IDENTIFICATION OF THE *LACTOBACILLUS* SPECIES ORIGINATED FROM HUMAN GUT BY PCR - ARDRA TECHNIQUE

by

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ABSTRACT

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Probiotic means 'living microorganisms' which have beneficial effects on their host's health. In recent years, interest in the probiotic lactobacilli has been stimulated by the use of these bacteria in products that are claimed to confer health benefits on the consumer. *Lactobacillus* is among the most important organisms with nearly 140 species at present, mostly of industrial and clinical importance. The identification of probiotic strain only in phenotypic and physiyological characteristics is often with a low level of discrimination. The identification of *Lactobacillus* at species level is becoming more and more required. Unfortunately, most of the methods are labor-intensive, costly, and time-consuming. The aim of this study is to identify and discriminate fourteen *Lactobacillus* strains that have been found in the human alimentary tract by the use of amplified 16S ribosomal DNA restriction analysis (ARDRA). In this study, using two universal primers (8AU and 1492R) against the 16S rRNA gene, 1.5 kb PCR products were obtained from the commercial strains of the manufacturers. To obtain species-specific restriction patterns, three restriction endonucleases (*FspB*I, *Hinf*I and *Dra*I) were chosen for the aligning of the 16S

rRNA gene sequences of fourteen *Lactobacillus* species retrieved from various databases. It was shown that 1.5 kb amplicon digested by these enzymes provided unique patterns of almost fourteen species. Digestion of the 1.5 kb PCR product with *FspB*I, *Hinf*I and *Dra*I endonucleases differentiated six, four and two species of *Lactobacillus* respectively. The present study has demonstrated that 1.5 kb 16S rRNA gene fragments can be identified and differentiated in a reliable, rapid and accurate manner for *Lactobacillus* species that are found in the human alimentary tract.

Keywords: Probiotics, *Lactobacillus*, amplified ribosomal DNA restriction analysis (ARDRA), 16S rRNA.

ÖZET

İNSAN BAĞIRSAĞI KAYNAKLI *LACTOBACILLUS* TÜRLERİNİN PCR-ARDRA TEKNİĞİ İLE TANIMLANMASI

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Konakçı sağlığı üzerinde olumlu etkiler gösteren canlı mikroorganizmalar probiyotik olarak adlandırılır. Son yıllarda, probiyotik olan laktobasillerin tüketicinin sağlığı için faydalı olduğu düşünülen ürünlerde kullanılmasıyla buna olan ilgi artmıştır. *Lactobacillus*lar çoğunlukla endüstriyel ve klinik öneme sahip olan şu anda yaklaşık 140 türü ile birlikte en önemli organizmalar arasındadır. Probiyotik suşun sadece fenotipik ve fizyolojik özellikleri bakımından tanımlanması genellikle çok fazla ayırt edici değildir. Tür seviyesinde *Lactobacillus*'un tanımlanması günden güne daha gerekli hale gelmektedir. Maalesef metodların çoğu yoğun iş yüklü, maliyetli ve zaman alıcıdır. Bu araştırmanın amacı çoğaltılmış 16S ribosomal DNA kesim analizlerinin (ARDRA) kullanımı ile insan sindirim sisteminde bulunan 14 *Lactobacillus* suşunu ayırt etmek ve tanımlamaktır. Bu çalışmada, 16S rRNA genine karşı iki üniversal primer (8UA ve 1492R) kullanılarak 1.5 kbç PZR ürünleri, üreticilerin ticari amaçla kullandığı suşlardan elde edilmiştir. Türe özgü restriksiyon parçaları oluşturmak için, çeşitli veri tabanlarından elde edilen 14 *Lactobacillus* türünün 16S rRNA gen dizilerinin eşleşmeleri sonucu üç adet restriksiyon endonukleaz (*FspB*I, *Hinf*I ve *Dra*I) seçilmiştir. PZR ürünlerinin bu üç enzim

tarafından kesilmesi sonucu oluşan fragmentlerin 14 tür için özgün olan fragmentler oluşturduğu görülmüştür. Ondört ticari *Lactobasillus* türünden elde edilen 1.5 kbç PZR ürünlerinin *FspB*I*, Hinf*I ve *Dra*I endonukleazları ile kesimi sonucunda sırası ile altı, dört ve iki *Lactobasillus* türü ayrıştırılmıştır. Sunulan bu çalışma 1.5 kbç 16S rRNA gen parçalarının insan sindirim sisteminde bulunan *Lactobacillus* türlerini etkili, hızlı ve uygun bir şekilde ayırt edilidiğini ve tanımlandığını göstermiştir.

Anahtar kelimeler: Probiyotikler, *Lactobacillus,* çoğaltılmış ribosomal DNA kesim analizi, 16S rRNA.

To my father, Sadi METERELLİYOZ

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ABBREVIATIONS

AFLP	Amplification Fragment Length Polymorphism
ARDRA	Amplified Ribosomal DNA Restriction Analysis
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden–Meyerhof–Parnas
GI	Gastrointestinal
GC	Guanine Cytosine
kb	Kilo base
kbç	Kilo baz çifti
LAB	Lactic acid bacteria
MRS	de Man, Rogosa and Sharpe
ng	Nanogram
NK	Natural killer
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RE	Restriction endonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
ΔTm	Temperature of melting

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CHAPTER I

1. INTRODUCTION

1.1. Probiotics

The gastrointestinal tract have diverse and concentrated microbial population and one of the key organs of the human body. It is ecosystems mediated numerous interactions like chemical and nutritional environment. Its mucosal surface increase by circular folds, intestinal villi, and microvilli which provides large area. This interaction associated with digestion, adhesion to the mucosal wall and colonization (Holzapfel et al., 1998). Intestinal flora can protect humans against and damage or disturbance of this flora can increase infection. Many in vivo and in vitro studies have shown that normal intestinal flora can have barrier. This barrier is effective against pathogenic and opportunistic microorganisms (Fuller, 1991).

Many microorganisms have been used or considered for use as probiotics. A probiotic preparation may contain one or various different strains of microorganisms. Because viable and active microorganisms are usually required target site in the host. It is essential that the probiotic be able to withstand and the host's natural barriers against ingested bacteria. The most commonly used probiotics are strains of lactic acid bacteria, e.g., *Lactobacillus, Bifidobacterium* and *Streptococcus*. Bacteria in *Lactobacillus* and *Bifidobacterium* which resist gasteric acid, bile salts and pancreatic enzymes, considered important components of the gastrointestinal flora

and relatively harmless. Lactic acid bacteria have been demostrated to inhibit the in vitro growth of many enteric pathogens including *Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Clostridium perfringens* and *Clostridium difficle* have been used in both humans and animals to treat a broad range of gastrointestinal disorders (Meurman et.al., 1995; Silva et.al., 1987).

1.2. History and Definition of Probiotics

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

The term "probiotic" firstly used in 1965 by Lily and Stillwell. They defined probiotics as substances produced by one microorganism that stimulated the growth of another microorganism. Another definition offered by Parker (1974) resembles more recent description of probiotics. He defined them as organisms and substances, which contribute to intestinal microbial balance. This definition was disputed by many authors since various substance even antibiotics might have been included. Late 1980s and 1990s saw a surge of different definitions of probiotics. Most

frequently cited definition is that of Fuller's (1992), who defined them as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". Today the universal meaning the term "probiotic" was established by the World Health Organization and the Food and Agriculure Organization of the United States. These two organizations defined probiotics as "live microorganisms which is administered in adequate amonunts, have a beneficial effect on health of the host organism" (Vasilijevic and Shah 2008).

Table 1.1. Microorganisms considered as probiotics (Holzapfel et. al., 2001)

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Nonlactic acid bacteria
Lb. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus var. toyoi*
Lb. amylovorus	*B. animalis*	*Enterococcus faecium*	*Echerichia coli strain nissle*
Lb. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
Lb. crispatus	*B. breve*	*Leuconostoc mesenteroides*	*Saccharomyces cerevisiae*
Lb. delbrueckii subsp. bulgaricus	*B. infantis*	*Pediococcus acidilactici*	*Saccharomyces boulardii*
Lb. gallinarum	*B. lactis*	*Sporolactobacillus inulinus*	
Lb. gasseri	*B. longum*	*Streptococcus thermophilus*	
Lb. johnsonii			
Lb. paracasei			
Lb. plantarum			
Lb. reuteri			
Lb. rhamnosus			

1.3. Selection Criteria for Probiotics

Oral consumption probiotics have to reach intestinal flora to effect host health. So that probiotic strain have stability to stomach acid and bile salt. This is the main remarks of probiotic selection criteria. In addition struggle with pathogens bacteriocin or bacteriocin derivatives antimicrobial compounds producing is a important criteria (Çakır, 2003). The significance of human origin has been debated recently, but most current successful strains are indicated to be of human origin. It can also be argued that a probiotic strain can function better in a similar environment (e.g. human gastrointestinal-tract) to where it was originally isolated from. Safety aspects include the following specifications (Lee and Salminen, 1995; Donohue and Salminen, 1996; Salminen et al., 1996b, 1998b; Adams, 1999):

1. Strains, preferably of human origin are used for human.
2. They are isolated from healthy human gastrointestinal (GI) tract.
3. They have a history of being non-pathogenic.
4. They have no history of association with diseases such as infective endocarditis or GI-disorders.
5. They do not carry transmissible antibiotic resistance genes.

The functional requirements of probiotics should be established by using in vitro methods and the results of these studies should be reflected in controlled human studies. While selecting a preferable probiotic strain several aspects of functionality have to be considered:

- Acid tolerance and tolerance to human gastric juice.
- Bile tolerance (an important property for survival in the small bowel).
- Adherence to epithelial surfaces and persistence in the human GI-tract.

- Immunostimulation, but no proinflammatory effect.
- Antagonistic activity against pathogens such as *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes* and *Clostridium difficile*.
- Antimutagenic and antigarcinogenic properties.

Feeding trials with different probiotic strains have shown that the probiotic strain usually disappears from the GI-tract within a couple of weeks after the ingestion is discontinued (Fukushima et al., 1998; Johansson et al., 1998; Alander et al., 1999; Donnet-Hughes et al., 1999). The role of the probiotic persistence in the human GI-tract has therefore been questioned. However, even temporary persistence, which has been noted for several ingested probiotic strains, may enhance their chances for beneficial functions in the GI-tract, and is therefore considered a desirable trait (Saarela et al., 2000).

Even though a probiotic strain fullfils the necessary safety and functional criteria the aspects related to probiotic production and processing are also of utmost importance. Several technological aspects have to be considered in probiotic selection. These include the following:

1. Good sensory properties.
2. Phage resistance.
3. Viability during processing.
4. Stability in the product and during storage.

Good viability and activity of probiotics are considered prerequisites for optimal functionality. However, several studies have shown that non-viable probiotics can have beneficial effects such as immune modulation and carcinogen binding in the host (Ouwehand and Salminen, 1998; Salminen et al., 1999). Thus, for certain

probiotic strains it might be sufficient that they grow well during initial production steps (to obtain high enough numbers in the product) but they do not necessarily need to retain good viability during storage (Saarela et al., 2000).

There is growing scientific evidence to support the concept that the maintenance of healthy gut microflora may provide protection against gastrointestinal disorders including gastrointestinal infections, inflammatory bowel diseases, and even cancer (Haenel and Bendig, 1975; Mitsuoka, 1982; Salminen et al., 1998a). The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria, and reinforces the body's natural defence mechanisms. Today, plenty of evidence exists on the positive effects of probiotics on human health (Salminen et al., 1998a).

1.4. Health Effects of Probiotics

Since Metchnikoff's era, a number of health benefits have been contributed to products containing probiotic organisms. Health benefits imparted by probiotic bacteria are very strain specific; therefore, there is no universal strain that would provide all proposed benefits, not even strains of the same species. Moreover, not all the strains of the same species are effective against defined health conditions. There have been hundreds of publications describing the use of probiotics to prevent and treat a variety of gastrointestinal disorders. However, only a few have contributed convincingly to our knowledge of the health effects of probiotics in humans. The majority of studies have been poorly designed (e.g., inadequately defined strains of microorganisms, variation in preparation and storage of probiotics, patient groups that are too small in size for statistical analysis or imprecise definitions of end points)

and therefore not reproducible by other investigators. Only a relatively few studies have been conducted with sufficient subjects, proper controls and statistical analysis of the results (Rolfe, 2000).

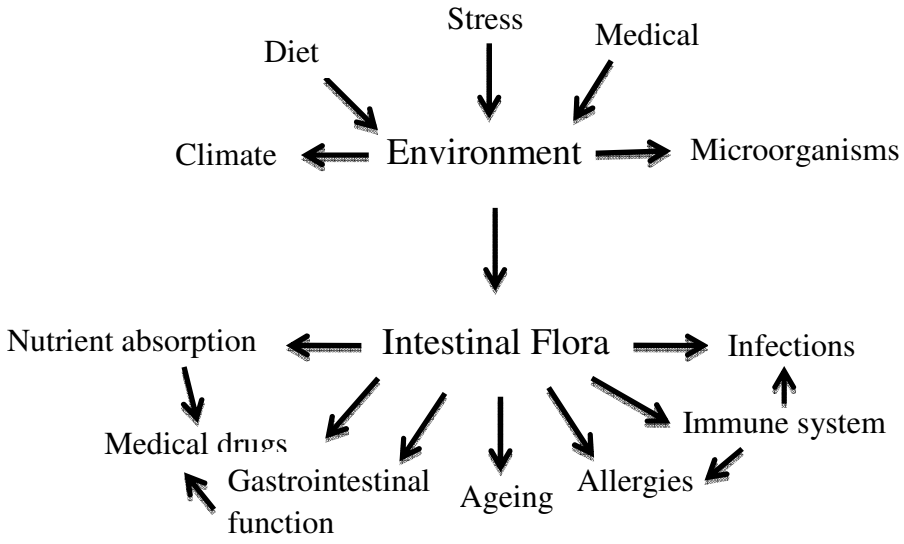

Figure 1.3. Factors affecting the intestinal tract flora (Cedgård, 2000)

1.4.1. Reduction of the Risk Associated with Mutagenicity and Carcinogenicity

Antigenotoxicity, antimutagenicity and anticarcinogenicity are important potential functional properties of probiotics. Mutagens are frequently formed during stress or due to viral or bacterial infections and phagocytosis but also commonly obtained via foods. Endogenous DNA damage is one of the contributors to ageing and age-related degenerative diseases. DNA irreversible damage is a critical factor of carcinogenesis and ageing. Antimutagencity could be described as a suppression of the mutation process, which manifests itself as a decrease in the level of spontaneous and induced mutations (Vasiljevic and Shah, 2008). Some epidemiological researches have emphasized that probiotic intake may be related to a reduced colon cancer incidence

(Hirayama and Rafter, 2000) and experimental studies showed the ability of lactobacilli and bifidobacteria to decrease the genotoxic activity of certain chemical compounds (Tavan et al., 2002) and increase in antimutagenic activity during the growth in selected media (Lo et al., 2004).

Live bacterial cells showed higher antimutagenicity than killed cells against the mutagens studied, which suggested that live bacterial cells were likely to be involved in metabolism of mutagens. The importance of consuming live probiotic bacteria and maintaining their viability in the intestine in order to provide efficient inhibition of mutagens. Several factors have been identified to be responsible for induction of colorectal cancer including bacteria and metabolic products such as genotoxic compounds (nitrosamine, heterocyclic amines, phenolic compounds, and ammonia). Epidemiological studies have shown that diet plays a role in the etiology of most large bowel cancers, implying that it is a potentially preventable disease (Vasiljevic and Shah, 2008).

Many different studies indicate that bile acids cause DNA damage, strongly suggesting mutagenic and carcinogenic potential. A rapid effect on cells of high bile acid exposure is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Increased production of ROS/RNS, can lead to increased mutation. For each organ of the GI tract, where available, on deletrious effects of bile acids, including the induction of ROS/RNS, induction of DNA damage, mutation and apoptosis, and the development of reduced apoptosis capacity. Reduced ability to undergo apoptosis is important because apoptosis is a beneficial process that can cause mutation. Reduced apoptosis capability has been linked to increased mutagenesis (Bernstein et al., 2009; Bernstein et al., 2005).

Bile acids cause DNA damage and may select for apoptosis-resistant cells (both leading to increased mutation), indicates that bile acids are likely carcinogens. In humans, an increased incidence of cancer of the upper aerodigestive tract, esophagus, stomach, pancreas, small intestine, colon associated with high levels of bile acids (Bernstein et al., 2005).

The mechanism of antimutagenicity and anticarcinogenicity of probiotic bacteria has not been clearly understood. It has been suggested that microbial binding of mutagens to the cell surface could be a possible mechanism of antimutagenicity (Orrhage et al., 1994). Other proposed mechanisms include alteration of intestinal microecology and intestinal metabolic activity, normalization of intestinal permeability and enhanced intestinal immunity (Shah, 2006).

1.4.2. Modulation of the Immune System

The complexity of the immune system is secondary only to that of the central nervous system and includes two principal components: innate and adaptive immunity, which work in concert to protect us from external and internal insults. The innate system is ancestral and is neither anticipatory nor clonal and does not respond to environmental changes. It represents the first line of defense with natural killer (NK) cells as the primary cells involved in the identification and spontaneous lysis of offensive targets (virus-infected cells, tumor cells, bone marrow stem cells and embryonic cells). An inverse relationship exists between the rise and fall of NK cells and the incidence of tumor growth (Dussault and Miller, 1996). In contrast, the adaptive system is acquired through interactions with the environment. It is subject to induction, anticipation (immune memory) and clonal expansion. Understanding these

responses is the key to understanding the mechanisms of allergy, autoimmunity, vaccination and carcinogenicity. The innate and adaptive systems are highly integrated and interdependent (Hoebe et al., 2004).

Humans as mammals have developed an extremely sophisticated adaptive immune system of both systemic and mucosal (local) type. Intestinal epithelial cells are in direct contact with the intestinal microflora and also interface and segregate the immune system. It has been suggested that the immune system might be beneficially affected in the presence of probiotics through the action of recognition receptors expressed on the surface of epithelial cells (Isolauri et al., 2001).

1.4.3. Prevention and Reduction of Diarrhoea Symptoms

One of the main applications of probiotics has been the treatment and prevention of antibiotic-associated diarrhoea, which is often caused by occurrence of *Clostridium difficile* after an antibiotic treatment. *Clostridium difficile* is an indigenous gastrointestinal organism usually encountered in low numbers in the healthy intestine; however, the antibiotic treatment may lead to a disruption of indigenous microflora and subsequently to an increase in the concentration of this organism and toxin production, which causes symptoms of diarrhoea. The administration of an exogenous probiotic preparation is required to restore the balance of the intestinal microflora. The application of probiotics in the clinical setting significantly reduced antibiotic-associated diarrhoea by 52%, reduced the risk of travellers' diarrhoea by 8% and that of acute diarrhoea of diverse causes by 34% (Vasiljevic and Shah, 2008).

1.4.4. Inhibition of *Helicobacter pylori* and Intestinal Pathogen

Probiotic cultures produce a wide range of antibacterial compounds including organic acids (e.g., lactic acid and acetic acid), hydrogen peroxide, bacteriocins, various low-molecular-mass peptides, and antifungal peptides/proteins, fatty acids, phenyllactic acid, and OH-phenyllactic acid. Lactic and acetic acids are the main organic acids produced during the growth of probiotics and their pH lowering effect in the gastrointestinal tract has a bacteriocidal or bacteriostatic effect. Low-molecular-mass compounds such as lactic acid have been reported to be inhibitory towards Gram-negative pathogenic bacteria (Alakomi et al., 2000). Probiotics like many other lactic acid bacteria can produce various bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides effective against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) with immunity to their own bacteriocins (Cotter et al., 2005). Many mechanisms have been suggested by which probiotics prevent the detrimental effect of intestinal pathogens including competition for limited nutrients, inhibition of epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, production of antimicrobial substances and/or the stimulation of mucosal immunity (Vasiljevic and Shah, 2008).

Helicobacter pylori is an intestinal pathogen, long-term infection by which leads to chronic gastritis, peptic ulcer and increases the risk of gastric malignancies (Plummer et al., 2004). Currently *Helicobacter pylori* infection is treated by a combined therapy consisting of two antibiotics and a proton pump inhibitor, which, although in many cases appeared very effective, presents a very expensive treatment with many side effects including antibiotic-associated diarrhoea and likelihood of induction of the antibiotic resistance in intestinal pathogens (Malfertheiner et al., 2002). The

clinical outcome of *Helicobacter pylori* infection depends on several factors including the strain of *Helicobacter pylori*, extent of inflammation and cell density (Ernst and Gold, 2000). The risk associated with the development of peptic ulcer and gastric cancer is directly proportional to the level of infection (Tokunaga et al., 2000). One of the measures, which may help reduce the rate of *Helicobacter pylori* infection, is a diet modulation with the inclusion of probiotics (Khulusi et al., 1995).

1.5. Main Terms and Concepts of Bacterial Taxonomy

It is possible to define taxonomy or systematics as the procedure in which biodiversity is classified since it is the scientific study on organism diversity, the final goal of which is to characterize and arrange them in an order (Schleifer and Ludwig, 1994). There are three individual, yet related, subdisciplines of taxonomy; classification, identification, and nomenclature. Clustering of organisms into taxonomic groups (taxa) based on the similarities thereof, or on their relationships with one another, is called the process of classification. Nomenclature, on the other hand, is the process of assigning names, in accordance with the international rules, to the taxonomic groups. Lastly, the process in which it is determined to what established and named taxa a new isolate belongs is the identification (Staley and Krieg, 1989). People are getting more and more interested in the field of bacterial taxonomy: it includes various basic scientific and applied fields; hence, it somehow underlies all biological research (Tautz et al., 2003). It is thought to have a 'philosophical' root which originates from human desire to realize and understand the world, which, in turn, necessitates the items to be ordered in a logical manner (Rosselló-Mora, 2005). Furthermore, its practical motivation is strong (Kandler,

1984): the schemes of categorizing may be predictive and permit characterization of novel isolates in a quick manner on the basis of similarity with the known taxa; the identity of the used strains may be confirmed thanks to the identification processes (e.g. in industrial processes having been patented); and finally, correct nomenclature permits labeling of the products comprising microorganisms unequivocally, as well as permitting scientific communication, thereby leading to customer and producer satisfaction. Taxa are ordered hierarchically within the classification system. Two prokaryotic domains are acknowledged today; Archaea and Bacteria. Domains are classified into phyla; the levels below the phylum are as follows: classes, orders (or subdivisions, depending on the group), families, genera, and species. For taxon names, different suffixes characterize different levels of taxonomy (Felis and Dellaglio, 2007).

Since the structure of microorganisms is not very complex and the informative characters thereof are fewer than those of the higher organisms (like morphology), bacterial taxonomy progress has never been independent of the technological advances; moreover, contemporary bacterial taxonomy is primarily based on molecular data. In addition to the developments in techniques for handling the smallest cell components, the discovery of DNA as the depositary material of genetic information has made the above data available (Colwell, 1970; Vandamme et al., 1996).

Species constitutes the basic unit of classification scheme. The concept referred to as the phylogenetic concept is the most acknowledged and suitable definition for the term ‘species’: separate organisms having a high level of resemblance in respect of numerous independent characteristics are clustered in a monophyletic and genomically coherent manner, which is regarded as the species; besides, it is possible

to diagnose the species with a discriminative phenotypic property (Rosselló-Mora and Amann, 2001). Species definition has some key points as follows; (i) the phylogenetic aspect, related organisms having a common ancestor; (ii) the phenetic aspect, i.e. the general similarity: the closer relatives are two taxa, the more likely they are to resemble one another. The similarity of the total DNA is an acknowledged measure for the mentioned resemblance: if two individuals share DNA–DNA relatedness at 70% or more (relative binding ratio assay) and/or the difference of the hybrid DNA duplex that they form in a DNA reassociation assay in the temperature of melting (ΔTm) is equal to or lower than 5°C, they are thought to be the member of the same species group. The reason why the mentioned threshold has been selected is that it has a good correlation with the other data according to what has been found (for example, phenotypic, chemotaxonomic, etc.). In taxonomic applications, the gold standard method for delineating bacterial species has been DNA–DNA similarity assays; however, multilocus sequencing approach has been developed and recommended, the potentials of which have not been explored thoroughly yet (Stackebrandt et al., 2002; Gevers et al., 2005).

Bacterial systematics has gone through a revolutionary phase due to the phylogenetic approach. The relations of the organisms with one another are predicted by comparing molecular sequences, in particular 16S rRNA encoding genes (Woese, 1987). A number of major assumptions providing the basis in this regard include (i) due to the vital role of the ribosomes in protein biosynthesis having been developed in the early phases of evolution of the organisms, rRNA genes are preserved to a great extent; (ii) those genes are not involved in horizontal gene transfer phenomena among organisms; and (iii) the rate of sequence similarity between different individuals represents the variance in their genomes (Felis and Dellaglio, 2007).

Analysis of the relationship between genomic and 16S rRNA gene sequence divergence has revealed an empirical non-linear correlation between sequence identity rates (%) and data regarding total DNA similarity (Keswani and Withman, 2001; Rosselló-Mora and Amann, 2001). In this case, it can be asserted, in general terms, that two organisms are distantly related at the genomic level, and hence they belong to different species if they share a 16S rRNA gene sequence identity less than 97%.

If the values of identity shared by two organisms is more than 97%, they must be regarded as being closely related, and also the data on total DNA–DNA hybridization and/or analyzing the other gene sequences that are more discriminative are definitive for the identification of species, even in case of identical sequences. Since DNA hybridization tests take too much time, are complicated and costly, phylogenetic analysis is more preferred over DNA hybridization tests. On the contrary, the identification conducted by means of sequence analysis is based on sequencing reaction, DNA amplification, and comparing with public databases, said processes being quite fast, much more reproducible and far less costly ones (Gevers et al., 2005).

1.6. Properties of Lactic Acid Bacteria

Lactic acid bacteria (LAB) used as probiotics. Bacteria producing lactic acid as the major metabolic product are generally grouped as “lactic acid bacteria” (Holzapfel, 1998). The most important LAB genera are: *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc and Bifidobacterium* (Vasiljevic and Shah, 2008). Lactic acid bacteria require rich environment for

growth, such as decaying plant material, food products (diary products, fermented meat, sour doughs, vegetables, fruits, beverages) respiratory tract, gastrointestinal tract, vagina of humans and animals and seawage (Felis and Dellaglio, 2007; Axelsson 1998). The GC content of DNA is usually between 32 and 51 mol%. They are aerotolerant or anaerobic and strictly fermentative. Glucose predominantly fermented to lactic acid (homofermentative strains), ethanol and/or acetic acid (heterofermentative strains) (Charteris et al., 1997).

The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, which results in decreasing the pH of the growth environment (Kuipers et al., 2000). A low pH induces the transformation of organic acids to soluble lipids, thereby making them diffuse through the cell membrane into the cytoplasm. LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins, (Rodriguez et al., 2003) some of which may act as antimicrobials. LAB are regarded as a major group of probiotics (Tannock, 1998). Several lactobacilli, lactococci and bifidobacteria are considered to be bacteria that are beneficial to health. However, not much is known about the probiotic mechanisms of gut microbiota (Gibson, 2000). Generally, LAB have a long history of safe use in a variety of food products. Members of the genera *Lactococcus*, *Lactobacillus* and *Bifidobacterium* have thus been accorded the status of being 'generally recognized as safe' (Salminen et al., 1998). Consequently, the most commonly studied intestinal bacteria for potential probiotic use are members of the genera *Lactobacillus* and *Bifidobacterium spp.* (Nagpal, 2007).

1.7. The Genus *Lactobacillus*

The genus *Lactobacillus* belongs to the large group of lactic acid bacteria (LAB) which produce lactic acid by fermentation (Kandler and Weiss, 1986). Lactobacilli are Gram-positive, non-spore-forming microorganisms. Considering cellular shape, they can occur as rods or coccobacilli. They are fermentative, microaerophylic and chemo-organotrophic, requiring rich media to grow. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. They are genetically diverse considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol%. They are almost ubiquitous: they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, gastrointestinal (GI) and genital tracts of humans and animals, and in sewage and plant material (Felis and Dellaglio, 2007). Lactobacilli are aerotolerant or anaerobic LAB, and are nutritionally fastidious. They are strictly fermentative and can be divided into three groups based on fermentation characteristics: obligately homofermentative, facultatively heterofermentative and obligately heterofermentative (Pot et al., 1994; Hammes and Vogel, 1995).

According to Taxonomic Outline of the Prokaryotes (Garrity et al., 2004), the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives, being grouped within the same family, are the genera *Paralactobacillus* and *Pediococcus*. The phylogenetically closest family appears to be the *Leuconostocaceae* family, which includes genera *Leuconostoc*, *Oenococcus* and *Weissella* (Hammes and Hertel, 2003). Seven species in the genus *Lactobacillus* comprise 2 subspecies or more: *Lactobacillus aviaries* (*Lb. aviaries* subsp. *aviaries* and *Lb. aviaries* subsp. *araffinosus*), *Lactobacillus*

coryniformis (Lb. coryniformis subsp. *coryniformisand Lb. coryniformis* subsp. *torquens), Lactobacillus delbrueckii (Lb. delbrueckii* subsp. *delbrueckii, Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *indicus*, and *Lb. delbrueckii* subsp. *lactis), Lactobacillus kefiranofaciens (Lb. kefiranofaciens* subsp. *kefiranofaciens and Lb. kefiranofaciens* subsp. *kefirgranum), Lactobacillus paracasei (Lb. paracasei* subsp. *paracasei and Lb. paracasei* subsp. *tolerans), Lactobacillus plantarum (Lb. plantarum* subsp. *plantarum and Lb. plantarum* subsp. *argentoratensis), and Lactobacillus sakei (Lb. sakei* subsp. *sakei and Lb. sakei* subsp. *carnosus)*, while the insubstantial separation in two subspecies for *Lb. salivarius* has been recently demonstrated and species description emended (Li et al., 2006). The first phylogenetic analysis of lactobacilli was performed in 1991 by Collins and co-workers, on the smaller number of species known at that time: they suggested to subdivide the genus *Lactobacillus* into three groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group and the *Leuconostoc* group, which also contained some lactobacilli. Schleifer and Ludwig (1995) confirmed these findings and *Lb. delbrueckii* group was given the name of *Lb. acidophilus* group; even if *Lb. delbrueckii* is the type species of the genus *Lactobacillus*, it was not as representative of its phylogenetic group as *Lb. acidophilus*. Moreover, these authors noted that the *Lb. casei-Pediococcus* group could be split into a further four subclusters. The description of a large number of species in recent years and the following phylogenetic re-examination of the genus have made splitting these groups into smaller groups more feasible (Hammes and Hertel, 2003).

The lack of correlation between phylogenetic arrangement and metabolic features is what is incoherent about the taxonomy of the genus *Lactobacillus*. Pot et al. (1994)

perfectly reviewed the historical subdivisions of the genus *Lactobacillus* based on the fermentation type drawing attention to the fact that different definitions are given to the terms including 'homofermentative', 'heterofermentative', 'obligately homofermentative', 'facultatively heterofermentative' and 'obligately heterofermentative' .

The acknowledged 'modern' definition is the one made by Hammes and Vogel (1995): obligately homofermentative lactobacilli are capable of fermenting hexoses nearly exclusively to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway whereas pentoses and gluconate are not fermented as they lacking phosphoketolase; facultatively heterofermentative lactobacilli degrade hexoses to lactic acid by the EMP pathway and they are also capable of degrading pentoses and generally gluconate as they possess not only aldolase, but also phosphoketolase; and finally, obligately heterofermentative degrade hexoses by the phosphogluconate pathway yielding lactate, ethanol or acetic acid and carbon dioxide; furthermore, pentoses are also fermented by said pathway. The combination of different methods of phylogenetic analysis and different models of phylogenetic inference allowed the recognition of a number of phylogenetic groups : *Lb. buncheri* group: *Lb. buncheri, Lb. diolivorans, Lb. ferintoshensis, Lb. fructivorans, Lb. hilgardii, Lb. homohiochii, Lb. kefiri, Lb. kunkeei, Lb. lindneri, Lb. parabuchneri, Lb. parakefiri* and *Lb. sanfranciscensis. Lb. delbrueckii* group: *Lb. acetotolerans, Lb. acidophilus, Lb. amylolyticus, Lb. amylophilus, Lb. amylovorus, Lb. crispatus, Lb. delbrueckii, Lb. fornicalis, Lb. gallinarum, Lb. gasseri, Lb. hamsteri, Lb. helviticus, Lb. iners, Lb. intestinalis, Lb. jensenii, Lb. johnsonii, Lb. kefiranofaciens, Lb. kefirgranum* and *Lb. psittaci. Lb. casei* group: *Lb. casei, Lb. manihotivorans, Lb. pantheris, Lb. paracasei, Lb. rhamnosus, Lb. sharpeae* and *Lb. zeae. Lb. plantarum* group: *Lb. alimentarius,*

Lb. arizonensis, Lb. collinoides, Lb. farciminis, Lb. kimchii, Lb. malefermentans, Lb. mindensis, Lb. paralimentarius, Lb. paraplantarum, Lb. pentasus, Lb. plantarum and *Lb. versmoldensis*. *Lb. reuteri* group: *Lb. coleohominis, Lb. durianis, Lb. fermentum, Lb. frumenti, Lb. ingluviei, Lb. mucosae, Lb. oris, Lb. panis, Lb. pontis, Lb. reuteri, Lb. suebicus, Lb. thermotolerans, Lb. vaccinostercus* and *Lb. vaginalis. Lb. sakei* group: *Lb. curvatus, Lb. fuchuensis, Lb. graminis* and *Lb. sakei. Lb. salivarus* group: *Lb. acidipiscis, Lb. agilis, Lb. algidus, Lb. animalis, Lb. aviarius, Lb. cypricasei, Lb. equi, Lb. mali, Lb. murinus, Lb. nagelii, Lb. ruminis, Lb. salivarus* and a number of species, i.e *Lb. bifermentans, Lb. brevis, Lb. coryniforms* and *Lb. perolens*, which do not belong to any group but form single branches.

Today, the process that includes sequencing of 16S rRNA gene partially and completely and the assessment of patterns of fermentations and other properties thereof only with respect to the closest relatives, is the easiest way to identify lactobacilli, in addition to bifidobacteria and bacteria in general. It should be kept in mind that, in case of analysis of closely related species, 16S rRNA-based identification could be deceptive. Being a molecule that diverges slowly, 16S rRNA is not capable of disclosing important differences between newly diverged species; e.g. *Lb. plantarum, Lb. paraplantarum and Lb. pentosus,* or *Lb. casei, Lb. rhamnosus* and *Lb. zeae,* in case of which the analysis of 16S rRNA gene sequence can demonstrate belonging to a group, rather than to a definite species. Protein-encoding genes can be sequenced successfully for a more detailed identification even at subspecies level (Felis et al., 2001, Torriani et al., 2001, Bringel et al., 2005), the resolutive technique is DNA–DNA hybridization, though. Genome GC content, the isomer type of the lactic acid produced, as well as peptidoglycan composition of the cell wall, are among the other parameters related to the genus *Lactobacillus*

attracting interest. What is interesting and conflicting about the genus *Lactobacillus* is the wide range of genome GC content of the defined species, ranging between 32 and 54% mol, an interval being twice as much as the one generally accepted for well-defined genera (Schleifer and Ludwig, 1995). Genome GC content is considered to be a feature linked with evolution, and so similar GC contents generally characterize sister taxa (Graur and Li, 1999). However, this may not be true for lactobacilli; even when taking *Lb. delbrueckii–Lb. acidophilus* group, which is the most well-described phylogenetic subgroup of the genus, into account, the range of genome GC content is from 32% to 50% (Felis and Dellaglio 2007).

The reviewed aspects of genus classification, open issues regarding particular species exist, which could have important implications for identification and nomenclature of strains. The most important case is that of the nomenclature of the species *Lb. casei*. The emended description of *Lb. casei* (Orla-Jensen 1919) comb. nov. by Hansen and Lessel (1971) designated ATCC 393 as the neotype strain of *Lb. casei* subsp. *casei*, on the basis of few phenotypic traits. Shortly after its designation, ATCC 393T was shown to be unsuitable as the neotype strain of *Lb. casei* on the basis of DNA-DNA hybridization experiments (Mills and Lessel, 1973; Dellaglio et al., 1975), as it shared very high genome similarity (84%) with *Lactobacillus casei* subsp. *rhamnosus* ATCC 15820, former type strain of '*Lactobacterium zeae'*(Kuznetsov, 1959), and it was not related to any of the other strains within the subspecies of *Lb. casei*. During the preparation of the Approved List of Bacterial Names in 1980, these data were neglected and Collins and co-workers (1989), on the basis of DNA-DNA hybridization data proposed to create the species *Lb. paracasei* for strains unrelated to *Lb. casei* ATCC 393T. However strain ATCC 334 was not included in the reclassification and it maintained the name *Lactobacillus casei* even if more related

to *Lb. paracasei* than to ATCC 393^T. A first request for ATCC 334 to be designated as the neotype strain of *Lb. casei* in place of ATCC 393^T and the rejection of the name *Lb. paracasei*, based on numerical analysis of total soluble cell protein and DNA–DNA hybridization experiments (Dellaglio et al., 1991), was denied (Wayne, 1994). After that pronouncement, however, a number of studies were published in which either ATCC 334 or ATCC 393 were indicated as the reference strain or the type strains for the species *Lb. casei*. Moreover, considering new isolates, identification procedures could result in the attribution of the name *Lb. casei* or *Lb. paracasei* depending on the reference strain used, *Lb. casei* ATCC 334 or *Lb. paracasei* subsp. *paracasei* NCDO 151T, respectively. However, these two strains are very similar, in certain analyses almost identical, although different from *Lb. casei* ATCC 393T. The status of valid species for *Lactobacillus zeae*, formerly '*Lactobacterium zeae'* (Kuznetsov, 1959) was accepted (Dicks et al.,1996), but the epithet did not included strain ATCC 393T. A number of studies (Mori et al., 1997; Zhong et al., 1998; Tynkkynen et al., 1999; Chen et al., 2000; Felis et al., 2001) supported the revision of the nomenclature of the *Lb. casei* species group, i.e. the reclassification of strain ATCC 393T as *Lb. zeae* and the rejection of the name *Lb. paracasei*. A detailed review of the data cited here and the formal request for the change in the nomenclature of the *Lb. casei* species group are reported in Dellaglio et al.(2002). However, at present, correct taxonomic procedure would imply the attribution of names on the basis of comparison with type strains, therefore strains could be named *Lb. casei* if they resemble more ATCC 393T than NCDO 151T, and the relationships with strain ATCC 334 has no formal meaning. The application of molecular techniques to the analysis of a larger collection of strains have revealed complex relationships between different strains (Vazquez et al., 2005) and common

misidentification of commercial strains belonging to the *Lb. casei* group (Deasi et al., 2006). *Lactobacillus acidophilus* PF01 was renamed *Lb. johnsonii* after 16S rRNA gene sequence comparison following whole genome sequencing of the PF01 strain (Lee et al., 2011).

1.8. Bacterial Identification Methods

1.8.1. Phenotypic Identification

Traditionally, LAB have been classified on the basis of their phenotypical properties, e.g., their morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, the fermentation of various carbohydrates, the methyl esters of fatty acids, (Decallone et al., 1991) and the pattern of proteins in the cell wall (Gatti et al., 1997) or in the whole cell (Tsakalidou et al., 1994). Unfortunately, these typing methods are not completely accurate (William and Sandler 1971; Morelli, 2001). Phenotypical methods have inherent limitations such as their poor reproducibility, the ambiguity of some techniques, the extensive logistics for large scale investigations and their poor discriminatory power. Another disadvantage of phenotypical analysis is that the whole information potential of a genome is never expressed, i.e., gene expression is directly related to the environmental conditions (e.g., the growth conditions in the laboratory). All these drawbacks adversely affect the reliability of phenotype-based methods for culture identification at the genus or species level (Mohania et al., 2008).

1.8.2. Biochemical Identification

The biochemical tests use specific growth media, nutrients, chemicals or growth conditions to elicit an observable or measurable biochemical response from the microorganism, thereby enabling its identification and characterization. These tests include: utilization of carbon and nitrogen sources, growth requirements (anaerobic or aerobic; temperature-optimum and range, pH optimum and range), preferred osmotic conditions, generation of fermentation products, production of enzymes, production of antimicrobial compounds, as well as sensitivity to metabolic inhibitors and antibiotics. Examples of recognized tests include: phenol red carbohydrate, catalase and oxidase tests, oxidation-fermentation tests, methyl red tests, Voges-Proskauer tests, nitrate reduction, starch hydrolysis, tryptophan hydrolysis, hydrogen sulfide production, citrate utilization, litmus milk reactions, etc. (Hammes and Hertel, 2006).

The analytical profile index or API is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. Because of this, only known bacteria can be identified. It was invented in the 1970s in the United States by Pierre Janin of Analytab Products, Inc. Presently, the API test system is manufactured by bioMérieux. The API range introduced a standardized, miniaturized version of existing techniques. But the identification of lactobacilli using biochemical methods is notoriously difficult largely due to the need for plenty of cumbersome biochemical tests along with the problems of highly resembling large number of species groups that are prone to transfer of plasmids among them. Hence, they alone are not sufficient for inter- and intra-species differentiation and need to be supplemented

with sensitive molecular methods to obtain more reliable identification (Singh, 2009).

1.8.3. Genotypic Identification

Several molecular typing techniques have been developed during the past decade for the identification and classification of bacteria at or near the strain level. Molecular techniques are important for the specific characterization and detection of strains (Amor 2007, Holzapfel 2001). The major advantages of these DNA-based typing methods lie in their discriminatory power (Farber, 1996) and in their universal applicability. Closely related strains with similar phenotypical features may now reliably be distinguished by DNA-based techniques such as randomly amplified polymorphic DNA (RAPD), RFLP, sequencing analysis, AFLP, Ribotyping, PFGE and amplification rDNA restriction analysis (ARDRA) (Mohania et al., 2008).

1.8.3.1. Restriction Fragment Length Polymorphism (RFLP)

Chromosomal DNA restriction analysis was the first of the chromosomal DNA-based typing schemes. The banding patterns that result after cutting and separating the DNA fragments by electrophoresis are referred to as DNA fingerprinting. Because of the high specificity of restriction enzymes and the stability of chromosomal DNA, a reproducible pattern of fragments is obtained after the complete digestion of the chromosomal DNA by a particular enzyme. These variations in the banding patterns between strains are described to basic differences in the DNA base composition of the organism examined. One general criticism about this method is the complexity of banding pattern. Nevertheless, there are researchers

who believe that using the right enzyme and specified conditions RFLP could still be a relatively rapid and reliable technique (Mohania et al., 2008).

1.8.3.2. Sequencing Analysis

Macromolecules have been described as documents of evolutionary history and for decades they have been used to explore the phylogenetic diversity and evolutionary relatedness of organisms. The 16S rRNA gene is the most common gene targeted in bacterial diversity studies. It is a well-conserved universal marker with constant and highly constrained functions that were established at early stages in its evolution and it is relatively unaffected by environmental pressures. These facts, along with the size of the gene, make it a good evolutionary clock (Kimura et al., 1997). Though the 16S rRNA gene is a well-conserved universal marker, however, there are some shortcomings associated with its use. First, the 16S rRNA genes are so well conserved that it results in a limited resolving power (Achenbach et al., 2001). Second, even though the 16S rRNA gene is a universal marker different bacterial species have different copy numbers of the gene. This leads to an over- and under-representation of some bacterial species when using 16S rRNA genes as targets. Additionally, many genes other than 16S rRNA genes have also been explored in bacterial diversity studies. Some of these are universal genes that every bacterium possesses, but with unique genetic sequential differences. Most of these universal genes are well conserved to the extent that they perform the similar functions in all bacteria. The advantage of using universal genes could be that they may have a more consistent copy number among bacterial species, thus giving a better quantitative representation of bacterial species. Some of these genes are taxa-specific, which reveals a greater genetic diversity between closely related species, i.e., such genes

provide much sharper phylogenetic resolution compared to universal genes (Chang et al., 2001).

1.8.3.3. Ribotyping

Ribotyping is a variation of the conventional RFLP analysis. It combines Southern hybridization of the DNA fingerprints, generated from the electrophoretic analysis of genomic DNA digests, with rDNA-targeted probing. The probes used in ribotyping vary from partial sequences of the rDNA genes the or intergenic spacer regions to the whole rDNA operon (Gatti et al., 1997). Ribotyping has been used to characterize strains of *Lactobacillus* and *Bifidobacterium* from commercial products as well as from human fecal samples (Tsakalidou et al., 1994; William and Sandler 1971). However, ribotyping provides high discriminatory power at the species and subspecies level rather than on the strain level. PFGE was shown to be more discriminatory in typing closely related *Lb. casei* and *Lb. rhamnosus* as well as *Lb. johnsonii* strains than either ribotyping or RAPD analysis (Gibson and Fuller , 2000; Zoetendal et al., 1998).

1.8.3.4. Pulse Field Gel Electrophoresis (PFGE)

PFGE employs an alternating field of electrophoresis to allow the separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes, with increasing pulse times throughout the run, and the resulting fingerprint profiles can be explored for culture identification. As such, the technique can be more time-consuming than other fingerprinting strategies (Holzapfel et al., 2001; O'Sullivan and Kullen, 1999). However, the profile generated by PFGE represents whole

genome and this technique has a discriminatory power that is superior to ribotyping. Indeed, excellent subspecies differentiation has been shown using PFGE for a number of organisms, including lactobacilli and bifidobacteria. In some cases PFGE has enabled the grouping of bacterial strains within a species, and there are various examples to assess the potential of this technique to characterize bacterial isolates as well. Further, the usefulness of PFGE has been adequately demonstrated in monitoring the changes in the predominant bifidobacterial and lactobacilli populations of human origin, both in individuals over time as well as between individuals (Kimura et al., 1997; O'Sullivan and Kullen, 1999).

1.8.3.5. Randomly Amplified Polymorphic DNA (RAPD)

The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in patterns of diagnostic value. In RAPD analysis, the target sequence(s) to be amplified is unknown and a primer with an arbitrary sequence is designed and synthesized. After these sequences have been synthesized they are used in PCR reactions with low-stringency annealing conditions, which results in the amplification of randomly sized DNA fragments. This method is currently being explored for the identification of LAB including probiotic strains. As the reproducibility of RAPD patterns is occasionally poor; this method needs to be performed under carefully controlled conditions. Various groups have adopted the use of RAPD to identify and characterize LAB strains from various sources, i.e., human, food and milk samples (Oh-Sik, 2002; Spano, 2002).

1.8.3.6. Amplification Fragment Length Polymorphism (AFLP)

AFLP analysis is based on the selective amplification of restriction fragments from total digests of genomic DNA, after which the DNA fragments are separated by polyacrylamide gel electrophoresis. AFLP methods rapidly generate hundreds of highly replicable markers from the DNA of the organism, thus allowing high-resolution genotyping of fingerprinting quality (Vos et al.,1995). The time, cost efficiency, replicability and resolution of AFLP is of high quality. Originally developed for plant systematics, AFLP has been found to be a very useful fingerprinting technique for bacteria that is applicable for both species resolution and strain differentiation. AFLP has been employed mostly in epidemiological studies and in investigations aiming to distinguish virulence markers in food-borne pathogens (Giraffa and Neviani, 2000).

1.8.3.7. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA is a technique combining the knowledge of ribosomal RNA sequences and their specific amplification (Amplified Ribosomal DNA) together with the characterization of strains by their restriction pattern (Restriction Analysis). The main advantage of this method is, that no sequence information about the amplified 16S rRNA is required. An advancement of this method is the characterization of pure culture rDNA for the analysis of natural microbial communities without cultivation (Weidner et al., 1996). Total community genomic DNA is extracted without culturing the participating microorganisms. The presence of universally conserved sequences at the 5' and 3' ends allows the amplification of nearly complete 16S rRNA genes fragments of the extracted DNA (Müller, 2000). ARDRA has been used to differentiate a variety of lactobacilli at species level, including *Lb. delbrueckii* and

its three subspecies (*bulgaricus, delbrueckii* and *lactis*), *Lb. acidophilus* and *Lb. helveticus* (Roy and Sirois, 2001).

1.9. Aim of This Study

Probiotics are gaining popularity and increasing the importance of their accurate speciation. *Lactobacillus* species are routinely used as probiotic strains mostly of clinical importance. Present knowledge indicates that at least 14 *Lactobacillus* species are associated with the human gut. Currently, researchers are interested in developing efficient techniques for screening and selecting probiotics bacteria, but unfortunately most of these methods are labor-intensive, costly and time-consuming. The identification of lactobacilli using biochemical and physiological methods are difficult and time-comsuming. Hence, they alone are not sufficient for differentiate species groups. Molecular methods used to obtain reliable identification. Closely related groups can be discriminated with molecular methods. In fact many *Lactobacillus* species have been reclassified on the basis of curent information from molecular techiques and their correct taxonomic status has been determined (Singh et al., 2009).

In this study, to develop an accurate, convenient and quick method for the genotype-based identification of the *Lactobacillus* species, located in human gastrointestinal tract, first 1.5 kb of 16S rDNA sequences of 14 *Lactobacillus* were collected from the Gene Bank, aligned, silico restricted and analyzed in respect to their restriction fragment polymorphism. Silico restriction analysis indicated that *FspB*I, *Hinf*I and *Dra*I restriction enzymes (RE) are convenient for differentiation of *Lactobacillus* species in human intestinal tract except *Lb. casei* and *Lb. paracasei*. To show our silico findings convenient with those obtained in practice, the whole 16S rRNA

isolated from 14 reference *Lactobacillus* species were digested with *FspB*I, *Hinf*I and *Dra*I REs. The patterns of our experimental findings completely confirmed our silico restriction patterns. Our results indicated that the 16S ARDRA technique is a simple, quick and highly discriminatory method to identify *Lactobacillus* bacteria isolated from human alimentary tract for probiotic use.

CHAPTER II

2. MATERIALS AND METHODS

2.1. Restriction Site and Nucleotide Sequences Homology Searching

All available complete 16S rRNA gene sequences from fourteen *Lactobacillus* species were obtained from the GenBank (http://ncbi.nlm.nih.gov) DNA database. All of the sequences were alinged with Clustal W (http://www.ebi.ac.uk/clustalW) and subjected to theorical restriction mapping with the webcutter (http://www.rna. lundberg.gu.edu) by comparing restriction profiles of more than 200 commercially available endonucleases. The resulting sequence alignement was edited by BioEdit, version 5.0.9. These sequences were performed based on the restriction sites for *FspB*I (CTAG), *Hinf*I (GANTC), and *Dra*I (TTTAAA) restriction endonucleases.

2.2. Bacterial Strains and Culture Conditions

Fourteen strains of *Lactobacillus* were collected from the culture collection of BCCM^{TM}/LMG Bacteria Collection and the bacteria collection of Abant İzzet Baysal University (Bolu, Turkey). All bacterial strains were grown in MRS (de Man, Rogosa and Sharpe) agars or broth at 30-37°C for 36-48 hours. Lactobacilli colonies, grown and reached the required values, were taken to the 30% glycerol/MRS broth at -80°C for stock culture. Some strains were cultured anaerobic conditions in MRS broth or MRS agar with L(+)-cysteine (0.5 mg/ml) at 37°C for 16-24 hours.

Table 2.2. Bacterial Strains and Culture Conditions

Name of *Lactobacillus* Species	Source of species	Culture Conditions
Lb. crispatus	ATCC33820	MRS agar and broth, 37ºC preferably anaerobic
Lb. acidophilus	ATCC11975	MRS agar and broth, 37ºC
Lb. casei	ATCC393	MRS agar and broth, 30ºC
Lb. paracasei	ATCC25302	MRS agar and broth, 30ºC
Lb. rhamnosus	AIBU-2[b]	MRS agar and broth, 30ºC
Lb. reuteri	B14171[ba]	MRS agar and broth, 37ºC
Lb. plantarum	AIBU-1[b]	MRS agar and broth, 37ºC
Lb. gasseri	ATCC33323	MRS agar and broth, 37ºC preferably anaerobic
Lb. johnsonii	B2178[a]	MRS agar and broth, 37ºC
Lb. salivarus	ATCC11741	MRS agar and broth, 30ºC
Lb. brevis	ATCC14869	MRS agar and broth, 30ºC
Lb. delbrueckii	ATCC9649	MRS agar and broth, 37ºC preferably anaerobic
Lb. sakei	ATCC15521	MRS agar and broth, 30ºC
Lb. ruminis	ATCC27780	MRS agar and broth with L-cysteine-hydrochloride, 37ºC definitaly anaerobic

*[a] and [b] , B14171, B2178, AIBU-1 and AIBU-2 *Lactobacillus* strains are from Abant Izzet Baysal University Culture Collection.

2.3. Genomic DNA Isolation

Bacterial cells were grown in 2 ml MRS broth for 36-48 hours at 30-37°C. Genomic DNA was isolated by Gene JET™ Genomic DNA purification kit (#K0721 Fermentas, Europe) or Wizard® Genomic DNA Purification kit (Cat.#A1120 Promega, USA).

Genomic DNA isolation protocol (Fermentas kit, Europe) : Bacterial cell cultures within 1.5 or 2 ml microcentrifuge tube were centifugated for 10 minutes at 7.000 rpm and supernatant was discarded. Later, bacterial pellets were resuspended in 180 µl of Gram-positive bacteria lysis buffer (Appendix B) and incubated at 37°C for 30 minutes. Then 200µl of lysis solution and 20 µl of proteinase K were added and then samples were incubated at 56°C for 30 minutes. Then 20µl of RNase A solution was added and incubated at room temperature for 10 minutes. 400µl ethanol (50%) was added and mixed by pipetting. After before lysates were not transferred to the genomic DNA purification column, the column was centrifuged for 1 minute at 8.000 rpm and the collection tube containing the flow-through solution was discarded. 500 µl wash buffer I was added and centrifuged for 1 minute at 10.000 rpm and discarded. 500 µl wash buffer II was added and centrifugeted for 3 minutes at 14.000 rpm. Finally genomic DNA was eluted within 200 µl elution buffer and it was incubated by incubating column at room temperature for 2 minutes and centrifuged for 1 minutes at 10.000 rpm. Purified DNA was stored at -20°C.

Genomic DNA isolation protocol (Promega kit, USA): 1.5 ml MRS mediums in eppendorf tubes were inoculated with desired *Lactobacillus* species and incubated at 30ºC or 37ºC for overnight. Bacterial cell cultures in microcentrifuge tube was centrifuged at 14.000 rpm at 25°C for 2 minutes and 200 µl EDTA (0.05 M) (Appendix B) was added. Then 100 µl lysozyme solution was added and incubated at

37°C for 45 minutes. Suspension was centrifuged at 14.000 rpm for 1 minute and supernatant was discarded. 850 µl nuclei lysis solution was added and pellet was gently mixed by pipeting. This step was performed by three-freeze thaw using liquid nitrogen and 80°C dry block by helding of samples for 30-60 seconds in liquid nitrogen and transfered to 80°C heat block for 1-2 minutes. 4 µl RNase solution was added and incubated at 37°C for 30 minutes with occasional inverting. Lysate were incubated on ice for 5 minutes. After the lysate was centrifugated at 14.000 rpm in room temperature for 5 minutes, supernatant was transfered to a clean eppendorf tube. Coloums were centrifuged at 14.000 rpm at 25°C for 5 minutes to remove any residual proteins from the extract. Supernatant was transfered to a clean tube and 600 µl isopropanol was added and mixed. Tube was centrifuged at 14.000 rpm at room temperature for 8 minutes. The supernatant was discarded and drained in the tube with clean absorbent paper. Then 600 µl ethanol (70%) mix was added and then centrifuged at 14.00 rpm centrifuged for 2 minutes. Ethanol was aspirated for 10-15 minutes on heat block. Finally the pellet was resuspended in 100 µl rehydration solution for 1 hour at 65°C.

2.4. Amplification of 16S rRNA Genes

The 16S rRNA gene fragments of all *Lactobacillus* species (approximately 1.5 kb) were amplified using the universal primers 8UA and 1492R (Table 2.2). Each PCR mixture (50 µl) contained a reaction mix of 50-100 ng template DNA, 1X buffer, 200 µ/mol of each dNTPs, 0.5 µ/mol of each primer, 1.5 mM $MgCl_2$ and 2.5 U/µl of the *Pfu* DNA Polymerase (Fermentas, Europe).

Table 2.2 Universal primers applied in PCR

Sequence	**Specificity**	**Primer**
5'-AGA GTT TGA TCC TGG CTC AG-3'	universal	8 UA
5'-TAC GGG TAC CTT GTT ACG ACT T-3'	universal	1492 R

PCR amplification was performed using a Techne 3000 PCR System (Barloworld Scientific, ABD) under the following PCR conditions; initial denaturation (95°C for 10 minutes), followed by 35 cycles of denaturation (94°C for 1 minutes), anneling (42°C for 1 minutes), extension (72°C for 3 minutes), and final extension (72°C for 10 minutes). Finally 1.5 kb PCR product was separeted and purified from a 0.7% agarose gel by electrophoresis at 80V for 45 minutes followed by ethidium bromide staining (10mg/ml).

2.5. Purification of PCR Product

PCR products were purified by using High Pure PCR Product Purification Kit (Roche, Germany). PCR reaction mixture were loaded on 0.8% agarose gel (Sigma, USA). Bands were identified in the agarose gel by staining with ethidium bromide solution for 10 minutes. Desired DNA bands were cut from gel using an ethanol-cleaned blade and agarose gel was excised in a sterile 1.5 microcentifuge tube. Gel mass was determined and 300 µl binding buffer was added to the microcentrifuge tube for every 100 mg agarose gel slice. Agarose gel was dissolved in order to release the DNA. Microcentrifuge tube was vortexed for 15-30 seconds to resuspend the gel slice in the binding buffer and suspension incubated for 10 minutes at 56°C. Tubes were vortexed briefly every 2-3 minutes during incubation. After the agarose gel slice was completely dissolved, 150 µl isopropanol for every 100 mg agarose gel

was added to the tubes. Supernatants were transfered from high pure filter columun into one collection tube. Entire contents were pipetted to the microcentrifuge tube into the upper reservoir of the filter tube. Filter tube was centrifuged in 30-60 seconds at 14000 rpm and supernatant was discarded. 500 µl wash buffer was added to the upper reservoir and centrifuged at 14000 rpm for 1 minute. Supernatant was discarded again and 200 µl wash buffer was added. Tubes were centrifuged for 1 minute at maximum speed. Solution was discarded and column was recombined with a clean 1.5 ml microcentrifuge tube. To obtain column bound DNA, 50-100 µl elution buffer was added to the upper reservoir of the column and centrifuged at 14000 rpm for 1 minute.

2.6. Restriction of PCR Product

Purified 16S PCR-ARDRA reaction mixture for each of the *Lactobacillus* strain (50µl) contained 0.3 µl of PCR amplicon and corresponding restriction enzyme buffer supplied by the manufacturers were restricted with *FspB*I, *Hinf*I and *Dra*I restriction endonucleases (Fermentas, Europe). 100ng 16S PCR product were digested with 2µl of *FspB*I and *Hinf*I restriction enzymes (10 U/µl) in 50 µl final volume at 37ºC for 8 hours. For *Dra*I digestion, 300ng 16S PCR products were digested with 2µl of restriction enzymes (10 U/µl) in 50µl final volume at 37ºC for overnight. The DNA fragments were separeted on 1.5% agarose gel at 80V for 90-120 minutes. Gels were stained with ethidium bromide solution (10mg/ml) for 10 minutes and visualized under UV light. 100bp and 1 kb DNA ladder (Fermentas, Europe) was used as a molecular marker.

2.7. Cluster Analaysis

Dendrograms of the 14 *Lactobacillus* species based on *FspB*I, *Hinf*I and *Dra*I restriction fragment length polymorphism of 16S rRNA genes from the agarose gel were obtained by UVP cluster analysis program (version 6.8.2, UVP, LLC, Upland, CA, USA) on the bases of complete linkage, Jaccard similarity and RF values.

CHAPTER III

3. RESULTS

3.1. Alignment of the 16S rRNA Genes of *Lactobacillus* Species Found in the Human Alimentary Tract

All available comlete 16S rRNA gene sequences (almost 200), ranging from 1500 to 1579 bp, of the 14 *Lactobacillus* species, were collected from GenBank. All of the complete 16S rRNA sequences from same species and closed species were aligned with clustalW program and similarity percents were determined (Table 3.1). It was found that 16S rRNA gene sequence of *Lb. acidophilus* and *Lb. crispatus*; *Lb. casei*, *Lb. rhamnosus* and *Lb. paracasei*; and *Lb. gasseri* and *Lb. johnsonii* closed each other (Table 3.1). On the other hand, *Lb. acidophilus* and *Lb. reuteri* were divided in two subgroups on the bases of 16S rRNA nucleotide similarity. It was found that16S rRNA gene sequence of *Lb. acidophilus*-b is very close to that of *Lb*. *crispatus*.

Table 3.1 16S rRNA gene sequence similarities of the reference *Lactobacillus* species

***Lactobacillus* (*Lb.*) species**	**Number of complete 16S rRNA available sequence**	**Similarity %**
Lb. acidophilus	4	98
Lb. crispatus	5	97.8
Lb. plantarum	16	99
Lb. salivarus	52	98.5
Lb. brevis	6	98
Lb. delbrueckii	6	99.4
Lb. ruminis	3	99.8
Lb. sakei	2	99
Lb. casei	4	98
Lb. paracasei	7	99
Lb. rhamnosus	20	99.4
Lb. reuteri	6	98.9
Lb. gasseri	14	98.5
Lb. johnsonii	7	98.9
Closely related *Lb*. species		**Similarity %**
Lb. acidophilus - Lb. crispatus		98
Lb. casei - Lb. paracasei		99.4
Lb. gasseri - Lb. johnsonii		99

3.2. Silico Restriction Analaysis of the 16S rRNA Genes of *Lactobacillus* Species

The silico size and silico restriction maps of the each 16S rRNA gene nucleotide sequence of 14 *Lactobacillus* species were obtained by using the Webcutter analysis program (Table 3.2). Restriction fragments smaller than 50 bp were not considered, as they were not determined on agarose gel. Several alternative restriction enzymes were tested. One restriction enzyme was not effective to discriminate fourteen *Lactobacillus* species. We found that *FspB*I*, Hinf*I and *Dra*I restriction endonucleas (RE) and their restriction profiles was given to the clearest and most reliable distinctions. This three endonucleases differentiate all of the *Lactobacillus* species expect *Lb. casei* and *Lb. paracasei*. According to 16S rRNA similarity *Lb. acidophilus* and *Lb. reuteri* species were divided into two groups. It was found that *FspB*I and *Hinf*I silico restriction profiles of *Lb. acidophilus*-b and *Lb. crispatus* were identical. But *Lb. acidophilus*-b and *Lb. crispatus Dra*I silico restriction profiles were different.

Table 3.2 Comparative silico restriction analysis of the *Lactobacillus*

Name of *Lactobacillus* species	rRNA size (bp)	The source of species	*FspBI* restriction sites and fragments	*HinfI* restriction sites and fragments	*DraI* restriction sites and fragments
Lb. crispatus	1528	LAB32	263, 1014, 1359	377, 1293, 1353, 1432	212
			169, 263,345,751	60, 79, 96, 377, 916	212, 1316
Lb. acidophilus-a	1527	1001H	263, 1013, 1358	75, 377, 1292, 1352, 1431	0
			170, 263, 345, 750	60, 75, 79, 96, 302, 915	1527
Lb. acidophilus-b	1528	KLDS 1.0738	263, 1014, 1359	377, 1293, 1353, 1432	0
			169, 263, 345, 751	60, 79, 96, 377, 916	1528
Lb. casei	1534	Zhang	266, 844, 1140, 1167, 1362	93, 380, 1356	0
			172, 195, 266, 296, 578	93, 178, 287, 976	1534
Lb. paracasei	1534	6W	266, 844, 1140, 1167, 1362	93, 380, 1356	0
			172, 195, 266, 296, 578	93, 178, 287, 976	1534
Lb. rhamnosus	1534	IDCC3201	266, 844, 1140, 1167, 1362	380, 1356	0
			172, 195, 266, 296, 578	178, 380, 976	1534
Lb. reuteri-a	1540	C10	274, 852, 1148, 1175, 1370	388, 1279, 1364	0
			170, 195, 274, 296, 578	85, 176, 388, 891	1540
Lb. reuteri-b	1540	*	852, 1175, 1370	388, 1279, 1364	0
			170, 195, 323, 852	85, 176, 388, 891	1540
Lb. plantarum	1531	*	265, 1361	3,791,355	0
			170, 264, 1096	176, 378, 976	1531
Lb. gasseri	1536	ATCC33323	69, 101, 202, 214, 270, 1365	384, 1359, 1438, 1478	222
			56, 69, 101, 171, 1095	58, 79, 384, 975	222, 1314
Lb. johnsonii	1537	NCC2822	69, 101, 202, 214, 270, 1366	384, 1360, 1439, 1479	0
			56, 69, 101,171, 1095	58, 79, 384, 975	1537
Lb. salivarus	1535	ATCC11741	261, 855, 1326	375, 1185, 1320, 1337	0
			209, 261, 471, 594	198, 375, 810	1535
Lb. brevis	1530	RO97	756, 1361	379, 1270, 1355, 1472	0
			169, 605, 756	58, 85, 89, 117, 379, 891	1530
Lb. delbrueckii	1530	*	102, 261, 840, 1026, 1359	193, 202, 375, 1353, 1472	0
			102, 171, 185, 200, 333, 580	58, 119, 173, 193, 978	1530
Lb. ruminis	1540	IMAUFB033	1358	376, 1267, 1352	0
			172, 1358	178, 376, 979	1540
Lb. sakei	1537	PSH-313	849, 1034, 1145, 1172, 1367	360, 385, 1361	0
			163, 185, 1 95, 848	169, 359, 977	1537

*16S ribosomal RNA gene, partial sequence

3.3. Amplification and Restriction Analysis of 16S rRNA Gene of *Lactobacillus* Species Founding in the Human Alimentary Tract

Genomic DNA was isolated from 14 reference *Lactobacillus* species of BCCM™/ LMG culture collection and Abant İzzet Baysal University culture collections (Figure 3.1). Approximately 1527-1540 bp 16S rRNA PCR amplicons were obtained

by using universal primers 8UA forward and 1492 R reverse primers (Figure 3.2). 16S rRNA gene fragments were purified by electrophoresis from 0.7% agarose gel (Figure 3.3).

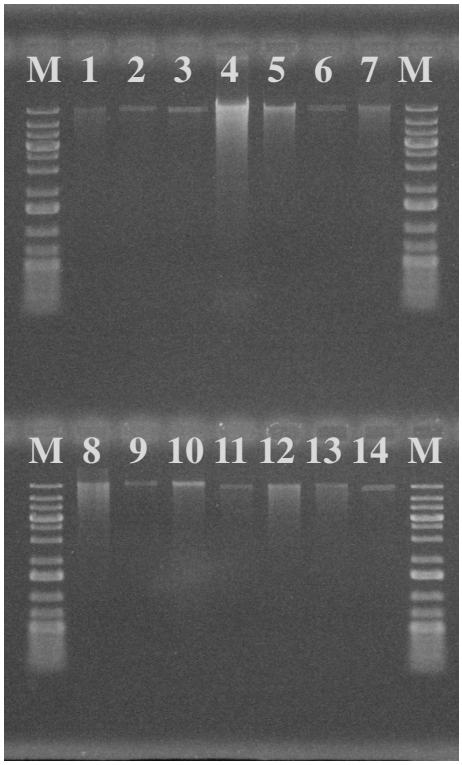

Figure 3.1 Genomic DNA of reference *Lactobacillus* species. Lanes; 1, *Lb. acidophilus;* 2, *Lb. crispatus;* 3, *Lb. plantarum;* 4, *Lb. salivarus;* 5, *Lb. brevis;* 6, *Lb. delbrueckii*; 7, *Lb. ruminis;* 8, *Lb. sake;* 9, *Lb. casei;* 10, *Lb. paracasei;* 11, *Lb. rhamnosus;* 12, *Lb. reuteri;* 13, *Lb. gasseri;* 14, *Lb. johnsonii.* M, molecular size marker (1 kb DNA ladder, Fermentas).

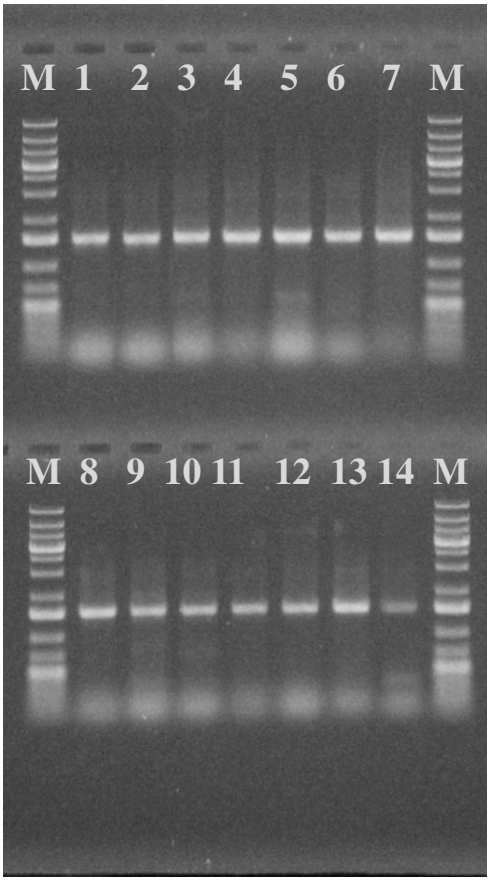

Figure 3.2 PCR amplified 16S rDNA of reference *Lactobacillus* species. Lanes; 1, *Lb. acidophilus;* 2, *Lb. crispatus;* 3, *Lb. plantarum;* 4, *Lb. salivarus;* 5, *Lb. brevis;* 6, *Lb. delbrueckii*; 7, *Lb. ruminis;* 8, *Lb. sakei;* 9, *Lb. casei;* 10, *Lb. paracasei;* 11, *Lb. rhamnosus;* 12, *Lb. reuteri;* 13, *Lb. gasseri;* 14, *Lb. johnsonii.* M, molecular size marker (1 kb DNA ladder, Fermentas).

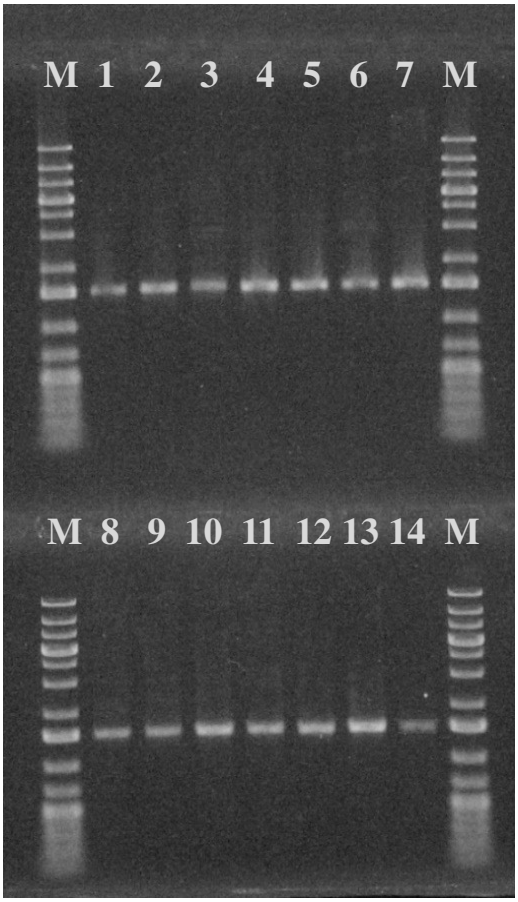

Figure 3.3 Purified 16S rDNA of reference *Lactobacillus* species. Lanes; 1, *Lb. acidophilus;* 2, *Lb. crispatus;* 3, *Lb. plantarum;* 4, *Lb. salivarus;* 5, *Lb. brevis;* 6, *Lb. delbrueckii*; 7, *Lb. ruminis;* 8, *Lb. sake;* 9, *Lb. casei;* 10, *Lb. paracasei;* 11, *Lb. rhamnosus;* 12, *Lb. reuteri;* 13, *Lb. gasseri;* 14, *Lb. johnsonii.* M, molecular size marker (1 kb DNA ladder, Fermentas).

16S rRNA nucleotide sequence were digested with *FspB*I*, Hinf*I and *Dra*I restriction endonucleases. The *FspB*I restriction endonuclease was found to give the clearest and most reliable distinction in experimental 16S ARDRA patterns, in order to differentiate the majority of reference *Lactobacillus* species; *Lb. plantarum* (AIBU-1[b]), *Lb. salivarus* (ATCC 11741), *Lb. brevis* (ATCC 14869), *Lb. delbrueckii* (ATCC 9649), *Lb. ruminis* (ATCC 27780) *Lb. reuteri* (B 14171[ba]) and *Lb. sakei* (ATCC 15521) (Figure 3.4 lane 3, 4, 5, 6, 7, 8 and 12). It was noticed that the *FspB*I restriction endonuclease pattern of the *Lb. acidophilus* (ATCC11975) looked quite similar to *Lb. crispatus* (ATCC 33820) and *Lb. rhamnosus* (AIBU-2[b]) were the same (Figure 3.4 lane 9, 10, 11). On the other hand the patterns of *Lb. gasseri* (ATCC 33323) and *Lb. johnsonii* (B 2178) were the same (Figure 3.4 lane 13, 14). Four of

the remaining unidentified reference *Lactobacillus* species; *Lb. acidophilus* (ATCC11975), *Lb. crispatus* (ATCC 33820), *Lb. rhamnosus* (AIBU-2[b]) and *Lb. reuteri* (B14171[ba]) were discriminated by *Hinf*I restriction endonuclease (Figure 3.5 lane 1,2,5 and 6). *Lb. gasseri* (ATCC 33323) and *Lb. johnsonii* (B 2178[a]) were discriminated with a *Dra*I restriction endonuclease. (Figure 3.6). *Lb. casei* (ATCC 393) and *Lb. paracasei* (ATCC25302) species could not be discriminated with any of these restriction enzymes as expected. We obtained clearly distinguishable experimental 16S ARDRA patterns, confirming the silico restriction profile.

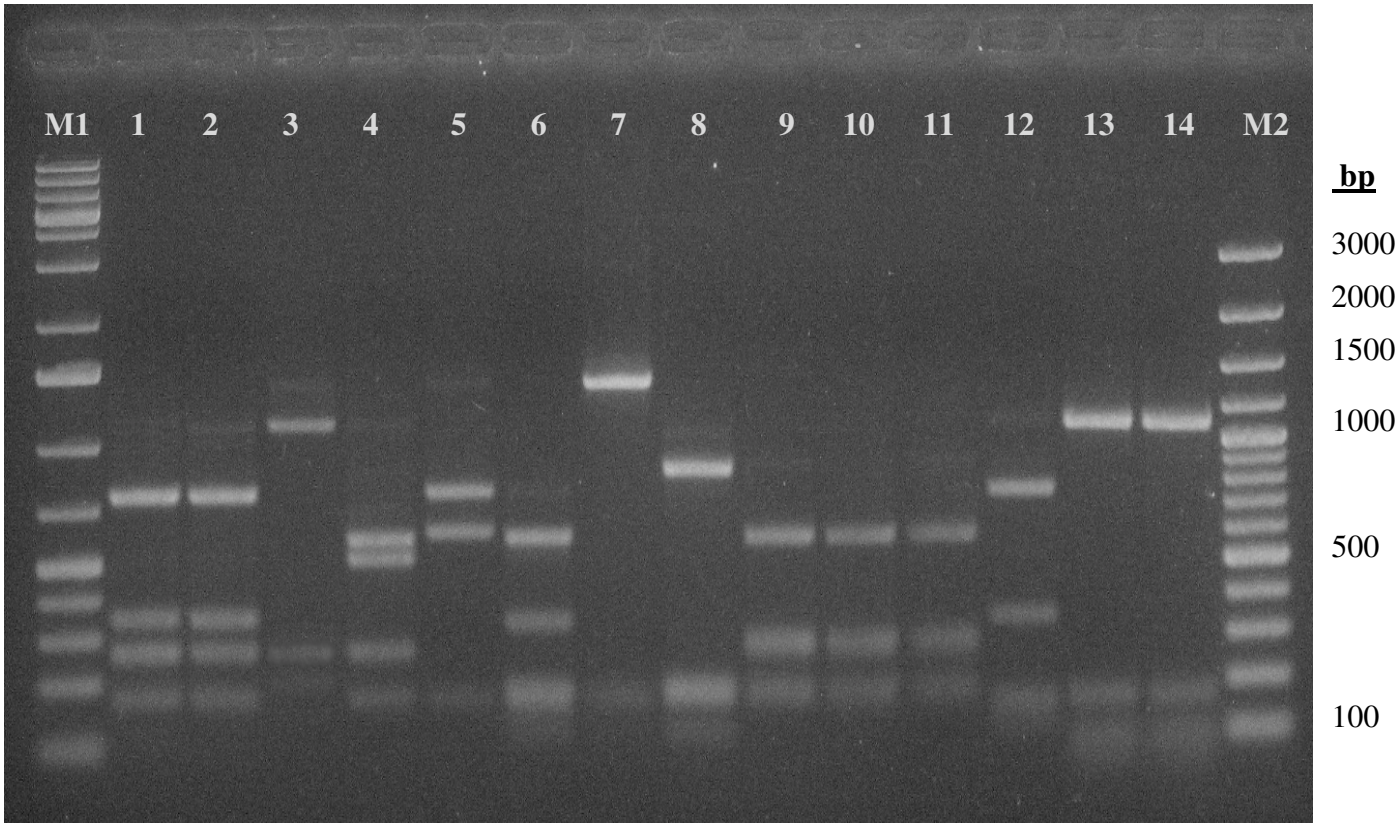

Figure 3.4 Digestion of 16S rRNA fragment of reference species with *FspB*I. Lanes; 1, *Lb. acidophilus;* 2, *Lb. crispatus;* 3, *Lb. plantarum;* 4, *Lb. salivarus;* 5, *Lb. brevis;* 6, *Lb. delbrueckii*; 7, *Lb. ruminis;* 8, *Lb. sakei;* 9, *Lb. casei;* 10, *Lb. paracasei;* 11, *Lb. rhamnosus;* 12, *Lb. reuteri;* 13, *Lb. gasseri;* 14, *Lb. johnsonii.* M1, 1 kb plus DNA ladder (Fermentas). M2, 100 bp plus DNA ladder (Fermentas).

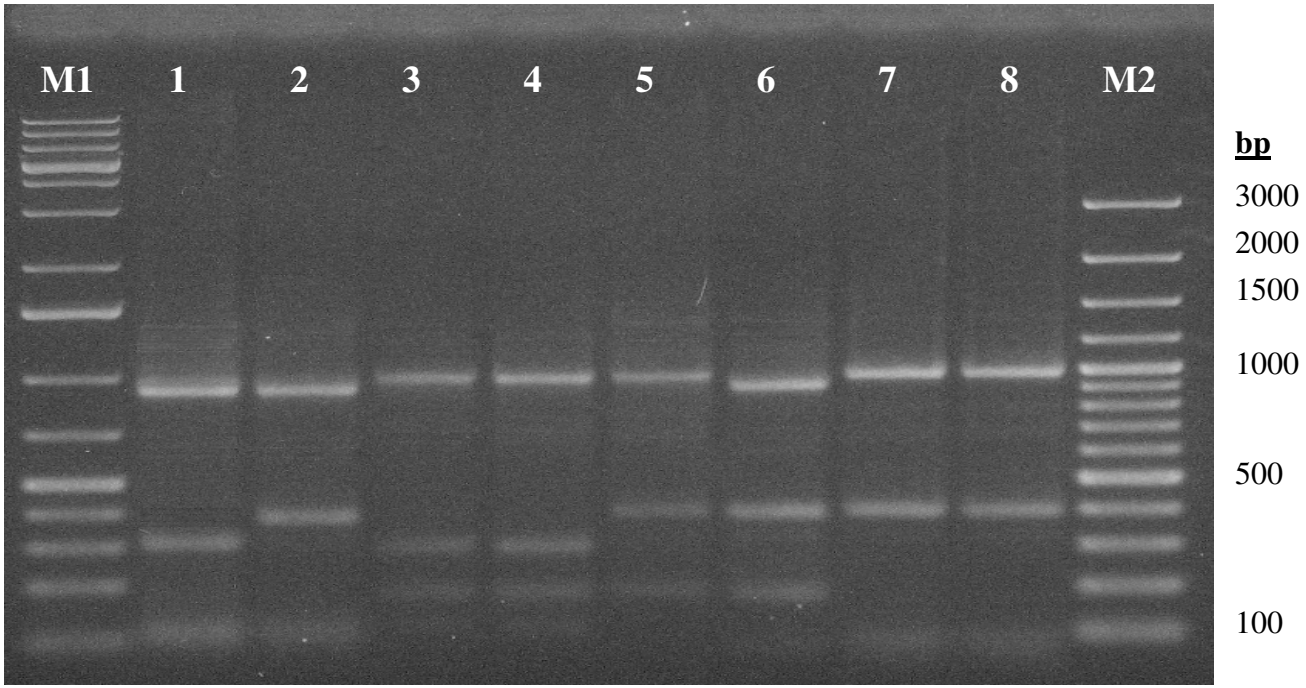

Figure 3.5 Digestion of 16S rRNA fragment of reference species with *Hinf*I. Lanes; 1, *Lb. acidophilus;* 2, *Lb. crispatus;* 3, *Lb. casei;* 4, *Lb. paracasei;* 5, *Lb. rhamnosus;* 6, *Lb. reuteri;* 7, *Lb. gasseri;* 8, *Lb. johnsonii;* M1, 1 kb plus DNA ladder (Fermentas). M2, 100 bp plus DNA ladder (Fermentas)

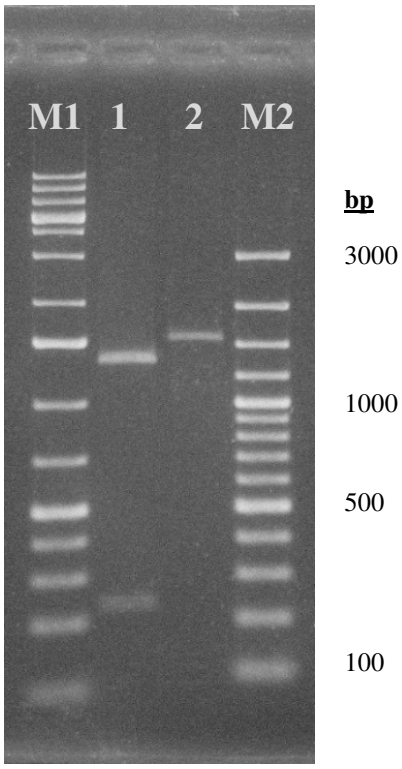

Figure 3.6 Digestion of 16S rRNA fragment of reference species with *Dra*I. Lanes; 1, *Lb. gasseri*; 2, *Lb. johnsonii;* M1, 1 kb plus DNA ladder (Fermentas). M2, 100 bp plus DNA ladder (Fermentas).

3.4. Comparion of 16S Amplified Ribosomal DNA Restriction Analysis (ARDRA) Patterns of Fourteen Reference *Lactobacillus* species

The silico patterns obtained from the almost complete 16S rRNA analysis of 14 *Lactobacillus* species were compared with experimental patterns of 14 reference species. Dendrograms of the 14 reference *Lactobacillus* species based on *FspB*I*, Hinf*I and *Dra*I restiriction fragment length polymorphism of 16S rRNA genes was constructed by UVP cluster analysis program (version 6.8.2, UVP, LLC, Upland, CA, USA) on the bases of complete linkage, Jaccard similarity and RF values. Figure 3.4, 3.5, and 3.6 showed the differences between 14 reference *Lactobacillus* species. The ARDRA pattern of *FspB*I dendrograme (Figure 3.7) showed seven clusters. The

differences between *Lactobacillus* species was evaluated based on 16S ARDRA profiles. Figure 3.9 showed that the reference *Lactobacillus* species were grouped into ten clusters at the difference level of over 3%. Seven clusters were well-defined and corresponded to seven separeted species of *Lactobacillus*; *Lb. reuteri, Lb. salivarus, Lb. brevis, Lb. ruminis, Lb. plantarum, Lb. sakei, Lb. delbrueckii* (Figure 3.4 lane 3,4,5,6,7,8, and 12). The ARDRA pattern of *FspB*I dendrograme showed that *FspB*I restriction enzyme could differentiate seven *Lactobacillus* species; *Lb. johnsonii*, *Lb. gasseri*; *Lb. crispatus*, *Lb. acidophilus*, *Lb. casei*, *Lb. rhamnosus* and *Lb. paracasei* respectively. The ARDRA pattern of *Hinf*I dendrograme showed six clusters (Figure 3.5). Four of these clusters were well-defined and corresponded to four separated species of *Lactobacillus*; *Lb. crispatus*, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. reuteri*. Cluster 3 and 4, contain identical *Lactobacillus* species *Lb. casei* and *Lb. paracasei*; *Lb. gasseri* and *Lb. johnsonii* respectively. The 16S ARDRA pattern of *Hinf*I dendrograme showed that *Hinf*I restriction enzyme could differentiate four *Lactobacillus* species. The ARDRA pattern of *Dra*I dendrograme showed two clusters indicating the diffentiation of *Lb. gasseri* and *Lb. johnsonii* species. Our experimental 16S ARDRA patterns of 14 reference *Lactobacillus* species are identical with those of silico profiles as expected.

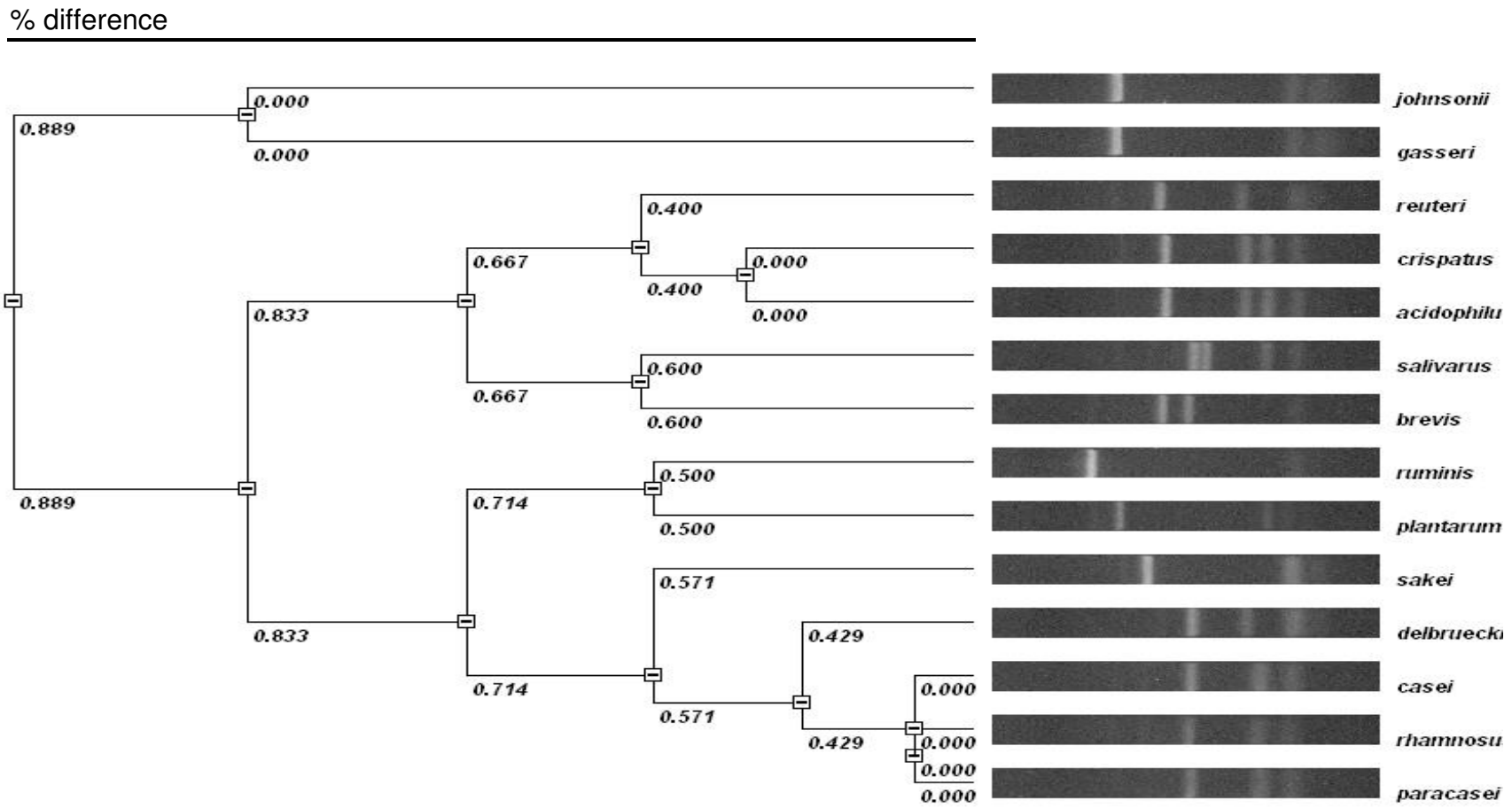

Figure 3.7 Dendrogram of *FspB*I digestion. Dendrogram of 14 *Lactobacillus* species based on ARDRA patterns of 16S rRNA gene isolated from commercial *Lactobacillus* species.

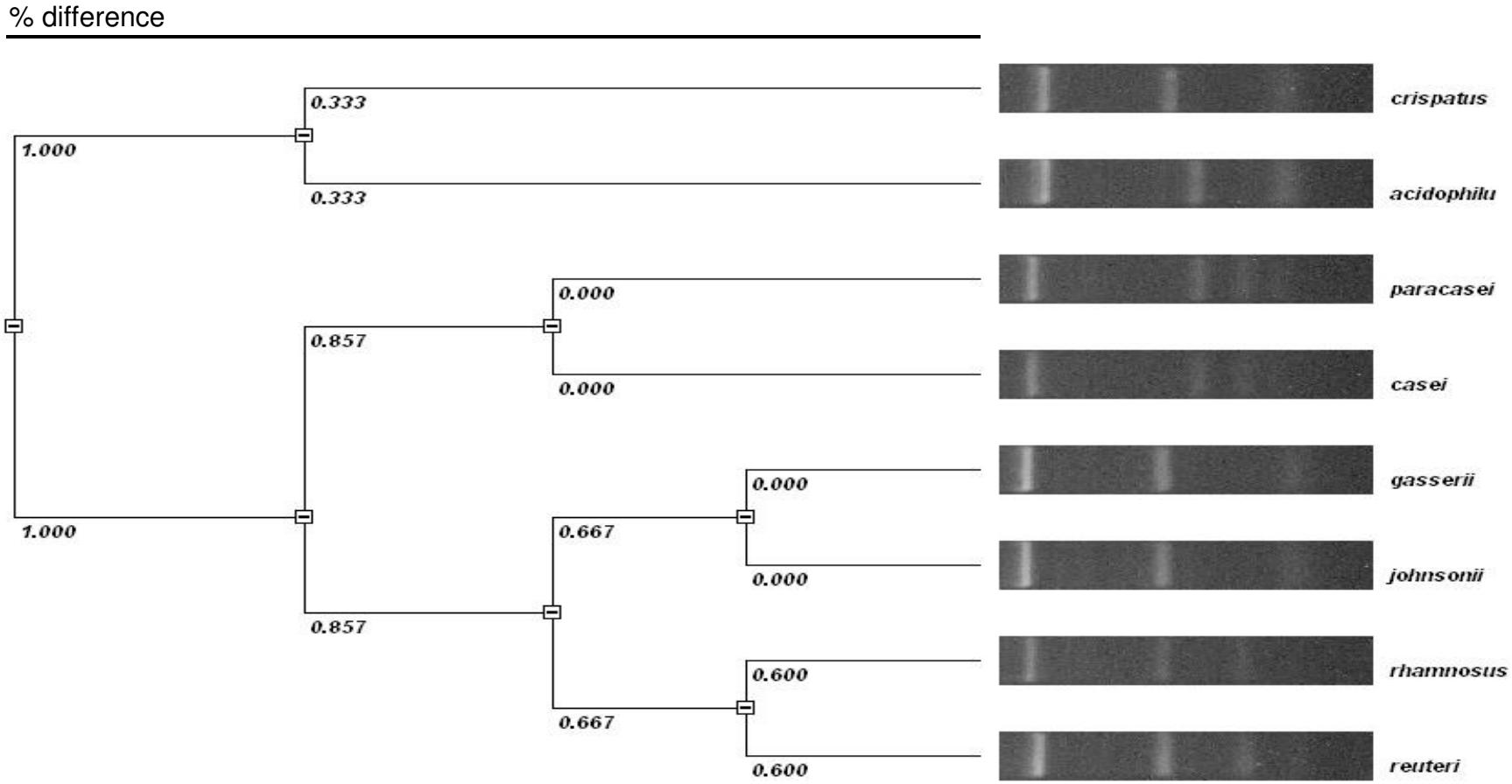

Figure 3.8 Dendrogram of *Hinf*I digestion**.** Dendrogram of 8 *Lactobacillus* species based on ARDRA patterns of 16S rRNA gene isolated from commercial *Lactobacillus* species.

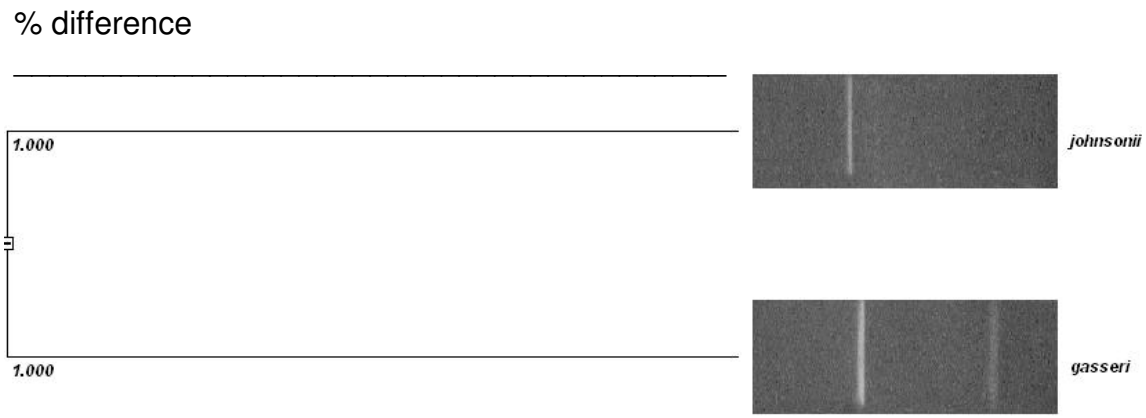

Figure 3.9 Dendrogram of *Dra*I digestion. Dendrogram of 2 *Lactobacillus* species based on ARDRA patterns of 16S rRNA gene isolated from commercial *Lactobacillus* species.

CHAPTER IV

4. DISCUSSION

Generally *Lactobacillus* used as probiotics and defined as viable microorganisms exhibit a beneficial effect on the health of the host. Present knowledge indicates that at least fourteen species associated with human gut (Table 3.2). Due to the similarity in phenotype and physiology of *Lactobacillus*, its taxonomy is still confusing, giving rise to incorrect identification in most instances (Dalezios and Siebert 2001; Hammes and Hertel, 2006). Based on the present taxonomy, *Lb. acidophilus* isolates are mostly divided into *Lb. gasseri* and *Lb. crispatus*, owing to the close similarity thereof.

Contrary to the phenotypic methods, molecular identification and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of phenotype. In fact, many *Lactobacillus* species have been reclassified on the basis of current information from advanced molecular techniques and their correct taxonomic status has been determined (Singh et al. 2009).

16S ARDRA method requires only universal primers for 16S rRNA genes that are widely used in studies of bacterial taxonomy. 16S PCR-ARDRA method is one of the adequate identification methods for determining *Lactobacillus* species. It could be of help to evaluate the differences between *Lactobacillus* in the human gastrointestinal tract. Compared with other methods, ARDRA is quick, cheap, less

laborious, discriminatory and gives reliable results in the identification of strains at the species level. In contemporary bacterial taxonomy, it is thought that the ribosomal gene sequences have the potential for the *Lactobacillus* species to be identified. Thus, complete sequencing of the 16S rRNA gene is the quickest means for identifying lactobacilli at present; however, in case of analyzing closely related species, the 16S rRNA sequencing based identification may not could be deceiving (Singh et al. 2009).

Heterogeneity among *Lactobacillus* species was detected by Hammes and Hartel (2003), who argued that seven phylogenetic groups could be obtained, the mentioned groups including *Lb. buchnerii*, *Lb. delbrueckii*, *Lb. casei*, *Lb. plantarum*, *Lb. reuteri*, *Lb. sakei* and *Lb. salivarus*. In a comprehensive study by Lerche and Reuter on the lactobacilli associated with human alimentary tract, however, homofermentative lactobacilli which are typical of the human host are classified into four groups (Holzapfel, 2001). The first group confirmed by the presence of six different species; i.e. *Lb. acidophilus*, *Lb. crispatus*, *Lb. gasseri* and *Lb. johnsonii*, is *Lb. acidophilus* (Cato et al., 1983). Due to the difficulty in distinguishing these species (Song et al., 1999), there exist some findings relating to the misidentification of a number of strains included in this group (Song et al. 2000; Yeung et al. 2002). Even with molecular techniques, it is sometimes hard to distinguish between *Lb. gasseri* and *Lb. johnsonii* (Walter et al. 2000). The second group is made up of *Lb. salivarus*, *Lb. ruminis* and *Lb. brevis*, the grouped being named *salivarus* group. The third one, on the other hand, is *Lb. delbrueckii* and the fourth group is *Lb. casei,* wherein *Lb. paracasei, Lb. casei* and *Lb. rhamnosus* are included. Our *Fsp*BI digested profiles (Figure 3.4) and dendrograms (Figure 3.7) indicated that although *Lb. plantarum*, *Lb. salivarius*, *Lb. brevis*, *Lb. delbrueckii*, *Lb. ruminis* and *Lb. sakei*

species were identified easily with *FspB*I restriction enzyme, *Lb. crispatus*, *Lb. acidophilus*, *Lb. rhamnosus, Lb. reuteri*, *Lb. casei*, *Lb. paracasei Lb. johnsonii* and *Lb. gasseri* remained unidentified because of their close similarity (Figure 3.4). The silico and experimental *Fsp*BI pattern of the *Lb. acidophilus* looked quite similar to those of *Lb. crispatus*. The pattern of *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* are the same (Figure 3.4 lanes 9, 10 and 11; and Table 3.2). On the other hand the patterns of *Lb. gasseri* and *Lb. johnsonii* are identical (Figure 3.4, lanes 13 and 14; and Table 3.2). In this work, remaining unidentified closely related species, *Lb. acidophilus, Lb. crispatus, Lb. rhamnosus* and *Lb. reuteri*, *Lb. gasseri* and *Lb. johnsonii* were easily distinguished by *Hinf*I and *Dra*I restriction enzymes. Therefore our *FspB*I digestion profiles and dendrograms of 16S rRNAs from 14 reference *Lactobacillus* species (Figure 3.7 and 3.9) almost confirm and support the Hammes and Hartels' (2003) classification.

Fourteen reference *Lactobacillus* species yielded apparently discernable 16S ARDRA patterns. Nearly all of the *Lactobacillus* species, found in human alimentary tract, gave rise to identical *FspB*I, *Hinf*I and *Dra*I patterns of their 16S ARDRA restriction profiles (Figure 3.4; Figure 3.5; Figure 3.6), in comparison with silico patterns (Table 3.2). In the present study, in differentiation of 14 reference *Lactobacillus* species, with the exception of *Lb. casei* and *Lb. paracasei*, 16S ARDRA analysis was performed with success. Having failed to differentiate between *Lb. casei* and *Lb. paracasei* species, some researchers (Chavagnat et al., 2002; Vasquez et al., 2001; Felis et al., 2001) recategorized some atypical *Lb. casei* species into *Lb. paracasei* by means of various molecular techniques. The results suggested that *Fsp*BI, *Hinf*I and *Dra*I are complementary, thus these enzymes could be used when one of them alone did not distinguish the strains.

It was proven using *FspB*I, *Hinf*I and *Dra*I enzymes that all *Lactobacillus* species, except for *Lb. casei* and *Lb. paracasei*, can be distinguished in human alimentary tract based on differences between the 16S ARDRA profiles with the known reference species. The results of this investigation have shown that 16S PCR-ARDRA analysis using *Fsp*BI, *Hinf*I and *Dra*I restriction enzymes are a reliable, rapid, and accurate method for the identification of human intestinal tract isolates of *Lactobacillus* species for probiotic use, especially when large numbers of isolates need to be identified and any laboratory equipped with a PCR machine. In addition, the 16S-ARDRA profiles suggest that further studies should be carried out to distinguish closely related (99, 4% similarity) *Lb. casei* and *Lb. paracasei* species.

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APPENDIX

A. Bacterial Growth Media

- **MRS broth**

  52,2 g MRS suspend (Merck) were dissolved in deionized water and sterilized at 121°C for 20 minutes.

- **MRS agar**

  For 1 L liquid MRS, 15 g agar (Merck) was added and sterilized at 121°C for 20 minutes.

- **MRS broth with L(+)-cysteine**

  26,6 g MRS suspend were dissolved in 500 ml deionized water and added 0,25 g L(+)-cysteine then sterilized at 121°C for 20 minutes.

- **MRS agar with L(+)-cysteine**

  For 500 ml liquid MRS with L(+)-cysteine, 7,5 g agar (Merck) was added sterilized at 121°C for 20 minutes.

B. Buffers and Solutions for Molecular Characterization

- **1M Tris-HCl pH 8.0**

  121.1 g of Tris base was dissolved in 800 ml of deionized H_2O. pH was adjusted to the 8.0 value by adding concentrated HCl.

- **0.5M EDTA pH 8.0**

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

- **50X TAE**

  242 g of Tris base was dissolved in deionized H_2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Final volume of solution was adjusted to 1 L with deionized water.

- **1X TAE**

  20 ml 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.

- **Ethidium Bromide Stock Solution (10mg/ml)**

  0.5 g ethidium bromide was dissolved in 50 ml deionized water and the solution was storred in dark bottle at room temperature or 4°C.

- **Lysis Buffer**

Ingredients	**µl**
1M Tris-HCl pH 8.0	40µl
0.5 M EDTA pH 8.0	80µl
Lysozyme 30 mg/ml	1333µl
Triton X-100	24µl
ddH_2O	523µl

C. Chemicals

Agar (Merck, 101614)

Agarose (Sigma, 9012-36-6)

EDTA [Ethylenediaminetetraacetic acid] (Sigma, 60-00-4)

EtBr [Ethidium Bromide] (Sigma, E-8751)

Glycerol, cell culture tested (Sigma, 6-2025)

Magnesium chloride [$MgCl_2$] (Merck, 442615)

MRS broth (Merck, 1.10661)

Sodium chloride [NaCl] (Merck, 106404)

D. Enzymes

1. Restriction Endonucleases

*FspB*I (*Bfa*I)	(Fermentas)	:cat.#ER1761
*Hinf*I	(Fermentas)	:cat.#ER0801
*Dra*I	(Fermentas)	:cat.#0221

2. Polymerase

Pfu DNA polymerase	(Fermentas)	:cat.#EP0501

3. dNTP set

dNTP	(Fermentas)	:cat#R1121

E. Equipments used in this study

PCR (Techne, TC 3000)

34°C and 37°C Incubators (Nuve EN 500, Nuve FN 500)

Electrophoresis system (Thermo Scientific)

Power supply (Thermo EC 250-90)

Dry block (VWR Digital Dry-Block)

Autoclave (Hirayana)

pH meter (HANNA HI 221)

Micropipettes (Finnipipette)

Desktop centrifuge (Hettich Micro 120)

+4°C refrigerators (Arçelik)

-20°C deepfreeze (Arçelik)

-80°C deepfreeze (Thermo scientific)

-80°C deepfreeze (Biolaps)

UV Transalluminator (UVP)

Imaging system (UVP Photo Doc-It ™)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

F. Programs used in this study

Clustal W Program

Web cutter Program

Bio Edit (version 5.0.9) Program

UVP Cluster Analysis (version 6.8.2) Program