter Combinations

Isolated strains with acidifying capability of 4.60-4.70 after 5h incubation were used for the production of yoghurt. Isolated *S. thermophilus* and *Lb. bulgaricus* strains in pairwise combinations and at 1:1 ratio, were inoculated into the 2% solutions of evaporated and pasteurized milk (%17-19 total- solids, Or-Köy Dairy Plant and Sakıpağa Dairy Plant). Incubation was carried out for 3-4h at 42 °C. Yoghurt samples obtained were then cooled immediately and stored at 4 °C overnight. Following day,

organoleptic properties were tested using the criteria as follows: appearance, consistency with spoon, consistency in mouth, flavor, aroma, and overall acceptability**.**

4.2.5.3.3. Yoghurt Analysis

The combinations of selected isolates which were used for yoghurt production were provided (Table 4.2).

Table 4.2. Sample codes and combination of yogurt starters

Sample Codes	Yoghurt Combinations	Sample Codes	Yoghurt Combinations
Y1	UN5 X UF6	Y9	UN9 X UF6
Y2	UN5 X UZ12	Y10	UN9 X UZ12
Y3	UN5 X UZ22	Y11	UN9 X UZ22
Y4	UN5 X DT54	Y12	UN9 X DT54
Y5	UN5 X DT62B	Y13	UN9 X DT62B
Y6	UN5 X UIIN24	Y14	UN9 X UIIN24
Y7	UN5 X UIIN26	Y15	UN9 UIIN26
Y8	UN5 X UIN42	Y16	UN9 X UIN42

UN5 and UN9: Strains of *S. thermophilus* cocci, and the others were strains of *Lb. bulgaricus*)

4.2.5.3.3.1. Determination of Titretable Acidity

For determination of titretable acidity, yoghurt samples (10 g) were weighed and diluted in distilled water to 100 g. A few drops of phenolphatlein solution were added as an indicator. The samples were then titrated by using standardized (F =1.1336) 0.1 N NaOH solutions until obtaining a pink appearance. The measurements were done in duplicate.

Titretable acidity values were calculated using the following Equation 4.5.

Lactic acid % = 0.1 N NaOH amount x 0.009 x 100/Sample amount (g) (4.5.)

4.2.5.3.3.2. Syneresis of Probiotic Yoghurt Samples

Syneresis of yogurt samples was determined in duplicate as described by Rodarte et al. (1993). Each of the samples (10 ml) was centrifuged (Hettich-Universal 320R, Tuttlingen, Germany) for 20 at 5 000 rpm min at 4 ºC. The clear supernatant was decanted and measured. Syneresis was based on the volume of clear supernatant per 100 ml yogurt.

4.2.5.3.3.3. Apparent Viscosity of Yoghurt Samples

Apparent viscosity was determined using circulating water (Haake Viscotester 550 LV4 spindle, Thermo Inc., Germany) at 100 rpm in bath at 10ºC. Measurements were taking in duplicates. Sixty milliliters of yoghurt samples were placed into the cylindrical container and were firstly stirred for 20s clockwise then 20s counter-clockwise. A concentric cylinder MV-DIN sensor was used for the analysis of viscosity. Apparent viscosity was calculated at m Pa.s. All measurements were done in duplicate (Chekanov, 2008; Derrick et al., 2008).

4.2.5.3.4. Aroma Profiles of Yoghurt Combinations

Aroma compounds, acetaldehyde, ethanol, acetone and diacetyl were identified and quantified in a gas chromatography (GC) system (Agilent 6890N, USA), equipped with an automated headspace sampler (Agilent 7694, USA) and a FID detector (Agilent 5973Nms, USA). An agilent HP-5 column (3m, 0.25 mm, 0.25 μm) were used for separation of the aroma compounds.

Ten grams of yoghurt samples were weighed in the 20 ml headspace vials (Agilent, USA). The vials were then sealed with 20 mm aluminum crimp caps with dark gray septa (Agilent, USA), and shaken homogeneously. In order to achieve volatilization of volatile compounds, the samples were equilibrated for 20 min at 80°C. The following conditions were adapted for the headspace sampler and GC/FID system: injector temperature 250ºC, carrier gas helium at constant flow mode, a flow rate of 1ml/min, oven temperature program initially held at 35ºC for 6 min, and then

programmed from 35 ºC to 250°C an elevation rate of 30°C/min held at 250°C for 3 min. Total run time was 16.17 min. The interface line to FID was set at 300°C. The column temperature was programmed to facilitate the separation of compounds which were then detected with the flame ionization detector (FID).

Standard stock solutions were prepared in deionized water: 10 000 mg/L acetaldehyde (Fluka, Spain), 4 000 mg/L ethanol (Merck, Germany), 1 000 mg/L acetone (Merck, Germany), and 500 mg/L diacetyl (Merck, Germany). Six calibration points were chosen. Calibration curves were calculated by least-square regression from these six points. The R^2 values for the linear calibration curves were about 0.999 (Baran, 2013).

4.2.5.3.5. Textural Analysis of Yoghurt Combinations

Texture profiles of the yoghurt samples were obtained by using a TA XT2 texture analyzer (Yang and Li, 2010). An SMS P/0.5 probe was used to measure the texture of the samples at room temperature. During the initial steps, including pretest, compression and relaxation of the samples, the speed of the probe was 1.0 mm/s, and texture profile data were obtained at 200 pps. During the analysis a sample thickness of 5 cm was used, and 40 % of the original depth was compressed during the first stage.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Isolation of Traditional Yoghurt Starter Strains

For the isolation of yoghurt starter bacteria, thirteen artisanal yoghurt samples were used (Section 4.1.2). Aliquots of the samples were inoculated either in MRS or M17 medium, and endogenous bacteria were allowed to grow for 3 d at 42ºC under anaerobic conditions. Resulting bacterial colonies were counted as the arithmetic means of the colony forming units (CFU). Arithmetic means of the colonies grown on MRS agar plates were higher than those of the M17 (Table 5.1). This was in accordance with the expectations (Tamime, 1985). The ratio of the sample US, UIIB and DT were found to be expected. But, none of the isolated strains from the sample US had probiotic features. Whereas, the ratio of samples UZ UIB, UIN, UIIN UF and GA were found to be lower than the expected value, isolates from these samples had probiotic properties.

In total, 453 colonies were picked and 170 of them were purified. After purification, isolated strains were stored within glycerol-broth medium for preservation at - 80°C.

Table 5.1. Colony counts on the MRS and M17 plates

Yoghurt Sample Codes	MRS Plates (CFU/ml)	M17 Plates (CFU/ml)	M17/MRS Ratio
GM	1.2×10^7	1.1×10^7	0.92
GS	1.3×10^7	1.1×10^7	0.85
UF	1.8×10^7	1.4×10^7	0.77
US	1.5×10^7	1.9×10^7	1.27
GG	2.4×10^7	1.7×10^7	0.71
GA	2.1×10^7	2.0×10^7	0.95
UZ	1.7×10^7	1.5×10^7	0.88
UIB	1.8×10^7	1.5×10^7	0.83
UIIB	$1,1 \times 10^7$	$1,6 \times 10^7$	1.45
UN	1.6×10^7	1.6×10^7	1.0
UIN	1.1×10^7	0.6×10^7	0.54
UIIN	0.6×10^7	0.4×10^7	0.66
DT	1.2×10^7	1.5×10^7	1.25

5.2. Identification of Starter Strains

5.2.1. Phenotypic Identification

The colony morphology of the isolates were recorded as yellowish, rounded colonies as expected.

Strains isolated from MRS agar plates all were bacilli with long and rounded ends, often forming a chain of 3-4 cells. The isolates of M17 agar plates mostly had spherical or ovoid morphology, and appeared as pairs- or chains of cells (Fig. 5.1).

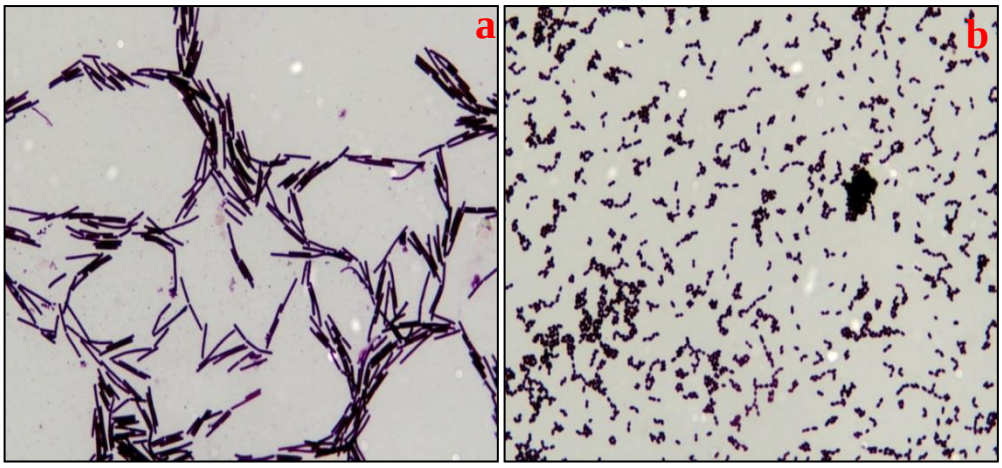

Figure 5.1. Gram stained representative isolates (a) bacilli (b) cocci

5.2.2. Presence of Catalase Activity

Catalase is an enzyme produced by many types of microorganisms, and it plays an important role in classification. Lactic acid bacteria (LABs) are generally known to be catalase negative and do not produce O_2.

All the bacilli isolates were found to be catalase negative, and only 18 of the cocci were catalase positive and were discarded. The remaining of the isolates was specified as LAB.

5.3. Preliminary Screening for Some of the Probiotic Characteristics

5.3.1. Tolerance to Low pH

Tolerance to low pH is one of the major selection criteria (Cakır 2003). Since, to reach the small intestine probiotics have to pass thorough from the stressful conditions of stomach (Bhatt, et al. 2012). Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays pH 3.0 has been preferred. The strains were screened using PBS, pH 3.0 (Bao et al., 2010). Only forty of the isolates tolerated this pH, most of which were bacilli (Table 5.2).

Table 5.2. Enumeration results of pH resistant isolates

Isolate Codes	Colony counts (cfu/ml)			Isolate Codes	Colony counts (cfu/ml)		
	10^{-6}	10^{-7}	10^{-8}		10^{-6}	10^{-7}	10^{-8}
GS18A	560	128	15	**UZ18**	102	11	4
UF2A	648	94	8	**UZ22**	146	20	2
UF4	240	30	6	**UZ32**	340	49	2
UF6	22	2	0	**UIN4B**	101	10	0
UF10	20	2	0	**UIN9**	642	178	36
GA12	83	8	0	**UIN22**	18	1	0
UN5	726	88	12	**UIN26**	118	19	2
UN9	692	152	32	**UIN42**	124	13	0
UN18	68	12	4	**DT54**	52	2	0
UN19	756	186	23	**DT62A**	784	152	28
UN22	12	3	0	**DT62B**	648	182	32
UN24	842	159	21	**DT66**	75	42	3
UN40	672	253	32	**DT74**	43	1	0
UIB2	788	164	14	**UIIN4**	48	2	0
UIB12	74	5	1	**UIIN18**	24	1	0
UIB14	894	116	79	**UIIN22**	4	0	0
UIB16	129	32	2	**UIIN24**	23	5	0
UIB31	860	160	26	**UIIN26**	19	3	0
UZ8	20	1	0	**UIIN28**	828	154	24
UZ12	14	2	0	**UIIN44**	25	4	0
UZ16	9	0	0				

These findings were also in accordance with those found in the literature (Pereira and Gibson, 2002), in which it has been shown that cocci were much more sensitive to low levels of pH than bacilli.

Hassandazadar et al. (2012) examined acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. They reported that, below pH 3.0 the number of bacteria in the medium decreased because of the loss of viability. At pH $\leq$ 2.0 none viable bacterial cells had been detected after the first hour.

Chan et al. (2011) reported that acids such as hydrochloric acids can be found in the human stomach disrupt the biomolecules of cells for example proteins, fatty acids and DNAs. The environment at low pH affected viability, growth and metabolism of *Lactobacilli*.

After examination, all of the isolates that were survived in pH 3.0 were taken to the next step.

5.3.2. Tolerance to the Simulated Human Gastric Juice

Simulated gastric juice medium was prepared and used for the screening, following the in vitro method reported by Musikasang et al. (2009). After 3h incubation, viability was determined by using standard pour plate method.

It was shown that only twenty nine of the acid tolerant isolates tolerated the simulated gastric human juice (Table 5.3). As can be seen, all of the cocci isolates (UN5, UN9, UN19, UIB31, and UIN9) survived the screening. Bacilli that did not survive were discarded (GS18A, UF2A, UF4, UF6, UF10, UN18, UN22, UN24, UN40, UIB2, UIB14 and UIB16).

Isolated strains, which resistant to acid and gastric environment, were selected for further analysis.

Table 5.3. Tolerance to simulated gastric juice

Isolate Codes	Colony counts (CFU/ml)			Isolate Codes	Colony counts (CFU/ml)		
	10^{-3}	10^{-4}	10^{-5}		10^{-3}	10^{-4}	10^{-5}
GS18A	0	0	0	**UZ18**	56	4	0
UF2A	0	0	0	**UZ22**	128	11	0
UF4	0	0	0	**UZ32**	98	5	0
UF6	248	38	0	**UIN4B**	253	29	0
UF10	0	0	0	**UIN9**	958	156	10
GA12	42	8	0	**UIN22**	2	0	0
UN5	56	2	0	**UIN26**	850	250	21
UN9	898	101	8	**UIN42**	71	10	0
UN18	0	0	0	**DT54**	86	5	0
UN19	890	352	100	**DT62A**	102	16	0
UN22	0	0	0	**DT62B**	118	9	0
UN24	0	0	0	**DT66**	181	10	0
UN40	0	0	0	**DT74**	205	19	0
UIB2	360	21	0	**UIIN4**	10	0	0
UIB12	0	0	0	**UIIN18**	29	1	0
UIB14	0	0	0	**UIIN22**	117	9	0
UIB16	0	0	0	**UIIN24**	48	3	0
UIB31	952	343	105	**UIIN26**	89	6	0
UZ8	978	148	22	**UIIN28**	893	256	32
UZ12	156	12	0	**UIIN44**	952	454	48
UZ16	187	21	0				

5.4. Physiological and Biochemical Characterization

Isolates which were tolerant both to low acid conditions and to gastric juice were subjected to biochemical identification.

5.4.1. Growth at Different Temperatures

The isolates were incubated at 10 °C, 45 °C, and 50 ºC. *Lb. delbrueckii* ssp. *bulgaricus* is not expected to grow at 10°C, while it can grow at 45°C. Surprisingly, however, three of the bacilli isolates, GA12, UIN22, and UIN26, did grow at 10°C (Table 5.4). One of the cocci also grew at this temperature (UIN9). All of the isolates did grow at 45°C, as expected, and at 50 ºC (Table 5.4). The growth at 50 ºC could be advantageous as it indicates that these bacteria could maintain their viability better at elevated temperatures, required at some stages of industrial yoghurt production.

5.4.2. Growth at Different NaCl Concentrations

Probiotic bacteria used as food adjuncts are commonly delivered in a food system and their journey starts from the mouth to lower intestinal tract. Therefore probiotic bacteria must overcome physical and chemical barriers such as bile and acid in the GI tract. Hence, tolerances to bile and acid and growth in presence of NaCl are the most important properties for selection of potential probiotic strains.

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. Current results show that *Lactobacillus* spp. isolated from yoghurts had tolerate 1-9% of NaCl and good growth was observed at 1% NaCl (Rana et al., 2012).

Cocci isolates were tested for their ability to grow in the presence of 2%, 4%, 6.5%, 8%, and 10% NaCl. They could all grow well in these conditions (Table 5.4).

Table 5.4. Growth at different temperatures and different NaCl concentrations

Isolate Codes	10°C	45°C	50°C	2% NaCl	4% NaCl	6.5% NaCl	8% NaCl	10% NaCl
UF6	-	+	+	+	+	+	+	+
GA12	+	+	+	+	+	+	+	+
UN5	-	+	+	+	+	+	+	+
UN9	-	+	+	+	+	+	+	+
UN19	-	+	+	+	+	+	+	+
UN26	-	+	+	+	+	+	+	+
UIB2	-	+	+	+	+	+	+	+
UIB31	-	+	+	+	+	+	+	+
UZ8	-	+	+	+	+	+	+	+
UZ12	-	+	+	+	+	+	+	+
UZ16	-	+	+	+	+	+	+	+
UZ18	-	+	+	+	+	+	+	+
UZ22	-	+	+	+	+	+	+	+
UZ32	-	+	+	+	+	+	+	+
UIN4B	-	+	+	+	+	+	+	+
UIN9	+	+	+	+	+	+	+	+
UIN22	+	+	+	+	+	+	+	+
UIN26	+	+	+	+	+	+	+	+
UIN42	-	+	+	+	+	+	+	+
DT54	-	+	+	+	+	+	+	+
DT62A	-	+	+	+	+	+	+	+
DT62B	-	+	+	+	+	+	+	+
DT66	-	+	+	+	+	+	+	+
DT74	-	+	+	+	+	+	+	+
UIN4	-	+	+	+	+	+	+	+
UIIN18	-	+	+	+	+	+	+	+
UIIN22	-	+	+	+	+	+	+	+
UIIN24	-	+	+	+	+	+	+	+
UIIN26	-	+	+	+	+	+	+	+
UIIN28	-	+	+	+	+	+	+	+
UIIN44	-	+	+	+	+	+	+	+
Lb.delb.* ssp.*bul.	-	+	+	+	+	+	+	+
St.therm.	+	+	+	+	+	+	+	+

(UN5, UN9, UN19, UIB31 and UIN9 were cocci and the others were bacilli isolates).

For the isolates of bacilli two NaCl concentrations, 4% and 6.5%, are generally used. Our isolates were grown at 2%, 4%, 6.5%, 8%, and 10% NaCl concentrations, and it was evidenced that all of the bacilli could grow at these salt concentrations (Table

5.4). These results were also in agreement with those of the literature (Hoque et al.,2010).

The growth abilities an extreme condition is very important for starters used in dairy industry. Drinking yoghurt, ayran, is another dairy product. There are two types of ayran process. Yoghurt is diluted with water after incubation and added salt. In other process, diluted milk is used for fermentation, after incubation, salt is added into the product. Toleration of high salt concentration can be an advantage for starter bacteria which is used for this type of products.

5.4.3. Sugar Fermentation

Carbohydrate fermentation appears to be the most discriminating biochemical method at strain level. All of the isolates of bacilli could utilise the sugars, arabinose, galactose, glucose, fructose, lactose, mannose, and ribose, as expected (Table 5.5). Only four of the bacilli (UF6, UZ8, UZ12 and UZ16) showed a negative fermentation pattern with maltose. Some of the bacilli, UF6, GA12, UIB2, UZ8, UZ18, and UZ22, could also not ferment the sugars mannitol, melibiose, salicin, and sucrose. Trehalose was fermented by only five of the bacilli (UIN42, DT54, DT62A, UIIN24, UIIN28, and UIIN44) (Table 5.5).

The isolates of cocci could ferment arabinose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, and sucrose, but they could not utilize glycerol, glycine, mannitol, melezitose, raffinose, rhamnose, sorbitol, or xylose (Table 5.5). Trehalose was also not metabolized. Only one of the cocci, UIN9, could use melibiose and salicin.

Table 5.5. Carbohydrate fermentation profiles of the isolates

Isolate Name	Arabinose	Fructose	Galactose	Glucose	Glycerol	Glycin	Lactose	Raffinnose	Rhamnone	Ribose	Maltose	Mannitol	Mannose	Melibiose	Melezitose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
UF6	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-
GA12	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
UN5	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	-
UN9	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	-
UN19	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	-
UN26	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
UIB2	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
UIB31	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	-
UZ8	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-
UZ12	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	-
UZ16	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	+	-	-
UZ18	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-
UZ22	+	+	+	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
UZ32	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	-
UIN4B	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-	+	-	+	-	-
UIN9	+	+	+	+	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-	-
UIN22	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	-	-
UIN26	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	-	-
UIN42	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	+	-
DT54	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	+	-
DT62A	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	+	-
DT62B	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	-	-
DT66	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	-	-

(cont. on next page)

Table 5.5. (cont.)

DT74	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	-	-
UIIN4	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	-	-	-
UIIN18	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	-	+	-	-
UIIN22	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-
UIIN24	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	-	+	-
UIIN26	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-
UIIN28	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	-	+	-
UIIN44	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	-	+	-
Lb.delb.ssp. bul.	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+	-
St. therm.	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+	+	-

(UN5, UN9, UN19, UIB31 and UIN9 were cocci and the others were bacilli isolates)

5.4.4. Gas Production

In order to differentiate the homofermentative and heterofermentative isolates, the presence of the activity of CO_2 production from glucose was investigated. All of the acidic pH resistant isolates were subjected to 5 d of incubation for the formation of CO_2.

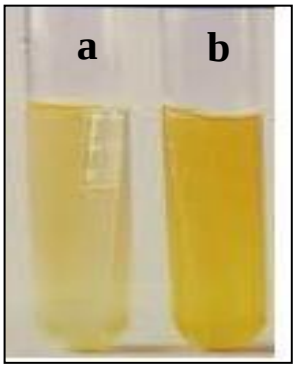

Figure 5.2. Glucose metabolism (a) Heterofermantative (b) Homofermentative

There was no gas accumulation in Durham tubes, although the cultures were well grown. This result confirmed the homofermentative behaviour of the isolates (Fig. 5.2b).

5.4.5. ß-Galactosidase Activity

Glycosidases are enzymes that are able to hydrolyse the glycosidic bonds. They are widely distributed in all organisms (Karasova et. al, 2002). β-Galactosidase, a glycosidase, is the enzyme widely used in dairy technologies. This enzyme hydrolyses lactose, the main carbohydrate in milk, into glucose and galactose (Gheytnchi et. al, 2010) to prevent the crystallization of lactose, to improve swetnes, to increase solubility of milk products and to produce lactose-free food products (Nyugen, 2010). It can also act on simple galactosides like o-nitrophenyl- β- D-galactopyronose (ONPG).

In humans, disfunction of the β-galactosidase enzyme causes digestive insufficiency, known as lactose intolerance. The existence or addition of bacteria, into dairy products, producing high amounts of ß- galactosidase enzyme can compensate for this insufficiency.

Another beneficial ability of β-galactosidases is the trans-galactosylation reaction which co-occurs during lactose hydrolysis resulting in the formation of galacto-oligoscharides (GOS) which posess prebiotic properties (Halbmayr et al., 2008).

To screen the presence of β-galactosidase activity, all of the isolates were grown in 5g/L lactose. When the incubation was ended, lactose hydrolysis was determined by using ONPG discs. The discs release a yellow chromogenic compound, o-nitrophenol, if galactose is free from lactose. Among our isolates only one of the cocci, UIN9, did not display ß- galactosidase activity. The type strains of *S. thermophilus* and *Lb. bulgaricus* used also did not appear to have a detectable ß-galactosidase activity (Fig. 5.3).

In one of the studies it has been reported that lactobacilli strains of human origin did not display any ß-galactosidase activity while those of the environmental strains did (Vindorella et al., 2008). Others have shown that *S. thermophilus* could exert β-galactosidase activity in human body (Iyer et al., 2010; Rizkalla et al., 2000).In this study, results were correlated with the literature.

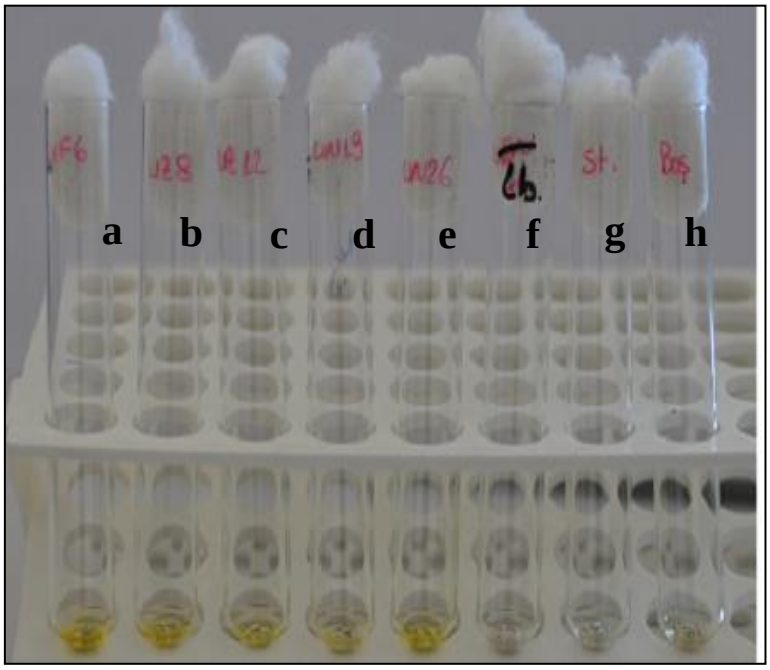

Figure5.3. ß-Galactosidase activity of some of the isolates and reference strains. Yellow colour (+) indicates the presence and white colour indicates (-) the absence of the activity. a: UF6, b:UZ8, c: UZ12, d:UN19, e: UN26, f: *Lb. delb.* ssp. *bul.* g: *S. therm.*, h: negative control.

5.4.6. Proteolytic Activity

There is a link between the presence of an efficient casein proteolytic system and fast growth, and acidification rate of LAB in milk. Casein breakdown is initiated by the cell-envelope proteinases (CEPs). Resulting oligopeptides are then transported into the cell where they are further hydrolysed by a set of various intracellular peptidases. LAB generally possess only one CEP, though some strains of Lb. delbrueckii ssp. bulgaricus can have more than one CEP-encoding genes (Dandoy et al., 2011).

Probiotic candidates were studied both for their milk acidification and proteolytic capacity. The isolates of cocci, identified as rapid acidifiers, also possessed CEP activity.

Proteolytic activity of isolated strains was also determined using another method using skimmed milk with bromocresol purple (0.01g/L) as the pH indicator. The yellow colonies indicated protease positive (Fig. 5.4).

A number of bacilli isolates, UN26, UZ32, UIN26, UIN42, DT54, DT62A, DT66, UIIN4, and UIIN28, identified as slow acidifiers, also displayed CEP activity.

Probiotics and especially *Bifidobacteria spp*. grow very slow in milk and often show a loss in viability at the end of shelf life of the products and it could be due to the lack of proteolytic activity in milk (Vinderola et al., 2000). In our study, isolated strains grow well in the skimmilk medium and determined as protease positive. This result was very important for bacterial survival into the milk environment and also for probiotic selection.

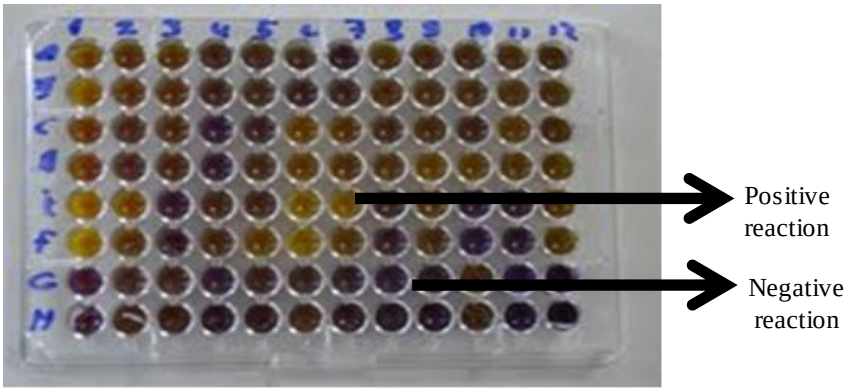

Figure 5.4. Proteolytic activity of isolated strains

5.4.7. Gelatinase Activity

Gelatinase activity of LAB is very important for dairy industry, especially in yoghurt production, because viscosity and gelling capacity of the products are positively affected by this activity.

Gelatinase activity was studied using the gelatin-medium liquefaction method (Sialedea et al. 2011). Our isolates appeared to have both the gelatinase and the proteolytic activity. Thus it could be thought that there was a positive correlation between these two activities.

Except for one of the bacilli, UIIN44, and the type strains of *S. thermophilus* and *Lb. bulgaricus*, all of the isolates tested, displayed gelatinase activity (Fig. 5.5).

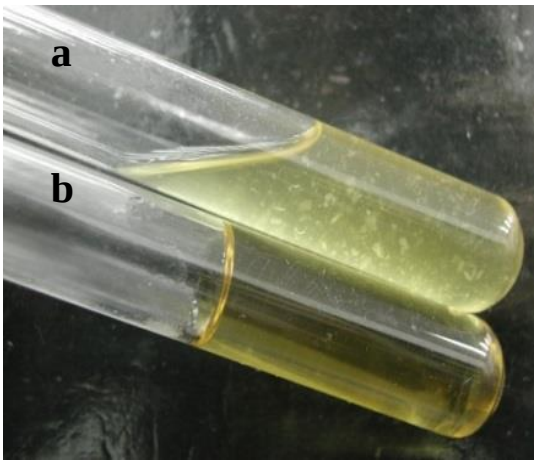

Figure5.5. Gelatinase activity (a) positive (UN5) (b) negative (UIIN4)

5.4.8. Urease Activity

Ammonia is produced by S. thermophilus as the breakdown product of urea in milk during fermentation of milk into yoghurt.

As expected, the cocci isolates, except one, UIN9, showed urease activity, and the isolates of bacilli did not have any detectable urease (Fig.5.6). These results were in accordance with those found in the literature, in that the colorimetric and conductimetric methods have been used (Mora et al., 2002).

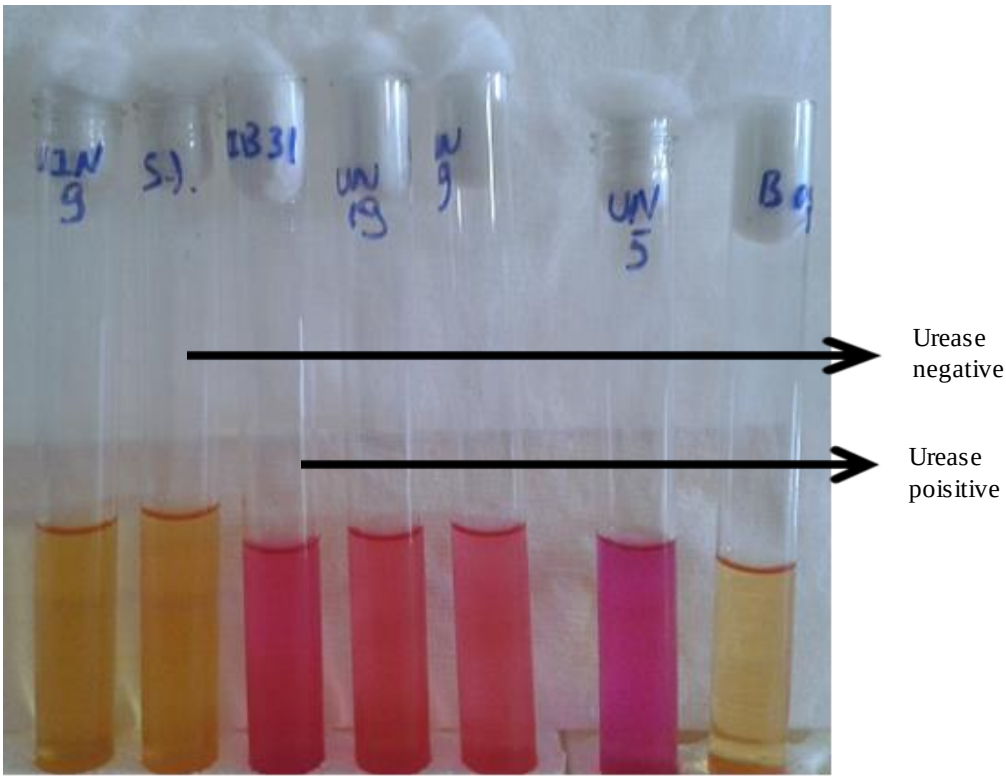

Figure5.6. Urease activity of cocci isolates

5.4.9. Indole Production

Indole is formed by the action tryptophanase. The enzyme is induced in the presence of tryptophan via the indole ring, and repressed by glucose in most colon bacteria. One of the by-products of this conversion is pyruvate, which can be utilized in the reactions of substrate-level of ATP synthesis. Diets rich in protein therefore strongly induce this activity. Lactic acid bacteria do not generally produce indole from tryptophan. None of our isolates did have indole producing activity (Fig. 5.7b). This was a very good result as it indicated the presence of another important probiotic feature.

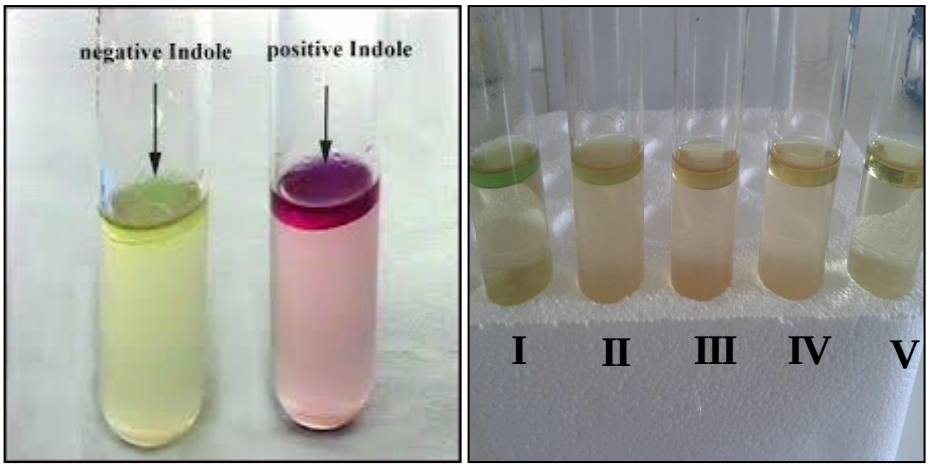

Figure 5.7. (a) Positive and negative indole reactions (b) Indole results of isolates. I. GA12, II. UN5, III. UN9, IV. UN26, V. UIB2

5.5. Further Screening for Probiotic Features

5.5.1. Bile Salt Tolerance

When fermented dairy products, mostly yoghurts, are supplemented with adjunct cultures of probiotic microorganisms, it must be taken into consideration that bacteria passing thorough the GI tract have to face the extremes of pH and salt environment and that most of them should remain alive in order to be able colonise the target organ, the intestines or the colon (Vindorella et al., 2008).

Bile salts, for example, are toxic for all cells, because they disrupt the structure of the cell membrane and therefore tolerance the bile salts is considered to be a prerequisite for the colonization and survival of bacteria in small and large intestine of the host (Mourad and Nour-Eddine, 2006; Guo et al., 2009). After bacterial exposure to bile salts, disruptions of cellular homeostasis occurred that caused the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in bacterial content leakage and finally death of cell (Mandal et al., 2006).

Bile salt concentration in GI is not static, ranging from 1.5 to 2 % (w/v) in the first hour digestion, and then decreasing approximately to 0.3% (w/v). The mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time is suggested to be 4 hours (Bhatt et. al., 2012).

Bile tolerance is considered to be one of the important properties required for high survival and as a consequence for a probiotic activity. There is no consensus about the precise concentration to which the selected strain should be tolerant (Rana et al., 2012).

In the study, the tolerance of the isolated strains to bile salts was investigated, using the range of concentration between 0.3 and 1.8%. MRS broth medium without bacteria was used as control along with the samples.

Results indicated that one percent concentration of bile salt was tolerated well by all of the cocci isolates (UN5, UN9, UN19 UIB31 and UIN9), which could also hydrolyze bile salts. Twelve of the bacilli isolates were also tolerant to 1% bile salt, and only three of them, GA12, DT66, and UIN22, could show the hydrolytic activity. The results indicated that, bile salts present in the bacteria cultures were much more effective on bacterial viability than effect of pH 3.0.

5.5.2. Bile Salt Hydrolytic (BSH) Activity

Bile salt hydrolysis is one of the desirable characteristics of a probiotic strain, as it plays a crucial role in the removal of cholesterol from the human body (Vindorela et al., 2008). Certain species of the indigenous microflora of the human intestine have evolved the deconjugate bile salts. This action is dependent on the presence of an enzyme known as bile salt hydrolase (BSH). It catalyzes the hydrolysis of glycine or taurine-conjugated bile salts into the amino acid residue and bile acid.

The presence of BSH activity was screened by spotting 10 µl aliquots of overnight cultures on MRS agar plates, containing 0.5% sodium taurodeoxcholic acid, and 0.37 g/L $CaCl_2$. Strains forming precipitation zones or opaque granular white colonies were regarded as BSH positive.

It was shown that all of the cocci isolates (UN5, UN9, UN19, UIB31, and UIN9) possessed BSH activity. This finding argued against those reported in the literature (Begley et al., 2006, McAuliffe et al., 2005). As was stated above, only three of the bacilli isolates (GA12, UIN22 and DT66) could precipitate sodium taurodeoxycholic acid and thus form precipitation zones, indicating BSH activity (Fig. 5.8)

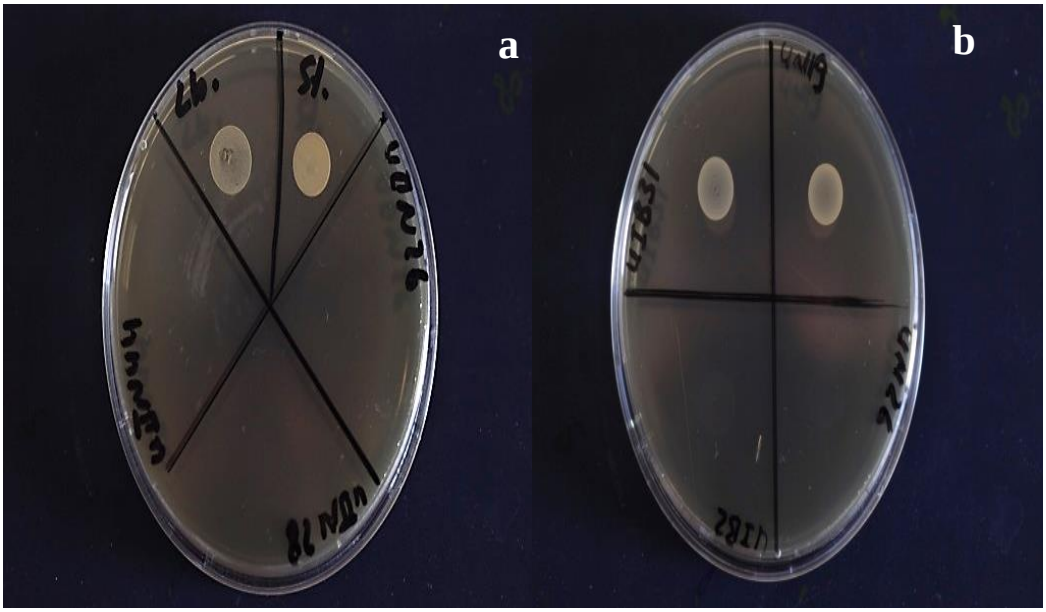

Figure 5.8. BSH activity of reference strains and some of the isolates.
a. Reference strains, and bacilli isolates, b. cocci isolates

5.5.3. Autoaggregation

Aggregation ability, termed as autoaggregation or self-aggregation, could be described as the clumping of cells of the same strain (Nikolic et al., 2010). Aggregation capacity, which is a function of cell surface hydrophobicity, is one of the most important probiotic features because it also determines the adherence capacity onto eukaryotic cells, specifically to the mucosal epithelia. Autoaggregation of the isolated strains was recorded at 600 nm after 24h of incubation at 37 ºC. All of the five cocci isolates (UN5, UN9, UN19, UIN9 and UIB31), and twelve of the bacilli isolates (GA12, UIIN24, UIIN44, UF10, DT62A, DT62B, UIIN4, UIN4B, UIIN28, DT74, DT66 and DT54) could self-aggregate (Fig. 5.9).

Kos et al (2003) studied autoaggregation ability of probiotic strain of *Lb. acidophilus* M92. Sedimentation rate of *Lb. acidophilus* M92 had been measured over a period of 5 h and they reported that the strain exhibited strong autoaggregation ability. The growth of the *Lb. acidophilus* M92 within the broth medium was better than on solid media could be the reason for better autoaggregation ability.

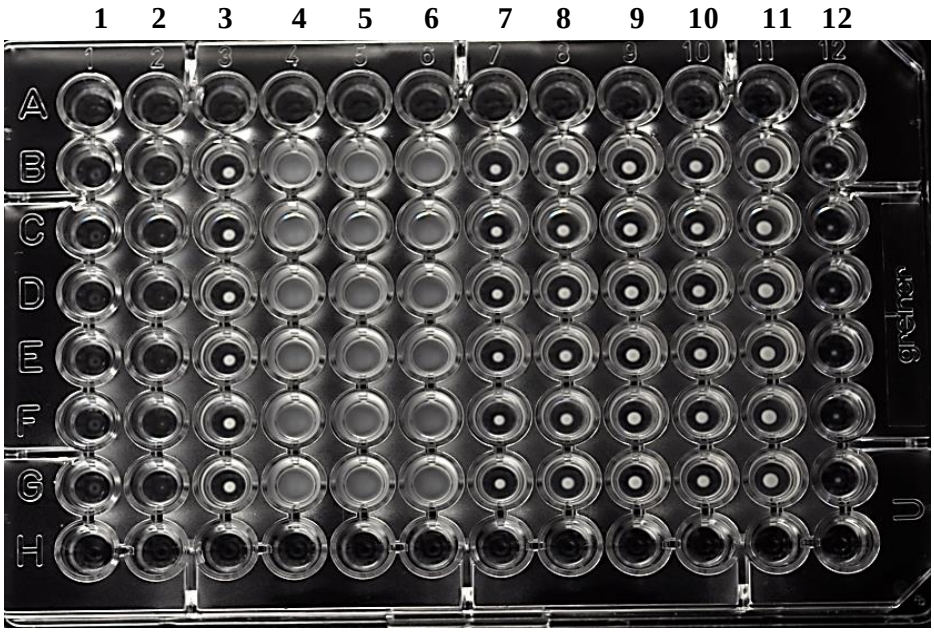

Figure5.9. Autoaggregation of some of the isolates. 1.UN24, 2.UZ12, 3.UN5, 4.UZ8, 5.UZ18, 6.UZ16, 7.UN9, 8.UN19, 9.UIN9, 10.UIB31, 11.GA12, 12.UZ32.

Rahman et al. (2008) reported that the pH of the medium has an effect on autoaggregation ability of bacteria. And they also suggested that bacteria loss autoaggregation ability when the assay applied on neutral media (PBS, pH 7.0) In the present study, the pH of the cultured media of all strains after 24 h incubation ranged from 3.5- 3.0.

5.5.4. Antibiotic Resistance Profiles of Strains

Antibiotic resistance patterns were produced by using the disc diffusion method (Mathara et al., 2008), and by following the NCCLS standards. The isolates grown onto Muller-Hinton agar plates (standard 25 ml) were screened against 13 different antibiotics (Table 5.6).

Table 5.6. Antibiotics used in experiments

Antibiotics		µg/disc
ß-Lactams	Penicllin G (P10)	10
	Amoxycillin (AML25)	25
	Amphicllin (AMP10)	10
	Cephalothin (KF30)	30
Aminoglycosides	Kanamycin (K30)	30
	Gentamicin (CM10)	10
	Lincomycin (MY2)	2
	Streptomycin (S10)	10
Broad Spectrum	Chloramphenicol (C30)	30
	Pefloxacin (PEF5)	5
Gram-positive	Azithromycin (AZM15)	15
Spectrum	Teicoplanin (TE30)	30
	Rifampicin (RD5)	5

A key requirement for the probiotic strains is that they should not carry transmissible antibiotic resistance genes. Because horizontal gene transfer taken place between the bacteria residing in the gut microflora could lead to the development of new antibiotic-resistance, ingestion of bacteria carrying such genes is undesirable (Zhou et al., 2005).

The results of antibiotics resistance were provided (Table 5.7). Some of the outstanding features of the Table could be summarized as follows: all of the isolates and references were sensitive to ampicillin, amoxicillin, teicoplanin, and penicillin; and again all of the cocci isolates produced resistance to kanamycin.

All isolates were tested for antibiotics and show different degree of resistance which was also observed by Bassyouni et al. (2012). Various reports indicate that LAB are normally resistant to the principal types of antibiotics, such as β-lactams, cephalosporin, aminoglycosides, quinolone, imidazole, nitrofurantoin and fluoroquinolines (Halami et al., 2000). However; .in this study, *Lb. bulgaricus* and *S. thermophilus* strains with reference strains were found as sensitive to β-lactams. Only DT62B, DT66 and UN22 were found to be resistant to amphicillin.

Table 5.7. Antibiotic resistance patterns of the reference strains and isolates

Isolate Codes	PEF5	AMP10	AML25	TE30	P10	AZM15	K30	RD5	S10	MY2	KF30	CM10	C30
Lb. bulg.	++	+++	+++	+++	+++	++	++	+++	+	+	+	++	+
S. therm.	+	+++	+++	+++	+++	+	+	++	+	+++	+++	+	+++
UIIN8	+	+++	+++	+++	+++	+++	++	+++	+	+++	+++	+++	+++
DT62B	+	+	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++
UN9	+++	+++	+++	+++	+++	+	++	+++	+	+++	+++	+++	+++
DT74	+	+++	+++	+++	+++	+++	+	+++	++	+++	+++	+	+++
UZ12	+	+++	+++	+++	+++	+++	+	+++	+	+	+++	+	+++
UN22	+	+	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
UIB2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
UIN42	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++
UN24	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++
UZ16	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
UIIN22	+	+++	+++	+++	+++	+	+	+++	+	+	+++	+	+++
UZ8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GA12	+	+++	+++	+++	+++	+++	+	+	+++	+	+++	+	+++

(cont. on next page)

Table 5.7. (cont.)

UZ22	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
UIN9	+	+++	+++	+++	+++	+	+	+++	+	+	+++	+	+++
UIN26	+++	+++	+++	+++	+++	+++	+	+++	++	+++	+++	+++	+++
UZ32	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++
DT54	+++	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++
DT66	+	+	+++	++	+++	+++	+	+++	+	+++	+++	+	+++
UIIN26	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++
UIIN44	+	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++	+++
DT62A	+++	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++
UN5	+++	+++	+++	+++	+++	+	++	+++	+	+++	+++	+++	+++
UIIN4	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++
UIN4B	+	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++
UIIN28	+	+++	+++	+++	+++	+++	++	+++	++	+++	+++	++	+++

+, resistant; ++, marginally susceptible; +++, susceptible

5.5. Cholesterol Assimilation

High concentration of cholesterol in the blood streams of humans has been recognized as a serious risk factor in the development of coronary heart diseases. The consumption of fermented dairy products containing probiotics has been claimed to lower the concentration of cholesterol to much healthier levels (Ahn et al., 2003), possibly by assimilating the cholesterol molecules during cell proliferation or by deconjugating bile salts (Ahn et al., 2000). Deconjugated bile salts are less soluble and excreted in faeces that must be replaced with new bile salts, formed from cholesterol in the body. Thus, the more bile salts excreted, the more cholesterol is removed from the body.

The percentage of cholesterol assimilated was determined during the 24h of incubation at 37°C in modified MRS broth. All tested bacilli strains were able to assimilate cholesterol within a percentage range between 19% and 54% (Fig. 5.10).

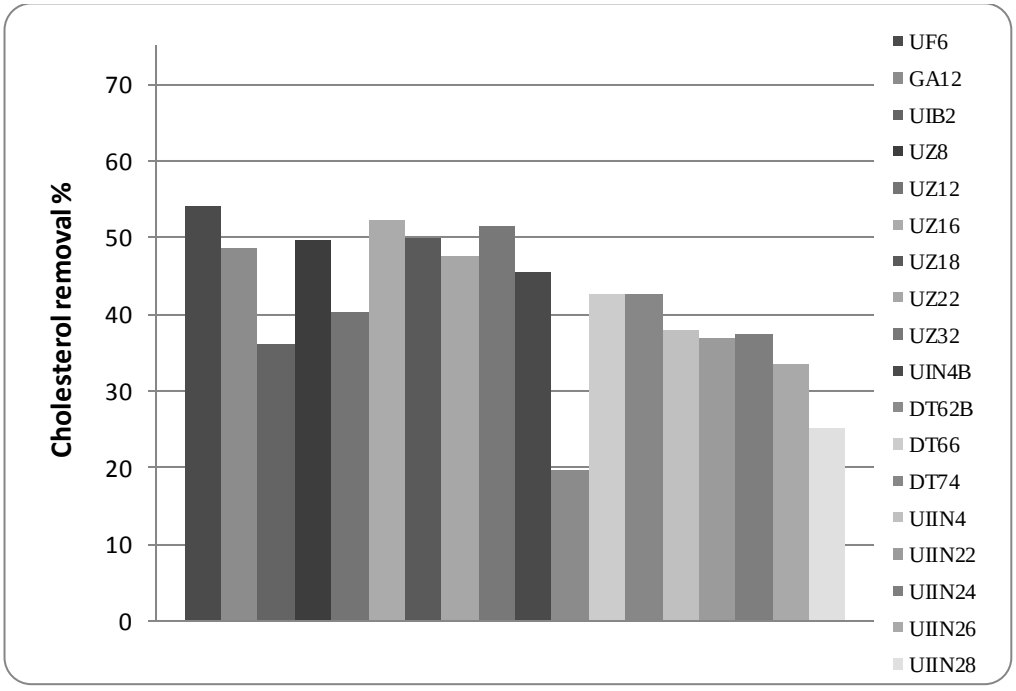

Figure 5.10. A histogram representation of the cholesterol removal percentage of bacilli isolates

Assimilation of cholesterol by cocci ranged between 50% and 44% (Fig. 5.11). These findings were in accordance with those found in the literature (Al-Saleh, 2006; Jain et al., 2009).

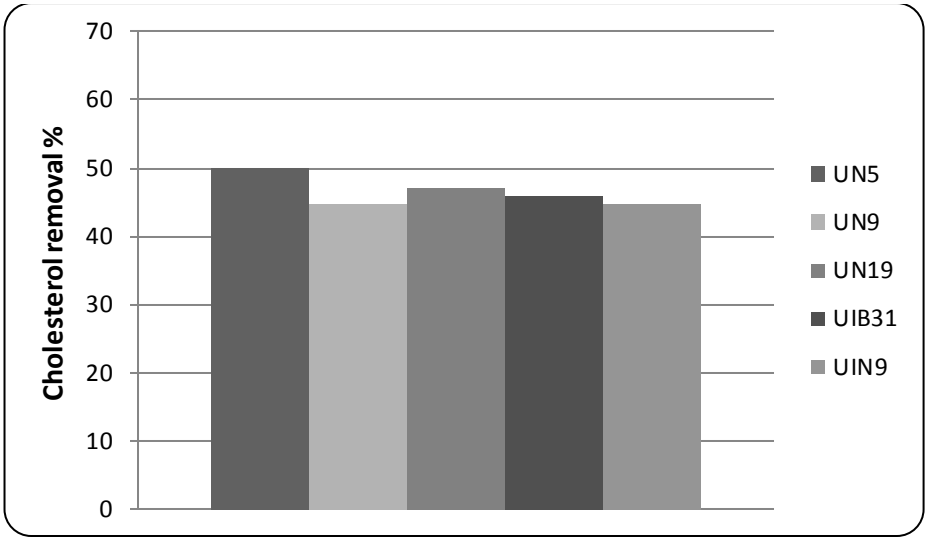

Figure 5.11. A histogram representation of the cholesterol removal percentage of cocci isolates

5.5.6. Cell Surface Hydrophobicity

Cell surface hydrophobicity has been associated with the bacterial attachment to a variety of surfaces. Greater hydrophobicity of cells resulted in greater levels of adhesion (Marin et al., 1997; Ly et al., 2008). Therefore, cell surface basically determine the interactions of a given microbial cell with its environment. These interactions play a very important role in the key processes of a microbial cell, such as cell division, growth, protection, and pathogenicity.

Cell surface hydrophobicity of the isolated strains was studied using two different hydrocarbons, hexane and xylene. It was found that the highest surface hydrophobicity percentage was 50% for the bacilli and 27% for the cocci isolates with hexane. The highest cell surface hydrophobicity with xylene was 36% for bacilli and 43% for cocci (Table5.8, Fig 5.12).

Table 5.8. % Cell surface hydrophobicy of isolates

Isolate Codes	**Hexane**	**Xylene**	**Isolate Codes**	**Hexane**	**Xylene**
UF6	16.07	24.84	UIN22	30.33	11.98
GA12	9.51	15.43	UIN26	3.64	10.22
UN5	5.84	10.95	DT54	21.58	34.25
UN9	2.28	10.07	DT62A	31.20	29.28
UN19	7.55	8.44	DT62B	7.82	12.77
UN26	3.71	9.79	DT66	2.84	9.5
UIB2	11.99	14.66	DT74	12.58	21.68
UIB31	26.45	16.34	UIIN4	9.89	5.13
UZ8	6.58	7.58	UIIN18	3.73	4.77
UZ12	3.27	6.03	UIIN24	20.47	19.05
UZ16	24.92	16.17	UIIN26	18.75	38.66
UZ18	49.72	11.87	UIIN28	5.86	5.74
UZ22	7.69	32.99	UIIN44	5.86	14.58
UZ32	49.91	35.97	*Lb. bul.*	18.81	18.61
UIN4B	19.68	22.38	*S. therm.*	12.48	12.48
UIN9	17.92	42.05			

Draksler et al. (2004) reported that the use of this assay was limited due to the harmful effects of hexane on the cell walls inducing lysis. However the use of xylene did not have negative effects on bacterial cells, and this organic solvent was recommended. This result was correlated with cell surface hydrophobicity with xylene in our research.

Cell surface hydrophobicity has also been considered a valuable reference when evaluating the adhesive properties of microorganisms. High hydrophobicity correlated with adhesion (Wadström et al., 1987). Lactobacilli with aggregation ability and hydrophobic cell surface could have more chance for adhesion to intestinal. It is reported that aggregative *Lactobacillus crispatus* is adhering better to Caco-2 cells than its non-aggregation mutant cells (Nikolic et al., 2010).

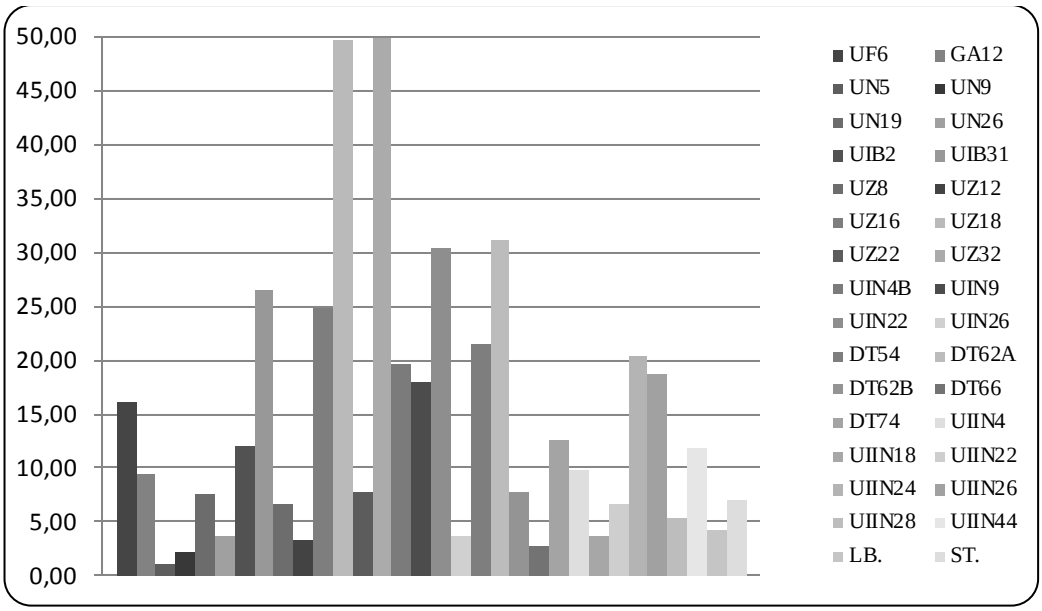

Figure 5.12. A histogram of cell surface hydrophobicity with hexane

Schillinger et al (2005) investigated cell surface hydrophobicity of lactobacillus strains using n-hexadecane. They reported that, the level of the cell surface hydrophobicity was different between species and strains. And they also indicated, the strain with a high cell surface hydrophobicity generally adhered to epithelium cells at a high level.

5.5.7. Growth in the Presence of Prebiotics

Human GI tract, especially the colon, harbours the heaviest load of microbes by serving as a combinatorial niche in which hundreds of species of commensal and symbiotic bacteria live together. Prebiotics are carbohydrates that selectively improve the survival and/or colonization of beneficial microflora.

Non-digestible oligosaccharides, fructo-oligosaccharides, have been shown to lead to a selective enrichment of lactic acid bacteria in the intestine. In the study, lactulose and inulin were used as prebiotics. The isolated strains were grown for 22 h at 37 °C in modified MRS broth media, supplemented with lactulose or inulin. Cell density was then recorded at 600 nm. It was found that all of the cocci isolates (UN5, UN9, UN19, UIB31 and UIN9) could grow in the presence of either of the prebiotics (Fig. 5.13).

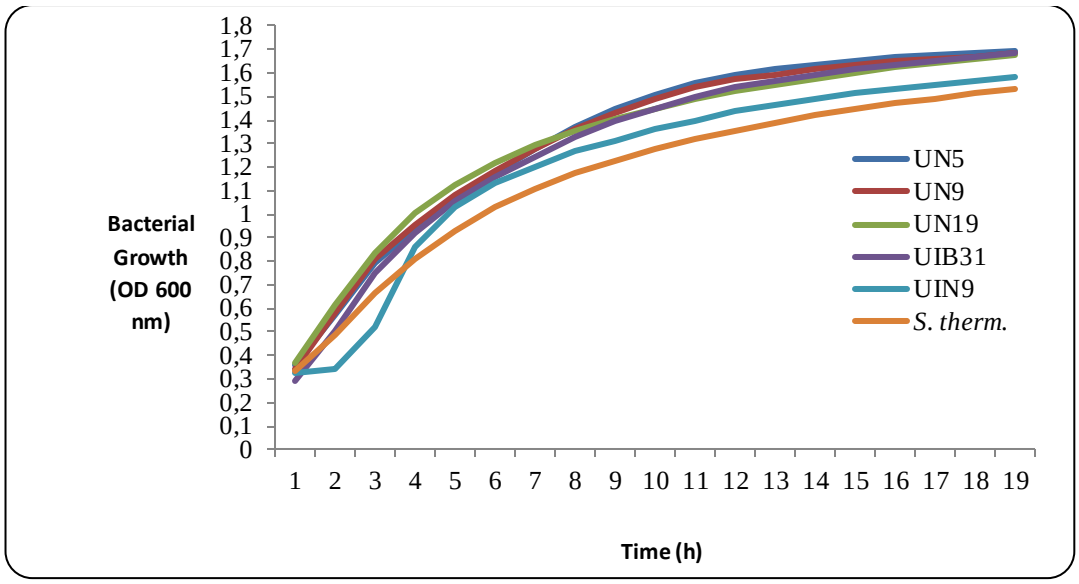

Figure 5.13. Growth rate of cocci in the presence of lactulose

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Most of the bacilli isolates also grew in the presence of lactulose or inulin (Fig. 5.13). It appeared that bacilli would need an adaptation period of 2 or 3 h before starting to grow in the presence of lactulose or inulin. The type strain of the *Lactobacillus bulgaricus* however did not appear to need an adaptation time (Fig. 5.14).

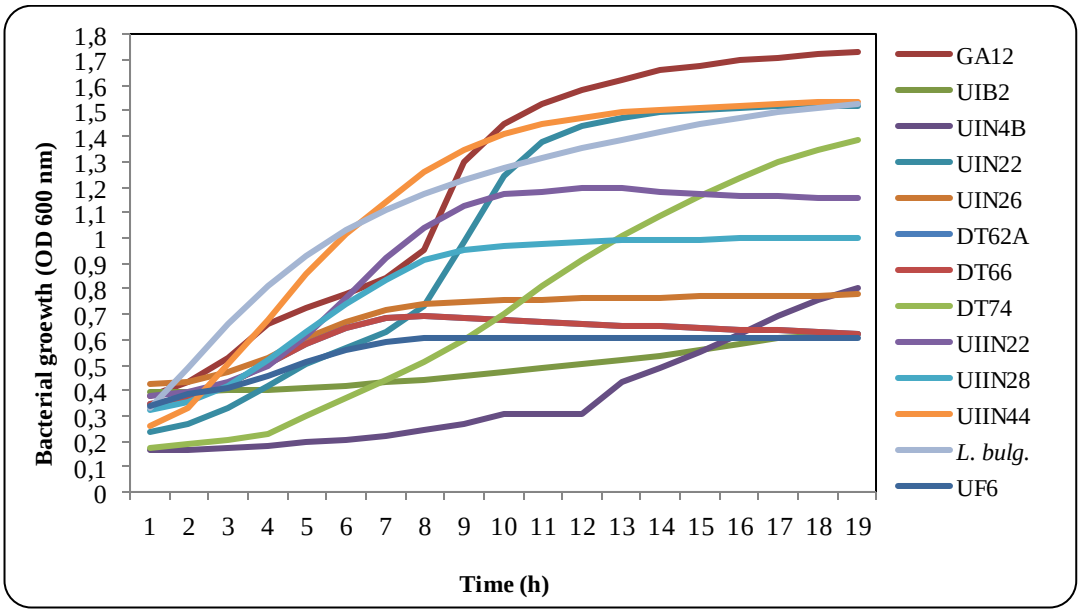

Figure5.14. Growth rate of bacilli in the presence of lactulose

5.5.8. Transit Tolerance Assay

5.5.8.1. Transit Tolerance to Gastric Juice

All of the isolated strains were exposed to pepsin for 4 h and their survival capacities were obtained by enumerating viable cell count.

The combined effect of pepsin-pH solution aims at stimulating the gastric juice, although it is not clear whether the decrease of viability conferred by the pepsin solution at low pH was due to the enzyme alone, or in synergy with low acidity. Probiotic microorganisms are generally consumed in the presence of milk proteins and they have a protective effect on starters and thus support bacterial survival in the acidic environment of the gastrointestinal system (Conway et al., 1987; Charteis et al., 1998)

In this study, strains were incubated for 4h in pepsin–PBS medium (pH 3.0). Results obtained indicated that this condition did not inhibit growth of any of the isolates completely (Table 5.9). The best survival obtained with the isolate UN19, and UIB31 also showed very high survival rate (Table 5.9). Reference strains of *S. thermophilus* also retained its viability (Table 5.9).

Table 5.9. Survival of the cocci isolates in the presence of pepsin, pH 3.0, expressed in log cfu/ml and survival percentage

	Pepsin (pH 3.0)				**Survival percentage**			
Strain	**0h**	**1h**	**2h**	**3h**	**1h**	**2h**	**3h**	**4h**
UN5	8.41	7.86	7.85	7.62	93.46	93.34	90.61	85.02
UN9	8.67	8.42	7.98	7.66	97.12	92.04	88.35	88.47
UN19	8.77	8.72	8.69	8.67	99.43	99.09	98.86	98.86
UIB31	6.65	6.40	6.30	6.30	96.24	94.74	94.74	94.74
UIN9	8.53	8.27	8.23	7.90	96.95	96.48	92.61	92.50
S. therm.	8.59	8.50	8.40	8.34	98.95	97.79	97.09	95.23

A graphical representation of the survival rate of the cocci isolates was also prepared (Fig.5.15).

Our findings on viability of the cocci isolates were in agreement with the data existed in the literature (Fernandez et al., 2003).

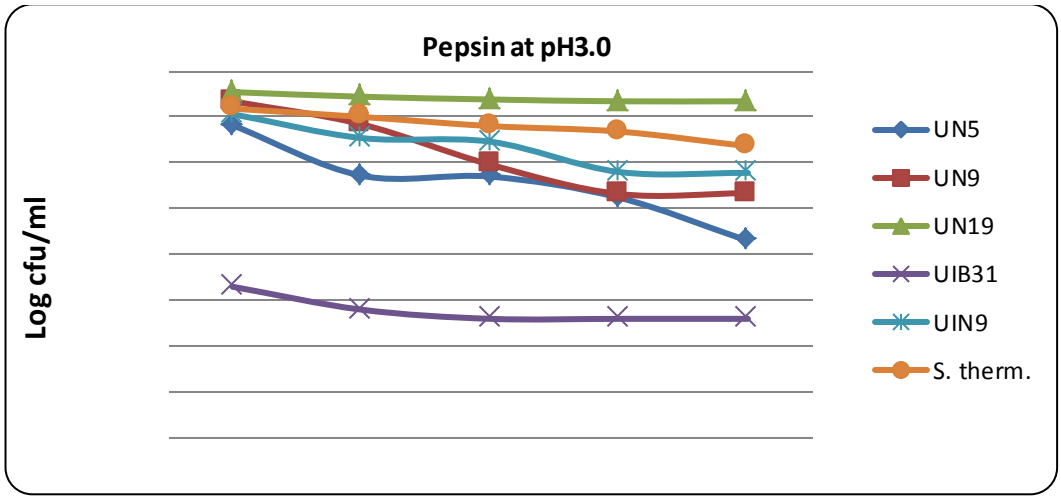

Figure 5.15. A graphical representation of the survival rates of cocci isolates in simulated gastric juice, pH 3.0

Survival rates of the bacilli isolates appeared to be quite varied (Table 5.10, Fig 5.16).

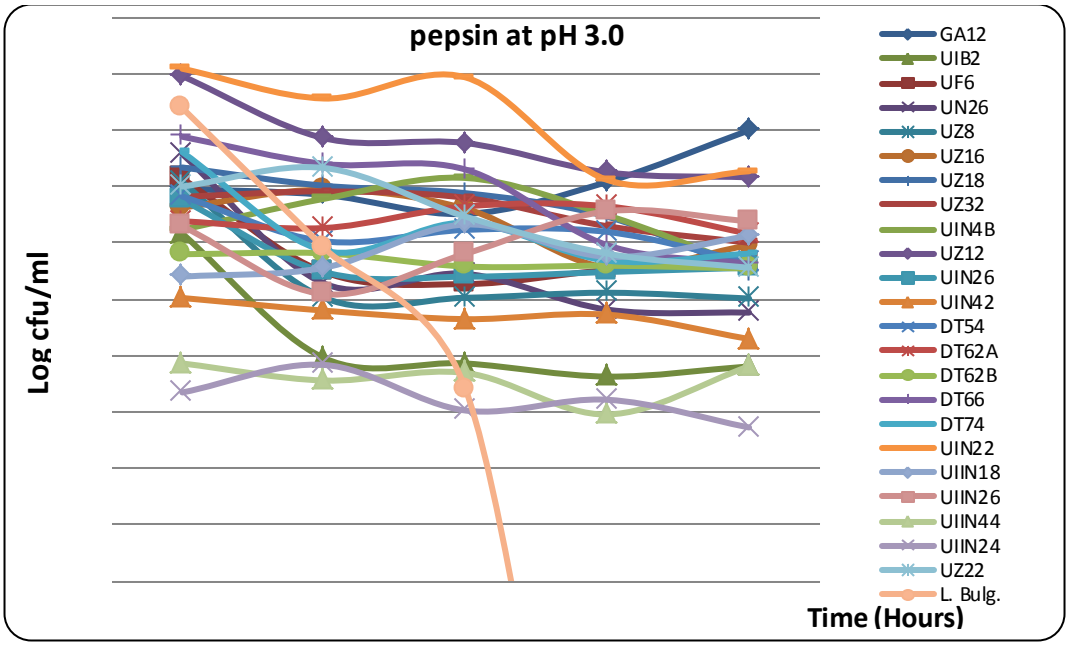

Figure 5.16. A graphical representation of the survival rates of bacilli isolates in simulated gastric juice, pH 3.0

Table 5.10. Survival rate of the bacilli isolates, expressed in log cfu/ml and percentage of survival

	Pepsin (pH 3.0)					Survival percentage			
Strain	**0h**	**1h**	**2h**	**3h**	**4h**	**1h**	**2h**	**3h**	**4h**
GA12	7.48	7.42	7.26	7.54	8.00	99.29	97.03	100.83	106.96
UF6	7.57	6.74	6.63	6.76	6.80	89.05	87.64	89.25	89.87
UIB2	7.08	5.98	5.93	5.81	5.90	84.42	83.74	82.09	83.37
UN26	7.79	6.64	6.72	6.41	6.38	85.26	86.36	82.28	81.94
UZ8	7.54	6.51	6.51	6.56	6.51	86.40	86.40	86.99	86.31
UZ16	7.31	7.47	7.30	6.78	6.98	102.16	99.85	92.70	95.43
UZ18	7.67	7.51	7.44	7.23	6.74	97.88	97.02	94.30	87.91
UZ32	7.41	7.45	7.40	7.15	7.00	100.65	99.88	96.48	94.51
UIN4B	7.11	7.39	7.57	7.24	6.78	103.87	106.47	101.81	95.28
UZ12	8.48	7.93	7.88	7.62	7.58	93.51	93.00	89.93	89.41
UIN26	7.38	6.74	6.70	6.74	6.78	91.33	90.77	91.33	91.84
UIN42	6.51	6.40	6.32	6.36	6.15	98.28	97.12	97.72	94.41
DT54	7.41	7.01	7.11	7.10	6.83	94.64	96.00	95.75	92.12
DT62A	7.19	7.13	7.32	7.32	7.08	99.17	101.83	101.83	98.45
DT62B	6.90	6.91	6.79	6.80	6.77	100.12	98.42	98.52	98.16
DT66	7.94	7.70	7.65	6.98	6.81	96.97	96.33	87.89	85.81
DT74	7.80	6.93	7.18	6.84	6.90	88.81	91.97	87.67	88.47
UIN22	8.54	8.28	8.47	7.56	7.63	96.91	99.09	88.45	89.36
UIIN18	6.70	6.78	7.16	6.88	7.06	101.18	106.90	102.63	105.40
UIIN26	7.15	6.54	6.90	7.28	7.19	91.58	96.60	101.86	100.62
UIIN44	5.93	5.78	5.85	5.48	5.90	97.45	98.58	92.37	99.56
UIIN24	5.67	5.92	5.51	5.61	5.36	104.31	97.18	98.86	94.53
UZ22	7.50	7.66	7.23	6.90	6.78	102.19	96.43	92.06	90.40
L. bulg.	8.19	6.95	5.70	0.00	0.00	84.86	69.55	0.00	0.00

A comparison of the figures obtained indicated that some of the bacilli isolates, GA12, UIIN26, and UIIN44, performed much better survival percentages in the simulated gastric environment in which the reference strain of *L. bulgaricus* did not.

In a study it has been reported that isolates of lactobacilli maintained their survival just for 30 min under the same conditions (Schillinger et al., 2005).

5.5.8.2. Transit Tolerance to Intestinal Juice

The survival percentages of the isolates were also screened using a trypsin –PBS solution, pH 8.0, and a time schedule of 1h, 2h, 3h, 4h, and 24 h. All of the cocci isolates were found to be tolerant to trypsin. Except for isolate UIB31, the remaining of

the strains, including the reference strain, could also grow in simulated intestinal juice for 4h. The isolate UN19 produced the most resistant profile and also retained its viability beyond 24 h (Table 5.11). Most of the remaining isolates appeared to display approximately 20% reduced survival rate at the end of 24th h. The reference strain of *L. bulgaricus* did not survive under this conditions.

Table 5.11. The survival rate of cocci after trypsin treatment at pH 8.0, expressed in log cfu/ml and survival percentage

	Trypsin (pH 8.0)						**Survival percentage**				
Strain	**0h**	**1h**	**2h**	**3h**	**4h**	**24h**	**1h**	**2h**	**3h**	**4h**	**24h**
UN5	7.24	7.33	7.30	7.29	7.29	6.48	101.24	100.83	100.69	100.69	89.50
UN9	7.31	7.57	7.54	7.54	7.50	6.20	103.56	103.15	103.15	102.60	89.19
UN19	7.98	8.34	8.24	8.34	8.26	6.73	104.51	103.26	104.51	103.51	84.34
UIB31	5.81	5.88	4.70	4.70	5.81	5.09	101.20	80.90	80.90	100.00	87.61
UIN9	8.17	8.40	8.58	8.56	8.68	6.88	102.82	105.02	104.77	106.24	84.21
S.therm	8.29	8.33	8.31	8.38	8.49	6.84	100.48	100.24	101.09	102.41	82.51

Survival percentage of each of the strains at 1st h, 2nd h, 3rd h, 4th h and 24th h were compared with that of 0h.

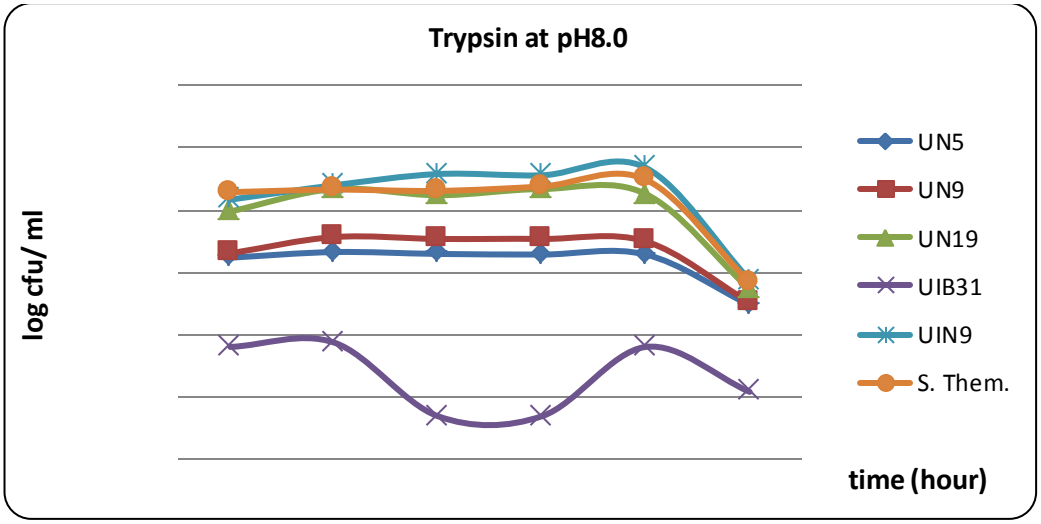

Figure 5.17. A graphical representation of the survival rates of cocci isolates in simulated intestinal juice, pH8.0

The cocci isolates could maintain a steady growth in simulated intestinal juice for the initial 4h of the incubation period, and survive for 24h. Survival time beyond

24h under these conditions is a very important criterion for the probiotics selection (Fig. 5.18).

Table 5.12. Survival percentage of bacilli in trypsin solution, pH 8.0

	Trypsin (pH 8.0)					Survival percentage			
Strain	**0h**	**1h**	**2h**	**3h**	**4h**	**1h**	**2h**	**3h**	**4h**
GA12	6.95	6.92	6.98	6.91	6.98	99.59	100.46	99.49	100.43
UF6	6.57	6.73	6.67	6.74	6.63	102.47	101.60	102.57	100.91
UIB2	5.47	5.54	5.81	4.95	0.00	101.36	106.27	90.57	0.00
UN26	6.48	6.50	6.29	6.30	5.69	100.22	97.08	97.13	87.86
UZ8	6.43	6.36	6.38	6.36	5.73	98.88	99.11	98.82	89.10
UZ16	6.62	6.43	6.69	6.62	6.47	97.18	101.02	100.00	97.76
UZ18	5.80	6.31	6.51	7.11	6.67	108.90	112.24	122.71	115.12
UZ32	7.18	7.04	7.13	7.11	7.13	97.98	99.23	99.00	99.26
UIN4B	6.94	6.63	6.83	6.73	6.70	95.59	98.41	96.96	96.53
UZ12	7.14	7.28	6.85	7.04	6.68	101.87	95.83	98.58	93.47
UIN26	6.20	6.45	6.35	6.15	6.15	103.92	102.39	99.07	99.07
UIN42	6.22	6.19	5.88	5.40	6.43	99.56	94.49	86.82	103.44
DT54	6.72	5.95	5.78	4.70	5.78	88.60	85.98	69.92	85.98
DT62A	6.71	6.72	6.76	6.76	6.71	100.06	100.66	100.77	99.94
DT62B	7.54	7.60	7.66	7.35	6.47	100.83	101.69	97.48	85.86
DT66	6.96	6.94	6.93	6.84	6.82	99.79	99.57	98.31	98.08
DT74	6.65	6.48	6.33	6.16	6.00	97.42	95.25	92.68	90.25
UIN22	7.22	7.57	7.56	7.44	7.51	104.92	104.75	103.08	104.01
UIIN18	7.52	6.22	6.13	6.06	6.20	82.67	81.51	80.58	82.49
UIIN26	6.64	6. 2	5.88	6.10	6.57	95.16	88.43	91.77	98.96
UIIN44	5.67	6.57	6.54	6.48	6.41	116.00	115.36	114.29	113.19
UIIN24	5.23	5.31	5.57	5.67	5.69	101.45	106.43	108.46	108.76
UZ22	6.15	6.53	6.39	6.37	6.06	106.17	103.95	103.66	98.61
Lb.delb. bul.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* Values were means of duplicates (SD 0.3-0.9)

Some of the lactobacilli isolates (GA12, UZ12, UZ32, DT62A , DT66 and UIIN26) could maintain their viability beyond 24th h of incubation at survival rates 3.52, 8.47, 3.32, 5.0, 5.74, and 6.47 log cfu/ml, respectively.

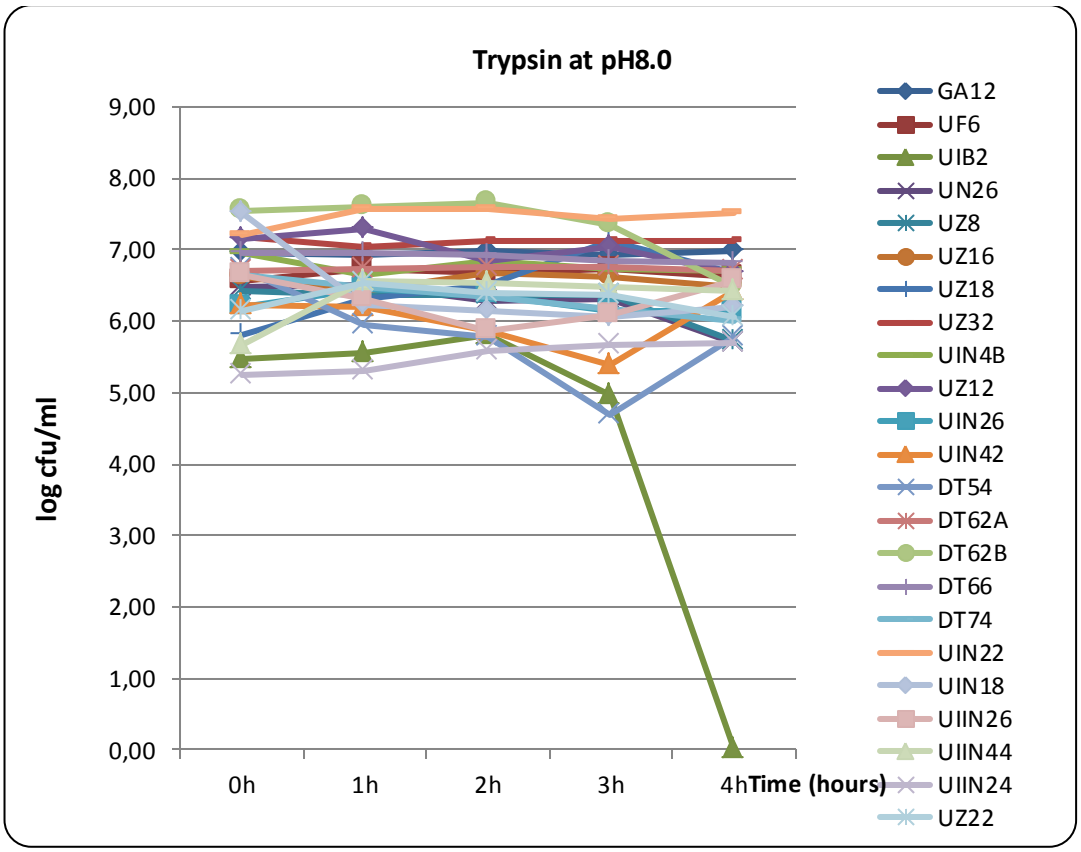

Figure 5.18. A graphical representation of the survival rates of bacilli isolates in simulated intestinal juice, pH8.0

These in vitro findings suggested that these probiotic candidates isolated from the traditional yoghurt samples could have the potential to successfully pass through the stomach and be able to lodge in the colon.Thus the strains deserve further investigations using animal models (Maragkaodakis et al., 2006).

5.5.9. Adhesion onto Eucaryotic Cells

A human cell line, Caco-2, was used in the adhesion experiments. It has been derived from human ephitelial colorectal adenocarcinoma. It is a population of heteregenous cell that, cultured under specific conditions, become differntiated and

resemble the enterocytes, lining the small intestine. It is generally used for the assessment of bacterial adherence to predict the absorption of orally administered probiotics (Sarem et al., 1996; Lin et al., 2006).

All of the strains tested could adhere to Caco-2 cells with a maximum adherence percentage of 75%, UIN9, (Table 5.13).

Table 5.13. Adhesion percentage of the cocci isolates onto Caco-2 cells

Strain	log cfu/ml before adhesion	Log cfu/ml after adhesion	% Adhesion
UN5	7.30	3.70	50.66
UN9	7.46	3.90	52.30
UN19	7.08	3.70	52.25
UIB31	7.69	3.60	46.84
UIN9	7.52	5.64	74.97
S. therm.	7.85	4.91	62.59

Bacilli isolates yielded higher adhesion percentages than those of the cocci isolates. Only one of the bacilli, UZ8, showed a maximum adherence percentage of 78.5% (Table 5.14). The reference starins of *Lb. delbrueckii* ssp. *bulgaricus* did not seem to adhere to Caco-2 cells.

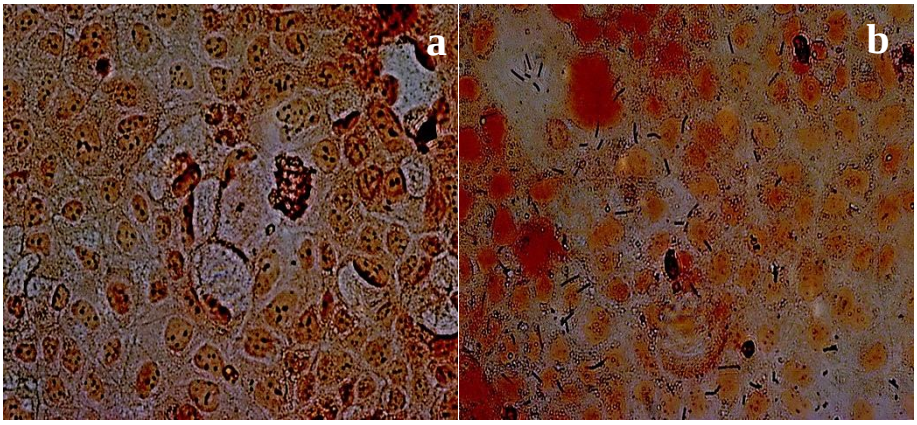

Figure 5.19. Gram staining of Caco-2 cells (a) before, and (b) after the adhesion

It appears that not all the environmental isolates of LAB can adhere to epithelial cells. In a study it has been reported that lactobacilli strains, isolated from commercial fermented products, were not able to adhere to ephitelium cells (Lin et al., 2006). Others, on the other hand, have demonstrated that lactobacilli isolates from dairy

products did show adherence but at much lower levels, 4%, to Caco-2 cells (Maragokoudakis et al., 2006).

Table 5.14. Percentages of adhesion of the bacilli isolates on Caco-2 cells

Strain	**log cfu/ml before adhesion**	**log cfu/ml after adhesion**	**Adhesion %**
UF6	6.11	3.00	49.07
GA12	6.76	4.91	72.73
UN26	6.98	5.02	71.90
UIB2	6.98	3.00	42.97
UZ8	6.57	5.16	78.54
UZ12	6.96	4.76	68.29
UZ16	6.54	5.09	77.72
UZ18	6.20	4.63	74.68
UZ22	7.05	4.04	57.33
UZ32	7.00	4.85	69.26
UIN4B	7.26	4.92	67.76
UIN22	7.01	5.12	73.01
UIN26	6.89	4.18	60.64
UIN42	6.76	5.07	75.07
DT54	5.85	3.85	65.78
DT62A	6.49	4.85	74.73
DT62B	6.54	4.08	62.33
DT66	6.20	4.65	75.00
DT74	6.91	4.41	63.86
UIIN4	6.15	4.32	70.32
UIIN18	6.54	4.58	69.98
UIN24	6.90	4.57	66.23
UIIN26	6.79	4.65	68.58
UIIN28	6.94	4.73	68.19
UIIN44	6.86	4.78	69.68
Lb. delb. bul.	0.00	0.00	0.00

5.5.10. Antimicrobial Activity of the Isolates

Antimicrobial activity is another important feature of the probiotic cells. Several researchers have observed that LAB strains can also produce antimicrobial substances which are active against pathogenic bacteria (Topisirovic et al., 2006). Thus, our isolates were examined for their antimicrobial activity against *E. coli* (RSHM 4024), *L. inocua* (NRRL-B 33314) and *S. aureus* (RSSK 1009) which are known to be common

foodborne pathogens. Antimicrobial effects were observed with a non-adjusted pH supernatant.

Results indicated that none of the cocci isolates, including the reference strains, could develop growth inhibition zones. And neither the cocci nor the bacilli isolates could show antimicrobial activities against *S. aureus*. Some of the bacilli could, however, display antimicrobial activity against both *E. coli* and *L. inocua* (Table 5.15). The activities were judged by the magnitude of the inhibition zone, and thus it was a qualitative assessment.

Through production of bacteriocins, H_2O_2, or organic acids, LAB could inhibit the growth of certain species of bacteria. Overnight cultures of our isolates appeared to have a pH range between 4.2 and 4.6. Thus, the isolates could inhibit the growth of acid-sensitive microorganisms at pH values below this range (Lin et al., 2007).

Koll-Klais et al. (2005) reported that the availability of substrate for fermentation seems to be one of the essential factors for the antimicrobial activity. When lactobacilli were grown on MRS agar with normal glucose content (20g/L), growth inhibition on pathogen is more pronounced.

Table 5.15. Antimicrobial activity results

Isolate Codes	*E.coli*	*L.innocua*	*S. aureus*
UF6	(—)	(—)	(—)
GA12	++	++	(—)
UN5	(—)	(—)	(—)
UN9	(—)	(—)	(—)
UN19	(—)	(—)	(—)
UN26	(—)	++	(—)
UIB2	++	++	(—)
UIB31	(—)	(—)	(—)
UZ8	+	++	(—)
UZ12	++	+	(—)
UZ16	++	+	(—)
UZ18	++	+	(—)
UZ22	+	+	(—)
UZ32	+	+	(—)
UIN4B	+	(—)	(—)
UIN9	(—)	(—)	(—)
UIN22	++	+	(—)
UIN26	(—)	(—)	(—)
UIN42	+	(—)	(—)
DT54	+	(—)	(—)
DT62A	++	++	(—)
DT62B	++	+	(—)
DT66	+++	++	(—)
DT74	(—)	(—)	(—)
UIIN4	+	+++	(—)
UIIN18	(—)	+++	(—)
UIIN22	(—)	++	(—)
UIIN24	(—)	++	(—)
UIIN26	+	+++	(—)
UIIN28	+	+++	(—)
UIIN44	+	+	(—)
Lb. delb.* ssp.*bul.	(—)	(—)	(—)
S. therm.	(—)	(—)	(—)

Symbols for diameter of zone inhibition: (—)no inhibition; (+): < 10 mm; (++): ≥10 mm; (+++): ≥15

5.6. Molecular Characterization of Candidate Probiotic Strains

5.6.1. Isolation of Genomic DNA

The quality of genomic DNA of the isolates was checked by agarose gel electrophoresis, and the purity was determined by using the ratios of the absorbance values (A_{260nm}/A_{280nm}) (Nanodrop, 8000-Thermo Scientific; Appendix D). Some of the genomic DNA samples were shown (Fig. 5.20). As can be seen, genomic DNA formed a continuous smear beginning at the wells.

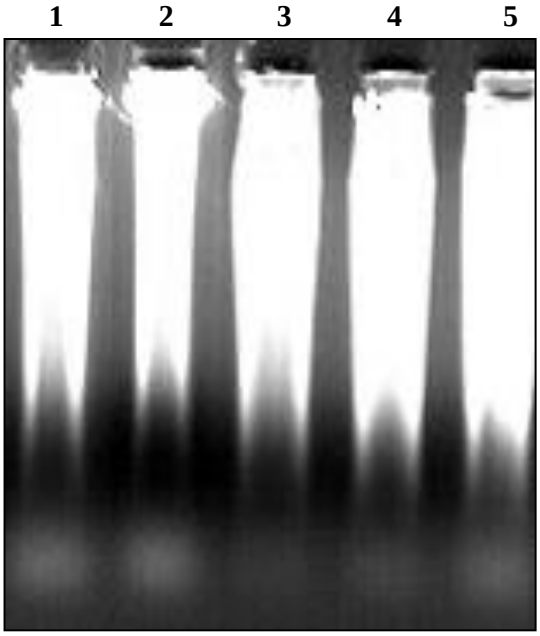

Figure 5.20. An image of genomic DNA samples

5.6.2. Amplification of 16S-ITS rDNA and 16S-ITS RFLP

The chromosomal region of approximately 2000 bp, comprising 16S rRNA gene and ITS sequence, was amplified by PCR. Some of the amplification products were shown (Fig. 5.21).

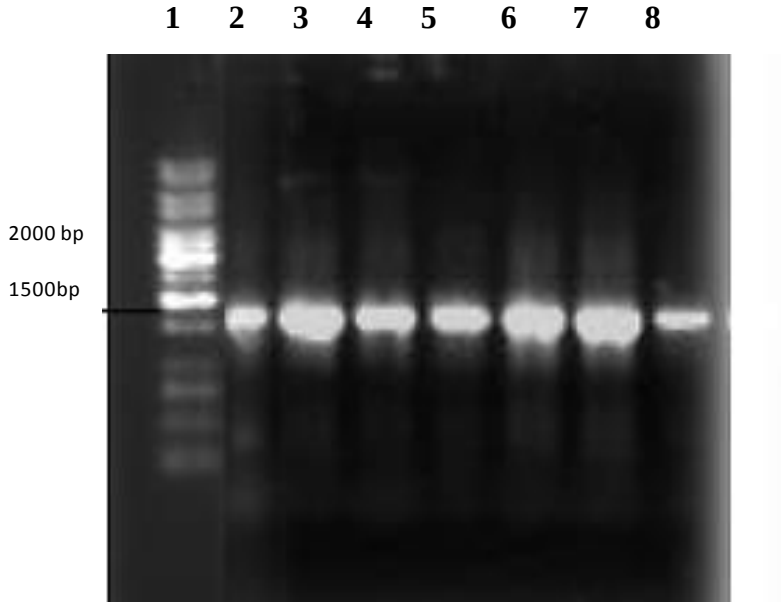

Figure5.21. Amplification products of 16SrDNA gene-ITS sequence of bacilli isolates: 1.1kb DNA marker, 2.UF6, 3.GA12, 4.UN26, 5.UIB2, 6. UIN22, 7. DT54, and 8. *Lb. delbrueckii.* ssp. *bulgaricus* CCM 7190.

Restriction fragment length polymorphism (RFLP) profiles were obtained by digesting the amplification products with *Eco*RI restriction endonuclease (Fig. 5.22).

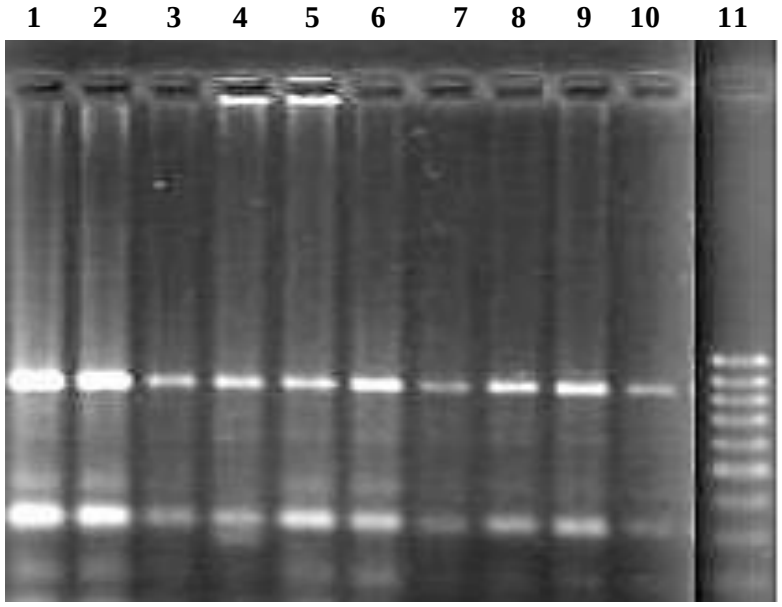

Figure 5.22. *Eco*RI-RFLP profiles of 16SrRNA gene-ITS sequence: 1. UF6, 2. GA12, 4. UN26, 5. UIB2, 6. UIN22, 7. DT54, 8. DT62A, 9. UIIN44, 10. *Lb. delbrueckii.* ssp. *bulgaricus* CCM 7190, and 11. 100 bp DNA marker

As it was obvious in the image that *Eco*RI restriction fragments yielded almost identical RFLP profiles for all of the isolates of bacilli (Fig. 5.22). Considering that the isolates were different in biochemical features, the RFLP results patterns could be taken as an indication that each of the bacilli isolates constituted a distinct strain of *Lb. delbrueckii.* ssp. *bulgaricus*.

5.6.3. Identification of the Isolates of Cocci by ARDRA

IYTE1 and IYTE 2 primers were used for the identification of cocci isolates by ARDRA. The length of the amplification products varied between 900 and 1000 bp (Fig. 5.23).

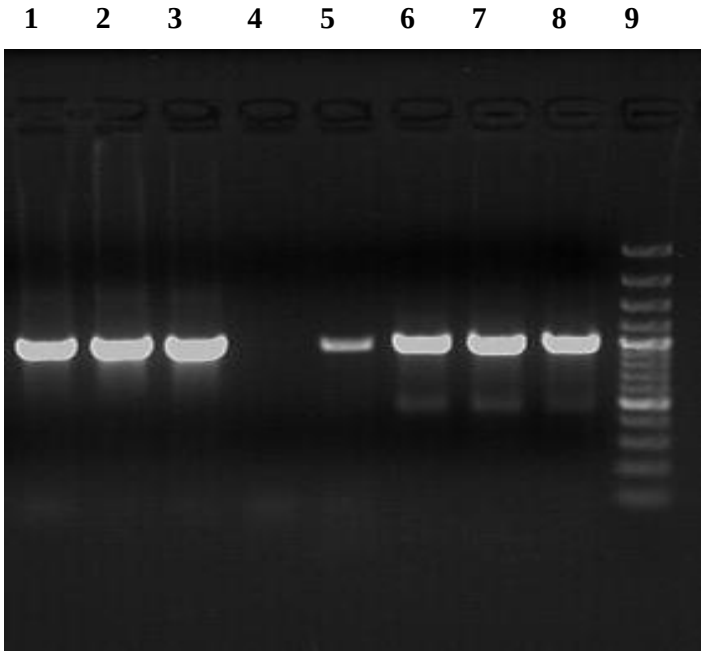

Figure 5.23. Amplification products of the cocci isolates and the reference strain: 1. UN5, 2.UN9, 3. UN19, 5. UIB31, 6. UIN9, 7 and 8. *S. therm*. CCM 4797, 9.100 bp DNA marker

The amplification products were treated with *Hae*III restriction enzyme and digestion products were resolved in 2% agarose gel (Fig. 5.24).

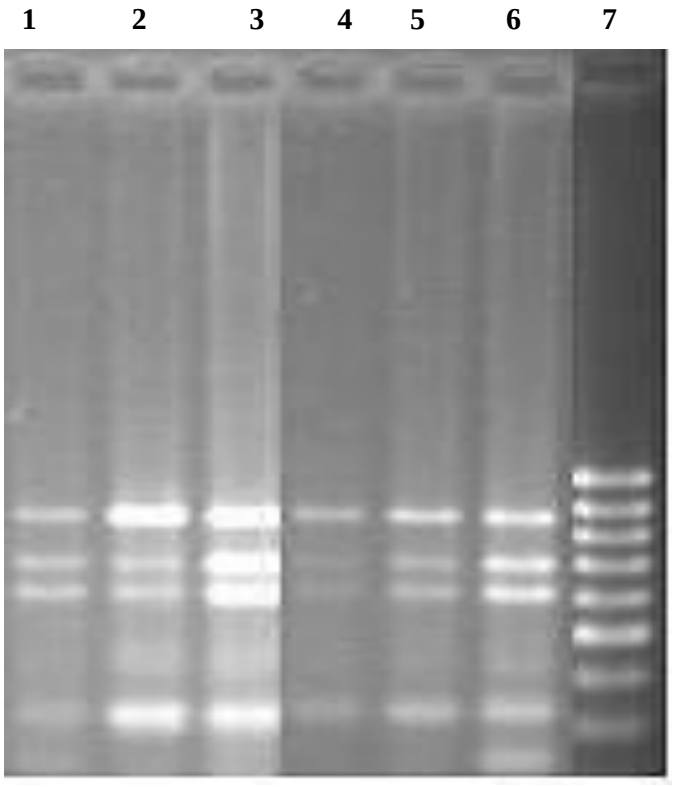

Figure 5.24. ARDRA patterns obtained with *Hae*III restriction enzyme: 1. UN5, 2. UN19, 3.UN19, 4.UIB31, 5. UIN9, 6. *Lb. delbrueckii.* ssp. *bulgaricus*, and 7. 100 bp DNA ladder

*Hae*III restriction enzyme produced identical ARDRA patterns and thus it was not possible to differentiate between the cocci isolates. Here again, because each of the cocci isolates yielded distinct carbohydrate profiles, it would be plausible to assume that each of the isolates formed a distinct strain of *S. thermophilus*. For example, mannose was fermented only by the isolate UIN9.

5.6.4. Pulsed Field Gel Electrophoresis (PFGE)

Molecular methods employed so far could not differentiate the candidate probiotic strains. Thus the isolates were subjected to a much more powerful discrimination procedure, known as PFGE.

RFLP fragments were obtained by digesting the chromosomal DNA was digested with *Sma*I restriction enzyme. PFGE could discriminate the cocci isolates as distinct strains (Fig. 5.25).

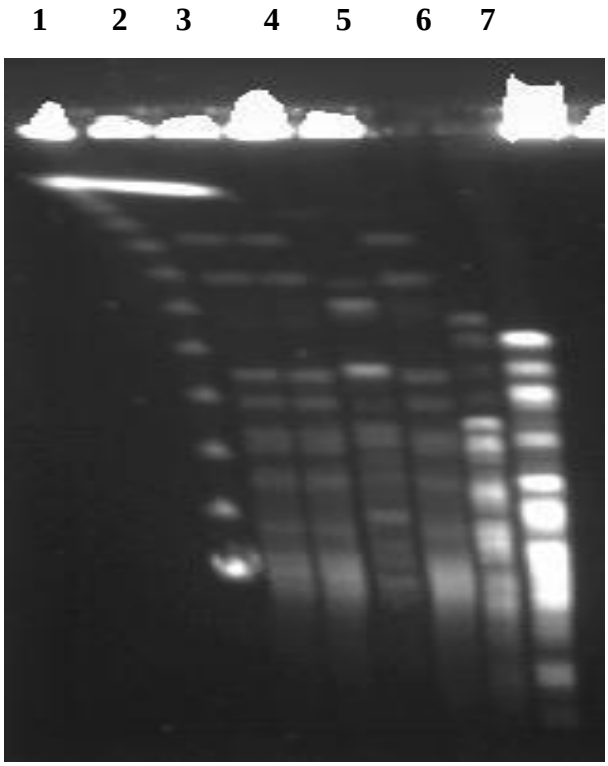

Figure 5.25. PFGE-RFLP profiles of the cocci isolates obtained with *Sma*I: 1. PFGE standart marker, 2. UN5, 3. UN9, 4. UN19, 5. UIB31, 6. UIN9, and 7. S. *thermophilus* CCM 4797.

PFGE profiles of *the cocci* isolates yielded three distinct homology groups. The reference strain also had a unique restriction profile. It could be seen that isolates with identical restriction profiles were clustered into the same homology group (Fig. 5.26).

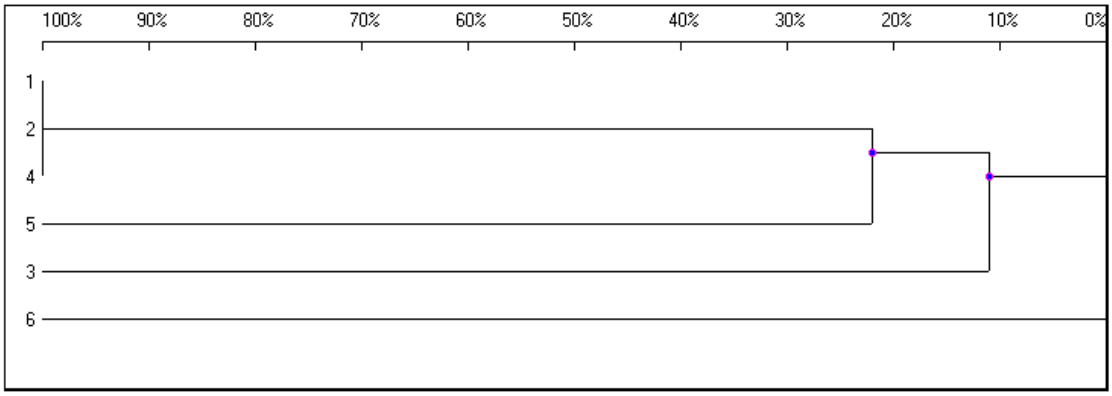

Figure 5.26. The dendrogram obtained from PFGE profiles: 1. UN5, 2. UN9, 3. UN19, 4. UIB31, 5. UIN9, 6. *S. thermophilus* CCM 4797

Thus, the isolates UN5, UN9 and UIB31 (1, 2, and 4) were identified as the same strain. UN5 and UN9 were isolated from the same location (Nohutalan) within

Urla, and UIB31 was collected from Birgi. The isolates UN19 collected from Nohutalan, was clustered into another homology group. In our laboratory, it has been demonstrated that the isolates of cocci each had a unique restriction pattern (Erkus et al., 2013).

Interpretable DNA profiles could not be obtained for the bacilli isolates. It was suspected that the cell wall degradation may have been insufficient, and chromosomal DNA could not be accessed by any of the following restriction enzymes: *Sma*I, *Apa*I, *Not*I, and *Pvu*II. To solve the problem, the amount of lysozyme was increased to 20 mg/mL, and sodium lauryl sarosinate and sodium deoxycholate were added into the lysis solutions. In the end, only three of the isolates yielded restriction patterns (Fig. 5.27).

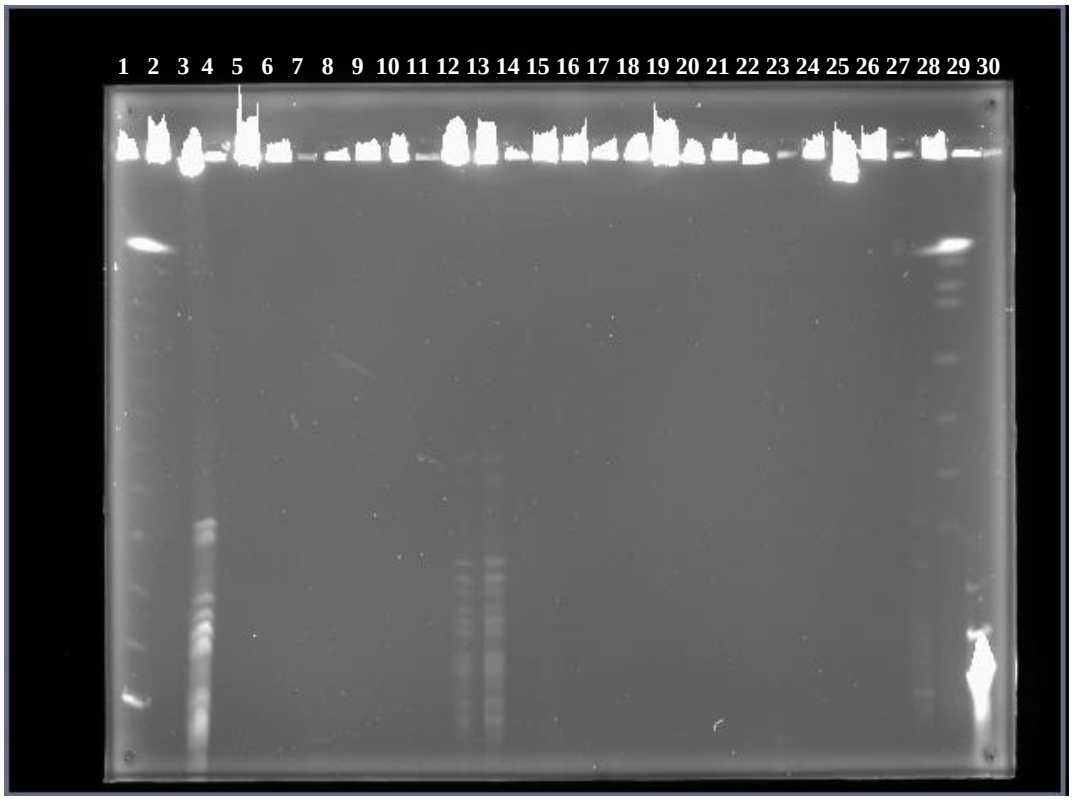

Figure 5.27. PFGE profiles of the isolates of bacilli: 1 PFGE standart marker, 3. GA12, 12. UIN4B, 13. UIN22, 28. *Lb. delbrueckii* ssp. *bulgaricus*, 29. PFGE standard: *Saccharomyces cerevisiae*

5.7. Selection of Probiotic Yoghurt Combinations

5.7.1. Coagulation of Milk Proteins

The selected isolates were inoculated into skimmed-milk prepared in a yeast extract medium, and then incubated at 42°C. All of the isolates showed positive coagulation results and decreased the pH to 4.6 at the 5^{th} h of incubation.

5.7.2. Rate of Acidification

5.7.2.1. Milk Acidifying Activity

The most important criterion for the isolates to be used as a starter strain is the rate of acidification of milk. Thus starter cultures are expected to decrease the pH of milk to 4.6 within the shortest fermentation time possible. And the acidification performance of a starter is basically a function of its proteolytic capacity (Table 5.16). The carbohydrate lactose was converted into lactic acid very fast by three of the cocci isolates, UN5, UN9 and UN19, while UIB31 and UIN19 appeared to be slow producers (Fig 5.27). Eight of the bacilli isolates were identified as fast converters (UF6, UZ12, UZ22, UIN22, DT62B, UIIN4, UIIN24, and UIIN26), and the remaining were slow acidifiers.

The carbohydrate lactose was converted into the lactic acid very fast by cocci isolates, UN5, UN9 and UN19, when compare to the reference strain. Milk acidification properties of UIB31and UIN19 were indicated as slow. The pH decrease for isolate UIN19 was not sufficient for yoghurt production. UIB31 decreased the pH of the skim-milk at 4.62 after 8 h incubation (Fig. 5.28).

Table 5.16. pH lowering rates of the isolated strains

Isolate Name	1h	2h	3h	4h	5h	6h	7h	8h	Time to pH 4,6
Skim milk	6.45	6.45	6.44	6.43	6.43	6.43	6.40	6.40	
UF6	6.26	6.25	6.05	5.48	4.98	4.37	4.12	4.03	4.60 (5h 30m)
GA12	6.26	6.26	6.21	6.06	5.88	5.48	5.06	4.64	4.64 (8h)
UN5	6.35	6.23	5.44	4.76	4.62	4.52	4.43	4.38	4.64 (4h 35m)
UN9	6.35	6.34	6.20	5.37	4.76	4.50	4.36	4.25	4.60 (5h 45m)
UN19	6.35	6.32	5.94	4.83	4.56	4.40	4.31	4.21	4.64 (4h 35m)
UN26	6.30	6.28	6.20	6.13	6.09	6.04	6.00	5.97	
UIB2	6.30	6.30	6.27	6.28	6.21	6.10	5.92	5.55	
UIB31	6.35	6.34	6.28	5.78	5.18	4.76	4.62	4.49	4.62 (7h)
UZ8	6.31	6.30	6.21	5.98	5.66	5.17	4.81	4.51	4.60 (7 h 30m)
UZ12	6.32	6.28	6.11	5.56	5.06	4.52	4.22	4.12	4.60 (5h 50m)
UZ16	6.31	6.31	6.25	6.15	5.88	5.45	5.00	4.57	4.57 (8h)
UZ18	6.29	6.28	6.21	6.01	5.71	5.24	4.84	4.54	4.54 (8h)
UZ22	6.31	6.28	6.16	5.68	5.15	4.53	4.21	4.06	4.60 (5h 50m)
UZ32	6.30	6.28	6.21	5.99	5.70	5.24	4.85	4.56	4.56 (8h)
UIN4B	6.29	6.26	6.18	6.00	5.70	5.17	4.72	4.40	4.6 (7h 30m)
UIN9	6.26	5.98	5.46	5.20	5.07	4.93	4.89	4.83	
UIN22	6.25	6.06	5.62	5.09	4.74	4.43	4.20	4.01	4.58 (5h 35m)
UIN26	6.23	6.17	5.95	5.56	5.28	4.99	4.78	4.58	4.58 (8h)
UIN42	6.26	6.19	5.93	5.40	4.90	4.54	4.29	4.05	4.54 (6h)
DT54	6.27	6.20	5.98	5.41	5.16	5.10	4.88	4.58	4.58 (8h)
DT62A	6.29	6.24	6.06	5.53	4.99	4.54	4.28	3.91	4.54 (6h)
DT62B	6.29	6.22	6.00	5.39	4.88	4.49	4.22	4.04	4.64 (5h 40m)
DT66	6.29	6.23	6.19	6.15	6.12	6.10	6.07	6.05	
DT74	6.26	6.23	6.19	6.06	5.94	5.78	5.58	5.55	
UIIN4	6.28	6.17	5.85	5.25	4.64	4.36	4.27	4.02	4.64 (5h)
UIIN18	6.27	6.18	6.01	5.70	5.37	5.23	5.02	4.89	
UIIN24	6.26	6.15	5.64	5.10	4.86	4.56	4.20	4.06	4.56 (6h)
UIIN26	6.21	5.95	5.21	4.56	4.21	4.09	3.95	3.85	4.56 (4h)
UIIN44	6.27	6.26	6.21	6.15	5.95	5.65	5.11	4.67	4.60 (8h 15m)
Lb. delb. ssp. bul	6.28	6.17	5.84	5.42	5.10	4.90	4.78	4.66	4.66 (8h)
S. therm.	6.27	6.07	5.59	5.32	5.12	5.02	4.94	4.83	

Eight bacilli isolates gave rapid acidification profiles (UF6, UZ12, UZ22, UIN22, DT62B, UIIN4, UIIN24 and UIIN26), the rest of bacilli isolates were indicated as slow acidifiers.

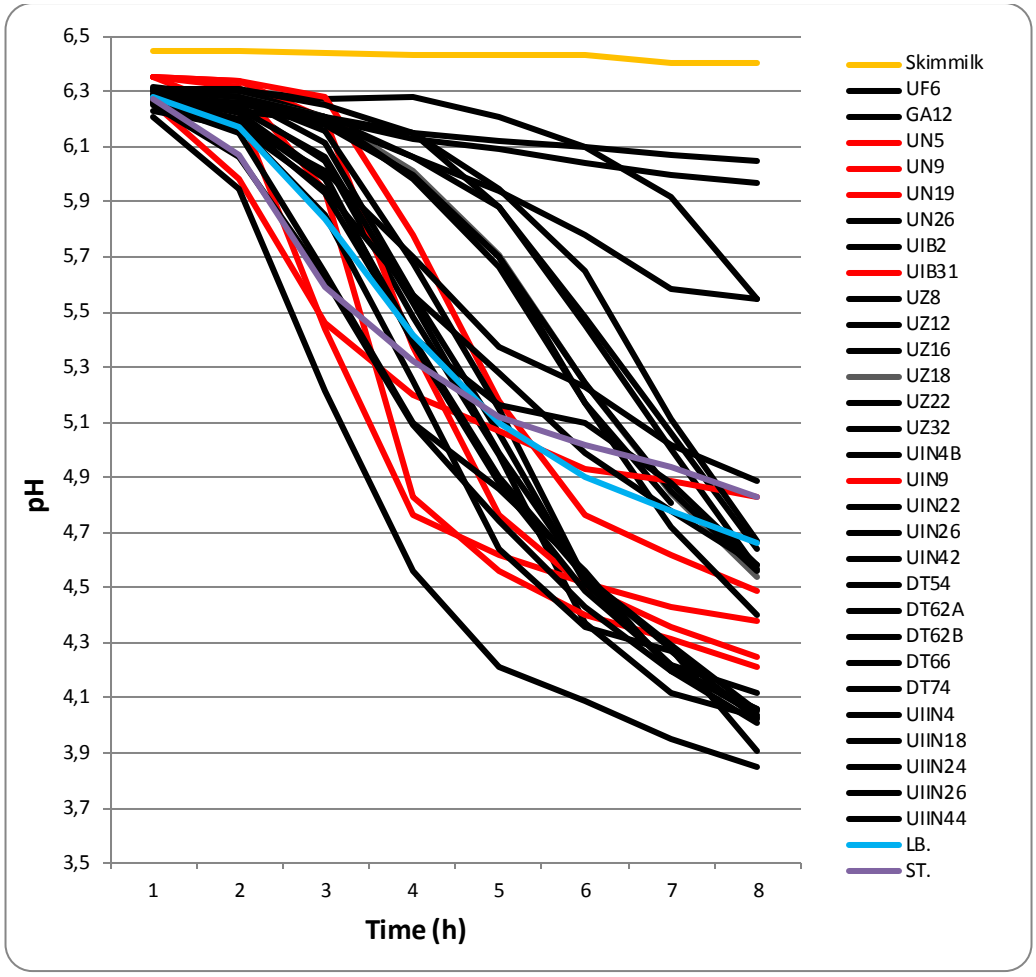

Figure 5.28. Acidification profiles of the selected isolates in 10g/100 mL sterile skimmed milk and 1% yeast extract

5.7.3. Optimum Starter Combinations for Yoghurt Production

The isolates which decreased pH to 4.60-4.70 within 5h were selected and combined. Five of the cocci isolates were combined with 26 of the bacilli isolates and in total 135 yoghurt samples were produced. Finally, 16 different combinations were chosen (Table 5.17). The initial pH of milk used for yoghurt production was 6.59. After the three or four hours of fermentation time, the final pH of the yoghurt samples dropped to the range between 4.60 and 4.70. The products were then evaluated for their organoleptic properties: appearance, consistency with spoon, consistency in mouth, flavour, and aroma, and overall acceptability.

Table 5.17. Optimum yoghurt combinations

Sample Codes	Yoghurt Combinations	Sample Codes	Yoghurt Combinations
Y1	UN5 X UF6	**Y9**	UN9 X UF6
Y2	UN5 X UZ12	**Y10**	UN9 X UZ12
Y3	UN5 X UZ22	**Y11**	UN9 X UZ22
Y4	UN5 X DT54	**Y12**	UN9 X DT54
Y5	UN5 X DT62B	**Y13**	UN9 X DT62B
Y6	UN5 X UIIN24	**Y14**	UN9 X UIIN24
Y7	UN5 X UIIN26	**Y15**	UN9 UIIN26
Y8	UN5 X UIN42	**Y16**	UN9 X UIN42

5.8. Characterization of Yoghurt Samples

5.8.1 pH and Titratable Acidity

Titratable acidity varied between 1.16±0.02 and 1.38±0.08 (Table 5.18, Fig.5.29). The acidity range was found to be in acceptable limits by CODEX Standart for Fermented Milks (CODEX STAN 243-2003 FAO/WHO 2001).

Table 5.18. Titratable acidity in the yoghurt samples (1-8 included UN5, and 9-16 included UN9

Sample Code	Yoghurt Combinations	Titretable Acidity (Lactic acid %)	Total Solids (%)	Syneresis (%)	Apperant Viscosity (mPa.s)
Y1	UN5-UF6	1.27± 0.11	19.10± 0.13	10.21±0.01	532.05± 6.15
Y2	UN5-UZ12	1.19 ± 0.01	18.69 ± 0.05	5.53±0.01	608.35± 2.47
Y3	UN5-UZ22	1.25± 0.09	19.84± 1.6	6.23±0.01	599.15± 33.87
Y4	UN5-DT54	1.29 ± 0.14	19.35 ± 0.04	6.43±0.02	597.50 ± 6.79
Y5	UN5-DT62B	1.17 ± 0.15	19.48 ± 0.34	12.83±0.02	543.05 ± 13.65
Y6	UN5-UIIN24	1.16 ± 0.02	18.33 ± 0.54	8.00±0.01	573.50 ± 59.11
Y7	UN5-UIIN26	1.21± 0.24	19.80 ± 0.27	11.66±0.02	522.95 ± 10.25
Y8	UN5-UIN42	1.38 ± 0.09	18.48 ± 0.09	9.56±0.03	563.20 ± 0.99
Y9	UN9-UF6	1.27± 0.11	21.15 ± 0.26	27.21±0.01	313.00 ± 1.84
Y10	UN9-UZ12	1.19 ± 0.01	19.69 ± 0.31	22.66±0.01	331.10 ± 29.70
Y11	UN9-UZ22	1.25± 0.10	18.34 ± 0.91	27.60±0.03	320.40 ± 2.83
Y12	UN9-DT54	1.29± 0.14	17.75 ± 0.13	24.23±0.03	261.25 ± 7.00
Y13	UN9-DT62B	1.17± 0.15	17.93 ± 0.39	27.51±0.02	292.00± 5.80
Y14	UN9-UIIN24	1.16± 0.02	19.93 ± 2.05	19.43±0.02	321.00 ± 7.92
Y15	UN9-UIIN26	1.21± 0.24	20.09 ± 1.8	24.83±0.03	342.60 ± 10.04
Y16	UN9-UIN42	1.38 ± 0.08	18.40 ± 0.24	22.86±0.01	321.95± 3.18

* Values were means of triplicates (SD 0.01-0.24 for titratable acidity, SD 0.09-1.6 for total solids, SD 0.01-0.03 for syneresis, SD 0.99-59.11 for viscosity)

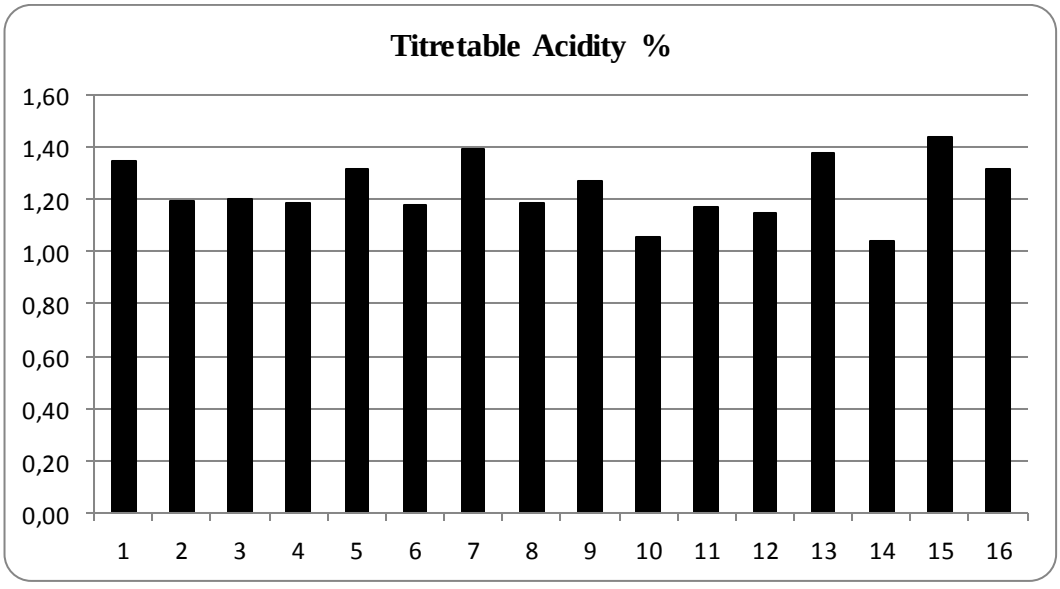

Figure 5.29. Titratable acidity (% lactic acid) of yoghurt samples (1-8 included UN5, and 9-16 included UN9)

5.8.2. Determination of Syneresis

The most important defect seen in industrial yoghurt products is the syneresis, the separation of whey that can be defined as the expulsion of liquid from the solid body (Lucey, 2004). The amount of syneresis in our yoghurt samples appeared to range from 5.53% to 27.60% (Table 5.18).

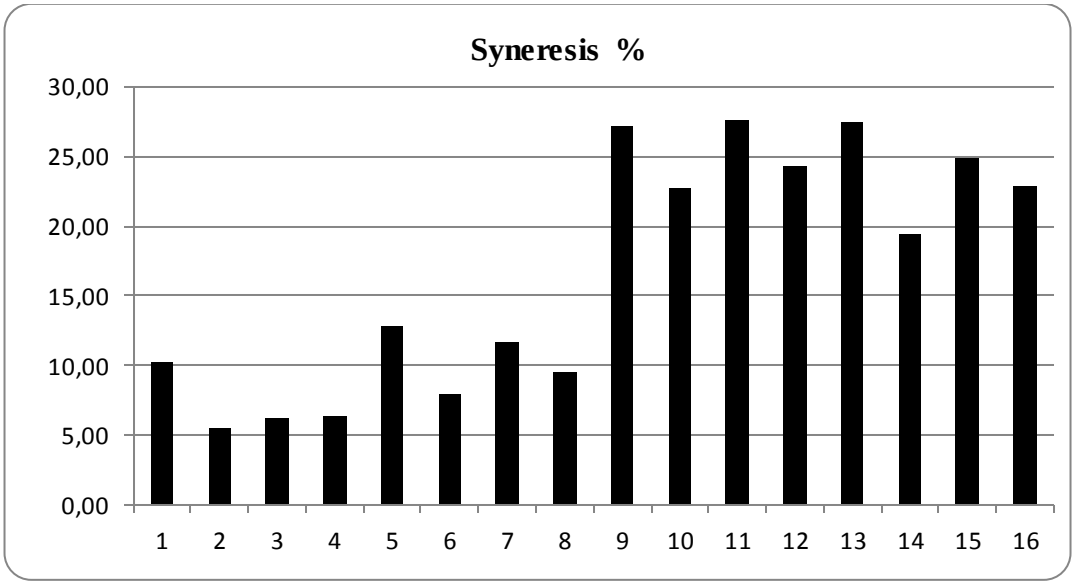

Figure 5.30. Syneresis in the yoghurt products; (1-8 included UN5, and 9-16 included UN9)

Yoghurt samples, produced by using the starter combinations shown in Table 5.17, were evaluated on the basis of the amounts of syneresis (whey-off) (Fig. 5.30). As can be seen, the amount of syneresis varied between 5.53% and 27.60%. Starter combinations including the cocci isolate UN9 appeared to release much higher amounts of whey.

Syneresis, also known as water holding capacity (WHC), of a protein gel is an important parameter in yogurt manufacturing. Lower WHC is desired and this favours the use of starter combinations including UN5 (Table 5.17) (Donkor et al., 2007; Yang and Li, 2010).

5.8.3. Apparent Viscosity of Yoghurt Samples

Viscosity of the yoghurt samples were calculated using the formula of apparent viscosity (Pa.s) and shear rate. (Fig. 5.31).

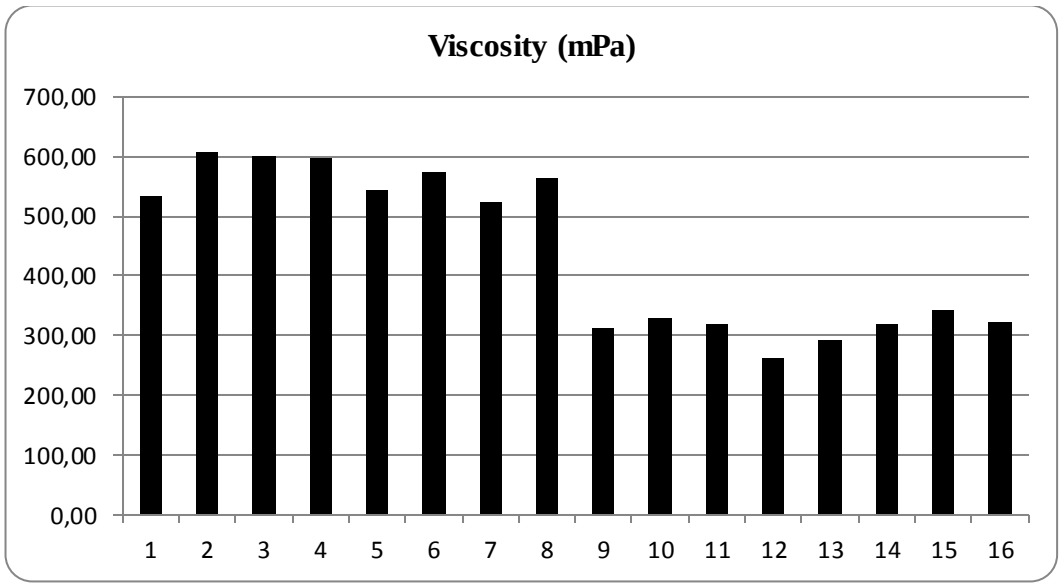

Figure 5.31. Apparent viscosity figures of the yoghurt samples (1-8 included UN5, and 9 -16 included UN9)

As could be seen, apparent viscosity values of the yoghurt samples ranged from 261.25 ± 7.00 and 608.35 ± 2.47 m Pa.s (Table 5.18). As expected, yoghurt samples with higher WHC values also displayed much higher viscosity values (Fig. 5.30).

5.8.4. Total Solid Content of Yoghurt Samples

The optimum total solid content of industrially produced yoghurts is expected to be within the range between 17% and 19%. The total solid content of our yogurt samples varied between 17.75 ± 0.13% and 21.15 ± 0.26 %. Therefore this was an encouraging result, as the total solid contents was found to be equal to or higher than those of the standard values (Fig. 5.32, Table 5.18).

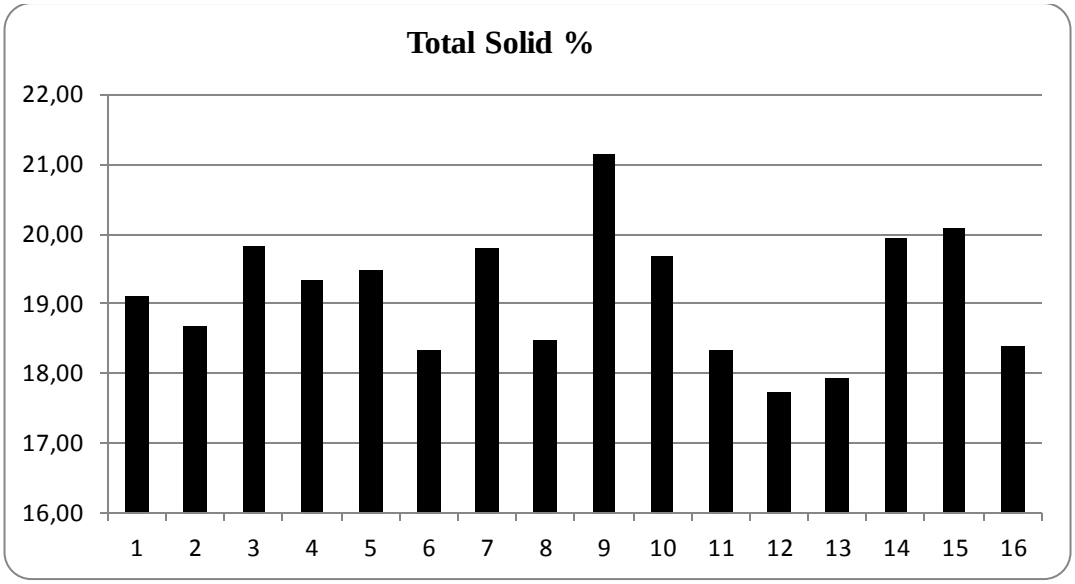

Figure 5.32. Total solid content of yoghurt samples (1-8 included UN5, and 9-16 included UN9)

5.8.5. Aromatic Features of the Yoghurt Samples

Aromatic compounds are usually volatile, and the most common flavour compounds found in a given yoghurt sample are acetaldehyde (2.0-41.0 mg/kg), diacetyl (0.2-2.3 mg/kg), ethanol (0.2-9.9 mg/kg), and acetone (1.8 to 3.4 mg/kg) (Rasic and Kurman, 1978; Kneifel et al., 1992). Aromatic content of our yoghurt samples were determined and they were mostly acetaldehyde, ethanol, acetone, and diacetyl (Fig. 5.33).

Acetaldehyde gives a fresh, fruity, pungent taste. It is produced by the yoghurt starters, but the mechanism of its production has not been well understood (Smit et al., 2005).

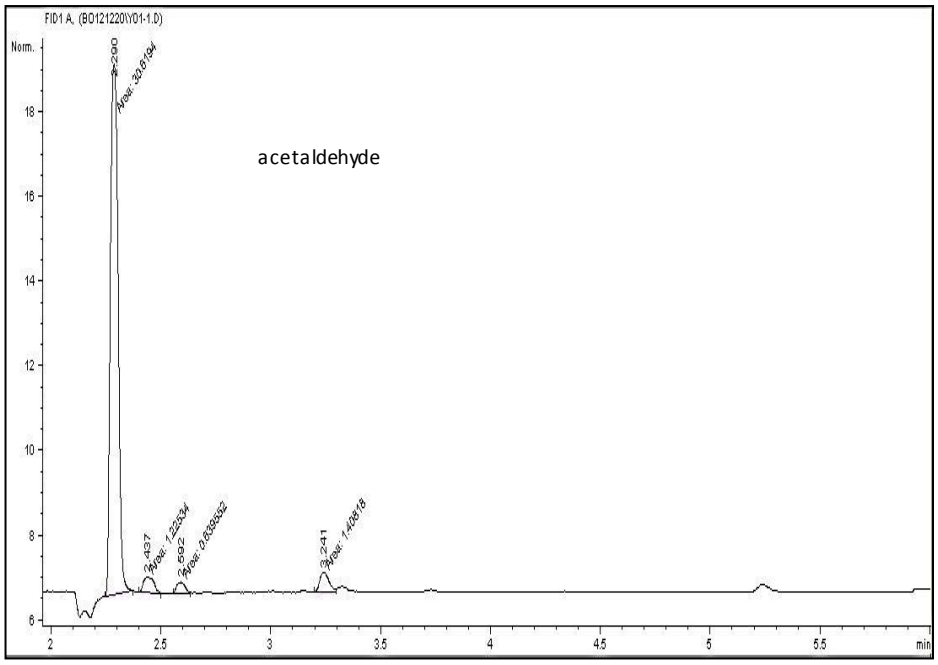

Figure 5.33. The content of volatile compounds in the yogurt samples (peaks from the left: acetaldehyde, ethanol, acetone, and diacetyl)

As it was shown, acetaldehyde content of our yoghurt samples varied between 5.61 and 15.38 mg/L (Table 5.19). And these values were in accordance with those found in the literature (Kneifel et al., 1992).

Higher amounts of acetaldehyde found in a given yoghurt sample indicates a relatively high metabolic activity of the starter combinations used. Oxidation of acetaldehyde results in the production of acetate, which can lower the pH of the yoghurt further (Tamime and Robinson, 2001).

Table 5.19. Aromatic content of the yoghurt samples; Y1-Y8 included UN5; and Y9-Y16 included UN9

Sample Codes	Acetaldehyde (mg/L)	Ethanol (mg/L)	Acetone (mg/L)	Diacetyl (mg/L)
Y1	12.08±0.01	2.96±0.81	0.32±0.00	1.12±0.02
Y2	10.61±0.02	3.53±0.64	0.31±0.01	0.84±0.04
Y3	9.4±0.01	3.3±0.16	0.3±0.02	0.74±0.03
Y4	7.87±0.03	5.01±0.21	0.27±0.03	1.36±0.01
Y5	12.1±0.06	5.35±0.23	0.35±0.01	1.0±0.01
Y6	6.34±0.01	7.52±0.75	0.36±0.01	0.96±0.01
Y7	12.16±0.02	6.72±0.18	0.29±0.00	1.08±0.02
Y8	10.81±0.01	3.3±0.12	0.31±0.02	1.48±0.01
Y9	10.06±0.02	3.19±0.35	0.28±0.01	1.24±0.01
Y10	8.51±0.02	0.68±0.06	0.28±0.01	1.8±0.02
Y11	8.46±0.02	0.59±0.01	0.26±0.02	0.92±0.03
Y12	8.06±0.03	0.6±0.02	0.26±0.02	1.56±0.02
Y13	15.65±0.05	0.9±0.05	0.33±0.04	0.67±0.01
Y14	5.61±0.01	1.06±0.12	0.25±0.01	1.08±0.03
Y15	15.38±0.01	1.1±0.10	0.28±0.01	0.83±0.01
Y16	11.32±0.01	1.05±0.15	0.3±0.00	0.59±0.01

* Values were means of duplicates (SD 0.01- 0.06 for acetaldehyde, SD 0.01-0.81 for ethanol, SD 0.00-0.003 for acetone, SD 0.01-0.04 for diacetyle)

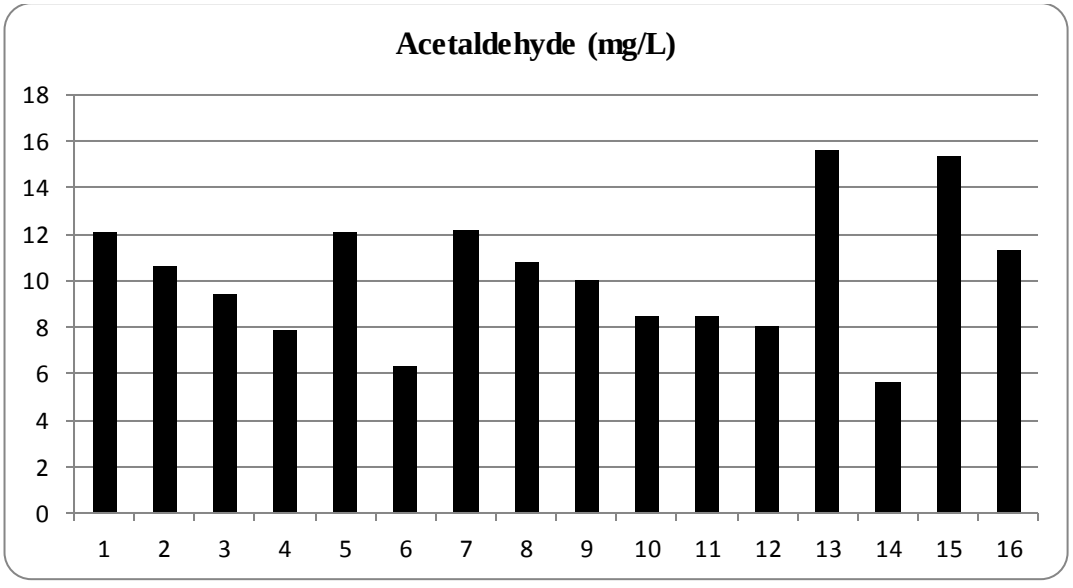

Figure 5.34. Acetaldehyde content of yoghurt samples (1-8 included UN5; and 9-16 included UN9)

Acetaldehyde can be metabolized into ethanol by the action of alcohol dehydrogenases. This activity has been mostly observed in the strains of *S. thermophilus* (Varga et al., 1998; Ozer et al., 2007). Ethanol concentration in our yoghurt samples were found to range between 0.6 and 7.32 mg/L (Table 5.19). As expected, the yoghurt samples including UN5 yielded higher amounts of ethanol (Fig. 5.35).

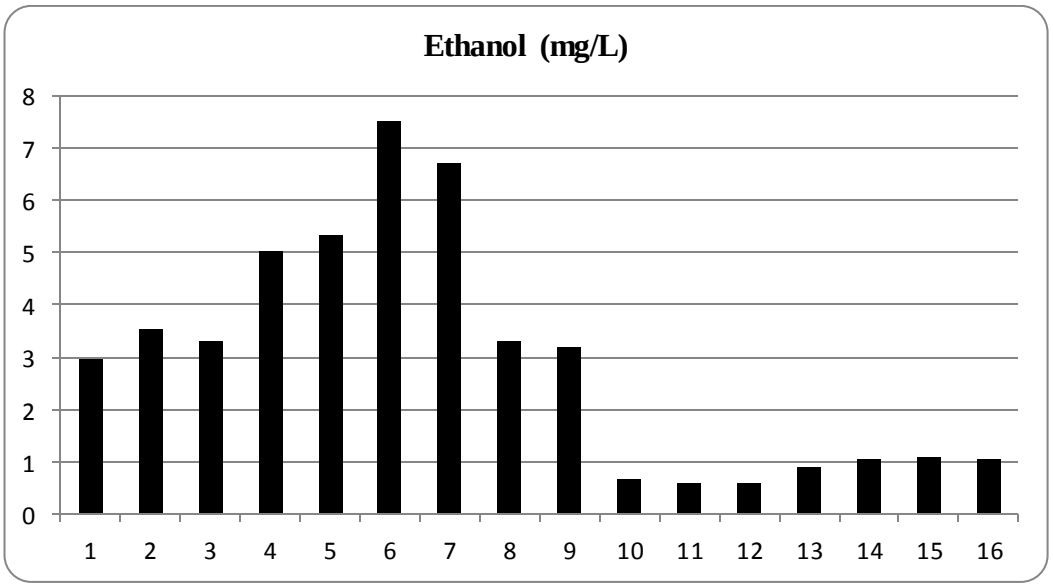

Figure 5.35. Ethanol concentration in yoghurt samples (1-8 included UN5, and 9-16 included UN9)

Diacetyl (buttery, fatty taste) is another main aroma compound found in yoghurt products. In cases when acetaldehyde content is low, diacetyl is expected to provide for full aroma and taste of yoghurt. Diacetyl concentration in our yoghurt samples seemed to range from 0.59 to 1.56 mg/L (Table5.19, Fig. 5.36).

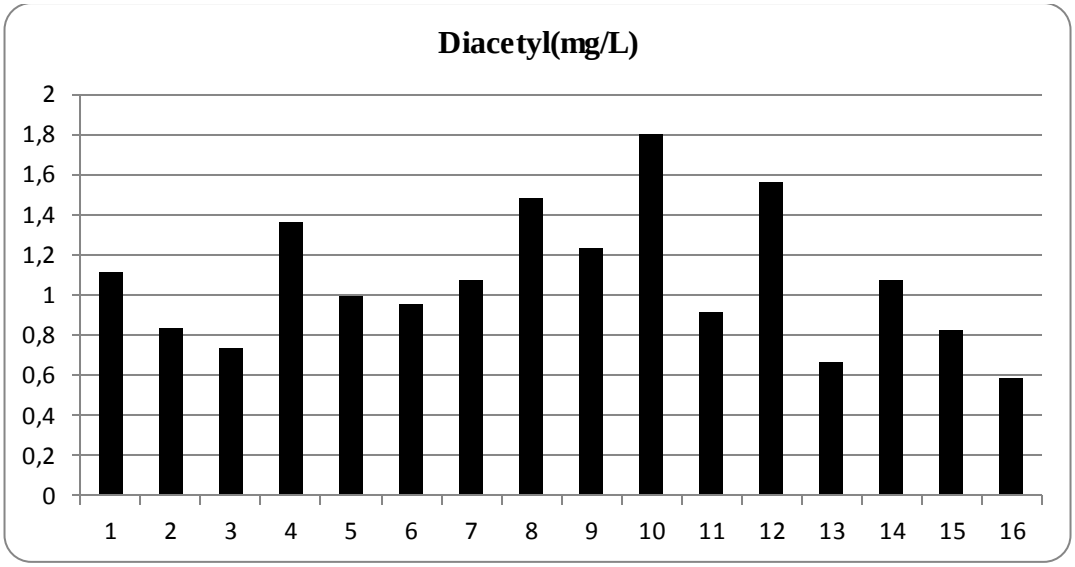

Figure 5.36. Diacetyl concentrations of yoghurt samples (1-8 included UN5, and 9-16 included UN9)

Acetone has a sweet, fruity aroma and is known to influence the aroma and flavor qualities of yogurt. The changes of acetone concentrations within all samples were ranging from 0.25 to 0.36 mg/L (Table 5.19, Fig. 5.37).

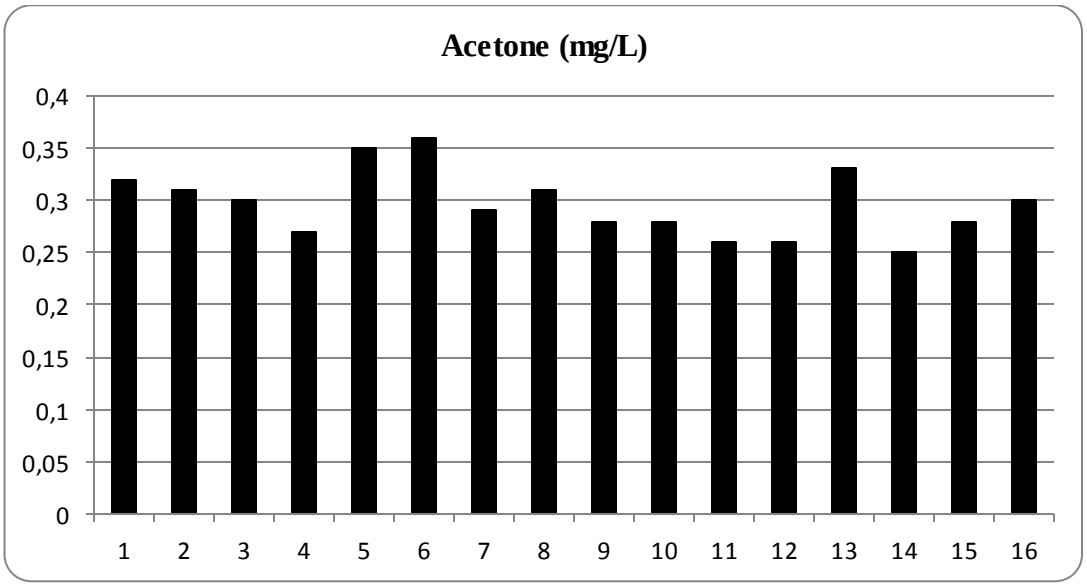

Figure 5.37. Acetone concentrations of yoghurt samples (1-8 included UN5, and 9-16 included UN9)

5.8.6. Screening for EPS Producing Strains

The main role of starter strains in the production of yogurt is acidification through the conversion of lactose into lactic acid, creation of the viscous texture by the production of exopolysaccharides (EPS), and development of yogurt specific flavour (Chaves et al., 2002).

In rheological terms, yoghurt is defined as viscoelastic and pseudoplastic. By viscoelasticity it was meant that yoghurt as a material is considered to show both solid and some of the ideal liquid behaviors at the same time (Lee and Lucey, 2010). Low viscosity and high syneresis cause serious problems for the industry because, such appearance and texture features can initiate consumer refusal of the product. These defects might be overcome either by increasing the total solid content or by the addition of stabilizers, or both, (e.g. locust beam gum). However, fortification of milk by such supplement compounds may adversely affect the taste of final product. Moreover, some stabilizers are also detrimental to human health. Thus, it would be much more beneficial to use starter strains that produce EPS, instead, that act as a bio-stabilizer (Marshall and Rawson, 1997).

Rheological features of polysaccharides are in part accounted for by their three-dimensional structure. Beside their viscosifying effect, EPSs can also interact with milk proteins, namely caseins. This interaction triggers casein aggregation, thereby improving the texture (Marshall and Rawson 1999).

In this study, EPS producing strains were screened by using skimmed-milk agar medium, supplemented with ruthenium red, and two different incubation temperatures, 37º C and 42 ºC. This medium was firstly used to differentiate between ropy, white colonies and others with pink appearance. All of the bacilli and cocci isolates produced white colonies in this medium at both temperatures. Some of the bacilli and only one of the cocci strains produced halo zones around the colonies (Fig. 5.38).

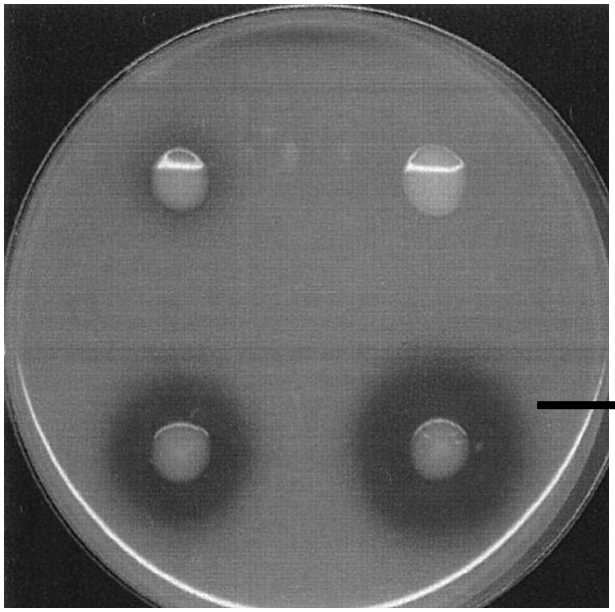

Figure 5.38. Bacilli isolate (DT66, UIN22, UZ8 and UZ12) with and without a halo zone

To further support these findings, the same observations were also made using MRS or M17 medium (Fig. 5.38). All of the cocci and bacilli isolates gave white colonies and all of them were indicated as ropy strains (Fig.5.39).

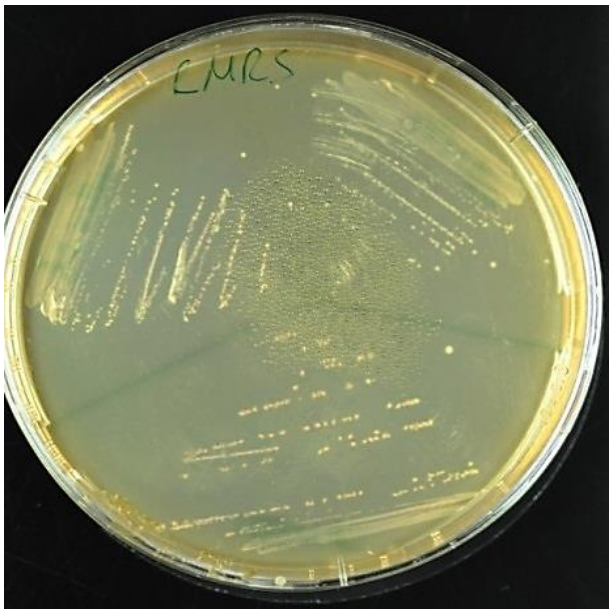

Figure 5.39. Ropy strains on modified MRS agar medium.

5.8.7. Texture Profile Analysis (TPA) of the Yoghurt Samples

Yoghurt is one of the most consumed dairy products. Flavor and consistency are its main quality parameters. Consistency of yoghurt is dependent on its structure, a protein network formed by casein micelles strings and fat globules. This network is relatively weak and it is formed by acidification of milk by a mixed culture of *Streptococcus thermophilus* and *Lb. delb*. ssp. bulgaricus (Vercet et al, 2002).

Texture profile analysis imitates the conditions in the mouth by compressing a product twice. Hardness, having been used to estimate the maximum force of the first compression, is a critical parameter in the evaluation of texture. It also reflects the syneresis capacity of the product. Protein matrix often constitutes the hardness (Vercet et al., 2002). Hardness of our yoghurt samples was determined using a similar strategy. Samples with the cocci strain UN5 yielded lower hardness values than those with UN9 (Table 5.20). These results were also in correlation with the syneresis results (Fig. 5.30).

Table 5.20. Texture Profile Analysis (TPA) results

Yoghurt Combinations	Hardness (N)	Adhesiveness	Cohesiveness	Springiness (mm)
UN5-DT62B	4.81±0.22	11.20±0.7	0.38±0.004	0.38±0.0
UN5-DT54	4.98±0.25	10.61±0.6	0.38±0.004	0.38±0.0
UN5-UIN42	4.53±0.16	9.81±0.8	0.37±0.004	0.38±0.0
UN5-UIIN24	3.94±0.53	8.95±0.2	0.37±0.003	0.46±0.001
UN5-UIIN26	5.37±0.32	12.46±0.6	0.38±0.004	0.38±0.0
UN5-UZ12	4.89±0.27	11.20±0.7	0.38±0.004	0.38±0.0
UN5-UZ22	4.74±0.28	10.65±0.8	0.38±0.004	0.46±0.001
UN9-UIN42	6.61±0.10	5.38±0.5	0.35±0.003	0.54±0.001
UN9-UIIN24	6.71±0.20	8.95±0.9	0.40±0.005	0.50±0.0
UN9-UIIN26	6.52±0.06	8.18±0.3	0.40±0.005	0.47±0.003
UN9-UZ12	6.37±0.05	4.88±0.1	0.41±0.004	0.42±0.0
UN9-DT62B	6.08±0.22	6.84±0.2	0.39±0.004	0.54±0.001
UN9-DT54	6.19±0.21	6.87±0.2	0.31±0.006	0.50±0.001
UN9-UZ22	4.66±0.33	9.11±0.1	0.49±0.005	0.42±0.001
UN9-UF6	6.42±0.24	4.39±0.3	0.32±0.004	0.42±0.001
UN5-UF6	3.81±0.18	7.62±0.4	0.35±0.003	0.38±0.0

Values were means of triplicates (SD 0.1- 0.9 for adhesiveness, SD 0.003 -0.005 for cohesiveness, SD 0.0- 0.003 for springiness, SD 0.06-0.53 for hardness)

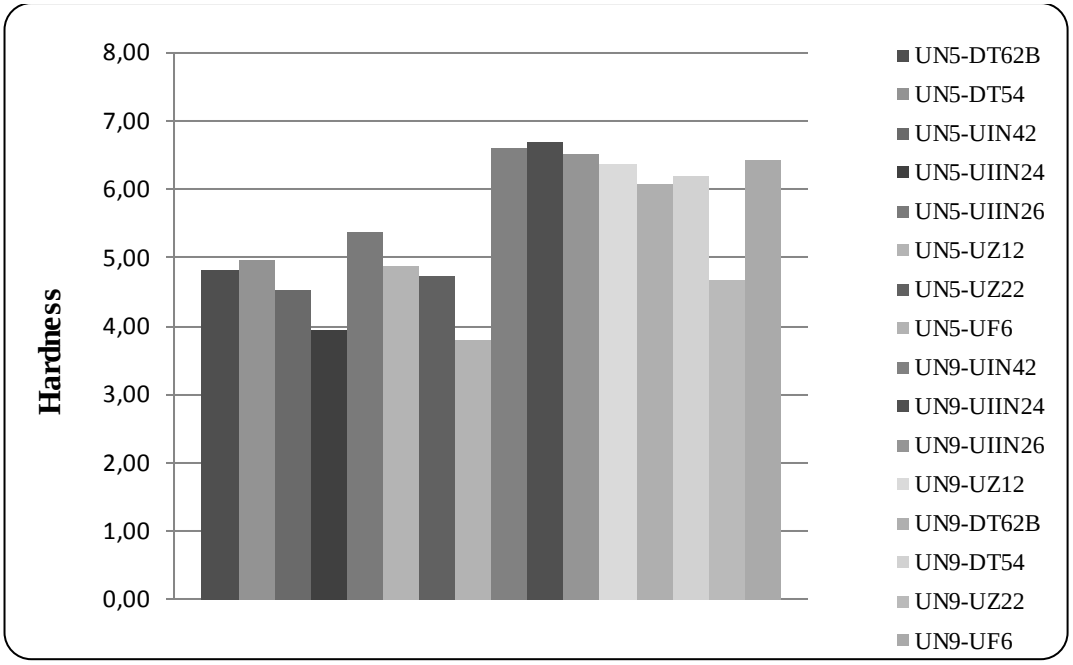

Figure 5.40. Hardness results as histograms

Cohesiveness is another important texture parameter and it is indicated by the ratio of first to second compression. In this study, the cohesiveness of the yoghurt samples including the cocci strain UN9, were found to be slightly higher than those including UN5 (Fig. 5.41). The highest cohesiveness value was obtained from the yoghurt sample Y11, produced by using the UN9-UZ22 combination (Table 5.20).

Average cohesiveness value was approximately 0.48, which was within the cohesiveness range, 0.38 - 0.54, indicated in the literature (Park et al., 2005).

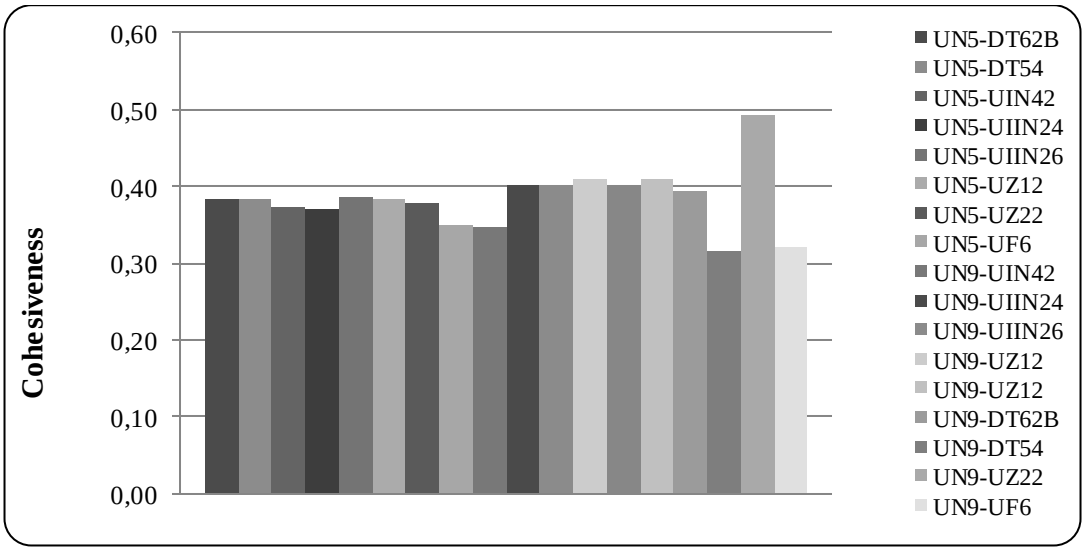

Figure 5.41. Cohesiveness results as histograms

Adhesiveness of the yoghurt samples were also studied and found to reside within a range between 4.39-12.46 (Fig. 5.42). One important observation should be mentioned that adhesiveness of the samples with UN5 was found higher than those with UN9.

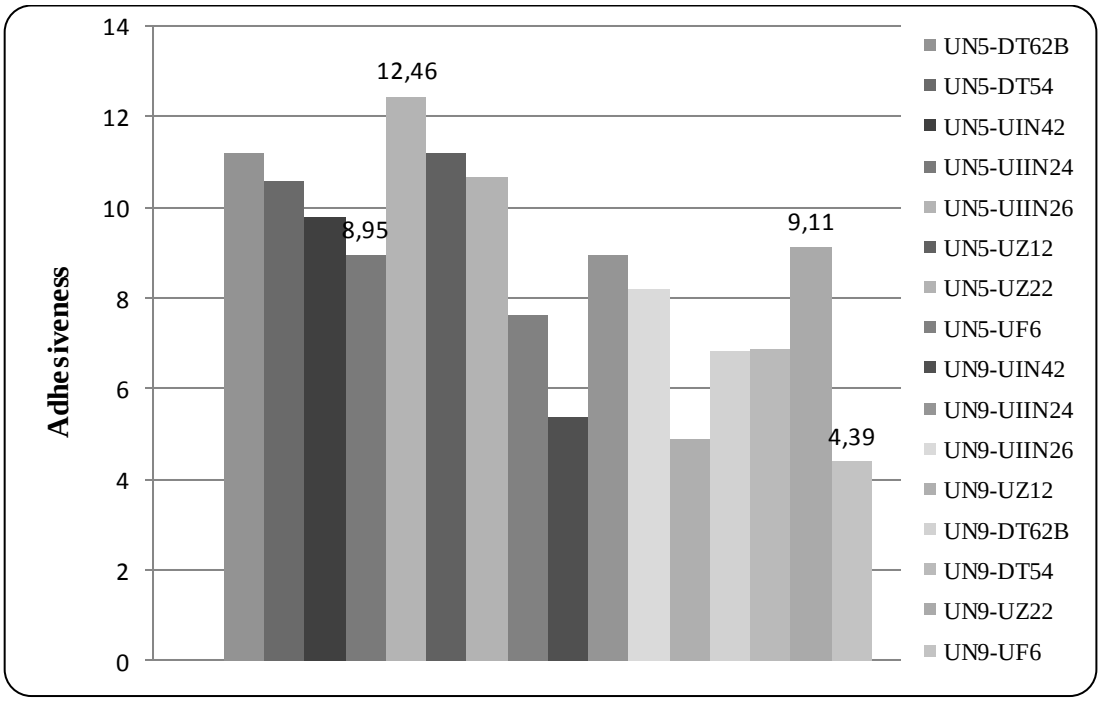

Figure 5.42**.** A histogram representation of adhesiveness values

Springiness can be defined as the capacity of a sample to gain its original form after the deforming force being removed. Results of the springiness test indicated that springiness values of the yoghurt samples including the cocci strain UN9 starter was slightly higher than those including the UN5 strain **(**Fig. 5.43).

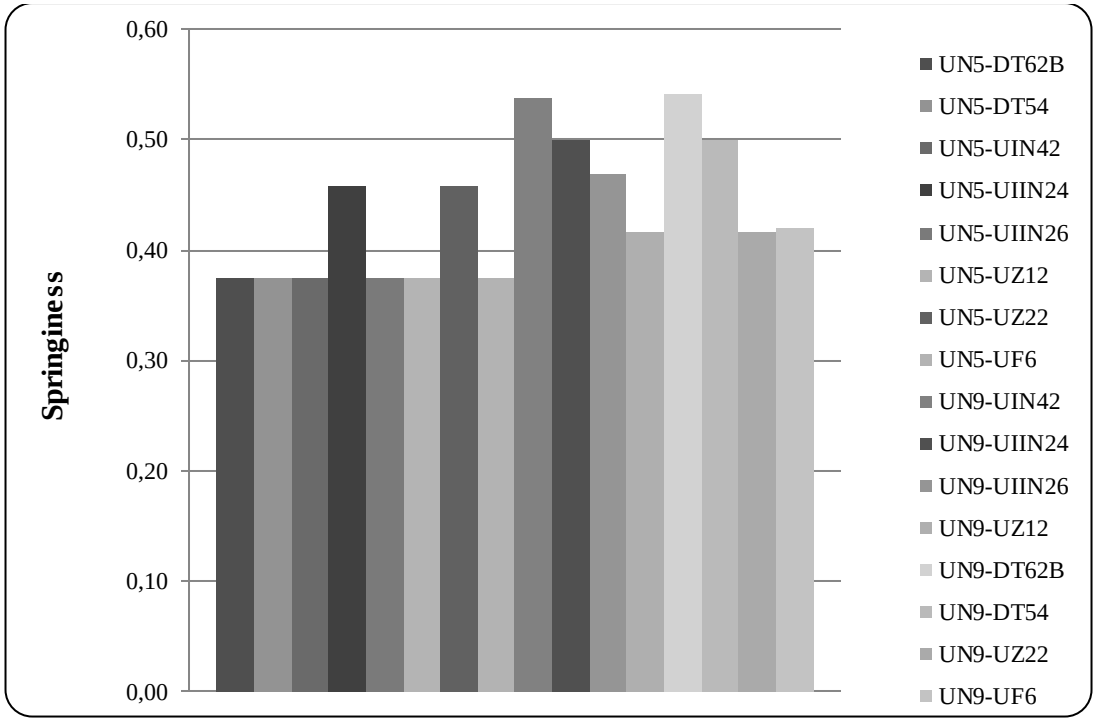

Figure 5.43. A histogram representation of springiness values

Total solid content of milk, starter culture types, and incubation temperatures affects the texture of the yoghurt (Bonczar and Regula, 2003). In this study, two different types of cocci strains used with 8 different bacilli strains. And, yoghurts produced with different cocci was separated each other according to the pyhysicochemical properties.

CHAPTER 6

CONCLUSIONS

Lactic acid bacterial isolates purified from artisanal yoghurt samples were identified and characterised by using biochemical, physiological, and molecular methods. At the end, a total of 5 cocci and 36 bacilli isolates were obtained.

The forty one isolates were then screened for their probiotic properties. All of the cocci isolates tolerated 1% (w/v) bile salt and they were also grown much better in the bile salts medium. And all of the cocci isolates produced precipitation zones in the BSH plate assay. BSH activity in part reflects the cholesterol removal capacity of a given strain. Thus, cholesterol removal capacity of the isolates were also studied and found to be within the percentage range between 44 and 50.

Simulated gastric acidic conditions were tolerated by all of the isolates. Only one isolate, UIB2, could not maintain its viability beyond 3rd h. And some of the bacilli isolates survived in this environment until the end of 24h incubation. All of the cocci isolates retained their viability after 24h incubation in tryptic environment.

Autoaggregation ability and cell surface hydrophobicity also constitute a highly sought after probiotic characteristic, reflecting the adhesive capacity of the strains. The cocci isolates appeared to have better autoaggregation capacity, and lower affinity to carbohydrates xylene and hexane. All of the cocci strains, including the reference strains, displayed much higher adhesion capacity to a human epithelial cell line, Caco-2.

Finally, probiotic yoghurts were produced using our probiotic candidates (Table 5.16). Sixteen starter combinations were made using the two cocci and eight bacilli isolates, and thus sixteen yoghurt products were produced and characterized by using physical, chemical, rheological, and by organoleptic methods. Acetaldehyde, ethanol, acetone and diactyl were the main aroma compounds of sample and the concentrations ranged from 5.61 and 15.38 mg/L for acetaldehyde, 0.59 mg/L and 7.52 mg/L for ethanol, 0.25 mg/L and 0.36 mg/L for acetone, 0.59 mg/L and 1.56 mg/L for diacetyl. Titratable acidity was main chemical characteristics of yoghurt and results ranged between 1.17 and 1.38. Consumer acceptances could be affected by syneresis of yoghurt and the results ranged between 5.33 and 27.60 as expected. Hardness, cohesiveness,

adhesiveness and springiness were the major texture characteristic. The results ranged from 3.81 and 6.71 N for hardness, 0.31 and 0.49 for cohesiveness, 4.39 and 12.46 for adhesiveness, 0.38 mm and 0.54 mm for springiness.

To conclude, some traditional yoghurt LAB were collected, isolated, and characterized to discover novel strains having both starter and probiotic features. A good number of cocci and bacilli were paired as probiotic/starter strain combinations that could perfectly be used for the production of functional yoghurt.

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APPENDIX A

CHEMICALS

A.1. Chemicals for Microbiological Experiments

No	Chemical Names	Codes
1	Agar	Merck 1.01613
2	Peptone	Oxoid LP037
3	Yeast Extract	Merck A 1.03753
4	Meat Extract	Oxoid LP029
5	Trimoniumcitrate	Sigma A1332
6	Glycerol	AppliChem A2926
7	NaCl	AppliChem A2942
8	Sodium Acetate	Sigma S2889
9	MRS Broth	Merck 1.10661
10	M17 Broth	Merck 1.15029
11	Bromocresolpurple	Merck 1.3025
12	Arginine monohydrochloride	Merck 1.1543
13	Glucose	AppliChem A3666
14	Lactose	Sigma L3750
15	Maltose	AppliChem A3891
16	Sucrose	AppliChem A3935
17	Fructose	AppliChem A3688
18	Mannose	Aldrich 1.258-5
19	Melizitose	Sigma M5375
20	Melibiose	Sigma M5500
21	Arabinose	Aldrich A,9190-6
22	Raffinose	AppliChem A6882
23	Galactose	Aldrich 11259-3
24	Ribose	Fluka 83860
25	Trehalose	Sigma T 9531
26	Crystal Violet	Sigma C3886
27	Potassium Iodide	Sigma C6757
28	Safranine O	Merck 1.15948
29	$MgSO_4.7H_2O$	Merck 1.05886
30	$MnSO_4.4H_2O$	Merck 1.02786
31	Ascorbic Acid	Merck 5.00074
32	Sodium phospahate dibasic	Merck 926870
33	K_2HPO_4	Sigma P8281

(cont. on next page)

Table A.1. (cont.)

34	Tween 80	AppliChem A1390
35	Sodium hydroxide	Merck 1.06498
36	Anaerogen	Oxoid AN25
37	H_2O_2 30%	Merck 1.07209
38	Rhamnose	AppliChem A4336
39	Bile salts	Oxoid LP0055
40	Ethanol	Merck 100986
41	Skimmilk	Enka
42	ONPG disc	Fluka 49940
43	Gelatine	Merck 1.04070
44	KH_2PO_4	SigmaP0662
45	Tryptone	Applichem A1553
46	Kovac's reagent	Merck 1.09293
47	Na_2HPO_4	Applichem 2943
48	Pepsin	Merck 1.07185
49	Trypsin	Sigma T3003
50	Sodium Taurocholic Acid	Sigma T4009
51	Sodium Thioglycholate	Applichem A0985
52	Sodium Taurodeoxcholic Acid	Sigma T0875
53	$CaCl_2$	Sigma 449709
54	Cholesterol	Applichem A0807
55	KOH	Sigma 221473
56	Hexane	Merck 1.04374
57	Xylene	Merck 1.08665
58	o-pyhtalaldehyde	Merck 821821
59	H_2SO_4	Merck 1.00729
60	Lactulose	Applichem A 0887
61	Foetal Bovine Serum	Sigma F3018
62	DMEM	Sigma D5546
63	Ruthenium Red	Sigma R5712
64	Phenolphatlein	Merck 1.07233
65	Acetaldehyde	Merck 8450010100
66	Acetone	Merck 100014100
67	Diacetyl	Merck 8035280100
68	Acetoine	Merck 8206640100
69	β-glyrecophosphate disodium salt	Sigma G-6376

A.2. Chemicals for Molecular Characterization Experiments

No	Chemical Names	Codes
1	Trisma Base	Sigma T6066
2	EDTA	Applichem A2937
3	Lysozyme	Applichem A3711
4	ProteinaseK	Applichem A3830
5	CTAB	Applichem A0805
6	Chloroform	Applichem A3830
7	Isoamylalcohol	Applichem A2610
8	RNase A	Applichem A3832
9	dNTP Set	Fermentas R0181
10	$MgCl_2$	
11	Taq DNA Polymerase	Fermentas EP0401
12	Agarose	Applichem A2114
13	Ethidium Bromide	Applichem A1151
14	Low Melting Temperature Agarose	Applichem A3762
15	N-laurylsarcosinate	Applichem A1163
16	Sodium Deoxycholate	Applichem A1531
17	Phenyl Methyl Sulfonyl Floride	Applichem A0999
18	SmaI	Fermentas ER0661
19	PFGE Grade Agarose	Bio-Rad 162-0134
20	Boric Acid	Applichem A2940
21	Glacial Acetic Acid	Merck 1.00056
22	Bromophenol Blue	Merck 1.08122
23	Isopropanol	AppliChem A3928
24	Phenol Crystaline	Applichem A1594

APPENDIX B

MEDIA

B.1. MRS Broth Medium

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Glucose	5
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

All ingredients are dissolved in distilled water by stirring with gentle heating. pH of the medium is adjusted to 6.2 - 6.6 and sterilized by autoclaving at 121°C for 15 min.

B.2. MRS Agar Medium

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Glucose	5
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15
Deionized water	1000 ml

All ingredients are dissolved in distilled water by stirring with gentle heating. pH of the medium is adjusted to 6.2 - 6.6 and sterilized by autoclaving at 121°C for 15 min

B.3. M17 Broth Medium

	g/L
Peptone	10
Lab-Lemco	5
Yeast Extract	5
Lactose	5
Ascorbic Acid	1
β-glyrecophosphate disodium salt	19
$MgSO_4$(0.1M).$7H_2O$	1 ml
Deionized water	1000 ml

Ingredients are dissolved in distilled water by stirring with gentle heating. Medium pH is adjusted to 7.15 ± 0.1 and sterilised by autoclaving at 121°C for 15 min.

B.4. M17 Agar Medium

	g/L
Peptone	10
Lab-Lemco	5
Yeast Extract	5
Lactose	5
Ascorbic Acid	1
β-glyrecophosphate disodium salt	19
$MgSO_4$(0.1M).$7H_2O$	1 ml
Agar	12
Deionized water	1000 ml

Ingredients except lactose are dissolved in 900 ml distilled water by stirring with gentle heating. pH is adjusted to 7.15 ± 0.1. Medium is sterilized by autoclaving at 121°C for 15 min. Lactose is dissolved in 100 ml deionized water, autoclaved at 121°C for 15 min. After sterilization lactose solution is added to medium.

B.5. Media for Growth at Different Temperatures

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Glucose	20
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromocresol Purple	0.04

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.6. Media for Growth at Different NaCl Concentrations

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Glucose	20
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromocresol Purple	0.04
NaCl	20, 40, 65 and 80
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.7. Media for Gas from Glucose

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Glucose	20
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. It was distrubuted into tubes containing inverted Durham tubes. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.8. Modified MRS for Carbohydrate Fermentations

	g/L
Peptone	10
Lab-Lemco	10
Yeast Extract	5
Tween 80	1 ml
K_2HPO_4	2
Sodium Acetate	5
Ammonium citrate tribasic	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Bromocresol Purple	0.04
NaCl	20, 40, 65 and 80
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.9 PBS Medium

	g/L
NaCl	8
KCl	0.2
Na_2HPO_4	1.64
KH_2PO_4	0.24
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 7.0-7.2. Medium was sterilized by autoclaving at 121°C for 15 minutes.

APPENDIX C

Absorbance Values of (A260nm/A280nm) Genomic DNA

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant
UF6	211.95	4.239	1.817	2.33	1.58	50
GA12	2696.18	53.924	24.249	2.22	2.25	50
UN5	165.66	3.313	1.421	2.33	1.46	50
UN9	182.51	3.65	1.593	2.29	1.41	50
UN19	147.37	2.947	1.307	2.26	1.23	50
UN26	1012.44	20.249	9.042	2.24	2.27	50
UIB2	584.71	11.694	5.156	2.27	2.09	50
UIB31	136.4	2.728	1.17	2.33	1.56	50
UZ8	37.54	0.751	0.328	2.29	0.8	50
UZ12	1474.44	29.489	12.645	2.33	2.28	50
UZ16	12.24	0.245	0.112	2.19	0.3	50
UZ18	55.0	1.1	0.484	2.27	1.13	50
UZ22	165.22	3.304	1.447	2.28	1.67	50
UZ32	1131.41	22.628	9.85	2.3	2.3	50
UIN4B	216.26	4.325	1.877	2.3	1.93	50
UIN9	542.22	10.844	4.788	2.27	2.08	50
UIN22	168.88	3.378	1.393	2.42	1.71	50
UIN26	575.27	11.505	4.974	2.31	1.96	50
UIN42	99.17	1.983	0.863	2.3	1.39	50
DT54	247.75	4.955	2.095	2.37	1.89	50
DT62A	118.77	2.375	1.041	2.28	1.57	50
DT62B	353.16	7.063	3.067	2.3	1.97	50
DT66	314.08	6.282	2.682	2.34	2.09	50
DT74	501.0	10.02	4.282	2.34	2.15	50
UIIN4	312.4	6.248	2.701	2.31	2.09	50
UIIN18	188.55	3.771	1.623	2.32	1.77	50
UIIN22	220.47	4.409	1.867	2.36	1.86	50
UIIN24	408.3	8.166	3.54	2.31	2.03	50
UIIN26	1025.72	20.514	9.001	2.28	2.24	50
UIIN28	135.57	2.711	1.16	2.34	1.66	50
UIIN44	82.68	1.654	0.715	2.31	1.33	50
Lb. bul.	4.59	0.092	0.028	3.28	0.18	50
S. therm.	417.24	8.345	3.614	2.31	1.97	50

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1. 1M Tris-HCl pH 8.0

121.1 g of Tris base was dissolved in 800 ml of deionized H2O. pH was adjusted to the desired value by adding concentrated HCl. 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.

D.2. 0.5M EDTA pH 8.0

186.1 g of disodium EDTA•2H2O was added to 800 ml of deionized H2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with pellets of NaOH. Volume was adjusted to 1 L with deionized water. It was dispensed into aliquots and sterilized by autoclaving.

D.3 50X TAE

242 g of Tris base was dissolved in deionized H2O. 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. 10X TBE Buffer

108g of Tris base and 55g of boric acid are mixed and dissolved in 800ml of deionized water. 40ml o 0.5M EDTA (pH 8) was added. The volume was adjusted to 1L with deionized water.

D.6. 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.7. 5M NaCl

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

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

Phenol is allowed to warm at room temperature, and melted at 68 °C. Equal volume of buffer (usually 0.5 M Tris-Cl pH 8.0, at room temperature) is added to the melted phenol. The mixture is stirred for 15 minutes. When the two phases have separated, the aqueous (upper) phase is removed using a separation funnel. Equal volume of 0.1 M Tris-Cl pH 8.0 is then added to the phenol. The mixture is again stirred for 15 minutes. The aqueous phase is removed as described below. The extractions are repeated until the pH of the phenolic phase is > 7.8. The pH is measured by using Ph paper slips. After the phenol is equilibrated, the mixture is divided into aliquots and they are stored under 100 mM Tris-Cl (pH 8.0) at 20 °C. When needed, the phenol is melted at room temperature. Hydroxyquinoline and mercaptoethanol are added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at -20 °C.

D.11. 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.12. 10% SODIUM DODECYL SULFATE (SDS)

100 g of SDS is dissolved in 900 ml of deionized water. Solution is heat to 68 °C to dissolve. The pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume is brought to 1000 ml with water.

D.13. 100 mM Phenyl Methyl Sulforyl Floride Stock Solution (PMSF)

17.4 mg of PMSF is dissolved in 1 ml isopropanol by stirring.

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