UNIVERSITÀ DEGLI STUDI DI CATANIA
Dipartimento di Scienze Biomediche e Biotecnologiche
Microbiology Section
DOTTORATO DI RICERCA INTERNAZIONALE IN
SCIENZE MICROBIOLOGICHE E BIOCHIMICHE
Ciclo XXVIII

Dott. ssa Virginia Fuochi

Amensalistic activity of *Lactobacillus* spp., isolated from human samples

PhD THESIS

Co-ordinator:
Prof.ssa Adriana Garozzo

Tutor:
Prof. Pio Maria Furneri

A C A D E M I C Y E A R S 2 0 1 3 / 2 0 1 5

Lasciatevi circondare solo da coloro
che pur non avendo vissuto le vostre esperienze,
riescono a percepire e comprendere appieno
le vostre gioie e le vostre paure.
Perché non è questione di età, sesso, forza
e nemmeno intelligenza,
è questione di empatia.
Siate empatici, sarete unici e meravigliosi.

Virginia Fuochi

ABSTRACT

Introduction

Lactobacillus are a bacterial genus belonging to LAB (Lactic Acid Bacteria) and they are among the most common probiotics. Recent guidelines on probiotics, issued by the Italian Ministry of Health, state that, on the basis of the available literature, the amount sufficient to obtain a temporary colonization of the intestine by a probiotic strain is at least 10^9 living cells.

A microorganism can be defined as a probiotic strain if it is of human origin, if survive to the gastrointestinal tract, resisting the acidity of the stomach and the action of bile, and it should have immunostimulant activity. In addition, the strain should be able to adhere to the mucosa causing no toxicity, and to produce substances with antibacterial activity against some pathogens.

Aims of the study and results

The aim of the work was the isolation and identification of lactobacilli of human origin. It was also deepened the study of their amensalistic properties, with particular attention to the resistance to gastrointestinal transit and their antagonism against pathogenic microorganisms.

Three hundred fifty-nine lactobacilli strains were isolated from swabs of healthy people and identified using molecular techniques based on the study of 16S rDNA. The identification of some strains was confirmed by further analysis DHPLC V1 and V3 of 16S rDNA. The strains were subjected to the evaluation of the resistance to bile salts and low pH, to the production of hydrogen peroxide and more particularly, it has been evaluated the ability to produce substances with antibacterial activity.

Finally, the attention was focused on the characterization of an active supernatant produced by an oral strain. The isolation of the substance provided chromatographic procedures such as SEC (Size Exclusion Chromatography using Sephadex 50) and SPE (Reverse Phase Chromatography using C18 column). The results were shown that the active fraction has a low molecular weight and for its chemical-physical characteristics is not a common bacteriocin, for this reason are on going further chromatographic studies using columns with increasing polarity (C4, phenyl, cyano, and amino).

Future outlooks

Future outlooks are focused on the identification of the molecule in question, by MALDI-TOF and ESI-TOF and then optimizing the whole process to standardize the entire method. In this way, the opportunity to bring to light new molecules will be possible, with the ultimate goal of being able to take advantage from these antibacterial substances.

ABSTRACT

Introduzione

I lattobacilli appartengono ai LAB (Lactic Acid Bacteria) e sono tra i più comuni microrganismi probiotici. Le recenti linee guida rilasciate dal Ministero della Salute, affermano che, sulla base della letteratura disponibile, la quantità sufficiente per ottenere una colonizzazione temporanea da parte di un ceppo probiotico è di almeno 10^9 cellule viventi. Un microrganismo per essere definito tale, deve essere di origine umana, avere attività immunostimolante e sopravvivere al tratto gastrointestinale, resistendo all'acidità dello stomaco e all'azione della bile. Inoltre, il ceppo deve essere in grado di aderire alla mucosa non causando tossicità, e di produrre sostanze ad attività antibatterica contro alcuni patogeni.

Obiettivi della ricerca e risultati

Scopo del lavoro è stato l'isolamento e l'identificazione di lattobacilli di origine umana. Inoltre, è stato approfondito lo studio delle proprietà amensalistiche, con particolare attenzione alla resistenza al transito gastro-intestinale e all'antagonismo nei confronti dei microorganismi patogeni.

Trecentocinquantanove ceppi di lattobacilli sono stati isolati da tamponi ottenuti da persone sane e identificati tramite tecniche molecolari basate sulla 16S rDNA. Per alcuni ceppi selezionati l'identificazione è stata ulteriormente confermata tramite la tecnica DHPLC applicata agli amplificati delle regioni V1 e V3 della 16S rDNA. In tutti ceppi è stata la valutata la resistenza ai sali biliari e al pH acido, la produzione di perossido di idrogeno, e infine è stata valutata la capacità di produrre sostanze ad attività antibatterica.

Particolare attenzione è stata posta alla caratterizzazione delle componenti "antibiotiche" di un surnatante prodotto da un ceppo di origine orale. L'isolamento della "sostanza inibitrice" è stato condotto mediante tecniche cromatografiche quali SEC (Size Exclusion Chromatography mediante Sephadex 50) e SPE (Reverse Phase Chromatography mediante colonna C18). I risultati hanno dimostrato che la sostanza contenuta nella "frazione attiva" possiede un peso molecolare basso e per le sue caratteristiche chimico-fisiche non è, molto probabilmente, una batteriocina, per questo

motivo sono in avanzamento ulteriori studi cromatografici con colonne a polarità crescente (C4, fenile, ciano e ammino).

Prospettive future

I progetti futuri sono focalizzati sull'identificazione della molecola in questione, tramite analisi MALDI-TOF ed ESI-TOF, e successivamente sull'ottimizzazione del processo di produzione e purificazione. In tal modo si avrà l'opportunità di portare alla luce nuove molecole con l'obiettivo finale di poter sfruttare l'attività antibatterica di tali sostanze.

Contents

1 The genus Lactobacillus 1

***1.1* Nutritional Characteristics and Metabolism of the genus *Lactobacillus* 2**

1.1.1 Obligately Homofermentative Lactobacilli 3

1.1.2 Facoltatively Heterofermentative Lactobacilli. 3

1.1.3 Obligately Heterofermentative Lactobacilli. 4

1.2 Habitat in Human Organism 7

1.2.1 Oral Cavity 7

1.2.2 Gastro-Intestinal (GI) Tract 8

1.2.3 Vagina 10

2 Isolation by Classic Cultural Techniques 12

2.1 Evaluation of Special Characteristics 14

2.1.1 Detection of bile salt hydrolysis 14

2.1.2 Detection of H_2O_2 production 14

2.1.3 Detection of lactic acid production 15

3 Taxonomy and the Importance of Phylogenetic Classification 16

4 Identification by Culture-Independent Techniques 26

4.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP) 26

4.2 Amplified Restriction Length Polymorphism (AFLP) 27

4.3 Amplified Ribosomal Restriction Fragment Analysis (ARDRA) 27

4.4 Randomly Amplified Polymorphic DNA (RAPD) 28

4.5 Denaturing Gradient Gel Electrophoresis (DGGE) 28

4.6 Loop Mediated Isothermal Amplification (LAMP) 29

4.7 Denaturing High Pressure Liquid Chromatography (DHPLC): a new culture-independent technique 29

5 16S rRNA as a Sequencing Gene, the Reason Why 33

6 Probiotic Properties 37

7 Bacteriocins 42

7.1 Classification 44

7.2 Biosynthesis and Mode of Action 46

7.3 Bacteriocins Applications 51

8 Introducing Proteomics 53

8.1 Size-Exclusion Chromatography (SEC) 54

8.2 Solid Phase Extraction (SPE) 56

8.3 Mass Spectrometry (MS) 59

9 Materials and Methods 60

9.1 Bacterial strains and culture conditions 60

9.2 Isolation from oral and fecal specimens 60

9.3 Isolation from vaginal specimens 62

9.4 Susceptibility testing 64

9.5 Evaluation of resistance to low pH and bile salts 65

9.6 Evaluation of the capacity of *Lactobacillus* strains to produce hydrogen peroxide 68

9.7 DNA Extraction 69

9.8 DNA Quantitation Using Spectrophotometer 70

9.9 16S rDNA/RFLP Amplifications 70

9.10 **Two-steps multiplex PCRs: *16S-ITS-23S* and the flanking region of *23S rDNA* 74**

9.11 *tuf* gene amplification 77

9.12 Nested PCR-amplifications of bacterial V1-V3: DHPLC (WAVE® System) analysis. 79

9.12.1 DHPLC collection protocol 81

9.12.2 Mutation analysis 82

9.13 Detection of inhibitory activity by agar-well diffusion assay 83

9.13.1 Sensitivity to different pH values 85

9.14 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain 86

9.14.1 Size Exclusion Chromatography 86

9.14.2 Reverse Phase Chromatography 87

9.14.3 1D SDS-PAGE Analysis 87

9.14.4 Evaluation of cytotoxicity by the producer strain on the BV2 cell line 88

10 Results 89

10.1 Molecular identification of *Lactobacillus* spp. 89

10.2 Discrimination of 14 type strains of *Lactobacillus* spp. by Denaturing High Performance Liquid Chromatography (DHPLC): pilot study 93

10.2.1 V1-V3 Analysis 95

10.2.2 V1 Species Discrimination 101

10.2.3 DHPLC analysis application to *Lactobacillus* strains 104

10.3 Susceptibility testing 109

10.4 Evaluation of resistance to low pH and bile salts 112

10.5 Evaluation of the capacity of *Lactobacillus* strains to produce hydrogen peroxide....115

10.6 Detection of inhibitory activity by agar-well diffusion assay....117

10.6.1 Sensitivity to different pH values....125

10.7 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain....126

10.7.1 SEC (Size-Exclusion Chromatography)....126

10.7.2 Reverse Phase Chromatography....126

10.7.3 1D SDS-PAGE Analysis....128

10.7.4 Evaluation of cytotoxicity by the producer strain on BV2 cell line....128

11 Discussion & Future outlooks....132

11.1 Identification....132

11.2 Susceptibility test....133

11.3 Amensalistic activity....134

11.4 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain....136

12 Funding....138

13 Acknowledgements....138

14 References....139

1 The genus Lactobacillus

The genus *Lactobacillus* comprises a homogenous group of organisms with morphological, biochemical and physiological characteristics that are very similar; some species, initially included in the *Lactobacillus* genus, have been separated by creating two new genera, *Weissella* and *Carnobacterium* (Collins et al., 1987; 1993).
With this separation *Lactobacillus* can be called a non-pathogenic bacterial genus. However, sometimes there are exceptions such as *L. psittaci,* which was isolated from an air bag of a dead parrot, but our knowledge about the infectivity or the habitats of this species is very poor (Lawson et al., 2001). Lactobacilli are part of our daily life, first of all, they are members of the human microbiome, and they are also indispensable fermentation agents of food and feed (Dworkin & Falkow, 2006; Vos et al., 1984-1989, 2009).
The cellular morphology of lactobacilli is variable, they resemble rods but they may differ in length among various species. For example, some heterofermentative species are short and stocky (coccobacillus) and they could be confused with *Leuconostoc*, on the other hand, some anaerobic homofermentative species are more similar to bifidobacteria. Sometimes they are curved, as in *L. curvatus*, or they have a spiral shape such as *L. delbrueckii subsp. bulgaricus*. In addition, within each species, the length varies according to the stage of development, the soil composition and the oxygen concentration (Dworkin & Falkow, 2006).

1.1 Nutritional Characteristics and Metabolism of the genus *Lactobacillus*

Lactobacilli are Gram-positive bacteria, non-spore forming, motionless, aero tolerant but whose optimal growth is achieved in microaerobic conditions (CO_2≈5%) or at low oxygen concentrations.

Most lactobacilli are mesophilic and they grow in an optimal range of temperature between 20-40°C; few species grow below 15°C or above 40°C with a maximum limit of 55 °C. Thanks to their metabolism lactobacilli cause a lowering of the pH values and this represents one of the factors with which they inhibit the growth of other bacteria favoring their establishment in many habitats. They are devoid of catalase, in fact, they consume oxygen by the action of flavoprotein (NADH-oxidase), and suppress the accumulation of H_2O_2 through the enzymatic activity of NADH-peroxidase. In addition, they possess nitrate reductase and cytochrome oxidase, and their metabolism is fermentative producing large quantities of lactic acid. However, these microorganisms are demanding and they are adapted to living on complex substrates using not only carbohydrates as a source of energy, but also amino acids, nucleotides, vitamins and ions (Kandler et al. 1986; Elli et al., 2000). The nutritional requirements change from one species to another and sometimes even from strain to strain, for example, biotin and vitamin B12 are required by a few strains, while acids such as pantothenic acid and nicotinic acid are required by all the species. Since the demands of nutrients are different, usually media for laboratory cultures contain carbohydrates, peptone, meat and yeast extracts with the addition of tomato juice, manganese acetate or Tween80, as in the most used medium; MRS (De Man, Rogosa, & Sharpe, 1960), besides, the optimum pH for growth is between 4.5-6.4, although there are exceptions, for example, *Lactobacillus suebicus* that grows even at extremely low pH (pH 2; Kleynmans, Heinzl, & Hamme, 1989).

Metabolically speaking, we can divide the genus *Lactobacillus* into three groups: (A) Obligately homofermentative lactobacilli, (B) Facultatively heterofermentative lactobacilli, and (C) Obligately heterofermentative lactobacilli (Vandamme et al., 1996).

1.1.1 Obligately Homofermentative Lactobacilli.

They degrade hexoses to lactic acid by the Embden-Meyerhof-Parnas pathway, but they cannot metabolize pentoses and gluconate.

The Embden-Meyerhof-Parnas pathway is the most common way for the degradation of glucose to pyruvate and it works in both cases, with or without oxygen. This pathway can be divided into two stages: in the first one, a molecule of six carbon atoms (glucose) is phosphorylated twice, and is converted into fructose 1,6-diphosphate, in this step two molecules of ATP are consumed. In the second phase, the enzyme fructose 1,6-diphosphatealdolase results in the cleavage of fructose 1,6-diphosphate into two halves, one half is dihydroxyacetone phosphate. Since dihydroxyacetone phosphate can be easily modified changing it into glyceraldehyde-3-phosphate, the two halves of fructose 1,6-bisphosphate are both used in this second phase. The two molecules of glyceraldehyde 3-phosphate will become, in a process of five steps, two molecules of pyruvate. The Embden-Meyerhof-Parnas pathway leads from the degradation of one molecule of glucose to the formation of two molecules of pyruvate (Fig. 1-2) (Willey, Sherwood, & Woolverton, 2008). Finally, the two molecules of pyruvate are reduced by lactic fermentation to two molecules of lactate (dissociated form of the lactic acid).

1.1.2 Facoltatively Heterofermentative Lactobacilli.

These lactobacilli convert the hexoses to lactic acid by the Embden-Meyerhof-Parnas pathway. Instead, if glucose is scarce they exploit the Warburg-Dickens-Horecker pathway, also known as the pentose phosphate cycle; it consists in the oxidation of glucose-6-phosphate to 6-phosphogluconate (with the reduction of one molecule of NAD); 6-phosphogluconate is subsequently decarboxylated producing a sugar at five atoms of carbon. Then, this sugar is converted into glyceraldehyde-3-phosphate and acetyl phosphate. Glyceraldehyde is converted to lactic acid with the same reactions of the second part of glycolysis while the acetyl-phosphate is converted into ethanol (Fig. 1-3) (Willey, Sherwood, & Woolverton, 2008).

1.1.3 Obligately Heterofermentative Lactobacilli.

Species belonging to this group only exploit the phosphogluconate pathway thanks to a phosphoketolase (Fig. 1-4) (Willey, Sherwood, & Woolverton, 2008).

The most important species of *Lactobacillus* are reported in Table 1.1, divided by type of metabolism.

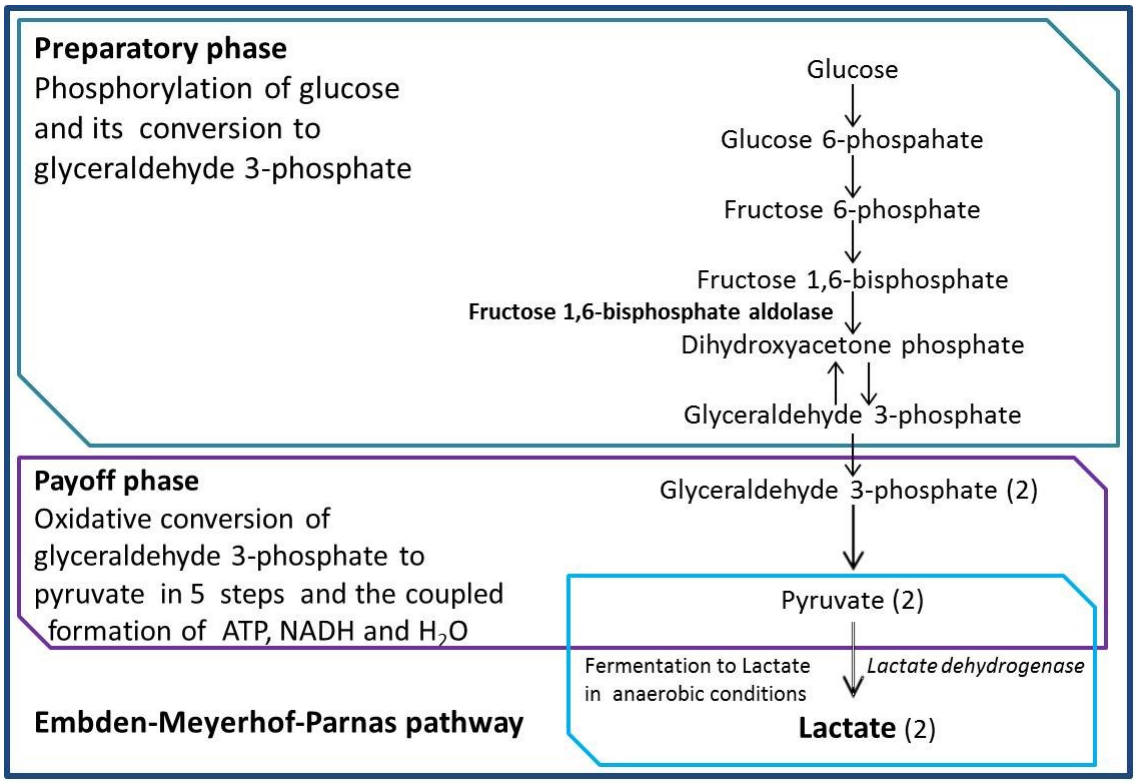

Figure 1-2.The Embden-Meyerhof-Parnas pathway in obligately homofermentative *Lactobacillus* spp. These species have only 1,6-bisphosphatealdolase, thus they cannot metabolize pentose and gluconate (Nelson, Lehninger, & Cox, 2008).

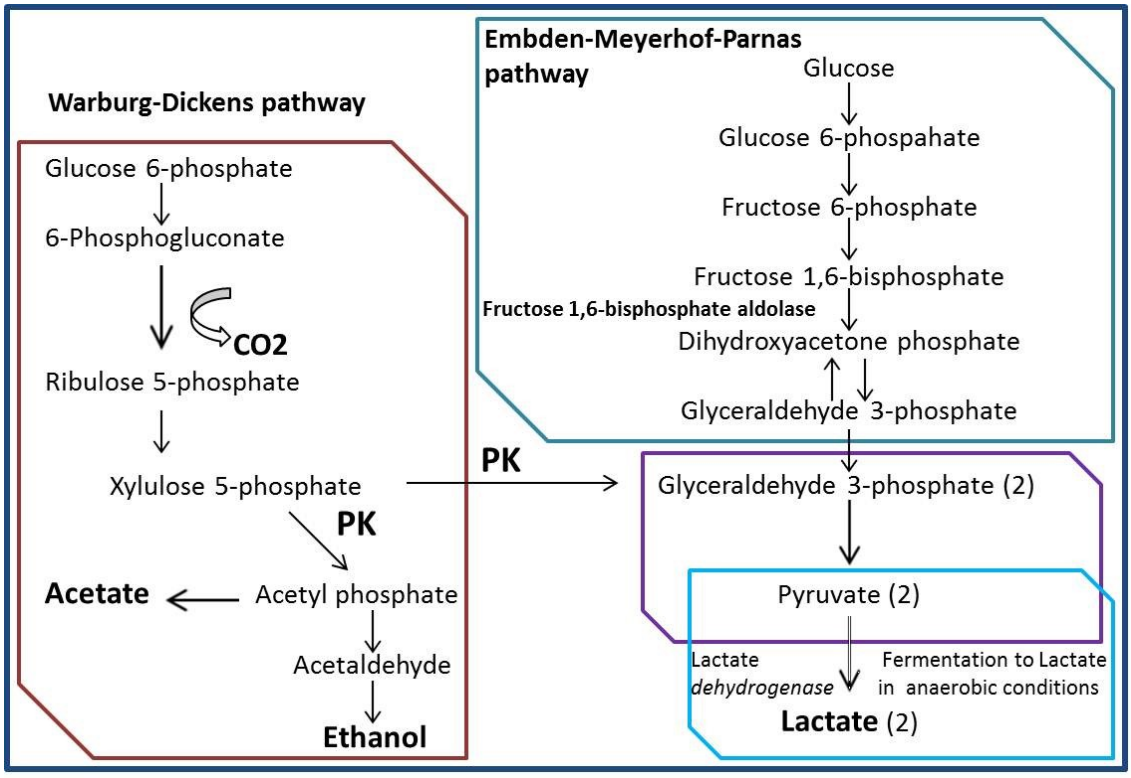

Figure 1-3. Facultatively heterofermentative *Lactobacillus* spp. have a 1,6-bisphosphatealdolase (Embden-Meyerhof-Parnas pathway) and an inducible phosphoketolase with pentoses acting as inducers (Warburg-Dickens pathway) (Nelson, Lehninger, & Cox, 2008).

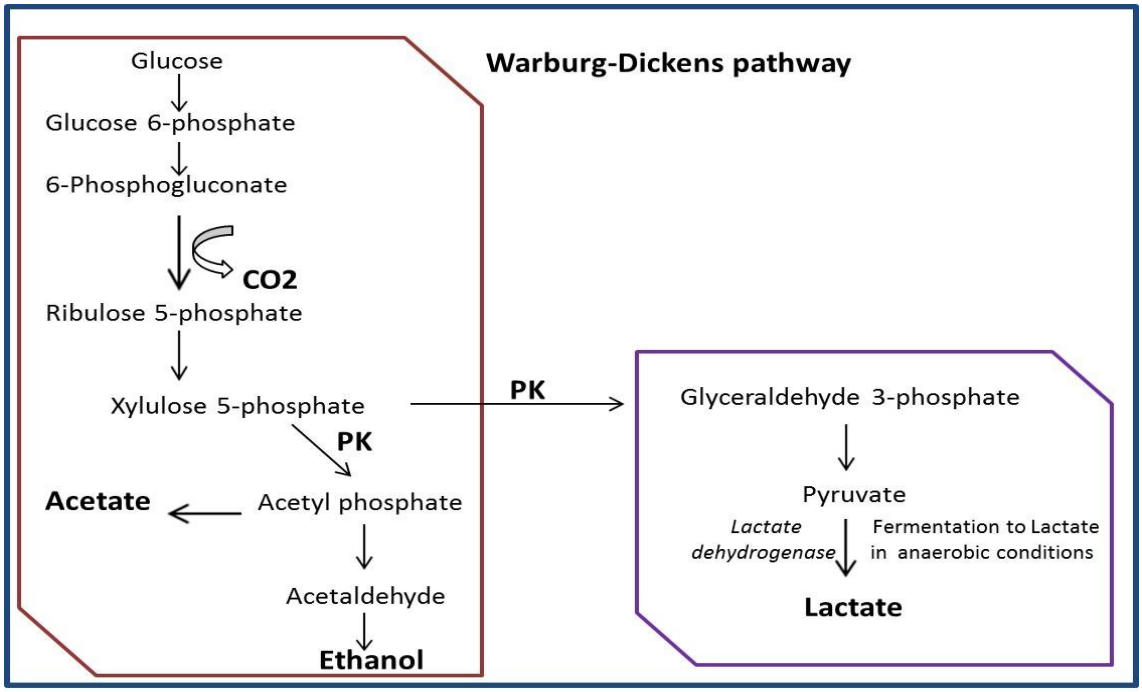

Figure 1-4. Obligately heterofermentative *Lactobacillus* spp. only Warburg-Dickens pathway using a phosphoketolase enzyme (Nelson, Lehninger, & Cox, 2008)

Table 1.1. List of the species of the genus *Lactobacillus* divided by type of metabolism (Vos, et al., 2009).

H/A: isolation from human or animal isolation; F/F*: involved in food fermentation/also in spoilage; P: pathogen

Obligately homofermentative	Facultatively heterofermentative	Obligately heterofermentative
	L. acidipiscis-F	*L. acidifarinae*-F
	L. agilis– H/A	*L. antri*– H/A
	L. alimentarius–F*	*L. brevis*–F*
L. delbrueckii subsp. delbrueckii-F	*L. animalis*– H/A	*L. buchneri*–F*
L. delbrueckii subsp. bulgaricus-F	*L. casei*-F	*L. coleohominis*– H/A
L. delbrueckii subsp. lactis-F	*L. coryniformis subsp. coryniformis*-F	*L. collinoides*–F*
L. delbrueckii subsp. indicus-F	*L. coryniformis subsp. torquens*-F	*L. diolivorans-F*
L. acidophilus– H/A; -F	*L. curvatus*– H/A; -F*	*L. durianis-F*
L. crispatus– H/A; -F	*L. cypricasei*-F	*L. ferintoshensis-F*
L. equi– H/A	*L. fornicalis*– H/A	*L. fermentum*–F*
L. gallinarum– H/A	*L. graminis*-F	*L. fructivorans*–F*
L. gasseri– H/A	*L. hamsteri*– H/A	*L. frumenti*-F
L. helveticus– F	*L. intestinalis*– H/A	*L. gastricus*– H/A
L. iners– H/A	*L. jensenii*– H/A	*L. hammesii*-F
L. johnsonii– H/A; -F	*L. kimchii*-F	*L. ingluviei*– H/A
L. kalixensis– H/A	*L. murinus*-F	*L. kefir* -F
L. kitasatonis– H/A	*L. paracasei subsp. paracasei*– H/A; -F*	*L. kunkeei*–F*
L. manihotivorans– F	*L. paracasei subsp. tolerans*–F*	*L. mucosae*– H/A; -F
L. mindensis– F	*L. paralimentarius*-F	*L. oris*– H/A
L. pantheris– H/A	*L. paraplantarum*–F*	*L. panis*-F
L. psittaci– H/A; -P	*L. pentosus*-F	*L. parabuchneri*– H/A
L. ruminis– H/A	*L. perolens*–F*	*L. paracollinoides*-F
L. saerimneri– H/A	*L. plantarum subsp. plantarum*–F*	*L. parakefiri*-F
L. salivarius subsp. salivarius– H/A	*L. plantarum subsp. argentoratensis*–F*	*L. reuteri*– H/A; -F
L. salivarius subsp. salicinius– H/A	*L. rhamnosus*-F	*L. rossii*-F
L. satsumensis– F	*L. sakei subsp. sakei*– H/A; -F*	*L. sanfranciscensis*-F
L. ultunensis– H/A	*L. sakei subsp. carnosus*–F*	*L. thermotolerans*– H/A
	L. versmoldensis-F	*L. vaccinostercus*– H/A
	L. zeae-F	*L. vaginalis*– H/A

1.2 Habitat in Human Organism

1.2.1 Oral Cavity

The oral cavity is a system constantly exposed to external factors because of respiration and nutrition. Here reside at least 700 microbial species (Moore & Moore, 1994; Aas et al., 2005), the composition and density is determined by kind of nutrition as well as sex, age, health status, composition of saliva and oral hygiene (Tannock, 1999; Slot & Chen, 1999).

As for the genus *Lactobacillus*, they make up about 1% of the bacteria in saliva, 0.1% of bacteria found on the epithelium of the cheek and tongue, and 0.005% of the intragingival plaque. The predominant species found both in childhood and adulthood are *L. casei, L. rhamnosus* and *L. fermentum*. To date, we know that many of the strains previously classified as *L. casei* might, instead, belong to the species *L. paracasei* (Ventura et al., 2003). *Lactobacillus brevis* strains isolated from human saliva (Hayward & Davis, 1956; Hayward, 1957) were then classified into a new species, *L. oris* (Farrow & Collins, 1988). Strangely, *L. salivarius* was isolated with a low incidence. In general, in the oral cavity lactobacilli are limited in number also because they are in an area that we can define "transient" (MacFarlane & Samaranayake, 2014).

As suggested by many articles, dental caries is caused by the metabolism of the bacteria such as Streptococci, Neisseria, Actinomyces, and Capnocytophaga that adhere to tooth enamel causing damage (Tannock, 1999). The progression of the disease is promoted mainly by *Streptococcus mutans*, but when the tooth has deteriorated, lactobacilli multiply inside. As already mentioned, Lactobacilli produce lactic acid by the metabolism of carbohydrates and thus alter the ecological conditions, resulting in selection of more acidophilic bacteria such as *L. casei*, *L. paracasei* and *L. rhamnosus* that will take possession of the niche. This helps us to understand that the high density of lactobacilli in the cases of caries is the result of the disease rather than the cause (Aas et al., 2005; Alaluusua et al, 1987; Wijeyeweera & Kleinberg, 1989).

1.2.2 Gastro-Intestinal (GI) Tract

The entire gastrointestinal tract is home to a substantial amount of bacteria. There are more than 400 bacterial species in the intestine but quantity and composition change from section to section: pH varies from values of 5.5 at proximal levels to values of 6.9 at the level of the distal colon, the temperature can change between 37-40°C; also, the constitution of the mucous membranes, the diet of the subject and not least, health, have always to be considered. All this involves a considerable variety of strains, even the anaerobic conditions are important, in fact, near the mucous there is a high oxygen tension which promotes the growth of facultative anaerobic bacteria (Dworkin & Falkow, 2006).

As for the lactobacilli, in the human stomach they are in low quantities, about $<10^3$cfu/ml and there are only species adapted to the extremely acidic environment such as *L. gastricus* and *L. antri*, or *L. kalixensis* and *L. ultunensis* (Roos, Engstrand, & Jonsson, 2005).

The number of bacteria in the intestines increases from $\approx10^4$cfu/ml in the duodenum, up to 10^8-10^9cfu/ml in the ileum with predominantly lactobacilli and enterococci (Fig. 1-5). The colon is the most colonized part of the human intestine and most of the knowledge acquired on the intestinal microbial populations is from stool analysis. Human feces contain $>10^{11}$cfu/g, which constitute about 50% of the solids within the colon (Tannock, 1995; Vos et al., 2009) but the percentage of lactobacilli isolated is only a low fraction (Tannock et al., 2000); these latter, in fact, are found in greater amounts in the proximal section of the colon while they are considerably reduced at the level of the distal colon, and then in the stool (Marteau et al., 2001).

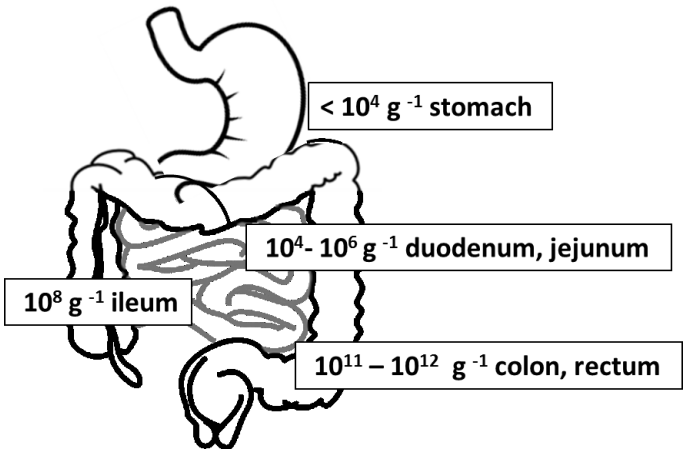

Figure 1-5. Numbers in individual sections depict the amount of bacteria per gram of intestinal content typically found in healthy individuals (Leser & Mølbak, 2009).

By molecular analysis it was found that *L. acidophilus*, *L. fermentum* and *L. salivarius*, *L. gasseri*, *L. crispatus* and *L. reuteri* are the dominant species (Mitsuoka, 1992; Reuter, 2001) while *L. paracasei, L. rhamnosus, L. delbrueckii, L. brevis* and *L. plantarum* are quite transient whose stay varies depending on the diet, age and health of the individual (Heilig et al., 2002).

The amount of lactobacilli decreases in cases of gastrointestinal diseases. Studies of patients with Crohn's disease and ulcerative colitis have shown a prevalence of unclassified strains of the phyla Bacteroidetes and Verrucomicrobia. In addition, an increase of enterobacteriaceae with particular attention to the presence of a non-commensal *Escherichia coli* has been found.

Finally, *Clostridium perfringens* was detected, too. The profiles of the analysis of biopsy, aspirations and fecal samples taken from each patient are similar. Instead, the results of stool samples of patients with pouchitis (inflammation of the ileal pouch) or familial adenomatous polyposis show profiles very different from each other, but even in these cases the diseases are associated with the presence of non-commensal bacteria in human gut; the administration of antibiotics brings an improvement of the pathologies in question (Tannock, 2008; 2010).

1.2.3Vagina

The vagina is a habitat in which, under normal conditions, there is a balance between different bacterial species in a commensal symbiosis with the host. Lactobacilli account for 50-90% of the vaginal aerobic microbiota in most Caucasian women (Zhou et al., 2007; Yamamoto et al., 2009) and they are present in concentrations of 10^7-10^8cfu/g of vaginal fluid, where glycogen is considered the maximum source of fermentable carbohydrates to be exploited for nourishment. Different ethnic women are at the heart of the most important differences observed in the composition of endogenous microbiota. Similar to the gut, there are many reasons why entire populations can differ from one another, variables can be included such as hygiene, sexual activity, number of sexual partners, diet, use of antibiotics or vaginal douches, cervical dysplasia, specific sampling site, age, and many other issues relating to socio-economic status (Marrazzo et al., 2002; Zhou et al., 2007; Yamamoto et al., 2009; Martínez-Peña et al., 2013; Pendharkar et al., 2013).

L. crispatus, L. gasseri, and *L. jensenii* were identified as predominant species in non-cultural analysis of samples, taken from healthy, sexually active women, while *L. rhamnosus, L. paracasei, L. fermentum, L. plantarum*, *L. vaginalis,* and *L. iners* have occasionally been identified (Antonio, Hawes, & Hillier, 1999; Falsen et al., 1999; Vásquez et al., 2002), finally, *L. coleohominis* was isolated only on a few occasions (Nikolaitchouk et al., 2001).

On the other hand, subsequent studies showed that the vaginal pH is already low at birth (Cook &Sobel, 1990; Boskey et al, 1999; Gorodeski et al., 2005), and therefore not correlated with the presence of lactobacilli (Boskey et al., 1999) but because of hormonal activity (Gorodeski et al., 2005), for these reasons the bacterial contribution for the vaginal pH is still debated. To create further confusion about the influence of the vaginal microbiota (mainly consisting of lactobacilli) on the pH was caused by Zhou et al. (2007), where the identification of eight different ecotypes of vaginal microbiome would seem to agree with Gorodeski et al. (2005) more than with Boskey et al. (1999). In cases of vaginal diseases the amount of lactobacilli decreases or, in cases where it remains predominant, the bacterial composition changes (Kuebrichet al., 2004; Vitali et al., 2007; Zhou et al., 2007).

The most common vaginal disorders are linked to bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and aerobic vaginitis (AV). Among these infections, the most common genital infection during reproductive age, which causes vaginal discharge characterized by bad odor, is BV. An illness caused by anaerobic bacteria such as *Mobiluncus, Gardnerella vaginalis,* and *Mycoplasma hominis*, which does not cause inflammation (Priestly, 1997; Beigi et al., 2004; Klebanoff et al., 2004).

VVC, however, is often caused by *Candida albicans*, a fungus and member of the vaginal microbiota in women of childbearing age, which is responsible for more than 90% of symptomatic cases of vaginitis. Nevertheless, in recent years, the incidence of vulvovaginitis can diasis due to non-*C.albicans* species has increased (Sobel et al., 1998; Wilson, 2005).

Finally, AV is pathology similar to BV but differs for different reasons: at the microbiological level there is an increase of Gram-positive cocci, such as *Streptococcus agalactiae*, staphylococci and enterococci and Gram-negative bacteria such as Enterobacteriaceae (in particular *E. coli*). The lactobacilli component of the microbiota decreases until it disappears completely (Donders et al., 2002; Kuebrich et al., 2004). From a clinical point of view, the infection is characterized by the presence of toxic leukocytes and parabasal cells, yellow secretion, foul odor (KOH negative test), elevated vaginal pH, redness, itching, burning and different degrees of dyspareunia (Donders et al., 2002; 2007; Kuebrich et al., 2004). Clinical studies have shown that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can reduce the risk of BV (Reid et al., 2001; 2003), not only by increasing the effectiveness of the antibiotic by oral administration of a probiotic, but they can also cure BV by direct intravaginal administration. Saunders et al. (2007) demonstrated that strains of *Lactobacillus spp*. have the ability to destroy and remove biofilms formed by *Gardnerella* spp., and they potentially reduce the need for antibiotics.

2 Isolation by Classic Cultural Techniques

The methods for lactobacilli isolation must consider, first of all, the nutritional needs complex, the pH (values between 4.5 and 6.4 will foster a better growth) and environmental conditions (microaerobicor sometimes strictly anaerobic conditions). Therefore, it is advisable to incubate the plates in microaerobic atmosphere using generating kits for H_2 and CO_2. Media should contain growth factors such as yeast extract, vitamins, peptone, manganese, acetate and Tween®80 considering any supplements as needed such as meat extract, tomato juice, malt extract or ethanol. These conditions encourage a higher similarity to the environment from which they must be isolated (Dworkin & Falkow, 2006; Vos et al., 2009).

Sources where Lactobacilli are the only present microorganisms are rare. When lactobacilli are the predominant bacteria, a non-selective media, such as MRS (De Man, Rogosa, & Sharpe, 1960), can be used. If the microbial population is more complex, you can use the medium proposed by Rogosa SL et al. in 1951, to isolate the lactobacilli from human samples (Rogosa, Mitchell, & Wiseman, 1951), but it should be considered that even other lactic acid bacteria (Leuconostoc, pediococci, enterococci, Weissella or Bifidobacterium) and yeasts could grow. The latter can be inhibited with the addition of cycloheximide (0.4 g/L) (Haley, Trandel, & Coyle, 1980).

Nowadays, to isolate the lactobacilli from human samples more specifically, according to the kind of sample (vaginal or oro-fecal) we can prepare a more selective media.

A useful media is LAMVAB (*Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol Green), highly selective because of low pH and vancomycin (20 mg/L) which inhibit competitive fecal microbiota such as enterobactiaceae, *Clostridium* spp., other Gram-negative bacteria, and also *Bifidobacterium* spp. and all other lactic acid bacteria. This medium contains cysteine–HCl to enhance anaerobic conditions and bromocresol green as an indicator, indeed, the colonies grow green or blue, and the medium loses the color (becoming yellowish) due to the production of acid (Hartemink, Domenech, & Rombouts, 1997).

Moreover, to isolate the lactobacilli from vaginal specimens, media containing blood is needed, in these cases the swabs are directly streaked onto MRS agar and Columbia agar with 5% horse blood.

In general, colonies grown on agar are small (2-5 mm diameter), convex, smooth, translucent or opaque, whose pigmentation depends on the medium used. In addition, many strains can show a weak proteolytic activity (caused by enzymes released from the cell) and lipolytic activity (caused by intracellular lipases). The edges are well-defined and the growth does not cause clear zones around the colonies. Finally, growth in broth appears homogeneous, smooth and whitish.

Generally, Lactobacilli develop a characteristic smell, more or less strong, caused by the production of lactic acid.

2.1 Evaluation of Special Characteristics

2.1.1 Detection of bile salt hydrolysis

The test is performed with the growth of *Lactobacillus* strains, in anaerobic conditions, on SL or MRS media by adding taurocholic acid, taurochenodeoxycholic acid, or taurodeoxycholic acid. Hydrolysis of bile salts is manifested by the formation of halos around white colonies, these latter may appear opaque and granular (Fig. 2-1). This test can provide a quick way to determine the functional activity of *Lactobacillus* species and a rapid identification of hydrolase-deficient strains (Dashkevicz & Feighner, 1989).

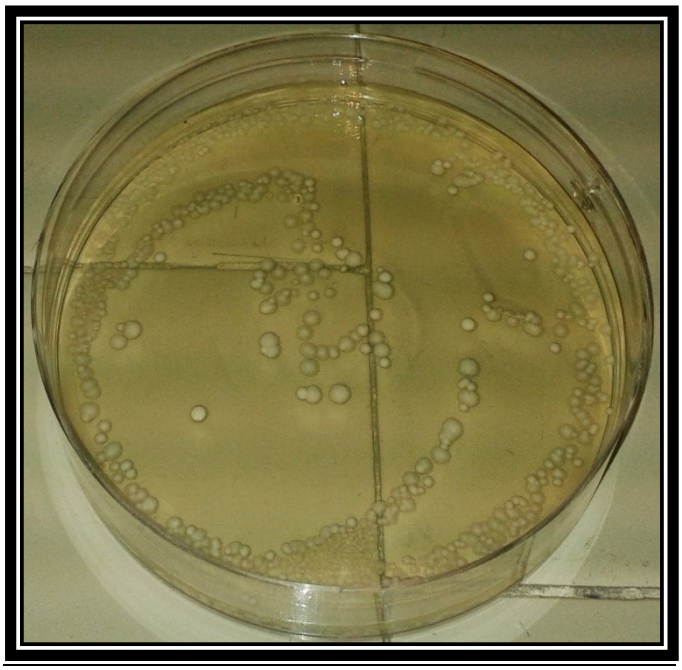

Figure 2-1. *Lactobacillus gastricus* isolated from a human rectal sample grown on MRS containing taurocholic acid.

2.1.2 Detection of H_2O_2 production

This test, for the detection of H_2O_2, exploits horseradish peroxidase, which is isolated from horseradish roots. It is a single chain polypeptide containing four disulfide bridges and 18% carbohydrates (galactose, arabinose, xylose, fucose etc.). It is used as a component of an MRS medium containing 3,3′,5,5′-tetramethylbenzidine (TMB); this latter acts as a chromogenic substrate of peroxidase. The peroxidase generates O_2 from the H_2O_2 produced by the lactobacilli, and then the TMB stains the colonies because it

reacts with oxygen The colonies that produce H_2O_2 appear dark green/blue (Fig. 2-2), while H_2O_2 not-producers appear colorless (Otero & Nader-Macías, 2006; Pascual et al, 2006).

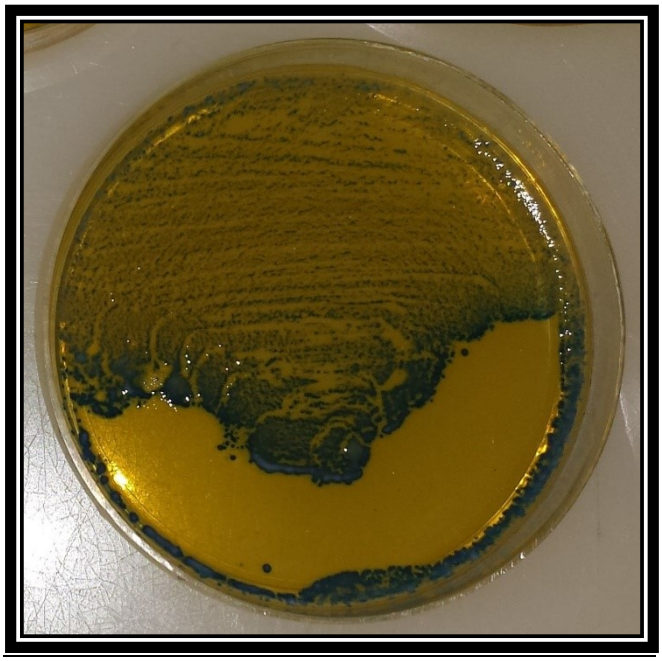

Figure 2-2. *Lactobacillus salivarius* isolated from a human oral sample grown on MRS containing horseradish peroxidase and TMB.

2.1.3 Detection of lactic acid production

There are various essays for such determination. One of these uses the following reactions: L-lactic acid is oxidized to pyruvate by NAD in the presence of L-lactate dehydrogenase (LDH-L). However, the equilibrium of this reaction is in favor of L-lactic acid, but it is possible to facilitate the balance shifting towards the formation of pyruvate and NADH with a second reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate. The increase in NADH is determined by its absorbance at 334, 340 or 365nm (Noll, 1966; Bergmeyer, 2012).

3 Taxonomy and the Importance of Phylogenetic Classification

Ταξι: taxis, "sort", and νομο: nomos, "norm" or "rule." Taxonomy is the science that deals with the classification, or the description, of an entity in a system, in order to facilitate the study of living beings. Each species has a scientific binary name of Latin or Greek origin that identifies it uniquely.

What about the genus *Lactobacillus*? The genus *Lactobacillus* belongs, together with the genera *Paralactobacillus* and *Pediococcus,* to the phylum *Firmicutes,* class *Bacilli,* order *Lactobacillales*, family *Lactobacillaceae* (Garrity et al., 2004). The phylogenetic structure of the family *Lactobacillaceae* considers *Lactococcus lactis* and *Streptococcus thermophilus* as limit groups.

The NCBI taxonomy database currently recognizes more than 200 species of *Lactobacillus;* therefore it is the most populous genus of the order *Lactobacillales*. Indeed, only this year seven new species have been described: *Lactobacillus formosensis* (Chang et al., 2015), *Lactobacillus plajomi* and *Lactobacillus modestisalitolerans* (Miyashita et al., 2015), *Lactobacillus mixtipabuli* (Tohno et al., 2015), *Lactobacillus insicii* (Ehrmann et al., 2015), *Lactobacillus herbarum* (Mao, Chen, & Horvath, 2015) and *Lactobacillus wasatchensis* (Oberg et al., 2015) (Table 3.1).

Historically, from Sharpe et al. (1979) to Kandler & Weiss (1986), morphological characteristics, fermentation of carbohydrates, nutritional requirements, the temperature dependence of growth and the properties of agglutination, were the principles of grouping of bacterial taxonomy.

The subdivision of the genus *Lactobacillus* was made on the basis of fermentation in three groups, but the terms about the different kind of fermentation have been described by many authors and in a different way and, for this reason, could be misleading. The metabolic terminology accepted is the following: obligately homofermentative Lactobacilli, which ferment hexoses almost exclusively to lactic acid by the Embden-Meyerhof-Parnas (EMP) pathway, but they lack phosphoketolase, therefore they are not able to ferment pentose and gluconate; facultatively heterofermentative Lactobacilli

possess the enzyme aldolase (EMP pathway) and are also able to degrade pentoses and gluconate through an inducible phosphoketolase; obligately heterofermentative Lactobacilli degrade hexoses and pentoses by the phosphogluconate pathway. These metabolic processes have already been described in the previous chapter (Hammes & Vogel, 1995).

A good method to identify bacteria is a polyphasic approach, combining phenotypic analysis to genetic analysis. The phylogenetic approach has revolutionized the study of bacteria and thus also the method of classification that has become more efficient and correct. The comparison of molecular sequences is able to precisely estimate the phylogenetic relationships among organisms.

Table 3.1 Species of *Lactobacillus* at the time of writing (November 2015)

Lactobacillus acetotolerans	*Lactobacillus crispatus*
Lactobacillus acidifarinae	*Lactobacillus crustorum*
Lactobacillus acidipiscis	*Lactobacillus curieae*
Lactobacillus acidophilus	*Lactobacillus curvatus*
Lactobacillus agilis	*Lactobacillus delbrueckii*
Lactobacillus algidus	*L. delbrueckii subsp. bulgaricus*
Lactobacillus alimentarius	*L. delbrueckii subsp. delbrueckii*
Lactobacillus alvei	*L. delbrueckii subsp. indicus*
Lactobacillus alvi	*L. delbrueckii subsp. jakobsenii*
Lactobacillus amylolyticus	*L. delbrueckii subsp. lactis*
Lactobacillus amylophilus	*L. delbrueckii subsp. sunkii*
Lactobacillus amylotrophicus	*Lactobacillus dextrinicus*
Lactobacillus amylovorus	*Lactobacillus diolivorans*
Lactobacillus animalis	*Lactobacillus equi*
Lactobacillus animata	*Lactobacillus equicursoris*
Lactobacillus antri	*Lactobacillus equigenerosi*
Lactobacillus apinorum	*Lactobacillus fabifermentans*
Lactobacillus apis	*Lactobacillus faecis*
Lactobacillus apodemi	*Lactobacillus faeni*
Lactobacillus aquaticus	*Lactobacillus farciminis*
Lactobacillus aviarius	*Lactobacillus farraginis*
L. aviarius subsp. araffinosus	*Lactobacillus fermentum*
L. aviarius subsp. aviarius	*Lactobacillus floricola*
Lactobacillus backii	*Lactobacillus florum*
Lactobacillus bifermentans	*Lactobacillus formosensis*
Lactobacillus bombi	*Lactobacillus fornicalis*
Lactobacillus bombicola	*Lactobacillus fructivorans*
Lactobacillus brantae	*Lactobacillus frumenti*
Lactobacillus brevis	*Lactobacillus fuchuensis*
Lactobacillus brevis subsp. coagulans	*Lactobacillus furfuricola*
Lactobacillus brevis subsp. gravesensis	*Lactobacillus futsaii*
Lactobacillus brevisimilis	*Lactobacillus gallinarum*
Lactobacillus buchneri	*Lactobacillus gasseri*
Lactobacillus cacaonum	*Lactobacillus gastricus*
Lactobacillus camelliae	*Lactobacillus ghanensis*
Lactobacillus capillatus	*Lactobacillus gigeriorum*
Lactobacillus casei	*Lactobacillus ginsenosidimutans*
Lactobacillus paracasei	*Lactobacillus gorillae*
L. paracasei subsp. paracasei	*Lactobacillus graminis*
L. paracasei subsp. tolerans	*Lactobacillus guizhouensis*
Lactobacillus zeae	*Lactobacillus halophilus*
Lactobacillus catenefornis	*Lactobacillus hammesii*
Lactobacillus ceti	*Lactobacillus hamsteri*
Lactobacillus coleohominis	*Lactobacillus harbinensis*
Lactobacillus collinoides	*Lactobacillus hayakitensis*
Lactobacillus composti	*Lactobacillus heilongjiangensis*
Lactobacillus concavus	*Lactobacillus helsingborgensis*
Lactobacillus coryniformis	*Lactobacillus helveticus*
L. coryniformis subsp. coryniformis	*L. helveticus subsp. jugurti*
L. coryniformis subsp. torquens	*Lactobacillus herbarum*

Lactobacillus heterohiochii
Lactobacillus hilgardii
Lactobacillus hokkaidonensis
Lactobacillus hominis
Lactobacillus homohiochii
Lactobacillus hordei
Lactobacillus iatae
Lactobacillus iners
Lactobacillus ingluviei
Lactobacillus insectis
Lactobacillus insicii
Lactobacillus intermedius
Lactobacillus intestinalis
Lactobacillus iwatensis
Lactobacillus japonicus
Lactobacillus jensenii
Lactobacillus johnsonii
Lactobacillus kalixensis
Lactobacillus kefiranofaciens
L. kefiranofaciens subsp. kefiranofaciens
L. kefiranofaciens subsp. kefirgranum
Lactobacillus kefiri
Lactobacillus kimbladii
Lactobacillus kimchicus
Lactobacillus kimchiensis
Lactobacillus kisonensis
Lactobacillus kitasatonis
Lactobacillus koreensis
Lactobacillus kullabergensis
Lactobacillus kunkeei
Lactobacillus larvae
Lactobacillus leichmannii
Lactobacillus letivazi
Lactobacillus lindneri
Lactobacillus malefermentans
Lactobacillus mali
Lactobacillus manihotivorans
Lactobacillus mellifer
Lactobacillus mellis
Lactobacillus melliventris
Lactobacillus mindensis
Lactobacillus mixtipabuli
Lactobacillus mobilis
Lactobacillus mucosae
Lactobacillus mudanjiangensis
Lactobacillus murinus
Lactobacillus nagelii
Lactobacillus namurensis
Lactobacillus nantensis
Lactobacillus nasuensis
Lactobacillus nenjiangensis
Lactobacillus odoratitofui
Lactobacillus oeni
Lactobacillus oligofermentans
Lactobacillus oris
Lactobacillus oryzae
Lactobacillus otakiensis
Lactobacillus ozensis
Lactobacillus panis
Lactobacillus pantheris
Lactobacillus parabrevis
Lactobacillus parabuchneri
Lactobacillus paracollinoides
Lactobacillus parafarraginis
Lactobacillus parakefiri
Lactobacillus paralimentarius
Lactobacillus paraplantarum
Lactobacillus pasteurii
Lactobacillus paucivorans
Lactobacillus pentosus
Lactobacillus perolens
Lactobacillus plantarum
L. plantarum subsp. argentoratensis
L. plantarum subsp. plantarum
Lactobacillus pobuzihii
Lactobacillus pontis
Lactobacillus porcinae
Lactobacillus psittaci
Lactobacillus rapi
Lactobacillus rennanquilfy
Lactobacillus reuteri
Lactobacillus rhamnosus
Lactobacillus rodentium
Lactobacillus rogosae
Lactobacillus rossiae
Lactobacillus ruminis
Lactobacillus saerimneri
Lactobacillus sakei
L. sakei subsp. carnosus
L. sakei subsp. sakei
Lactobacillus salivarius
Lactobacillus sanfranciscensis
Lactobacillus saniviri
Lactobacillus satsumensis
Lactobacillus secaliphilus
Lactobacillus selangorensis
Lactobacillus senioris
Lactobacillus senmaizukei
Lactobacillus sharpeae
Lactobacillus shenzhenensis
Lactobacillus sicerae
Lactobacillus silagei

Lactobacillus nodensis	*Lactobacillus siliginis*
Lactobacillus similis	*Lactobacillus uvarum*
Lactobacillus songhuajiangensis	*Lactobacillus vaccinostercus*
Lactobacillus spicheri	*Lactobacillus vaginalis*
Lactobacillus sucicola	*Lactobacillus vermiforme*
Lactobacillus suebicus	*Lactobacillus versmoldensis*
Lactobacillus sunkii	*Lactobacillus vini*
Lactobacillus taiwanensis	*Lactobacillus wasatchensis*
Lactobacillus thailandensis	*Lactobacillus xiangfangensis*
Lactobacillus tucceti	*Lactobacillus yonginensis*
Lactobacillus ultunensis	*Lactobacillus zymae*

Nowadays, in fact, the identification is no longer based only on metabolism, but above all on the study of genomic analysis. One of the fastest methods for the identification of *Lactobacillus* spp. is the sequencing of the 16S rRNA gene, but remembering that closely related species cannot be discriminated, such as *L. plantarum*, *L. pentosus* and *L. paraplantarum* (99.7 to 99. 9%) or *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. kimchii* and *L. paralimentarius* (99.9%), *L. mindensis* and *L. farciminis* (99.9%), or also, *L. animalis* and *L. murinus* (99. 7%).

The first phylogenetic analysis of lactobacilli was carried out by Collins et al. (1991), and the genus *Lactobacillus* was divided into 3 groups: *L. delbrueckii* group, *L. casei-Pediococcus* group, and *Leuconostoc* group, which contained some *Lactobacillus*. In 1995, Schleifer & Ludwig attributed to the *L delbrueckii* group the name *L. acidophilus*, and they divided the *L. casei-Pediococcus* group into four sub-clusters (*L. salivarius* group, *L. reuteri* group, *L. buchneri* group and *L. plantarum* group). Instead, all lactobacilli of the *Leuconostoc* group are now reclassified as species of the genera *Leuconostoc* or *Weissella*.

The recent identification of a large number of species has led to revisions of the genus, allowing the reorganization of these groups in a better and more flexible way. Subsequently, Hammes & Hertel (2006), through the study of the 16S rRNA gene, divided the genus *Lactobacillus* into 7 different groups: *Lactobacillus buchneri* group, *Lactobacillus casei* group, *Lactobacillus delbrueckii* group, *Lactobacillus plantarum* group, *Lactobacillus reuteri* group, *Lactobacillus sakei* group and *Lactobacillus salivarius* group. Finally, *L. brevis*, *L. perolens*, *L. bifermentans* and *L. coryneformis* were positioned separately in the phylogenetic tree of lactobacilli (Table 3.2) (Felis & Dellaglio, 2007).

Nevertheless, a closer study reveals that only three distinct phylogenetic groups can be defined: *L. delbrueckii* group, *L. reuteri* group, and *L. salivarius* group, because, as was said above, the discriminatory power of the 16S rRNA gene sequences is limited (Garrity et al., 2004).

The *L. delbrueckii* group contains mainly obligately homofermentative Lactobacilli, the range of G+C is rather wide from 34 up to 51 mol % and the type of peptidoglycan is Lys–D-Asp; it contains four subspecies, and *Lactobacillus kefiranofaciens* has two subspecies which cannot be reliably differentiated by means of 16S rDNA analysis.

The *L. reuteri* group contains only obligately heterofermentative Lactobacilli, the range of G+C varies among species belonging to this group (38-54 mol%) and they possess two types of peptidoglycan, Lys-D-Asp-Orn or D-Asp.

Finally, *L. salivarius* group contains both obligately homofermentative and facultatively heterofermentative Lactobacilli. This group possesses a content of G+C between 32-44 mol%, and two types of peptidoglycan, Lys-D-Asp and meso-DPM-direct (Garrity et al., 2004).

However, the analysis of 16S rDNA sequences (especially the first 900 bases) is still a fast tool that can be used in combination with other techniques or together with the study of other molecular targets, in order to identify bacterial species.

Table 3.2. Phylogenetic grouping (Felis & Dellaglio, 2007).

Group	Hammes & Hertel, 2006	Dellaglio & Felis, 2005	Felis & Dellaglio, 2007
L. delbrueckii (delb)	*L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasseri, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kefiranofaciens, L. kefirgranum, L. psittaci*	*L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L .gallinarum, L. gasseri, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefiranofaciens, L. kefirgranum, L. kitasatonis, L. psittaci, L. suntoryeus, L. ultunensis*	*L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylotrophicus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasseri, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefiranofaciens, L. kitasatonis, L. psittaci, L. sobrius, L. ultunensis*
L. salivarius	*L. acidipiscis, L. agilis, L. algidus, L. animalis, L. aviarius, L. cypricasei, L. equi, L. mali, L. murinus, L. nagelii, L. ruminis, L. salivarius*	*L. acidipiscis, L. agilis, L. algidus, L. animalis, L. aviarius, L. cypricasei, L. equi, L. mali, L. murinus, L. nagelii, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis*	*L. acidipiscis, L. agilis, L. algidus*, L. animalis, L. apodemi, L. aviarius, L. equi, L. mali, L. murinus, L. nageli, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis, L. vini*

L. reuteri (reu)	*L. coleohominis, L. durianis, L. fermentum, L. frumenti, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. suebicus, L. thermotolerans, L. vaccinostercus, L. vaginalis*	*L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. thermotolerans, L. vaginalis (L. reuteri group-a) associated with L. durianis, L. vaccinostercus, L. suebicus, L. rossii (L. reuteri group-b)*	*L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. secaliphilus, L. vaginalis*
L. buchneri (buch)	*L. buchneri, L. diolivorans, L. ferintoshensis, L. fructivorans, L. hilgardii, L. homohiochii, L. kefiri, L. kunkeei, L. lindneri, L. parabuchneri, L. parakefiri, L. sanfranciscensis*	*L. buchneri, L. diolivorans, L. ferintoshensis, L. hilgardii, L. kefiri, L. parabuchneri, L. parakefiri (L. buchneri group-a)* associated with *L. fructivorans, L. homohiochii, L. lindneri, L. sanfranciscensis (L. buchneri group-b)*	*L. buchneri, L. diolivorans, L. farraginis, L. hilgardii, L. kefiri, L. parabuchneri, L. parafarraginis, L. parakefiri associated with L. acidifarinae, L. namurensis, L. spicheri, and L. zymae (which form a robust group)*
L. alimentarius -L. farciminis (al-far)	/	/	*L. alimentarius, L. farciminis, L. kimchii, L. mindensis, L. nantensis, L. paralimentarius, L. tucceti, L. versmoldensis*
L. casei (cas)	*L. casei, L. manihotivorans, L. pantheris, L. paracasei, L. rhamnosus, L. sharpeae, L. zeae*	*L. casei, L. paracasei, L. rhamnosus, L. zeae (L. casei group-a) L. manihotivorans, L. pantheris, L. sharpeae (L. casei group-b) appear as distinct clusters, not robustly associated with each other*	*L. casei, L. paracasei, L. rhamnosus, L. zeae*
L. sakei (sakei)	*L. curvatus, L. fuchuensis, L. graminis, L. sakei*	*L. curvatus, L. fuchuensis, L. graminis, L. sakei*	*L. curvatus, L. fuchuensis, L. graminis, L. sakei*

***L. fructivorans* (fru)**	/	/	*L. fructivorans, L. homohiochii, L. lindneri, L. sanfranciscensis*
***L. coryniformis* (cor)**	/	/	*L. bifermentans, L. coryniformis, L. rennini,* not robustly associated with *L. composti*
***L. plantarum* group (plan)**	*L. alimentarius, L. arizonensis, L. collinoides, L. farciminis, L. kimchii, L. malefermentans, L. mindensis, L. paralimentarius, L. paraplantarum, L. pentosus, L. plantarum, L. versmoldensis*	*L. arizonensis, L. collinoides, L. paraplantarum, L. pentosus, L. plantarum (L. plantarum group-a)* associated with *L. alimentarius, L. farciminis, L. kimchii, L. mindensis, L. paralimentarius, L. versmoldensis (L. plantarum group-b)* the affiliation of *L. collinoides* was poorly supported	*L. plantarum, L. paraplantarum, L. pentosus*
***L. perolens* group (per)**	/	/	*L. perolens, L. harbinensis, L. paracollinoides*
***L. brevis* group (bre)**	/	*L. acidifarinae, L. brevis, L. hammesii, L. spicheri, L. zymae*	*L. brevis, L. hammesii, L. parabrevis*
***Pediococcus dextrinicus* group (Pdex)**			*P. dextrinicus, L. concavus, L. oligofermentans* (the latter sometimes poorly supported)

Pediococcus	*Not reported*	1 single cluster (not including *P. dextrinicus)*	2 clusters, not associated: the first comprises *P. cellicola, P. damnosus P. parvulus, P. inopinatus, while the second includes P. acidilactici, P. claussenii, P. pentosaceus and P. stilesii*
Couples (couple)			*L. rossiae-L. siliginis* *L. vaccinostercus-L. suebicus* *L. manihotivorans-L. collinoides*
Single species (ss)	*L. bifermentans, L. brevis, L. coryniformis and L. perolens*	*L. algidus, L. kunkeei, L. malefermentans, L. paracollinoides, L. perolens, Paralactobacillus selangorensis*	*L. kunkeei, L. malefermentans, L. pantheris, L. sharpeae, Paralactobacillus selangorensis*

4 Identification by Culture-Independent Techniques

PCR is a molecular technique effective for the identification of bacterial species, also when they are strongly correlated. In particular, there are various techniques based on PCR each with different characteristics that can be used individually, or in combination, depending on the discriminatory power that is desired. Some of the most used techniques are described below.

4.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

It is the first molecular typing method to have been used. The profiles in bands that result from the enzymatic cuts, and the subsequent separation into DNA fragments, constitute the DNA fingerprinting. Because of the high specificity of the endonuclease, and the stability of the chromosomal DNA, the profiles obtained after complete digestion of the DNA, are reproducible. The electrophoretic profiles are consisted of approximately 20-40 bands for each restriction enzyme used, and the complexity of this profile represents a limit of the method; nevertheless, it is believed that the careful choice of particular enzymes, and the use of specific conditions, can make the RFLP technique relatively rapid and easily accomplished (Satokari et al., 2003; Scialpi & Mengoni, 2008).

Ribotyping is a variation of the RFLP technique, where certain fragments are recognized by probes to obtain less complex profiles, which are easier to interpret. The fragments, obtained with this method, are derived from the rRNA gene operon and adjacent regions, which are hybridized by the specific- probes. The discriminatory power of this technique is at the species level but not at the strains level; the discrimination depends on the size of the probe and on the restriction enzymes used (Mohania et al., 2008). The effectiveness of this technique was assessed by Zhong et al. (1998) on *Lactobacillus* type strains.

4.2 Amplified Restriction Length Polymorphism (AFLP)

It is a technique based on restriction and DNA amplification with which it is possible to generate a high number of markers with a single combination of restriction enzyme-primers. It is exploited for bacterial and vegetal fingerprinting analysis, but also to search for markers. The DNA is treated with restriction enzymes so as to produce fragments <1kb which are then joined together using T4 DNA ligase to double stranded oligonucleotide adapters which have ends complementary to the restriction sites so that all fragments have the same end. Then, each fragment formed is amplified by PCR. The products are separated by capillary electrophoresis. The results are manifold, also 100 markers per reaction, so the analysis of the data takes place by software, such as GelComparII or Genotyper, excluding fragments of sizes smaller than 50 bp and peaks below 50 units of fluorescence (Scialpi & Mengoni, 2008). As concerns lactobacilli, the AFLP technique is used in clinical trials and for the typing of strains such as *L. acidophilus* (Gancheva et al., 1999).

4.3 Amplified Ribosomal Restriction Fragment Analysis (ARDRA)

It is an RFLP analysis carried out on a PCR product, it is specific for the genes encoding ribosomal RNAs and it is exploited for bacterial taxonomic discrimination. The technique is based on the presence of SNPs in the region of interest. After this the filaments are amplified, they are then digested with restriction enzymes and since the areas where there are the SNPs are digested in a different way, this determines the changes in length of the fragments and the effectiveness of the method in distinguishing between highly correlated species and subspecies. The technique produces an electrophoretic profile of few bands allowing the bacterial strain identification by comparison with reference species. The difficulty of the technique is in the interpretation of data, and then it is always useful to use the same race conditions and the same molecular weight marker (Scialpi & Mengoni, 2008).

4.4 Randomly Amplified Polymorphic DNA (RAPD)

This technique is also known as AP-PCR (Arbitrarily Primed PCR); it amplifies unknown segments of any genome using a single nucleotide of around 10bp as a primer. The amplification is made possible because the annealing temperature is very low and the concentration of $MgCl_2$ is very high. The profile that is obtained consists of an array of "anonymous" genic amplicons. Generally this method is used to discriminate the species and sometimes different strains of the same species. The technique produces an electrophoretic profile of 5-20 bands for each sample; also in this case it is useful to use a software package for data analysis (Scialpi & Mengoni, 2008). The method is simple to perform and it is rapid, but the reproducibility of results is poor: use of different thermal cyclers, different DNA polymerases, various methods of DNA extraction, different concentrations of the primers or $MgCl_2$, can cause variations in the RAPD pattern and, consequently, the profiles are often not comparable. The technique has been exploited for the typing of lactobacilli (Nigatu, Ahrné, & Molin, 2001).

4.5 Denaturing Gradient Gel Electrophoresis (DGGE)

It is a technique based for the separation of DNA fragments, which have the same length but different sequence, on a polyacrylamide gel. This gel has a linear gradient given by a mixture of 7M urea and 40% formamide (Scialpi & Mengoni, 2008). The principle is based on the stroke of the DNA molecules that stops when the conditions of denaturing gradient permit the passage of molecules partially single stranded and not double stranded. The technique is used in the screening of heterogeneity in rDNA between bacterial species and the fingerprinting of bacterial communities (Meroth et al., 2003; Meroth et al., 2004). In addition, the technique helps to identify the species of *Lactobacillus* in samples where these represent the predominant organisms, as in the vaginal microbiota. *Lactobacillus iners* is the most frequent species in the bacterial vaginal microbiota but it is difficult to grow because it does not grow on selective media used for the isolation of lactobacilli; the use of culture-independent techniques helps in the detection of this species in human samples (Burton et al., 2003).

4.6 Loop Mediated Isothermal Amplification (LAMP)

It is an alternative to the classic PCR which obtains the amplified DNA in isothermal conditions, and it is often used for the identification of pathogenic microorganisms (Notomi et al., 2000; Goto et al., 2007). For this type of PCR four primers are drawn (two outer and two inner), which recognize six different regions of the molecular target in the first steps and four in the last steps. These particular primers do not lead to the amplification of identical copies but of a mixture of amplicons. Another particular is the DNA polymerase (Bst polymerase); it does not work at high temperatures (63-65°C). The electrophoretic profile is constituted by a smear of bands with apparent precise dimensions but only if the target was present at the beginning of the reaction, otherwise there will be no trace of any amplification (Scialpi & Mengoni, 2008).

4.7 Denaturing High Pressure Liquid Chromatography (DHPLC): a new culture-independent technique

DHPLC (ADS Biotec Inc., Omaha, NE, USA) is a chromatography method which uses amplified PCR for the detection of deletions, insertions and point mutations in DNA (Prof. Cavalli-Sforza, Stanford University). It can be used as a technique for the detection of new mutations (SNPs, insertions, deletions and tandem repeats) or for screening populations for the detection of a specific mutation. Moreover, it can purify the PCR products to eliminate contamination, and for separations of DNA fragments cut with restriction enzymes. In the clinical analysis, this method is exploited for the detection of DNA mutations that are the cause of disease or determine predisposition to diseases such as tumors. It is a rapid and automatable method, with sensitivity close to 100%.

In practice, the technique uses an inert HPLC system, free of metallic elements in contact with the hydraulic and chromatographic circuits. It uses a controlled system of temperature extended to multiple items (not only the column as in traditional HPLC); the method is based on the fast migration of genomic molecules along the column. The DNA undergoes a partial denaturation and a re-annealing, and then any variation

between the original and the mutated molecule leads to a heteroduplex that behaves differently, chromatographically, from the omoduplex (non-mutated) with different retention times. DHPLC reveals the presence of the mutation within the fragment but is not able to define which of amending it is. The peaks obtained are recorded and analyzed, in the form of a chromatogram, by the WAVE SYSTEM. You can also record standard chromatograms of mutational patterns for creating a database of already identified mutations. The system also has a fraction collector through which you can retrieve the peaks of interest for further analysis (Xiao & Oefner, 2001).

In microbiology, the first culture-independent 16S rDNA study with DHPLC was carried out to identify bacteria in urinary tract samples (Fig. 4-1). Similarly sized amplicons encompassing the V6 to V8 region of 16S rDNA were analyzed with this technique (HDPLC, WAVE® System) (Domann, et al., 2003); more than 100 samples were analyzed, peak shaped products revealed the presence of pathogens such as *Anaerococcus lactolyticus*, *Bacteroides vulgatus*, *Dialister invisus*, *Fusobacterium nucleatum* and others, whose identities were confirmed by sequencing of the collected peaks (Domann, et al., 2003).

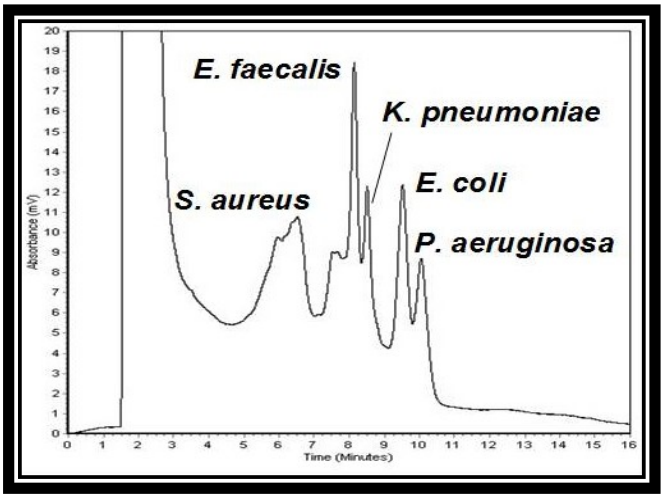

Figure 4-1. Courtesy of Dr. M. Sbalzarini and Dr. S. Papadimitriou (ADS Biotec Ltd): peak profiles produced by amplified 16S rRNA gene of UT samples 16S r RNA of UT samples (Domann, et al., 2003).

Subsequently, the technique was exploited by Imirzalioglu et al. (2008) on 1,449 samples of urine. The majority of UTIs examined were caused by *Escherichia coli*, *Enterococcus faecalis* and by miscellaneous bacteria, which were detected only by using PCR-HDPLC but they were not detected by classic cultural methods.

An interesting study was the investigation that showed how the PCR- DHPLC technique can be used to analyze changes of the gut microbiota in fecal samples from patients before, during and after antibiotic therapy (Goldenberg et al., 2007).

A further example was made by Ercolini et al. (2008) who used PCR-DHPLC to study the variety of bacteria of natural whey cultures (NWCs) for the manufacture of Caciocavallo Silano cheese PDO accurately revealing the presence of LAB (lactic acid bacteria) such as *Lactobacillus* spp., *Streptococcus thermophilus* and *Lactococcus lactis*.

All these previously culture-independent molecular methods are based upon 16S rRNA gene analysis (Fig. 4-2, 4-3).

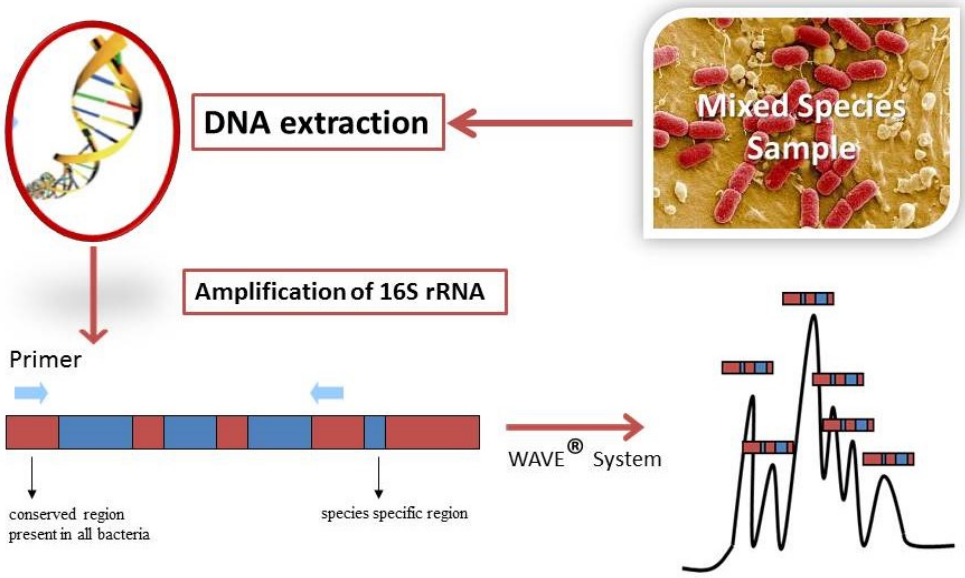

Figure 4-2. HDPLC-WAVE® System based upon 16S rRNA gene analysis.

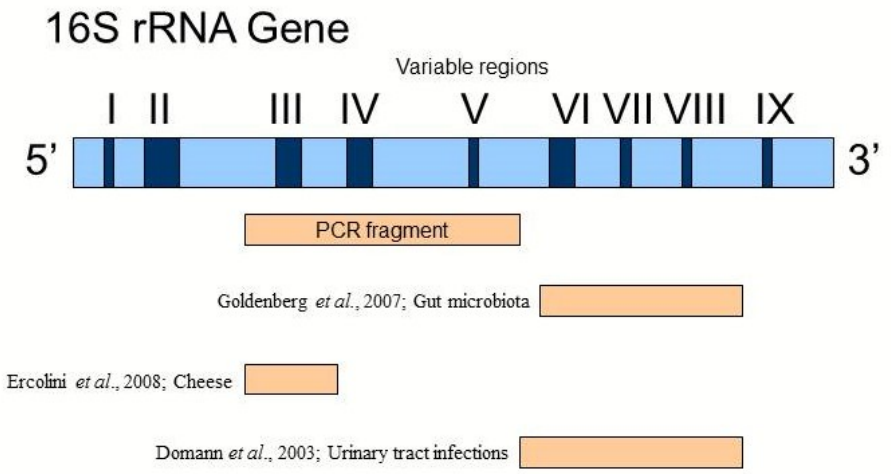

Figure 4-3. Courtesy of Dr. M. Sbalzarini and Dr. S. Papadimitriou (ADS Biotec Ltd): 16S rRNA gene analysis by amplification of its variable regions in the studies previously described.

5 16S rRNA as a Sequencing Gene, the Reason Why

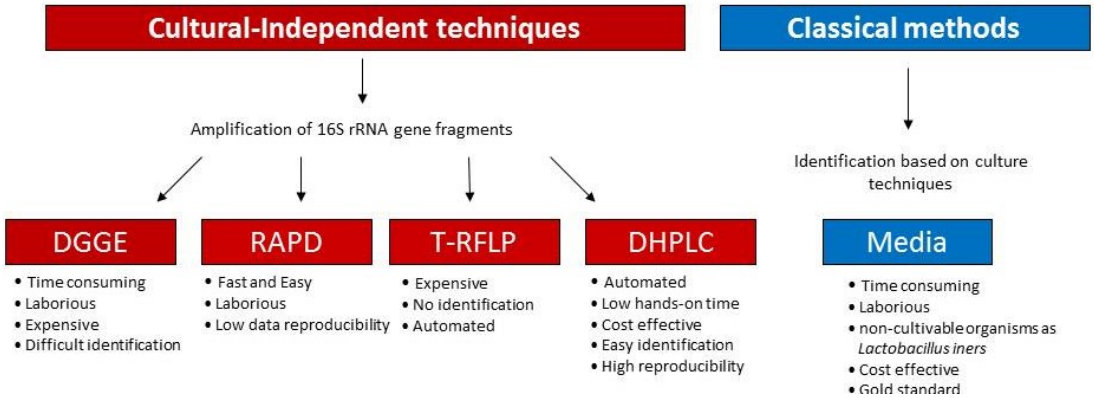

Figure 5-1. Advantages and disadvantages of the various techniques used: cultural-independent techniques are more accurate managing to identify species that are not cultivable.

Because of the restrictions to which the structure of rDNA is subject, as it must assume a defined secondary structure and interact with different proteins to form a functional ribosome, the degree of variation is significantly lower than other genes. Therefore, it is possible to determine the phylogenetic relationships over large evolutionary distances. Then, this kind of function, as a molecular clock, allows accurate determination of the phylogenetic distances. The 16S rRNA gene is composed of variable regions as well as conserved regions, and the entire sequence consists of about 1,550 bp (Fig. 5-2).

Figure 5-2. Scheme of division about hypervariable regions within the sequence of the 16S rRNA gene.

Pairs of universal primers are designed so as to be complementary to conserved regions, which are located at the beginning of the gene and in the region over 540bp or at the end of the sequence. The high conservation of genes among phylogenetically close species belonging to the genus *Bacillus* was observed by Dubnau et al. (1965) and subsequently, as a result of a study by Woese et al. (1987), the sequence of 16S rRNA gene began to spread to the studies of bacterial taxonomy. The nine hypervariable

regions, contained within the gene, are used in these comparison studies (Relman, 1999).

Sometimes, if the differences among gene sequences are not unevenly distributed but concentrated in some hypervariable regions, this could create uncertainty about assigning taxonomy (Stackebrandt & Goebel, 1994).

In addition, it has been demonstrated that some of the nine hypervariable regions are more informative than others allowing a more reliable taxonomic assignment (Wang et al., 2007; Liu et al., 2008; Youssef et al., 2009).

Therefore, you need a comparison and a choice of the hypervariable regions more suitable to study when making an analysis of clinical samples, also depending on the type of sample and the method that you choose to make the assay, in order to obtain reliable results (Kim et al., 2011).

In this case, the study is performed on human oro-fecal and vaginal samples, then examining the hypervariable V1-V3 regions will give better results (Fig. 5-3). Also because in public databases there are a large number of sequences that correspond to these regions: thus, the partial sequences obtained will have more sequences in the database to be compared, greatly facilitating identification.

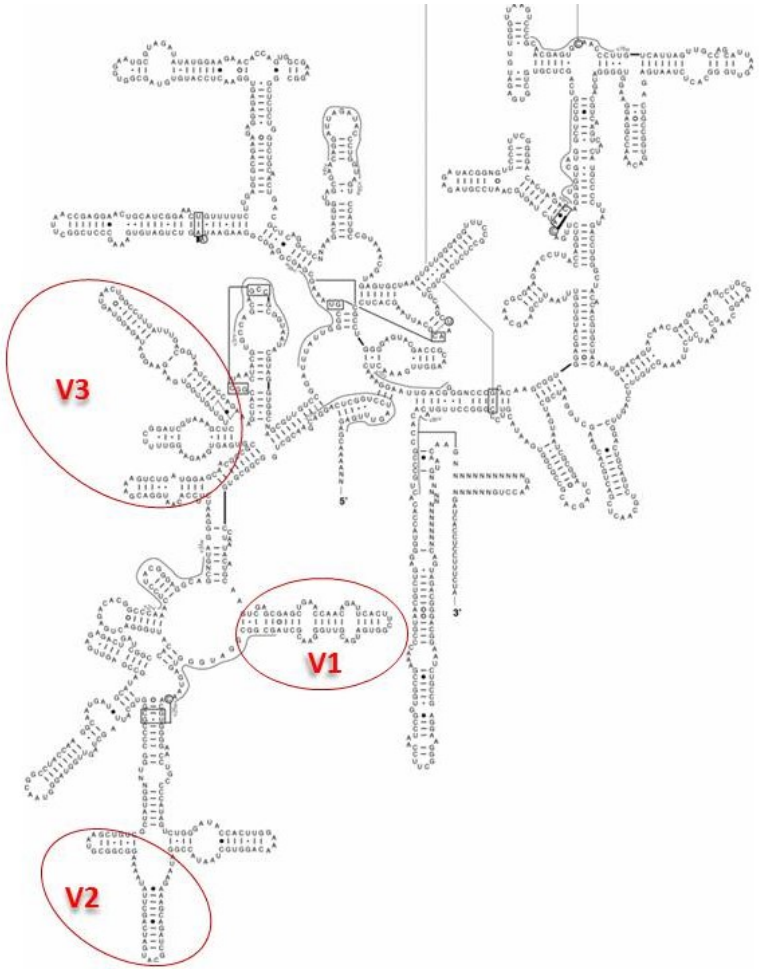

Figure 5-3. The hypervariable regions V1, V2 and V3 of the 16S rRNA gene sequence (image modified from http://bce.bioetanol.cnpem.br/node/43)

Many other genomic regions have been used to study the phylogenetic relationships between bacteria. The intergenic sequences, between the 16S and 23S rRNA genes, have been used to distinguish *Mycobacterium* spp.. This has brought many advantages in the distinction between species that were not discriminated by the mere analysis of the 16S rRNA gene (Roth et al., 1998). The analysis of 23S rRNA gene sequences, on the other hand, has been particularly useful for the distinction of species belonging to the genus *Streptococcus* (Rantakokko-Jalava et al., 2000). The gene encoding a heat shock protein of 65 kDa is highly conserved in mycobacteria and it was therefore used to define the taxonomic relationships among species of this genus (Tortoli, 2003).

Song et al. (2000) designed a two-step protocol multiplex PCR for the rapid identification of 11 species of intestinal lactobacilli isolated from human feces. It uses a first distinction in phylogenetic groups, and then, through the designation of species-

specific primers obtained from the ISR-sequence 16S-23S and the 23S rRNA flanking the region, it identifies at the species level. This region, in fact, shows a higher variability compared to the sequence of the 16S rDNA, which makes it more advantageous to use to discriminate closely related species from the phylogenetic point of view.

In addition, Ventura et al. (2003), analyzed the *tuf* gene, coding for the elongation factor Tu, to discern the species of *Lactobacillus* and *Bifidobacterium*; and they showed that the phylogeny generated from these sequences *tuf* is consistent with that derived from the analysis of 16S rRNA gene. The search for a multiple sequence alignment TUF has revealed conserved regions between strains of the same species, but distinct among other species. Through this study we can discern the species of the *Lactobacillus casei* group: *L. casei, L. paracasei* and *L. rhamnosus*.

6 Probiotic Properties

What are probiotics? According to the official definition of the FAO and the WHO, probiotics are "live organisms which, when administered in adequate amounts, bring benefit to the health of the host" (FAO, 2001).

From the etymological point of view, the term "probiotic" comes from the union of the Latin preposition pro ("on behalf of") and the adjective greek βιωτικός (biotic), resulting in turn from the noun βίος (bios, "life") (Hamilton-Miller, Gibson, & Bruck, 2003).

Lactobacilli, and bifidobacteria, are the most common types of probiotic microorganisms, which are consumed every day together with fermented foods that contain them, such as yogurt. Many of the strains present in these products, however, play no beneficial role in the human body, because they die as soon as they come into contact with the gastric juices because they cannot survive acidity. According to the guidelines of the FAO/WHO, probiotics are defined as only those strains that prove capable, when administered in adequate amounts, to exercise functions beneficial for the body.

Thus a microorganism can be defined as a probiotic strain if it is of human origin, resists the acidity of the stomach and the action of bile, survives in the gastrointestinal tract, adheres to the intestinal mucosa where it can reproduce, and also, it must be tolerable and have beneficial effects for health antagonizing pathogenic microorganisms and producing antimicrobial substances (Table 6.1). Recent guidelines on probiotics, issued by the Italian Ministry of Health, state that, on the basis of the available literature, the amount sufficient to obtain a temporary colonization of the intestine by a probiotic strain is at least 10^9 living cells. This quantity of cells must be present in the daily intake of the food or supplement for at least one strain among those present in the product (Ministero della Salute, 2015).

When it comes to probiotic strains, one of the most important feedbacks is the determination of the profile off susceptibility/resistance to antibiotics that must be assessed for each bacterial strain to be used: in this way all those strains that could convey resistance to some antibiotics can be eliminated (Dughera, 2012).

Nevertheless, the EFSA (European Food Safety Authority) has recently rejected most of the claims used by food companies on food labels and supplements (Table 6.2).
Therefore, it is important that the study of strains of interest must be significantly extended so that they really respond to all the requirements.

Table 6.1. The most important features that a probiotic strain must have (Fuochi et al., 2015).

Essential Requirements	References
• it must survive gastrointestinal transit, and then resist the low pH in the stomach and bile salts released into the intestine	Cote & Holt, 2006; Ministero della Salute, 2015
• it must compete for receptors and adhere to cells and then colonize and stay alive in the intestine	Sengupta et al., 2013
• it must effectively compete with pathogenic bacteria already present through acidification and production of antimicrobial compounds	Alakomi et al., 2000; Servin, 2004
• it must affect the enzymatic modification of the receptors for bacterial toxins	Chen et al., 2006
• it must improve immune defenses against microorganisms	Wold, 2001

Table 6.2. Some claims rejected by EFSA (Fuochi et al., 2015).

Strains	No cause: administration-effect correlation
L. casei Shirota (Tetens, 2010a)	Maintenance of immune defenses of the upper respiratory tract against pathogens
L. fermentum CECT5716 (Tetens, 2010b)	
Combination *L. gasseri* PA 16/8, *B. bifidum* M20/5 and *B. longum* SP 07/3 (Tetens, 2012a)	
L. rhamnosus LB21 NCIMB 40564 (Tetens, 2010c)	Reduction of microorganisms pathogenic to the health of the digestive tract; reduction of *Streptococcus mutans* in the oral cavity
L. paracasei IMC 502, *L. rhamnosus IMC 501* (Tetens, 2010d)	Reduction of pathogenic microorganisms, maintenance of normal intestinal transit time and reduction of gastrointestinal disorders
L. plantarum 299v (Tetens, 2011a)	Reducing flatulence and bloating; protection of DNA, proteins, and lipids from oxidative damage
L. johnsonii NCC 533 (La1) (CNCM I-1225) (Tetens, 2011b)	Improvement of the immune system against gastrointestinal microorganisms; skin protection from damage caused by UV rays
Combination *L. paracasei* CNCM I-1688 and *L. salivarius* CNCMI-1794 (Tetens, 2012b)	Reduction of gastro-intestinal disorders, decrease of potentially pathogenic gastro-intestinal microorganisms, increased lactose digestion, and increased production of IL-10

Probiotics represent a therapeutic-nutritional approach that is economic and free from adverse side effects in the long term. According to clinical practice guidelines of the World Gastroenterology Organization the beneficial effects given by probiotics can be divided into two groups according to the mechanism of action: effects given by the immune mechanism type and effects given by non-immune mechanisms (Guarner et al., 2012).

The first category comprises:

- activation of macrophages to increase presentation of the antigen to lymphocytes B and to increase local and systematic production of immunoglobulin A (IgA);
- modulation of cytokine profiles (reduction of inflammatory cytokines and increase of anti-inflammatory cytokines);
- induction of a low response to food antigens.

The second category comprises:

- food digestion and competition for nutrients with pathogens such as *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp. and enteropathogenic *Escherichia coli* strains;
- alteration of the pH to create an unfavorable environment for pathogens;
- competition for adhesion to epithelial cells;
- production of bacteriocins to inhibit pathogens;
- elimination of superoxide radicals;
- stimulating of epithelial mucin production;
- intensification of the intestinal barrier function;
- modification of the toxins produced by pathogenic strains.

It must be specified, however, that the administration of probiotic species does not have the same effect in different diseases and in different age groups. Recent clinical data show that specific *Bifidobacterium* or *Lactobacillus* strains are not equally effective in the treatment of the same disease. Each strain has specific mechanisms of action and effectiveness is primarily determined by the characteristics of the host organism. For example, *Lactobacillus* strains show very strong action in immunological subsets such as food allergy and lactose intolerance. Moreover, this activity is more evident in children and young people (Chapman et al., 2011).

Table 6.3. Clinical Application (Guarner et al., 2012).

Pathology	Therapeutic evidence	Ref.
Colorectal cancer	Administration of probiotics in patients with colorectal cancer could lead to a reduction in the incidence and severity of gastrointestinal toxicity; *Lactobacillus salivarius* Ren as potential agent for colon cancer prevention based on the modulation of intestinal microbiota	Mego et al., 2015; Zhang et al., 2015
Acute diarrhea or diarrhea caused by antibiotics	*Lactobacillus reuteri* ATCC 55730, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* DN-114 001, e *Saccharomyces cerevisiae (boulardii)*, are useful for reducing the severity and duration of acute infectious diarrhea in children; *L. casei* DN-114 001 is useful for prevention of diarrhea caused by antibiotics or caused by *Clostridium difficile* in adults	Lee et al., 2001; Allen et al., 2003; Hickson et al., 2007
Helicobacter pylory eradication	Reduction of side effects of antibiotic therapy and improved patient compliance, but no effect on the rate of eradication; instead, the integration of antibiotics anti-*H. pylori* associated with probiotics could be effective in increasing the rate of eradication	Sýkora et al., 2005; Tong et al., 2007
Irritable Bowel Syndrome (IBS)	*Escherichia coli* Nissle may be equivalent to mesalazine in maintaining remission of ulcerative colitis; significant therapeutic improvements compared to placebo for the treatment of abdominal bloating and flatulence; there are conflicting results about Crohn's disease	Kruis et al., 2004; Kim et al., 2005; Gawrońska et al., 2007
Lactose maldigestion	*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* improve lactose digestion and reduce symptoms of intolerance	Montalto et al., 2006
Necrotizing enterocolitis	There is strong evidence of the benefits conferred by the use of certain strains of probiotics in preterm infants	Deshpande et al., 2007

7 Bacteriocins

A large number of Gram positive (Tagg, Dajana, & Wannamaker, 1976) and Gram negative (Oudega, Mooi, & de Graaf, 1984) becteria produce protein substances, defined bacteriocins. Their molecular weight is usually less than 10kDa, although there are larger bacteriocins, such as lattococcina G, and helveticina I (Beasley & Saris, 2004; Rogne et al., 2008). Usually bacteriocins are cationic and amphipatic molecules with many residues of lysine and arginine; they are synthesized during the primary growth phase, sensitive to protease, with maximum activity at low pH (Nissen-Meyer & Nes, 1997; Rodriguez et al., 2000; Ennahar et al., 2000; Martı, Horn, & Dodd, 2003). In solvents such as trifluoroethanol or in the presence of anionic phospholipids they are in a helical form, while in aqueous solutions they are unstructured (Moll, Konings, & Driessen, 1999). The particular attention to the LAB (Lactic Acid Bacteria) is also due to their ability to produce these bacteriocins, which have antimicrobial activity and for this reason they are often erroneously classified in the category of antibiotics (Ross, Morgan, & Hill, 2002; Todorov & Dicks, 2004, 2005a, 2005b). The latter in fact have the characteristics to be secondary metabolites active against strains from different species that produce them, on the contrary, bacteriocins often have a more limited scope to related species or even strains of the same species. In special cases, however, we have the good fortune to observe a broad spectrum of action.

In 1925, colicina V was discovered and isolated from *Escherichia coli*, it is a substance with antibacterial activity against other strains of *E. coli* (Gratia, 1925), for that time, in fact, the activity against strains of the same species was a new concept. The term bacteriocin arrived in 1953 (Jacob et al., 1953), which initially included only the substances with the characteristic of having a limited spectrum; it will only be at the beginning of the 1990s, after the first results of activity against different species from the producer (Hamon & Peron, 1963), that "bacteriocin" will include all substances with antimicrobial activity (Klaenhammer, 1993). The special feature of limited spectrum is associated with the competition that many bacteria are facing for nourishment and for the space when their ecosystem is overcrowded (Tagg, Dajana, & Wannamaker, 1976, Baba & Schneewind, 1998).

Therefore, these strains should protect themselves from the action of their own deadly bacteriocin and this defense takes place with the production of a specific immunity protein whose coding genes are often found on the same operon (Venema et al., 1994; Cleveland et al., 2001; Deegan et al.,2006).

There are various methods of screening to know if a strain produces bacteriocins. The most widely used is the agar-well diffusion test: the supernatants of the producing strains are placed in wells in the agar where a sensitive microorganism is inoculated (Tagg & McGiven, 1971). It is believed that all LAB produce at least one bacteriocin as a defense mechanism, but as previously mentioned, some show a broad spectrum of activity while others have a very restricted spectrum. Accordingly, when we screen producing strains, we must tread carefully examining the supernatants of many potentially sensitive strains, to avoid excluding a producer. Moreover, the production of bacteriocins does not occur when the environmental conditions are optimum, however they can be produced though very bland; in fact, there is the necessity to subject the strains to stress conditions to mimic possible environmental conditions, and, also, choose culture media with different characteristics.

On the other hand, the appearance of inhibition zones does not give absolute certainty of the presence of a bacteriocin in the supernatant, since there are other substances that can act with antibacterial activity: organic acids, lactic acid, hydrogen peroxide, and bacteriophages which may also be the reason for the development of inhibition zones . To avoid this, you have to neutralize the pH of the supernatant and perform a test in order to exclude the inhibitory effect due to the lactic acid or hydrogen peroxide. The production of hydrogen peroxide can also be inhibited by anaerobic incubation. It is also recommended to always test a supernatant of a susceptible strain as negative control.

Finally, to obtain the certainty to be in the presence of a proteinaceous substance it is necessary to verify the sensitivity to substances as trypsin, α-chymotrypsin and pepsin from which they are partially or totally inactivated.

The unit of activity of a bacteriocin is defined UA (units of activity) or BU (unit bacteriocins), it is usually defined as the reciprocal of the dilution showing 50% inhibition of growth compared to a control sample without bacteriocins (turbidimetric method) as well as the reciprocal of the highest dilution resulting in a clear zone of

growth inhibition of the indicator (agar well diffusion assay), always under standardized conditions.

7.1 Classification

For what concerns the names of bacteriocins, even if it depends on the research group that isolated it for the first time, they often have the suffix "-cin". Also, generally there is the rule to insert the name of the bacterial strain which produces it.

The classification, in four different groups, takes place according to their thermal resistance, size, physical properties and chemical properties, spectrum of action and the presence of modified amino acids.

CLASS I: in this group we find lantibiotics, as the most popular nisin (Berridge, Newton, & Abraham, 1952) or the newest suicin (Vaillancourt et al. 2015). They are small peptides resistant to heat that contain modified amino acids: polycyclic thioether amino acids lanthionine, methyl lanthionine, or unsaturated amino acids 2-aminoisobutyric acid and dehydroalanine (Martì, Horn, & Dodd, 2003; Rodriguez et al., 2000; Chen & Hoover, 2003).

They are small peptides with a molecular mass between 2 to 5 kDa that are in turn divided in two subclasses: type A and type B. The first type includes longer, flexible, amphipatic molecules (3-5 kDa) with a positive charge and screw shaped. The mode of action is through pore formation and membrane depolarization of the cytoplasmic membrane. The molecule belonging to the second type (2-3 kDa) have a globular structure, negative or no net charge, and mode of action through cellular enzymatic reactions (Cleveland et al., 2001; Deegan et al., 2006).

CLASS II: these active peptides are slightly longer (<10 kDa) but less stable to heat and they have no modified amino acids. Bacteriocin couples are prepared to form amphiphilic helices with variable regions of hydrophobia, it also seems that the secondary structure of the native forms possess β sheets. This class is further divided into three subclasses:

- Subclass IIa: bacteriocins of this subclass are the most important of group II as they are active against Listeria. Moreover, they are characterized by a consensus sequence in the N-terminal region as YGNGV that resulting highly conserved leads to a similarity between 40 and 60% in bacteriocins produced by different

bacteria: PA-1 (Henderson, Chopko, & Van Wassenaar, 1992), sakacin A (Holck et al., 1992), sakacin P (Holck et al., 1992; Tichaczek et al., 1992), leucocin A-UAL187 (Hastings et al., 1991) and curvacin A (Tichaczek t al., 1992), carnobatteriocin BM1 and B2 (Quadri et al., 1994), enterocin A (Aymerich et al., 1996) and piscicolina 123 (Jack et al., 1996; Patton & van der Donk, 2005).

- Subclass IIb: these bacteriocins are unique because they are constituted by two different peptides (20-40 amino acids each); moreover it is necessary that both are present in equal amounts so that the antimicrobial activity of the protein is explicated. Lactococcin G (Moll et al., 1996, 1998), plantaricin E/F (Moll et al., 1999), plantaricin J/K (Moll et al., 1999), lactacin F (Muriana & Klaenhammer, 1991) and lactocin 705 (Cuozzo et al., 2003) are bacteriocins of this subclass. Two-peptide bacteriocins make cell membranes more permeable to a wide variety of monovalent cations (Na+, K+, Li+, Cs+) excluding H+, but not to divalent cations (Mg++) and anions (such as phosphate); instead, as regards the lactococcin G, plantaricin E/F and plantaricin J/K, they make membranes permeable also to H+ (Moll et al., 1996, 1998, 1999). In this way the mechanism of action causes the dissipation of the electric transmembrane potential and the pH gradient leading to cell death (Daw & Falkiner, 1996; Nissen-Meyer et al, 1992; Mulet-Powell et al, 1998).
- Subclass IIc: they are peptides which require cysteine residues reduced to be active, such as lactococcin B (Venema et al., 1993).

CLASS III: these proteins are bigger than other bacteriocins (>30 kDa) and labile to heat. Helveticin I produced by *Lactobacillus helveticus* and enterolysin A produced by *Enterococcus faecalis* LMG 2333 are examples of molecules of this group (Joerger & Klaenhammer, 1986; Nilsen, Nes, & Holo, 2003).

CLASS IV: these bacteriocins are active only if linked to a non-protein part, a lipid or a sugar, such as pediocin SJ-1 and lactocin 27 (Upreti & Hinsdill, 1975; Schved et al., 1993). The validity of this category is often questioned, or even not accepted, because the characterization of the molecules is not detailed enough and it is based mainly on the observation of the antimicrobial activity (Fiani, 2007).

7.2 Biosynthesis and Mode of Action

In Gram positive bacteria the genetic information coding for the production of bacteriocins can be contained on plasmids such as at the chromosomal level, instead for Gram-negative bacteria the genes involved are located only at the plasmidic level, such as colicin (Nissen-Meyer and Nes, 1997). Bacteriocins are ribosomally synthesized and post-translational modified peptides (Chen & Hoover, 2003; Patton & van der Donk, 2005; Deegan et al., 2006). A particularity of bacteriocin biosynthesis is that these molecules are not synthesized as active proteins, in fact, ribosomal production makes inactive prepeptides containing an N-terminal extension (18-24 amino acids); these pre-peptides are then modified before export from the cell (Cleeveland et al., 2001).

Active sites and structure–function relationships can be examined simply by genetic manipulation because bacteriocins are encoded by one structural gene. Molecular techniques also allow studying analogues with increased activity or with altered specificity, unlike antibiotics, which must be chemically synthesized. Moreover, there is a difficulty to manipulate the genes because of the increased number of genes involved.

As already mentioned, most bacteriocins cause the formation of pores on the membrane-target leading increased permeabilization and subsequent loss of cellular compounds and dissipation of the proton motive force (Nissen-Meyer & Nes, 1997); some of these bacteriocins have also shown activity against artificial lipid membranes, suggesting that the action is not receptor mediated. One example concerns subclass IIa bacteriocins (Ennahar et al., 2000), which are thoroughly investigated because they are active against serious pathogenic bacteria such as *Listeria* (Héchard et al., 1992). Nevertheless, there are known different modes of action, for example colicin E3 leads to specific inhibition of protein synthesis or colicin E2 leads to specific inhibition of DNA synthesis and induces DNA degradation (Herschman & Helinski, 1967).

Focus on bacteriocins produced by *Lactobacillus* spp. Here is a brief overview of some known molecules (Table 7.1).

Table 7.1 Characteristics of bacteriocins produced by *Lactobacillus* strains.

Strain	Name	Spectrum	AA	kDa	Susceptibility	pH	Heat	Ref.
L. fermentum 466	Bacteriocin 466	*Lactobacillus* spp	16		trypsin and pepsin and insensitive to lysozyme		96°C for 30 min	De Klerk & Coetzee, 1961; De Klerk, 1967; Smit, 1967
L. helveticus LP27	Lactocin 27	*Lactobacillus* spp			trypsin and pepsin and insensitive to lysozyme		96°C for 30 min	Upreti & Hinsdill, 1973, 1975
L.acidophilus IFO 3205		*Escherichia coli*	11	3.5				Hosono et al., 1977
L.acidophilus AC1		both Gram-positive and Gram-negative bacteria		5.4	trypsin and a-chymotrypsin,	4.0-7.5	inactivation within 20 min at 50°C	Mehta et al., 1982, 1983
L.acidophilus N2	Lactacin B	both Gram-positive and Gram-negative bacteria		6.2	protease-sensitive		60min at 100°C and pH 5·0	Barefoot & Klaenhammer, 1983, 1984
L. helveticus 481	Helveticin J	*L. helveticus, L. delbrueckii subsp. bulgaricus* and *L.delbrueckii subsp. lactis*		37	proteolytic enzymes	5.0–7.0	30 min at 100°C	Joerger & Klaenhammer, 1986
L. plantarum NCFB 1193	Plantaricin B	*L. plantarum* NCFB 30 and NCFB 1752, *Leuconostoc mesenteroides subsp. mesenteroides* NCFB L8015 and *Pediococcus damnosus* NCFB 1832			pronase, pepsin, trypsin, α-chymotrypsin, lipase and α-amylase			West & Warner,1988
L. rhamnosus GR-l		*Escherichia coli*		12-14			heat-labile	McGroarty & Reid, 1988

L. reuteri	Reuterin **	*Salmonella, Shigella*. *Clostridium, Staphylococcus* and *Listeria* spp. and also fungi and protozoa		< 200	resistant to protease activity			Talarico et al., 1988; Axelsson et al., 1989; Talarico & Dobrogosz, 1989
L. sake 706	Sakacin A	lactic acid bacteria and *Listeria monocytogenes*			trypsin and pepsin		20 min at 100°C	Schillinger & Lucke, 1989
L. plantarum LPCO-10	Plataricin S	*Pediococcus*, *Carnobacterium*, *Clostiridium* and *Propionobacterium*	26-27		proteolytic, glycolytic and lipolytic enzymes		30 min at 100°C	Jimenez-Diaz et al., 1990
L. plantarum C11	Plantaricin A	*Enterococcus, Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* spp.		>6.0	proteolytic enzymes	4.0-6.5	30 min at 100°C	Daeschel et al., 1990
L. sake L45	Lactocin S	*Lactobacillus, Leuconostoc and Pediococcus* spp.	37		sensitive to proteases		<50% after1 h at 100°C	Mortvedt & Nes, 1990
L. brevis B 37	Brevicin 37	many lactic acid bacteria and *Nocardia corallina*			proteolytic enzymes; inactivated under alkaline conditions	1.0-12.0	121°C for at least 1 h	Rammelsberg & Radler, 1990
L. casei	Caseicin 80	*Lactobacillus casei* B 109		≈4.1	proteolytic enzymes; under alkaline conditions	3.0-9.0	above 60°C within 10 min	Rammelsberg & Radler, 1990
L. acidophilus 11088	Lactacin F	*Lactobacillus acidophilus, Lactobacillus fermentum* and *Enterococcus faecalis*	56	2.5	Proteinase K, subtilisin, trypsin, and ficin		121°C for 15 min	Muriana & Klaenhammer,1991

L. reuteri LA 6	Reutericin 6	*L. acidophilus, L. delbrueckii subsp. bulgaricus* and *L. delbrueckii subsp. factis*		200	actinase E and trypsin	4.0-10.0	60 min at 100°C	Toba et al., 1991a
L.acidophilus LAPT 1060	Acidophilucin A				trypsin and actinase E		10 min at 60-70°C	Toba et al., 1991b
L. casei LHS	Casein LHS							Dicks, Van Jaarsveld, & Van Vuuren, 1992
L. curvatus LTH 1174	Curvacin A	*Carnobacterium* spp., *Enterococcus faecalis* and *Listeria* spp		3.0	Proteinase K and trypsin		30 min at 100°C	Tichaczek et al., 1992
L. sake LTH673	Sakacin P	closely *Lactobacillus* spp, *Carnobacterium* spp., *Enterococcus faecalis* and *Listeria* spp	41	5.0	proteinase K and trypsin		30 min at 100°C	Tichaczek et al., 1992
L. gasseri LA39	Gassericin A		35	3.8				Kawai et al., 1994
L. plantarum	two-peptide Plantaricin JK	*Pediococcus*, *Carnobacterium*, *Clostiridium* and *Propionobacterium*	30-40					Moll, et al.,1999; Rogne, et al., 2009
L. plantarum	two-peptide Plantaricin EF	*Pediococcus*, *Carnobacterium*, *Clostiridium* and *Propionobacterium*	30-40					Moll, et al.,1999; Fimland, et al., 2008

L. gasseri, KT7	Gassericin KT7	*Bacillus cereus*, *Lactococcus* spp., *Leuconostoc*, *Carnobacterium, Clostridium perfringens*, *Cl. botulinum Listeria monocytogenes* and *Enterococcus* spp., *Staphylococcus aureus*	≈38	4.5±5.0	proteolytic enzymes	2.0-10.0	30 min at 90°C	Zhu, Liu, & Wu, 2000
L. plantarum	two-peptide Plantaricin W	*Pediococcus*, *Carnobacterium*, *Clostiridium* and *Propionobacterium*	29-32	3.3				Holo, et al., 2001
L. salivarius CRL 1328	Two-peptide Salivaricin CRL 1328	urogenital pathogens		4.5				Pingitore, et al., 2009
L. salivarius BGHO1	Bacteriocin LS2	*Listeria* spp	41	4.1				Busarcevic & Dalgalarrondo, 2012

7.3 Bacteriocins Applications

To preserve food various techniques are used such as pasteurization, dehydration or preservatives are added such as sorbate, benzoate, and propionate. Nowadays, however, there are more requests for products without preservatives, and which had not been previously treated. And here lactic acid bacteria come into play, in fact, it has been shown that bacteriocins, such as nisin (Deegan et al., 2006), can be used in the food industry (milk, meat and vegetables) to offset any growth of pathogens.

The application is through the growth of the protector strain inside the food at the beginning of the production process or, even when the product is ready for consumption; another method is to add the purified bacteriocin directly as a food additive to the product; to date, however, the only bacteriocin that is used in many countries as a food additive is nisin A (lantibiotic type I) (Fiani, 2007).

This is because the use of bacteriocins has disadvantages:

- it is not compatible if there are other fermenting strains because it may inhibit their growth;
- the use of only bacteriocins as food preservatives does not ensure complete safety because they might not inhibit the growth of pathogenic Gram-negative strains;
- it has been shown that some chemical-physical parameters can influence activity, such as the varying pH.

There are also other uses of bacteriocins, always in the food industry; in fact, they can be used as bioactive packaging to protect food from external contaminants.

For example, cheeses begin to degrade from the surface, thus the use of bacteriocins in combination with artificial packaging can improve the preservation state of the product (Ross, & Morgan Hill, 2002).

The clinical and medical use for bacteriocins is still rather uncertain. First of all, the resistance to them is developed easily and also because during treatment the level of pathogens is numerically very high. On the other hand, you can use the power of probiotic producering strains. For example, in a local treatment such as anti-caries therapy: taking a strain which competes for the ecological oral niche, it could displace *Streptococcus mutans* (Twomey et al., 2000). Another example comes from the

veterinary world, where lactococcin 3147 (Ryan et al., 1998, 1999) has had a positive effect on the treatment of mastitis in bovines (Fiani, 2007; Schirru, 2009).

8 Introducing Proteomics

Proteomics is a scientific discipline that studies the proteome (proteins encoded by the genome) that aims to purify, identify and study proteins. To achieve this, it has an essential separation of the protein from the biological sample in order to determine in detail the physico-chemical properties and the amino acid composition.

When we study an already known protein it is useful to exploit its properties (size, charge, hydrophobicity, any specificity for ligands) so that the isolation is successful. In any case, it must minimize sample handling, the use of additives and the number of steps in order to obtain a better result in terms of quality and quantity (Doonan, 1996; Wilson & Walker, 2000).

The purification of proteins is a complex process which generally requires several steps. A classic approach includes:

1. cell destruction (by blenders, presses, sonication or enzymes)
2. ultracentrifugation to remove the insoluble material from the original homogenate (organelles, membrane fragments, etc.)
3. use of DNase and RNase to eliminate the possible presence of nucleic acids
4. low resolution fractionating with ammonium sulphate (the addition of salt removes water molecules on the protein surface, allowing aggregation, and then precipitation. The very hydrophobic proteins precipitate by low concentrations of salt, the less hydrophobic proteins precipitate by high salt concentrations), using heat (the mixture is heated to 5-10°C lower than the critical temperature for 15-30 minutes and then, all denatured proteins are removed by centrifugation), organic solvents (depending on the solubility of the protein), or two-dimensional gel (exploits both pI and size of the protein).
5. the mixture is purified enough, and now you can exploit a unique feature of the protein to separate it clearly from the others (chromatographic techniques) (Doonan, 1996; Scopes, 2013).

The requirement to separate unknown proteins from complex mixtures led to the development of numerous techniques that take advantage of chemical differences in a mixture so as to allow an initial efficient separation.

8.1 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography is a kind of chromatography that separates molecules in solution by their size. The method is usually applied to large molecules such as proteins in aqueous solution. In this case, the technique is known as gel-filtration chromatography.

The main application is the fractionation of proteins by trapping smaller molecules in the pores of the adsorbent material ("stationary phases"). The larger molecules pass out from the pores because they are too large to enter. Therefore, the largest molecules flow through the column more quickly than smaller molecules. These latter, indeed, have a longer retention time.

In this type of chromatography, an important requirement is that the analyte has no electrostatic or chemical interactions with the surface of the stationary phase, so that the elution time is based only on the volume of the molecules that make up the sample. In this way, the smaller molecules penetrate into the interstices of the porous mass, and elute outside of the system with a volume of solvent approx. equal to 80% of the total volume of the column. The very large molecules, instead, will exit with a volume of solvent equal to ~ 35% of the volume of the column, and will be collected first. Proteins with the same size are eluted together, provided that the entire sample is loaded on the column at the same time.

The range of fractionation depends on the size of the pores of the particles, which in turn is inversely proportional to the amount of agent used to cause the transom ties. The various kinds of gels are characterized in terms of their values of reabsorption of water, which define the amount of water (in ml) that is absorbed by a gram of granules of dry gel. In the case of Sephadex® the characteristic numbers correspond to ten times the value of water reabsorption .

Sephadex is a cross-linked dextran gel used for SEC. These highly specialized gels are composed of macroscopic beads synthetically derived from the polysaccharide, dextran. The organic chains are cross-linked to give a three-dimensional network having functional ionic groups attached by ether linkages to glucose units of the polysaccharide chains. By varying the degree of cross-linking, the fractionation properties of the gel

can be altered. For example, Sephadex G-10 has a value of reabsorption equal to one, and Sephadex G-200 has a value of reabsorption equal to twenty.

Therefore, each size exclusion column has a range of molecular weights that can be separated.

Sephadex® with small absorption values possesses small sized pores, thus good for the fractionation of small molecules, while the types with large values of reabsorption are used for compounds with high molecular weight. Referring to Sephadex, G-10 fractionates substances with molecular weights up to 700, instead, G-200 fractionates substances with molecular weights ranging between 5,000 and 500,000 (Table 8.1).

Table 8.1. Fractionation range of Sephadex gels as reported on the manufacturer's manual.

Sephadex	Fractionation Range (Da)
G-10	≤700
G-15	≤1500
G-25	1000 – 5000
G-50	1500 – 30,000
G-75	3000 – 80,000
G-100	4000 – 150,000
G-150	5000 – 300,000
G-200	5000 – 600,000

The kind of Sephadex useful to separate small molecules, such as G10, G-25 and G-50, obey Darcy's Law:

$$U = K \times \Delta P \times L^{-1} \qquad \text{Eq.1.}$$

U = linear flow rate (cm/h)
ΔP = pressure drop over the packed bed expressed in cm water
L = bed height (cm)
K = constant of proportionality depending on the properties of the bed material and the buffer

Assuming a buffer with viscosity calculated by Equation 2: 1 cP: $U = K_0 \times \Delta P \times L^{-1}$

K_0 = the specific permeability depending on the particle size of the medium and the water regain.

Theoretical flow has been calculated by equation 2, assuming that K_0 is equal as reported in Table 8.2.

Table 8.2. Specific permeabilities of Sephadex as reported in appendix 2 on the manufacturer's manual.

Permeability K	Sephadex type
19	G-10
9	G-25 superfine
30	G-25 fine
80	G-25 medium
290	G-25 coarse
36	G-50 fine

8.2 Solid Phase Extraction (SPE)

A technique currently under development is Solid Phase Extraction (Spencer & de Spencer, 2001).

This process is used mainly when the protein to be studied is present in low concentrations in the starting sample, which thus undergoes a phase that we can define pre-concentration or enrichment, which will allow an increase of the concentration appropriate for subsequent analyzes.

The SPE technique uses polypropylene columns that contain an absorbent packaged material (solid phase), to which the solute that you want to purify binds.

After the passage of the "dirty" sample through the column, a few washes should be performed that eliminate the interfering compounds from the solid phase.

Now, to recover the protein of interest which is still bound to the solid phase, the solvent suitable to untie it is added (Notardonato, 2010).

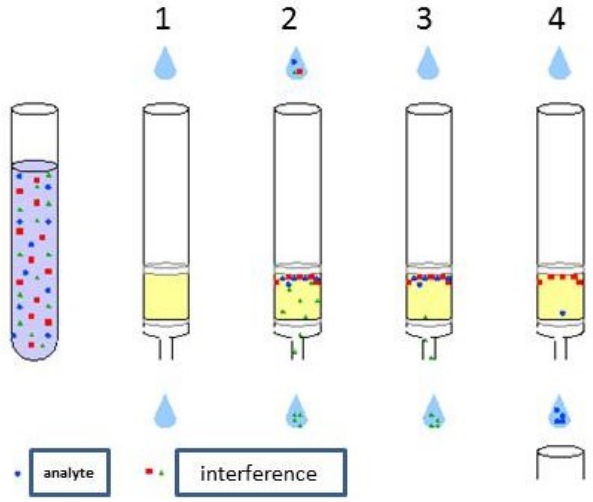

Figure 8-1. Representation of SPE analysis.

The choice of the absorbent is the crucial step in the study of separation, after which the procedure has the following steps (Fig. 8-1):

1. initial conditioning of the column that prepares the stationary phase so as to ensure optimum retention;
2. passage of the sample, where the protein is separated from the liquid phase by binding to the adsorbent stationary phase (therefore, it is necessary that the bond strength between the analyte and the active site of the stationary phase is higher than that existing between analyte and the liquid phase);
3. washings, whose solvent must be chosen so as to eliminate interference but allows the analyte to remain bound to the stationary phase;
4. elution, where the solvent establishes a stronger bond with the protein, and allows it to spill from the column.

In addition, SPE conducted on prepacked columns has many advantages such as the use of a lesser amount of solvent, rapid analysis and good selectivity due to the variety of solid adsorbents that you can choose (Table 8.3).

Table 8.3. Various types of stationary phase that can be exploited for good selectivity of the analyte (Notardonato, 2010).

STATIONARY PHASE	
Non modified Polar Adsorbent	Silica (SiOH), Allumina (Al2O3)
Modified Polar Adsorbent (cromatografia a fase normale)	Silica –ciano group Silica –ammino group
Modified Apolar Adsorbent (Reverse Phase Chromatography)	Silica – C4 Silica – C18
Adsorbent Charged (Ion Exchange Chromatography)	Silica – CH2-N+(CH3)2-C18 Silica – CH2-CH2-CH2-SO3-

Thanks to this technique numerous steps are eliminated and it has good capacity and resolution to produce a purified solution which can be studied directly by HPLC, FPLC (Fast Protein Liquid Chromatography) or by the more sophisticated ÄKTA-FPLC.

ÄKTA™ pure is a flexible and quick chromatography system for the purification of proteins, peptides and nucleic acids which makes use of the UNICORN™ software (Oppegård et al., 2015).

The use of the ÄKTA system allows monitoring of unbound material, so when it was entirely washed away the final elution starts. It also allows the use of two (or more) columns to take advantage of different properties of the protein and give a purer product: for example, it executes a step of affinity chromatography to which a step of ion exchange chromatography follows.

Now, the protein is purified and you can perform the subsequent experiments in order to study it.

8.3 Mass Spectrometry (MS)

Assuming that we are face to face with an unknown protein, the next step in the proteomic world is mass spectrometry: a powerful analytical technique used to identify unknowns, for quantitative determinations of known compounds and to clarify the structural and chemical properties of molecules (Hiraoka, 2013).

One of the great advantages of the method is that it can be carried out with extremely limited sample amounts, even less than a picogram (Goodlett & Aebersold, 2001).

A mass spectrometer is an instrument used to measure the mass of a molecule after it has been ionized; actually, the tool does not directly measure the molecular mass, but the mass/charge ratio of ions that form the molecule under investigation (Fig. 8-2).

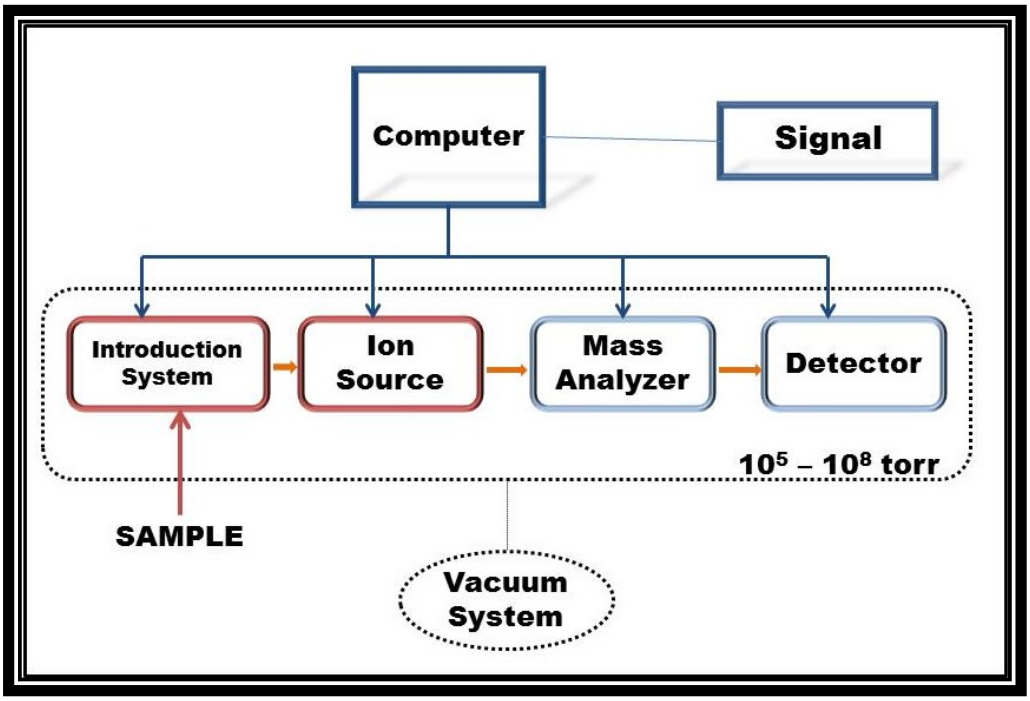

Figure 8-2. Scheme of a mass spectrometer.

9 Materials and Methods

9.1 Bacterial strains and culture conditions

The bacterial strains used in this study included 28 reference strains of *Lactobacillus* spp. or subspp. from the collection of our Laboratory of Applied Microbiology (Department of Biomedical and Biotechnological Sciences, Università degli Studi di Catania) (Table 9.1) and 359 clinical strains isolated from Italian volunteers (healthy men and women): oral, fecal and vaginal specimens (respectively 122, 125 and 112 samples).

9.2 Isolation from oral and fecal specimens

Oral swabs were collected from healthy volunteers by inserting the swab into the mouth and gently rubbing the cheek. Sampling was carried out on an empty stomach without performing operations of oral hygiene (brushing teeth or use of mouthwash). The swabs were placed in a tube containing Stuart transport medium (Oxoid, Thermo Fisher Scientific Inc.) and preserved at 4°C until inoculation (within 24 h). The swabs were streaked onto Man Rogosa Sharpe (MRS; Oxoid, Thermo Fisher Scientific Inc.) and Columbia blood (5% horse blood; bioMérieux) agar plates.

Rectal swabs were collected from healthy volunteers by inserting the swab into the rectum, through the anus, to a depth of about 2-4 cm, and then rubbing to make the fecal material adhere. The swab was maintained in the rectal bulb for 30 seconds, continuing to move and rotate it against the walls of the intestine; after which it was extracted and placed in a tube containing Stuart transport medium (Oxoid, Thermo Fisher Scientific Inc.) and preserved at 4°C until inoculation (within 24 h). Before storing, it was ascertained that the swab showed significant traces of fecal material.

The swabs were streaked onto LAMVAB (*Lactobacillus* Anaerobic MRS with vancomycin and bromocresol green) agar plates (home-made), highly selective because of low pH and vancomycin (20 mg/L) which inhibit competitive fecal microbiota such as enterobactiaceae, *Clostridium* spp., other Gram-negatives and also *Bifidobacterium* spp..

All plates were incubated at 37°C with partial CO_2 tension for 48 h. Some colonies were selected and picked from each plate where bacteria were grown and all of these were examined by Gram staining (Fig. 9-1), oxidase test and catalase test. 247 strains, which corresponded phenotypically with *Lactobacillus*, were preserved in MRS broth 10% glycerol at -80°C until DNA extraction.

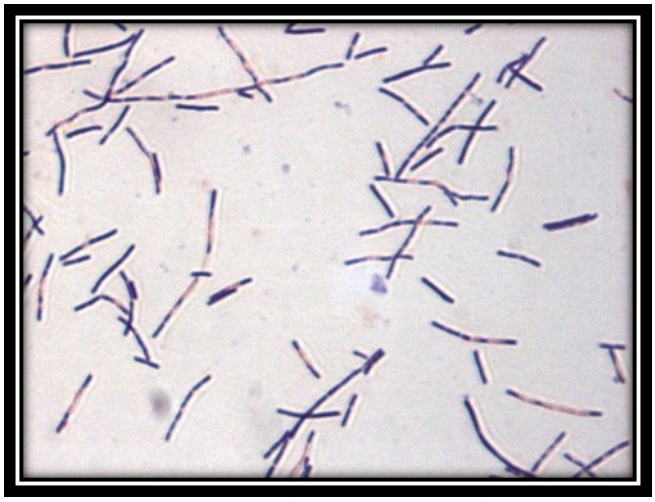

Figure 9-1. Lactobacilli seen under the microscope after Gram staining.

9.3 Isolation from vaginal specimens

The healthy volunteer women were between 18 and 45 years old; pregnant women, women under antibiotic treatment within a month and those that had had sexual activity within 5 days, were excluded. All samples were taken from the posterior zone of the fornix of the vagina using a sterile swab, which was placed in a tube containing Stuart transport medium (Oxoid, Thermo Fisher Scientific Inc.) and preserved at 4°C until inoculation (24 h). The swabs were streaked onto MRS (Oxoid, Thermo Fisher Scientific Inc.) and Columbia blood (5% horse blood; bioMérieux) agar plates. A thin smear was examined by Gram staining (Fig. 9-2). Plates were incubated at 37°C with partial CO_2 tension for 48 h.

Some colonies were selected and picked from each plate where bacteria were grown and all of these were examined by Gram staining, oxidase test and catalase test.

112 strains, which corresponded phenotypically with *Lactobacillus* species, were preserved in MRS broth 15% glycerol at -80°C until DNA extraction.

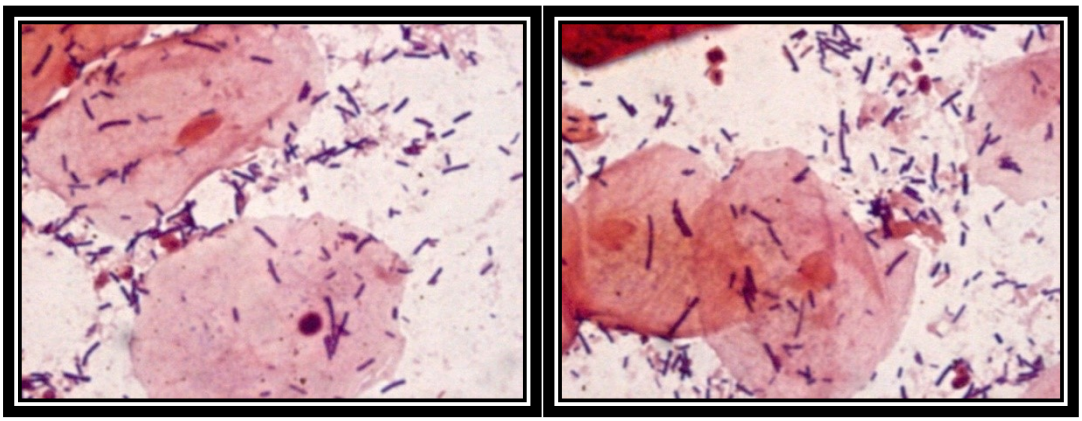

Figure 9-2. Vaginal smears examined under the microscope after Gram staining.

Table 9.3. List of Reference strains from the collection of Laboratory of Applied Microbiology.

Reference strain	DSMZ code	Other collection codes
Lactobacillus acidophilus	20079	ATCC 4356
Lactobacillus antri	16041	CCUG 48456
Lactobacillus brevis	20054	ATCC 14869
Lactobacillus casei	20011	ATCC 393
Lactobacillus coloheminis	14050	CCUG 44007
Lactobacillus concavus	17758	AS 1.5017
Lactobacillus crispatus	20584	ATCC 33820
Lactobacillus delbrueckii subsp. bulgaricus	20081	ATCC 11842
Lactobacillus delbrueckii subsp. delbrueckii	20074	ATCC 9649
Lactobacillus delbrueckii subsp. indicus	15996	LMG 22083
Lactobacillus delbrueckii subsp. lactis	20072	ATCC 12315
Lactobacillus fermentum	20052	ATCC 14931
Lactobacillus gasseri	20243	ATCC 33323
Lactobacillus gastricus	16045	CCUG 48454
Lactobacillus gastricus "wild"	16046	CCUG 48455
Lactobacillus intestinalis	6629	ATCC 49335
Lactobacillus jensenii	20557	ATCC 25258
Lactobacillus johnsonii	10533	ATCC 33200
Lactobacillus oris	4854	ATCC 49062
Lactobacillus parabuckneri	5707	ATCC 49374
Lactobacillus paracasei subsp. paracasei	5622	ATCC 25302
Lactobacillus paraplantarum	10667	CIP 104668
Lactobacillus plantarum	20174	ATCC 14917
Lactobacillus reuteri	20016	ATCC 23272
Lactobacillus rhamnosus	20021	ATCC 7469
Lactobacillus salivarius	20555	ATCC 11741
Lactobacillus vaginalis	5837	ATCC 49540
Lactobacillus zeae	20178	ATCC 15820

9.4 Susceptibility testing

Broth dilution test was used to perform antimicrobial susceptibility testing of *Lactobacillus* spp. isolates.

Broth dilution test to determine MIC values (Minimum Inhibitory Concentration), were carried out in 96 well polystyrene microplates (Bibby Sterilin), by microdilution assay as reported in the CLSI M100 – S24 (CLSI, 2014). CAMHB (Cation Adjusted Muller Hinton Broth, with 2.5-5% of horse lysed blood) was replaced with LSM broth (Oxoid, Thermo Fisher Scientific Inc.) because most of the *Lactobacillus* strains grow poorly in CAMHB (Klare et al., 2005). For each strain, a 0.5 McFarland bacterial suspension was set up, and starting from the latter dilutions in broth were prepared, in order to obtain a final concentration of 10^3-10^4CFU/mL. The stock solution of antibiotics (erythromycin, clindamycin, ampicillin and kanamycin), were prepared as reported in the CLSI guidelines: antibiotics in powder were dissolved in suitable solvent, to give a final concentration of 5120 μg/mL. All dilutions were set up in macrovolumes, and then distributed in the microplates according to CLSI. The concentration range studied for all antibiotics started at the values of cut-off considered by EFSA (EFSA FEEDAP), and eight dilutions only were tested: i.e. with the cut-off of the range was 1 μg/mL – 0.008 μg/mL. The microplates were inoculated with 100 μl of each dilution from column 1 to column 8, columns 9 and 10 were inoculated sterility controls, and finally in column 11 was inoculated the growth control (the inoculated strain in broth in the absence of antibiotic). The strains were tested six times for each antibiotic drug, and six additional times in a different day for all antibiotics to ascertain the reproducibility.

Enterococcus faecalis ATCC 29212 was used as positive internal control (CLSI, M100-S22). The microplates were incubated at 37°C overnight in microaerobic conditions. The MICs were read as the lowest concentration of the antibiotic that inhibited the growth of the microorganism.

9.5 Evaluation of resistance to low pH and bile salts

The method chosen to evaluate the resistance to low pH, and bile salts was established following our pilot project, (Fuochi et al., 2015) that included bacterial counts and relative survival rate after stressed growth under acidic conditions and in the presence of different concentrations of porcine bile salts, following a modified protocol by Bolado-Martinez et al. (2009) (Fig. 9-3).

Each strain of *Lactobacillus* spp. and one strain of *E. coli* ATCC 25218, used as positive control, were grown in two different sets for 48 h at 37°C in 10 mL of LSM broth (0.05% cysteine w/v). After incubation, the tubes were centrifuged at room temperature for about 20 min. For the first set of tubes:

- supernatants were removed, and the pellets were suspended in 10 mL saline;
- serial ten-fold dilutions in NaCl (0.85% w/v) were done;
- each dilution was spread on LSM agar (cysteine 0.05% w/v) as a control, and on LSM agar (cysteine 0.05% w/v) with porcine bile salts at different concentrations (0.5%, 0.25%, and 0.12% w/v) to ascertain the resistance to bile salts;
- all plates were incubated for 48 h at 37°C in microaerobic atmosphere.

Resistance to bile salts was expressed as the percentage of surviving cells: CFUs found on LSM agar with porcine bile salts against colonies grown on LSM agar control.

For the second set of tubes, after centrifugation, supernatants were discarded and the pellets were suspended in LSM broth at pH 3.0. The cultures were incubated for 1 h at 37°C under stirring (to simulate the transit through the stomach). Then, as for the first set, supernatants were removed by centrifugation, each pellet was suspended in 10 mL saline, serial ten-fold dilutions in NaCl (0.85% w/v) were done, and each dilution was spread on LSM agar (cysteine 0.05% w/v) as a control, and on LSM agar (cysteine 0.05% w/v) with porcine bile salts (0.5%, 0.25%, and 0.12% w/v).

Finally, the plates were incubated at 37°C in a microaerobic atmosphere for 48 h, at the end of which the counts were made. For each strain, ten independent experiments were performed. For each independent experiment two different runs were performed. One run was carried out with an initial controlled concentration of the strains (10^6 CFU/mL)

but plates had no visible colonies, thus swabs were taken from the plates where colonies were not visible and spread onto LSM agar. This was done to evaluate if the effect of bile salts were bactericidal or bacteriostatic. The plates were incubated for 24 h at 37°C in microaerobic atmosphere, after which visible colonies of lactobacilli were evaluated. Therefore, there was a bacteriostatic effect even at the maximum concentration of bile salts (0.5%). Because of the lack of growth, we proceeded to the second run of the experiment with LSM agar in decreasing concentrations of bile salts (0.5%, 0.25%, 0.12% w/v), but in the absence of cysteine and having an initial concentration between 10^7-10^{11} CFU/ml (Fuochi et al., 2015).

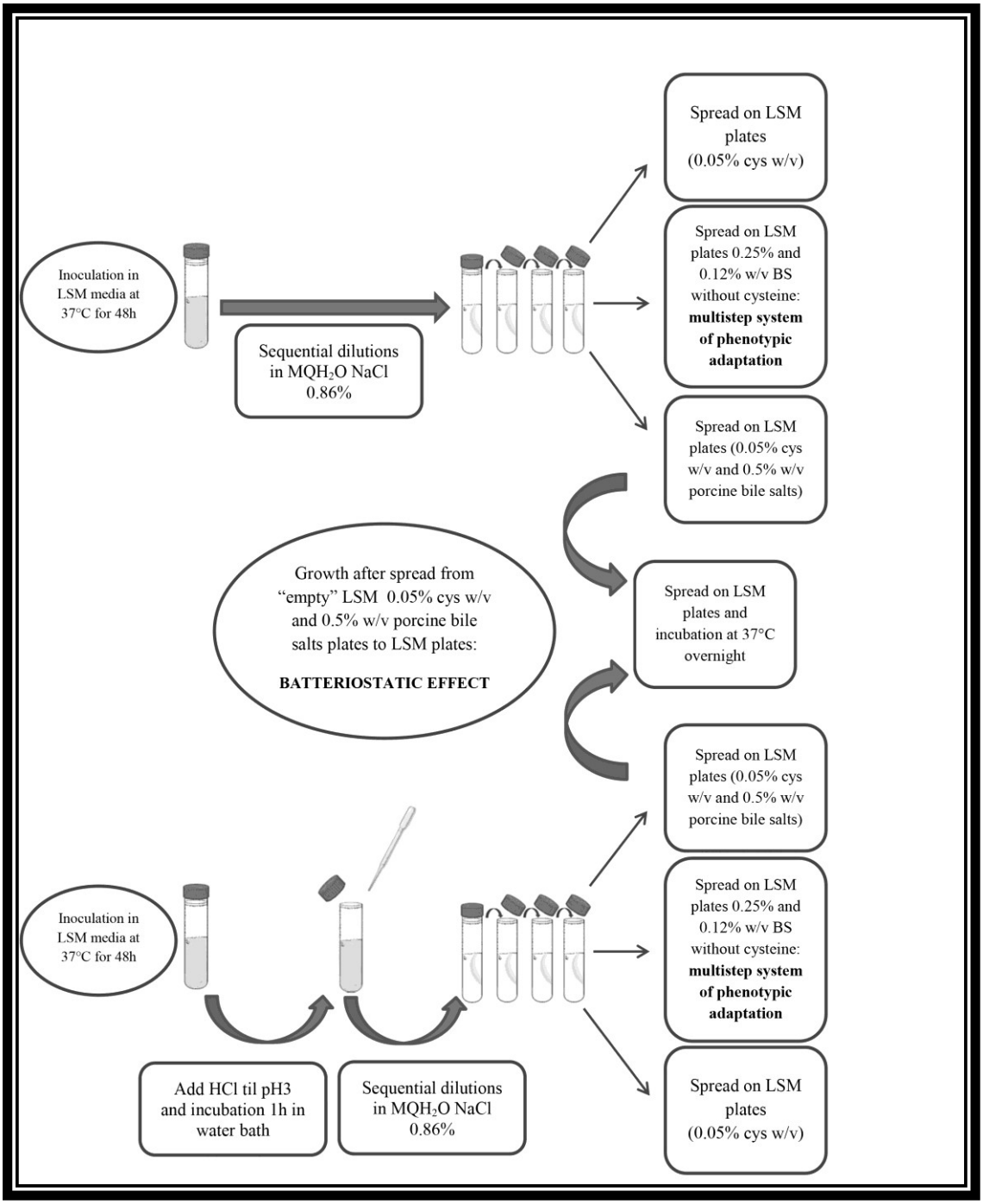

Figure 9-3. Tolerance evaluation procedure to low pH and bile salts (Fuochi et al., 2015).

9.6 Evaluation of the capacity of *Lactobacillus* strains to produce hydrogen peroxide.

MRS agar medium with horseradish peroxidase and TMB was used for the evaluation and quantification of the production of hydrogen peroxide by isolated Lactobacilli strains. To prepare 1 liter of MRS agar medium (Oxoid, Thermo Fisher Scientific Inc.) 62 g of powder were dissolved in MQ water and sterilized by autoclaving at 121°C for 15 min (Solution A). Separately, 10.0 mg of peroxidase from horseradish (Sigma P6782) were dissolved in 10.0 mL of MQ water at room temperature, and sterilized by filtration using a 0.22 µl filter, and finally, 250.0 mg of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma T2885) were dissolved in ≈5 mL of ethanol ≥99.8 %, slightly heated, (Solutions B and C, respectively). After sterilization, solution A was cooled to 50°C, and then solutions B and C were added.

The plates were filled immediately after thorough mixing and stored, protected from light, at +4°C before use (stable for max 14 days).

The test was performed by applying a 0.5 McFarland bacterial inoculum (approx. $1.5x10^8$ CFU/mL) to the surface of MRS agar modified and incubated at 37°C for 48 h with partial CO_2 tension, and finally, at room temperature for 24 h in the dark.

Negative or positive classification of the colony for hydrogen peroxide production was performed as follows: white colonies as negative, and colonies of any other color (from brown to dark blue) as positive. Instead, the rate of accumulation was calculated as the mean percentage fraction of positive colonies from the total number of CFU.

9.7 DNA Extraction

DNA was extracted with RTP Bacterial DNA Mini Kit (Stratec Biomedical AG) following the manufacturer's protocol:

- an aliquot of the bacteria culture was taken and spun down at 11,000 g for 3 min, then the supernatant was carefully removed;
- 400 µl of Resuspension Buffer R were added to the pellet and resuspended by pipetting up and down. The samples were transferred into the Extraction Tube and gently vortexed;
- Each of the samples was incubated in a thermomixer for 10 min at 37°C, at 65°C for 10 min and 95°C for 8-10 min;
- 400 µl of Binding Buffer B6 were added to the samples;
- The samples were loaded onto the RTA Spin Filter Set and incubated for 1 min, then they were centrifuged at 11,000g for 2 min. The filtrates were discarded;
- 500 µl of Wash Buffer I were added and centrifuged at 11,000 g for 1 min. The filtrates were discarded;
- 600 µl of Wash Buffer II were added and centrifuged at 11,000 g for 1 min. The filtrates were discarded, and then a final centrifugation for 4 min at max. speed to completely remove the ethanol, was carried out.
- RTA Spin Filters were placed into new 1.5 ml Receiver Tubes and 200 µl of Elution Buffer were added. They were incubated for 1 min at room temperature, centrifuged for 1 min at 11,000 g and finally the eluted bacterial DNA was stored at -20°C.

DNA samples were visualized by electrophoresis (70V) in agarose gel 1.5% in 1X TAE buffer (Tris, Acetic Acid, EDTA), stained with Sybr Safe® 1X (Invitrogen ™) and observed on transilluminator Safe Imager (Invitrogen™).

9.8 DNA Quantitation Using Spectrophotometer

DNA samples were subjected to a spectrophotometric analysis (NanoDrop® 2000, Thermo Scientific NanoDrop Products). 2µl of DNA were placed on the lower optical surface, and the measurement was done.

Ratios 260/280 nm and 260/230 nm were analyzed in combination to accurately assess the quality of the sample. Pure DNA gives a ratio of 260/280 nm equal to ~ 1.8 ng/mL. The ratio of 260/230 nm is a second measurement of purity; values of a pure nucleic acid enter in the range of 1.8-2.2 ng/mL.

9.9 16S rDNA/RFLP Amplifications

To each sample a reaction mix (100 µL) was set up, containing reagents described in Table 9.2:

Table 9.2. Reagents used for rDNA/RFLP reaction mix.

Reagents	**Concentration**
Buffer (Biotools™) [10mM Tris-HCl (pH 8.0), 50 mM, KCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)]	1X
MgCl2 (Biotools™)	3mM
dNTPmix (TaKaRa™)	0.2mM
Primers (Invitrogen™) 7FW (5'-AGAGTTTGATC/TA/CTGGCTCAG-3') 1510REV (5'-ACGGC/TTACCTTGTTACGACTT-3')	0.4mM
Taq polimerase (Biotools™)	1.5U
H_2O	Up to vol.
DNA	15-20ng

The amplification program followed is described in Table 9.3 (Randazzo et al., 2004).

Table 9.3. Amplification Program.

Step		**°C**	**Time**
Denaturation		94	3 min.
30 cycles	Denaturation	94	30 sec.
	Annealing	52	30 sec.
	Elongation	72	90 sec.
Final elongation		72	7 min.

The amplifications obtained (approx. 1500 bp) were visualized by electrophoresis (70V) in agarose gel 1.5% in 1X TAE buffer (Tris, Acetic Acid, EDTA), stained with Sybr Safe® 1X (Invitrogen ™) and observed on a transilluminator Safe Imager (Invitrogen™). DNA ladder 1Kb (BioRad™) was used (Fig. 9-4). The amplifiers were digested at 37°C for 2 h, with the following restriction enzymes: HaeIII (Fig. 9-5), AluI, MspI (Fig. 9-6) (Invitrogen™). The mix of digestion (20µL) contained 10µL of PCR product, 1U of restriction endonuclease with the appropriate buffer (1X) and ultrapure water up to volume. The digested product was fully loaded in agarose gel 1.5%, colored with Sybr Safe® 1X (Invitrogen ™) and observed on a transilluminator Safe Imager (Invitrogen™). The restriction profiles of the strains were compared with those obtained for *Lactobacillus* type strains (Table 9.1) in order to identify each strain.

The cleavage sites, of the three restriction enzymes, are respectively: 5 'GC ↑ ... CC ... 3' for HaeIII, 5 '... AG ↑ CT ... 3' for MspI,5 '... C ↑ CGG ...3' for AluI.

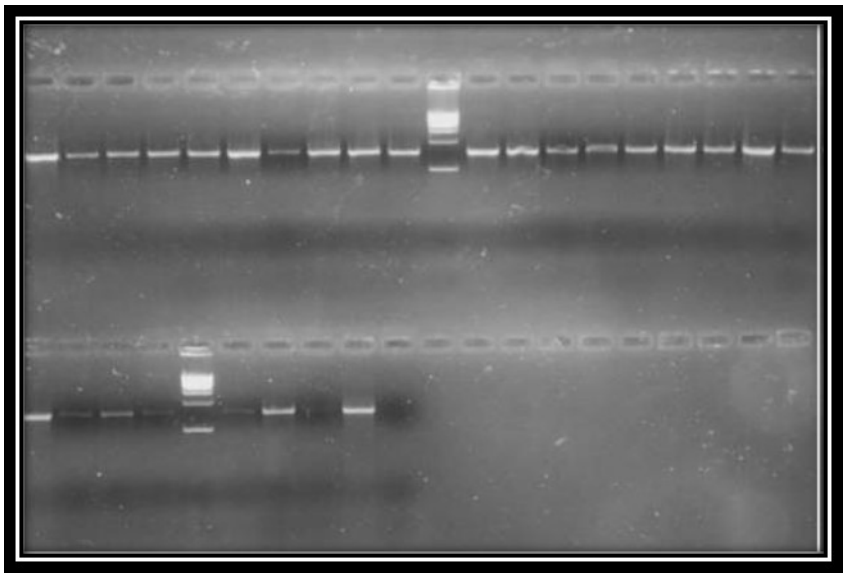

Figure 9-4. Electrophoretic run of 16S rDNA, amplified.

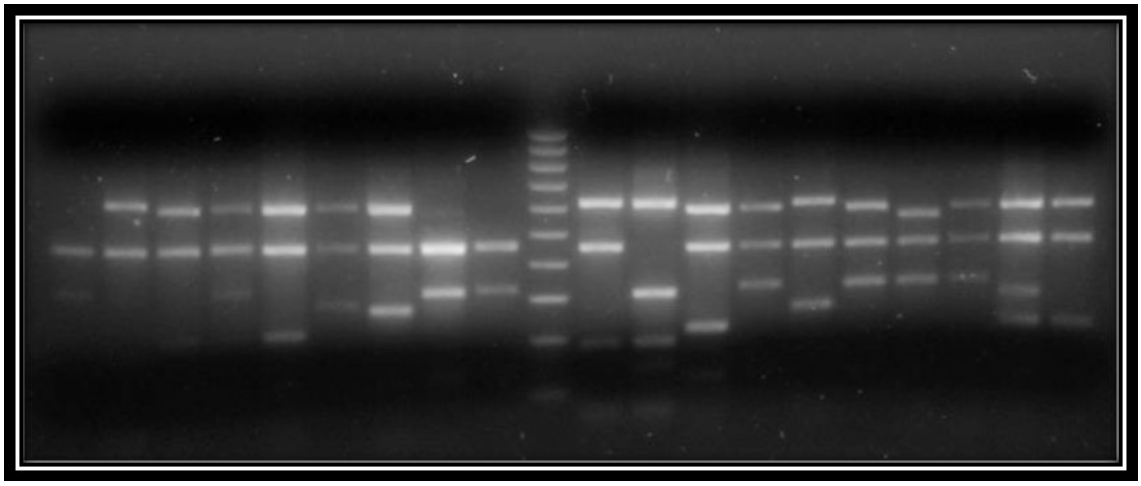

Figure 9-5. Electrophoretic run after digestion with *HaeIII*.

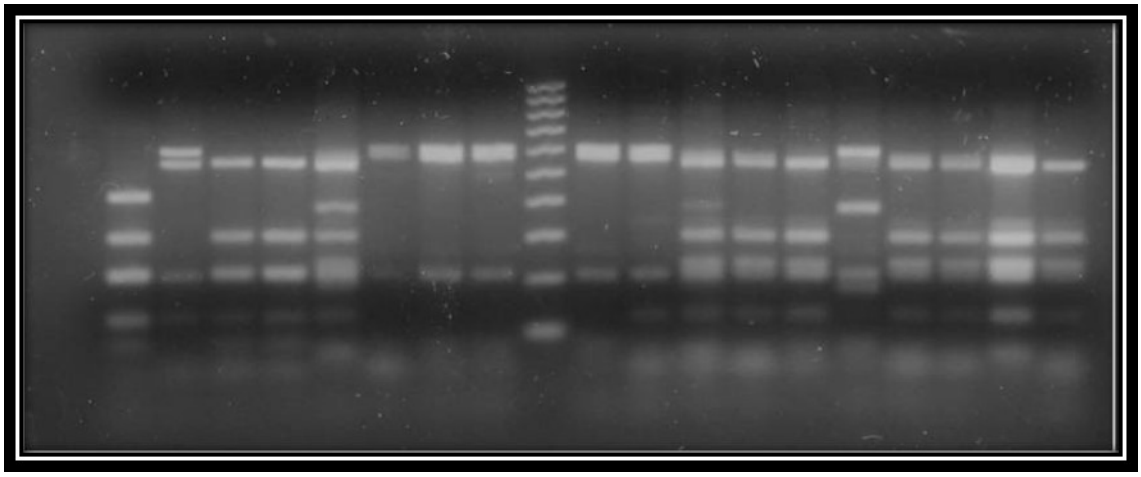

Figure 9-6. Electrophoretic run after digestion with MspI.

Table 9.4. Virtual restriction maps of 16S rRNA gene deposited in NCBI database.

Species	*16S rDNA* (bp)	HaeIII*	MspI*	AluI*
L. acidophilus	1553	(8) 242-35-44-22-139-459-457-55-100	(6) 177-389-606-11-211-53-106	(9) 59-146-20-33-186-429-221-44-208
L. antri	1520	(7) 62-191-78-620-312-145-55-57	(8) 24-552-88-518-11-71-48-92-116	(7) 268-186-429-105-102-189-204-37
L. brevis	1430	(5) 227-44-22-618-457-82	(6) 516-68-538-11-211-53-33	(5) 823-105-102-207-193
L. delbrueckii subsp. bulgaricus	1561	(5) 280-44-22-598-457-160	(6) 180-389-289-317-11-211-164	(7) 64-164-33-186-636-221-44-213
L. casei	1517	(4) 320-22-598-457-120	(5) 565-606-11-211-53-71	(5) 224-33-615-207-207-231
L. colehominis	1564	(7) 65-191-78-23-596-457-55-99	(8) 27-551-68-234-304-11-211-158	(6) 271-494-120-105-102-207-265
L. concavus	1528	(5) 313-22-139-458-457-139	(5) 558-605-11-211-53-90	(7) 217-33-615-206-207-14-169-67
L. confusus	1525	(2) 347-22-1156	(7) 437-155-376-11-211-53-52	(6) 96-188-370-245-207-207-212
L. crispatus	1518	(7) 226-79-22-139-459-457-55-81	(6) 161-389-606-11-211-53-87	(8) 189-20-33-219-615-207-221-233
L. fermentum	1364	(5) 67-225-44-22-598-408	(6) 29-552-68-234-304-11-166	(5) 273-186-429-105-269
L. gasseri	1747	(5) 405-22-139-459-457-261	(7) 266-388-68-538-11-211-53-212	(8) 40-15-85-206-186-429-207-275-314
L. gastricus	1550	(6) 48-224-44-22-598-457-157	(6) 10-551-302-304-11-211-161	(5) 253-186-429-105-102-475
L. iners	1539	(5) 276-44-22-598-457-142	(8) 177-388-68-96-442-11-211-53-93	(6) 257-186-217-212-207-265-195
L. intestinalis	1513	(6) 306-22-139-459-457-55-75	(7) 162-389-59-547-11-211-53-81	(9) 44-134-32-33-186-429-207-221-44-183
L. jensenii	1496	(5) 288-22-139-459-457-131	(5) 144-389-606-11-211-135	(10) 26-40-88-38-32-187-429-207-221-44-184
L. johnsonii	1487	(5) 302-22-139-459-457-108	(7) 159-388-68-538-11-211-53-59	(6) 33-206-186-429-207-265-161
L. oris	1359	(7) 134-78-234-384-311-145-55-18	(9) 457-88-375-140-11-71-48-92-60-17	(4) 149-364-455-186-205
L. parabuchneri	1497	(5) 272-44-22-598-458-103	(6) 561-68-539-11-211-53-54	(4) 253-615-208-207-214
L. paracasei	1558	(4) 153-457-598-22-328	(5) 102-53-211-11-606-575	(5) 264-207-207-615-33-232
L. paraplantarum	1502	(5) 309-22-564-34-457-116	(4) 554-606-11-211-120	(5) 246-615-105-102-207-227
L. plantarum	1555	(5) 327-22-564-34-457-151	(4) 572-606-11-211-155	(6) 213-51-615-105-102-207-262
L. reuteri	1571	(5) 67-269-620-457-55-103	(8) 29-125-427-88-214-304-11-211-162	(7) 273-186-429-105-102-195-14-269
L. rhamnosus	1540	(4) 306-22-597-457-158	(5) 550-606-11-211-53-109	(5) 210-33-614-207-207-269
L. salivarius	1570	(4) 279-44-620-457-170	(7) 568-68-538-11-211-53-117-4	(4) 875-207-207-268-13
L. vaginalis	1541	(6) 42-190-78-621-457-55-98	(6) 555-89-214-304-11-211-157	(7) 247-186-430-105-102-207-231-33
L. zeae	1559	(4) 324-22-598-457-158	(5) 569-606-11-211-53-109	(5) 228-33-615-207-207-269

***in brackets the number of cuts.**

9.10 Two-steps multiplex PCRs: *16S-ITS-23S* and the flanking region of *23S rDNA*

On the basis of nucleotide sequences in the ISR region and 23S rDNA, different primers were designed in order to split *Lactobacillus* species into 4 groups (Multiplex PCR-G). The subsequent multiplex PCRs (II-1, II-2, III and IV) were carried out with the use of primers designed on the basis of the alignments of the 16S-ITS-23S rDNA sequences and the flanking region of 23S rDNA. Indeed, Song et al. (2000) were able to draw species-specific primers, which generate amplicons of significantly different amplitudes, to discern eleven different species (Fig. 9-7).

Table 9.5. Primer sequences for multiplex PCRs (Song et al., 2000).

Primers	Sequence (5'→3')		Multiplex
Ldel-7	ACAGATGGATGGAGAGCAGA	FW	G
LU-1'	ATTGTAGAGCGCGACCGAGAAG	FW	G
LU-3'	AAACCGGAGAACACCGCGTT	FW	G
LU-5	CTAGCGGGTGCGACTTTGTT	FW	G; III
Laci-2	CCTCTTCGCTCGCCGCTACT	REV	G
Laci-1	TGCAAAGTGGTAGCGTAAGC	FW	IIA
Ljens-3	AAGAAGGCACTGAGTACGGA	FW	IIA
2310-C	CCTTTCCCTCACGGTACTG	REV	IIA
Lcri-1	AGGATATGGAGAGCAGGAT	FW	IIB
Lcri-2	CAACTATCTCTCTTACACTGCC	REV	IIB
Lgas-2	TGCTATCGCTTCAAGTGCTT	FW	IIB
Lgas-3	AGCGACCGAGAAGAGAGAGA	REV	IIB
Lpar-4	GGCCAGCTATGTATTCACTGA	REV	III
RhaII	GCGATGCGAATTTCTATTATT	REV	III
Lfer-3	ACTAACTTGACTGATCTACGA	FW	IV
Lfer-4	TTCACTGCTCAAGTAATCATC	REV	IV
Lpla-3	ATTCATAGTCTAGTTGGAGGT	FW	IV
Lpla-2	CCTGAACTGAGAGAATTTGA	REV	IV
Lreu-1	CAGACAATCTTTGATTGTTTAG	FW	IV
Lreu-4	GCTTGTTGGTTTGGGCTCTTC	REV	IV
Lsal-1	AATCGCTAAACTCATAACCT	FW	IV
Lsal-2	CACTCTCTTTGGCTAATCTT	REV	IV

In particular, the amplified products obtained with the multiplex PCR-G are 450 bp, 300bp, 400bp, 350bp, for Group I, II, III and IV, respectively.

For Group I other multiplexes were not set up, since the members belonging to this group have all been brought back to *delbrueckii* species, and it was not possible to discriminate subspecies. For Group II, two separated mixes were prepared, making two multiplex-PCR (II-1 and II-2), and getting the discrimination of the species *L. acidophilus* (210bp) and *L. jensenii* (700bp) with the multiplex II-1; *L. crispatus* (522bp) and *L. gasseri* (360bp) with the multiplex II-2. The species *L. rhamnosus* (113bp) and *L. paracasei* (312bp) were identified with the multiplex III. Finally, the multiplex IV identified four species, *L. fermentum* (192bp), *L. salivarius* (411bp), *L. reuteri* (303bp) and *L. plantarum* (248bp). The mixes (50µL each) were prepared as shown in Table 9.6, and the amplification programs, used for each multiplex PCR, are described in Table 9.7.

Table 9.6. Reagents used for multiplex reaction mixes.

Reagents	**Concentration**	**MultiplexPCR**
Buffer (Biotools™) [10mM Tris-HCl (pH 8.0), 50 mM, KCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)]	1X	G, IIA, IIB, III, IV
MgCl2 (Biotools™)	2mM	G
	2.5mM	IIA, IIA, IIB, III, IV
dNTP (TaKaRa™)	1mM	G, IIA, IIB, III, IV
Primer (Invitrogen™)	0.4mM	G
	0.3mM	IIA, IIB, III, IV
Taq polimerase (Biotools™)	1U	G, IIA, IIB, III, IV
H_2O	Up to volume	G, IIA, IIB, III, IV
DNA	10-15ng	G, IIA, IIB, III, IV

Table 9.7. Amplification Programs for the multiplex PCRs.

Step		°C	Time (min)	Multiplex-PCR
Denaturation		94	5	G, IIa,IIb, III, IV
30 cycles	Denaturation	95	0.5	G, IIa,IIb, III, IV
	Annealing	55	2	G
		68		IIA
		65		IIB
		62		III
		60		IV
	Elongation	72	0.5	G, IIa, IIb, III, IV
Final elongation		72	10	G, IIa, IIb, III, IV

The amplifications obtained were visualized by electrophoresis (70V) in agarose gel 1.5% in 1X TAE buffer (Tris, Acetic Acid, EDTA), stained with Sybr Safe® 1X (Invitrogen ™) and observed on a transilluminator Safe Imager (Invitrogen ™). DNA ladder 1Kb (BioRad ™) was used as reference.

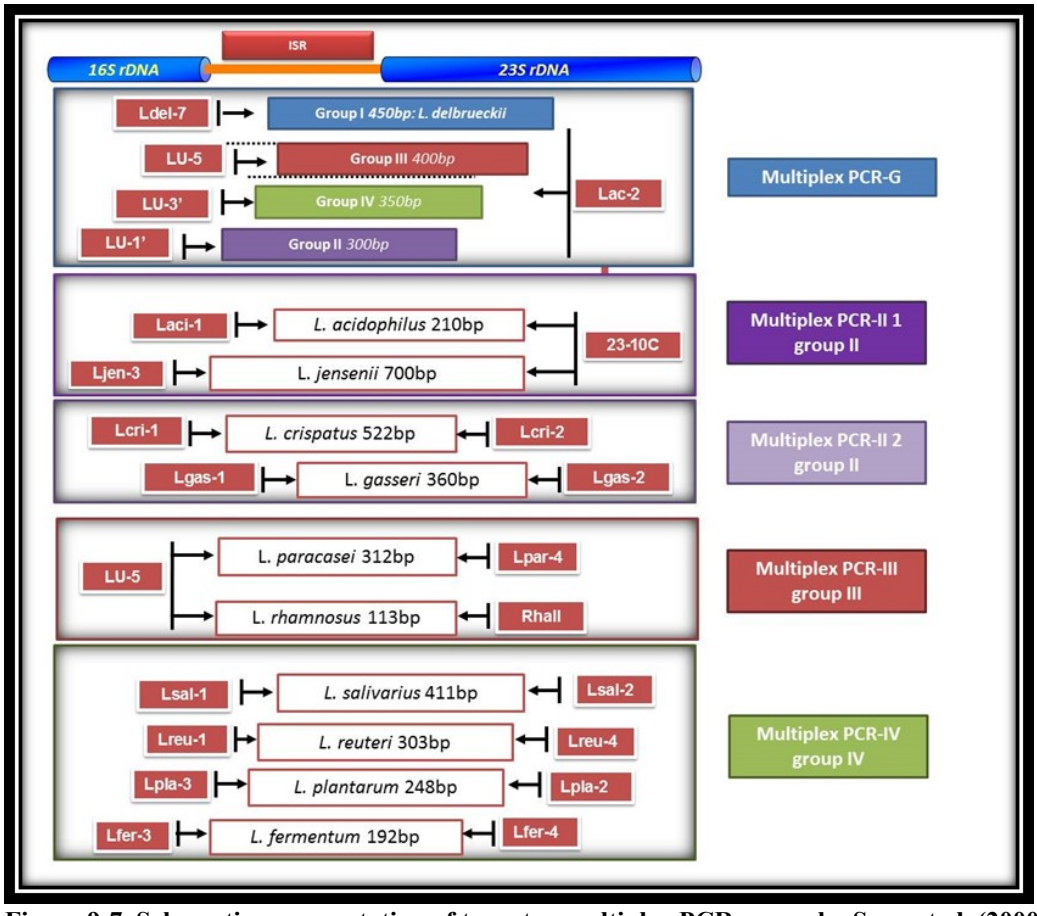

Figure 9-7. Schematic representation of two-step multiplex PCR assays by Song et al. (2000).

9.11 *tuf* gene amplification

The reaction mix (50 µL), for amplification of regions contained within the *tuf* gene, is described in Table 9.9 using the primers reported in Table 9.8 following the amplification program shown in Table 9.10 (Ventura et al., 2003).

Table 9.8. Primer sequences for *tuf* gene PCR (Ventura et al., 2003).

Primers		**Sequence 5'→3'**
CPR	REV	CAANTGGATNGAACCTGGCTTT
Par	FW	GACGGTTAAGATTGGTGAC
Cas	FW	ACTGAAGGCGACAAGGA
Rha	FW	GCGTCAGGTTGGTGTTG

Table 9.9. Reagents used for reaction mix.

Components	**Concentration**
Buffer (Biotools™) [10mM Tris-HCl (pH 8.0), 50 mMKCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)]	1X
$MgCl_2$ (Biotools™)	1.5mM
dNTPmix (TaKaRa™)	0.8mM
Primers (Invitrogen™)	0.2mM
Taq polimerase (Biotools™)	1.5U
H_2O	Up to vol.
DNA	10-15ng

Table 9.10. Amplification program for *tuf* gene PCR.

Step		**°C**	**Time**
Denaturation		95	5 min.
30 cycles	Denaturation	95	30 sec.
	Annealing	60	30 sec.
	Elongation	72	90 sec.
Final elongation		72	10 min.

The amplifications obtained were visualized by electrophoresis (70V) in agarose gel 2.5% in 1X TAE buffer (Tris, Acetic Acid, EDTA), stained with Sybr Safe® 1X (Invitrogen™) and observed on a transilluminator Safe Imager (Invitrogen™). The amplified products obtained were 540 bp for *L. rhamnosus,* 350 bp for *L. casei* and 200 bp for *L. paracasei* (Fig. 9-8).

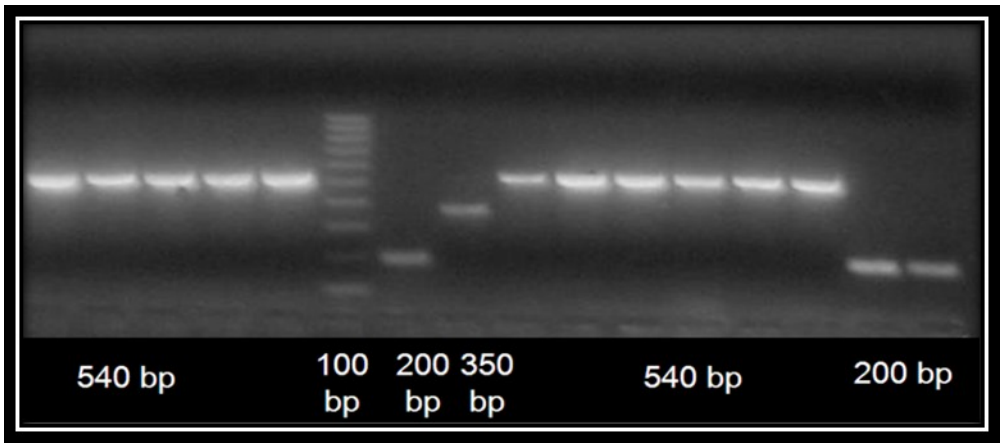

Figure 9-8. Amplification products of *tuf* gene: *L. rhamnosus* (540 bp), *L. casei* (350bp) and *L. paracasei* (200 bp).

9.12 Nested PCR-amplifications of bacterial V1-V3: DHPLC (WAVE® System) analysis.

Prior to DHPLC analysis on the WAVE® Microbial Analysis System (ADS Biotec, Omaha, NE), bacterial 16S rDNA was PCR-amplified using a nested PCR protocol:

- A first amplicon of ≈500bp (V1-V3) were generated using 15-20 ng of bacterial genomic DNA and LAC-V1F and LAC-V3R as respectively FW and Rev primers (Table 9.11).

  Amplicons length were controlled by electrophoresis run in agarose gel 0.9%

  V1-V3 amplicons were collected by DHPLC collector FCW 200 (ADS Biotec, Omaha, NE); see below collection protocol. 5-10 ng of these products were used as substrate for the second PCR (V1-V1) after spectrophotometric analysis by NanoDrop® 2000 (Thermo Scientific NanoDrop Products). Amplification was carried out using LAC-V1F and LAC-V1R as primers to generate an amplicons of ≈130bp. Amplicons length were controlled by electrophoresis run in agarose gel 0.9%.

All primers are complementary to regions flanking the variable regions V1–V3 of 16S rRNA gene (Fig. 9-9) and they were designed on the basis of 16 rDNA sequences of 25 *Lactobacillus* type strains previously reported (Table 9.1). For this purpose software MEGA ver.7 (Molecular Evolutionary Genetics Analysis) and Primer3Plus were used.

PCR reaction mix is described in Table 9.12 and the amplification protocol used is shown in Table 9.13.

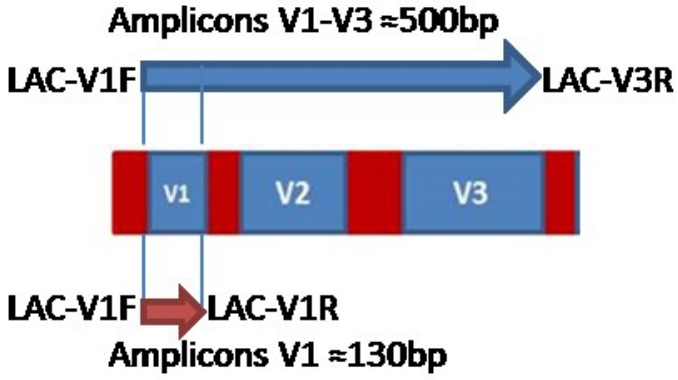

Figure 9-9. Nested PCR V1-V3 16S rDNA.

Table 9.11. 16S rRNA gene primers used for DHPLC analysis.

Primers		Target Region*	Sequence 5'→3'	Ref.
LAC-V1F	FW	33-53 (V1)	GGCGTGCCTAATACATGCAA	Similar to Klijn, Weerkamp, & de Vos, 1991
LAC-V1R	REV	100-119 (V1)	TGCCCACGTGTTACTCACC	This study
LAC-V3R	REV	499-519 (V3)	GGCTGCTGGCACGTAGTTAG	This study

* Escherichia coli ATCC25922 16S rRNA gene numbering.

Table 9.12. Reagents used for 16S rRNA gene amplification mix.

Components	Concentration
Buffer 5X iProof HF (BioRad™)	1X
dNTPmix (TaKaRa™)	0.2mM
Primers (Invitrogen™)	0.5mM
iProof DNA Polymerase (BioRad™)	0.02U/µl
H_2O	Up to vol.
DNA	10-15ng (V1-V3) 5-10ng (V1)

Table 9.13. Amplification Protocol V1-V3 and V1 16S rRNA gene for DHPLC analysis.

Step		°C	Time
Denaturation		98	30 sec.
31 cycles	Denaturation	90	10 sec.
	Annealing	60	15 sec.
	Elongation	72	30 sec.
Final elongation		72	5 min.

9.12.1 DHPLC collection protocol

10 µl of purified PCR products containing bacterial V1-V3 16S rRNA gene segments were size fractionated by DHPLC on a DNASep® HT cartridge (ADS Biotec, Omaha, NE), and collected with the fragment collector FCW 200 (ADS Biotec, Omaha, NE). The collection was achieved by application of a specifically developed gradient (Table 9.14) under non-denaturing conditions (50°C) and a flow rate of 0.9 mL/min.

All gradients in this study were formed with WAVE® Optimized Buffer A (0.1 M TEAA) and WAVE® Optimized Buffer B (0.1 MTEAA in 25% acetonitrile v/v). Collected PCR products were detected and visualized with a UVL2400 detector. The analysis was performed with Navigator™ software ver. 3.1.0 (ADS Biotec, Omaha, NE).

Positive fractions were dried under vacuum until complete removal of the eluent (Concentrator 5301, Eppendorf, Hamburg, Germany) and reconstituted in water to a final concentration of 5-10ng/µl.

Table 9.14. Collection gradient-run.

Gradient	Time (min)	Buffer A%	Buffer B%	Flow rate (mL/min)	Gradient duration time (min)	Temperature
Loading	0.0	43.9	56.1	0.9		50°C
Start Gradient	0.5	38.9	61.1		4.5	
Stop Gradient	5.0	29.9	70.1			
Start Clean	5.1	43.9	56.1			
Stop Clean	5.2	43.9	56.1			
Start Equilibrate	5.3	43.9	56.1			
Stop Equilibrate	5.8	43.9	56.1			

9.12.2 Mutation analysis

V1-V3 and V1 amplicons were analyzed by DHPLC under denaturing conditions with a gradient run as described in Table 9.15 and Table 9.16 respectively.

Table 9.15. V1-V3 analysis gradient-run.

Gradient	Time (min)	Buffer A%	Buffer B%	Flow rate (mL/min)	Gradient duration time (min)	Temperature
Loading	0.0	46.4	53.6	0.9		59°C
Start Gradient	0.5	41.4	48.6		4.5	
Stop Gradient	5.0	32.4	67.6			
Start Clean	5.1	46.4	53.6			
Stop Clean	5.2	46.4	53.6			
Start Equilibrate	5.3	46.4	53.6			
Stop Equilibrate	5.8	46.4	53.6			

Table 9.16. V1 analysis gradient-run.

Gradient	Time (min)	Buffer A%	Buffer B%	Flow rate (mL/min)	Gradient duration time (min)	Temperature
Loading	0.0	59.6	40.4	0.9		59.5°C
Start Gradient	0.5	54.6	45.4		4.5	
Stop Gradient	5.0	45.6	54.4			
Start Clean	5.1	59.6	40.4			
Stop Clean	5.2	59.6	40.4			
Start Equilibrate	5.3	59.6	40.4			
Stop Equilibrate	5.6	59.6	40.4			

9.13 Detection of inhibitory activity by agar-well diffusion assay

Ten human clinical isolates were investigated for the production of substances with antibacterial activity: *L. crispatus* (1), *L. fermentum* (2), *L. gasseri* (2), *L. gastricus* (2), *L. paraplantarum* (1), *L. reuteri* (1), and *L. salivarius* (1).

The related type strains (*L. crispatus* DSMZ 20584, *L. fermentum* DSMZ 20052, *L. gasseri* DSMZ 20243, *L. gastricus* DSMZ 16045, *L. gastricus* DSMZ 16046, *L. paraplantarum* DSMZ 10667, *L. reuteri* DSMZ 20016, *L. salivarius* DSMZ 20555) were investigated with the same procedures.

These strains were grown by induction of stressed conditions (33°C, 18 h), in different media such as MRS, LSM, and M17, and also, in the presence of an "inducer" (pathogens killed by tyndallization (Table 9.17) and/or glycerol). The inducer with pathogens was constituted by MRS containing type strains such as *S. aureus*, *P. aeruginosa, E. coli* and *E. faecalis* in a concentration equal to $1.5x10^4$.

In particular, different mediums were prepared: LSM (90% Isosensitest broth, 10% MRS broth w/v), LSM 20% glycerol, MRS, MRS 20% glycerol, MRS 20% glycerol 0.12% pathogens GM17 (M17 0,4% glucose), GM17 20% glycerol, and GM17 20% glycerol 0.12% pathogens.

After 18 h at 33°C, the cells were separated by centrifugation at 8,000 rpm for 20 min at 4°C. Cells were stored separately with H_2O (0.9% NaCl, 15% glycerol) at -30°C. Wells (8 mm of diameter) were made on pre-inoculated MH agar media with stainless steel rods (Fig. 9-10, 9-11), and each well was inoculated with 200 µl of cell-free supernatant (filtered with 0.22 µm pore size) after neutralization with NaOH (Toba et al., 1991). Inhibitory activity was performed against certain Gram positive and Gram negative organisms: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Sarcina lutea* ATCC 9341 and *Staphylococcus aureus* ATCC 25923. Some supernatants were also tested against *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 43816.

For quality control non fastidious pathogens ciprofloxacin was used as positive internal antibiotic-control (CLSI, M100-S22).

The plates were incubated at 37°C overnight and then the zones of inhibition were measured in mm by a gauge.

Figure 9-10. Stainless steel rods 8mm diameter.

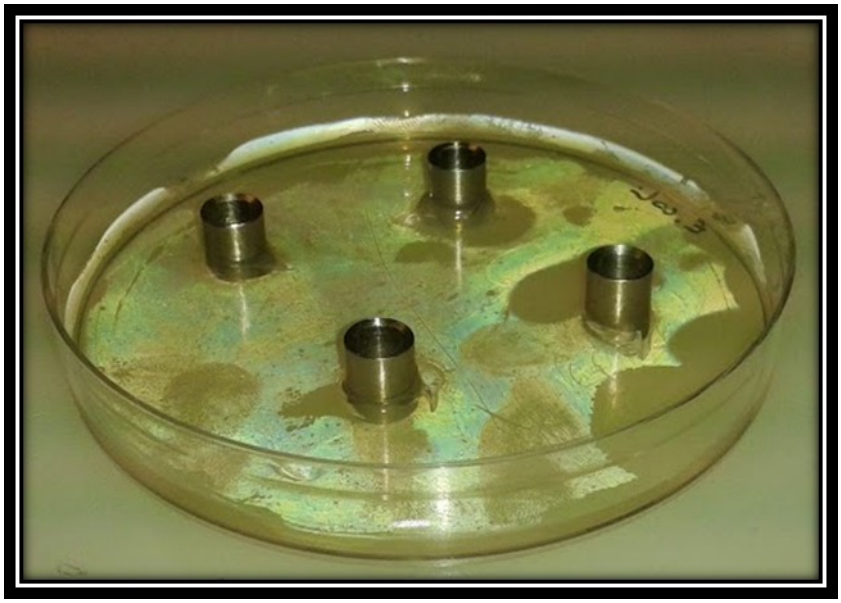

Figure 9-11. MH agar containing E. coli ATCC 25922.

Table 9.17. Tyndallization Program

Temperature	Time
99°C	1 h
37°C	9 h
99°C	1 h
37°C	9 h
99°C	1 h
4°C	For ever

9.13.1 Sensitivity to different pH values

The pH of cell-free supernatants was adjusted to 3.0, 4.5, 7.0 and 9.0 and then kept at room temperature for some hours. Residual activity was determined by the agar-spot method as described (Larsen, Vogensen, & Josephsen, 1993).

9.14 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain

In order to understand the best methodology to isolate the substances, with antibacterial activity, produced by these strains isolated from human samples, I chose the cell-free supernatant with the highest antibacterial activity and a broad spectrum of activity. For this purpose, various analyses were used.

9.14.1 Size Exclusion Chromatography

SEC, based on Sephadex (GE Healthcare), was performed. It enables group separation of biomolecoles that are above the exclusion limit of the medium, from contaminants such as salts. Sephadex is prepared by cross-linking dextran with epichlorohydrin. To prepare the column packing , an empty XK 16/20 (GE Healthcare) column was used, and a quantity of fine Sephadex 50, up to five-fold the volume of the sample, was swelled for 3 h at 20°C for a good packing. Then, an aliquot of cell-free supernatant was eluted with buffer and several fractions were collected.

Sephadex G 50 obeys Darcy's Law:

$$\text{Eq.1.} \qquad U = K \times \Delta P \times L^{-1}$$

U = linear flow rate (cm/h)
ΔP = pressure drop over the packed bed expressed in cm water
L = bed height (cm)
K = constant of proportionality depending on the properties of the bed material and the buffer

Assuming a buffer with viscosity calculated by Equation 2: 1 cP: $U = K_0 \times \Delta P \times L^{-1}$

K_0 = the specific permeability depending on the particle size of the medium and the water regain. Theoretical flow was calculated by equation 2, assuming that K_0 for Sephadex G 50 is equal to 36.

Agar well diffusion test was performed for each desalted fraction in order to know what the active fraction was. Inhibitory activity was performed against certain Gram positive and Gram negative organisms: *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. aureus* ATCC 25923 and *Sarcina lutea* ATCC 9341.

The plates were incubated at 37°C overnight and then the zones of inhibition were measured in mm by a gauge.

9.14.2 Reverse Phase Chromatography

Reverse phase chromatography analysis was also performed, exploiting a C18 column (Grace Davison Discovery Sciences, Alltech® Extract-Clean™).

The elution gradient used for this analysis is shown in Table 9.18.

Table 9.18. Elution gradient used for the reverse phase chromatography.

Step	A%*	B%**	C%***	Temperature
Conditioning	0	0	100	20°C
Washing	100	0	0	
Flow through	0	0	0	
Washing	100	0	0	
Start Gradient	50	50	0	
Stop Gradient	98	2	0	

* H_2O 0.1% TFA
** Acetonitrile
***CH_3OH

The fractions were evaporated to dryness by using an evaporator centrifuge (Concentrator Plus, Eppendorf) and solubilized in HPLC water for subsequent analyses.

Broth dilution tests, to determine MIC values for each fraction (Minimum Inhibitory Concentration), were carried out in 96 well polystyrene microplates (Bibby Sterilin), in order to know what the active fraction was. *Enterococcus faecalis* ATCC 29212, *Sarcina lutea* ATCC 9341 and *Pseudomonas aeruginosa* ATCC 27853 were used as pathogens for this purpose.

For each strain, a 0.5 McFarland bacterial suspension was set up and starting from the latter dilutions in broth were prepared, to obtain a final concentration of 10^3-10^4CFU/mL.

The microplates were incubated at 37°C overnight in microaerobic conditions. The MICs were read as the lowest concentration of the fraction that inhibited the growth of the microorganism.

9.14.3 1D SDS-PAGE Analysis

Active fractions were separated by one-dimensional SDS-PAGE using a Mini-PROTEAN electrophoresis cell (Bio-Rad®). Samples were reconstituted in 70 µL of

Tris–HCl (pH 6.8; 50 mM);2% SDS; 10% glycerol; 0.49 ml DTT; 0.02% bromophenol blue. SDS-PAGE Molecular Weight Standards (Bio-Rad®) were loaded onto the first SDS-PAGE well of a 12% precast polyacrylamide gel (Bio-Rad®) and the samples were loaded into the following wells. Tris–Base (pH 8.5; 2.5 mM), 19.2 mM glycine and 0.01% SDS have constituted the running buffer. At the end of the electrophoretic run, the gel was stained by Coomassie Blue, in order to visualize the separation of the sample.

9.14.4 Evaluation of cytotoxicity by the producer strain on the BV2 cell line

- Cell culture: the BV2 cell line was cultured in high glucose (4.5 g/LD glucose) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin, and streptomycin at 37°C in a humidified incubator with 5% CO_2. Confluent cultures were passaged by trypsinization. BV2 microglia were plated overnight with two different concentrations of *Lactobacillus* producer strain, killed by tyndallization(Table 11) (10^4CFU/mL, 10^2CFU/mL), and then cultivated using a chamber under normoxia (20% O_2, 5% CO_2) at 37 °C overnight.
- Cell viability and Apoptosis Measurement: cell viability measurements were performed by a Muse® Cell Analyzer (Merck Millipore Corporation). The measurement is based on differential permeability of two DNA-binding dyes. The nuclear dye stains only nucleated cells while the viability dye brightly stains dying and dead cells. 180 µl of Muse® Count & Viability Reagent were added to 20 µl of suspended cells. Cells were incubated at room temperature for 5 min and then the measurements were performed. Apoptosis measurements were performed by a Muse® Cell Analyzer (Merck Millipore Corporation). The assay relies on the binding of fluorescently labeled Annexin V to phosphatidylserine (PS) which translocates to the outer surface of the cell membrane upon the onset of apoptosis. 100 µl of Muse® Annexin V & Dead Cell Reagent were added to100 µl of suspended cells. Cells were incubated for 30 min at room temperature and then the measurements were performed.

10 Results

10.1 Molecular identification of *Lactobacillus* spp.

For the molecular identification were exploited the techniques such as 16S rDNA PCR/RFLP (Randazzo et al., 2004), the multiplex-PCRs to amplify different locus of the gene region 16S-23S and ITS-region flanking the gene 23S rDNA (Song et al., 2000) and finally, the amplification based on the gene *tuf* to discriminate the strains of *L. casei* group (Ventura et al., 2003) since the profiles of restriction HaeIII, MspI, and AluI, obtained with RFLP method, were indistinguishable.

Most of the strains were divided into 4 groups by the first multiplex PCR (PCR-G), on the basis on the amplified obtained for each, as shown in Figure 10-1.

In detail, 19 strains were belonged to Group I, and were identified as *L. delbrueckii*; 169 strains were assigned to Group II, 35 strains to Group III and finally 81 strains to Group IV. For each multiplex type strains were used as positive references.

Overall, thanks to the multiplex PCRs 304 of 359 were identified (84.7%).

55 strains, however, did not give amplified but were identified by 16rDNA / RFLP such as *L. gastricus* (30) and *L. vaginalis* (25) (15.3%).

The multiplex PCR on *tuf* gene was conducted to the strains identified as *L. paracasei* and *L. rhamnosus*; bands for each strain were viewed, and confirmed the above species.

Table 10.1 shows the identification of the strains in reference to the sample of isolation; instead the Fig. 10-2, 10-3 and 10-4 show the distribution in percentage.

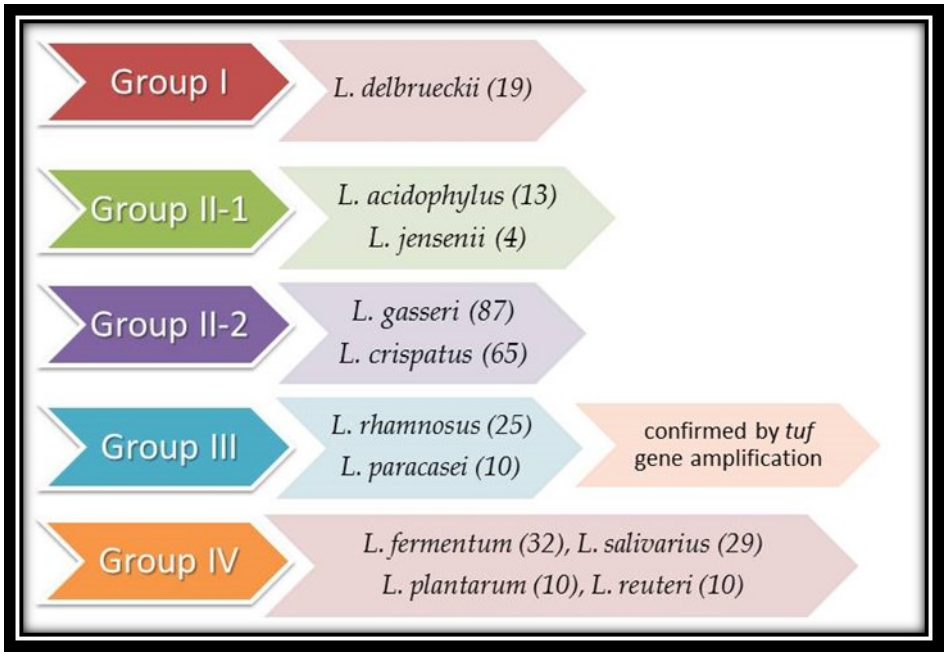

Figure 10-1. Multiplex PCR-Grouping (Song et al., 2000).

Table 10.1. Number of identified strains, subdivided in basis to origin, from oral, fecal or vaginal samples.

Species	Oral origin	Fecal origin	Vaginal origin
L. acidophilus	13	//	//
L. crispatus	17	//	48
L. delbrueckii	11	//	8
L. fermentum	8	20	4
L. gasseri	25	30	32
L. gastricus	//	30	//
L. jensenii	//	//	4
L. paracasei	10	//	//
L. plantarum	//	10	//
L. reuteri	10		
L. rhamnosus	13	//	12
L. salivarius	//	25	4
L. vaginalis	15	10	//

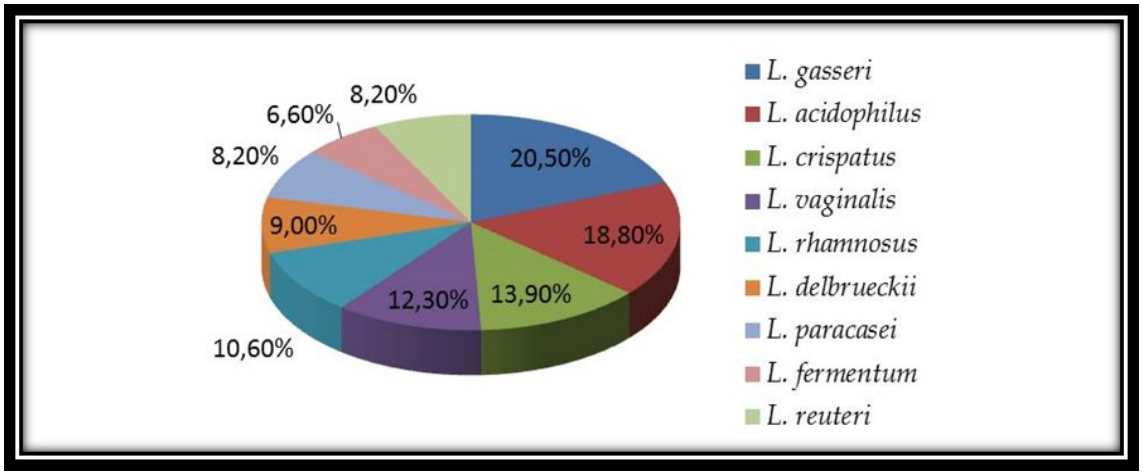

Figure 10-2. Molecular identification of oral samples.

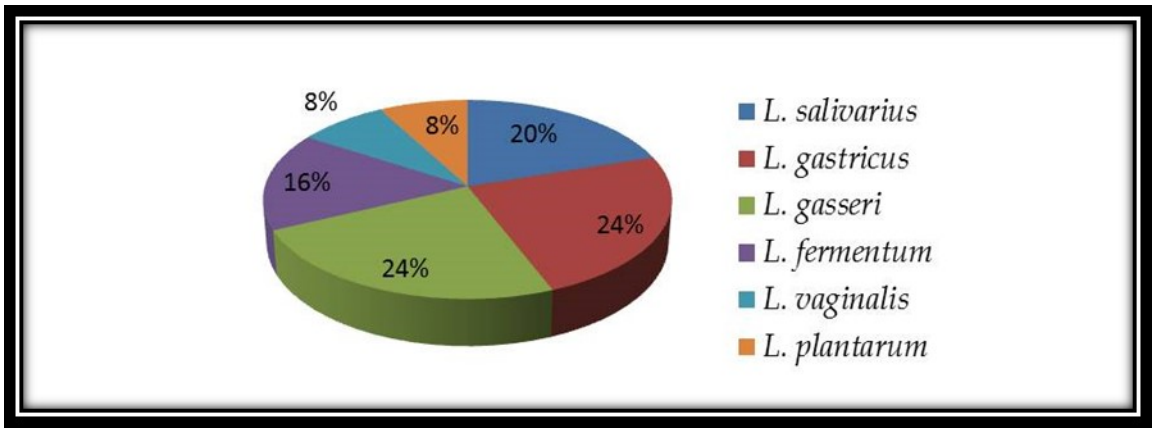

Figure 10-3. Molecular identification of fecal samples.

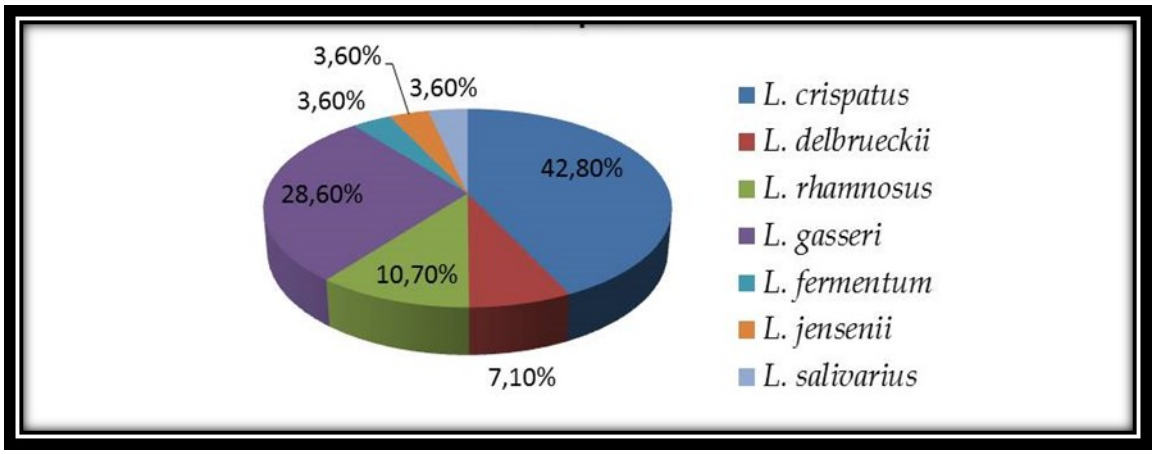

Figure 10-4. Molecular identification of vaginal samples.

The methods, used for clinical isolates, were also used to identify the commercial strain used as reference for the assays of evaluation of resistance to low pH and bile salts. Thanks to the use *tuf* gene PCR method the commercial strain was identify: it was *L.*

paracasei (but it was marketed as *L. casei*). We repeated the experiments more times to be sure of the final result (Fig. 10-5).

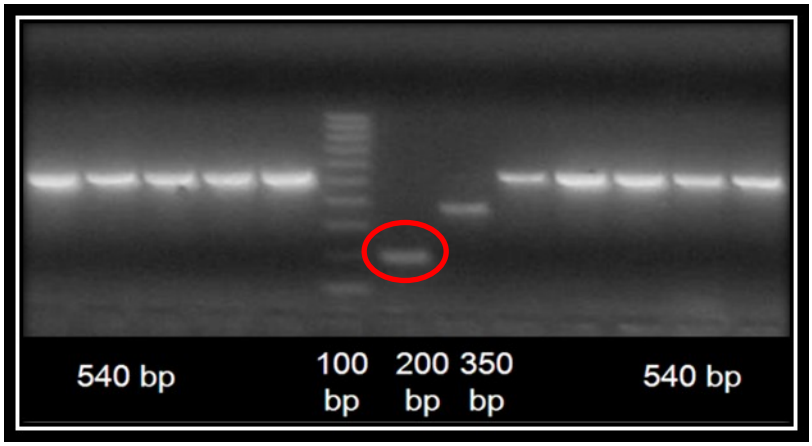

Figure 10-5. Strain of *Lactobacillus* isolated from a well-known commercial product and identified as *L. paracasei* (200bp).

10.2 Discrimination of 14 type strains of *Lactobacillus* spp. by Denaturing High Performance Liquid Chromatography (DHPLC): pilot study

The Denaturing High Performance Liquid Chromatography technique was used to discriminate 14 *Lactobacillus* species (isolated in this study; Table 10.2).

Table 10.2. List of the14 type strains examined by DHPLC.

Type Strain	DSMZ Code	DHPLC code
L. casei	20011	04 Casei
L. crispatus	20584	07 Crispatus
L. delbrueckii subsp. indicus	15996	10 Delbrueckii sub. indicus
L. fermentum	20052	12 Fermentum
L. gasseri	20243	13 Gasseri
L. gastricus	16045	14 Gastricus
L. jensenii	20557	16 Jensenii
L. paracasei	5622	20 Paracasei
L. paraplantarum	10667	21 Paraplantarum
L. plantarum	20174	22 Plantarum
L. reuteri	20016	23 Reuteri
L. rhamnosus	20021	24 Rhamnosus
L. salivarius	20555	25 Salivarius
L. vaginalis	5837	26 Vaginalis

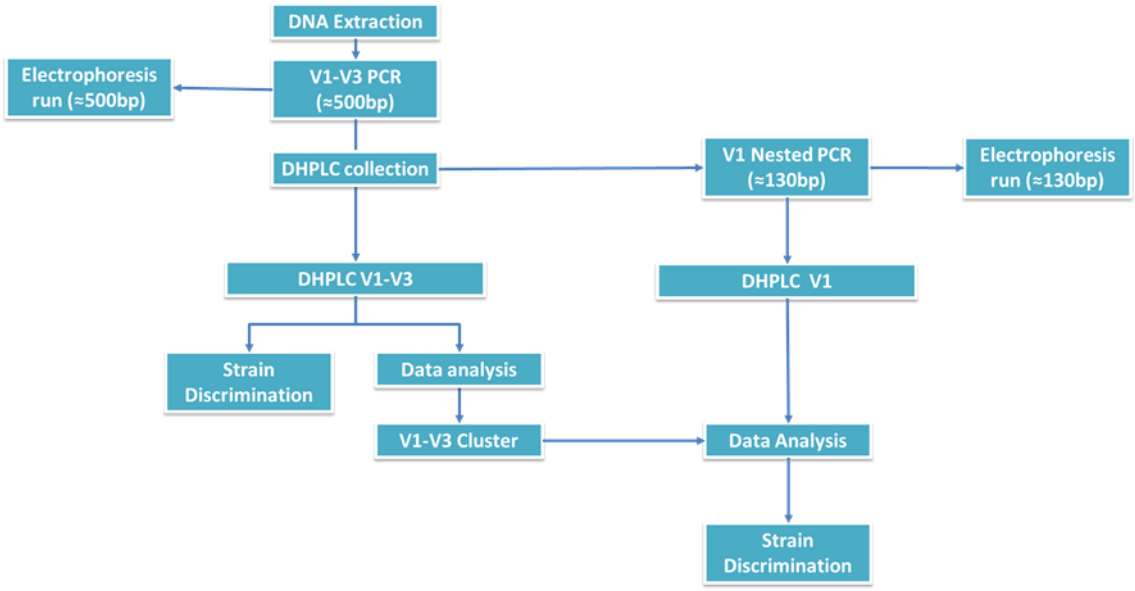

Figure 10-6. Work flow used to identify the species of *Lactobacillus*.

The discrimination of the 14 species under consideration is based on the comparison of the chromatographic peaks generated by nested PCR (V1-V3 and V1) in various denaturing conditions as shown in Fig 10-6.

The two amplicons (≈500bp and ≈130bp) were obtained using the methods described earlier (Chapter 9.12). First of all, the larger fragment was analyzed (V1-V3 Analysis), allowing the identification of three species, and at the same time, the grouping of the remaining species in clusters.

Afterwards, for each strain that belongs to the same cluster, the analysis of the internal fragment (V1 Species Discrimination) has been analyzed; this allowed the discrimination of the species in question.

10.2.1 V1-V3 Analysis

Figure 10-7 shows the chromatogram of V1-V3 amplicons (500bp), eluted by DHPLC following the protocol previously described (Table 9.15).

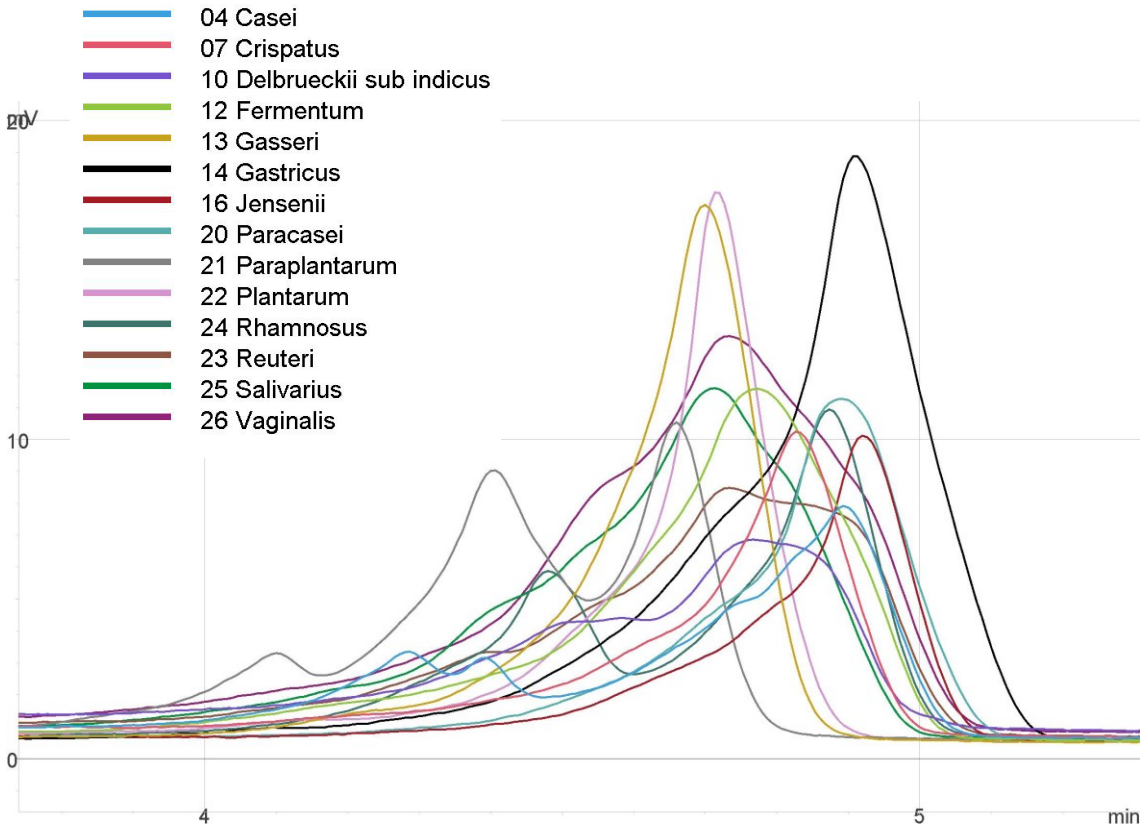

Figure 10-7. V1-V3 chromatogram of *Lactobacillus* type strains.

Because of curves overlapping, discrimination of all species was not possible, according to retention times (between 4.66 min. and 4.92 min.). Nevertheless, three species (04 Casei, 21 Paraplantarum and 24 Rhamnosus) showed more than one peak and for this reason they were easy to discriminate (Fig.s 10-8, 10-9 and 10-10).

Table 10.3. Retention times of V1-V3 chromatogram for *Lactobacillus* type strains.

DHPLC code	Rightmost peak retention time (min)
04 Casei*	4.89
07 Crispatus	4.83
10 Delbrueckii sub. indicus	4.77
12 Fermentum	4.77
13 Gasseri	4.70
14 Gastricus	4.91
16 Jensenii	4.92
20 Paracasei	4.89
21 Paraplantarum*	4.66
22 Plantarum	4.71
23 Reuteri	4.73
24 Rhamnosus*	4.87
25 Salivarius	4.71
26 Vaginalis	4.73

*samples which have more than one peak.

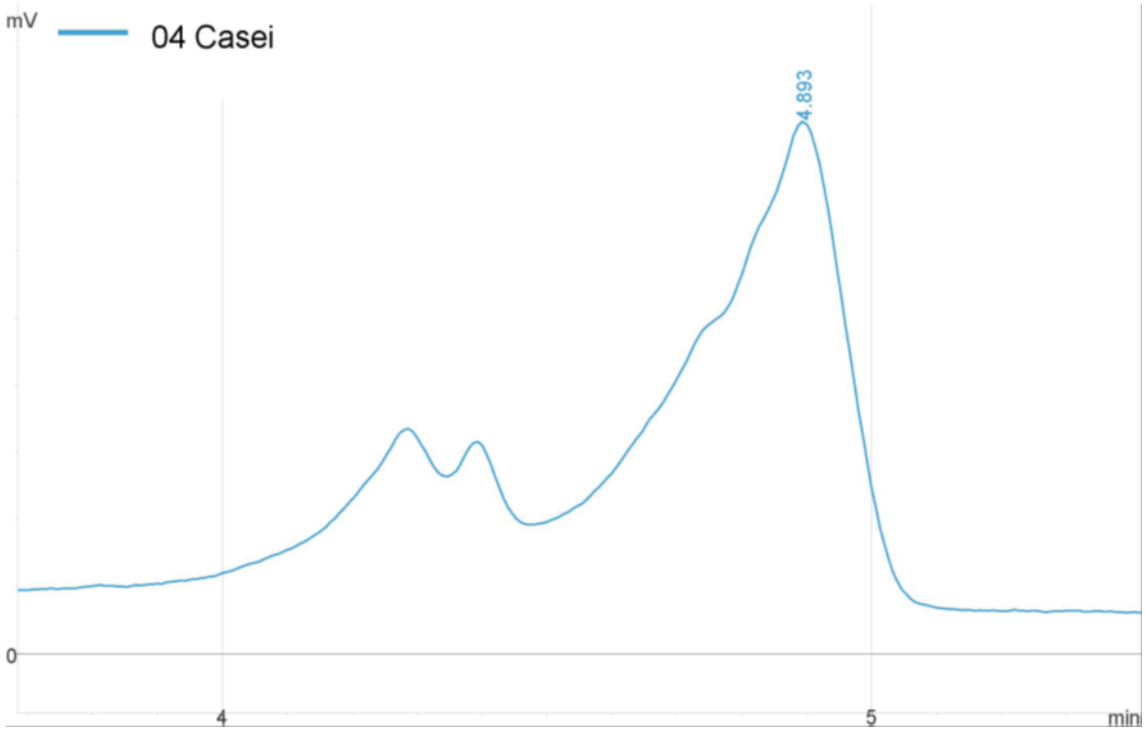

Figure 10-8. V1-V3 chromatogram of *L. casei* type strain, only retention time of rightmost peak is shown.

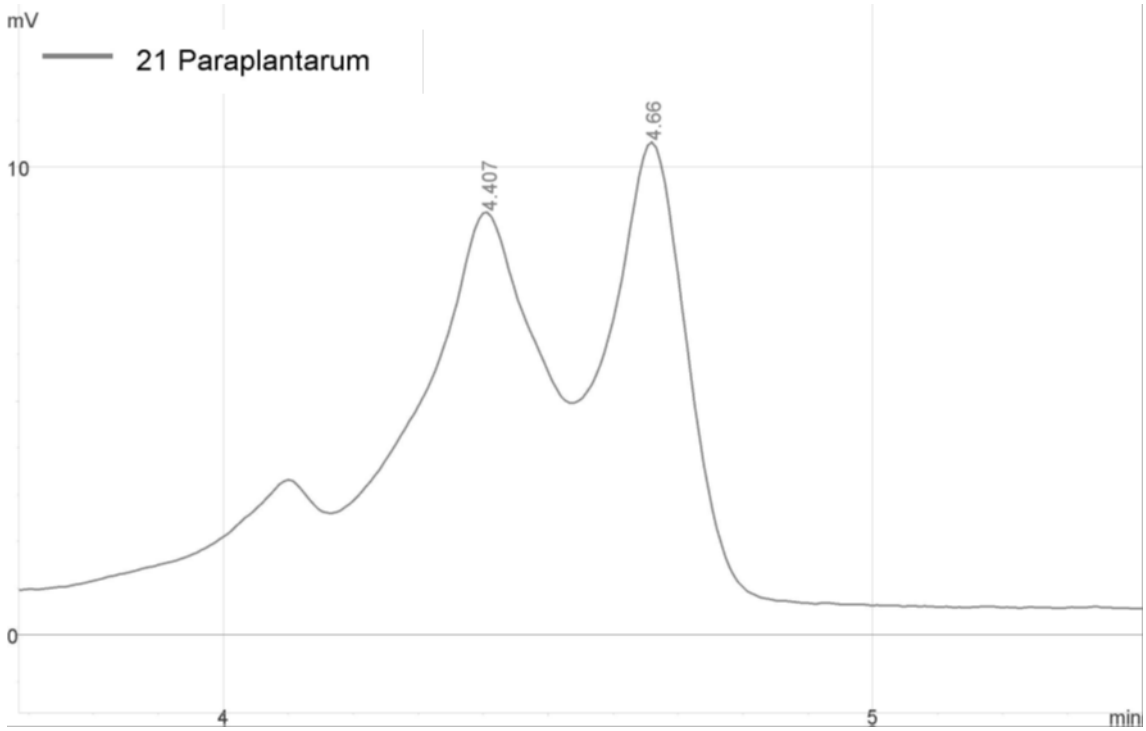

Figure 10-9. V1-V3 chromatogram of *L. paraplatarum* type strain.

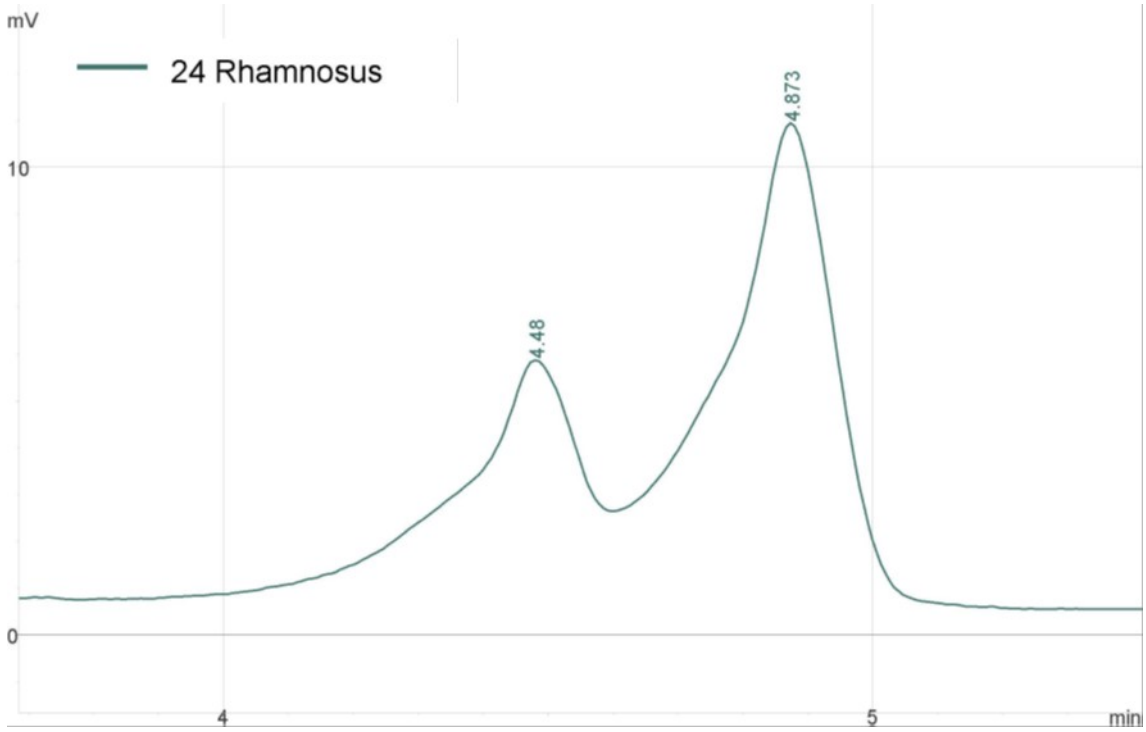

Figure 10-10. V1-V3 chromatogram of *L. rhamnosus* type strain.

The remaining eleven species were analysed using Navigator^{TM} Software according to curves profiles by mutation calling analysis within specified timeframe (between 4.00

min. – 5.03 min). The largest peak was found in each chromatogram and has been used to align all chromatograms, both horizontally and vertically.

Selected tracks were normalized and then, the differences were compared between points mathematically optimal along those tracks.

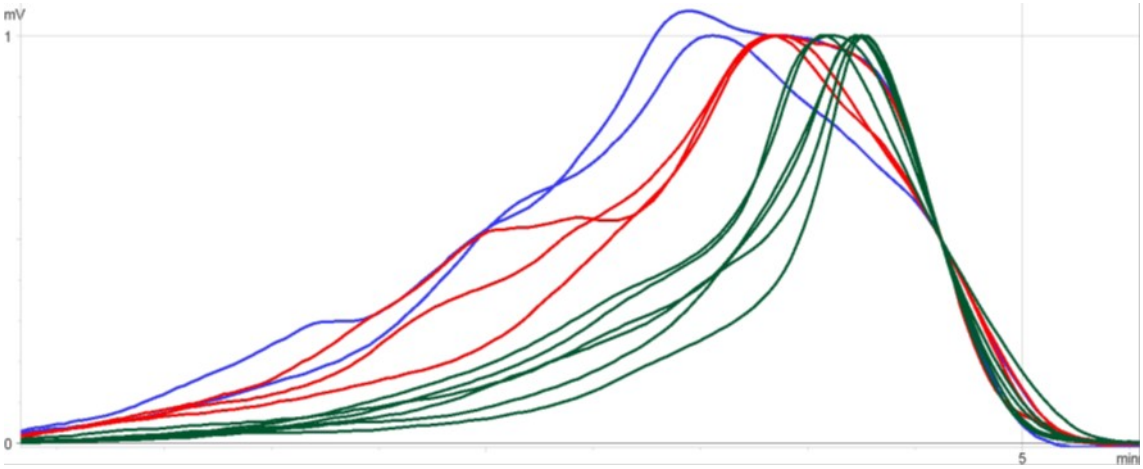

Figure 10-11. V1-V3 chromatogram after mutation calling analysis: cluster 1 (green curves), cluster 2 (red curves) and cluster 3 (blue curves).

Therefore, in the same group similar traces have been grouped together, generating three different clusters:

Cluster 1: 07 Crispatus, 13 Gasseri, 14 Gastricus, 16 Jensenii, 20 Paracasei and 22 Plantarum (Fig. 10-12);

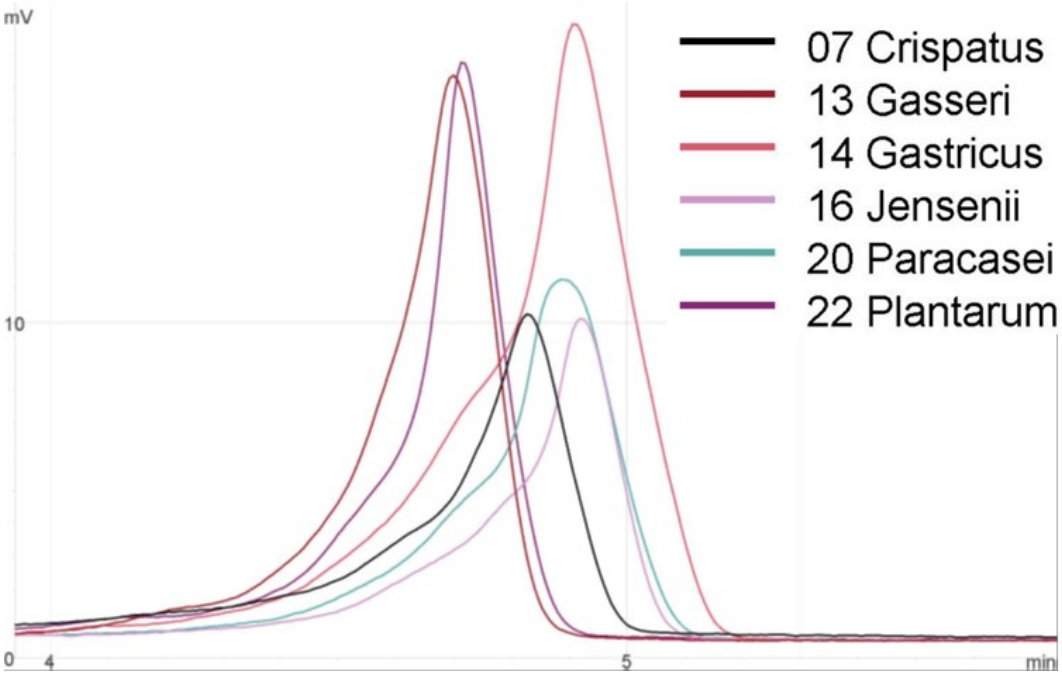

Figure 10-12. V1-V3 chromatogram Cluster 1.

Cluster 2: 10 Delbrueckii sub indicus, 12 Fermentum and 25 Salivarius (Fig. 10-13);

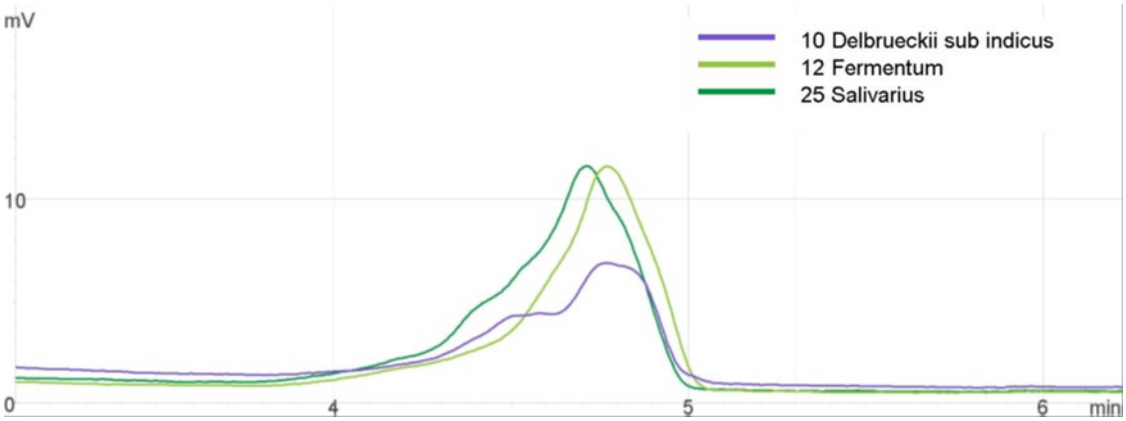

Figure 10-13. V1-V3 chromatogram Cluster 2.

Cluster 3: 23 Reuteri and 26 Vaginalis (Fig. 10-14).

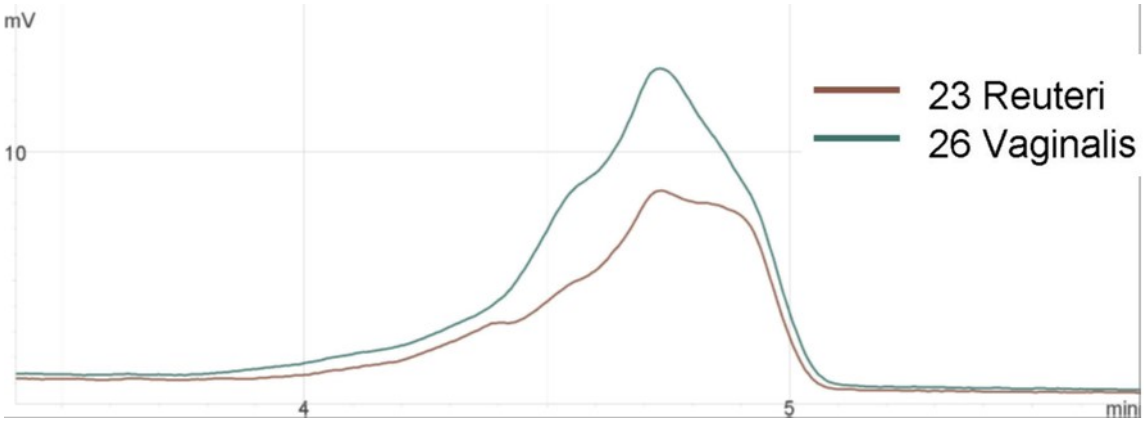

Figure 10-14. V1-V3 chromatogram Cluster 3.

In addition to clustering, the results were represented by means of a three-dimensional scattergraph (Fig 10-15), where each track has been represented in a 3D space. The dots into the scattergraph represent the traces analysed: the near points corresponded to similar profiles, while those separated accounted traces significantly different among them. In the end, the results were not separated only by the clustering, but the mutation calling analysis also visually depicted the variance among the clusters.

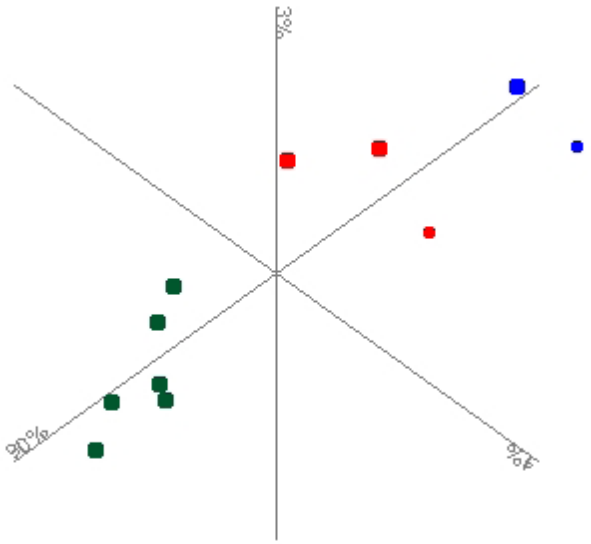

Figure 10-15. 3D scattergraph of V1-V3 chromatogram.

After mutation calling analysis, curves overlapping was extremely reduced, therefore it was possible to separate Cluster 1 in two subclasses: 1A (07 Crispatus, 14 Gastricus, 16 Jensenii and 20 Platarum) and 1B (13 Gasseri and 22 Platarum) as shown in Fig 10-16.

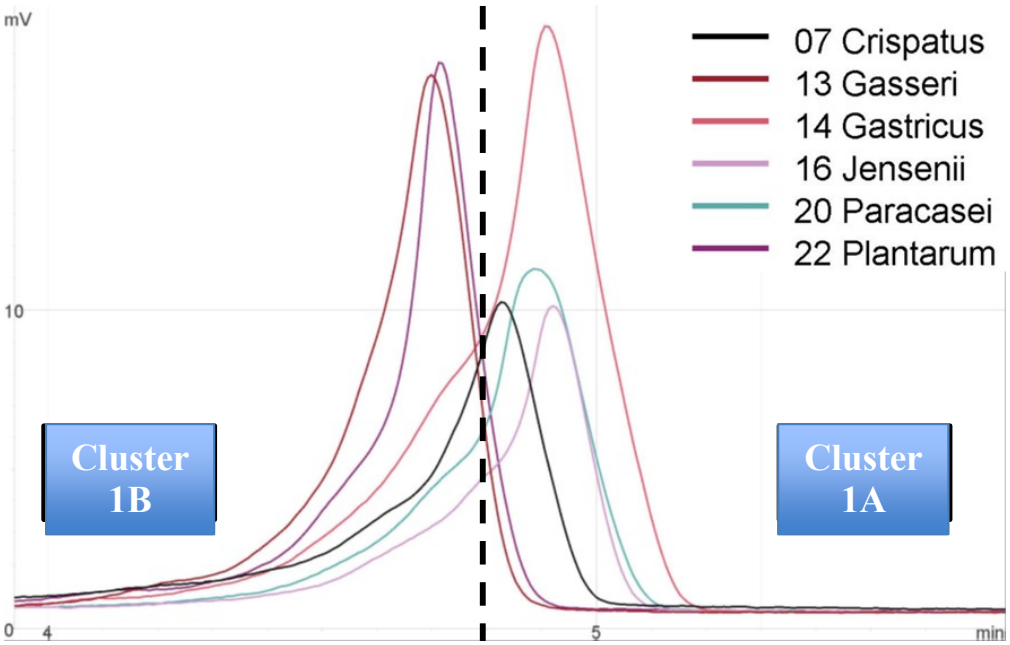

Figure 10-16. V1-V3 Cluster 1 chromatogram: separation in cluster 1A and cluster 1B according to retention times.

10.2.2 V1 Species Discrimination

- **CLUSTER 1A-1B**

V1 fragments were analyzed for the species belonging to cluster 1A (Fig. 10-17) and to cluster 1B (Fig. 10-18). In both cases, the best resolution of chromatograms has allowed the discrimination.

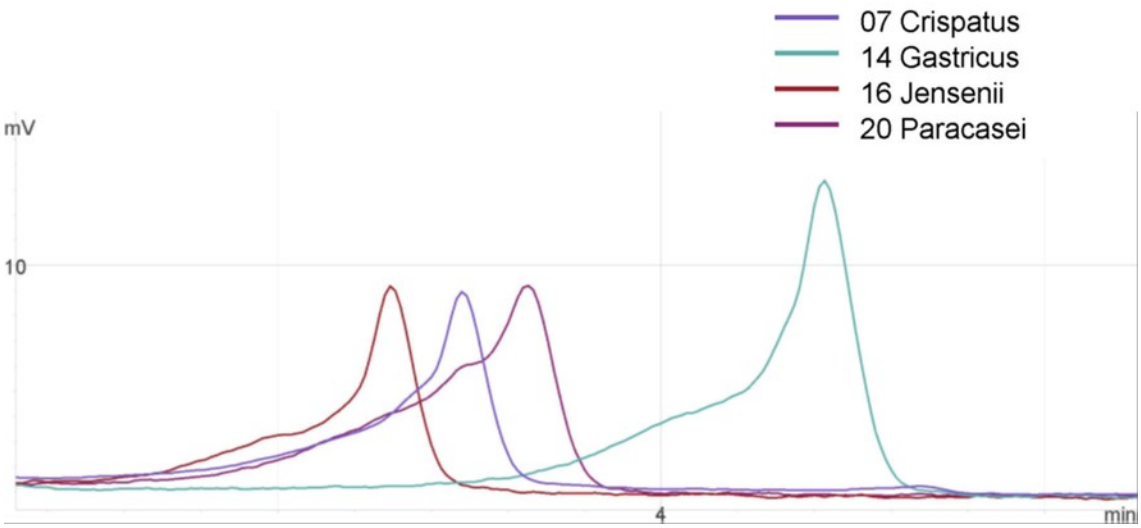

Figure 10-17. V1 chromatogram cluster 1A.

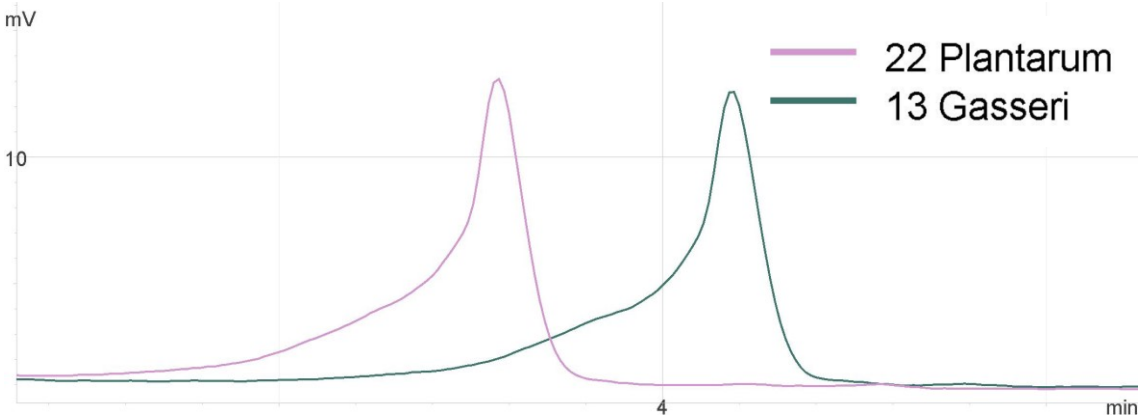

Figure 10-18. V1 chromatogram cluster 1B.

- **CLUSTER 2 and CLUSTER 3**

V1 fragments were analyzed for the species belonging to cluster 2 and 3 (Fig.s 10-19A, 10-20A).

In this case, the increased resolution has not allowed the complete discrimination of all species. For this reason, scatterographs were created (Fig.s 10-19B, 10-20B) by mutation calling analysis to obtain discrimination of overlap curves.

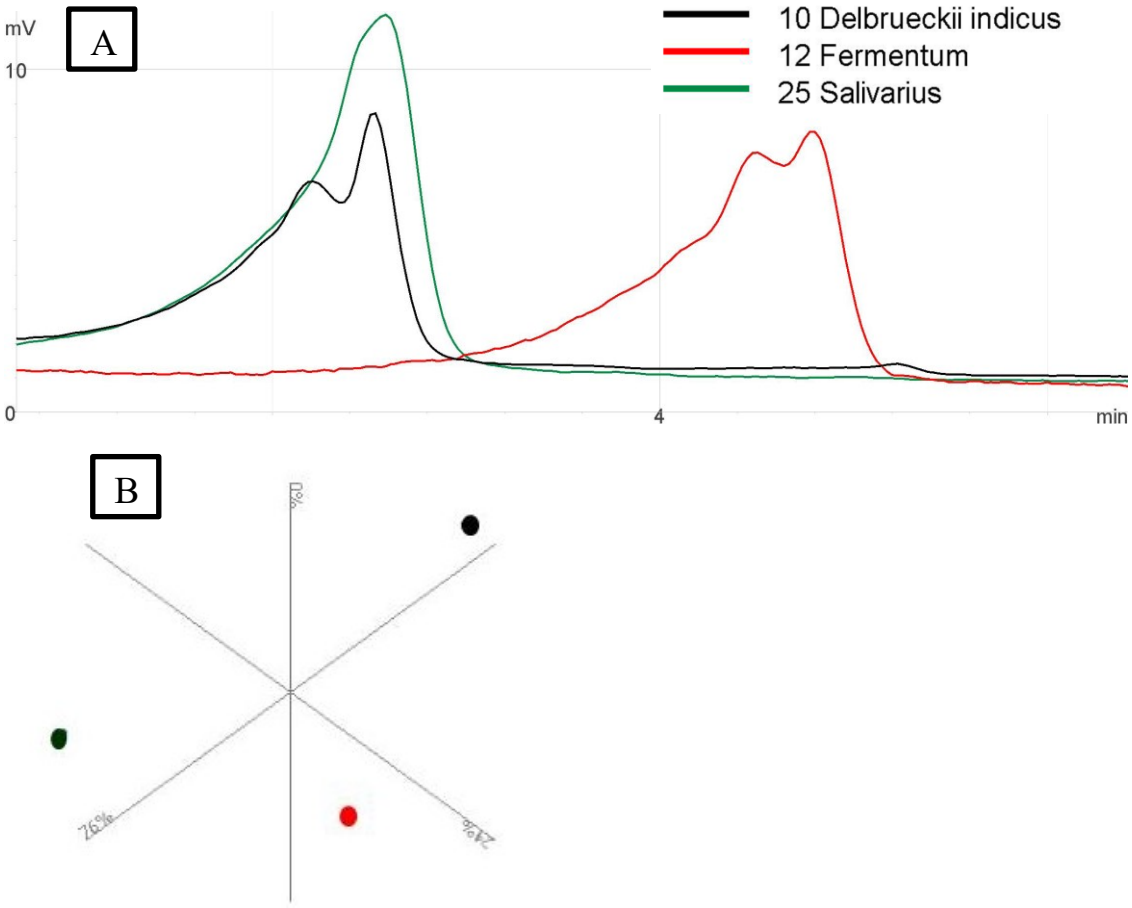

Figure 10-19. A: chromatogram cluster 2; B: scattergraph cluster 2.

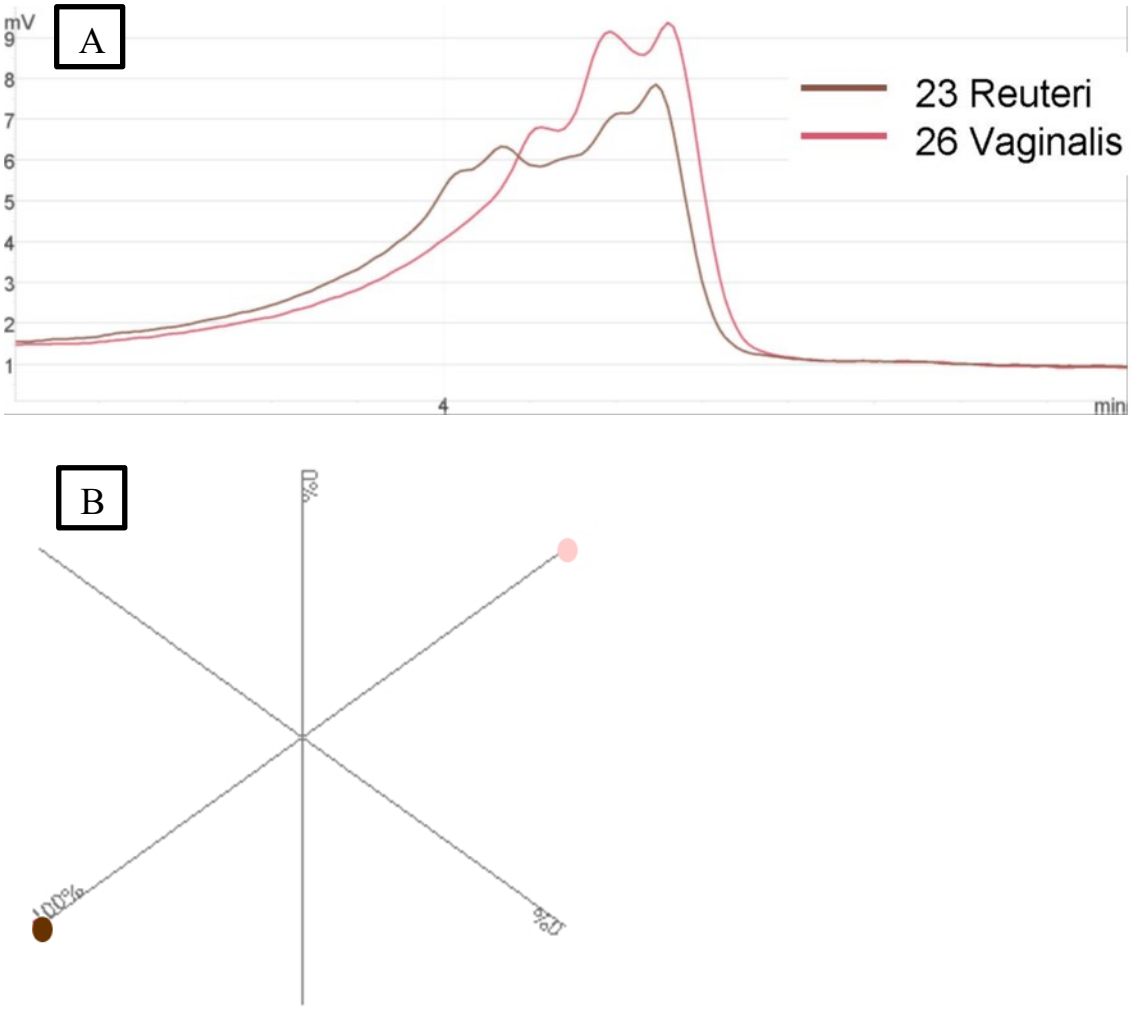

Figure 10-20. A: chromatogram cluster 3; B: scattergraph cluster 3.

10.2.3 DHPLC analysis application to *Lactobacillus* strains

The method just described was applied to six *Lactobacillus* strains isolated in this study and identified with other molecular methods as previously described (Chapter 10.1).

Table 10.4. List of the six strains chosen for DHPLC analysis application.

Strain	Previously identification	DHPLC identification
AD1	*L. rhamnosus*	*L. rhamnosus*
EB4	*L. crispatus*	*L. crispatus*
ENT	*L. paracasei*	*L. paracasei*
GW	*L. gastricus*	*L. gastricus*
LAC 28	*L. gasseri*	*L. gasseri*
LAC 38	*L. paraplantarum*	*L. paraplantarum*

- **Strain AD1 and LAC 38**

Strains AD 1 and LAC 38 were directly identified by analysis V1-V3. Figs. 10-21 and 10-22 show the chromatograms related to the type strains *L. rhamnosus* DSMZ 20021 and *L. paraplantarum* DSMZ 10667 (blue and red continuous lines, respectively) and clinical isolates (blue dot and red dash lines, respectively). Retention times are not exactly coincident, but the peculiar profile of the curves allows the identification.

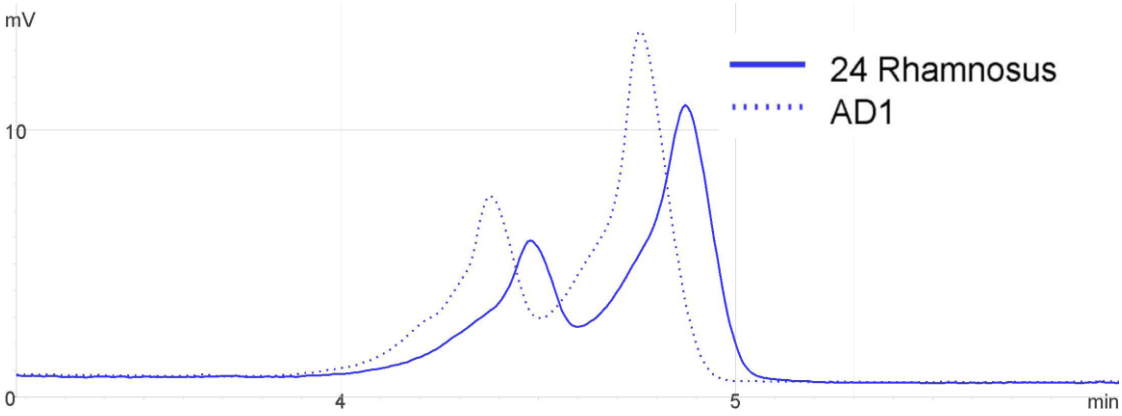

Figure 10-21. Chromatogram of *L. rhamnosus* DSMZ 20021 and clinical isolate AD1 (vaginal origin).

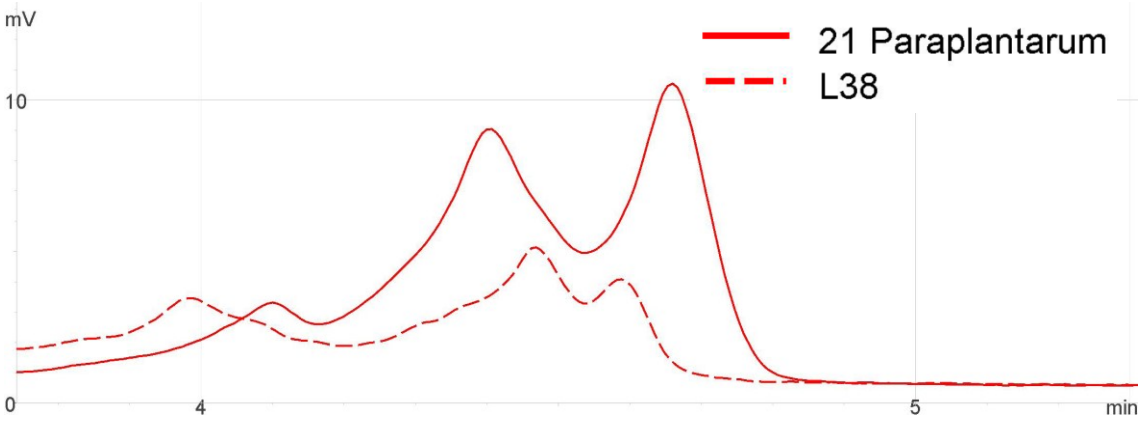

Figure 10-22. Chromatogram of *L. paraplantarum* DSMZ 10667 and clinical isolate LAC38 (oral origin).

- **Strains EB4, ENT, LAC 28 and GW**

Figure 10-23 shows V1-V3 chromatograms of four clinical isolates. In this case the curves are highly overlapped, so clustering with type strains was necessary.

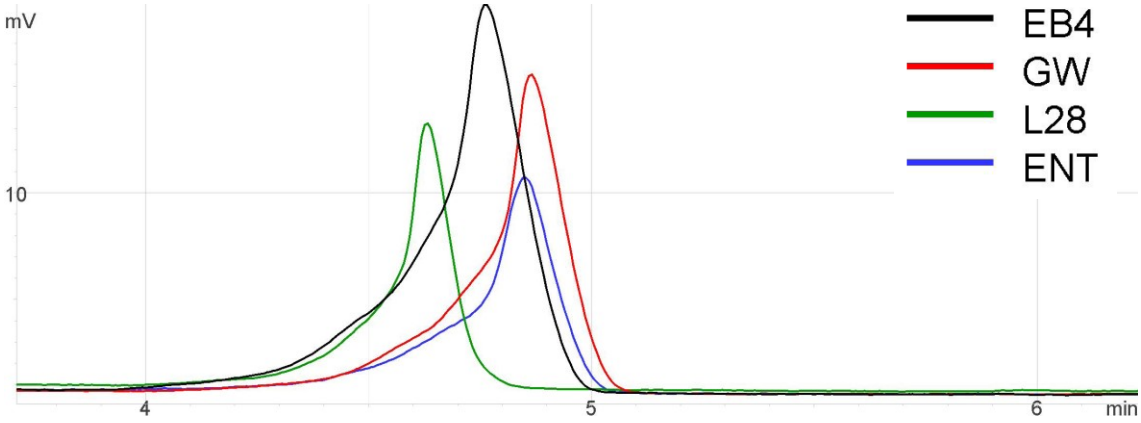

Figure 10-23. V1-V3 chromatogram of four clinical isolates EB4, GW, LAC28 and ENT.

Cluster analysis shows the membership of the four isolated strains to cluster 1 (Fig. 10-24).

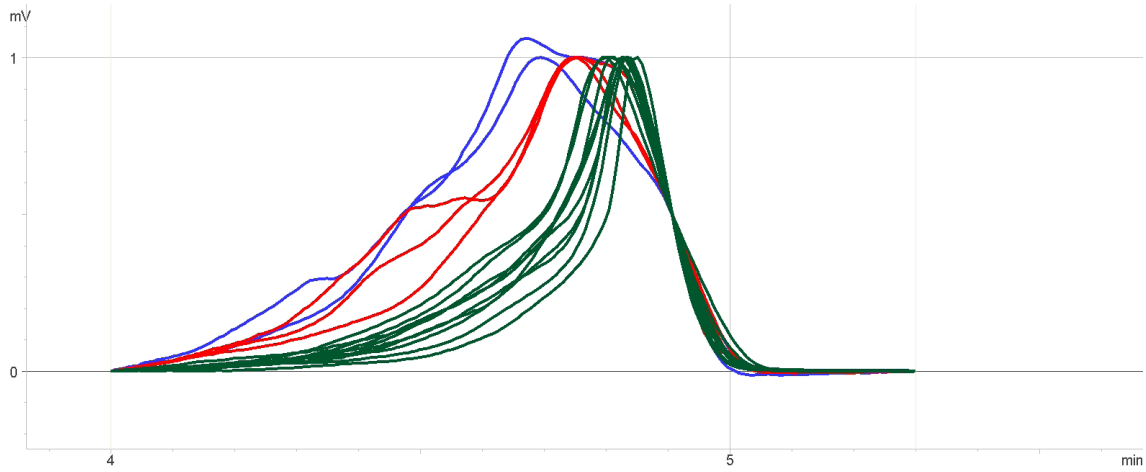

Figure 10-24. Clustering of four clinical isolates together with type strains. Green curves belonged to cluster 1: 07 Crispatus, 13 Gasseri, 14 Gastricus, 16 Jensenii, 20 Paracasei and 22 Plantarum, EB4, LAC 28, ENT and GW.

Subsequently the four strains were discriminated in subclusters:

- cluster 1A EB4, GW, ENT (Fig. 10-25)
- cluster 1B LAC28 (Fig. 10-26)

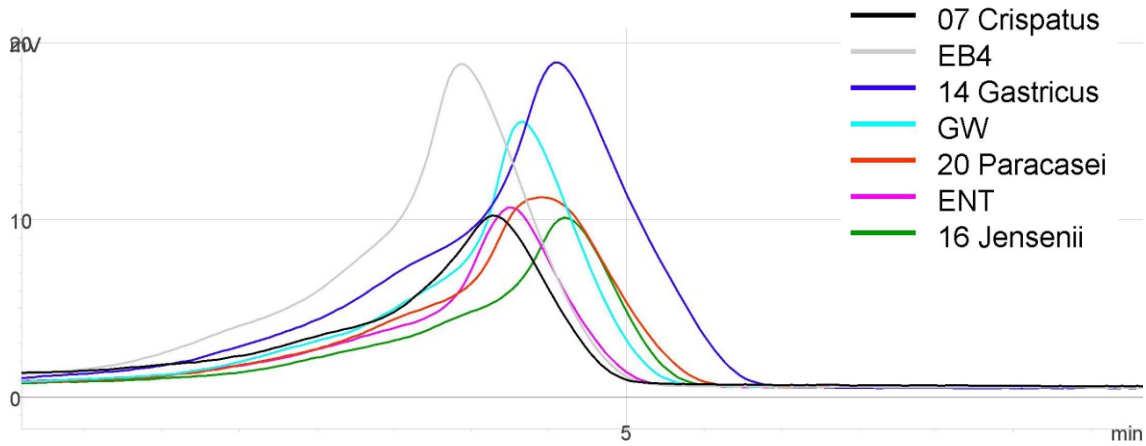

Figure 10-25. V1-V3 chromatogram cluster 1A: type strains and clinical isolates EB4 (vaginal origin), GW (fecal origin) and ENT (oral origin).

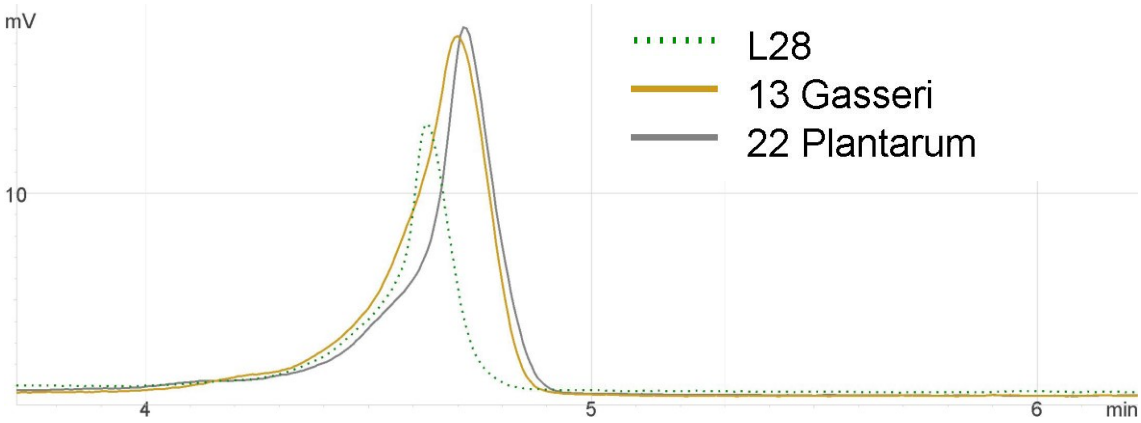

Figure 10-26. V1-V3 chromatogram cluster 1A: type strains and clinical isolate LAC28 (vaginal origin).

Finally, V1 fragments were analyzed to identify the four isolates. Fig. 10-27 shows V1 chromatogram related to the type strains (*L. crispatus* DSMZ 20584, *L. gastricus* DSMZ 16045, *L. paracasei* DSMZ 5622 and *L. jensenii* DSMZ 20557) and clinical isolates (EB4, GW and ENT), while Fig. 10-28 shows V1 chromatogram of type strains *L. gasseri* DSMZ 20243 and *L. plantarum* DSMZ 20173 together to clinical isolate LAC 28 showing that the latter is *L. gasseri.*

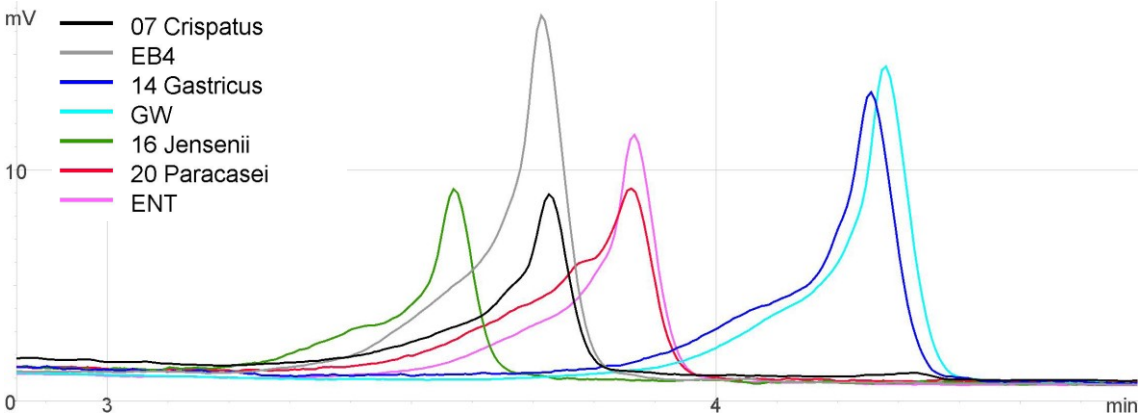

Figure 10-27. V1 chromatogram of cluster 1A.

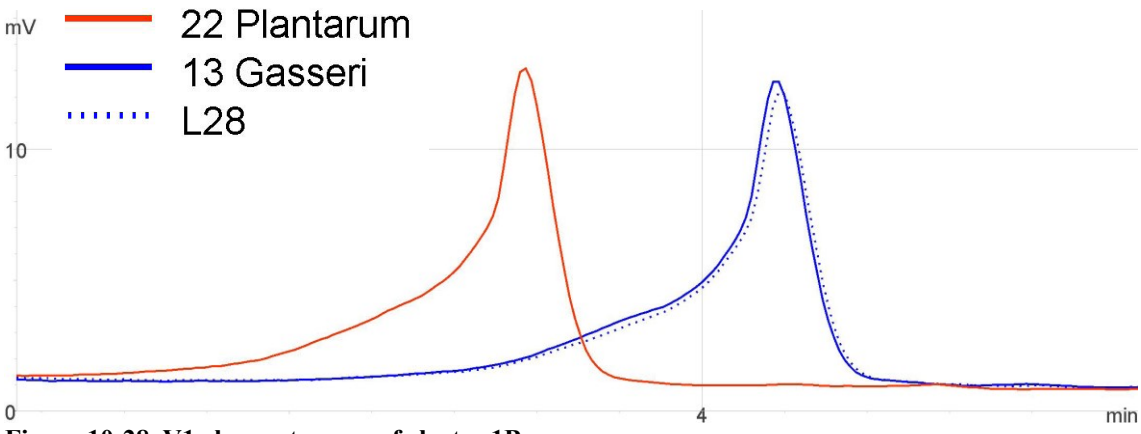

Figure 10-28. V1 chromatogram of cluster 1B.

10.3 Susceptibility testing

In vitro activity of antibiotics, such as ampicillin, kanamycin, erythromycin and clindamycin, by microdilution broth method was evaluated.

Table 10.5 shows microbiological cut-off values of *Lactobacillus* spp. proposed by EFSA (EFSA FEEDDAP, 2012), which were subdivided by their metabolism (homofermentative, facultative heterofermentative and obligate heterofermentative). *L. acidophilus, L. delbruekii* and *L. helveticus* were classified as *L. acidophilus* group. *L. plantarum*/*pentosus* and *L. casei/paracesei* were classified in two different groups, while *L. reuteri* and *L. rhamnosus* were classified alone.

Tables 10.6 and 10.7 show for each species of identified *Lactobacillus* spp., the MIC 50/90 µg/mL for the four antibiotics tested, according to EFSA FEEDAP microbiological cut-off values. The results showed that 100% of the strains were resistant to aminoglycoside kanamycin and fluoroquinolone ciprofloxacin. All strains were susceptible to beta-lactam ampicillin, to clindamycin and to erythromycin.

Table 10.5. Microbiological cut-off values (µg/mL); EFSA FEEDAP, 2012.

Strain	**Antibiotic**			
	Ampicillin	**Kanamycin**	**Erythromycin**	**Clindamycin**
Lactobacillus obligate homofermentative[a]	1	16	1	1
Lactobacillus acidophilus group	1	64	1	1
Lactobacillus obligate heterofermentative[b]	2	32	1	1
Lactobacillus reuteri	2	64	1	1
Lactobacillus facultative heterofermentative[c]	4	64	1	1
Lactobacillus plantarum/pentosus	2	64	1	2
Lactobacillus rhamnosus	4	64	1	1
Lactobacillus casei /paracasei	4	64	1	1

a including *L. delbrueckii, L. helveticus*

b including *L. fermentum*

c including the homofermentative species *L. salivarius.*

Table 10.6. MIC50 and MIC90 (µg/mL) for ampicillin (AMP) and kanamycin (KAN).

Species	Numbers of isolates	AMP		KAN	
		MIC50	MIC90	MIC50	MIC90
L. acidophilus [d]	13	≤0.03	0.5	>16	>16
L. crispatus [a]	65	≤0.03	0.5	>16	>16
L. delbrueckii [a]	19	≤0.03	0.5	>16	>16
L. fermentum [b]	32	≤0.03	0.5	>16	>16
L. gasseri [a]	87	≤0.03	0.5	>16	>16
L. gastricus [b]	30	≤0.03	0.5	>16	>16
L. jensenii [a]	4	≤0.03	0.5	>16	>16
L. paracasei [e]	10	≤0.03	0.5	>16	>16
L. plantarum [f]	10	≤0.03	0.5	>16	>16
L. reuteri [b]	10	≤0.03	0.5	>16	>16
L. rhamnosus [g]	25	≤0.03	0.5	>16	>16
L. salivarius [c]	29	≤0.03	0.5	>16	>16
L. vaginalis [b]	25	≤0.03	0.5	>16	>16

AMP: ampicillin; KAN: kanamycin.

a including *L. delbrueckii, L. helveticus*; **b** including *L. fermentum*; **c** including the homofermentative species *L. salivarius;* **d** acidophilus group; **e** *L. casei/paracasei*; **f** *L. plantarum/pentosus;* **g** *L. rhamnosus.*

Table 10.7. MIC50 and MIC90 (µg/mL) for erythromycin (ERY), clindamycin (DA) and ciprofloxacin (CIP).

Species	Numbers of isolates	ERY		DA		CIP*	
		MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
L. acidophilus [d]	13	0.06	0.06	0.06	0.06	>1	>1
L. crispatus a	65	0.12	0.25	0.12	0.25	>1	>1
L. delbrueckii [a]	19	0.12	0.25	0.12	0.25	>1	>1
L. fermentum [b]	32	0.12	0.25	0.12	0.25	>1	>1
L. gasseri [a]	87	0.12	0.5	0.12	0.5	>1	>1
L. gastricus [b]	30	0.12	0.25	0.12	0.25	>1	>1
L. jensenii [a]	4	0.12	0.25	0.12	0.25	>1	>1
L. paracasei [e]	10	0.12	0.25	0.12	0.25	>1	>1
L. plantarum [f]	10	0.12	0.25	0.12	0.25	>1	>1
L. reuteri [b]	10	0.12	0.25	0.12	0.25	>1	>1
L. rhamnosus [g]	25	0.25	0.5	0.25	0.5	>1	>1
L. salivarius [c]	29	0.25	0.5	0.25	0.5	>1	>1
L. vaginalis [b]	25	0.12	0.5	0.12	0.5	>1	>1

E: erythromycina; DA: clindamycin; CIP*: ciprofloxacin, as reported CLSI M 45A.

10.4 Evaluation of resistance to low pH and bile salts

A pilot experiment was performed with few strains chosen randomly; results are shown in Table 10.8 (Fuochi et al., 2015).

Table 10.8. Survival percentage *pre*- and *post*-treatment at low pH and in the presence of bile salts (BS) at different concentrations (% w/v).

percentage survival (%) *pre*-treatment at low pH

Strain	**LSM**	**BS 0.5% Cys 0.05%**	**BS 0.5%**	**BS 0.25%**	**BS 0.12%**
L. jensenii	$1.0x10^{7}$	1.5	2	2.5	4
L. crispatus	$1.0x10^{8}$	10	20	40	80
L. delbruekii	$2.0x10^{7}$	2.4	25	35	40
L. gasseri	$10x10^{11}$	20	40	50	70
L. salivarius	$7.0x10^{11}$	14.2	28.6	40	43
L. rhamnosus	$6.0x10^{7}$	16	33	58	75
L. fermentum	$8.0x10^{7}$	18	38	50	62.5
L. paracasei trademark	$1.0x10^{9}$	1	3	4.5	5

percentage survival (%) *post*-treatment at low pH

Strain	**LSM**	**BS 0.5% Cys 0.05%**	**BS 0.5%**	**BS 0.25%**	**BS 0.12%**
L. jensenii	$1.0x10^{3}$	0	0.2	0.4	1
L. crispatus	$1.0x10^{3}$	0	10	15	30
L. delbruekii	$1.0x10^{3}$	0	20	20	20
L. gasseri	$5.0x10^{7}$	0	15	30	40
L. salivarius	$1.0x10^{3}$	0	40	55	60
L. rhamnosus	$2.0x10^{5}$	0	10	20	25
L. fermentum	$3.0x10^{5}$	0	10	15	20
L. paracasei trademark	$1.4x10^{3}$	0	0	0	0

The same experiment was performed for all strains.

All strains were grown on LSM agar without supplements, the strain of *E. coli* ATCC 35218, chosen as internal positive control, grew in all conditions considered invariably.

All strains belonging to *L. gastricus,* and *L. gasseri* species were particularly resistant to the treatment at pH 3.0; instead strains belonging to *L. acidophilus*, *L. crispatus*, *L. delbruekii, L. salivarius, L. reuteri, L. paracasei* and *L. plantarum* were reduced significantly. Finally, strains belonging to *L. vaginalis, L. rhamnosus*, *L. fermentum* and *L. jensenii* have shown good tolerability.

Regarding the sensitivity to bile salts, the first run of the experiment was performed with bacterial cultures having an initial concentration of 10^6 CFU/mL. It was observed that all strains were sensitive to treatment BS 0.5% - Cys 0.05% w/v both pre- and post-treatment at low pH. Furthermore, the bacteriostatic action, demonstrated *in vitro* by high concentrations of bile salts, showed that the strain was still alive though not able to multiply. Instead, when we proceeded to the second run of the experiment with decreasing concentrations of bile salts (0.5%, 0.25%, 0.12% w/v) and an initial concentration of bacteria 10^7CFU/mL, the results indicated that they were able to multiply even in the presence of the highest concentration of bile salts (pre- low pH treatment) but with a strain-dependent resistance/sensitivity (Tables 10.9, 10.10). However, after 1 h at a low pH, the bacterial concentration was drastically reduced, obtaining similar results to those of the first run of the experiment.

Table 10.9. Survival average percentage *pre*- treatment at low pH and in the presence of bile salts (BS) at different concentrations (% w/v).

percentage survival (%) *pre*-treatment at low pH

Strain	**LSM**	**BS 0.5% Cys 0.05%**	**BS 0.5%**	**BS 0.25%**	**BS 0.12%**
L. acidophilus	$1.0x10^{7}$	1	2	3	4
L. crispatus	$1.0x10^{8}$	10	20	40	80
L. delbrueckii	$2.0x10^{7}$	2.4	25	35	40
L. fermentum	$8.0x10^{7}$	18	38	50	65
L. gasseri	$9.0x10^{11}$	20	40	50	70
L. gastricus	$6.0x10^{7}$	10	20	40	80
L. jensenii	$1.0x10^{7}$	2	2	4	6
L. paracasei	$1.0x10^{9}$	20	40	50	70
L. plantarum	$10x10^{11}$	16	27	40	43
L. reuteri	$7.0x10^{11}$	2.4	25	35	40
L. rhamnosus	$6.0x10^{7}$	16	33	58	75
L. salivarius	$7.0x10^{11}$	13	29	40	43
L. vaginalis	$7.0x10^{11}$	20	40	50	70

Table 10.10. Survival average percentage *post*- treatment at low pH and in the presence of bile salts (BS) at different concentrations (% w/v).

percentage survival (%) *post*-treatment at low pH

Strain	**LSM**	**BS 0.5% Cys 0.05%**	**BS 0.5%**	**BS 0.25%**	**BS 0.12%**
L. acidophilus	$1.0x10^{3}$	0	3	1	5
L. crispatus	$1.0x10^{3}$	0	10	15	20
L. delbrueckii	$1.0x10^{3}$	0	20	20	20
L. fermentum	$3.0x10^{5}$	0	20	25	40
L. gasseri	$5.0x10^{7}$	0	35	40	50
L. gastricus	$5.0x10^{7}$	0	50	65	70
L. jensenii	$3.0x10^{5}$	0	15	30	40
L. paracasei	$1.4x10^{3}$	0	3	1	5
L. plantarum	$1.0x10^{5}$	0	10	15	20
L. reuteri	$1.0x10^{3}$	0	15	20	20
L. rhamnosus	$3.0x10^{5}$	0	15	30	40
L. salivarius	$1.0x10^{3}$	0	10	15	20
L. vaginalis	$3.0x10^{5}$	0	15	30	40

10.5 Evaluation of the capacity of *Lactobacillus* strains to produce hydrogen peroxide

Fourteen *Lactobacillus* type strains, and 308 wild strains were grown on MRS medium with horseradish peroxide and TMB, instead 6 strains of *L. acidophilus*, 9 of *L. crispatus*, 29 of *L. gasseri*, and 7 of *L. rhamnosus* were not grown on this medium (Table 10.11).

One hundred seventeen strains did not accumulate hydrogen peroxide (25 of *L. crispatus*, 10 of *L. fermentum*, 29 of *L. gasseri*, 10 of *L. gastricus*, 4 of *L. jensenii*, 18 of *L. rhamnosus* and 21 of *L. salivarius*).

One hundred fiftyeight strains were exhibited a weakly positive reaction (7 of *L. acidophilus,* 31 of *L. crispatus,* 19 of *L. delbrueckii,* 22 of *L. fermentum,* 29 of *L. gasseri,* 18 of *L. gastricus,* 10 of *L. paracasei,* 10 of *L. reuteri* and 12 of *L. vaginalis*).

Twenty-three strains were exhibited a moderate positive accumulation of hydrogen peroxide (10 of *L. plantarum* and 13 of *L. vaginalis*).

Accumulation was greatest by the strains of *L. gastricus* (2) and *L. salivarius* (8) from fecal origin (Fig. 10-29).

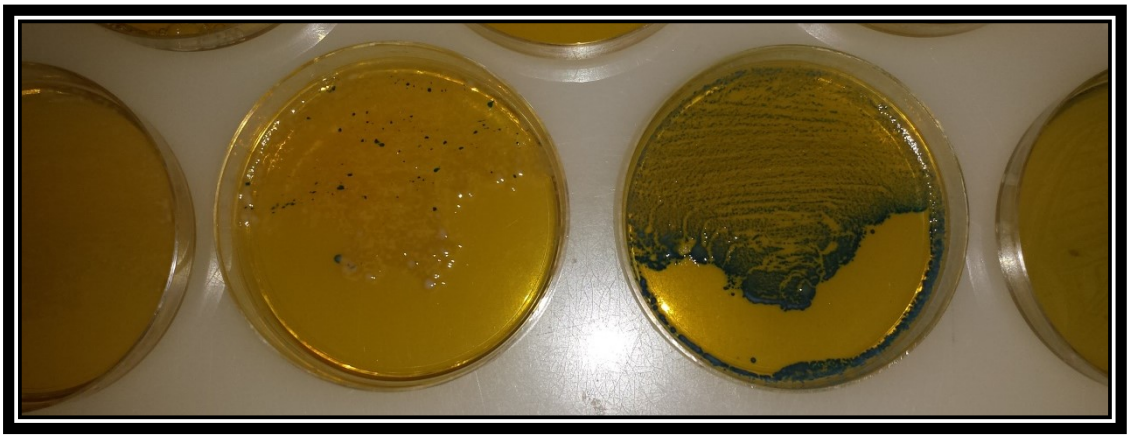

Figure 10-29. Two different strains of the same species with different hydrogen peroxide accumulation.

Table 10.11. Growth and hydrogen peroxide accumulation of *Lactobacillus* type strains and *Lactobacillus* wild strains isolated from human samples.

NG: not grown; ND: not determined; -:negative; +: slightly positive; ++:moderate; +++:strong. (between brackets the number of strains).

Type Strain	H_2O_2	Wild Strain	H_2O_2
L. acidophilus	+	*L. acidophilus* (6)	*NG*
		L. acidophilus (7)	+
L. crispatus	++	*L. crispatus* (9)	*NG*
		L. crispatus (25)	-
		L. crispatus (31)	+
L. delbrueckii	+	*L. delbrueckii* (19)	+
L. fermentum	++	*L. fermentum* (10)	-
		L. fermentum (22)	+
L. gasseri	-	*L. gasseri* (29)	*NG*
		L. gasseri (29)	-
		L. gasseri (29)	+
L. gastricus	++	*L. gastricus* (10)	-
		L. gastricus (18)	+
		L. gastricus (2)	+++
L. jensenii	-	*L. jensenii* (4)	-
L. paracasei	+	*L. paracasei* (10)	+
L. plantarum	+	*L. plantarum* (10)	++
L. paraplantarum	++	*L. paraplantarum*	*ND*
L. reuteri	+	*L. reuteri* (10)	+
L. rhamnosus	-	*L. rhamnosus* (7)	*NG*
		L. rhamnosus (18)	-
L. salivarius	++	*L. salivarius* (21)	-
		L. salivarius (8)	+++
L. vaginalis	++	*L. vaginalis* (12)	+
		L. vaginalis (13)	++

10.6 Detection of inhibitory activity by agar-well diffusion assay

Diameter of inhibition zones were determined by gange, and the measurements are shown in Tables 10.12 – 10.17.

In particular, the supernatants obtained by the type strains grown in five media (LSM, LSM 20% glycerol, MRS, MRS 20% glycerol, MRS 20% glycerol/0,12% pathogens killed by tyndallization) have not given zones of inhibition on all microorganisms tested (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 25212 and *Sarcina lutea* ATCC 9341), instead in the other three media (GM17, GM17 20% glycerol, GM17 20% glycerol/0,12% pathogens killed by tyndallization) the strains did not grow at all, and consequently the supernatants were not tested.

With regard to the strains isolated from human samples, they produce substances with antibacterial activity as demonstrated by inhibition zones in the Fig.s 10-30 - 10-32.

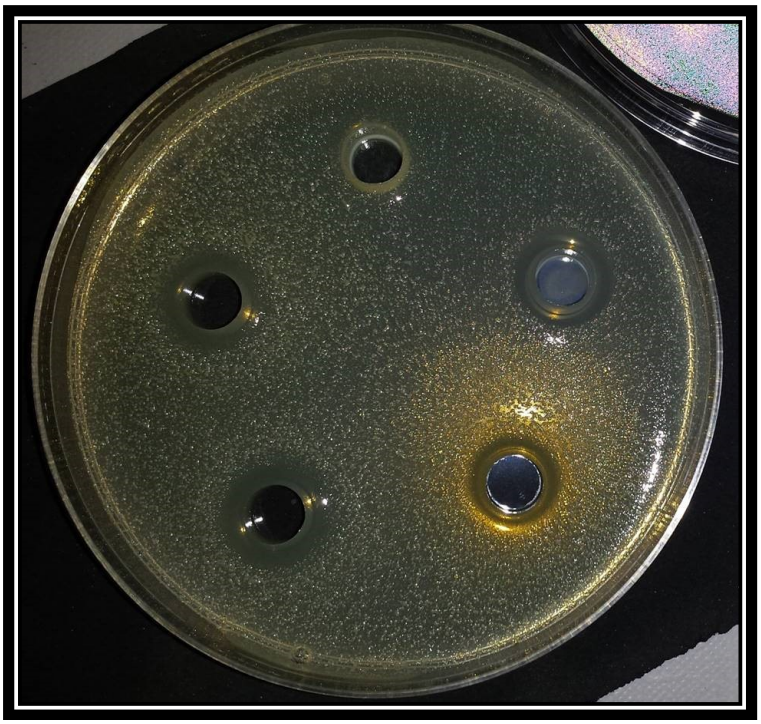

Figure 10-30. Clockwise starting from the top: ML76IV (12mm), LAC42 (16mm) and LAC26 (14mm) against *E. faecalis* ATCC 29212.

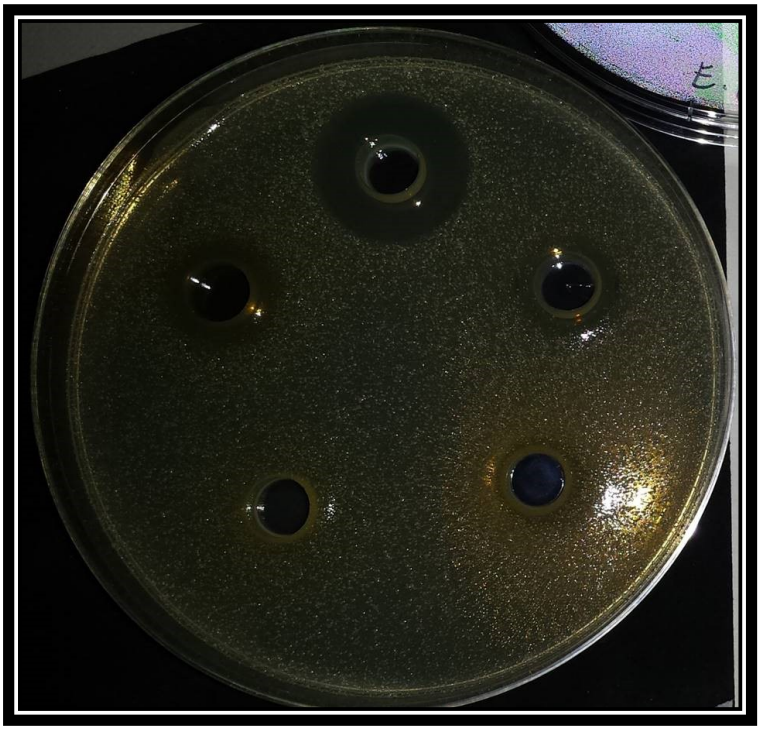

Figure 10-31. Clockwise starting from the top: LAC9 (21mm) and LAC8 (13mm) against E. faecalis ATCC 29212.

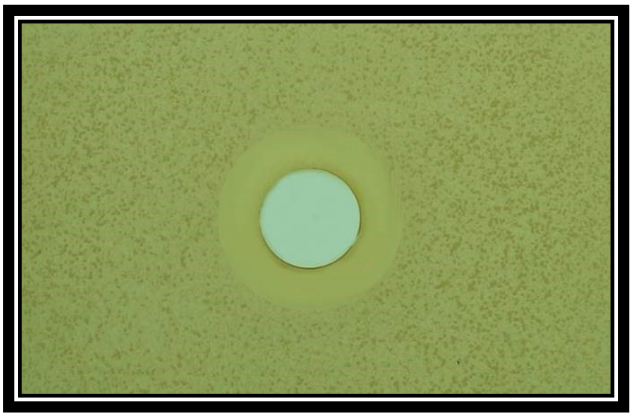

Figure 10-32. LAC26 (15mm) against *E. coli* ATCC 25922.

Secondly, depending on the broth where the strains grew, the supernatants of the same strain were given inhibition zones of different sizes on different pathogens.

In general, the results (Table 10.11 – 10.14) have shown that in conditions more stringent there is more production of antibacterial substance; but this is true until a breaking point where the environment is so hostile that there is no production of the substance or even the growth of the strain.

For example, GM17 broth and kindred have proven to be too basic for the growth of lactobacilli, probably the growth of bacteria was inhibited also because of low

temperature and short time. For all strains not grown on these media, the supernatants were not tested, of course.

Instead, the best medium was MRS with supplements such as glycerol and pathogens killed by tyndallizzation. This could be due to a combination of glycerol as possible precursor, as well as the presence at very low concentration of primary pathogens that it's possible found in the human gut. In the latter case, in fact, the lactobacilli may have received a signal due to the presence of dead bacteria membranes, changing their metabolism and producing antibacterial substance against them. We could describe it as a defense production.

The strains, *L. fermentum* LAC42, *L. gastricus* LAC9, *L. gastricus* LAC47 and *L. paraplantarum* LAC38, grown in MRS 20G and MRS 20G-T, have given the best results, and for this reason the respective supernatants were tested also against *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 21316 (Table 10.15).

Table 10.12. Zones of inhibition measured in mm (diameter) caused by type strains' cell-free supernatants.

Type Strain	***Escherichia coli* ATCC 25922, *Sarcina Lutea* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212**							
	LSM	LSM 20G	MRS	MRS 20G	MRS 20G-T	GM17	GM17 20G	GM17 20G-T
L. crispatus DSMZ 20584	/	/	/	/	/	ND	ND	ND
L. fermentum DSMZ 20052	/	/	/	/	/	ND	ND	ND
L. gasseri DSMZ 20243	/	/	/	/	/	ND	ND	ND
L. gastricus DSMZ 16045	/	/	/	/	/	ND	ND	ND
L. gastricus DSMZ 16046	/	/	/	/	/	ND	ND	ND
L. paraplantarum DSMZ 10667	/	/	/	/	/	ND	ND	ND
L. reuteri DSMZ 20016	/	/	/	/	/	ND	ND	ND
L. salivarius DSMZ 20555	/	/	/	/	/	ND	ND	ND

Table 10.13. Zones of inhibition measured in mm (diameter) caused by human isolates' cell-free supernatants.

Human isolates	***Escherichia coli* ATCC 25922**							
	LSM	LSM 20G	MRS	MRS 20G	MRS 20G-T	GM17	GM17 20G	GM17 20G-T
L. crispatus ML 76 IV	/	15	18	20	22	/	ND	ND
L. fermentum LAC 42	13	18	20	23	25	/	ND	ND
L. fermentum LAC 35	/	/	/	/	/	/	ND	ND
L. gasseri SA IV	/	13	14	15	18	/	ND	ND
L. gasseri LAC 44	/	22	21	23	24	/	ND	ND
L. gastricus LAC 9	/	/	18	20	25	/	ND	ND
L. gastricus LAC 47	/	/	/	/	13	/	ND	ND
L. paraplantarum LAC 38	20	21	20	23	25	/	ND	ND
L. reuteri LAC 8	/	20	21	23	25	/	ND	ND
L. salivarius LAC 26	15	/	/	/	15	/	ND	ND

Table 10.14. Zones of inhibition measured in mm (diameter) caused by human isolates' cell-free supernatants.

Human isolates	***Sarcina lutea* ATCC 9341**							
	LSM	LSM 20G	MRS	MRS 20G	MRS 20G-T	GM17	GM17 20G	GM17 20G-T
L. crispatus ML 76 IV	21	28	30	30	*28*	/	ND	ND
L. fermentum LAC 42	30	28	24	29	*30*	/	ND	ND
L. fermentum LAC 35	30	25	25	30	30	/	ND	ND
L. gasseri SA IV	16	27	18	/	25	/	ND	ND
L. gasseri LAC 44	20	29	26	28	28	/	ND	ND
L. gastricus LAC 9	20	22	25	26	26	/	ND	ND
L. gastricus LAC 47	14	13	16	20	22	/	ND	ND
L. paraplantarum LAC 38	22	23	22	25	28	/	ND	ND
L. reuteri LAC 8	30	27	22	27	/	/	ND	ND
L. salivarius LAC 26	25	/	20	22	25	/	ND	ND

Table 10.15. Zones of inhibition measured in mm (diameter) caused by human isolates' cell-free supernatants.

Human isolates	***Staphylococcus aureus* ATCC 25923**							
	LSM	LSM 20G	MRS	MRS 20G	MRS 20G-T	GM17	GM17 20G	GM17 20G-T
L. crispatus ML 76 IV	/	/	/	/	13	/	ND	ND
L. fermentum LAC 42	16	17	/	/	16	/	ND	ND
L. fermentum LAC 35	/	/	/	/	15	/	ND	ND
L. gasseri SA IV	/	/	/	/	/	/	ND	ND
L. gasseri LAC 44	/	/	/	/	/	/	ND	ND
L. gastricus LAC 9	/	/	15	15	15	/	ND	ND
L. gastricus LAC 47	/	/	/	/	14	/	ND	ND
L. paraplantarum LAC 38	14	14	16	16	18	/	ND	ND
L. reuteri LAC 8	/	/	/	/	12	/	ND	ND
L. salivarius LAC 26	15	13	/	/	15	/	ND	ND

Table 10.16. Zones of inhibition measured in mm (diameter) caused by human isolates' cell-free supernatants.

Human isolates	***Enterococcus faecalis* ATCC 29212**							
	LSM	LSM 20G	MRS	MRS 20G	MRS 20G-T	GM17	GM17 20G	GM17 20G-T
L. crispatus ML 76 IV	/	/	12	12	12	/	ND	ND
L. fermentum LAC 42	11	13	11	11	16	/	ND	ND
L. fermentum LAC 35	/	/	12	13	13	/	ND	ND
L. gasseri SA IV	/	/	11	13	16	/	ND	ND
L. gasseri LAC 44	/	/	12	12	16	/	ND	ND
L. gastricus LAC 9	/	/	12	12	21	/	ND	ND
L. gastricus LAC 47	/	/	12	11	13	/	ND	ND
L. paraplantarum LAC 38	12	13	12	13	15	/	ND	ND
L. reuteri LAC 8	/	/	11	11	13	/	ND	ND
L. salivarius LAC 26	11	12	12	12	14	/	ND	ND

Table 10.17. Zones of inhibition measured in mm (diameter) caused by human isolates' cell-free supernatants.

Human isolates	*Pseudomonas aeruginosa* ATCC 27853		*Klebsiella pneumoniae* ATCC 21316	
	MRS 20G	MRS 20G-T	MRS 20G	MRS 20G-T
L. fermentum LAC 42	13	16	13	16
L. gastricus LAC 9	14	18	14	16
L. gastricus LAC 47	11	14	12	15
L. paraplantarum LAC 38	12	15	15	18

10.6.1 Sensitivity to different pH values

By varying the pH from 3.0 to 9.0, the supernatants did not lose activity, but the diameter of inhibition zones varied. The activity was not linear with increasing or decreasing of pH; an example is LAC9 which possesses a greater antibacterial activity at pH 6.9 (Fig. 10-33). For this reason there are on-going more detailed studies.

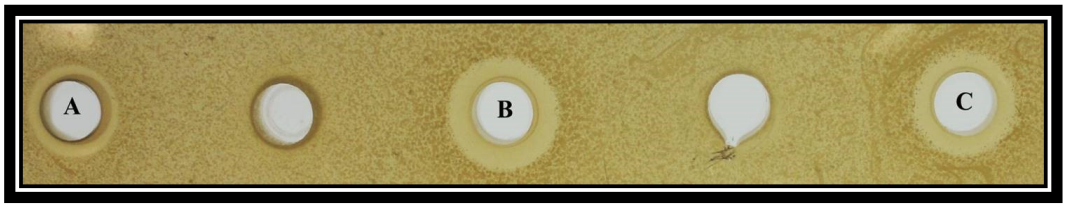

Figure 10-33. LAC9 pH 4.5 (12mm), LAC9 pH 6.9 (18mm) and LAC9 pH 8.0 (15mm) against S. *aureus* ATCC 25923.

10.7 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain

For this pilot study cell-free supernatant of LAC 42 was subjected at the following chromatographic analysis.

10.7.1 SEC (Size-Exclusion Chromatography)

Void volume was calculated as 30% of total volume of gel column:

r^2 x 3,14 x h = $1,25^2$ x 3,14 x 12,3= 60,35 cm^3; 30% = 19,91 mL

70 fractions in total were collected, after passage in Sephadex 50, in the following way: the first two fraction of 10mL (in order to collected even the void volume), then 30 fractions of 1mL, and finally, other two fractions of 10mL.

All fractions were tested, to detect inhibitory activity, against certain Gram positive and Gram negative organisms: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and *Sarcina lutea* ATCC 9341 by agar-well diffusion test. No inhibition zones were detected.

In fact, this result, apparently negative, indicates that the molecule having antibacterial activity has a molecular weight <1500Da, and this is a very important data

10.7.2 Reverse Phase Chromatography

At the same time, the supernatant was subjected to Reverse Phase Chromatography analysis. In particular, a C18 column was used for this purpose. The following fractions were collected:

- 1 mL of flow through (total volume injected)
- 4 mL washing water
- 1 mL of eluate 50/50 (Acetonitrile/ H_2O 0.1% TFA)
- 1 mL of eluate 60/40 (Acetonitrile/ H_2O 0.1% TFA)
- 1 mL of eluate 70/30 (Acetonitrile/ H_2O 0.1% TFA)
- 1 mL of eluate 80/20 (Acetonitrile/ H_2O 0.1% TFA)
- 1 mL of eluate 98/02 (Acetonitrile/ H_2O 0.1% TFA)

The fractions were evaporated to dryness by using an evaporator centrifuge (Concentrator Plus, Eppendorf) and solubilized in water (same starting volume) to determine MIC values. The results obtained are shown in Table 10.18.

Table 10.18. MIC values of fractions against pathogens.

Strain	Flow through	Washing	Eluate 50/50	Eluate 60/40	Eluate 70/30	Eluate 80/20	Eluate 98/02
E. coli ATCC 25922	**1/4**	/	/	/	/	/	/
S. aureus ATCC 25923	**1/8**	/	/	/	/	/	/
E. faecalis ATCC 25212	**1/32**	/	/	/	/	/	/
P. aeruginosa ATCC 27853	**1/4**	/	/	/	/	/	/

The only active fraction was the flow through against all pathogens tested: the more sensitive pathogen was *Enterococcus faecalis* ATCC 25212. The activity against *Pseudomonas aeruginosa* ATCC 27853 is particularly interesting.

10.7.3 1D SDS-PAGE Analysis

One-dimensional SDS-PAGE has shown only a smear on the gel but no a clear band (Fig. 10-34). This is on other demonstration of the hypothesis about small size and hydrophilic nature of the molecule.

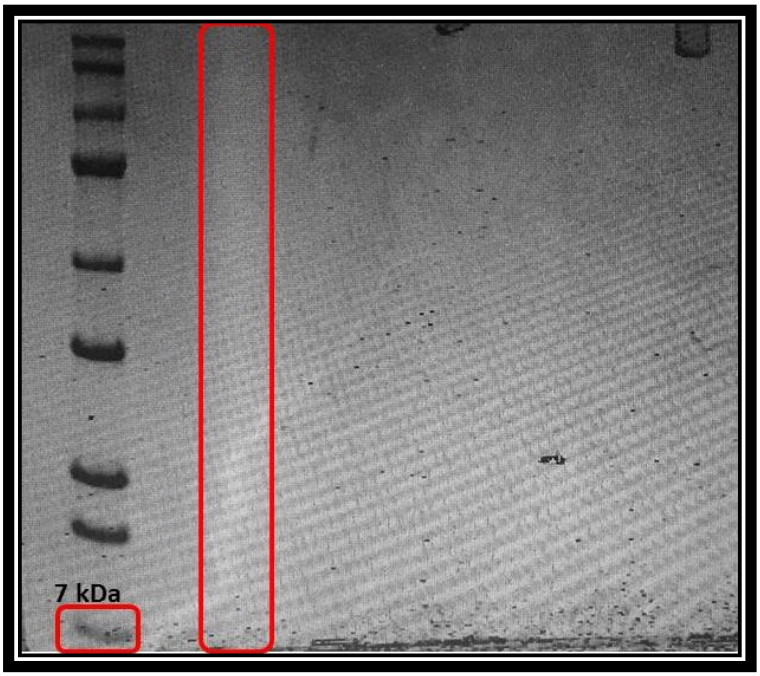

Figure 10-34. Monodimensional gel of the active fraction. It is visible only a smear.

10.7.4 Evaluation of cytotoxicity by the producer strain on BV2 cell line

- **Cell viability**

Fig. 10-35 shows the microglial cells after 24 h in normoxia. Looking at Fig.s 10-36 and 10-37 is possible notice that *Lactobacillus* cells at the concentrations tested ($1.5x10^5$ and $1.5x10^3$) were not toxic, with a survival of BV2 cells of 95.40% and 95.60%, respectively (Table 19.10), almost identical to the positive control.

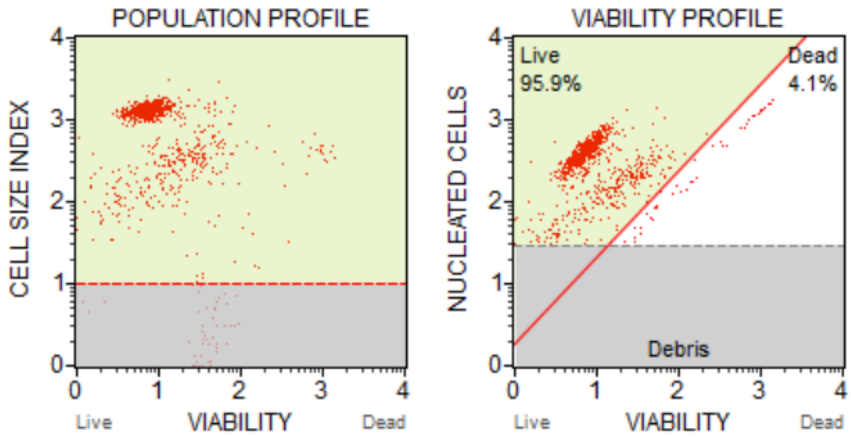

Figure 10-35. Positive control of BV2 cell culture after 24 h in normoxia.

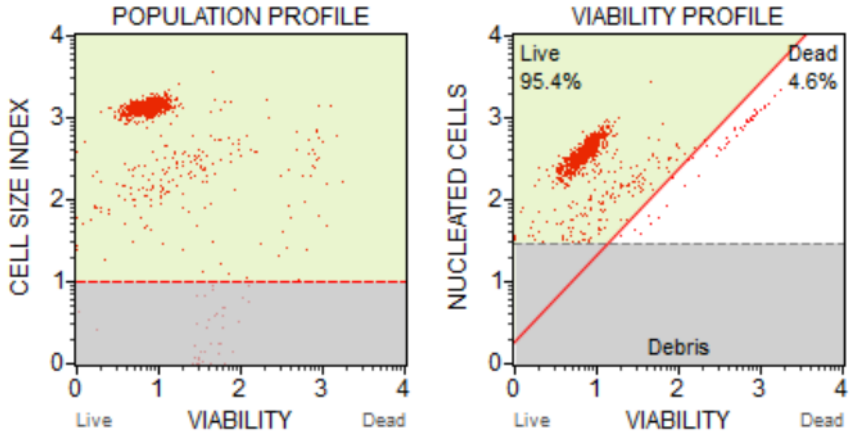

Figure 10-36. . BV2 cell culture viability after 24 h in the presence of $1.5x10^5$ CFU/mL of *Lactobacillus* LAC42.

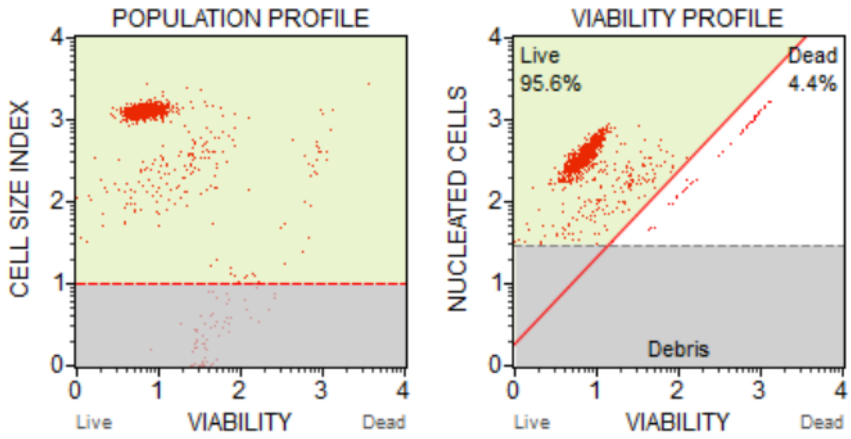

Figure 10-37. BV2 cell culture viability after 24 h in the presence of $1.5x10^3$ CFU/mL of *Lactobacillus* LAC42.

Table 10.19. Viability of BV2 cells after 24 h in the presence of LAC42 cells.

Cell BV2	Total cell	Viable cells	Viability %
CTR+	$2.17x10^6$	$2.08x10^6$	95.90
$1,5x10^5$ CFU/mL	$1,50x10^6$	$1.43x10^6$	95.40
$1,5x10^3$CFU/mL	$2.23x10^6$	$2.14x10^6$	95.60

- **Apoptosis**

The level of apoptotic cell BV2 was calculated after the same treatment: Fig. 10-38 shows the positive control, apoptotic cells after 24 h in normoxia were to 0.30%. Fig.s 10-39 and 10-40 of BV2 after 24 h of tratment with *Lactobacillus* LAC42 at concentrations of $1.5x10^5$ CFU/mL and $1.5x10^3$ CFU/mL, respectively, showing a slight increase of apoptosis for the highest concentration, but 0% for the lowest concentration; even a better result respect to positive control (Table 10.20).

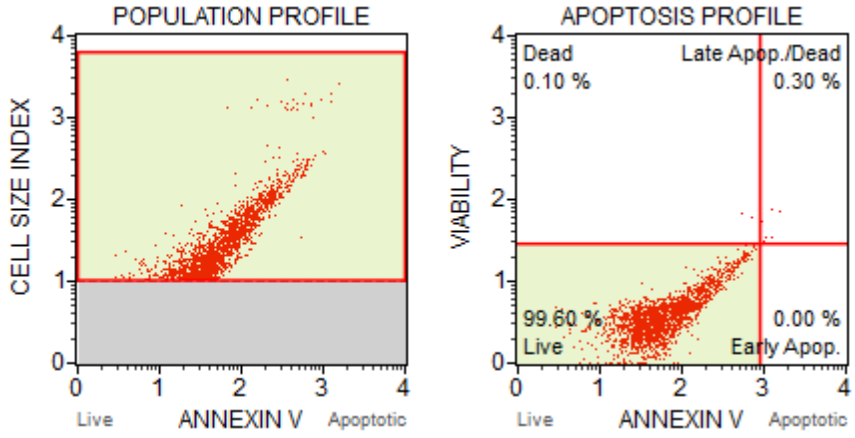

Figure 10-38. Positive control of BV2 cell culture after 24 h in normoxia.

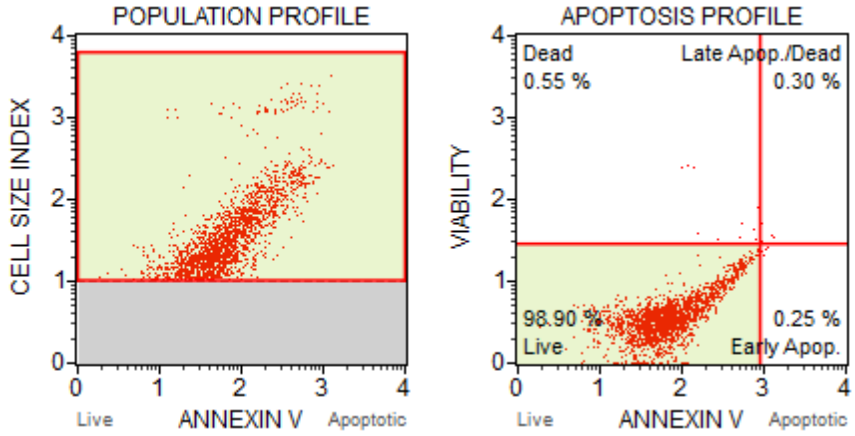

Figure 10-39. BV cell culture after 24 h in normoxia in the presence of $1.5x10^5$ CFU/mL of *Lactobacillus* LAC42.

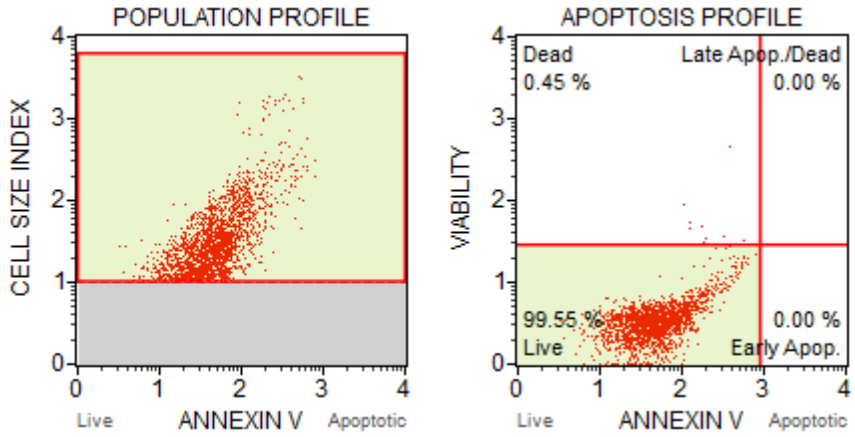

Figure 10-40. BV cell culture after 24 h in normoxia in the presence of $1.5x10^3$ CFU/mL of *Lactobacillus* LAC42.

Table 10.20. Apoptotic state of BV2 cells after 24 h in the presence of LAC42 cells.

Cell BV2	Live	Early apoptotic %	Late apoptotic	Debris	Total apoptotic
CTR+	$1.21x10^6$	0	$3.65x10^30$	$1.22x10^3$	0.30
$1,5x10^5$ CFU/mL	$1.12x10^6$	0.25	$3.41x10^3$	$6.26x10^3$	0.55
$1,5x10^3$CFU/mL	$1.04x10^6$	0	0	$4.71x10^3$	0

11 Discussion & Future outlooks

The aim of this work was the isolation and identification of lactobacilli of human origin. It was also deepened the study of their amensalistic properties, with particular attention to the resistance to gastrointestinal transit and their antagonism against pathogenic microorganisms.

11.1 Identification

As discussed earlier (Chapter 6.0), the identification of the species of *Lactobacillus* isolated in this study was conducted by the use of three different methods based on the analysis of the bacterial 16S rDNA:

- 16S rDNA/RFLP (Randazzo et al., 2004)
- Multiplex PCRs (Song et al., 2000)
- *tuf* gene PCR (Ventura et al., 2003)

The need to use more methods resides in the limits that they possess, individually. Regarding 16S rDNA/RFLP (Randazzo et al., 2004), the electrophoretic profiles consist of approximately 20-40 bands for each restriction enzyme used; the complexity of this profile represents a limit of the method. In my case, the restriction profiles of *L. casei* group were very similar, and for this reason it was difficult to discriminate the species that belong to it. On the other hand, the use of a multiplex PRCs (Song et al., 2000) allows a rapid identification, but it is possible identify only eleven species of lactobacilli. In addition, it does not allow the distinction between *L. casei* and *L. paracasei*, making necessary the use of the third method: specific primers for the *tuf* gene (Ventura et al., 2003). Despite the limitations of the methods listed above, their concomitant use has allowed the identification of 359 strains subject of this study.

The distribution of the species identified is shown in Fig.s 10-2, 10-3 and 10-4. The results show that the most species present in the oro-fecal tract is *L. gasseri*, while the species most representative of the vagina is *L. crispatus*.

In an attempt to address the limitations described above (related to the use of multiple molecular methods), a pilot project based on "Denaturing High Performance Liquid

Chromatography" technology , for the discrimination of 14 species of *Lactobacillus* (Table 10.2) has been developed. The method was successfully applied to confirm the identification of six species of *Lactobacillus* isolated in this study (*L. rhamnosus, L. crispatus, L. paracasei, L. gastricus, L. gasseri* and *L. paraplantarum*).

Indeed, in the literature there are few studies that describe the method for the identification of microbial communities of human origin. For example, some studies were carried out to identify bacteria in urinary tract samples (Domann, et al., 2003) or to analyze changes of the gut microbiota in fecal samples from patients before, during and after antibiotic therapy (Goldenberg et al., 2007). Nevertheless there are no studies that use the method to discriminate among different strains at the species level of *Lactobacillus* human isolates.

11.2 Susceptibility test

When it comes to probiotic strains, one of the most important feedbacks is the determination of the profile off susceptibility/resistance to antibiotics that must be assessed for each bacterial strain to be used: in this way all those strains that could convey resistance to some antibiotics can be eliminated (Dughera, 2012). The EFSA (European Food Safety Authority) document “Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance” has reported a list of antibiotic that should be tested for this aim. MIC values, of all 359 strains of human origin, for ampicillin, erythromycin and clindamycin, were below the microbiological cut-off (Table 10.6 and 10.7); while all strains were resistant to kanamycin.

In addition, all strains tested were also resistant to ciprofloxacin (CLSI M45A, 2006). Although ciprofloxacin is not present in the list of antibiotics reported by EFSA, this was chosen as a representative of fluoroquinolones. This choice was made for the important role that this class of antibiotics plays in maintaining the vaginal microbiota in case of antibiotic therapies (Tempera et al., 2009; Tempera & Furneri, 2010). Indeed, as reported by Tempera & Furneri (2010) the therapeutic choice for ‘aerobic vaginitis’ should take into consideration an antibiotic characterized by an intrinsic activity against the majority of bacteria of fecal origin. The antibiotic should also have bactericidal effect, and poor or absent interference with the vaginal microbiota. Another example is

the fluoroquinolone prulifloxacin, that not affect the lactobacillus component of the vaginal flora in healthy fertile women (Tempera et al., 2009).

11.3 Amensalistic activity

The intestinal mucosa is an enormous surface area exposed intensively to a high number of antigens that come into the lumen. For this reason, the immune system of the intestine must be able to protect the mucosa from pathogens, but at the same time, it must avoid hypersensitivity reactions to food proteins and to the normal intestinal microbiota.

The colonization of the intestine by these bacteria is important, since it contributes also to the development of the mucosal immune system. In fact, in the absence of the intestinal microbiota, the intestinal immune system does not develop properly and the intestinal morphology is greatly impaired (Lu & Walker, 2001). The colonization of the gastrointestinal tract begins with the birth, when the newborn is in contact with the vaginal microbiota maternal and subsequently with the external environment. Since this moment and until adulthood, the microbiota of a person may vary because of external factors (environmental or food), and internal factors, in the case of diseases and alteration of health (Britti, 2006).

The role of the microbiota has been revalued since LAB (Lactic acid bacteria) have shown beneficial effects. In recent years, the interest for the oral administration of probiotics has grown considerably; in fact, the use of these bacteria for the prevention towards some food allergies has been suggested (Montalto et al., 2006).

All this is possible only if the probiotics are able to survive at the gastric transit and if they manage to colonize, even transiently, the intestinal mucosa. Here probiotic bacteria should to integrate with enteric bacteria so as to maintain or induce a balanced microbiota (Cote & Holt, 2006; Ministero della Salute, 2015). The isolation of lactobacilli in fecal samples is indicative of a large intestinal colonization. I extensively discussed the demonstration of the survival of lactobacilli in the intestinal transit and the cassation by EFSA of some claims related to various probiotics (Tetens I, 2010a, 2010b, 2010c, 2010d, 2011a, 2011b, 2012a, 2012b).

It’s the reason why one of the most important experiments of this study was to evaluate the behavior of the strains in typical conditions of the intestinal tract.

Resist at the extremely low pH of the stomach is a fundamental step, since it is the essential requirement to access the intestine: in this study only two species showed a high resistance, *L. gastricus* and *L. gasseri* species. Instead, the strains belonging to *L. acidophilus*, *L. crispatus*, *L. delbruekii*, *L. salivarius*, *L. reuteri*, *L. paracasei* and *L. plantarum* were reduced significantly. Finally, the strains belonging to *L. vaginalis, L. rhamnosus, L. fermentum* and *L. jensenii* have shown good tolerability.

Regarding the sensitivity to bile salts, they strains were able to multiply even in the presence of the highest concentration of bile salts with a strain-dependent resistance/sensitivity if they were not previously treated at low pH. In the other case, after 1 h at pH 3, the bacterial concentration was drastically reduced.

In conclusion, if these strains were administered by oral administration as probiotics, at a concentration of $1,5x10^{11}$ CFU/mL, *L. gastricus* and *L. gasseri* strains (32,6%) would arrive in the intestine, after passing through the stomach, in appropriate concentrations for a local colonization. Instead, the strains of *L. acidophilus* and *L. paracasei* most likely would not be able to reach the intestine in adequate amounts for colonization.

Once colonized the intestines probiotics can perform their beneficial functions, such as the inhibition of pathogenic bacteria. Such an action occurs thanks to the production of substances such as bacteriocins, organic acids, lactic acid and hydrogen peroxide.

The evaluation of the production of hydrogen peroxide was analyzed in this study with the following results: 117 strains (32,6%) did not accumulate hydrogen peroxide, 158 (44%) strains were exhibited a weakly positive reaction, 23 (6,4%) strains were exhibited a moderate positive accumulation of hydrogen peroxide. Accumulation was great by the strains of *L. gastricus* and *L. salivarius* from fecal origin (2,8%).

The heart of this project comes now: evaluate the production of substances with antibacterial activity by the strains isolated from human samples. To avoid the possibility to confuse the inhibitory activity against the pathogens tested caused by the production of hydrogen peroxide and lactic acid, with the production of antibacterial substances, all strains were grown under anaerobic conditions and the supernatants were buffered to pH 6.9.

The molecules with antibacterial activity produced by *Lactobacillus* spp. more commonly found in the literature are of protein nature, the so-called bacteriocins.

It is believed that all LAB produce at least one bacteriocin as a defense mechanism. Accordingly, in this study I proceeded carefully, examining the supernatants of many potentially sensitive strains, to avoid excluding a producer. Moreover, the production of bacteriocins does not occur when the environmental conditions are optimum; in fact, there is the necessity to subject the strains to stress conditions to mimic possible environmental conditions, and, also, choose culture media with different characteristics.
Only ten strains (*L. crispatus* ML 76 IV, *L. fermentum* LAC 42, *L. fermentum* LAC 35, *L. gasseri* SA IV, *L. gasseri* LAC 44, *L. gastricus* LAC 9, *L. gastricus* LAC 47, *L. paraplantarum* LAC 38, *L. reuteri* LAC 8, *L. salivarius* LAC 26) on 359 had given a good antimicrobial activity.
Their supernatants were active against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 25212 and *Sarcina lutea* ATCC 9341 (Table 10.13 – 10.16). The strains *L. fermentum* LAC42, *L. gastricus* LAC9, *L. gastricus* LAC47 and *L. paraplantarum* LAC38 had given the best results, indeed their supernatants were active even against *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 21316 (Table 10.17).
Looking at an overview of the results obtained, it appears that the species *L. gastricus*, *L. gasseri, L. fermentum* and *L. paraplantarum* have excellent amensalistic activities.

11.4 Partial chemical characterization of the antimicrobial fraction produced by an oral origin strain

I chose the cell-free supernatant with the highest antibacterial activity and a broad spectrum of activity, in order to understand the best methodology to isolate the substance. The large number of bacteriocins (Tagg, Dajana, & Wannamaker, 1976) produced by *Lactobacillus* strains makes the isolation of these substances a difficult procedure. This latter is greatly increased by the fact that the chemical and physical properties of bacteriocins are very different depending on the substance in question.
Their molecular weight is usually less than 10kDa, although there are larger bacteriocins, such as lattococcina G, and helveticina I (Beasley & Saris, 2004; Rogne et al., 2008). Usually bacteriocins are cationic and amphipatic molecules with many

residues of lysine and arginine; they are synthesized during the primary growth phase, sensitive to protease, with maximum activity at low pH (Nissen-Meyer & Nes, 1997; Rodriguez et al., 2000; Ennahar et al., 2000; Martı, Horn, & Dodd, 2003); but it is also true that anionic molecules or molecules active only if linked to a non-protein part, a lipid or a sugar, such as pediocin SJ-1 and lactocin 27 (Upreti & Hinsdill, 1975; Schved et al., 1993), were isolated from LAB's supernatants.

The results obtained by the chromatographic methods used, such as SEC (Size-Exclusion Chromatography) and SPE (Solid Phase Extraction, precisely a Reverse Phase Chromatography), indicate that the molecule having antibacterial activity has a very low molecular weight and it has a component particularly hydrophilic. For this reason are ongoing further chromatographic studies using columns with increasing polarity (C4, phenyl, cyano, and amino).

It is possible to find the BLIS acronym (bacteriocin-like inhibitory substance) to refer to those bacteriocins whose amino-acid sequences have not yet been elucidated (Zaeim, Soleimanian-Zad, & Sheikh-Zeinoddin, 2014).

Future outlooks are focused on the identification of the molecule in question, by MALDI-TOF and ESI-TOF and then optimizing the whole process to standardize the entire method. In this way, the opportunity to bring to light new molecules will be possible, with the ultimate goal of being able to take advantage from these antibacterial substances.

Moreover, the BLIS will be investigate for the activity against GBS for probiotical potential and prevent infections in newborns and pregnant women as already studied by Ruìz et al. (2012), or for treatment of acne against *Propionibacterium acne* (Bowe et al., 2006).

Further studies for the production of probiotics and functional foods could be developed thanks to the isolation of BLIS with suitable characteristics for industrial applications.

12 Funding

This work was supported by **Bio-nanotech Research and Innovation Tower (BRIT) Project PON a3_00136** of which I am a recipient of the PhD fellowship, and **PO FESR 2007/2013 Project**.

13 Acknowledgements

First of all, I would like to express my sincere gratitude to my tutor Prof. P.M. Furneri for the continuous support of my researches, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better tutor and mentor for my PhD. study.

I would like to thank my co-ordinator Prof. A. Garozzo for following me and my colleagues during these three years. Also, I'd like to thank all teachers of Microbiology section: Prof. G. Tempera, Prof. G. Blandino, Prof. M.A. Toscano, Prof. A. Stivala, Prof. S. Oliveri, Prof. M. Salmeri, Prof. D. Nicolosi and Prof. A. Morello. A big thank to our head of Department Prof. F. Drago.

My sincere thanks also goes to Prof. S. Foti and Dr. S. Gallina (Dip. di Scienze Chimiche, Università degli Studi di Catania), Prof. G. Li Volti and Dr. K. Heiss (Dip. di Scienze Biomediche e Biotecnologiche, sez. Biochimica Medica, Università degli Studi di Catania), Prof. S. Travali (Dip. di Scienze Biomediche e Biotecnologiche, sez. Patologia Generale, Università degli Studi di Catania) and finally, Prof. A. Spadaro and Dr. S. Ronsisvalle (Dip. di Scienze del Farmaco, Università degli Studi di Catania) who provided me an opportunity to join their team as intern, and who gave access to their laboratories. It would not be possible to conduct this research without their precious support. I thank my colleague and friend Dr. G. Petronio Petronio for the sleepless nights we were working together before deadlines. Also I thank my international colleagues and friends of the Universitetet i Oslo (Faculty of Mathematics and Natural Sciences, Dep. of Biosciences). In particular, I am grateful to Prof. J. Nissen-Meyer and Dr. C. Oppergård to enlightening me particular points of research. Moreover, I would like to thank ADS Biotec Team such as Dr. S. Papadimitriou and Dr. M. Sbalzarini.

Last but not the least, I would like to thank my parents for supporting me spiritually throughout my life.

14 References

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of clinical microbiology*, *43*(11), 5721-5732.

Aebersold, R., & Goodlett, D. R. (2001). Mass spectrometry in proteomics. *Chemical reviews*, *101*(2), 269-296.

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and environmental microbiology*, *66*(5), 2001-2005.

Alaluusua, S., Kleemola-Kujala, E., Nystrom, M., Evalahti, M., & Gronroos, L. (1987). Caries in the primary teeth and salivary Streptococcus mutans and lactobacillus levels as indicators of caries in permanent teeth. *Pediatr Dent*, *9*(2), 126-30.

Allen, S. J., Okoko, B., Martinez, E., Gregorio, G., & Dans, L. F. (2003). Probiotics for treating infectious diarrhoea. *Cochrane Database of Systematic Reviews*, *4*.

Antonio, M. A., Hawes, S. E., & Hillier, S. L. (1999). The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. *Journal of Infectious Diseases*, *180*(6), 1950-1956.

Axelsson, L. T., Chung, T. C., Dobrogosz, W. J., & Lindgren, S. E. (1989). Production of a broad spectrum antimicrobial substance by Lactobacillus reuteri. *Microbial Ecology in Health and Disease*, *2*(2), 131-136.

Aymerich, T., Holo, H., Håvarstein, L. S., Hugas, M., Garriga, M., & Nes, I. F. (1996). Biochemical and genetic characterization of enterocin A from Enterococcus faecium, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology*, *62*(5), 1676-1682.

Baba, T., & Schneewind, O. (1998). Instruments of microbial warfare: bacteriocin synthesis, toxicity and immunity. *Trends in microbiology*, *6*(2), 66-71.

Barefoot, S. F., & Klaenhammer, T. R. (1983). Detection and activity of lactacin B, a bacteriocin produced by Lactobacillus acidophilus. *Applied and Environmental microbiology*, *45*(6), 1808-1815.

Barefoot, S. F., & Klaenhammer, T. R. (1984). Purification and characterization of the Lactobacillus acidophilus bacteriocin lactacin B. *Antimicrobial Agents and Chemotherapy*, *26*(3), 328-334.

Beasley, S. S., & Saris, P. E. (2004). Nisin-producing Lactococcus lactis strains isolated from human milk. *Applied and environmental microbiology*,*70*(8), 5051-5053.

Beigi, R. H., Austin, M. N., Meyn, L. A., Krohn, M. A., & Hillier, S. L. (2004). Antimicrobial resistance associated with the treatment of bacterial vaginosis. *American journal of obstetrics and gynecology*, *191*(4), 1124-1129.

Bergmeyer, H. U. (Ed.). (2012). *Methods of enzymatic analysis*. Elsevier.

Berridge, N. J., Newton, G. G. F., & Abraham, E. P. (1952). Purification and nature of the antibiotic nisin. *Biochemical Journal*, *52*(4), 529.

Bolado-Martínez, E., Pérez-Morales, R., & Acedo-Félix, E. (2009). An Alternative Method for Evaluation of Resistance to Low ph and Bile Salts in Probiotic Characterization of Lactobacillus Reuteri Strains. A Technical Note. *Revista Científica*, *19*(1).

Boskey, E. R., Telsch, K. M., Whaley, K. J., Moench, T. R., & Cone, R. A. (1999). Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infection and immunity*, *67*(10), 5170-5175.

Bowe, W. P., Filip, J. C., DiRienzo, J. M., Volgina, A., & Margolis, D. J. (2006). Inhibition of propionibacterium acnes by bacteriocin-like inhibitory substances (BLIS) produced by Streptococcus salivarius. *Journal of drugs in dermatology: JDD*, *5*(9), 868.)

Britti, M. S. (2006). Studio dell'effetto dei probiotici sulla modulazione della risposta immunitaria intestinale.

Burton, J. P., Cadieux, P. A., & Reid, G. (2003). Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. *Applied and environmental microbiology*, *69*(1), 97-101.

Busarcevic, M., & Dalgalarrondo, M. (2012). Purification and genetic characterisation of the novel bacteriocin LS2 produced by the human oral strain Lactobacillus salivarius BGHO1. *International journal of antimicrobial agents*, *40*(2), 127-134.

Chapman, C. M. C., Gibson, G. R., & Rowland, I. (2011). Health benefits of probiotics: are mixtures more effective than single strains?. *European journal of nutrition*, *50*(1), 1-17.

Chen, H., & Hoover, D. G. (2003). Bacteriocins and their food applications. *Comprehensive reviews in food science and food safety*, *2*(3), 82-100.

Chen, X., Kokkotou, E. G., Mustafa, N., Bhaskar, K. R., Sougioultzis, S., O'Brien, M., ... & Kelly, C. P. (2006). Saccharomyces boulardii inhibits ERK1/2 mitogen-activated protein kinase activation both in vitro and in vivo and protects against Clostridium difficile toxin A-induced enteritis. *Journal of Biological Chemistry*, *281*(34), 24449-24454.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International journal of food microbiology*, *71*(1), 1-20.

CLSI; 2014. *Clinical and Laboratory Standards Institute (CLSI). M100-S22.* Performance Standards for Antimicrobial Susceptibility Testing, 24.

CLSI; 2014. *Clinical and Laboratory Standards Institute (CLSI). M100-S24.* Performance Standards for Antimicrobial Susceptibility Testing, 24.

CLSI; 2006 *Clinical Standard Laboratory Institute (CLSI).* Methods for antimicrobial and disk susceptibility testing of infrequently isolated or fastidious bacteria. Approved standard M45-A. Clinical Laboratory Standard Institute, Wayne, PA, USA 2006

Cole, R. B. (Ed.). (2011). *Electrospray and MALDI mass spectrometry: fundamentals, instrumentation, practicalities, and biological applications*. John Wiley & Sons.

Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S., & Jones, D. (1987). Classification of Lactobacillus divergens, Lactobacillus piscicola, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, Carnobacterium. *International Journal of Systematic Bacteriology*, *37*(4), 310-316.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., ... & Wallbanks, S. (1991). Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters*, *77*(1), 5-12.

Collins, M. D., Samelis, J., Metaxopoulos, J., &Wallbanks, S. (1993). Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus Weissella for the Leuconostoc paramesenteroides group of species. Journal of Applied Bacteriology, 75(6), 595-603.

Cook, R. L., & Sobel, J. D. (1990). Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Review of Infectious Diseases*, *12*(5), 856-872.

Cote, G. L., & Holt, S. M. (2006). Probiotics in food: health and nutritional properties and guidelines for evaluation. Corp Author (s): Food and Agriculture Organization of the United Nations. *World Health Organization*.

Cunsolo, V., Muccilli, V., Saletti, R., & Foti, S. (2013). MALDI-TOF mass spectrometry for the monitoring of she-donkey's milk contamination or adulteration. *Journal of Mass Spectrometry*, *48*(2), 148-153.

Cuozzo, S. A., Castellano, P., Sesma, F. J., Vignolo, G. M., & Raya, R. R. (2003). Differential roles of the two-component peptides of lactocin 705 in antimicrobial activity. *Current microbiology*, *46*(3), 0180-0183.

Daeschel, M. A., McKenney, M. C., & McDonald, L. C. (1990). Bacteriocidal activity of Lactobacillus plantarum C-11. *Food Microbiology*, *7*(2), 91-98.

Dashkevicz, M. P., & Feighner, S. D. (1989). Development of a differential medium for bile salt hydrolase-active Lactobacillus spp. *Applied and Environmental Microbiology*, *55*(1), 11-16.

Daw, M. A., & Falkiner, F. R. (1996). Bacteriocins: nature, function and structure. *Micron*, *27*(6), 467-479.

De Klerk, H. C. (1967). Bacteriocinogeny in Lactobacillus fermenti.

De Klerk, H. C., & Coetzee, J. N. (1961). Antibiosis among lactobacilli. *Nature*,*192*, 340-341.

De Man, J. C., Rogosa, D., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. Journal of applied Bacteriology, 23(1), 130-135.

Deegan, L. H., Cotter, P. D., Hill, C., & Ross, P. (2006). Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International dairy journal*, *16*(9), 1058-1071.

Dellaglio, F., & Felis, G. E. (2005). Taxonomy of Lactobacilli and Bifidobacteria. *Probiotics and prebiotics: scientific aspects*, 25.

Deshpande, G., Rao, S., & Patole, S. (2007). Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. *The Lancet*, *369*(9573), 1614-1620.

Dicks, L. M. T., Van Jaarsveld, D. E., & Van Vuuren, H. J. J. (1992). Casein LHS, a broad-spectrum bacteriocin produced by Lactobacillus casei.

Domann, E., Hong, G., Imirzalioglu, C., Turschner, S., Kühle, J., Watzel, C., ... & Chakraborty, T. (2003). Culture-independent identification of pathogenic bacteria and polymicrobial infections in the genitourinary tract of renal transplant recipients. *Journal of clinical microbiology*, *41*(12), 5500-5510.

Donders, G. G. (2007). Definition and classification of abnormal vaginal flora.*Best Practice & Research Clinical Obstetrics &Gynaecology*, *21*(3), 355-373.

Donders, G. G., Vereecken, A., Bosmans, E., Dekeersmaecker, A., Salembier, G., & Spitz, B. (2002). Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis.*BJOG: An International Journal of Obstetrics &Gynaecology*, *109*(1), 34-43.

Doonan, S. (Ed.). (1996). *Protein purification protocols*. Springer Science & Business Media.

Dubnau, D., Smith, I., Morell, P., & Marmur, J. (1965). Gene conservation in Bacillus species. I. Conserved genetic and nucleic acid base sequence homologies. Proceedings of the National Academy of Sciences of the United States of America, 54(2), 491.

Dughera, L. (2012). Probiotici, prebiotici e disturbi funzionali intestinali.

Dworkin, M., & Falkow, S. (2006). The Prokaryotes: (Vol. 4: Bacteria: Firmicutes, Cyanobacteria, pp. 320-403). New York, NY:Springer Science & Business Media.

EFSA-FEEDAP. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J,10, 2740-2749.

Elli, M., Zink, R., Rytz, A., Reniero, R., & Morelli, L. (2000). Iron requirement of Lactobacillus spp. in completely chemically defined growth media. *Journal of Applied Microbiology*, *88*(4), 695-703.

Elmer, G. W. (2001). Probiotics:" living drugs". *American Journal of Health System Pharmacy*, *58*(12), 1101-1109.

Ennahar, S., Sashihara, T., Sonomoto, K., & Ishizaki, A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS microbiology reviews*, *24*(1), 85-106.

Ercolini, D., Frisso, G., Mauriello, G., Salvatore, F., & Coppola, S. (2008). Microbial diversity in natural whey cultures used for the production of Caciocavallo Silano PDO cheese. International journal of food microbiology, 124(2), 164-170.

Falsen, E., Pascual, C., Sjödén, B., Ohlén, M., & Collins, M. D. (1999). Phenotypic and phylogenetic characterization of a novel Lactobacillus species from human sources: description of Lactobacillus iners sp. nov. *International journal of systematic bacteriology*, *49*(1), 217-221.

FAO, W. (2001). Evaluation of health and nutritional properties of probiotics in food, including powder milk with live lactic acid bacteria. *Food and Agricultural Organization of United Nations and World Health Organization Expert Consultation Report*.

Farrow, J. A., & COLLINS, M. D. (1988). Notes: Lactobacillus oris sp. nov. from the Human Oral Cavity. International journal of systematic bacteriology, 38(1), 116-118.

Felis, G. E., & Dellaglio, F. (2007). Taxonomy of lactobacilli and bifidobacteria. *Current issues in intestinal microbiology*, *8*(2), 44.

Fiani, T. (2007). Studio della produzione di batteriocine da parte di batteri lattici isolati da Boza e Koumis.

Fimland, N., Rogne, P., Fimland, G., Nissen-Meyer, J., & Kristiansen, P. E. (2008). Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin plantaricin EF. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1784*(11), 1711-1719.

Gancheva, A., Pot, B., Vanhonacker, K., Hoste, B., & Kersters, K. (1999). A polyphasic approach towards the identification of strains belonging to Lactobacillus.

Garrity, G. M., Bell, J. A., & Lilburn, T. G. (2004). Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology. *Springer, New York, Berlin, Heidelberg*.

Gawrońska, A., Dziechciarz, P., Horvath, A., & Szajewska, H. (2007). A randomized double-blind placebo-controlled trial of Lactobacillus GG for abdominal pain disorders in children. *Alimentary pharmacology & therapeutics*, *25*(2), 177-184.

Goldenberg, O., Herrmann, S., Marjoram, G., Noyer-Weidner, M., Hong, G., Bereswill, S., & Göbel, U. B. (2007). Molecular monitoring of the intestinal flora by denaturing high performance liquid chromatography. *Journal of microbiological methods*, *68*(1), 94-105.

Goto, M., Hayashidani, H., Takatori, K., & Hara-Kudo, Y. (2007). Rapid detection of enterotoxigenic Staphylococcus aureus harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. Letters in applied microbiology, 45(1), 100-107.

Gratia, A. (1925). Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. *CR Soc Biol*, *93*, 1040-1041.

Guarner, F., Khan, A. G., Garisch, J., Eliakim, R., Gangl, A., Thomson, A., ... & Kim, N. (2012). World gastroenterology organisation global guidelines: probiotics and prebiotics october 2011. *Journal of clinical gastroenterology*, *46*(6), 468-481.

Haley, L. D., Trandel, H., & Coyle, M. B. (1980). Cumitech 11, Practical methods for culture and identification of fungi in the clinical mycology laboratory. *Coordinating ed., JC Sherris. American Society for Microbiology, Washington, DC*.

Hamilton-Miller, J. M. T., Gibson, G. R., & Bruck, W. (2003). Some insights into the derivation and early uses of the word ‘probiotic’. *British Journal of Nutrition*, *90*(04), 845-845.

Hammes, W. P., & Hertel, C. (2006). The genera Lactobacillus and Carnobacterium. In *The prokaryotes* (pp. 320-403). Springer US.

Hammes, W. P., & Vogel, R. F. (1995). The genus lactobacillus. In *The genera of lactic acid bacteria* (pp. 19-54). Springer US.

Hamon, Y., & Peron, Y. (1963). Bacteriologie-Quelques Remarques Sur Les Bacteriocines Produites Par Les Microbes Gram-Positifs. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences*, *257*(5), 1191.

Hartemink, R., Domenech, V. R., &Rombouts, F. M. (1997). LAMVAB — a new selective medium for the isolation of lactobacilli from faeces. *Journal of microbiologicalmethods*, *29*(2), 77-84.

Hastings, J. W., Sailer, M., Johnson, K., Roy, K. L., Vederas, J. C., & Stiles, M. E. (1991). Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from Leuconostoc gelidum. *Journal of Bacteriology*, *173*(23), 7491-7500.

Hayward, A. C. (1957). A comparison of Lactobacillus species from human saliva with those from other natural sources. *Br. Dent. J*, 102, 450-451.

Hayward, A. C., & Davis, G. H. G. (1956). The isolation and classification of Lactobacillus strains from Italian saliva samples. *Br. Dent. J*,101, 2733-2741.

Héchard, Y., Dérijard, B., Letellier, F., & Cenatiempo, Y. (1992). Characterization and purification of mesentericin Y105, an anti-Listeria bacteriocin from Leuconostoc mesenteroides. *Journal of General Microbiology*, *138*(12), 2725-2731.

Heilig, H. G., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermans, A. D., & de Vos, W. M. (2002). Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Applied and environmental microbiology*, *68*(1), 114-123.

Henderson, J. T., Chopko, A. L., & Van Wassenaar, P. D. (1992). Purification and primary structure of pediocin PA-1 produced by Pediococcus acidilactici PAC-1.0. *Archives of Biochemistry and Biophysics*, *295*(1), 5-12.

Herschman, H. R., & Helinski, D. R. (1967). Purification and characterization of colicin E2 and colicin E3. *Journal of Biological Chemistry*, *242*(22), 5360-5368.

Hickson, M., D'Souza, A. L., Muthu, N., Rogers, T. R., Want, S., Rajkumar, C., & Bulpitt, C. J. (2007). Use of probiotic Lactobacillus preparation to prevent diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. *Bmj*, *335*(7610), 80.

Hiraoka, K. (2013). *Fundamentals of Mass Spectrometry*. Springer.

Holck, A., Axelsson, L., Birkeland, S. E., Aukrust, T., & Blom, H. (1992). Purification and amino acid sequence of sakacin A, a bacteriocin from Lactobacillus sake Lb706. *Journal of General Microbiology*, *138*(12), 2715-2720.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S., & Nes, I. F. (2001). Plantaricin W from Lactobacillus plantarum belongs to a new family of two-peptide lantibiotics. *Microbiology*, *147*(3), 643-651.

Hosono, A., Yastuki, K., & Tokita, F. (1977). Isolation and characterization of an inhibitory substance against Escherichia coli produced by Lactobacillus acidophilus [milk and cream]. *Milchwissenschaft*.

Imirzalioglu, C., Hain, T., Chakraborty, T., & Domann, E. (2008). Hidden pathogens uncovered: metagenomic analysis of urinary tract infections. *Andrologia*, *40*(2), 66-71.

Jack, R. W., Wan, J., Gordon, J., Harmark, K., Davidson, B. E., Hillier, A. J., ... & Coventry, M. J. (1996). Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by Carnobacterium piscicola JG126. *Applied and Environmental Microbiology*, *62*(8), 2897-2903.

Jacob, F., Lwoff, A., Siminovitch, A., & Wollman, E. (1953, January). Définition de quelques termes relatifs à la lysogénie. In *Annales De L Institut Pasteur* (Vol. 84, No. 1, Pp. 222-224). 120 Blvd Saint-Germain, 75280 PARIS 06, FRANCE: MASSON EDITEUR.

Jimenez-Diaz, R., Piard, J. C., Ruiz-Barba, J. L., & Desmazeaud, M. J. (1990). Isolation of a bacteriocin-producing Lactobacillus plantarum strain from a green olive fermentation. *FEMS Microbiol. Rev*, *87*, 91.

Joerger, M. C., & Klaenhammer, T. R. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by Lactobacillus helveticus 481. *Journal of bacteriology*, *167*(2), 439-446.

Jorgensen, J. H., & Clinical and Laboratory Standards Institute. (2010). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria: Approved Guideline*. National Committee for Clinical Laboratory Standards.

Kandler, O., & Weiss, N. (1986). Regular, nonsporing gram-positive rods. *Bergey's manual of systematic bacteriology*, *2*(2), 1208-1234.

Kawai, Y., Saito, T., Toba, T., Samant, S. K., & Itoh, T. (1994). Isolation and characterization of a highly hydrophobic new bacteriocin (gassericin A) from Lactobacillus gasseri LA39. *Bioscience, biotechnology, and biochemistry*, *58*(7), 1218-1221.

Kim M., Morrison M., Yu Z., 2011. Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. Journal of Microbiological Methods 84: 81–87.

Kim, H. J., Vazquez Roque, M. I., Camilleri, M., Stephens, D., Burton, D. D., Baxter, K., ... & Zinsmeister, A. R. (2005). A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterology & Motility*, *17*(5), 687-696.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*, *12*(1-3), 39-85.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., ... & Witte, W. (2005). Evaluation of new broth media for microdilution antibiotic

susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Applied and Environmental Microbiology, 71(12), 8982-8986.

Klebanoff, M. A., Schwebke, J. R., Zhang, J., Nansel, T. R., Yu, K. F., & Andrews, W. W. (2004). Vulvovaginal symptoms in women with bacterial vaginosis. *Obstetrics & Gynecology*, *104*(2), 267-272.

Kleynmans, U., Heinzl, H., &Hammes, W. P. (1989). Lactobacillus suebicus sp. nov., an obligatelyheterofermentative Lactobacillus species isolated from fruit mashes.*Systematic and applied microbiology*, *11*(3), 267-271.

Klijn, N., Weerkamp, A. H., & de Vos, W. M. (1991). Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes.Applied and Environmental Microbiology, 57(11), 3390-3393.

Kruis, W., Frič, P., Pokrotnieks, J., Lukáš, M., Fixa, B., Kaščák, M., ... & Schulze, J. (2004). Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. *Gut*, *53*(11), 1617-1623.

Kuebrich, C. T., Sobel, J. D., Wiesenfeld, H. C., & Martens, M. (2004). Chronic vulvovaginal candidiasis. *The New England journal of medicine*, *351*(24), 2554-6.

Larsen, A. G., Vogensen, F. K., & Josephsen, J. (1993). Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricin A, a bacteriocin produced by Lactobacillus bavaricus MI401. *Journal of Applied Bacteriology*,*75*(2), 113-122.

Lawson, P. A., Wacher, C., Hansson, I., Falsen, E., & Collins, M. D. (2001). Lactobacillus psittaci sp. nov., isolated from a hyacinth macaw (Anodorhynchushyacinthinus).*International journal of systematic and evolutionary microbiology*, *51*(3), 967-970.

Lee, M. C., Lin, L. H., Hung, K. L., & Wu, H. Y. (2001). Oral bacterial therapy promotes recovery from acute diarrhea in children. *Acta paediatrica taiwanica*, *42*(5), 301-305.

Leser, T. D., & Mølbak, L. (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental microbiology*, *11*(9), 2194-2206.

Liu, Z., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2008). Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. *Nucleic acids research*, *36*(18), e120-e120.

Lu, L., & Walker, W. A. (2001). Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *The American journal of clinical nutrition*, *73*(6), 1124S-1130S.

MacFarlane, T. W., & Samaranayake, L. P. (2014). *Clinical oral microbiology*. Butterworth-Heinemann.

Marrazzo, J. M., Koutsky, L. A., Eschenbach, D. A., Agnew, K., Stine, K., & Hillier, S. L. (2002). Characterization of vaginal flora and bacterial vaginosis in women who have sex with women.*Journal of Infectious Diseases*, *185*(9), 1307-1313.

Marteau, P., Pochart, P., Doré, J., Béra-Maillet, C., Bernalier, A., & Corthier, G. (2001). Comparative study of bacterial groups within the human cecal and fecal microbiota.*Applied and environmental microbiology*, *67*(10), 4939-4942.

Martì, M. I., Horn, N., & Dodd, H. M. (2003). Heterologous production of bacteriocins by lactic acid bacteria. *International journal of food microbiology*, *80*(2), 101-116.

Martínez-Peña, M. D., Castro-Escarpulli, G., & Aguilera-Arreola, M. G. (2013). Lactobacillus species isolated from vaginal secretions of healthy and bacterial vaginosis-intermediate Mexican women: a prospective study. *BMC infectious diseases*, *13*(1), 189.

McGroarty, J. A., & Reid, G. (1988). Detection of a Lactobacillus substance that inhibits Escherichia coli. *Canadian Journal of Microbiology*, *34*(8), 974-978.

Mego, M., Chovanec, J., Vochyanova-Andrezalova, I., Konkolovsky, P., Mikulova, M., Reckova, M., ... & Drgona, L. (2015). Prevention of irinotecan induced diarrhea by probiotics: A randomized double blind, placebo controlled pilot study. *Complementary Therapies in Medicine*.

Mehta, A. M., Patel, K. A., & Dave, P. J. (1982). Purification and properties of the inhibitory protein isolated from Lactobacillus acidophilus AC1. *Microbios*,*38*(152), 73-81.

Mehta, A. M., Patel, K. A., & Dave, P. J. (1983). Isolation and purification of an inhibitory protein from Lactobacillus acidophilus AC1. *Microbios*.

Meroth, C. B., Hammes, W. P., & Hertel, C. (2004). Characterisation of the microbiota of rice sourdoughs and description of Lactobacillus spicheri sp. nov. *Systematic and applied microbiology*, *27*(2), 151-159.

Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J., & Hammes, W. P. (2003). Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology, 69(1), 475-482.

Ministero della Salute (2015). Commissione unica per la nutrizione e la dietetica. Guidelines on probiotics and prebiotics. *Ministero della Salute*.

Mitsuoka, E. T. (1992). The human gastrointestinal tract. In *The Lactic Acid Bacteria Volume 1* (pp. 69-114). New York, NY: Springer Science & Business Media.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., ... & Yadav, H. (2008). Molecular approaches for identification and characterization of lactic acid bacteria. *Journal of digestive Diseases*, *9*(4), 190-198.

Moll, G. N., Konings, W. N., & Driessen, A. J. (1999). Bacteriocins: mechanism of membrane insertion and pore formation. In *Lactic acid bacteria: Genetics, metabolism and applications* (pp. 185-198). Springer Netherlands.

Moll, G. N., van den Akker, E., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., & Driessen, A. J. (1999). Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. *Journal of bacteriology*, *181*(16), 4848-4852.

Moll, G., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., & Driessen, A. J. (1998). Mechanistic properties of the two-component bacteriocin lactococcin G. *Journal of bacteriology*, *180*(1), 96-99.

Moll, G., Ubbink-Kok, T., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., & Driessen, A. J. (1996). Lactococcin G is a potassium ion-conducting, two-component bacteriocin. *Journal of bacteriology*, *178*(3), 600-605.

Montalto, M., Curigliano, V., Santoro, L., Vastola, M., Cammarota, G., Manna, R., ... & Gasbarrini, G. (2006). Management and treatment of lactose malabsorption. *World journal of gastroenterology: WJG*, *12*(2), 187-191.

Moore, W. E. C., & Moore, L. V. (1994). The bacteria of periodontal diseases. *Periodontology 2000*,*5*(1), 66-77.

Mørtvedt, C. I., & Nes, I. F. (1990). Plasmid-associated bacteriocin production by a Lactobacillus sake strain. *Journal of general microbiology*, *136*(8), 1601-1607.

Mulet-Powell, N., Lacoste-Armynot, A. M., Vinas, M., & De Buochberg, M. S. (1998). Interactions between pairs of bacteriocins from lactic bacteria. *Journal of Food Protection®*, *61*(9), 1210-1212.

Muriana, P. M., & Klaenhammer, T. R. (1991). Purification and partial characterization of lactacin F, a bacteriocin produced by Lactobacillus acidophilus 11088. *Applied and Environmental Microbiology*, *57*(1), 114-121.

Nelson, D. L., Lehninger, A. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry*. Macmillan.

Nigatu, A., Ahrné, S., & Molin, G. (2001). Randomly amplified polymorphic DNA (RAPD) profiles for the distinction of Lactobacillus species. *Antonie van Leeuwenhoek*, *79*(1), 1-6.

Nikolaitchouk, N., Wacher, C., Falsen, E., Andersch, B., Collins, M. D., & Lawson, P. A. (2001). Lactobacillus coleohominis sp. nov., isolated from human sources. International journal of systematic and evolutionary microbiology, 51(6), 2081-2085.

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from Enterococcus faecalis LMG 2333. *Applied and environmental microbiology*, *69*(5), 2975-2984.

Nissen-Meyer J., Holo H., Håvarstein S. L., Sletten K., Nes I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J. Bacteriol. 174, 5686-5692

Nissen-Meyer, J., & Nes, I. F. (1997). Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. *Archives of Microbiology*, *167*(2-3), 67-77.

Noll, F. (1966). Methode zur quantitativen Bestimmung von L (+)-Lactat mittels Lactat-Dehydrogenase und Glutamat-Pyruvat-Transaminase. *Biochem. Z*, *346*, 41-49.

Notardonato, I. (2010). Sviluppo di metodi analitici per determinazioni affidabili in campo ambientale ed alimentare.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research*, *28*(12), e63-e63.

Oppegård, C., Fimland, G., Haug Anonsen, J., & Nissen-Meyer, J. (2015). The pediocin PA-1 accessory protein ensures correct disulfide bond formation in the antimicrobial peptide pediocin PA-1. *Biochemistry*.

Otero, M. C., &Nader-Macías, M. E. (2006). Inhibition of Staphylococcusaureus by H 2 O 2-producing Lactobacillus gasseri isolated from the vaginal tract of cattle. *Animal reproduction science*, *96*(1), 35-46.

Oudega, B., Mooi, F. R., & de Graaf, F. K. (1984). Excretion of proteins by gram-negative bacteria: export of bacteriocins and fimbrial proteins by Escherichia coli. *Antonie Van Leeuwenhoek*, *50*(5-6), 569-584.

Pascual, L. M., Daniele, M. B., Pájaro, C., & Barberis, L. (2006). Lactobacillus species isolated from the vagina: identification, hydrogen peroxide production and nonoxynol-9 resistance. Contraception, 73(1), 78-81.

Patton, G. C., & van der Donk, W. A. (2005). New developments in lantibiotic biosynthesis and mode of action. *Current opinion in microbiology*, *8*(5), 543-551.

Pendharkar, S., Magopane, T., Larsson, P. G., de Bruyn, G., Gray, G. E., Hammarström, L., & Marcotte, H. (2013). Identification and characterisation of vaginal lactobacilli from South African women. *BMC infectious diseases*, *13*(1), 43.

Pingitore, E. V., Hébert, E. M., Nader-Macías, M. E., & Sesma, F. (2009). Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by Lactobacillus salivarius CRL 1328 isolated from the human vagina. *Research in microbiology*, *160*(6), 401-408.

Priestly, C., & Dhar, J. (1997). What is normal vaginal flora? Reply. *Genitourinary Medicine*, *73*(3), 230-230.

Quadri, L. E., Sailer, M., Roy, K. L., Vederas, J. C., & Stiles, M. E. (1994). Chemical and genetic characterization of bacteriocins produced by Carnobacterium piscicola LV17B. *Journal of Biological Chemistry*, *269*(16), 12204-12211.

Rammelsberg, M., & Radler, F. (1990). Antibacterial polypeptides of Lactobacillus species. *Journal of Applied Bacteriology*, *69*(2), 177-184.

Randazzo, C. L., Restuccia, C., Romano, A. D., & Caggia, C. (2004). Lactobacillus casei, dominant species in naturally fermented Sicilian green olives.*International Journal of FoodMicrobiology*,*90*(1), 9-14.

Rantakokko-Jalava, K., Nikkari, S., Jalava, J., Eerola, E., Skurnik, M., Meurman, O., ... & Kotilainen, P. (2000). Direct amplification of rRNA genes in diagnosis of bacterial infections. Journal of clinical microbiology, 38(1), 32-39.

Reid, G., Beuerman, D., Heinemann, C., & Bruce, A. W. (2001). Probiotic Lactobacillus dose required to restore and maintain a normal vaginal flora. *FEMS Immunology & Medical Microbiology*, *32*(1), 37-41.

Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R., & Bruce, A. W. (2003). Oral use of Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunology & Medical Microbiology*, *35*(2), 131-134.

Relman, D. A. (1999). The search for unrecognized pathogens. Science, 284(5418), 1308-1310.

Reuter, G. (2001). The Lactobacillus and Bifidobacteriummicroflora of the human intestine: composition and succession. *Current issues in intestinal microbiology*, *2*(2), 43-53.

Rodríguez, E., González, B., Gaya, P., Nuñez, M., & Medina, M. (2000). Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal*, *10*(1), 7-15.

Rogne, P., Fimland, G., Nissen-Meyer, J., & Kristiansen, P. E. (2008). Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin lactococcin G. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1784*(3), 543-554.

Rogne, P., Haugen, C., Fimland, G., Nissen-Meyer, J., & Kristiansen, P. E. (2009). Three-dimensional structure of the two-peptide bacteriocin plantaricin JK. *Peptides*, *30*(9), 1613-1621.

Rogosa, M., Mitchell, J. A., & Wiseman, R. F. (1951). A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *Journal of Bacteriology*, *62*(1), 132.

Roos, S., Engstrand, L., & Jonsson, H. (2005). Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis sp. nov., isolated from human stomach mucosa. *International journal of systematic and evolutionary microbiology*, *55*(1), 77-82.

Ross, R. P., Morgan, S., & Hill, C. (2002). Preservation and fermentation: past, present and future. *International journal of food microbiology*, *79*(1), 3-16.

Roth, A., Fischer, M., Hamid, M. E., Michalke, S., Ludwig, W., & Mauch, H. (1998). Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *Journal of Clinical Microbiology*, *36*(1), 139-147.

Ruíz, F. O., Gerbaldo, G., García, M. J., Giordano, W., Pascual, L., & Barberis, I. L. (2012). Synergistic effect between two bacteriocin-like inhibitory substances produced by lactobacilli strains with inhibitory activity for Streptococcus agalactiae. *Current microbiology*, *64*(4), 349-356.)

Ryan, M. P., Flynn, J., Hill, C., Ross, R. P., & Meaney, W. J. (1999). The natural food grade inhibitor, lacticin 3147, reduced the incidence of mastitis after experimental challenge with Streptococcus dysgalactiae in nonlactating dairy cows. *Journal of dairy science*, *82*(10), 2108-2114.

Ryan, M. P., Meaney, W. J., Ross, R. P., & Hill, C. (1998). Evaluation of lacticin 3147 and a teat seal containing this bacteriocin for inhibition of mastitis pathogens. *Applied and Environmental Microbiology*, *64*(6), 2287-2290.

Sanders, M. E., Akkermans, L. M., Haller, D., Hammerman, C., Heimbach, J. T., Hörmannsperger, G., & Huys, G. (2010). Safety assessment of probiotics for human use. *Gut Microbes*, *1*(3), 164-185.

Satokari, R. M., Vaughan, E. E., Smidt, H., Saarela, M., Mättö, J., & de Vos, W. M. (2003). Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. *Systematic and applied microbiology*, *26*(4), 572-584.

Saunders, S., Bocking, A., Challis, J., & Reid, G. (2007). Effect of Lactobacillus challenge on Gardnerellavaginalis biofilms. *Colloids and Surfaces B: Biointerfaces*, *55*(2), 138-142.

Schillinger, U., & Lücke, F. K. (1989). Antibacterial activity of Lactobacillus sake isolated from meat. *Applied and Environmental Microbiology*, *55*(8), 1901-1906.

Schirru, S. (2009). Batteriocine da enterococchi isolati da latte di capra. *Appl. Microbiol. Biotechnol*, *53*, 159-166.

Schleifer, K. H., & Ludwig, W. (1995). Phylogeny of the genus Lactobacillus and related genera. Systematic and Applied Microbiology, 18(4), 461-467.

Schved, F., Lalazar, A., Henis, Y., & Juven, B. J. (1993). Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocin produced by Pediococcus acidilactici. *Journal of Applied Bacteriology*, *74*(1), 67-77.

Scialpi, A., & Mengoni, A. (Eds.). (2008). *La PCR e le sue varianti. Quaderno di laboratorio* (Vol. 2). Firenze University Press.

Scopes, R. K. (2013). Protein purification: principles and practice. Springer Science & Business Media.

Sengupta, R., Altermann, E., Anderson, R. C., McNabb, W. C., Moughan, P. J., & Roy, N. C. (2013). The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. *Mediators of inflammation*, *2013*.

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS microbiology reviews*, *28*(4), 405-440.

Sharpe, M. E., Fryer, T. F., & Smith, D. G. (1979). Identification of the lactic acid bacteria. *Identification methods for microbiologists*, *157*, 233-259.

Slots, J., & Chen, C. (1999). The oral microflora and human periodontal disease. In *Medical importance of the normal microflora*(pp. 101-127). Springer US.

Smit, J. (1967). Properties of a Lactobacillus fermenti bacteriocin. *Journal of general microbiology*, *48*(2), 309-316.

Sobel, J. D., Faro, S., Force, R. W., Foxman, B., Ledger, W. J., Nyirjesy, P. R., ... & Summers, P. R. (1998). Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *American journal of obstetrics and gynecology*, *178*(2), 203-211.

Song, Y. L., Kato, N., Liu, C. X., Matsumiya, Y., Kato, H., & Watanabe, K. (2000). Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group-and species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiology Letters*, *187*(2), 167-173.

Spencer, J. F., & de Spencer, A. L. R. (2001). *Food microbiology protocols* (Vol. 14). Springer Science & Business Media.

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, *44*(4), 846-849.

Sýkora, J., Valecková, K., Amlerová, J., Siala, K., Dedek, P., Watkins, S., ... & Schwarz, J. (2005). Effects of a specially designed fermented milk product containing probiotic Lactobacillus casei DN-114 001 and the eradication of H. pylori in children: a prospective randomized double-blind study. *Journal of clinical gastroenterology*, *39*(8), 692-698.

Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriological reviews*, *40*(3), 722.

Tagg, J., & McGiven, A. R. (1971). Assay system for bacteriocins. *Applied microbiology*, *21*(5), 943.

Talarico, T. L., & Dobrogosz, W. J. (1989). Chemical characterization of an antimicrobial substance produced by Lactobacillus reuteri. *Antimicrobial agents and chemotherapy*, *33*(5), 674-679.

Talarico, T. L., Casas, I. A., Chung, T. C., & Dobrogosz, W. J. (1988). Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri. *Antimicrobial agents and chemotherapy*, *32*(12), 1854-1858.

Tannock, G. W. (1995). *Normal microflora: an introduction to microbes inhabiting the human body*. New York, NY:Springer Science & Business Media.

Tannock, G. W. (1999). The normal microflora: an introduction. *Medical importance of the Normal microflora* (pp. 1-23). Springer US.

Tannock, G. W. (2008). Molecular analysis of the intestinal microflora in IBD. *Mucosal immunology*, *1*, S15-S18.

Tannock, G. W. (2010). The bowel microbiota and inflammatory bowel diseases. *International journal of inflammation*, 2010.

Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J., & Gopal, P. K. (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. *Applied and environmental microbiology*, *66*(6), 2578-2588.

Tempera, G., Furneri, P. M., Cianci, A., Incognito, T., Marano, M. R., & Drago, F. (2009). The impact of prulifloxacin on vaginal lactobacillus microflora: an in vivo study. *Journal of Chemotherapy*, *21*(6), 646-650.

Tempera, G., & Furneri, P. M. (2010). Management of aerobic vaginitis. *Gynecologic and obstetric investigation*, *70*(4), 244-249.

Tetens, I (2011b). *Scientific Opinion on the substantiation of health claims related to Lactobacillus johnsonii NCC 533 (La1) (CNCM I-1225) and improving immune defence against pathogenic gastrointestinal microorganisms (ID 896), and protection of the skin from UV-induced damage (ID 900) pursuant to Article 13(1) of Regulation (EC) No 1924/2006.*

Tetens, I. (2012b). *Scientific Opinion on the substantiation of health claims related to a combination of Lactobacillus paracasei CNCM I-1688 and Lactobacillus salivarius CNCM I-1794 and reduction of gastro-intestinal discomfort (ID 2972), decreasing potentially pathogenic gastro-intestinal microorganisms (ID 2972), improved lactose digestion (ID 2972) and increasing IL-10 production (ID 2973) (further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006.*

Tetens, I. (2010a) *Scientific Opinion on the substantiation of a health claim related to Lactobacillus casei strain Shirota and maintenance of the upper respiratory tract defence against pathogens by maintaining immune defences pursuant to Article 13 (5) of Regulation (EC) No 1924/2006: EFSA-Q-2010-00137*. European Food Safety Authority.

Tetens, I. (2010b). *Scientific Opinion on the substantiation of health claims related to Lactobacillus fermentum CECT5716 and maintenance of the upper respiratory tract defence against pathogens by maintaining immune defences (ID 916) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006: EFSA-Q-2008-1703* European Food Safety Authority.

Tetens, I. (2010c). *Scientific Opinion on the substantiation of health claims related to Lactobacillus rhamnosus LB21 NCIMB 40564 and decreasing potentially pathogenic intestinal microorganisms (ID 1064), digestive health (ID 1064), and reduction of mutans streptococci in the oral cavity (ID 1064) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006: EFSA-Q-2008-1803*. European Food Safety Authority.

Tetens, I. (2010d). *Scientific Opinion on the substantiation of a health claim related to Synbio, a combination of Lactobacillus rhamnosus IMC 501R and Lactobacillus paracasei IMC 502R, and maintenance and improvement of intestinal wellbeing pursuant to Article 13(5) of Regulation (EC) No 1924/2006.*

Tetens, I. (2011a). *Scientific Opinion on the substantiation of health claims related to Lactobacillus plantarum 299v and reduction of flatulence and bloating (ID 902), and protection of DNA, proteins and lipids from oxidative damage (ID 1083) pursuant to Article 13(1) of Regulation (EC) No 1924/2006.*

Tetens, I. (2012a). *Scientific Opinion on the substantiation of health claims related to a combination of Lactobacillus gasseri PA 16/8, Bifidobacterium bifidum M 20/5 and Bifidobacterium longum SP 07/3 and maintenance of upper respiratory tract defence against pathogens (ID 931, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006* European Food Safety Authority.

Tichaczek, P. S., Nissen-Meyer, J., Nes, I. F., Vogel, R. F., & Hammes, W. P. (1992). Characterization of the bacteriocins curvacin A from Lactobacillus curvatus LTH1174 and sakacin P from L. sake LTH673. *Systematic and Applied Microbiology*, *15*(3), 460-468.

Toba, T., Samant, S. K., Yoshioka, E., & Itoh, T. (1991a). Reutericin 6, a new bacteriocin produced by Lactobacillus reuteri LA 6. *Letters in applied microbiology*, *13*(6), 281-286.

Toba, T., Yoshioka, E., & Itoh, T. (1991b). Acidophilucin A, a new heat-labile bacteriocin produced by Lactobacillus acidophilus LAPT 1060. *Letters in applied microbiology*, *12*(4), 106-108.

Todorov, S. D., & Dicks, L. M. T. (2004). Influence of growth conditions on the production of a bacteriocin by Lactococcus lactis subsp. lactis ST34BR, a strain isolated from barley beer. *Journal of Basic Microbiology*, *44*(4), 305-316.

Todorov, S. D., & Dicks, L. M. T. (2005a). Lactobacillus plantarum isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology*, *36*(2), 318-326.

Todorov, S. D., & Dicks, L. M. T. (2005b). Effect of Growth Medium on Bacteriocin Production by Lactobacillus plantarum ST194 BZ, a Strain Isolated from Boza. *Food Technology and Biotechnology*, *43*(2), 165-173.

Tong, J. L., Ran, Z. H., Shen, J., Zhang, C. X., & Xiao, S. D. (2007). Meta-analysis: the effect of supplementation with probiotics on eradication rates and adverse events during Helicobacter pylori eradication therapy. *Alimentary pharmacology & therapeutics*, *25*(2), 155-168.

Tortoli, E. (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clinical microbiology reviews*, *16*(2), 319-354.

Twomey, D. P., Wheelock, A. I., Flynn, J., Meaney, W. J., Hill, C., & Ross, R. P. (2000). Protection against Staphylococcus aureus mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. *Journal of Dairy Science*, *83*(9), 1981-1988.

Upreti, G. C., & Hinsdill, R. D. (1973). Isolation and characterization of a bacteriocin from a homofermentative Lactobacillus. *Antimicrobial agents and chemotherapy*, *4*(4), 487-494.

Upreti, G. C., & Hinsdill, R. D. (1975). Production and mode of action of lactocin 27: bacteriocin from a homofermentative Lactobacillus. *Antimicrobial agents and chemotherapy*, *7*(2), 139-145.

Vaillancourt, K., LeBel, G., Frenette, M., Gottschalk, M., & Grenier, D. (2015). Suicin 3908, a New Lantibiotic Produced by a Strain of Streptococcus suis Serotype 2 Isolated from a Healthy Carrier Pig. *PloS one*, *10*(2), e0117245.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological reviews, 60(2), 407-438.

Vásquez, A., Jakobsson, T., Ahrné, S., Forsum, U., & Molin, G. (2002). Vaginal Lactobacillus flora of healthy Swedish women. *Journal of clinical microbiology*, *40*(8), 2746-2749.

Venema, K., Abee, T., Haandrikman, A. J., Leenhouts, K. J., Kok, J., Konings, W. N., & Venema, G. (1993). Mode of action of lactococcin B, a thiol-activated bacteriocin from Lactococcus lactis. *Applied and environmental Microbiology*, *59*(4), 1041-1048.

Venema, K., Haverkort, R. E., Abee, T., Haandrikman, A. J., Leenhouts, K. J., Leij, L. D., ... & Kok, J. (1994). Mode of action of LciA, the lactococcin A immunity protein. *Molecular microbiology*, *14*(3), 521-532.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T. R., & Zink, R. (2003). Analysis, characterization, and loci of the tuf genes in Lactobacillus and Bifidobacterium species and their direct application for species identification. *Applied and environmental microbiology*, *69*(11), 6908-6922.

Vitali, B., Pugliese, C., Biagi, E., Candela, M., Turroni, S., Bellen, G., ... & Brigidi, P. (2007). Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis, or no infection, analyzed by PCR-denaturing gradient gel electrophoresis and real-time PCR. *Applied and environmental microbiology*, *73*(18), 5731-5741.

Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., ... & Whitman, W. B. (Eds.). (2009). Bergey's manual of systematic bacteriology: (Vol. 3: The Firmicutes, pp. 465-511) New York, NY: SpringerScience & Business Media.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*, *73*(16), 5261-5267.

West, C. A., & Warner, P. J. (1988). Plantacin B, a bacteriocin produced by Lactobacillus plantarum NCDO 1193. *FEMS Microbiology Letters*, *49*(2), 163-165.

Wijeyeweera, R. L., & Kleinberg, I. (1989). Arginolytic and ureolytic activities of pure cultures of human oral bacteria and their effects on the pH response of salivary sediment and dental plaque in vitro. *Archives of oralbiology*, *34*(1), 43-53.

Willey, J. M., Sherwood L. M., & WoolvertonC. J. (2008). *Prescott, Harley, and Klein's Microbiology-7th international ed./Joanne M. Willey, Linda M. Sherwood, Christopher J. Woolverton*. New York [etc.]: McGraw-Hill Higher Education.

Wilson, C. (2005). Recurrent vulvovaginitis candidiasis; an overview of traditional and alternative therapies. *Advance for nurse practitioners*, *13*(5), 24-9.

Wilson, K., & Walker, J. M. (2000). *Principles and techniques of practical biochemistry*. Cambridge University Press.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews*, *51*(2), 221.

Wold, A. E. (2001). Immune effects of probiotics. *Scand J Nutr/Naringsforskning*, *2*, 01.

Xiao, W., & Oefner, P. J. (2001). Denaturing high-performance liquid chromatography: A review. *Human mutation*, *17*(6), 439-474.

Yamamoto, T., Zhou, X., Williams, C. J., Hochwalt, A., & Forney, L. J. (2009). Bacterial populations in the vaginas of healthy adolescent women. *Journal of pediatric and adolescent gynecology*, *22*(1), 11-18.

Youssef, N., Sheik, C. S., Krumholz, L. R., Najar, F. Z., Roe, B. A., & Elshahed, M. S. (2009). Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Applied and environmental microbiology*, *75*(16), 5227-5236.

Zaeim, D., Soleimanian-Zad, S., & Sheikh-Zeinoddin, M. (2014). Identification and Partial Characterization of a Bacteriocin-Like Inhibitory Substance (BLIS) from Lb.

Bulgaricus K41 Isolated from Indigenous Yogurts. *Journal of food science*, *79*(1), M67-M73.

Zhang, M., Fan, X., Fang, B., Zhu, C., Zhu, J., & Ren, F. (2015). Effects of Lactobacillus salivarius Ren on cancer prevention and intestinal microbiota in 1, 2-dimethylhydrazine-induced rat model. *Journal of Microbiology*, *53*(6), 398-405.

Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., & Reid, G. (1998). Differentiation of Lactobacillus species by molecular typing. *Applied and environmental microbiology*, *64*(7), 2418-2423.

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. C., Hansmann, M. A., Joyce, P., ... & Forney, L. J. (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *The ISME journal*, *1*(2), 121-133.

Zhu, W. M., Liu, W., & Wu, D. Q. (2000). Isolation and characterization of a new bacteriocin from Lactobacillus gasseri KT7. *Journal of Applied Microbiology*, *88*(5), 877-886.