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**Biotechnological and Spectroscopical Evaluation of Selected  
*Lactobacillus plantarum* Strains with Probiotic and  
Nutraceutical Potentialities**

**Scientific field AGR/16**

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**ABSTRACT:** Probiotics are non-pathogenic microorganisms that, when ingested in adequate amounts, exert a positive influence on their host's health. A variety of microorganisms, typically food grade lactic acid bacteria (LAB), have been evaluated for their probiotic potential and are applied as adjunct cultures in various types of food products or in therapeutic preparations. Within the genus *Lactobacillus*, *Lactobacillus plantarum* is a member of the facultatively heterofermentative group of lactobacilli. It is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. *L. plantarum* strains have also been found in many cheese varieties. Moreover, strains of *L. plantarum* have proven ability to survive gastric transit and colonize the intestinal tract of humans and other mammals. Thus, the aim of this Ph.D. thesis was to select and characterize strains belonging to *Lactobacillus plantarum* species for their probiotic and nutraceutical potentialities which could be added to other foods for exploitation as carrier of functional supplements.

Eighty *Lactobacillus plantarum* strains isolated from different matrixes were evaluated for their probiotic and nutraceutical potential. After a preliminary subtractive screening based on the presence of *msa* and *bsh* genes, the strains which showed the presence of these two genes, were characterized for their nutraceutical features dealing with their ability to produce riboflavin and to remove cholesterol from growth medium.

At the same time, a spectroscopical characterization of these bacteria was carried out. Strains were tested for their ability to grow in milk whey and the fermentation parameters, such as microbial growth, lactose decrease and lactic acid increase, were monitored by a NIR optic probe. Results obtained show how this non-destructive technique could be a viable alternative to traditional methods. In fact, the NIR proposed regression models were very satisfactory for all of the three considered analytes showing a high correlation coefficient coupled with low errors both in calibration and prediction.

Thanks to this technique, it was verified whether strains with different ability to remove cholesterol can show changes in the cell wall. Preliminary data obtained by PCA analysis showed the suitability of these techniques for the study of the bacterial cell walls: changes in the spectra seemed to highlight the possibility to distinguish not only different strains but also different effects produced by the addition of cholesterol in the medium.

The potential of NIR coupled with Aquaphotomics and multivariate techniques to identify species has been investigated for evaluating its feasibility in microbiology. Spectral data analysis revealed very promising results for discrimination and classification of samples, demonstrating how this technique successfully distinguished between different bacteria.

The research of lactic acid bacteria with probiotic and nutraceutical potentialities offer the opportunity to improve the nutritional quality of many fermented foods by increasing their added value. The development of new functional foods by fermentation may contribute to a further expansion of the market for this class of products.

These new functional foods could pave the way for the development of food products "functional" for specific consumer groups with special needs such as the elderly, adolescents, pregnant women, children, athletes and vegetarians.

**RIASSUNTO:** I probiotici sono microrganismi non patogeni che, se ingeriti in quantità adeguate, esercitano un influsso positivo sulla salute dell'ospite. All'interno del genere *Lactobacillus*, *Lactobacillus plantarum* è un membro del gruppo degli eterofermentanti facoltativi. Si tratta di una specie eterogenea e versatile che è presente in una varietà di nicchie ambientali, tra cui latticini, carne, pesce, verdure e molti o fermentazioni vegetali. Inoltre, ceppi di *L. plantarum* hanno mostrato la capacità di sopravvivere di transito gastrico e colonizzare il tratto intestinale sia di esseri umani che di altri mammiferi. Pertanto, l'obiettivo della presente tesi di dottorato è stato quello di selezionare e caratterizzare i ceppi appartenenti alla specie

*Lactobacillus plantarum* per le loro potenzialità probiotiche e nutraceutiche al fine di poter utilizzare questi ceppi come carrier di integratori funzionali.

Ottanta ceppi di *Lactobacillus plantarum* isolati da diverse matrici sono stati valutati per il loro potenziale probiotico e nutraceutico. Dopo uno screening preliminare basato sulla presenza dei geni *msa* e *bsh*, i ceppi che mostravano la presenza di questi due geni, sono stati caratterizzati per le loro caratteristiche nutraceutiche riguardanti la capacità di produrre riboflavina e di rimuovere il colesterolo dal mezzo di crescita.

Allo stesso tempo è stata effettuata una caratterizzazione spettroscopica. I ceppi sono stati testati per la loro capacità di crescere in siero del latte e i parametri di fermentazione, come la crescita microbica, la diminuzione lattosio e l'aumento dell'acido lattico sono stati monitorati da una sonda ottica NIR. I risultati ottenuti mostrano come questa tecnica non distruttiva possa essere una valida alternativa ai metodi tradizionali. Infatti, i modelli di regressione proposti sono molto soddisfacenti per tutti i tre analiti considerati mostrando un alto coefficiente di correlazione accoppiato con bassi errori sia in calibrazione che in predizione.

Grazie a questa tecnica, si è verificato se i ceppi con differente capacità di rimuovere il colesterolo possano mostrare cambiamenti nella parete cellulare. Dati preliminari ottenuti dall'analisi PCA hanno evidenziato l'idoneità di tali tecniche per lo studio delle pareti cellulari batteriche: le variazioni negli spettri sembravano evidenziare la possibilità di distinguere non solo ceppi diversi, ma anche diversi effetti prodotti dalla dipendenza di colesterolo nel mezzo.

Inoltre è stata studiata la capacità della tecnica NIR accoppiata ad Aquaphotomics e alla statistica multivariata di identificare specie batteriche.

L'analisi dei dati spettrali ha rivelato risultati molto promettenti per la discriminazione e classificazione dei ceppi dimostrando come questa tecnica sia in grado di distinguere batteri diversi.

La ricerca di batteri lattici con potenzialità probiotiche e nutraceutiche offre l'opportunità di migliorare la qualità nutrizionale di molti alimenti fermentati, aumentandone il valore aggiunto. Lo sviluppo di nuovi alimenti funzionali mediante fermentazione può contribuire ad una ulteriore espansione del mercato per questa classe di prodotti.

Questi nuovi alimenti potrebbero aprire la strada per lo sviluppo di prodotti alimentari "funzionali" per gruppi specifici di consumatori con esigenze speciali, come gli anziani, adolescenti, donne incinte, bambini, atleti e vegetariani

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## **1. INTRODUCTION**

# 1.1 LACTIC ACID BACTERIA

## 1.1.1 General characteristics and technological applications

Lactic acid bacteria (LAB) are a heterogeneous group of microorganisms, whose species may possess different morphology, metabolism, adaptability and growth characteristics. They are Gram +, ubiquitous, rods or cocci shaped, non-motile, non-sporeforming, facultative or obligate anaerobic, heterotrophic, fermenting sugars according to different metabolic processes, however, mainly producing lactic acid.

They are divided into different families and in particular:

- Family Lactobacillaceae: genus *Lactobacillus* and *Pediococcus*;
- Family Streptococcaceae: genus *Streptococcus* and *Lactococcus*;
- Family Leuconostocaceae: genus *Leuconostoc*, *Oenococcus* and *Weissella*

The optimum growth temperature is variable between different species and according to this classification, LAB are divided in mesophilic or thermophilic bacteria.

In relation to their ability to ferment sugars, they are classified into:

- Homofermentative: ferment only sugars to produce lactic acid as a primary metabolite;
- Heterofermentative: ferment sugars to produce, in addition to lactic acid, acetic acid, ethyl alcohol and CO<sub>2</sub> as primary metabolites.

The microorganisms belonging to this group are, in general, very demanding from the nutritional point of view and require complex substrates for their growth. For this reason, despite their ubiquity, LAB tend to colonize only those matrices which possess a composition rich in constituents and suitable for their multiplication.

The metabolites associated with the multiplication of LAB are the basis of fermentation processes of different fermented foods such as dairy products (cheese, cream and yoghurt), salami and sausages, vegetables (sauerkraut), sourdoughs and wine.

The production of lactic acid and other primary metabolites may induce changes in the structure of proteins, in food rheology and inhibits pathogenic and alternative microorganisms. Furthermore, it extends the shelf life of products.

Concerning lactic acid bacteria, in order of their use in food technology, it is useful to deepen the study of different phenotypic traits.

To evaluate the technological functionality of LAB, it is necessary to study their phenotypic traits such as:

- the ability to ferment lactose and galactose or the ability to use other carbon substrates (citric acid, carbohydrate fractions associated with casein);
- the possibility to use different nitrogen fractions (peptides and amino acids);
- resistance to osmotic stresses;
- the ability to grow in different conditions of temperature characteristics of a particular process;
- the ability to interact positively with other microorganisms or to inhibit them;
- the proteolytic activity,
- the phage resistance;
- the autolytic ability;
- the capability to produce extracellular polysaccharides .

The development of phenotypic and genotypic techniques has favored the possibility of recognizing the presence of more biotypes within the same species. It has been frequently highlighted as strains belonging to the same species, characterized by significant genetic polymorphisms, may have different metabolic attitudes. This assumes, therefore, a technological relief: it is possible to use strains with different characteristics useful for the transformation or to prepare mixtures of different biotypes able to interact and cooperate during the fermentation process. In this sense, the biodiversity observed in certain species of

microorganism can be considered an opportunity of practical interest (Mucchetti et al., 2006).

### 1.1.2 Lactic acid bacteria and metabolism

LAB have a simple metabolism and even though they require complex precursors present in the growth medium to supply their nutritional requirements. The nutritional requirements of LAB usually include, in addition to carbon sources, amino acids, vitamins, nucleic acids and salts. Environments designed to provide microorganisms this pool of substances available in free form are not many, and despite the ubiquity of LAB, only a relatively small group of natural substrates (mainly milk, vegetables and meat) is designed to promote a massive growth.

#### 1.1.2.1 Carbohydrates fermentation

An essential aspect of the metabolism of LAB is carbohydrates fermentation in order to obtain the needed energy for cells biosynthetic processes. LAB are able to use different monosaccharides and disaccharides. This attitude varies in relation to the enzyme equipment of each species and it presents different efficiency in relation to the physiological characteristics of the biotype and environmental conditions.

The fermentation process that derives from the development of the LAB lactic microflora involves the total or partial utilization of fermentable sugars present, that are subtracted to the potential use by germs spoilage, and induces the modification of the chemical-physical characteristics of the matrix resulting in the accumulation of metabolites primary such as lactic acid, ethanol and CO<sub>2</sub> (Figure 1.1).

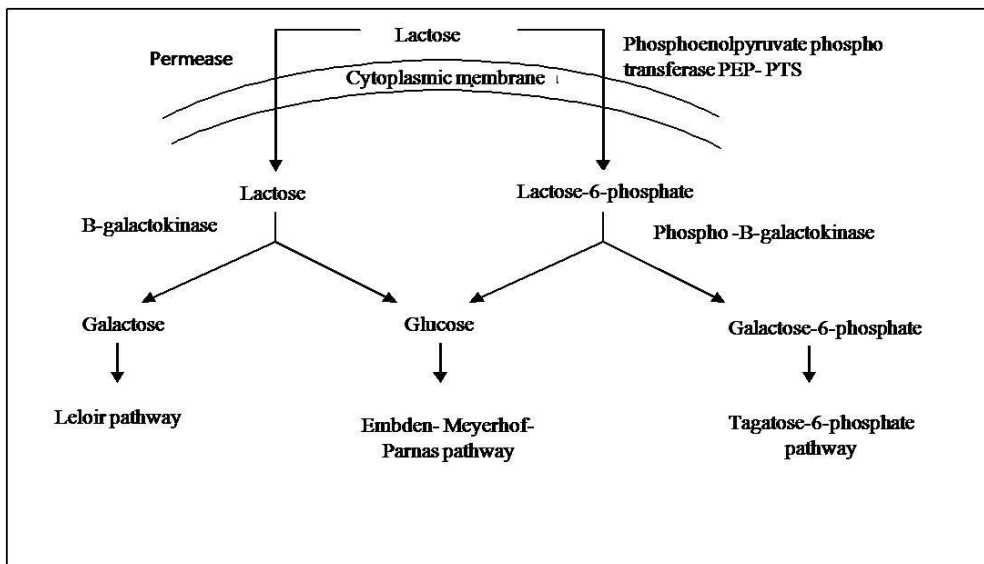


Figure 1.1: Lactic acid bacteria homolactic fermentation pattern

LAB can be divided into two groups: homofermentative and heterofermentative depending on which of the two main fermentative pattern they use. In the first case, from a molecule of a monosaccharide, such as glucose, it is possible to obtain a theoretical yield of two molecules of pyruvate which, in normal conditions (presence of sugars and limited presence of oxygen), are reduced to lactic acid (Figure 1.2).



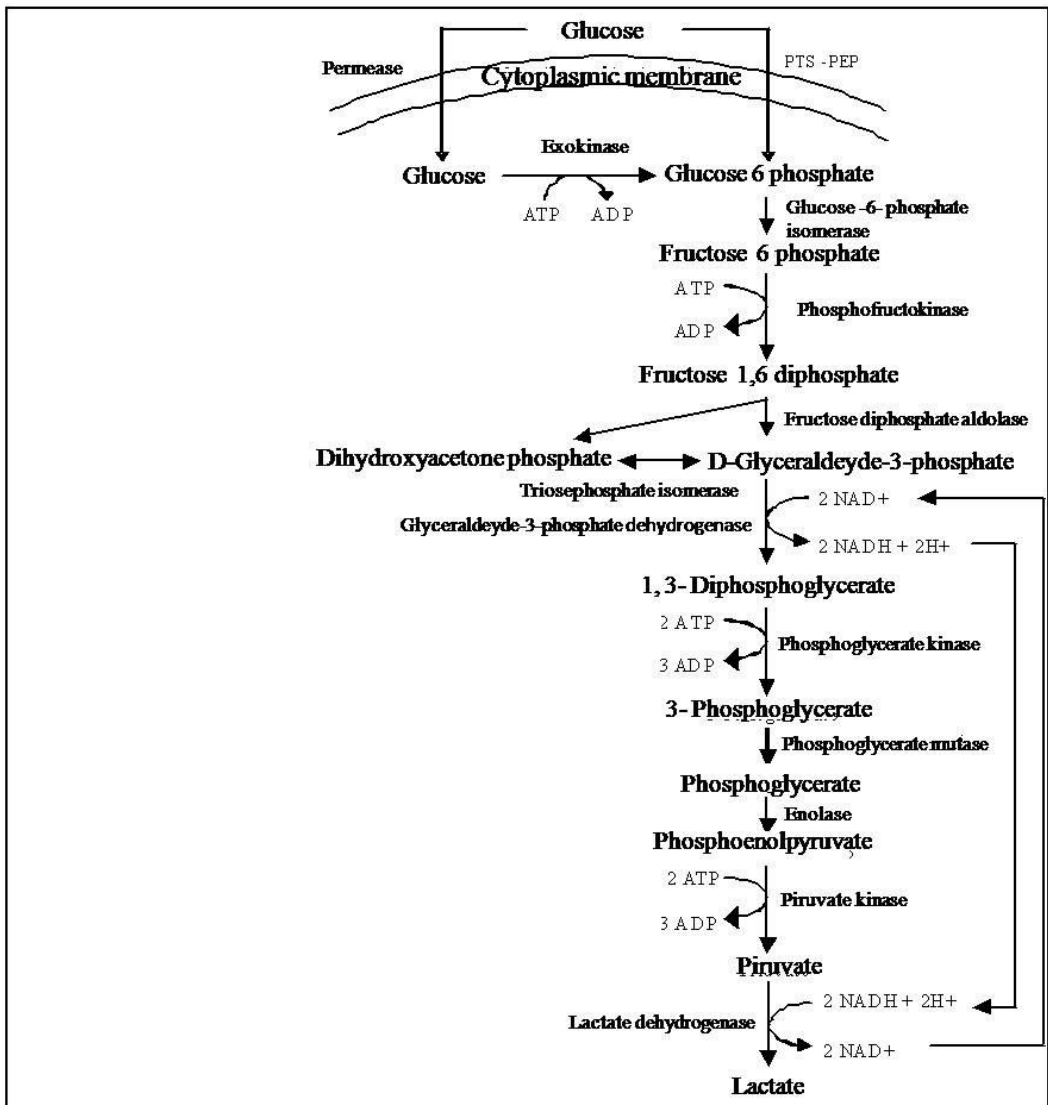


Figure 1.2: Homolactic fermentation: Embden-Meyerhof-Parnas pattern

Different monosaccharides (galactose, fructose, mannose etc..) may be fermented by LAB through the way of glucose-6-phosphate pathway or alternatively, through the way of the tagatose-6-phosphate although less widespread among LAB (Figure 1.3).

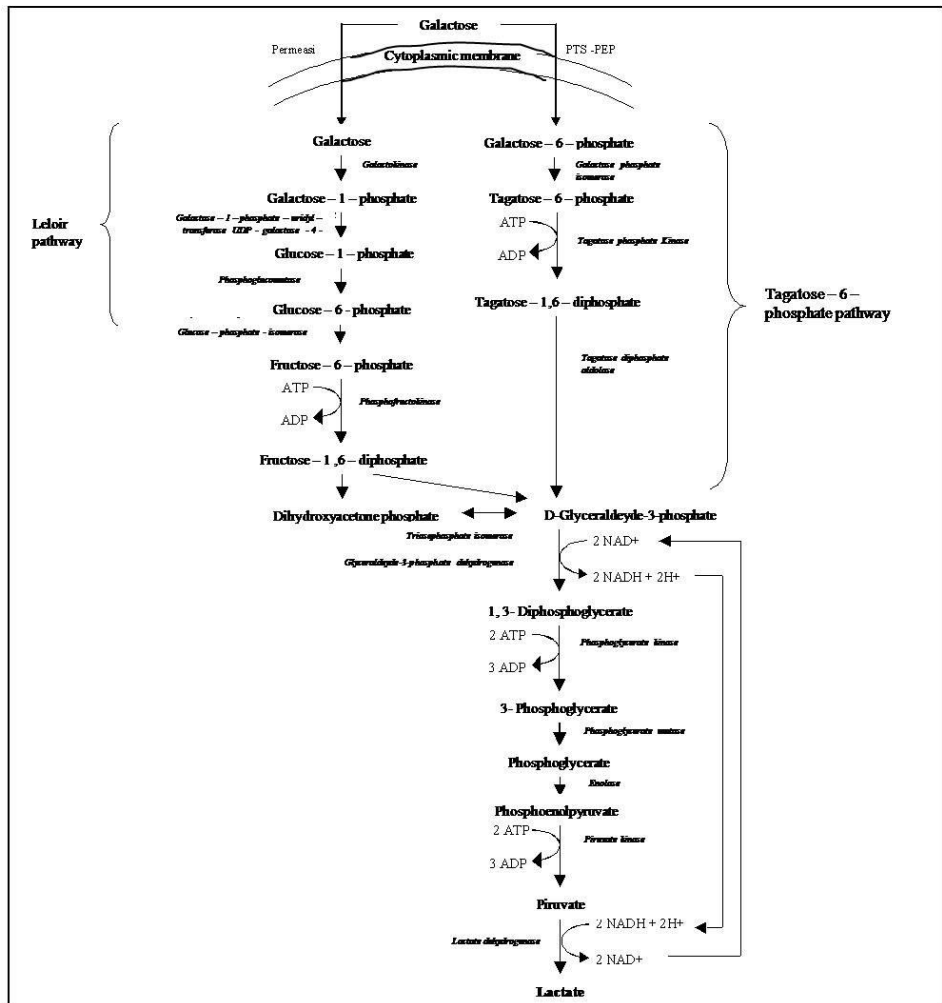


Figure 1.3: Galactose fermentation pathway

Decarboxylation is a characteristic process of heterolactic fermentation which causes the release of CO<sub>2</sub> and the theoretical equimolar production of lactic acid and ethanol or acetaldehyde. The yield of lactic acid is halved compared to that produced during homolactic fermentation (Figure 1.4).

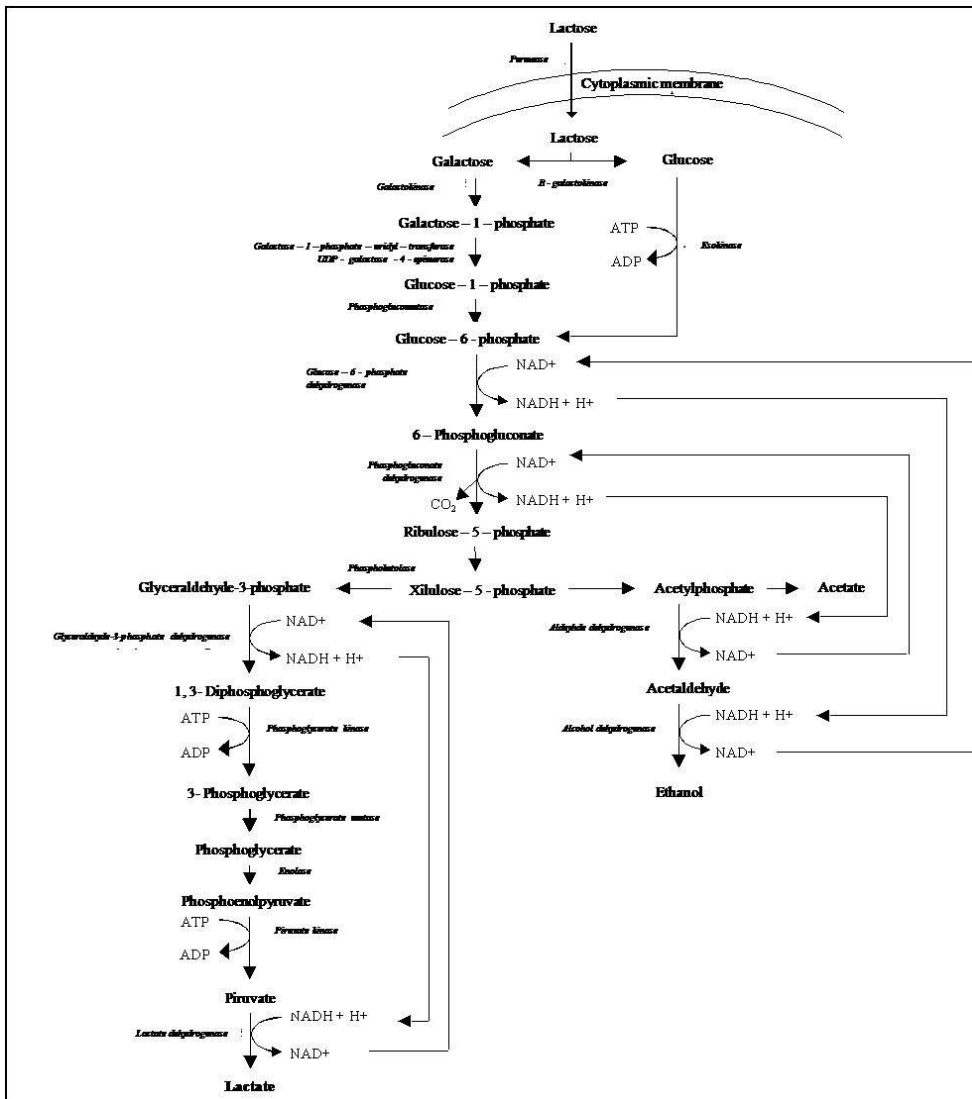


Figure 1.4: Heterolactic fermentation: phosphoketolase pathway

Many species of LAB, not only heterofermentative, are able to also use the pentose sugars. In general, specific permease located in cytoplasmic membrane are involved in the transportation of these sugars inside the cell. In the cytoplasm, pentoses are phosphorylated and converted by specific epimerase or isomerase to ribulose-5-phosphate.

Citrate is an important carbon source, among all carbon sources present in the matrix, for LAB and different species are able to transform citrate in aromatic compounds. It is not directly used as electrons acceptor but it is converted, by citrate lyase, in acetate and oxalacetate from whose decarboxylation, piruvate is obtained (Figure 1.5).

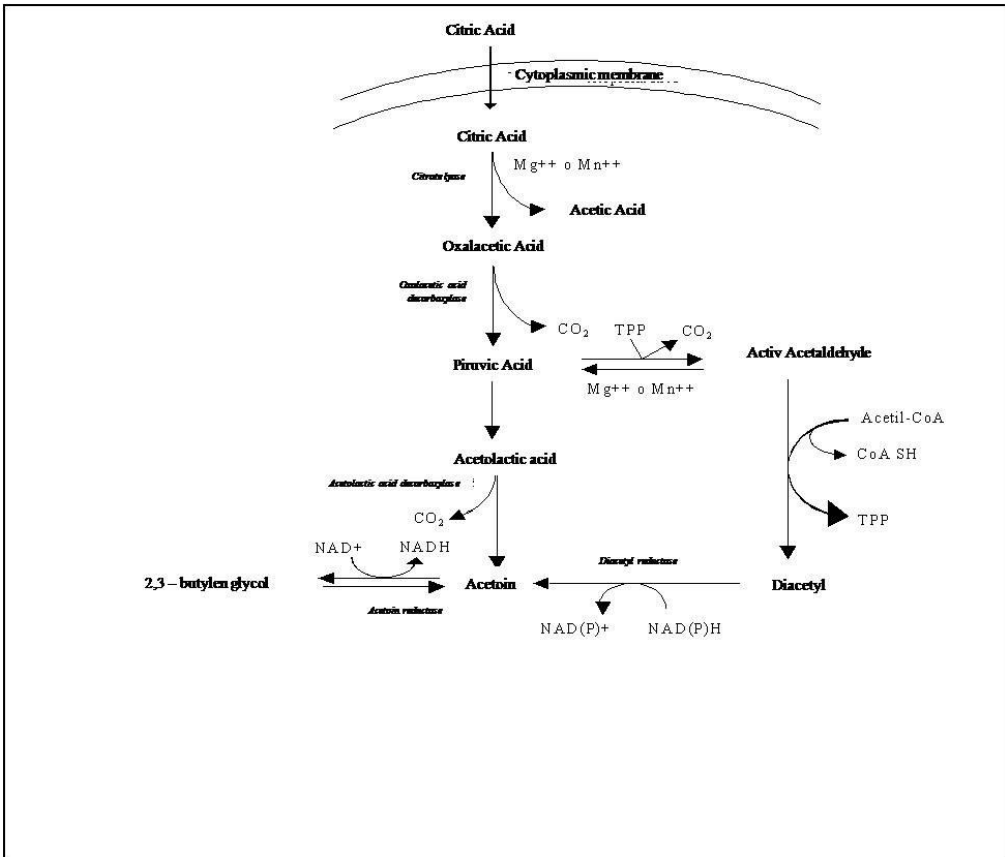


Figure 1.5: Citric acid utilization from LAB

### 1.1.2.2 Proteolysis

LAB are particularly demanding in amino acids. This nutritional need is variable among different species and, in some cases, is linked to specific characteristics of the single strain. Milk proteins, in particular casein, are too large to be able to permeate through the membrane into the cytoplasm of the bacterial cell. At the same time, the nitrogenous non-proteic fraction, with a lower molecular weight, composed by peptides with different sizes, free amino acids, urea and nitrogen bases, is not always sufficient to allow an adequate bacterial growth.

LAB, therefore, utilize endo and exopeptidases to hydrolyze casein and whey proteins, which allow the production of peptides with compatible size with transport across the cell membrane. Then, the peptides will transform in simple compounds in the cytoplasm. Amino acids and oligopeptides can be used by the cell for biosynthetic finality or alternatively, demolished to acids or amines and/or processed through other metabolic pathways, if they are in excess of the cellular needs. The catabolism of amino acids is considered one of the metabolisms of major importance for the formation of flavor and aroma in cheese.

### 1.1.2.3 Lipolysis and polysaccharides production

The attitude of LAB to degrade the lipidic fraction of milk is less deepened because it is generally believed that the lactic bacteria are not able to hydrolyse triglycerides characteristic of the fat fraction of milk even though it is recognized an esterase activity against diglycerides and monoglycerides.

Many strains of LAB are also capable of producing different exopolysaccharides, which may get stuck on the cell wall to form a sort of capsule or released in the medium. The role of these substances is of considerable interest, in particular in relation to the possibility to obtain yoghurt and/or fermented milk-based beverages with different rheological characteristics and viscosity.

#### **1.1.2.4 Bacteriocins production**

Bacteriocins are secondary metabolites, which are produced in larger amounts after the exponential phase of bacterial growth. It is a heterogeneous group of molecules characterized by different spectrum of inhibition, structure, molecular weight, conditions of activity and stability (Mucchetti et al., 2006).

## 1.2 PROBIOTICS

### 1.2.1 Functional aspects

Probiotics are defined as "live microorganisms which when administered in adequate amounts, exert beneficial effects on the health of the consumer" (Guarner et al., 2005). Nowadays, most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*. However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces* and *Propionibacterium* are also considered as probiotic microorganisms (Table 1.1) (Vinderola et al., 2003).

<i>Lactobacillus</i> sp.	<i>Bifidobacterium</i> sp.	<i>Enterococcus</i>	<i>Lactococcus</i> sp.
<i>L. acidophilus</i>	<i>B. bifidum</i>	<i>Ent. faecalis</i>	<i>Lc. lactis</i> subsp. <i>lactis</i>
<i>L. casei</i>	<i>B. adolescentis</i>	<i>Ent. faecium</i>	<i>Lc. lactis</i> subsp. <i>cremoris</i>
<i>L. delbruekii</i> spp. <i>bulgaricus</i>	<i>B. animalis</i>		<i>Lc. lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i>
<i>L. cellobiosus</i>	<i>B. infantis</i>		
<i>L. curvatus</i>	<i>B. thermophilus</i>		
<i>L. fermentum</i>	<i>B. longum</i>		
<i>L. lactis</i>			
<b><i>L. plantarum</i></b>			
<i>L. reuteri</i>			
<i>L. brevis</i>			

Table 1.1: The most commonly used species of lactic acid bacteria in probiotic preparations (Parvez et al., 2006)

The direct target of the action of these organisms is the intestine, but the whole body is indirectly the beneficial effects of probiotics. The function is to promote the proliferation and the balance of the bacterial composition that constitutes the intestinal ecosystem. The intestinal microbiota is made up of hundreds of different bacterial species, whose multiple metabolic activities affect the state of health of the host. In particular, during the evolution of the human species, it has established an important and delicate balance of mutual benefit, between man and bacteria, which may, however, be compromised by sudden changes. The intestinal bacterial population and its biochemistry, affect many aspects of the pathophysiology of the host, such as digestion, lipid metabolism and resistance to the invasion of pathogenic microorganisms. In addition, probiotics are able to manifest beneficial activities on organs and tissues, regardless of the mode and route of administration. The latter effect can be explained by the ability of probiotics to implement the immunocompetence of the intestinal mucosa and regulate the permeability of the intestinal wall, which in pathological situations may be less, increasing the risk of dangerous pathogens can pass into the blood stream (Vertuani et al., 2001).

The selection of probiotic microorganisms must consider important aspects:

- the security (the origin of the microorganisms used, non-pathogenicity and the absence of transferable antibiotic-resistance);
- the technological characteristics (fermentation ability, adaptability to various food matrices);
- the functional characteristics (viability and persistence in the gastro-intestinal tract, immunomodulation, antagonistic and antimutagenic properties).

There are numerous factors that can affect the functionality of probiotics (Figure 1.8). The most important features that a probiotic must have are:

- strong resistance to gastric acid;
- tolerance to bile;
- ability to adhere to intestinal epithelia and persistence in the gastro-intestinal tract;
- attitude immunostimulant;
- antagonistic activity against pathogens such as *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes*, *Clostridium difficile*;

- resistance to phages;
- high vitality and stability during the preparation and storage of food in which they are conveyed (Mattila-Sandholm et al., 2002).

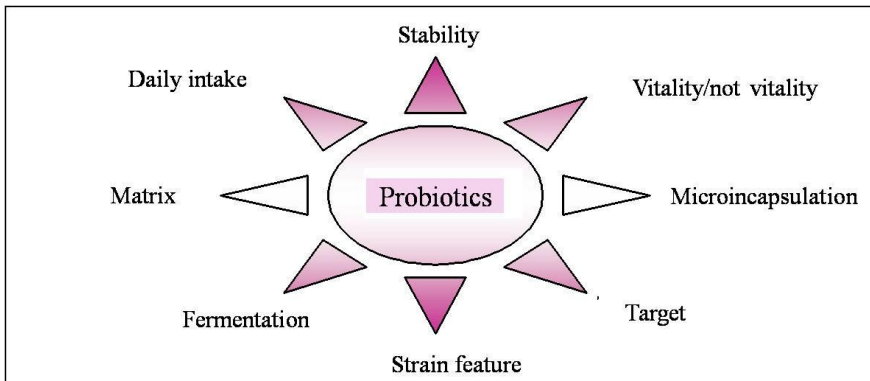


Figure 1.8: Main technological factors that influence the functionality of probiotics according to Mattila-Sandholm et al., 2002.

Many probiotic bacteria are of human origin. This choice is based on the belief that these strains, when ingested with foods fermented by them, are better adapted to colonize the human intestine. Currently there is little scientific evidence to support this observation, and recent studies have shown that the character of a probiotic strain is not dependent on its origin but it is a species-and strain-specific characteristic (Bude Ugarte et al., 2006; Schillinger 2005; Minelli et al., 2004).

## 1.3 LACTOBACILLUS PLANTARUM

### 1.3.1 General aspects

The genus *Lactobacillus* comprises more than 50 species, many of which are often used as starter cultures for the production of fermented foods as they contribute to the conservation, to the flavor and the to the rheology of the products. In addition, many species of this genus, including *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus plantarum*, are able to colonize the human and animal gut.

*Lactobacillus plantarum* belongs to the phylogenetic group of *Lactobacillus casei* - *Pediococcus* of the genus *Lactobacillus* and in particular to the phylogenetic subgroup of *L. plantarum* (Bringel et al., 2005).

It is a Gram-positive bacterium, facultative heterofermentative, non-mobile, non-spore-forming, rod-shaped with rounded ends, usually from 0.9 to 1.2 µm at 3-8 µm, facultative anaerobic, able to grow at 15°C but not at 45°C (de Vries et al., 2006). Some strains, in certain conditions, are capable of nitrate reduction.

The fermentation profile is very wide: this species is, in fact, able to ferment many carbohydrates, except for rhamnose. Lactic acid is produced both in the D and L forms (Bishop et al., 1993).

*Lactobacillus plantarum* is one of the most frequently microorganisms isolated from sourdoughs, and in a wide range of environmental niches, including dairy, meat products such as salami, and many fermented vegetables (olives and sauerkraut).

At this microorganism is recognized an important role in the process of fermentation due to its ability to rapidly acidify the medium and the ability to produce bacteriocins, preventing the growth of pathogenic microorganisms and spoilage, improving the hygienic safety and food preservation (de Vries et al., 2006).

### 1.3.2 Probiotic aspects

*Lactobacillus plantarum* is a microorganism that grows abundantly in many environmental niches. Many clinical tests were conducted to evaluate the effects due to the ingestion of *Lactobacillus plantarum* by subjects suffering from various diseases. Recent studies have demonstrated that the recruitment of adequate quantities of *Lactobacillus plantarum* 299v on the part of subjects characterized by high levels of cholesterol in the blood, leads to a significant reduction of LDL and fibrinogen, which are risk factors for the development of heart disease (Parvez et al., 2006).

A considerable attention has been paid to the ability of probiotics to inhibit intestinal infections caused by pathogens. *Lactobacillus plantarum* is able, among the constituents of the natural bacterial flora of the whole intestinal tract, to bind to the intestinal villi and to prevent access of pathogenic bacteria to the intestinal wall, keeping the balance of the endogenous flora and modulating the immune system associated with it (GALT: gut-associated lymphoid tissue or gut-associated lymphoid tissue) (de Vries et al., 2006).

Some strains of *Lactobacillus plantarum* are capable of interacting with the specific receptor of mannose present on the surface of the intestinal cells (erythrocytes). This bond promotes adhesion to the intestinal epithelium and its colonization by *Lactobacillus plantarum*, and causes an inhibitory effect against pathogens causing intestinal infections, especially those caused by *Escherichia coli*, due to competition for binding to mannose receptors present on erythrocytes (Pretzer et al., 2005).

*Lactobacillus plantarum* has demonstrated therapeutic efficacy in infections associated with *Clostridium difficile*, and its administration (in association with other lactobacilli) in capsules or in functional foods (yoghurt) has been proposed in association with antibiotic treatment for



preventive purposes (de Vries et al., 2006).

*Lactobacillus plantarum* in fact, is able to produce different substances capable of inhibiting the development of pathogenic bacteria. This effect is due to the production of organic acids which reduce the pH of the medium, and the production of anti-microbial metabolites such as hydrogen peroxide and bacteriocins (Bishop et al., 1993).

### 1.3.3 Technological aspects

Probiotics most widely used in food belong to the genera *Lactobacillus* and *Bifidobacterium*. In particular for the genus *Lactobacillus* species most commercially used are *L. casei/paracasei*, *L. acidophilus* and *L. rhamnosus* (Stanton et al., 2003).

The dairy industry has used probiotic cultures as a tool for the development of new products: some dairy products, in fact, have proved good vehicles of probiotics for human consumption (Champagne & Garner, 2005), although some of the probiotic species mentioned above do not develop well in milk or remain viable during the life of the product, limiting its industrial application.

This justifies the need for a continuous search for new strains with probiotic potential, able to diversify the range of dairy products available on the market (Farmworth, 2003).

*L. plantarum* has the ability to change cyclically habitat (from the gastro-intestinal human to fermented foods, and vice versa) demonstrating its adaptability and competitiveness, which in turn justified by the plasticity of its genome recently sequenced. The large number of membrane proteins suggests that *L. plantarum* has the ability to adhere to different surfaces and to potential substrates for growth. Moreover, the high number of genes encoding the regulatory functions, indicates a marked ability to adapt to many and different environmental conditions. All this explains the ability of *L. plantarum* to grow in wide range of food ecosystems (de Vries et al., 2006).

These characteristics make *Lactobacillus plantarum* potential and interesting candidate for possible applications in the field of fermented milks and justify their interest for further study on its probiotic properties.

## 1.4 INFRARED SPECTROSCOPY

### 1.4.1 Theoretical principles of infrared spectroscopy (Workman & Weyer, 2008; Burns & Ciurczak, 2001; Siesler, 2002)

Infrared spectroscopy can be defined as the analysis of materials regarding their tendency to absorb light in a certain area of the electromagnetic radiation. In particular, it is used to indicate the separation, detection and recording of changes in energy (resonance peaks) involving nuclei, atoms or entire molecules. These energetic variations are due to the interaction between radiation and matter, specifically the emission, absorption or diffusion of electromagnetic radiation or particles. Infrared spectroscopy is applied for quantitative and qualitative analysis. Its most important and characteristic application field is the identification of organic compounds that give rise, especially in the mid-infrared region, to generally complex spectra with several maxima and minima absorption peaks. In many cases, in fact, the infrared spectrum of an organic compound provides a unique fingerprint that is easily distinguishable from other compounds.

The high selectivity of the methodology often allows the quantitative determination of an analyte in a complex mixture without prior separation. The theoretical basis of the interaction between matter and radiation is the quantum nature of energy transfer from the radiation to matter and vice versa. In fact both the matter and the electromagnetic field have a “dual nature”, i.e. the ability to behave both as waves and as particles. Electromagnetic radiations, the best known of which is light, are nothing but a form of transport of energy electromagnetic thought space. According to studies of James Clerck Maxwell, the movement of electrical charges can generate waves of radiant energy in space. They are the result of the superposition of an electric field and a magnetic field orthogonal mutually coupled: each of them is the source of the other and propagates with a sinusoidal movement in both space and time (Figure 1.10).

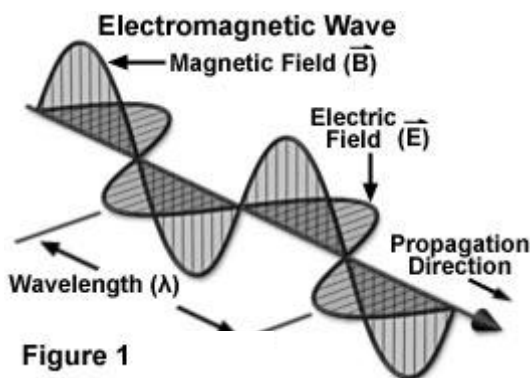


Figure 1.10: Representative model of the electromagnetic radiation.

The directions of oscillation in space of electric and magnetic fields are perpendicular to the direction of propagation which has a wave nature. The wave character of the electromagnetic radiation is commonly described by its wavelength ( $\lambda$ ), measured in nanometers ( $\text{nm} = 10^{-9} \text{ m}$ ), the wave number ( $\nu$ ), which represents the number of waves present in a unit length, measured in reciprocal centimeters ( $\text{cm}^{-1}$ ), the speed (V) with the wave advance, and the number of wavelengths that pass in a given point per unit of time, frequency ( $\nu$ ), measured in hertz ( $\text{Hz} = \text{s}^{-1}$ ). The relationship between these quantities is given by the expression formula

$$v = \frac{l}{\lambda} = \frac{\nu}{V}$$

where  $V$  is the velocity of the electromagnetic wave in vacuum, i.e. the rate of radiation diffusion. Maxwell discovered that the propagation speed was constant for all the electromagnetic waves in vacuum, and it was equal to  $2.998 \times 10^{10} \text{ cm s}^{-1}$ , i.e. the speed of light. Thus, being the speed propagation constant, the frequency can be deduced from the wavelength and vice versa. The entire electromagnetic spectrum is composed of several areas defined by specific wavelengths as shown in Figure 1.11. This division gives rise to five major groups: the visible region, the ultraviolet and ionizing radiation, characterized by high frequencies and short wavelengths, and the infrared and radio waves, characterized by low frequency and high wavelengths. The infrared region of the spectrum comprises radiation with wave numbers ranging from about 12500 to  $10 \text{ cm}^{-1}$ . It's usually divided into three regions: the higher energy near-IR (NIR), ( $4000\text{-}10000 \text{ cm}^{-1}$ ) exciting overtone or harmonic vibrations; the mid-infrared (IR), ( $4000\text{-}400 \text{ cm}^{-1}$ ) used to study fundamental vibrations and associated rotational-vibrational structure; the far-infrared (FIR) ( $400\text{-}10 \text{ cm}^{-1}$ ) used for rotational spectroscopy.

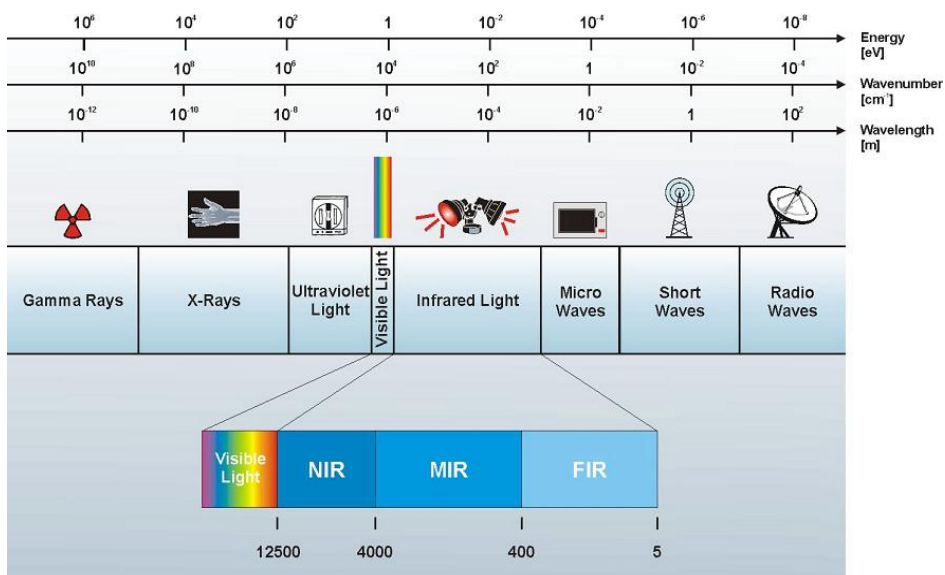


Figure 1.11: Electromagnetic spectrum.

The radiation shows its particle nature when interacts with matter. It does not transmit a continuous quantity of energy, as in classical physics, but “packets” of quantized energy. It can therefore be considered as a stream of particles called photons. The interaction between radiation and matter happens when the quantum energy transfer occurs between the electromagnetic wave and the energy states of matter and vice versa. By considering that the radiation consists of photons and the energy transmitted by a photon is proportional to the frequency of the electromagnetic wave, the amount of energy that a photon of a certain wave transmits to the matter can be calculated through the Einstein -Planck relation:

$$E = h\nu = \frac{hc}{\lambda} = hc\nu$$

where  $E$  is the energy in Joules,  $h$  is Planck's constant ( $6.62 \times 10^{-34}$  J / s) and  $\nu$  is the frequency of the radiation in Hertz.

This function shows that the energy of a photon or of a monochromatic radiation (single frequency) depends on its wavelength ( $\lambda$ ) or by its frequency ( $\nu$ ). A radiation beam can have an intensity more or less strong depending on the amount of photons per time unit and area unit, but the quantum energy ( $E$ ) is always the same for a given frequency of radiation. The electromagnetic spectrum is the radiation set consisting in a series of photons or electromagnetic waves at increasing energy and it can therefore be divided into regions, corresponding to well-defined fields of energy. Thus, the electromagnetic radiation it is not distributed in a continuous way but in a quantized way and consequently also the energetic events occurring at the atomic or molecular level. From these considerations, Bohr (Burns & Ciurczak, 2001) in 1914 laid the foundation for a correct interpretation of the spectra of atoms and molecules with the following postulates:

1. The atomic systems exist in stable states, without emitting electromagnetic energy.
2. The absorption or the emission of electromagnetic energy occurs when an atomic system changes from one energy state to another.

3. The process of absorption or emission corresponds to a photon of radiant energy

$h\nu = E' - E''$ , where  $E' - E''$  is the difference in energy between two states of an atomic system.

So, according to quantum physics, a molecule can not rotate or vibrate freely with any value of energy, but it is subjected to what are called quantum restrictions. So when the energy of a radiation goes through the energy of a molecule that is vibrating, there is a transfer of energy that can be measured and graphically represented as a variation of energy (in the ordinate) and wavelength (in the abscissa) as a spectrum. According to the third postulate of Bohr (Burns & Ciurczak, 2001), the passage of energy from a photon to a molecule can take place only if the photon has a frequency, and therefore energy, equal to that is necessary to move the molecule from the ground to the excited state.

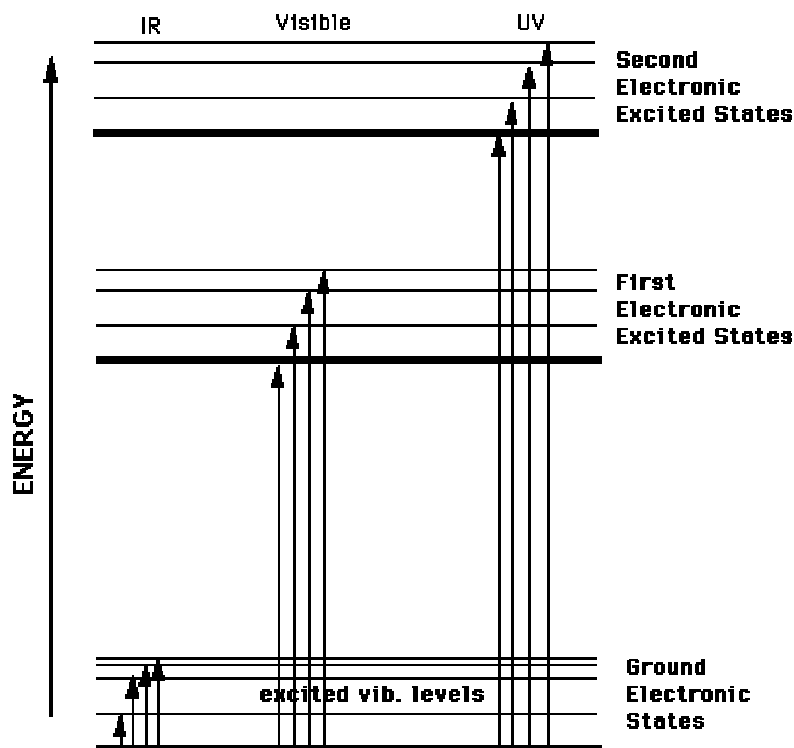


Figure 1.12: Atomic quantum jumps

The three groups of lines, represented in Figure 1.12, correspond to three different arrangements of electrons. The lowest energy corresponds to the most stable configuration, called basic configuration. The next level corresponds to the first excited level. If a photon, with an energy equal to the difference between the two considered configurations, strikes the molecule, an electron in the basic state has a certain probability to move to the next level. Thus, the photon is absorbed by the molecule. After some time, typically  $10^{-8}$  seconds, the electron returns to its basic state with the emission of a photon of energy equal to the jump in energy between the two levels. Higher energy photons can lead the electron to a second level or to subsequent levels of excitement. High energy photons in the ultraviolet region can also split the electron from the atom which remains positively charged (ionized). In the infrared region, with low energy, photons are not able to excite the molecule, but they may induce vibrational motions of electrons. Even in this case energies associated with various modes of vibration are quantized. Energy of the ground state and excited states are flanked by vibrational states. The system of the possible levels jumping greatly increases and gives rise to very complicated emission and absorption spectra. Electromagnetic radiations in the microwave, even less energy, are not able to induce vibrations but only the rotation of the molecule. So the effects of radiation on matter vary depending on the frequency of the radiation, as represented in Figure 1.13.

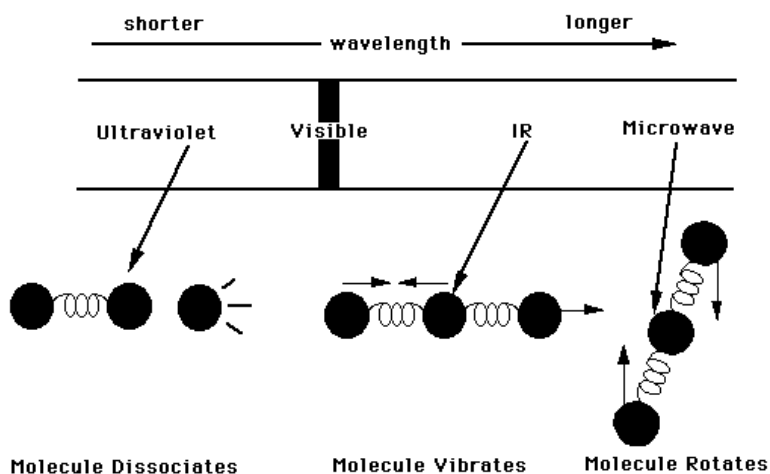


Figure 1.13: Molecular effects of UV, VIS, IR and microwave radiations.

For these reasons the absorption or the emission of energy by matter is one of the most important identification marks provided by nature. When a beam of radiation is passed through an absorbent material, the intensity of the incident radiation ( $I$ ) will be greater than that emitted ( $I_0$ ). So it is possible to go back to the frequency of the radiation that was absorbed and thus to the jump of energy of the molecule. Jumps with a given energy level may be restricted to certain molecules; thus it's possible to understand what molecules make up the matter. The total energy of a molecule can be considered as the sum of the contributions of the electronic, rotational and vibrational energies:  $E_{tot} = E_{el} + E_{rot} + E_{vib}$

In the atomic spectra, electronic interactions regarding the electrons in the valence shell are the only possible; regarding molecules, for each electronic state, usually several vibrational and rotational states are possible. In the case of NIR, even combinations of these and the presence of overtones occur.

A photon, that has an amount of energy that is two or three times the energy required to bring a molecule to a higher energy level, will produce changes in the second or third level, thus forming the second or third overtone.

Consequently, the number of possible energy levels for a molecule is much larger than that for an atomic particle. That is why the atomic spectra appear as lines, while those molecular consist of hundreds or thousands of absorption lines so closed together that they appear as bands of absorption.

In the area of the electromagnetic spectrum defined as near-infrared, the energies involved seem to result in a change in the vibrational motion of molecules and in particular of the links they contain. In fact, absorptions of the ground states usually fall in the region between 2500 and 1500 nm ( $4000-660 \text{ cm}^{-1}$ ) defined as mid-infrared (MIR), while absorptions of states with multiple frequencies to those of the ground state, called overtones, are characteristic of the area of NIR.

A molecule absorbs infrared radiation when it vibrates in such a way that its electric dipole moment changes during vibration. The electric dipole moment  $\mu$  is a vector quantity  $\mu = qd$ , where  $q$  is the electric charge and  $d$  is the vectorial distance of charge  $q$  from a defined origin point of coordinates for the molecule. When the molecule vibrates, its charge distribution, with respect to this origin, may change or remain unchanged, depending on the structure of the molecule. Not all the vibrations of a particular molecular structure necessarily absorb infrared radiation, but only those vibrations that are changing the electric dipole moment of the molecule. Models to explain the vibrations are based on the concept of "harmonic oscillator",

which consists of two masses connected by a spring (Figure 1.14):

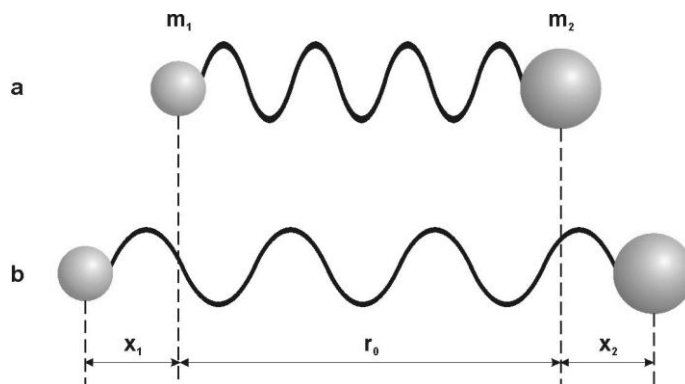


Figure 1.14: Harmonic oscillator

When set in motion, the system will oscillate or vibrate back and forward along the axis determined by the spring, at a certain frequency, depending on the masses of the spheres and the stiffness of the spring. A sphere with small mass is lighter and easier to move than one with a large mass. So the smaller masses oscillate at higher frequencies than large masses. A very stiff spring is difficult to deform and quickly returns to its original shape when the force of deformation is removed. On the other hand, a weak spring is easily deformed; in addition, a stiffer spring will oscillate at frequencies higher than a weak spring. A generic chemical bond between two atoms can be considered as a simple harmonic oscillator. The link is the spring, and the two atoms or groups of atoms, held together by the binding, are the masses. Each atom has a different mass, and single, double and triple bonds have different degrees of stiffness, so that each combination of atoms and bonds has its particular harmonic frequency. Mathematically, the system behavior is described by Hooke (Burns & Ciurczak, 2001):

$$\nu = \frac{1}{2\pi c} \sqrt{\frac{k(m_1+m_2)}{m_1m_2}}$$

where  $c$  is the speed of light,  $k$  is the spring constant (dyne \* 5 \* 10<sup>5</sup> cm<sup>-1</sup>) and  $m_1$  and  $m_2$  are the masses of the involved atoms.

At any temperature above absolute zero, all the small and simple harmonic oscillators that make up any molecule vibrate intensely. The frequency of vibration of the molecules matches the frequencies that characterize the infrared radiation. If a vibrating molecule is hit with IR light, the molecule could absorb energy delivered by radiation, if this exactly combines with the frequencies of the different harmonic oscillators that make up the molecule. When light is absorbed in the small molecule, oscillators continue to vibrate at the same frequency, but since they have absorbed the energy of light, have greater amplitude, resulting in a lengthening of the “spring”. The absorption intensity is also influenced by the polarity of the bond on which the radiation affects: the more polarity of a bond, the greater is its absorption. This model represents, with a good approximation, only the symmetric diatomic molecules. Although the harmonic model is often used to explain the vibrational spectroscopy, it has some limitations because it fails to describe the possible energetic transitions that can occur in a molecule that has a large number of atoms and especially not symmetrically arranged, as in most of organic molecules in food.

The infrared radiation is absorbed by a molecule when the radiation has enough energy to induce vibrational transitions on the molecule itself. The basic types of vibration caused by the incidence of IR radiation are divided into two categories: stretching and bending, as shown in

Figure 1.15 Stretching is the vibration of the bond along the plane, due to the inter-atomic distance which varies rhythmically and can be symmetrical or asymmetrical; the bending vibrations are characterized by a variation of the angle between two atoms in the plane (scissoring and rocking) and out of the plane (wagging and twisting).

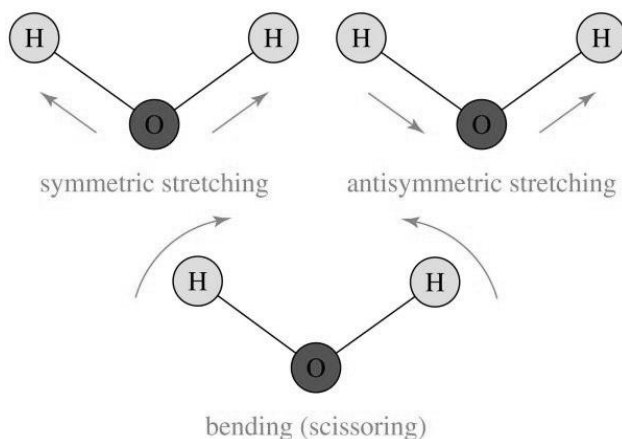


Figure 1.15: Molecular vibrations in a water molecule

The vibrational frequencies can be approximately related to molecular properties by means of Hooke's law, already described in the previous paragraph. This approximation is valid for diatomic molecules, but can also be applied, without significant differences of the average values, for stretching and bending vibrations of two atoms in a polyatomic molecule.

Since the values of the reduced mass of the groups -OH, -NH and -CH are quite similar, the spectral information is determined primarily by the  $k$  value, which depends not only on the length and strength of the bond, but also from the surrounding environment, thus creating differences in energy of absorption for each bond, making them specific, and are used in the spectrum interpretation. Actually, however, we analyze diatomic asymmetrical molecules, which change their responses to excitation caused by the incident radiation. The phenomena of mechanical anharmonicity, or the loss of equidistance between different energy levels, and of electrical anharmonicity, i.e. the change of the equation of electric dipole moment, move away from the ideal conditions. The anharmonicity leads to the appearance of overtone bands, or non-harmonic band, whose frequency is not a numeral multiple of the fundamental frequency in which the dipole bond of the molecule ranges.

These phenomena are even more dominant in a polyatomic molecule in which the mutual influences between atoms increase exponentially. In bonds involving hydrogen atoms, which have a very small mass, the lack of harmonicity is even more evident, and leads to a vibration with a great amplitude and more intense absorption bands. The absorption of electromagnetic radiation in the NIR region is therefore mainly due to overtones and their combination arising from the absorptions of the fundamental vibrations in the mid-infrared. The intensity of these bands decreases significantly when the probability that the corresponding transitions occur decreases. Since the absorptions are up to 10-100 times weaker than the intensity of the fundamental bands, only the first 2 or 3 overtones for each fundamental band are observable. They are progressively less intense, located at higher and higher frequencies and therefore absorb at lower wavelengths.

The stretching motion of the hydrogen atom, because of its small mass and then the big difference with the other atom involved in the binding (usually oxygen, carbon or nitrogen) shows a particularly high deviation from the harmonic behavior. This implies that the



fundamental stretching bands located in the mid-infrared between 3000 and 2400  $\text{cm}^{-1}$ , at the limit with the NIR region, induce overtones and combination bands in the NIR region, thus making the absorption related to the secondary vibrational modes of hydrogen, the main feature of a near infrared spectrum. In fact most of the absorptions in this region are derived from the first, second and third overtones, corresponding to the fundamental vibrations of the bonds -CH, -NH, -OH, -SH, and their combination bands. The interactions due to the presence of hydrogen bonds between molecules of the sample are particularly noticeable, since they cause enlargements of the bands and shifts to lower frequencies. Very weak bands are related to the vibrations of C-C, C-F and C-Cl bonds.

The low-intensity of absorption in the NIR region may at first seem to be a limit, since it seems to decrease the sensitivity of the technique. Actually, at a practical level, this is a big advantage because it allows the direct analysis of a sample, without diluting or dispersing it into inert matrices as normally happens in traditional spectroscopic techniques, and also to obtain representative spectra of the whole sample, since the optical paths used are very long. Moreover, even if NIR bands are larger and liable to overlap more than in other spectral regions, chemometric techniques available today are able to extract a lot of information even from complex spectra such as NIR spectra.

IR measurements can be performed both in transmission and in reflectance mode; in the case of transmission mode, the intensity of light transmitted through the sample compared to the intensity of incident light is measured:

$$T = I/I_0$$

Lambert and Beer (Burns & Ciurczak, 2001) observed that the amount of radiation absorbed or transmitted from a solution or a medium was the exponential function of the concentration of the adsorbent and the radiation path length through the sample:

$$A = \log I/T = k_a cl$$

where  $k_a$  is the molar extinction coefficient,  $c$  is the concentration and  $l$  is the optical path length of incident radiation through the sample. In the case of acquisition of reflectance data, the intensity of the reflected light compared to the intensity of incident light is measured:

$$R = I_{rif}/I_0$$

According to the Kubelka-Munk law (Burns & Ciurczak, 2001), reflectance depends on the coefficient of absorption  $k_r$  and the coefficient of dispersion of a sample  $s$ :

$$f(R_\infty) = k_r/s$$

where  $R_\infty$  is the absolute reflectance.

Experimentally the relative reflectance, i.e. the intensity of light that is reflected from the sample compared to the intensity of the reflected light in a reference material with a high and constant absolute reflectance, will be measured; examples of used materials are Teflon, MgO, discs of high purity ceramic materials. In practice, the relative reflectance is often converted into apparent absorbance  $A'$ , using an empirical relationship between analyte concentration and reflectance, similar to the Lambert and Beer's law:

$$A' = \log I/R = a'c$$

where  $c$  is the concentration and  $a'$  is a constant of proportionality.

However, if the matrix is highly absorbent or the analyte shows intense absorption bands, the linear relationship between absorbance and concentration fails. Both for the transmittance and the reflectance mode, the proposed equations are obtained from ideal situations, and are applicable only when the absorptions are weak or the product between concentration and molar extension coefficient is small. In the case of NIR spectroscopy, the matrix, which cannot be

separated from the analyte, has the major absorption and can absorb at the same wavelengths of the analyte.

Often, the spectroscopic measurement is affected by scattering phenomena, or light diffusion on the surface, especially in the case of the acquisition of solid samples: in fact, the more the incident radiation is scattered, the less the beam penetrates deep into and therefore the lower will be the absorbance (apparent or real). The scattering phenomena depend primarily on the physical properties of the sample (particle size, crystal environment) and can cause shifts in the baseline of the spectrum and lead to phenomena of collinearity at different wavelengths.

The signal dependence of the signal from the physical properties of the sample is a significant disadvantage when NIR is used for qualitative determinations such as product identification or monitoring of chemical parameters during a process (humidity, homogeneity), and quantitative analysis of one or more components. To avoid this, some mathematical spectra pre-treatments have been developed to be applied before the data processing.

#### **1.4.2 FT-NIR instrumentation** (Settle, 1997; Da-Wen Sun, 2008)

A generic FT-NIR instrument is formed by a number of basic components: the source of radiation, a wavelength selector, a system of sample exposure to radiation and a detector.

##### **Radiation sources**

For FTIR instrumentation nichrome coil source is commonly used. Helium neon laser source is used for timing operations in an FTIR.

The NIR radiation sources are mainly incandescent bulbs or emitting diodes (LEDs). Each source has a specified emission range of wavelengths; for example, incandescence sources are effective for visible radiation while LEDs are limited to specific wavelengths depending on the material used. For each of these sources, especially with incandescent lamps, filters must be still used to eliminate the portion of radiation irrelevant for the analysis purposes and which can lead to excessive sample heating. For some very specific applications the use of lasers is emerging.

##### **Wavelength selectors**

The selection refers to the method used to separate specific wavelengths, in order to obtain the best resolution. The most common method for selecting wavelengths is the use of filters, made by layers of clear or colored glass and covered with aluminum, in order to pass only specific wavelengths or groups of them. More filters can be put together to make more accurate selections. In modern systems, the diffraction grating is used: this is a surface that reflects infrared radiation and is engraved with a number of parallel lines, which leads, for the diffraction, the division of the incident radiation into separate wavelengths. The selection of wavelengths to be addressed to the sample or to the detector occurs by rotating the grating and thus changing the incident angle of the radiation source. The critical point of the system is just the rotation mechanism, which must be extremely precise.

Another category of monochromators, which today is widely used for being fast and precise, and which characterizes all the instruments based on the Fourier transform (FT), is the interferometers. The traditional model of spectrometer is modified by replacing the monochromator with the interferometer discovered by Michelson in 1891, which is still widely used in most of the NIR instruments (Figure 1.16)

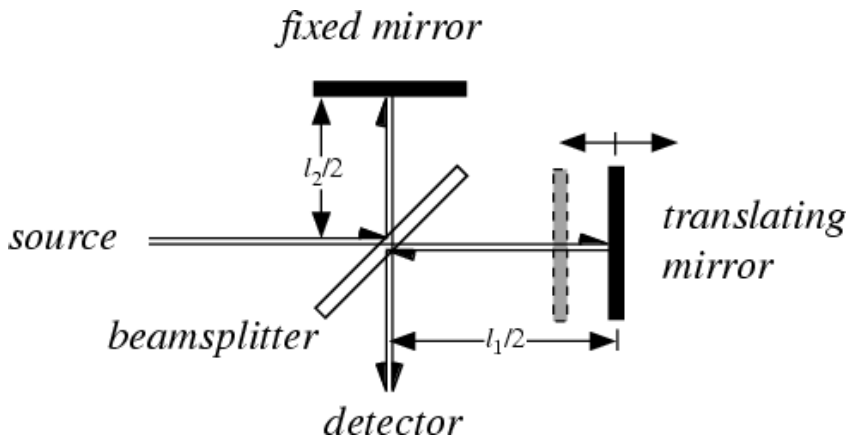


Figura 1.16: Michelson inteferometer.

It is based on the principle that two different waves can add up and then combined constructively each other in a peak with maximum intensity when they are involved, or they can combine annulling each other when are out of phase (Figure 1.17). To do so, the radius from the source is split into two parts so that they have the same propagation conditions. The first part will be reflected on a fixed mirror, while the second on a moving mirror. The two mirrors are positioned at right angles to each other and the two parts of the radius from the source are orthogonally directed and separated with a semi-transparent mirror (beamsplitter) with reflectivity equal to 50%. Once reflected, the two parts are recombined, but with different phases, since the displacement of the moving mirror causes a delay which in turn induces the out of phase of the fixed mirror. After recombination, only a certain wavelength will be enhanced with a peak, while the other will be deleted (Figure 1.17).

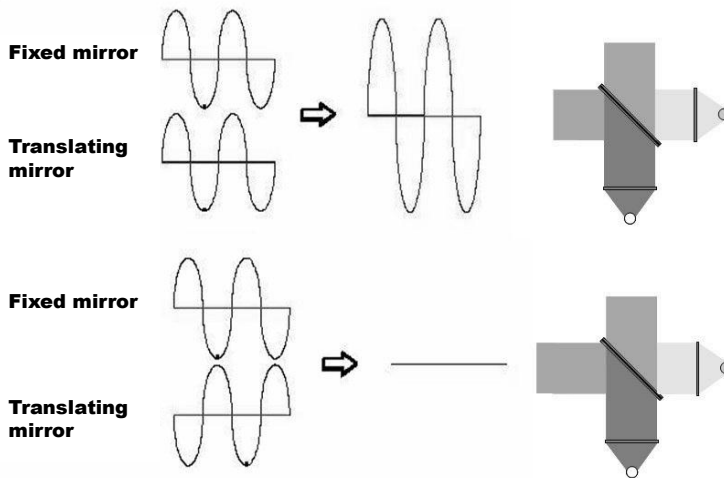


Figure 1.17: Generation of interference through the interferometer.

Therefore, the moving mirror is able to select all the wavelengths in a fixed range. The transmitted, diffused or reflected light reaches the detector, which sends a signal to the analog-digital converter that converts the signal into digital data which are then analyzed by a software that, applying the Fourier transform, translates the data into a spectral interferogram.

FT spectroscopy has the advantage to simultaneously analyze all frequencies; conversely,

traditional spectroscopy, using the grating monochromator, sends to the detector a single wavelength at each time.

### 1.4.3 NIR Sample presentation systems (Burns & Ciurczak, 2001)

When a sample is exposed to radiation, interaction with matter can occur in several ways: the light can be absorbed by the sample, reflected, and in part or completely transmitted through the sample (Figure 1.18).

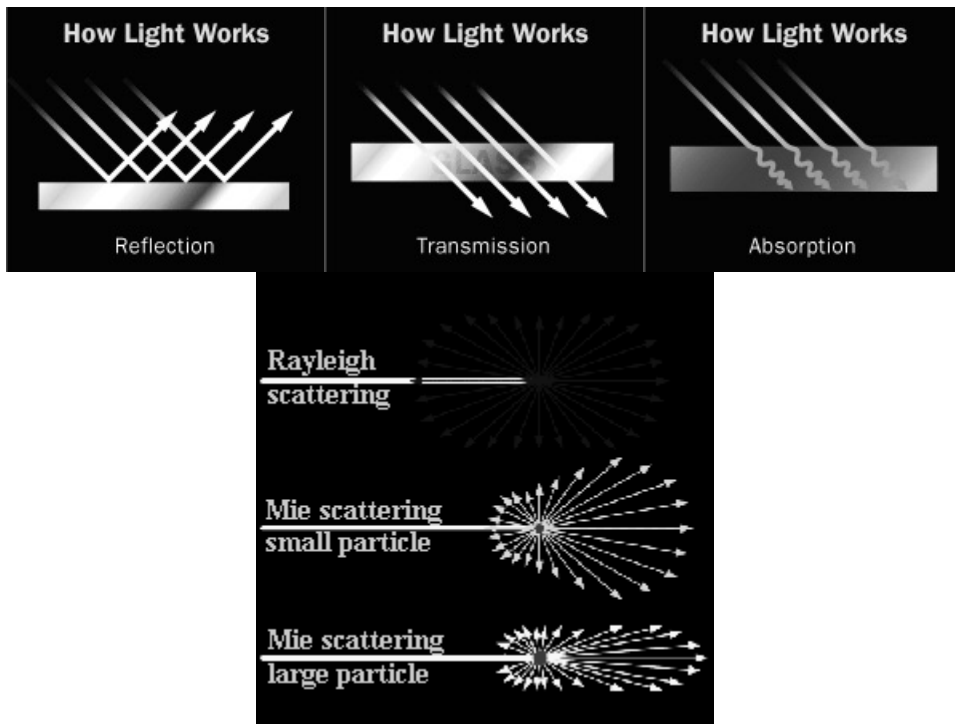


Figure 1.18: Light and matter interaction modes

The mode and the degree to which these effects occur depends on the physical state of the sample and the reading system used. The radiation transmission systems are mainly used for liquid samples or for thin layers solids, while the reflection mode is most useful for solid samples. If the sample does not reflect or transmit radiation well enough, the transmittance can be used as a measuring parameter. In this mode, the radiation penetrates the sample, part is absorbed, the rest is then reflected on a non-absorbent surface on the bottom of the cell and re-transmitted through the sample to the detector. Several types of presentation systems are currently available, strictly dependent on the construction technology.

#### *Fiber-optics*

NIR instrumentation is a growing field, thanks to the development of optical fibers that allow the direct and simple acquisition of spectra by placing the tip of the fiber on the surface and / or inside the sample. The fibers can have two different optical geometries: diffuse reflectance fibers (for solid matrices) and transmission fibers (for liquid matrices). The operating principle of diffuse reflectance fibers is illustrated in Figure 1.19: the radiation beam from the NIR source strikes the sample, the fraction not absorbed by the sample is reflected and reaches the detector.

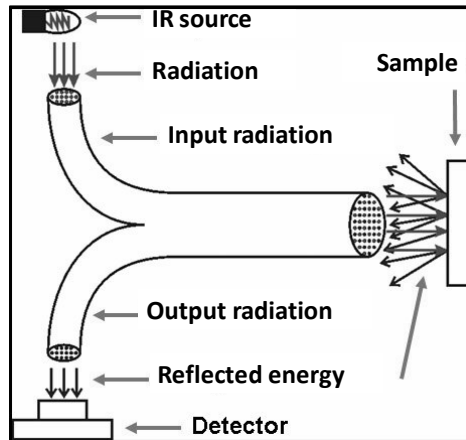


Figure 1.19: Operating principle of diffuse reflectance fiber optic.

Using transmission fibers (Figure 1.20), the light beam strikes the sample, passes through it and it is collected by the detector. In this optical geometry, the light passes through the sample once.

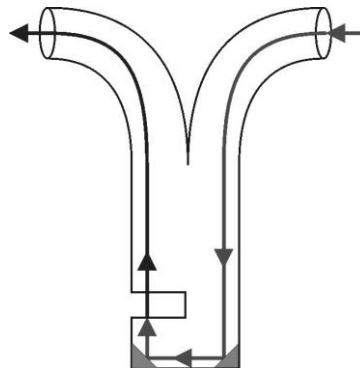


Figure 1.20: Operating principle of transmission optical fiber.

Optical fibers withstand stressful environmental conditions, are easily integrated into machines and allow conveying the signal unchanged for tens of meters, permitting the centralization of the measurement devices into a single structure. Moreover, the use of optical fiber, positioned directly on the sample surface, allows non-invasive, non destructive and in line measurements.

### *Integrating sphere*

For measurements on heterogeneous solid samples, the most suitable sampling system is the integrating sphere, whose operating principle is illustrated in Figure 1.21.

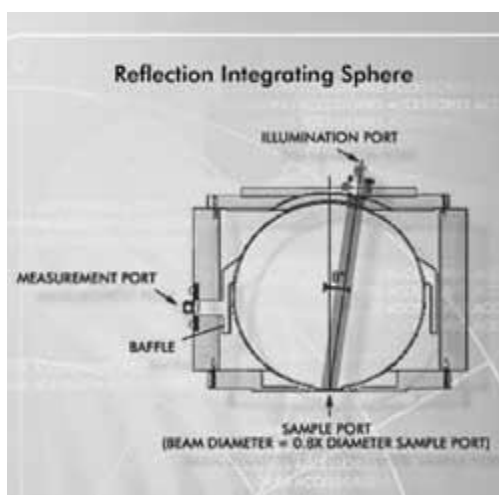


Figure 1.21: Operating principle of the integrating sphere

The radiation strikes a mirror outside the sphere which in turn directs the radiation to the sample. The part of radiation not absorbed by the sample is reflected back to the inner surface of the sphere to be collected by the detector. The sample is usually collected in a container with a bottom transparent to radiation, and equipped with a geometry that allows the rotation of the sample, which is necessary when working with non-homogeneous samples.

#### *Scanning transmission system for solids*

Although transmission systems are mainly used for liquid samples or for thin layers of solids, it is possible to scan solid samples in transmission mode. In this case, the light beam strikes the sample, passes through it and it is collected by the detector. This sampling technique is used to measure the whole mass of the sample, especially to determine hardness or composition.

Table 1.2 shows the advantages and disadvantages between diffuse reflectance and transmission measurements on solid samples. As the table shows, diffuse reflectance measurements allow the analysis of the whole spectral range and therefore the collection of information on combination bands and overtone spectral region, while transmission measurements, characterized by a low energy, allow the acquisition of spectra in a smaller range of wavelengths.

<b>Diffuse reflectance measurements</b>	<b>Transmission measurements</b>
High energy	Low energy due to the high sample absorption
The analysis requires sample homogeneity (the surface should be representative of the whole mass of the sample)	The whole mass of the sample is measured
Analysis lasting from 1 to 30 seconds	Analysis lasting from 10 seconds to 2 minutes
Whole spectral range: from 12500 to 3600 $\text{cm}^{-1}$ (800-2780 nm)	Reduced spectral range: from 12500 to 7000 $\text{cm}^{-1}$ (800-1400 nm)
Combination bands and overtone working zone	Overtone bands working zone
Usually applied to samples surface (coatings etc.)	Usually applied to the sample core (hardness or composition etc.)

Table 1.2: Comparison between diffuse reflectance and transmission measurements.

#### 1.4.4 NIR detectors (Burns & Ciurczak, 2001)

The most characteristic element for the instrument is the detector. Its task is to receive the radiation from the sample and turn it into an electrical signal. The detector must be sensitive to the wavelength of interest: generally sulphide or lead selenide detector are used for the spectral region between 1100 and 2500 nm and silicon detectors for the NIR region at short length (SWNIR) between 400 and 1100 nm. Most applications of the NIR technique are intended to obtain the spectrum of a sample, i.e. the graphical representation of absorbance or transmittance as a function of wavelength or wavenumber. An additional request is the ability of the tool to acquire a white or a background (baseline) to subtract from the sample spectrum. This can be done before the scan or, in some cases, i.e. in dual-beam instruments, continuously and simultaneously with the reading.

#### 1.4.5 NIR advantages (Burns & Ciurczak, 2001)

The practical advantages offered by FT-NIR are:

- Improved signal to noise ratio. The high value of this ratio allows the well resolved spectra with fast scans in few seconds
- Less energy loss and hence a greater energy to the detector. The optics of the FT-NIR in fact allows the availability of a passing energy greater than that of dispersive instruments, where the available energy is limited by the need of the use of splits.
- Improved accuracy and precision in the wavelengths discrimination.
- Increased speed of spectra collection.

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**2. PRELIMINARY SELECTION OF *LACTOBACILLUS***  
***PLANTARUM* STRAINS WITH PROBIOTIC**  
**POTENTIALITIES**

## 2.1 INTRODUCTION

### 2.1.1 Probiotics: beneficial effects on human health

The beneficial effects of probiotics on the human organism are numerous. Several studies have shown that probiotic bacteria may decrease the incidence, duration and severity of some gastro-intestinal diseases (Parvez et al., 2006).

The considerable interest in this subject is confirmed by the scientific literature, resulting from exploration of the therapeutic potential of probiotics (Figure 2.2) in various contexts, which will be hereafter considered.

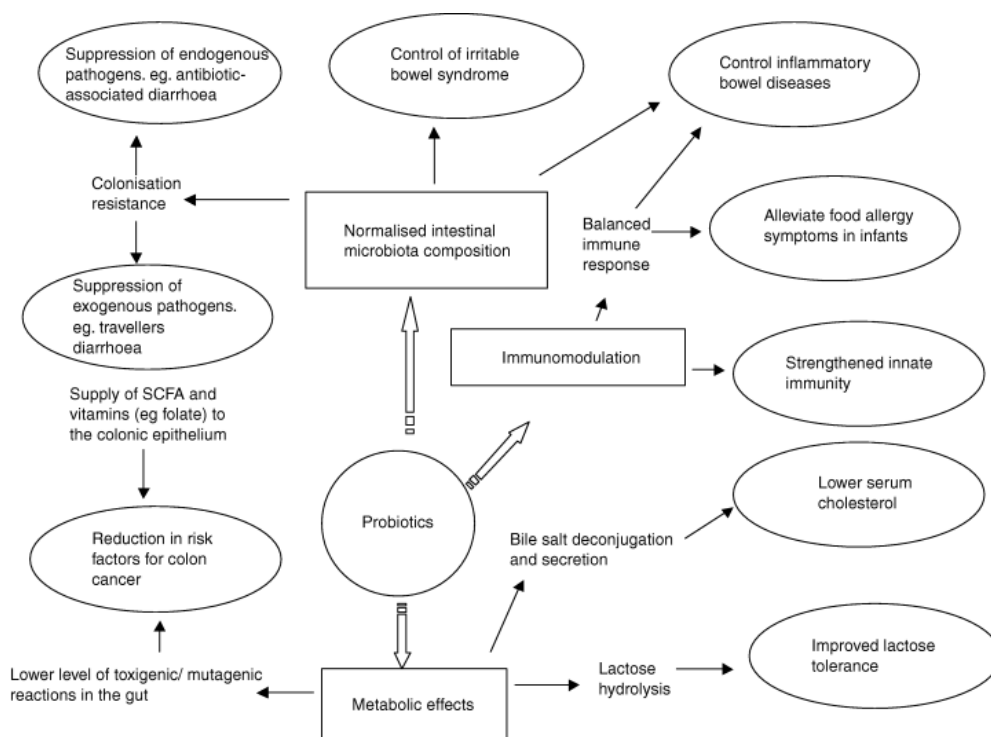


Figure 2.1. Main beneficial effects due to the consumption of probiotics (Parvez et al., 2006)

1. **Detoxification properties.** The intestine plays a central role in the process of detoxification of the body through its function as double barrier, both mechanical and immunological. Probiotics are considered detoxifying agents, able to counteract the generation of harmful metabolites through direct activity (degradation of toxic agents) and indirect (decreased activity of enzymes pro-toxin).
2. **Reduction of lactose intolerance.** Approximately 70% of the world population is intolerant to lactose because of a low  $\beta$ -galactosidase activity, responsible for the conversion of lactose into glucose and galactose (Vertuani et al., 2001). The beneficial effects due to the assumption of probiotics results from their ability to ferment lactose, resulting in higher tolerance in intolerant people (Parvez et al., 2006).
3. **Immunomodulation.** Probiotics are able to influence various mechanisms of the immune response, such as humoral or cell-mediated immunity. Regarding the humoral response, numerous scientific studies show that a treatment with probiotics belonging to *Lactobacillus casei* and *Lactobacillus acidophilus* determines a rise in the production of IgA, which improves the function of the gut as a barrier. An interesting aspect of the

modulation of the immune system by probiotics is their ability to influence the mediated response by T cells in the intestinal epithelium, through the production of cytokines. It has also been shown that lactobacilli are able to stimulate the activity of macrophages against different species of bacteria. Probably this effect is determined by absorption through the intestinal walls of a soluble antigen or translocation of lactobacilli in the bloodstream. The immunomodulating action of probiotics also explains the decrease in the symptoms associated with food allergies, in which there is an increase in intestinal permeability to allergens, caused by chronic inflammation of the mucosa.

4. Rheumatoid arthritis. It is an auto-immune disease that affects joints and other internal organs. The etiology is not entirely clear, but there are studies that indicate this disease as a consequence of intestinal infection or a food allergens action. In these circumstances, the intestinal mucosa is damaged, resulting in increased permeability to antibodies, which attack the body's structures and trigger an inflammatory process. The consumption of probiotics by patients suffering from this disease, showed an improvement of their clinical situation (Parvez et al., 2006).
5. Anti-infective activities. An important application of probiotics is the prevention of opportunistic infections resulting from antibiotic treatment. A considerable part of the intestinal microflora is destroyed by antibiotics, thus promoting the development of pathogenic organisms such as *Candida albicans* and *Clostridium difficile*, which can lead to infection, sepsis, colitis and diarrhea. Numerous studies confirm that the intake of probiotics, concomitantly with antibiotic therapy, is able to reduce the incidence of opportunistic infections and to restore, more quickly, the physiological structure of the intestinal microflora.
6. Intestinal disorders. The use of probiotics has a positive influence on inflammatory and infectious diseases of the intestine. It has been hypothesized that the mechanism involved include the reduction of intestinal pH through the stimulation of lactic acid production by the intestinal microflora, the direct effects of antagonism of pathogenic microorganisms and the process of immunostimulation.
7. Chemo-protective activities. The scientific data available concern the reduction of the risk of developing colon cancer, which is one of the most important causes of mortality. The experiments, conducted on animal treated with probiotic lactobacilli and bifidobacteria, after treatment with chemical carcinogens, showed the decrease of specific tumor markers. An hypothetic explanation of this effects on the development of tumors is given by the ability of lactobacilli to suppress the growth of bacterial species which convert pro-carcinogens into carcinogens. This property determines the reduction of the concentration of carcinogenic substances in the gut. Moreover, lactobacilli subtract mutagenic compounds at the intestinal level, thus preventing them from being absorbed. There is no direct experimental evidence on men yet, but the increase of consideration of probiotics as potentially useful in the prevention of neoplastic diseases encourage further research (Vertuani et al., 2001).
8. Hypertension. Preliminary studies indicate that probiotics or fermented products may affect the control of blood pressure. In this regard, clinical studies have been conducted. These studies allowed us to document the anti-hypertensive effect caused by the ingestion of probiotics.
9. Control of cholesterol. Cholesterol is essential for many body functions. It acts as a precursor of various hormones and vitamins and is a component of biological membranes. High levels of cholesterol or other fats in the blood, however, are considered risk factors for the development of heart disease. The cholesterol-lowering activity of probiotics is controversial (Parvez et al., 2006), it may be due to a direct action of cellular uptake of cholesterol or indirectly, by hydrolysis of bile salts.

The functional requirements of probiotics should be established by using in vitro methods and the results of these studies should be reflected in controlled human studies.

Among the most important features that a probiotic must have, the ability to adhere to intestinal epithelia and the tolerance to bile were in depth investigated.

Adhesion of probiotic strains to the intestinal surface and the subsequent colonization of the human GI-tract has been suggested as an important prerequisite for probiotic action. Adherent strains of probiotic bacteria are likely to persist longer in the intestinal tract and thus have better possibilities of showing metabolic and immunomodulatory effects than non-adhering strains (Saarela et al., 2000).

Adhesion provides an interaction with the mucosal surface facilitating the contact with gut associated lymphoid tissue mediating local and systemic immune effects. Thus, only adherent probiotics have been thought to effectively induce immune effects and to stabilize the intestinal mucosal barrier (Salminen et al., 1998).

*Lactobacillus plantarum* is encountered inhabitant of the human intestinal tract, and some strains are marketed as probiotics. Their ability to adhere to mannose residues is a potentially interesting characteristic with regard to proposed probiotic features such as colonization of the intestinal surface and competitive exclusion of pathogens (Pretzer et al., 2005).

The capacity to recognize the same receptor sites has been proposed to enable probiotic microorganisms to inhibit colonization of pathogens by competitive exclusion, which might prevent infections in the small intestine (Reid & Burton, 2002). Studies reported how the adherent bacteria can inhibit the intestinal infections by enterotoxigenic *Escherichia coli* (ETEC), which causes travelers' diarrhea by the recognition of the same adherence sites on the intestinal epithelial surface (Pretzer et al., 2005).

Recently, the potentially 'probiotic gene' (*msa*) encoding the mannose-specific adhesin (MSA) of *L. plantarum* has been identified, using a biodiversity-based approach including phenotype-genotype correlation and mutation analysis in *L. plantarum* strain WCFS1 (Pretzer et al., 2005).

In recent years, the ability of probiotic strains to produce bile salt hydrolase (BSH) has become the focus of attention on account of its influence on cholesterol metabolism and hence BSH activity can be explored as a functional probiotic biomarker for the selection of the probiotic strains (Kumar et al., 2012).

Bile salt hydrolase (BSH) is an enzyme produced by several bacterial species in the human or animal gastrointestinal tract that catalyzes the glycine- or taurine-linked bile salt deconjugation reaction (Patel et al., 2009).

The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and a number of bile salt hydrolases (BSHs) have been identified and characterized.

BSHs are very specific for certain bile types, and their duration of contact to bile ensures the bacterial survival into varying bile environments. Studies carried out in *Lactobacillus plantarum* WCFS1, which had four *bsh* genes, and *L. acidophilus* NCFM, which had two *bsh* genes, supported this assumption (Patel et al., 2009).

BSH activity involves deconjugation of bile salts to primary bile salts: primary bile acids are synthesized in the liver from cholesterol and are conjugated with either glycine or taurine before their secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond. (Figure 2.2a)

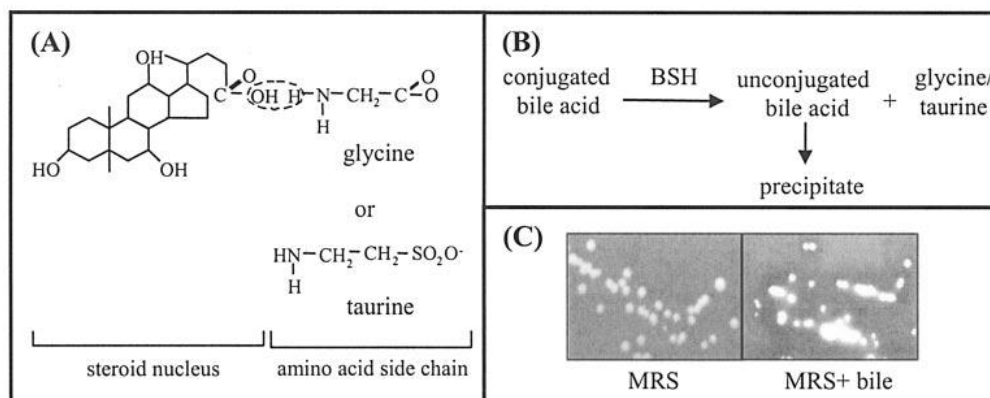


Figure 2.2 (A) Chemical structure of bile acids. (B) Reaction catalyzed by BSH enzymes. BSHs cleave the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH. (C) Detection of BSH activity. The white precipitates around colonies and the clearing of the medium are indicative of BSH activity (Begley et al., 2006).

This activity is strictly related to the ability of strains to remove cholesterol. Many authors reported how the cholesterol removal ability is linked to bile salt deconjugation and co-precipitation (Liong et al., 2006).

Moreover, BSH activity protects bacteria from the toxicity of conjugated bile salts (they repress bacterial growth), being considered a detoxification mechanism of vital importance to bacterial communities (such as lactobacilli), which are typically associated to the human GIT (Zago et al., 2011).

Thus, the aim of this study was to investigate the presence of *msa* and *bsh* genes in *L.plantarum* strains involving in MSA and BSH activity that are considered to be associated with probiotic features. Moreover, positive strains were tested for BSH activity using a phenotypic assay.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Strain selection

A total of 80 strains were used in this study. Seventy-nine strains of *Lactobacillus plantarum*, belonging to the collection of the CRA-FLC (Fodder and Dairy Productions Research Centre, Lodi), were isolated from different matrixes (23 strains from dairy products, 38 strains from olive brines and 18 strains from grape marcs); the type strain ATCC 14917<sup>T</sup>, obtained from the American Type Culture Collection (Rockville, MD) was used as reference strain. Strains were kept as frozen stocks at -80 °C in MRS broth (Merck, Darmstadt, Germany) added with 20% (v/v) glycerol. Frozen stock cultures were thawed and subcultured twice (2% inoculum) in MRS at 30 °C overnight before use.

### 2.2.2 Molecular typing

Bacterial DNA was extracted using a Chelex<sup>®</sup> 100 method described by Walsh et al. 1991. Two-hundred µl of culture were washed twice with 500 µl di TE<sub>0.1</sub> pH 8.0 (TE<sub>0.1</sub>: Tris-HCl 10 mM, EDTA 0.1 mM) and the pellet was harvested and resuspended in 245 µl of the same buffer and 5 µl of lysozyme (stock solution 50 mg/ml). After that, the strains were incubated at 56°C for 45 min.

To each eppendorf were added 218.7 µl of TE<sub>0.1</sub> pH 8.0, 5 µl of DTT 1 M, 10 µl of EDTA 0.5 M pH 8.0, 12.5 µl of 20% SDS and finally 3.8 µl of proteinase K (stock solution 20 mg/ml). The strains were incubated for 1 hour at 37°C and than 500 µl of a 5% Chelex<sup>®</sup> 100 (Sigma –

Aldrich) solution were added. This ionic resin allows the DNA purification by precipitation of all impurities generated by bacterial lysis. The extracted DNA was diluted 1:10 and used as a template for PCR reactions.

RAPD-PCR was performed using M13 primer (5' – GAGGGTGGCGGTTCT – 3') as described by Rossetti and Giraffa (2005). Amplification reactions were carried out in 25 µl volume containing 2 mM of M13 primer, 2.5 units/100 µl of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 3 mM MgCl<sub>2</sub>, 2.5 ng/µl of total DNA, and 0.2 mM of each dNTP. PCR amplifications were performed in a Perkin–Elmer thermal cycler (model 9700 Applied Biosystem). The cycling program consisted of an initial denaturation step at 94 °C for 120 s and then 40 cycles of: 94 °C for 60 s, 45 °C for 20 s, and 72 °C for 120 s. The final elongation was performed at 72 °C for 10 min.

PCR products were analyzed by electrophoresis through 1.5% agarose gels at 26 V for 12 h in 1× Tris-acetate EDTA (TAE) buffer (1 × TAE: 40 mmol/l Tris acetate, 1 mmol/l EDTA, pH 8.0) stained with ethidium bromide, and visualized under UV light.

The fingerprinting profiles were processed using *Bionumerics* software (5.1 Applied Maths, Belgium). Similarity among different profiles was evaluated by applying the Pearson's correlation coefficient. Strains representative of different genotypic profiles were selected and their taxonomic affiliation was confirmed by PCR according to Torriani et al. (2001).

### 2.2.3 Research for *msa* and *bsh* genes

Total DNAs were also used to detect the presence of *bsh* (bile salt hydrolase) and *msa* (mannose specific adhesing) genes, encoding for the bile salt hydrolase (BSH) and the mannose-specific adhesin (MSA), respectively (Zago et al., 2011). PCR primers were designed on the basis of the gene sequences of *bsh* (GenBank accession number AL935262.1) and *msa* (GenBank accession number AL935255.1) of *L. plantarum* WCFS1. The primers had the following sequences: 5'-CGTATCCAAGTGCTCATGGTTTAA-3' (*bsh* for, nucleotide position 150568 to 150593 of the *bsh* gene), 5'-ATGTGTAAGTCCATAACTTATCAATCTT-3' (*bsh* rev, nucleotide position 151487 to 151460 of the *bsh* gene), 5'-GCTATTATGGGGATTACGTTG-3' (*msa* for, nucleotide position 133061 to 133081 of the *msa* gene) and 5'-CTGTCTTGACAATAGCCATATA-3' (*msa* rev, nucleotide position 134801 to 134781). The primers were designed to obtain PCR product lengths of 919 bp (for *bsh*) and 1740 bp (for *msa*). The strain LMG 9211 was used as positive control for both PCR amplifications.

PCR amplifications were performed in 20 µl volumes with 0.5 mM of each primer (Biotez, Berlin, Germany), 2.5 units/100 µl of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 1.5 mM MgCl<sub>2</sub>, 20 ng of total DNA, and 200 mM of each dNTP. DNA amplifications were performed by a Perkin Elmer thermal cycler (mod. 9700, Applied Biosystems) and were set as follows: 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 64°C (*bsh* for/rev) or 52°C (*msa* for/rev), and 1 min at 72°C; the final extension step consisted of 7 min at 72°C. The PCR products were separated on a 1% agarose gel in Tris-Acetate EDTA buffer, prepared as previous described.

### 2.2.4 BSH activity

BSH activity of the strains was detected using a plate screening procedure according to Zago et al. (2011). Briefly, overnight cultures were spotted on MRS agar plates containing 0.37 g/l of CaCl<sub>2</sub> and 0.5% sodium salt of taurodeoxycholic acid (TDCA) or 0.5% sodium salt of glycodeoxycholic acid (GDCA) (Sigma-Aldrich). Plates were anaerobically incubated at 37°C for 72 h. The presence of halos around colonies or white opaque colonies indicated BSH activity. *Enterococcus faecium* LMG 16170 was used as BSH-positive strain. The inoculums of each strain in MRS without supplementation was included as negative control.

## 2.3 RESULTS AND DISCUSSION

Several functional attributes have already been demonstrated for *L. plantarum* strains isolated from fermented foods (Adlerberth et al., 1996; Nguyen et al., 2007; Georgieva et al., 2008; Belviso et al., 2009).

Considering the role that *L. plantarum* plays in food systems, the aim of this work was to select potentially probiotic *L. plantarum* strains from a wide collection of strains isolated from different matrixes..

In the present study 80 *L. plantarum* strains were investigated for their probiotic properties. These strains, identified on the basis of species-specific PCR for *L. plantarum* group, with different M13 PCR fingerprinting profiles and coming from different matrixes, were screened for the presence of *msa* and *bsh* genes, that are considered to be associated with probiotic features. The *msa* gene was selected to indicate the mannose-specific adhesion of *L. plantarum*, being one of the first identified genes associated with a particular probiotic effect (Pretzer et al., 2005). The *bsh* gene, coding for the BSH activity, was selected for its importance in bile salt hydrolysis which, ultimately, leads to lower blood serum cholesterol levels (Begley et al., 2006).

Thirty-four (Table 2.1) out of the 80 strains showed the expected amplicons for both genes. These 33 strains and the *L. plantarum* type strain ATCC14917<sup>T</sup> were selected for further characterization.

Strains	Origin	Strains	Origin	Strains	Origin
Lp751	Pecorino Pisano Cheese	50339	Olive Brine	50357	Olive Brine
Lp752	Pecorino Pisano Cheese	50341	Olive Brine	50358	Olive Brine
Lp755	Pecorino Pisano Cheese	50343	Olive Brine	50360	Olive Brine
Lp793	Canestrato Pugliese Cheese	50346	Olive Brine	50361	Olive Brine
Lp794	Monte Veronese Cheese	50347	Olive Brine	50362	Olive Brine
Lp797	Morlacco Cheese	50348	Olive Brine	50363	Olive Brine
Lp799	Monte Veronese Cheese	50350	Olive Brine	50366	Olive Brine
Lp804	Monte Veronese Cheese	50351	Olive Brine	50368	Olive Brine
Lp813	Spessa Cheese	50352	Olive Brine	50369	Olive Brine
Lp994	Argentinian Cheese	50353	Olive Brine	50371	Olive Brine
50337	Olive Brine	50356	Olive Brine	50372	Olive Brine
				ATCC 14917	Type Strain

Table 2.1: *Lactobacillus plantarum* strains used for characterisation

The ability to adhere to mannose residues is a potentially interesting characteristic with regard to colonization of the intestinal surface and competitive exclusion of pathogens. The product of the *msa* gene, identified as a mannose-specific adhesin, was found to mediate adhesion to human colonic cell line HT-29 (Adlerberth et al., 1996), and to agglutinate *S. cerevisiae* cells (Pretzer et al., 2005). All the strains carried the *msa* gene.

A relevant property of candidate probiotics is BSH activity, which involves deconjugation of bile salts to primary bile salts: all 34 strains, which possessed *bsh* gene, demonstrated the ability

to hydrolyze both sodium glycodeoxycholate and sodium taurodeoxycholate, as shown by halos around colonies after growth in MRS-GDCA and by the appearance of white opaque colonies after growth in MRS-TDCA, respectively.

The deconjugation activity would play a role in maintaining the equilibrium of the gut microflora. In fact, the inhibition of common intestinal bacteria has been related to the presence of free (deconjugated) bile acids rather than the conjugated ones. It has been also suggested that bile salts hydrolase enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress (Vinderola & Reinheimer, 2003).

Another phenomenon related to the presence of the deconjugation activity is the reduction of serum cholesterol. In this sense, bile salt deconjugation is a desirable property when selecting strains to be used as a dietary adjunct (Liong et al., 2006).

As shown above, all 34 *L. plantarum* strains possessed the *bsh* gene and expressed BSH activity. Schmidt et al. (2001) also showed that, at least in lactobacilli, bile salt resistance could not be related to the presence of BSH. BSH activity, however, is a widespread trait in *L. plantarum* (Zago et al., 2011).

## 2.4 CONCLUSIONS

This study showed that potentially probiotic *L. plantarum* strains could be isolated from dairy products and that strains of human origin are not necessarily the most suitable for probiotic application. This seems corroborated by the wide finding on genotypic traits (such as adhesion ability and capacity to hydrolase bile salts) which are considered to be typical of probiotic bacteria, within the *L. plantarum* dairy isolates studied here.

Further phenotypic analysis to confirm expression of the studied genes and *in vitro* (such as adhesion ability, antibiotic resistance, and cell hydrophobicity) and *in vivo* studies are required to better detect really *L. plantarum* candidates to be used in functional foods.

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**3 EVALUATION OF RIBOFLAVIN PRODUCTION IN  
LACTOBACILLUS PLANTARUM STRAINS**

### 3.1 INTRODUCTION

The relationship between nutrition and health is constantly developing and linking the concepts of "functional food" (where functional refers to the nutraceutical and healthy aspects of food) to the new trend of achieving wellness consuming foods with high added value. Food is considered "functional" if, in addition to a high nutritional value, proves to have beneficial effects on the body, to preserve the health of the individual, to mitigate the risk of the onset of disease. Foods enriched in probiotics are certainly the model of functional food.

Besides probiotic LAB, certain strains of LAB are able to produce/release and/or increase specific beneficial compounds in foods. These functional ingredients are sometimes referred to as nutraceuticals, a term that describes "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease". These ingredients can be macronutrients, micronutrients (such as vitamins) or non-nutritive compounds and can be naturally present in certain foods or added during processing (Le Blanc et al., 2011).

Vitamins are micronutrients that are essential for the metabolism of all living organisms. They are found as precursors of intracellular coenzymes that are necessary to regulate vital biochemical reactions in the cell. Humans are incapable of synthesizing most vitamins, and they consequently have to be obtained exogenously (i.e. from their diet). Although most vitamins are present in a variety of foods, vitamin deficiencies still exist in many countries including highly industrialized nations mainly because of malnutrition, not only as a result of insufficient food intake but also because of unbalanced diets (Le Blanc et al., 2011).

Vitamins are generally classified into two groups, the fat-soluble vitamins, which include vitamins A, D, E and K, and the water-soluble vitamins, which include vitamin C, biotin (vitamin H or B7) and a series of B vitamins — thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), folic acid (B9) and cobalamin (B12). The fat-soluble vitamins appear to function as integral parts of cell membranes while, the water-soluble vitamins act as coenzymes, often as carriers of a particular chemical group (Burgess et al., 2009).

The ability of lactic acid bacteria (*Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc* spp. and *Lactobacillus plantarum* are the most studied species) to produce spontaneously vitamins can be commercially exploited in view of the high cost of chemical synthesis or extraction from the raw materials of these important molecules. The aim of the development of the research of strains able to produce vitamins is to use the fermented food, obtained with strains selected for the ability to produce these compounds, as carrier of these natural substances with biological value reducing the costs of artificial fortification with synthetic vitamins.

The B vitamins produced by microorganisms are riboflavin (vitamin B2), folic acid and folate (vitamin B9) and cyanocobalamin (vitamin B12).

Riboflavin (vitamin B2) is an essential component of basic cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The latter two biomolecules play a central role in metabolism acting as hydrogen carriers in biological redox reactions involving enzymes such as NADH dehydrogenase (Burgess et al., 2004).

Many microorganisms, plants, and fungi possess the biosynthetic ability to produce riboflavin. However, vertebrates, including humans, lack this ability and must therefore obtain this vitamin from their diet. Dietary, riboflavin is present in liver, egg yolk, milk, and meat, whereas the vitamin is commercially synthesized for nutritional use in the fortification of various food products such as bread and breakfast cereals.

Riboflavin is synthesized by many bacteria and its biosynthetic pathway has been studied extensively in *Bacillus subtilis* and *Escherichia coli*.

Riboflavin biosynthesis (Figure 3.1) requires the precursors guanosine 5'-triphosphate (GTP) and ribulose 5-phosphate. The first step of the GTP-dependent branch of the biosynthetic pathway is encoded by *ribA* in *E. coli*. In *B. subtilis* it is also encoded by *ribA* but in this case *ribA* is a bifunctional enzyme which also catalyses the formation of 3,4-dihydroxy-2-butanone 4-phosphate from ribulose 5-phosphate. *RibA* overexpression in *B. subtilis* increases riboflavin yield up to 25%, suggesting that this enzyme is rate-limiting in riboflavin biosynthesis (Burgess et al., 2009).

However, in *Lactococcus lactis*, overexpression of *ribA* alone does not lead to increased riboflavin production (Burgess et al., 2004).

To be biologically active, riboflavin must be converted to its coenzyme forms, FMN and FAD. This is accomplished by a bifunctional flavokinase/FAD synthetase, which is encoded in *B. subtilis* by *ribC* or *ribF* in *E. coli*. Certain mutations in the *B. subtilis* *ribC* gene have been found to result in riboflavin overproduction (Coquard et al., 1997; Kreneva and Perumov, 1990). *B. subtilis* also encodes a separate monofunctional riboflavin kinase encoding gene, *ribR*, which can suppress the effect of *ribC* mutations, restoring normal riboflavin production (Burgess et al., 2009).

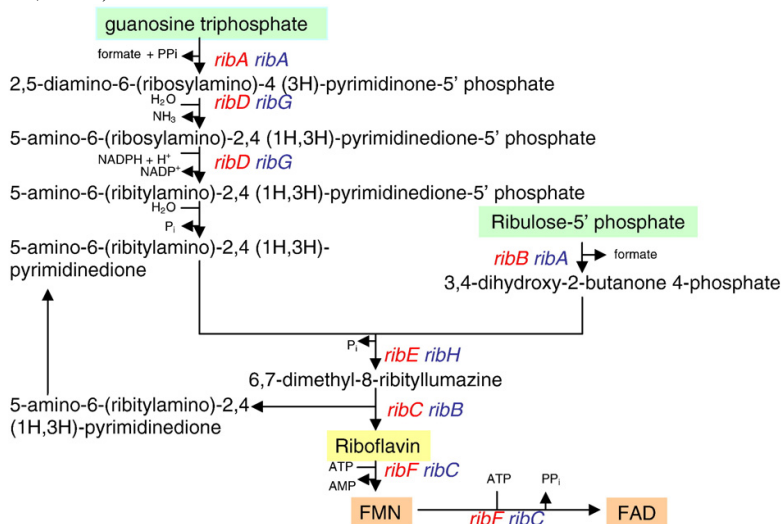


Figure 3.1: Riboflavin biosynthesis (Burgess et al., 2009).

Some bacteria and fungi are capable of riboflavin overproduction. In bacteria this trait can be achieved either by metabolic engineering or by exposure to purine analogues and/or the toxic riboflavin analogue roseoflavin. Particularly, in *Lactococcus lactis* both of these approaches have been used with success (Burgess et al., 2004). Recent studies have reported on the selection of riboflavin overproducing strains for potential food applications, for example, the manufacture of vitamin B2-enriched dairy products, which were found to improve the riboflavin status of deficient rats (Capozzi et al., 2011).

Thus, the present study reports on the selection of food-grade, riboflavin-producing LAB isolated from different matrices.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Strains selection

A total of 34 strains were used in this study: 33 strains of *Lactobacillus plantarum*, belonging to the collection of the CRA-FLC (Fodder and Dairy Productions Research Centre, Lodi), were isolated from different matrixes (10 strains from dairy products and 23 strains from olive

brines); the type strain ATCC 14917<sup>T</sup>, obtained from the American Type Culture Collection (Rockville, MD) was used as reference strain.

Strains were kept as frozen stocks at -80 °C in MRS broth (Merck, Darmstadt, Germany) added with 20% (v/v) glycerol. Frozen stock cultures were thawed and subcultured twice (2% inoculum) in MRS at 30 °C overnight before use (Table 3.1).

Strains	Origin	Strains	Origin	Strains	Origin
Lp751	Pecorino Pisano Cheese	50339	Olive Brine	50357	Olive Brine
Lp752	Pecorino Pisano Cheese	50341	Olive Brine	50358	Olive Brine
Lp755	Pecorino Pisano Cheese	50343	Olive Brine	50360	Olive Brine
Lp793	Canestrato Pugliese Cheese	50346	Olive Brine	50361	Olive Brine
Lp794	Monte Veronese Cheese	50347	Olive Brine	50362	Olive Brine
Lp797	Morlacco Cheese	50348	Olive Brine	50363	Olive Brine
Lp799	Monte Veronese Cheese	50350	Olive Brine	50366	Olive Brine
Lp804	Monte Veronese Cheese	50351	Olive Brine	50368	Olive Brine
Lp813	Spessa Cheese	50352	Olive Brine	50369	Olive Brine
Lp994	Argentinian Cheese	50353	Olive Brine	50371	Olive Brine
50337	Olive Brine	50356	Olive Brine	50372	Olive Brine
				ATCC 14917	Type Strain

Table 3.1: *Lactobacillus plantarum* strains used for the characterization

### 3.2.2 Detection of the *rib* operon

Strains were tested by PCR to search for the presence of genes of the *rib* operon encoding for the production of riboflavin (vitamin B2).

Bacterial DNA was extracted using a Chelex<sup>®</sup> 100 method described by Walsh et al. 1991. Two-hundred µl of culture were washed twice with 500 µl di TE<sub>0.1</sub> pH 8.0 (TE<sub>0.1</sub>: Tris-HCl 10 mM, EDTA 0.1 mM) and the pellet was harvested and resuspended in 245 µl of the same buffer and 5 µl of lysozyme (stock solution 50 mg/ml). After that, the strains were incubated at 56°C for 45 min.

To each eppendorf were added 218.7 µl of TE<sub>0.1</sub> pH 8.0, 5 µl of DTT 1M, 10 µl of EDTA 0.5 M pH 8.0, 12.5 µl of 20% SDS and finally 3.8 µl of proteinase K (stock solution 20 mg/ml). The strains were incubated for 1 hour at 37°C and than 500 µl of a 5% Chelex<sup>®</sup> 100 (Sigma – Aldrich) solution were added. This ionic resin allows the DNA purification by precipitation of all impurities generated by bacterial lysis. The extracted DNA was diluted 1:10 and used as a template for PCR reactions.

Total DNAs were used to detect the presence of *rib* operon which encode for the production of riboflavin.

PCR primers were designed on the basis of the alignment of operon sequences of *L. plantarum* WCFS1 (GenBank Accession number: AL 935263.1) and JDM1 (GenBank Accession number: CP001617.1). The primers had the following sequences: 5' – GGGTGGCGAGCTACCAAAT – 3' (*rib* for) and 3' – CGTGGTTAAAACGCCGAACATTA – 5' (*rib* rev). The primers were designed to obtain a PCR product length of 3190 bp. *L. plantarum* WCFS1 which contains an

incomplete *rib* operon, in which the entire *ribG* and part of *ribB* are absent from the genome, was used as control strain.

PCR amplifications were performed in 20 µl volumes with 0.5 mM of each primer, 2.5 units/100 µl of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 1.5 mM MgCl<sub>2</sub>, 20 ng of total DNA, and 200 mM of each dNTP. DNA amplifications were performed by a Perkin Elmer thermal cycler (mod. 9700, Applied Biosystems) and were set as follows: 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; the final extension step consisted of 7 min at 72°C. The PCR products were separated on a 1% agarose gel in Tris-Acetate EDTA buffer (1 × TAE: 40 mmol/l Tris acetate, 1 mmol/l EDTA, pH 8.0) stained with ethidium bromide, and visualized under UV light.

### **3.2.3: Evaluation of *rib* operon expression by real time PCR**

#### **3.2.3.1 Reverse PCR**

Total RNA was isolated from 1.5 ml of an overnight culture, by using TRIzol reagent (1 ml) (Invitrogen, New Zealand). TRIzol is a mono-phasic solution, phenol-based which maintains, during the sample homogenization, the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform (200 µl) and the subsequent centrifugation (12000 rpm x 15 min at 4°C) allow the separation of the solution into an aqueous phase and organic phase. RNA remains exclusively in the aqueous phase. After transfer the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol (500 µl), it is free of proteins and DNA contamination and stored at -80°C. RNA concentration was measured using a spectrophotometer (Jasco, Japan).

One µg/100 µl of total RNA were transcribed into cDNA using High Capacity cDNA reverse Transcription Kit (Life Technologies, Italy). Retrotranscription was performed in 20 µl volumes with 1X of random primer, 50 units/µl of Reverse Transcriptase, 20 units/µl of RNase inhibitor, and 1X of each dNTP. The amplification was performed by a Perkin Elmer thermal cycler (mod. 9700, Applied Biosystems) and were set as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min.

#### **3.2.3.2 Real - time PCR**

PCR primers to detect the *ribG* gene, the endogenous reference (glyceraldehydes - 3- phosphate gene) and the internal Taqman Minor Groove Binder (MGB) probe were designed with Primer Express version 3.0 (Life Technologies, Italy). The probe was labelled at the 5' end with a the reporter dye FAM.

Forward and reverse primers for the *rib* gene detection had the following sequences respectively: primer for: 5' – GGAATTCGGCAAGTCGTGATTG – 3', rev: 5' – CAAAAGATACTGACGTCCCTTACCA – 3'. The probe sequence was 5' – (FAM) TCCCCATCCAATCGTG – 3'. RT-PCR amplified an internal fragment of the *ribG* gene with size of 75 bp.

Forward and reverse primers for the endogenous gene detection had the following sequence, respectively: for: 5' – CCCTGTACGTGGTGGTAACTT – 3', rev: 5' – CACCAGTTGAATGAGGAATA – 3' The probe sequence was 5' – (FAM) CTGCCGGTGTAACAC – 3'. RT-PCR amplified an internal fragment of the endogenous gene with the size of 71 bp.

Real time PCR was carried out in a 25 µL volume and contained TaqMan universal PCR master mix, No AmpErase UNG (Life Technologies, Italy), Gene Expression Assay Mix (final concentration 1X) and 2.5 µL of template cDNA. Thermal cycling conditions were the following: 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Thermal cycling, data collection and data analysis were carried out with the ABI 7300 Real time PCR System

(Life Technologies, Italy). The amplification were normalized to the constantly expressed housekeeping gene and relative transcript levels were calculated applying the equation  $2^{-\Delta\Delta Ct}$ .

### 3.2.4 Detection of riboflavin production

The evaluation of riboflavin production was carried out with strains which expressed the entire *rib* operon by a microbiological assay using the *Lactobacillus rhamnosus* ATCC 7469 as test strain because it needs riboflavin to grow. Riboflavin Assay Medium (RAM; Difco, USA) free from riboflavin but containing all other nutrients and vitamins essential for the growth of *L. rhamnosus* ATCC 7469 was used (Salveti et al., 2003). The inoculum for assay was prepared in MRS for 16-24 h at 35-37 °C.

The cells were recovered by centrifugation at 9000 g for 10 min, washed three times and resuspended in 10 ml of sterile 0.85% saline solution. The cell suspension was diluted to a turbidity of 35-40% of transmittance, corresponding to  $0.45 \pm 0.05$  of absorbance at 660 nm. Fifty  $\mu\text{L}$  of this suspension were used to inoculate tubes containing RAM. A standard curve was constructed before performing the assay according to a reference procedure. The standard curve was obtained by using different concentration of riboflavin (Sigma, Italy), ranging from 0 to 30 ng/ml. Tubes were incubated at 37 °C overnight, and after growth the absorbance was measured to evaluate the linearity of the curve.

To detect the riboflavin production, the *L. plantarum* strains were cultivated in RAM at 37 °C overnight, cells eliminated by centrifugation, and spent broths filter sterilized. Different concentrations (2%, 5%, 10%) of spent broths were added to RAM inoculated with the test strain to detect the presence of riboflavin and to determine the amount by interpolation from the standard curve.

## 3.3 RESULTS AND DISCUSSION

In order to investigate the potentiality of the 34 *L. plantarum* strains to produce riboflavin, a PCR based method was applied. Among all the strains analysed, only 10, from dairy matrix, and type strain ATCC 14917 highlighted the presence of complete *rib* operon and these strains were subject of further studies of gene expression. Transcription analysis of *rib* operon revealed that the strain ATCC14917 showed the high level of expression followed by Lp794. Lp 813 was used as calibrator to normalize the expression level (Figure 3.1). Statistical analysis (one way ANOVA and Duncan's test) confirmed the obtained results highlighting a significance for  $P < 0.05$ .

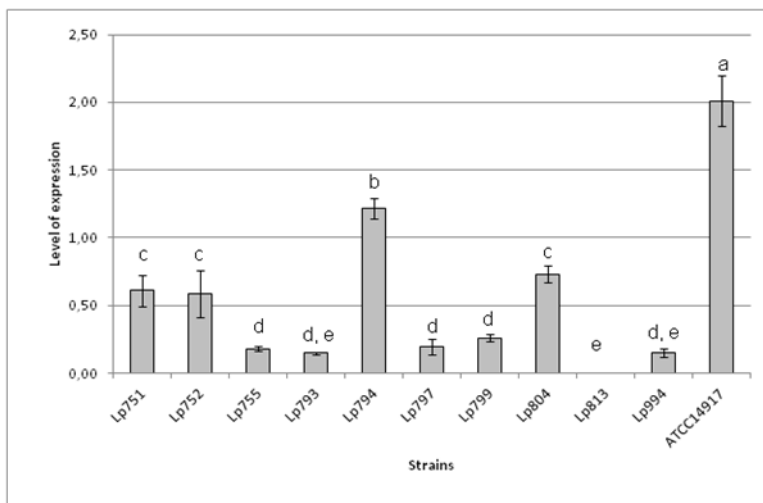


Figure 3.1: Expression level of tested strains

These strains were first tested to verify their ability to grow in RAM medium which not contain riboflavin. All strains grew, and the amount of the riboflavin produced and released into RAM were detected by a microbiological assay using a strain auxotroph for riboflavin. The standard curve carried out with *L. rhamnosus* ATCC 7469 on the basis of OD<sub>660</sub> values obtained with increasing amounts of riboflavin in RAM medium gave a linear response ( $R^2 = 0.9719$ ). The interpolation of OD<sub>660</sub> values, obtained by adding 2%, 5% or 10% of *L. plantarum* spent broths, to RAM on the standard curve allowed to determine the amount of riboflavin produced by each strains.

Figure 3.2 shows that *L. plantarum* ATCC 14917 is the highest producer of free riboflavin, whereas Lp 813 was the lowest. The amount of riboflavin produced is in accordance with the relative level of gene expression determined by RT-qPCR.

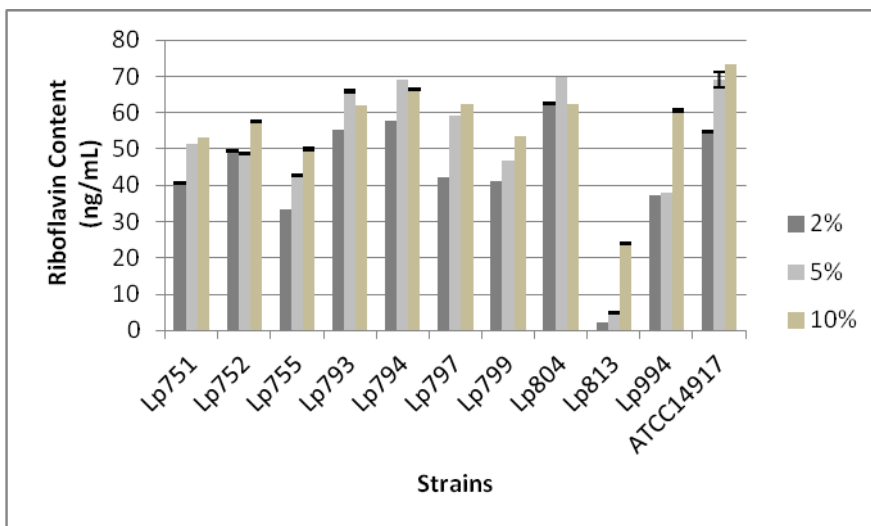


Figure 3.2: Riboflavin content for the tested strains

Results obtained were confirmed by the evaluation of the kinetic of production during 24 hours of growth for the stains ATCC 14917 and Lp813 (Figure 3.3).



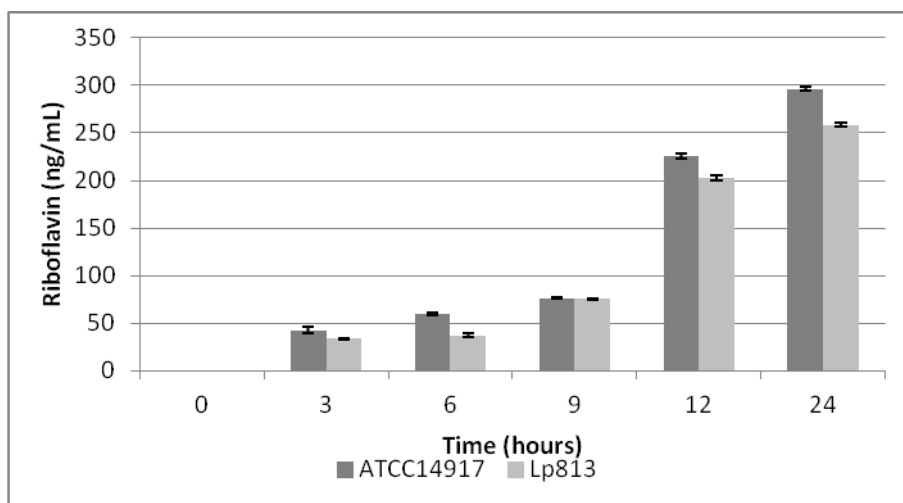


Figure 3.3: Kinetic of riboflavin production

The results obtained are in accordance with those reported in literature. Many authors reported how LAB are able to produce riboflavin for food enrichment. Le Blanc et al. (2001) reported how riboflavin concentrations can sometimes vary in certain dairy products because of processing technologies and through the action of micro-organisms utilized during food processing. This author reported the case of buttermilk and yogurt where riboflavin levels increased significantly (1.7 and 2.0 mg/l) compared with unfermented milk (1.2 mg/l). It has been shown that most yogurt starter cultures decreased riboflavin concentrations whereas others can increase the levels of this essential vitamin up to 60% of the initial concentration present in unfermented milk (Le Blanc et al., 2011).

The study of genes which regulate the biosynthesis of this vitamin carried out in *Lactococcus lactis* highlights that the presence of the biosynthetic genes and thus biosynthetic ability is not conserved within LAB. The presence or absence of the *rib* biosynthetic genes does not appear to be linked to whether the LAB in question is a hetero- or homofermentative species, whether they are pathogenic species or phylogenetically closely related (Burgess et al., 2004).

### 3.4 CONCLUSIONS

The research of lactic acid bacteria capable of producing vitamins offers the opportunity to improve the nutritional quality of many fermented foods by increasing their added value. The development of new functional foods naturally rich in vitamins by fermentation may contribute to a further expansion of the market for this class of products.

The availability of complete sequences of microbial genomes offers an overview of possibilities to fast select microorganisms of biotechnological interest. Through genetic selection strategies is now possible to screen the genomes of entire microbial collections and identify, by searching gene expression of genes involved in the biosynthesis of vitamins and functional genomics studies, strains capable of producing these and other compounds of nutritional interest.

The use of lactic acid bacteria to produce fermented foods are characterized by a high content of vitamins of the group B may restrict or prevent the use of a fortification of these products with synthetic compounds. These new functional foods could help people with illnesses from vitamin deficiency, but could also pave the way for the development of food products "functional" for specific consumer groups with special needs such as the elderly, adolescents, pregnant women, children, athletes and vegetarians.

### 3.5 REFERENCES

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**4 LACTIC ACID BACTERIA CHOLESTEROL  
REMOVAL CAPABILITY AND RELATED CELL  
MEMBRANE FATTY ACID MODIFICATIONS**

## 4.1 INTRODUCTION

Cholesterol is a polycyclic alcohol having a secondary -OH group at position 3'. It plays an important role as a component of cell membranes in vertebrates and, in many of the normal metabolic pathways as a precursor of steroid hormones, bile acids and vitamin D. An intricate balance is physiologically maintained between the biosynthesis, utilization and transport of cholesterol in a healthy organism (Yazid et al.1999). When the endogenous cholesterol produced by the liver and the cholesterol dietary intake exceeds the amount required by the body, serum cholesterol concentration increases and may represent a risk factor for cardiovascular diseases. The growing awareness of consumers about eating food with an excessive ratio of cholesterol and saturated fatty acids leads to the consumption of food with low cholesterol content (Parodi 2009). Milk and dairy products also represent a source of lipids and cholesterol, even if they play an important role in a healthy diet, because of their high nutritional value (Belviso et al. 2009). Several treatments both physical and chemical (Aloğlu et al. 2006; Kim et al. 2004), have been developed to reduce the cholesterol level in milk and dairy products, but these methods resulted expensive and often involved changes in the sensory characteristics of the products. On the other hand some reports indicate that fermented dairy products, like kefir, possess hypocholesterolaemic properties. (Guzel-Seydim et al. 2011). This property is due to the presence of certain strains of lactic acid bacteria (LAB) that determine a reduction of bile salts levels in the intestine, acting directly on bile acids in the gastrointestinal tract thanks to the bile salt hydrolase (*bsh*) activity. Therefore, new bile is synthesized in the liver from serum cholesterol, causing the hypocholesterolemic effect (Mann and Spoerry 1974, Rossi et al. 2000; Nguyen et al. 2007; Zhang et al. 2008). These findings have aroused great interest in the understanding of the mechanism by which these products were able to exert cholesterol-lowering action and in particular the role played by LAB. The exact mechanism by which certain strains of LAB are able to reduce cholesterol is not completely clear. Different hypotheses have been suggested to explain the hypocholesterolemic properties, including assimilation by growing cells, binding to bacterial cellular surface, deconjugation of bile acids catalyzed by the *bsh* activity, disruption of cholesterol micelle, co-precipitation of cholesterol with deconjugated bile salts, and conversion of cholesterol removed (Pereira et al. 2002; Lambert et al. 2008; Liong et al. 2006; Lye et al. 2010a; Lye et al. 2010b; Johnsson et al. 1995). Concerning this hypothesis, several reports have been published on cholesterol removal from laboratory media containing cholesterol. Kimoto et al. (2002) investigated the ability to remove cholesterol from laboratory media of growing cells, resting cells and heat-killed cells. The author observed that bacterial growth stimulated the removing of cholesterol, which also occurred in the presence of the heat-killed cells. Moreover the same authors detected differences in the fatty acid distribution pattern for cells grown with and without cholesterol, suggesting that cholesterol removed was incorporated into the cellular membrane. Recently, the cholesterol attachment to bacterial cells studied by electron microscopy, showed that cholesterol was adhered to the surface of lactobacilli cells upon fermentation and it was removed together with the cells from the media upon centrifugation (Lye et al. 2010a). More information is thus needed to strengthen the hypotheses raised. Therefore, the objective of this study was to test and compare the cholesterol removing abilities of different LAB species, some of which have never been studied for this property before, and to investigate the capacity of this molecule to change the cellular fatty acids composition of microorganisms to which it adheres.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Bacterial strains and culture conditions

Fifty-eight strains were used in this study (Table 4.1). Forty-three strains, belonging to the collection of the CRA-FLC, were isolated from different dairy products. Fourteen strains were obtained from culture collections and used as reference strains. One strain, *Lactobacillus acidophilus* LA-5<sup>®</sup>, a commercial starter preparation (Chr. Hansen, Hørsholm, Denmark), was used as a positive control (Belviso et al. 2009). Strains were kept as frozen stocks at -80 °C in MRS broth (Merck, Darmstadt, Germany) for lactobacilli or in M17 broth (Merck) for streptococci and enterococci with added 20% (v/v) glycerol. Frozen stock cultures were thawed and subcultured twice (2% inoculum) in MRS or M17 at 37 °C overnight before use.

Species	Cholesterol reduction (%)		
	≤ 15	> 15 ≤ 20	> 20
<i>Lactobacillus plantarum</i>	803, 995, ATCC14917	997, 752	885
<i>Lactobacillus delbr. ssp. bulgaricus</i>	ATCC11842	-	V15
<i>Lactobacillus acidophilus</i>	-	-	LA-5 <sup>®</sup>
<i>Lactobacillus helveticus</i>	9, 46, ATCC15009	1030, 75, 153	-
<i>Lactobacillus paracasei</i>	955, 1230, 1236, DSMZ5622	1235, 913	-
<i>Lactobacillus delbr. ssp. lactis</i>	A21, 223, 255, 544, ATCC12315	-	-
<i>Streptococcus macedonicus</i>	-	631, 661 627, 638, 656, LMG18488	-
<i>Streptococcus thermophilus</i>	374	402, 408, 478, 563, ATCC19258	-
<i>Enterococcus gilvus</i>	DSMZ 15689	993, LMG 13601	-
<i>Enterococcus italicus</i>	985, 991, 22039	LMG 987, 988	989
<i>Enterococcus faecium</i>	677, 1275, 3F7, F14, ATCC 19434	710	-
<i>Enterococcus mundtii</i>	-	LMG 10748	-
<i>Enterococcus casseliflavus</i>	2364, LMG14406, LMG10745	2370, -	-

Table 4.1: Cholesterol reduction ability of the LAB strains studied. Strains were divided into three classes according to the percentage of cholesterol removed from the culture media.

### 4.2.2 Evaluation of cholesterol removal from the growth media

#### 4.2.2.1 Preliminary screening

A stock solution of cholesterol (1 g/l) was prepared by dissolving cholesterol at 99% purity (Sigma-Aldrich, Milan, Italy) in an aqueous solution containing 12% of Tween 80 and 26.4% of ethanol (Sigma-Aldrich) (Belviso et al. 2009). The stock solution was filter sterilized and added to MRS or M17 broth to obtain a final concentration of 100 mg/l (MRS-Ch, M17-Ch). Each strain was inoculated at 2% in duplicate into MRS-Ch or M17-Ch broths and incubated at 37 °C for 24 h. After centrifugation at 5000 x g for 10 min, the supernatants were collected and stored

at 4 °C for the following cholesterol determination.

#### 4.2.2.2 Influence of the growth phase on cholesterol removal

Strains showing the best cholesterol lowering capability were inoculated (2%) in MRS-Ch or M17-Ch broths, incubated at 37 °C and sampled at 0, 10, 16, 24 and 48 h. At the same time, viable cells were enumerated by plate count on MRS or M17 agar media at 37 °C for 24 h. Results were expressed as CFU/ml. At each sampling time, cultures were centrifuged at 5000 x g for 10 min, and the supernatants were collected and stored at 4 °C for cholesterol determination with the GC.

#### 4.2.2.3 Cholesterol determination

Cholesterol levels in supernatant were determined by the method of Fletouris et al. (1998) with modifications. An aliquot (0.4 ml) of the supernatant was mixed with 4 mL of methanolic KOH 0.5 mol/l, vortexed for 15 s, heated at 80 °C for 15 min and cooled. After cooling, 1 ml of sterile distilled water and 4 ml of the internal standard solution of 5- $\alpha$ -cholestane (0.02 mg 5- $\alpha$ -cholestane/ml hexane) were added. The mixture was vortexed for 1 min and centrifuged at 2000 x g for 1 min. The hexane layer was separated, dried under nitrogen, diluted with 1 ml of hexane and injected into the GC. The GC analysis was carried out by a Trace GC (ThermoFisher, Rodano, Italy) provided with the Ultrafast module (UFM) and an automatic sampler. A 5% phenyl, 95% dimethylpolysiloxane column, 2.5 m in length and with an internal diameter of 0.32 mm and film thickness of 0.05  $\mu$ m, was used. One  $\mu$ L was injected in the Programmed Temperature Vaporisation (PTV) split injector (split ratio 1:20). The following PTV temperature program was adopted: 60 °C, for 0.1 min, rate at 12 °C/s up to 320 °C, held for 4 min. Oven conditions were: 80 °C for 1 min, rate 60 °C/min up to 240 °C, held for 1 min. The carrier gas was hydrogen (2.4 ml/min, constant flow) and the detector was a FAST flame ionisation detector (FID) kept at 320 °C. Data processing was performed by the Chrom-Card software (ThermoElectron, Milan, Italy). The concentration (mg/l) of cholesterol was calculated using the internal standard method and applying the correction factor cholesterol/5- $\alpha$ -cholestane, previously calculated by analysing solutions of known concentration of both constituents. All assays were repeated in duplicate. The amounts of cholesterol removed by the test cultures were expressed as percentages of cholesterol reduction compared to the cholesterol content determined in the uninoculated control broth and calculated as follows:  $(100 \times B)/A$ , where B = average of cholesterol (mg/l) in culture supernatant; A = average cholesterol (mg/l) in uninoculated control broth.

#### 4.2.3 Detection of the bile salt hydrolase (*bsh*) gene

Strains showing high ability of cholesterol reduction were tested for the presence of the *bsh* gene by PCR amplification. PCR primers were designed on the basis of the gene sequences of *bsh* (GenBank accession number AL935262.1) of *L. plantarum* WCFS1. The primers had the following sequences: 5'-CGTATCCAAGTGCTCATGGTTTAA-3' (*bsh* for, nucleotide position 150568 to 150593 of the *bsh* gene) and 5'-ATGTGTACTGCCATAACTTATCAATCTT-3' (*bsh* rev, nucleotide position 151487 to 151460 of the *bsh* gene). The strain LMG 9211 was used as positive control for both PCR amplifications.

PCR amplifications were performed in 20  $\mu$ l volumes with 0.5 mM of each primer (Biotez, Berlin, Germany), 2.5 units/100 $\mu$ l of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 1.5 mM MgCl<sub>2</sub>, 20 ng of total DNA, and 200 mM of each dNTP. DNA amplifications were performed by a Perkin Elmer thermal cycler (mod. 9700, Applied Biosystems) and were set as follows: 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 64°C (*bsh* for/rev) or 52°C (*msa* for/rev), and 1 min at 72°C; the final extension step consisted of 7 min at 72°C. The PCR

products were separated on a 1% agarose gel in Tris-Acetate EDTA buffer.

#### **4.2.4 Phenotypic assays**

##### **4.2.4.1 BSH activity**

BSH activity of the strains was detected using a plate screening procedure according to Zago et al. (2011). Briefly, overnight cultures were spotted on MRS agar plates containing 0.37g/l of CaCl<sub>2</sub> and 0.5% sodium salt of taurodeoxycholic acid (TDCA) or 0.5% sodium salt of glycodeoxycholic acid (GDCA) (Sigma-Aldrich). Plates were anaerobically incubated at 37°C for 72 h. The presence of halos around colonies or white opaque colonies indicated BSH activity. *Enterococcus faecium* LMG 16170 was used as BSH-positive strain. The inoculums of each strain in MRS without supplementation was included as negative control.

##### **4.2.4.2 Bile resistance**

The ability of strains to grow in the presence of 0.3%, 0.5% and 1.0% of bile (Sigma-Aldrich) was determined according to the method of Vinderola & Reihneimer (2003). Each strain was inoculated (2% v/v) into MRS broth on M17 broth with 0.3, 0.5 or 1% (w/v) of bile (Sigma Chemical Co., St. Louis, MO USA). Cultures were incubated at 37°C (25°C for lactococci) and, after 24 h, A<sub>560</sub> nm was measured and compared to a control culture (without bile salts).

The results were expressed as the percentage of growth (A<sub>560</sub> nm) in the presence of bile salts compared to the control.

##### **4.2.5 Cellular fatty acid composition**

Cellular fatty acid composition was determined on colonies harvested from a culture plate at the stationary growth phase, as described by Morandi et al (2012). About 40 mg bacterial cells were saponified with 1 ml basic methanol (45 g NaOH dissolved in 300 ml deionized water:methanol, 1/1, v/v); tubes containing the saponified mixture were vortexed for 5–10 s and kept in a boiling water bath for 30 min. After cooling to room temperature, 2 ml 6 M HCl/methanol (325:275, v/v) was added and the sample was heated in an 80 uC water bath for 10 min. The sample was cooled rapidly, 1.25 ml methyl tertbutyl ether/hexane (1:1, v/v) was added and the tube was turned end over end for about 10 min. The lower aqueous phase was discarded by pipetting, 3 ml 0.3 M NaOH was added and the tube was turned end over end for 5 min. Then, the organic phase was transferred into a 2 ml glass vial. Fatty acids methyl esters were injected into a gas chromatograph (Thermo Fischer) equipped with a flame ionisation detector and a high polar fused silica capillary column (SP 2560 Supelco, Bellefonte, PA, USA) of 100-m length, with an internal diameter of 0.25 mm and a film thickness of 0.20 µm. The temperature program was as follows: 45 °C for 8 min, 12 °C/min up to 173 °C, 47 min holding, 4 °C/min up to 220 °C, 20 min holding time. The split injector and the FID were held at 250 °C. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min and the split ratio was 1:80. The identification of the fatty acid methyl esters in the samples was performed using the BAME Mix standard (Sigma-Aldrich) and FAME mix standard cod. 47885-U (Supelco). Data processing was carried out by the Chrom-Card software (ThermoElectron).

##### **4.2.6 Statistical analysis**

Data analysis was carried out with the SPSS software (version 17.0; SPSS Inc., Chicago, IL). One-way ANOVA analysis of variance was used to test the effect of the different variables on the measured factors. Duncan's test was used to perform multiple comparisons between the means. All data are presented as mean ± standard deviation.

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Cholesterol removal capability of LAB

The 58 strains showed different levels of abilities in removing the cholesterol added to the growth media. After 24 h of growth, a reduction of the cholesterol in the cells free culture supernatants was determined. The amount of cholesterol removed from the substrate ranged from a minimum of 0% to a maximum of 33.31%. The cholesterol reduction was strain-specific and four strains of different species (i.e., *Lact. plantarum* 885, *Lact. acidophilus* LA-5<sup>®</sup>, *Lact. delbrueckii* ssp. *bulgaricus* V15 and *Ent. italicus* 989) showed the highest percentage of the cholesterol removal capability (Table 4.1). Several *Streptococcus* and *Enterococcus* strains also caused a decrease in cholesterol, whereas *Lact. delbrueckii* ssp. *lactis* and *Ent. casseliflavus* strains showed the lowest cholesterol removal ability.

### 4.3.2. Influence of the growth phase on cholesterol removal

The four strains that showed the highest activity on cholesterol were selected to study the trend of cholesterol removal during a complete cycle of growth (Table 4.2). The strains cultivated in growth media in the presence of cholesterol showed the highest increase in cell population after 16 h (*Lact. acidophilus* LA-5<sup>®</sup> and *Ent. italicus* 989) or 24 h (*Lact. plantarum* 885 and *Lact. delbrueckii* ssp. *bulgaricus* V15). The number of viable cells then decreased until the end of the 48-h incubation period. The amount of cholesterol found in the cell-free culture supernatants at each time was related to growth and viability of the bacterial cells.

The strains also showed an increased ability to remove cholesterol during the bacterial exponential growth phase. The level of cholesterol in the medium increased with a decreasing number of viable and cultivable cells, resulting in lower removal ability. This behaviour was recorded for *Lact. plantarum* 885, *Ent. italicus* 989 and *Lact. acidophilus* LA-5<sup>®</sup>. For *Lact. delbrueckii* ssp. *bulgaricus* V15, the amount of cholesterol removed did not change when the cells entered the mortality of growth phase. The significance of these results were confirmed by statistical analysis, which showed the influence of the time of growth on the percentage of cholesterol capture. *Lact. plantarum* 885 and *Lact delbrueckii* subsp. *bulgaricus* V15 showed the highest ability to remove cholesterol from the medium after 24 hours, whereas *Lact. acidophilus* LA-5<sup>®</sup> and *Ent. Italicus* 989 exhibited their highest ability after 16 h (Table 4.2). To verify the possible presence of products of cholesterol conversion, a GC/MS analysis of the cell-free culture supernatant was performed. No compounds identifiable as cholesterol derivatives were detected (data not shown).



Strains	Time (h)								Total amount of cholesterol removed (%)	Sig
	10		16		24		48			
	Removal	$\Delta \log$ CFU/ml	Removal	$\Delta \log$ CFU/ml	Removal	$\Delta \log$ CFU/ml	Removal	$\Delta \log$ CFU/ml		
<i>Lp</i> 885	0.61±0.86 <sup>c</sup>	0.87±0.07	21.12±6.12 <sup>b</sup>	1±0.03	33.31±0.22 <sup>a</sup>	1.28±0.02	20.67±0.33 <sup>b</sup>	0.38±0.01	75,71	**
<i>La</i> LA-5 <sup>®</sup>	18.97±0.88 <sup>b</sup>	1.65±0.04	24.09±0.73 <sup>a</sup>	2.04±0.01	21.03±1.38 <sup>b</sup>	1.09±0.01	10.69±0.03 <sup>c</sup>	0.38±0.02	74,78	**
<i>Ldb</i> V15	7.24±2.23 <sup>b</sup>	1.16±0.25	15.60±2.57 <sup>ab</sup>	1.42±0.11	19.29±1.07 <sup>a</sup>	1.62±0.02	18.80±4.87 <sup>a</sup>	0.76±0.03	60,93	*
<i>Eit</i> 989	7.59±3.89 <sup>b</sup>	0.73±0.05	26.45±2.17 <sup>a</sup>	1.33±0.03	13.90±2.96 <sup>b</sup>	1.06±0.01	10.59±1.47 <sup>b</sup>	0.63±0.02	58,53	**

Table 4.2: Comparison of cholesterol removal capability and growth of the tested strains related to the time of growth. Values expressed as percentage of reduction for cholesterol removal and as  $\Delta \log$  CFU/ml for bacterial growth. Data are showed as means and standard deviations. Data followed by different letters are significantly different. \*significance at  $P < 0.05$ ; \*\*significance at  $P < 0.01$ .

### 4.3.3. Bile resistance and BSH activity

The growth of *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup> was not substantially inhibited by the presence of increasing concentration of bile (Figure 4.1). However, *Ent. italicus* 989 and *Lact. delbrueckii* ssp. *bulgaricus* V15 were less bile-resistant and growth was already strongly inhibited by the presence of 0.3% bile. Among these strains, the *bsh* gene was detected only in *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup>, and BSH activity was confirmed by phenotypic tests.

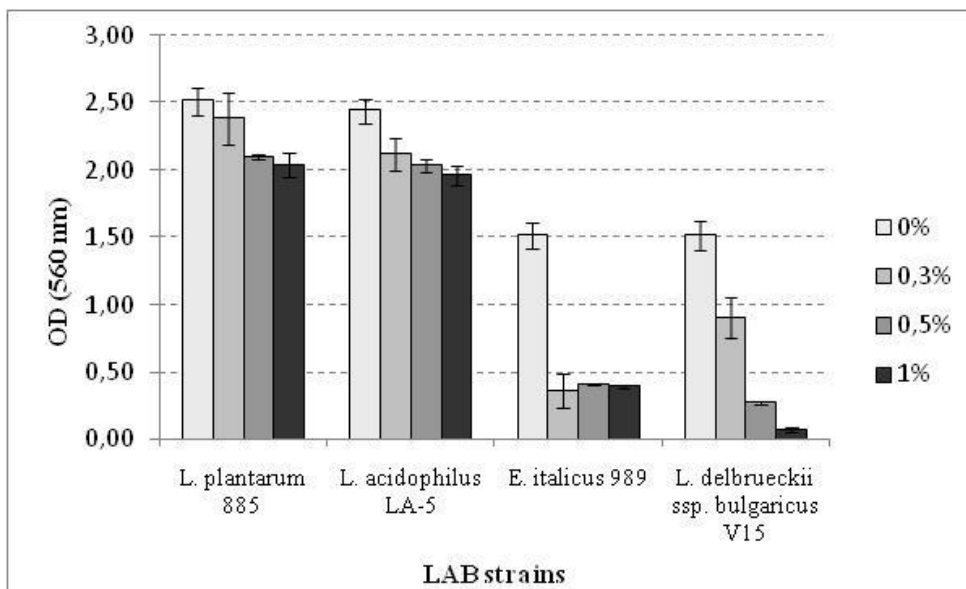


Figure 4.1: Bile tolerance of the tested LAB strains. Values reported as OD<sub>560</sub> of strain cultures after 24 h in the presence of different amounts of bile salts. Data are shown as means ± standard deviation.

Among the 58 strains investigated, two strains of lactobacilli, *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup>, demonstrated the highest cholesterol removal ability.

As presented above, bile tolerance, cholesterol removal and deconjugation of bile salts should all be considered when selecting strains as potential cholesterol-reducing bacteria. As reviewed by Maire et al. (2006), deconjugation of bile salts *in vivo* could lead to a reduction in serum cholesterol either by increasing the demand for cholesterol in the synthesis of bile acids to replace those lost in faeces or by reducing cholesterol solubility and thereby, absorption of cholesterol through the intestinal lumen (Zhang et al. 2008). Therefore, *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup> both have high abilities of bile salt deconjugation and cholesterol removal. These results strengthen the high bile resistance property of these strains showed by *in vitro* tests and are in accordance with other studies that indicate, for the same strains of lactobacilli, a possible correlation between the production of BSH and the reduction of serum cholesterol (Brashcars et al. 1998; Usman and Hozono 1999; Zhang et al. 2008).

### 4.3.4. Effect of cholesterol on cellular fatty acid composition

The cellular fatty acid compositions of the two most interesting strains which showed the highest ability to remove cholesterol, *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup>, are reported in Table 4.3.

Fatty acids	<i>Lact. plantarum</i> 885		<i>Lact. acidophilus</i> LA-5 <sup>®</sup>	
	- cholesterol	+ cholesterol	- cholesterol	+ cholesterol
C12	2.49±0.128 *	1.92±0.056	3.84±0.054 **	4.39±0.043
C14	18.12±0.555 *	15.64±0.220	8.47±0.124 **	7.19±0.055
C14:1	0.15±0.042 ns	0.24±0.013	0.51±0.042 *	0.31±0.017
C15	0.36±0.040 ns	0.49±0.007	0.77±0.103 ns	0.50±0.025
C16	19.99±0.475 *	18.09±0.147	20.83±0.114 **	17.86±0.224
C16:1 <i>cis</i> 9	2.71±0.022 ns	2.78±0.030	2.51±0.082 ns	2.81±0.093
C18	0.57±0.003 ns	0.54±0.009	1.78±0.063 **	0.66±0.026
C18:1 <i>trans</i>	1.21±0.193 ns	1.34±0.028	1.27±0.039 ns	1.50±0.069
C18:1 <i>cis</i> 9	24.46±0.495 **	35.44±0.091	36.42±0.017 ns	35.13±0.185
C18:1 <i>cis</i> 11	2.76±0.099 ns	2.46±0.063	1.61±0.052 ns	1.57±0.155
C18:2	0.58±0.056 *	0.35±0.007	0.91±0.132 ns	0.79±0.020
C19 (dihydrosterculic)	20.91±0.039 **	16.81±0.057	17.39±0.071 **	23.11±0.137
Others	5.68±0.076 *	3.91±0.130	3.71±0.137 ns	4.18±0.084
Saturated	62.45±0.206 **	53.49±0.349	53.07±0.056 ns	53.71±0.408
Unsaturated	31.87±0.281 **	42.61±0.219	43.22±0.082 ns	42.10±0.499

Table 4.3: Cellular fatty acids composition of LAB strains grown in medium with or without cholesterol. Values expressed as percentage of total fatty acids.

The two strains showed differences independently of the presence of cholesterol. *Lact. plantarum* 885 grown in the absence of cholesterol was characterized by a higher percentage of saturated fatty acids (SFA) than *Lact. acidophilus* LA-5<sup>®</sup> (+9.38%), mainly due to the high amount of C14. As for the other SFA, C16 was present in a rather similar amount in the two strains, whereas C18 was higher in *Lact. acidophilus* LA-5<sup>®</sup>. Moreover, the latter strain showed the highest amount of unsaturated fatty acids (+11.35%), especially C18:1 c9.

The presence of cholesterol in the medium of *Lact. plantarum* 885 elicited a highly significant ( $P < 0.01$ ) decrease in the overall percentage of SFA and a corresponding increase in the unsaturated ones. The main contribution to this result was due to the variation of C19 cyc and C18:1 cis9, respectively (Table 4.3).

According to Kimoto et al. (2002), who suggested that cholesterol incorporation into the cellular membrane alter the fatty acid profiles, changes in hexadecanoic, octadecanoic and total saturated and unsaturated acids were observed.

Saturated fatty acids confer rigidity to the lipids in the membranes, whereas the double bonds in the unsaturated fatty acids are responsible for the increase in this physical property. The balance between saturated and unsaturated fatty acids is important in maintaining the optimum degree of membrane fluidity and in bacterial membranes, branched-chain and cyclopropane fatty acids behave like unsaturated ones (Dowhan et al. 2008). Cholesterol plays an important role in biological membranes since it is believed to modulate their fluidity by interacting with their complex lipid components, like phospholipids (Dowhan et al. 2008). A membrane composed

only of unsaturated lipids is in a fluid state, characterized by a degree of disorder, whereas a membrane composed of more saturated lipids is in a more solid gel state. Cholesterol increases the degree of order in the former and renders the latter more fluid (Dowhan et al. 2008). The changes in lipid composition enable the microorganisms to maintain the membrane characteristics and functions in response to environmental conditions (Aricha et al. 2004). The incorporation of cholesterol to the bacterial membrane induces a change in the balance between saturated and unsaturated fatty acids. This modification was more evident in *Lact. plantarum* 885, in which the original fatty acid composition was richer in saturated acids than that of *Lact. acidophilus* LA-5<sup>®</sup>. This result leads to the hypothesis that the incorporation of cholesterol increases the amount of unsaturated fatty acids to maintain membrane fluidity. On the contrary, the high percentage of unsaturated fatty acids in *Lact. acidophilus* LA-5<sup>®</sup> appeared to be able to maintain the membrane's physical characteristics, even in the presence of cholesterol.

#### 4.4 CONCLUSION

Some LAB strains confirmed the ability to remove cholesterol from culture media. The amount of cholesterol decreased during growth phase and increased during the resting or mortality phase. The release of cholesterol when the cells reached the resting or mortality phase suggested that the cholesterol was reversibly incorporated within the bacterial cell membranes. Regarding the changes in fatty acid composition, as affected by the presence of cholesterol in the culture media, the two strains with the highest cholesterol removal ability, *Lact. plantarum* 885 and *Lact. acidophilus* LA-5<sup>®</sup> showed a different behaviour that appeared to be related to the needs of the cells to maintain membrane fluidity, while depending on the original fatty acid composition.

These preliminary findings will lead to further studies aimed at better characterizing LAB strains for use in developing fermented dairy products with reduced cholesterol content or able to induce hypocholesterolemic effects *in vivo*.

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**5. THE USE OF NIR SPECTROSCOPY FOR  
MONITORING MILK - WHEY  
BIOTRANSFORMATION PROCESSES USING  
*LACTOBACILLUS PLANTARUM***

## 5.1 INTRODUCTION

The dairy industry generates significant liquid waste, whose disposal requires a large amount of capital investment. The majority of total milk used for manufacturing cheese is discarded as whey, which retains a lot of nutrients (Jalen, 2002). The most abundant nutrients are lactose, soluble proteins, organic acids and salts. The disposal of whey as waste can have a high environmental impact. To overcome this problem, a better alternative could be to process whey in order to obtain new added value products. Whey contains approximately 4.5% lactose, 0.8% proteins, 1% salts and 0.1-0.8% lactic acid (Wenge et al., 1999) Availability of a high amount of lactose in whey and the presence of other essential nutrients for Lactic Acid Bacteria (LAB) growth make whey a potential raw material for the production of different bio-products using biotechnological means, such as fermentation processes.

LAB are Gram positive micro-organisms, non-spore forming, catalase negative, non-motile, microaerophilic, rod or cocci that ferment sugars to mainly produce lactic acid (John et al., 2007).

Among LAB, selected species of the *Lactobacillus* genus are widely used as probiotics in dairy products. Probiotics are defined as active microorganisms that show health benefits for the host by improving the properties of indigenous microbial community when consumed in adequate amounts. They should have technological characteristics to allow their production on a large scale and their incorporation into food products without losing viability and functionality but, at the same time, without creating unpleasant flavours (Guarner et al., 2005).

One of these species, *Lactobacillus plantarum*, is industrially important and is involved in many vegetable fermentations as well as being a frequent inhabitant of the human intestinal tract. Some *Lactobacillus plantarum* strains are already on the market as probiotics. High acid tolerance is an important feature that prevents contamination during fermentation processes. *L. plantarum* is a fair lactic acid producer.

Bioprocess optimization is often limited by the inability to measure biomass, nutrients and products concentration in a time frame that allows process adjustment.

For accurate control of fermentation processes, it is very important to monitor accurately and rapidly substrate and product concentrations during incubation. However, the analysis of these compounds requires sample preparations, and in some cases several steps of purification are needed, which delays obtaining the final results from analyses.

Near-infrared transmittance spectroscopy (NIRS) offers the advantage of rapid sample processing with results available within 5 min. It is a nondestructive technique that requires no sample preparation and can measure several broth constituents simultaneously. Furthermore, authentic standards are not necessary after the prediction equation is developed. With the use of NIRS as a routine analysis method, opportunities for on-line control processes and fault analysis are much greater than can presently be achieved with existing measurements, such as HPLC (Guarner et al., 2005; Vaccari et al., 1994). Nevertheless, the NIRS method also has disadvantages, such as interference caused by unexpected components in the sample, with overlapping spectral features, which can introduce errors in the analysis. However, the main advantage in using NIRS is its ability to monitor in real time the progress of fermentation. This allows the optimization of the harvesting time during batch culture, the selection of feeding times with growth-limiting substrate during feed-batch fermentation, and the detection of steady state for continuous fermentation. In all cases NIRS could save medium and operation time . NIRS has mainly been used for the evaluation of nutritional components in animal feed. Recently it has been used for determining natural products in plants, monitoring cell and biomass concentrations, characterizing polymers, enhancing production of lactic acid in a fully automated plant-controlled process, and also for analyzing dairy foods (Macedo et al., 2002).

The main advantage in using NIRS is its ability to monitor in real time the progress of

fermentation allowing the optimization of the starter culture during processes. Thus, this study aimed to develop a control system for monitoring fermentation using NIR techniques recognized as rapid and accurate methods to also correct as quickly as possible anomalies during the milk fermentation processes. This can give way to new added value products starting from milk whey recovery (Bouzas et al., 1991).

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Inoculum preparation**

Tests were performed using strains of *Lactobacillus plantarum* belonging to the internal collection of CRA-FLC (Lodi, Italy). The strains were reactivated from frozen stock at  $-80^{\circ}\text{C}$ , revitalized in MRS broth (Merk, Germany) and incubated at  $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$  overnight. Culture was centrifuged for 10 min at 7000 rpm, at  $4^{\circ}\text{C}\pm 1^{\circ}\text{C}$ . The supernatant was removed, and the cellular pellet suspended in equal volume of Ringer solution (Oxoid, Basingstoke, England). Microbial count was determined for direct counts with optical microscope (Leitz, Wetzlar, Germany). The optimal value of microbial count, established in  $9.0\pm 0.2$  log CFU/ml, was normalized by diluting or concentrating cellular pellet. The standardized cell suspension was added at a rate of 1% to reconstituted whey, used as substrate of microbial growth.

### **5.2.2 Substrate preparation**

Whey was prepared in accordance with the method proposed by Parnesar et al. (2007) with some modifications. Whey powder was reconstituted at 7% with deionized water and subjected to agitation for dissolution. After preliminary tests, the percentage of recovery was established when the final percentage of lactose equal to  $4.5\pm 0.1$  was reached. After heat treatment with flowing steam for 10 minutes to allow the precipitation of proteins, and spinning at 8000 rpm for 10 minutes at  $4^{\circ}\text{C}\pm 1^{\circ}\text{C}$ , 0.75% of yeast extract (Merck, Germany) and 1% of calcium carbonate (Carlo Erba, Italy) were added to the supernatant whey (clarified whey). Then, the substrate was pasteurized in flowing steam for 30 min and cooled to  $4^{\circ}\text{C}\pm 1^{\circ}\text{C}$ .

### **5.2.3 Fermentation tests**

Pasteurized whey was inoculated and incubated in thermostatically-controlled water bath at  $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$  for 48 hours. Aliquots of 2 ml were taken at fixed intervals (0, 6, 8: 11, 15, 18, 24, 28, 32 and 48 hours) and microbial count was determined using MRS agar, after the plates incubation for 48 hours at  $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$  in anaerobic conditions.

### **5.2.4 Determination of lactose and lactic acid content by HPLC**

One gram of fermented whey was retrieved with sulphuric acid 0,009 N, centrifuged, filtered and injected into an ion exchanger column (Aminex HPX 87H, 300 mm x 7.8 mm, 9 mm diameter average Biorad, U.S.A) in isocratic conditions ( $65^{\circ}\text{C}\pm 0.1$ ) with HPLC system (Kontron "instrument", Switzerland). Components were eluted with an aqueous solution of sulphuric acid 0.009 N at a speed of 0.6 ml/min (Bouzas et al., 1991). Refractometer REFRACTIVE INDEX 475 (Kontron "instrument", Switzerland) was used as detector. Data processing was made using Kromasystem 2000 software (Kontron "instrument", Switzerland) and quantification was made by comparison with an external standard consisting of a solution of acids and sugars at known concentrations.<sup>7</sup>

### **5.2.5 Near infrared Spectroscopy**

FT-NIR spectra were acquired in transflectance mode using a FT-NIR® - NIRFlex N-500 (BUCHI Assago, Milan, Italy) spectrometer. Analysis were conducted using an optic fiber with an optical path of 0.08 mm, submerged directly in the whey samples at established intervals, at



the optimum growth temperature ( $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ ). Replicated measurements were made (three replicates for sample) and spectra were collected in the whole NIR range, from 4000 to 10 000  $\text{cm}^{-1}$  using NIRWare software 1.2 Operator (BUCHI, Italy).

### 5.2.6 Data processing

The dataset was divided into two independent subsets composed of 30 (calibration) and 20 samples (prediction) respectively. All spectra were processed using the Unscrambler software v. 9.2 (Camo Inc., OSLO, Norway). Calibration curves for the determination of lactose and lactic acid contents, and biomass expressed as log CFU/ml were calculated by applying the method of partial least squares regression (PLSR) on the averaged and mean centered data.

## 5.3 RESULTS AND DISCUSSION

Adaptability of *Lactobacillus plantarum* strains tested on the substrate was demonstrated by the fermentation trends reported in Figure 5.1, and further supported by the microbial growth values resulting from sampling performed. Within 48 hours, the maximum fermentation period, strains reached growth values of order 9 log CFU/ml, demonstrating how this species is able to multiply even in non-optimal growth conditions, thereby justifying its presence in many environmental niches, as reported in literature (De Vries et al., 2006).

Monitoring the lactose degradation and the corresponding production of lactic acid showed how each strain has a different behavior than others of the same species: starting from an initial value of lactose standardized (4.5%), final values were achieved ranges from 3.56 to 0.24 g/100 g depending on the strain used. Similarly, the lactic acid production reached a maximum value of 3.49 g/100 g and a minimum value of 1.093 g/100 g (data not shown). An example is reported in Figure 5.2.

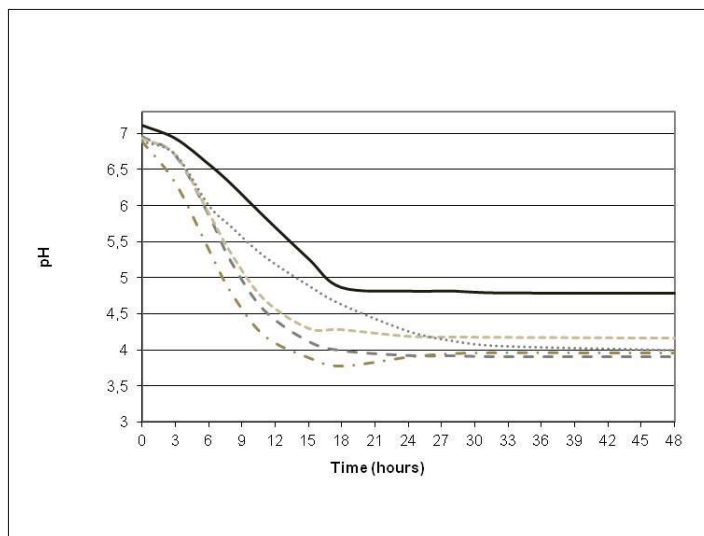


Figure 5.1: Example of a *Lactobacillus plantarum* acidification curve

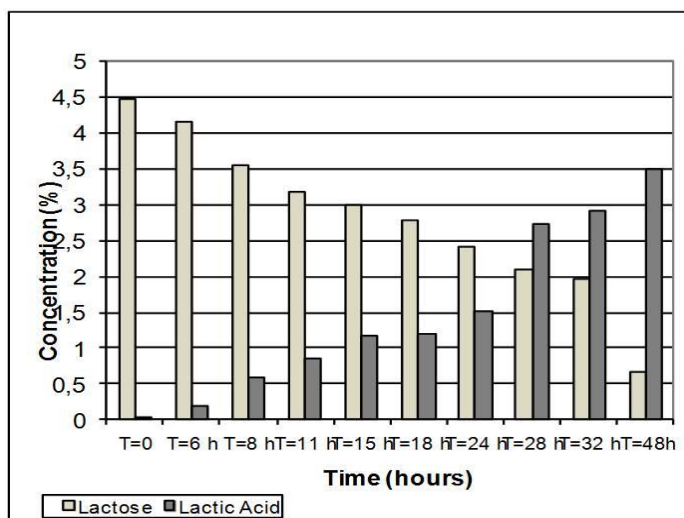


Figure 5.2: Example of Lactose degradation and Lactic Acid formation

It was noted that not all the available lactose was metabolized to lactic acid: *L. plantarum* is an optional heterofermentative microorganism, therefore the degradation of this sugar leads to the formation of traces of other compounds (such as galactose, acetic acid and succinic acid) that have not been considered here.

Results, obtained by applying PLS regression on the pre-processed data (smoothing Savitzky-Golay: 15 points, polynomial order 2), proved to be very satisfactory for all parameters, as shown in Table 5.1. In all cases it was possible to obtain high correlation coefficients and good RMSEP values in validation, using models with a low number of principal components (PCs). Based on these statistical indexes, the NIR prediction was aligned with data resulting from the use of the reference methods.

Parameter	Range	Sample set					
		Calibration			Validation		
		R	RMSEC (g/100g)	SEC (g/100g)	R	RMSEP (g/100g)	SEP (g/100g)
Lactose	0.24-3.56 (g/100g)	0.9885	0.0451	0.0459	0.9224	0.1802	0.1713
Lactic Acid	1.093-3.49 (g/100g)	0.9893	0.0579	0.0590	0.9585	0.1245	0.1270
Biomass	7-9 (log CFU/mL)	0.8902	0.2531	0.2586	0.9222	0.2070	0.2130

Table 5.1: Statistical parameters for NIRS Calibration and Validation curves for the determination of Lactose, Lactic Acid and Biomass

The proposed regression models were very satisfactory for all of three analytes. Usually SEP (Standard Error in Prediction) is higher than SEC (Standard Error in Calibration), but in the case of biomass produced with *Lactobacillus plantarum* it turned out to actually be lower. This information demonstrated that the developed model was an adequate representation of the fermentation process (Tamburini et al., 2003).

To identify the specific absorption bands, related to the determination of lactic acid and lactose, and the evaluation of biomass content, the second derivative of NIR spectra was computed: an example is reported in Figure 5.3.

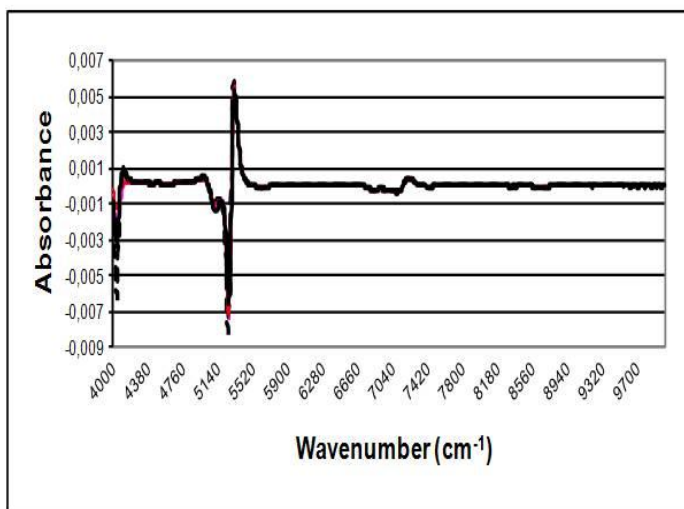


Figure 5.3: Example of NIR second derivative spectra obtained from a microbial starter in whey as a function of time.

The spectral region between 6500 and 7500  $\text{cm}^{-1}$  appeared to be super-imposable to all tested times. Indeed, in this area it was possible to identify the absorption bands related to the stretching and bending of C–H bond (6960 and 7084  $\text{cm}^{-1}$ ), and the absorption band of the first overtone of O–H stretching, positioned at 6840  $\text{cm}^{-1}$ . Another common area was identified around 4400  $\text{cm}^{-1}$ , ascribable to the stretching of O–H and C–H bonds; furthermore, the second overtone of C–H stretching was identified around 5115  $\text{cm}^{-1}$  (Williams 2001).

In particular, as reported in Figure 5.4, a relationship between bands intensity and concentration was noticed: the NIR bands that increased with the incubation time were located in the same spectral region for all the calibrated parameters (5000 – 5200  $\text{cm}^{-1}$ ). The evidence found by the correspondance with the main whey constituents demonstrated how, even in complex matrices, quantitative answers relating to a single constituent, can be optimized.

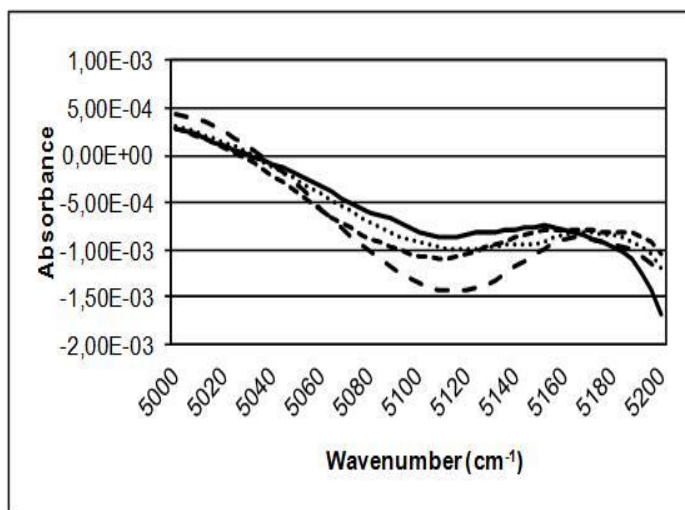


Figure 5.4: A more in depth portion of the NIR second derivative spectra obtained from a microbial starter in whey as a function of time.

## 5.4 CONCLUSIONS

On the basis of the obtained statistical parameters for each studied index determined by both reference methods of analysis and FT-NIR technology, it was possible to confirm the applicability of NIRS for the direct determination of lactose, lactic acid, and microbial count in milk whey. The use of an optic probe proved to be a viable alternative to existing methods of fermentation monitoring, particularly with respect to biomass determination. A complex analytical matrix, such as a culture medium with suspended cells, at varying concentration wasn't an impediment for a rapid, reliable, and accurate determination of the studied components.

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**6. NIR AND MIR SPECTROSCOPY IN THE STUDY OF  
CHOLESTEROL REMOVAL ABILITY OF  
*LACTOBACILLUS PLANTARUM* STRAINS**

## 6.1 INTRODUCTION

Cholesterol is a polycyclic alcohol having a secondary -OH group at position 3'. It plays an important role as a component of cell membranes in vertebrates and, in many of the normal metabolic pathways as a precursor of steroid hormones, bile acids and vitamin D.

The consumption of food rich in lipids, especially saturated fatty acids and cholesterol, has a predominant role in the rise in the heart disease. Thus, the consumer demand on food with lower amount of these components and with additional healthy properties is increasing (Belviso et al., 2009).

Dairy products play an important role in a healthy diet because of their high nutritional value, but since in some cases they also represent a source of lipids, many attempts were carried out to reduce their fat concentration. As the consumption of fermented milk containing bacterial strains provides beneficial effects, such as the reduction of serum cholesterol levels on human health (Gilliland et al., 1985), several researches on bacterial strains with potential hypocholesterolaemic properties were then carried out.

In particular, the consumption of dairy products containing probiotic bacteria has been proposed as a means to lower cholesterol in serum (Ling & Shah, 2005).

Probiotics are defined as a "live microbial supplement that beneficially affects the host by improving its intestinal microbial balance". Many authors have shown how these bacteria are able to decrease blood cholesterol content, proposing several in vitro studies about the mechanism of cholesterol-lowering action of probiotic bacteria even if the exact mechanism(s) remain unclear (Liong & Shah, 2005; Gilliland et al., 1985; Tahri et al., 1996). Recent papers tried to show how strains isolated from dairy matrixes have the ability to remove cholesterol during their growth. In particular, Belviso et al. (2009) tried to do this with *Lactobacillus plantarum* hypothesizing the co-precipitation of cholesterol with bile salts.

Within the genus *Lactobacillus*, *L. plantarum* is a member of the facultative heterofermentative group of lactobacilli and it is present in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. Moreover, strains of *L. plantarum* have proven ability to survive gastric transit and colonize the intestinal tract of humans and other mammals (Zago et al., 2011).

Methods have been proposed to investigate whether cholesterol was really assimilated by strains or whether it remains free in culture medium. Many authors used the method of Rudel & Morris (1973): cholesterol can be estimated by visible spectroscopy using *o*-phthalaldehyde reagent after extraction of unsaponified matter (cholesterol) with an organic solvent such as hexane. The application of this method requires a long time and so spectroscopic methods have been proposed using MIR and NIR techniques<sup>7-9</sup>.

Infrared spectroscopic methods are simple, cost-effective, rapid, nondestructive and have the potential for routine analysis if proper calibration and validation procedures with data acquisition protocols could be established. Budinova et al. (1997) have used the FTIR spectral region between 950 and 1100 cm for the detection of cholesterol in blood and serum and Shaw & Mantsch (2000) used MIR attenuated total reflectance (ATR) technique (in the spectral region between 2800 and 3000 cm) to develop a statistical model using eight partial least square (PLS) factors. This study is one of the first attempts to use FTIR-ATR spectroscopy to estimate cholesterol in milk products.

The application of chemometrics to analyse the spectra is an important step in spectroscopy. The most commonly used multivariate calibration methods is principal component analysis (PCA). Discriminant analysis is commonly used in classification to group objects or data of similar characteristics into clusters based on statistical measure. The success of these methods depends upon the choice of proper spectral data and the number of variables employed in the calibration model.

The aim of this study was to verify, by the use of NIR and MIR spectroscopic techniques, whether strains with different ability to remove cholesterol can show changes in the cell wall and if it was possible to determine the amount of the cholesterol removed.

## 6.2 MATERIALS AND METHODS

### 6.2.1. Bacterial strains and culture conditions

Four *L. plantarum* strain (Lp752, Lp885, Lp995, Lp997) belonging to the collection of CRA-FLC (Lodi, Italy) and isolated from Italian dairy products, and the type strain ATCC14917<sup>T</sup>, stored as frozen stock at  $-80^{\circ}\text{C}$ , were cultivated overnight in MRS broth (Merck, Germany) at  $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ . After growth, 2% (v/v) of fresh culture was transferred in MRS and MRS broth supplemented with a stock solution of cholesterol (10% w/v) to obtain a final concentration of 0.1 g/l.

Cholesterol stock solution (1 g/l) was prepared by dissolving 100 mg of cholesterol (Sigma-Aldrich, Milan) in an aqueous solution of 26,4% ethanol and 12% Tween 80 (polyoxyethylene sorbitan monooleate) (Sigma-Aldrich, Milan). After growth for 24 h at  $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ , the following samples were analyzed: A) cells harvested (7000 rpm for 10 min at  $4^{\circ}\text{C}$ ) by centrifugation and washed twice with ultrapure water B) culture supernatant. The pH measured on samples B was  $3.9 \pm 0.2$ .

### 6.2.2 MIR spectroscopy

One gram of sample A was distributed on ZnSe tablets ( $\varnothing$  13mm, h 2mm, PIKE Technologies, USA) to collect, at room temperature, ATR-FTIR spectra (128 scans, resolution  $4\text{ cm}^{-1}$ ) using a FT-IR 420 spectrometer (JASCO Europe, Cremella, Como, Italy), in the range between 900 and  $1750\text{ cm}^{-1}$ .

### 6.2.3 NIR spectroscopy

FT-NIR spectra were collected on samples B in transmission mode (pathlength 0.2 mm, 32 scans, resolution  $8\text{ cm}^{-1}$ , 6 replicates) with FT-NIR@ NIRFlex N-500 spectrometer (BUCHI, Italy) in the whole NIR range ( $4000\text{--}10000\text{ cm}^{-1}$ ) with 1.2 NIRWare Operator (BUCHI, Italy) at controlled temperature ( $40^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ). Data were processed with Unscrambler v. 9.2 (Camo Inc., OSLO, Norway).

### 6.2.4 Data processing

MIR average spectra of sample A (5 replicates), after subtraction of an average spectrum of water or supernatant, depending on the matrix, were pretreated with smoothing (Savitzky Golay, 7 points, polynomial order 0). NIR data were processed by using Unscrambler software v. 9.2 (Camo Inc., OSLO, Norway) in order to evaluate qualitative differences between culture supernatants (in absence and in presence of cholesterol). PCA analyses were performed by pretreating spectral data with SNV (Standard Normal Variate), Savitzky – Golay second derivative (8 points, polynomial order 2) and mean centering.

## 6.3 RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) are Gram+ microorganisms and their cell wall is primarily made up of peptidoglycan (ca. 40-80% of the dry weight of the wall), which is a polymer of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). Other important constituents are: teichoic acid, a polymer of glycopyranosyl glycerol phosphate, teichuronic acid, which is similar to teichoic acid, but replaces the phosphate functional groups with carboxyls, and surface proteins<sup>6</sup>. Therefore, the principal functional groups found in MIR spectra were: carboxyl, hydroxyl, phosphate and amide. In particular, phosphate group in bacteria can exist



both in inorganic forms such as orthophosphate and its oligomers and in organic forms such as phosphate mono- and di-esters. The principal absorption bands, identified in the second derivative of MIR spectra, are shown in Table 6.1 (Jang et al., 2004; Leone et al., 2007).

Absorption bands (cm <sup>-1</sup> )	Vibrational modes
950-1150	Asymmetric and symmetric stretching of PO <sub>2</sub> <sup>-</sup>
1153	C-O stretching in C-OH group
900-1200	C-O-C and C-O of various polysaccharides
1215	Interaction of O-H bending and C-O stretching in C-O-H group, C-OH stretching vibration of carboxylic acid groups <sup>11</sup> , stretching of P=O bond in phosphate
1220-1330	Amide III band components of proteins <sup>1</sup> : 1220-1250cm <sup>-1</sup> β sheets, 1250-1270 cm <sup>-1</sup> random coils, 1270-1295 cm <sup>-1</sup> β turns, 1295-1330 cm <sup>-1</sup> α helix
1240	PO <sub>2</sub> <sup>-</sup> stretching of phosphodiesteres, C-O stretching in carboxylic acid
1372	Interaction of O-H bending and C-O stretching in C-O-H group, CH <sub>3</sub> symmetric bending
1396	Symmetric stretching of COO <sup>-</sup>
1407	=C-H in-plane bending
1418	C-O-H in-plane bending, carboxylic acids
1454	CH <sub>2</sub> scissoring
1566	C=C stretching in alkenes
1548	Amide II band
1637	Amide I of β-pleated sheet structure
1743	C=O stretching of esters

Table 6.1: Absorption bands of functional groups recognizable in the spectra of *Lactobacillus plantarum* before and after the addition of cholesterol.

Preliminary measurements were performed on the five considered strains of *L. plantarum* (Lp752, Lp885 Lp995, Lp997 and ATCC14917<sup>T</sup>), cultivated in absence and in presence of cholesterol. MIR spectra, acquired on the pellets obtained by centrifugation and subtracted by the contribution of the matrix, showed an interesting change in the signals in the range from 1180 to 1300 cm<sup>-1</sup> whose entity depended on the strain characteristics. In particular, a shift of the signal centred at 1240 cm<sup>-1</sup> towards 1217 cm<sup>-1</sup> was observed.

The formation of H bond between phosphate group and the hydroxyl group of cholesterol determined this shift. It is well known, in fact, that the formation of hydrogen bonds produces a weakening of all the bonds involved and a shift of absorption bands towards lower energy.

As shown in Figure 6.1, and more in detail in Figure 6.2, this effect was very low for Lp995, Lp997 and ATCC14917 and more evident for Lp752 and Lp885 strains. This result was in agreement with our results, which demonstrates an increased capacity of these last strains to remove cholesterol (Paladino, 2010) It is also interesting to note that the initial shape of the bands differed for different strains: it can be related to a different cell wall composition that could explain their own capacity to remove cholesterol from the culture broth. Many more evident variations were visible in the range from 900 to 1150 cm<sup>-1</sup>: further measurements, performed on washed cells of Lp885 seemed to demonstrate that these changes, disappearing after washing, were due to an interference of the matrix (data not shown). It was, instead, confirmed the variations observed in the range from 1180 to 1300 cm<sup>-1</sup>.

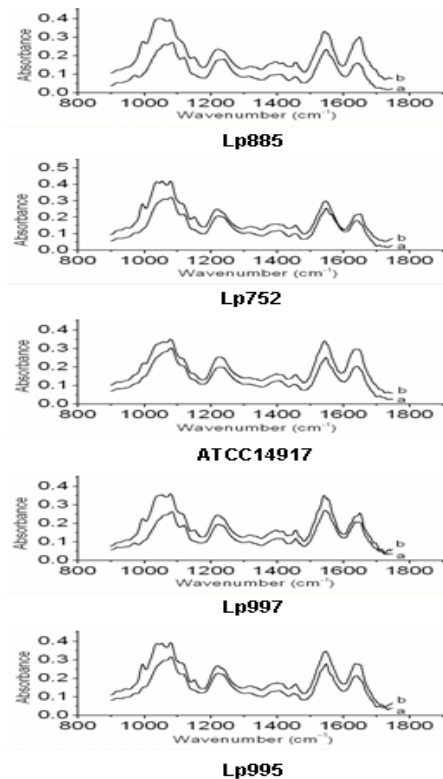


Figure 6.1. MIR spectra of Lp995, Lp997, ATCC14917, Lp752, Lp885 strains cultivated in absence (a) and in presence (b) of cholesterol (the spectra were shifted on the absorbance axis to facilitate the lecture).

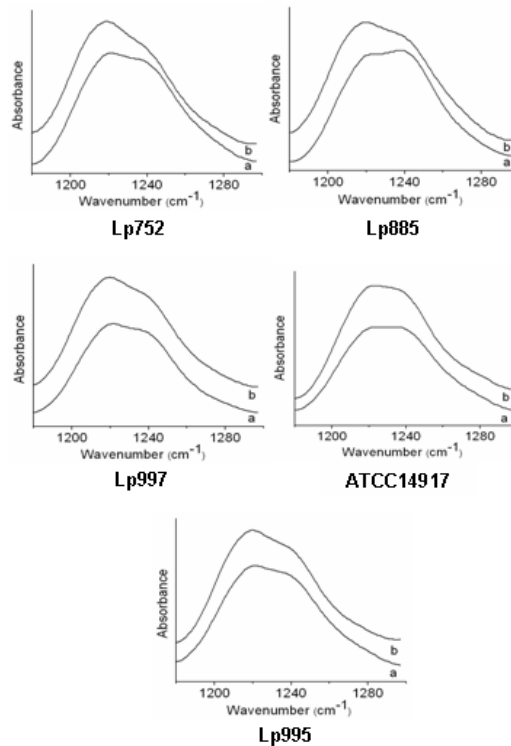


Figure 6.2. Detail of MIR spectra in the range from 1180 to 1300 cm<sup>-1</sup> of Lp995, Lp997, ATCC14917, Lp752, Lp885 strains cultivated in absence (a) and in presence (b) of cholesterol (the spectra were shifted on the absorbance axis to facilitate the lecture).

The supernatants obtained after centrifugation of three strains (Lp885, Lp995, ATCC14917) cultivated in absence and in presence of cholesterol were analyzed by NIR spectroscopy. Preliminary data, processed by PCA, showed the possibility to separate the supernatants obtained both in absence (supernatant a) (Figure 6.3) and in presence (supernatant b) of cholesterol (Figure 6.4), highlighting differences in metabolism and in capacity to remove cholesterol among strains.

The difference between strains in Figures 3 and 4 was observed along the PC1 (97% of total variance explained) for ATCC14917 and for the samples sets Lp885 and Lp995; separation for Lp885 and Lp995 was essentially obtained along the PC2 (3% of total variance explained).

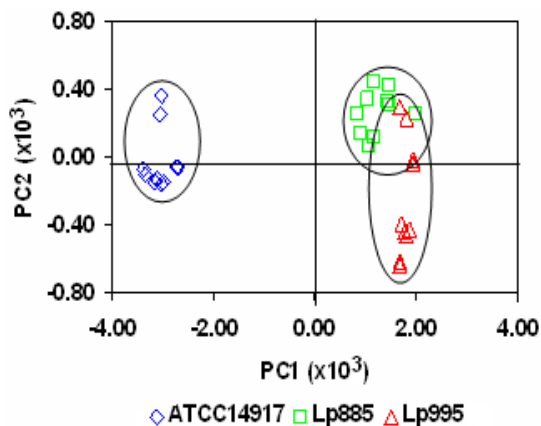


Figure 6.3. Scores plot obtained from the PCA analysis performed on 30 samples (10 of ATCC14917, 10 of Lp885 and 10 of Lp995) of supernatant a.

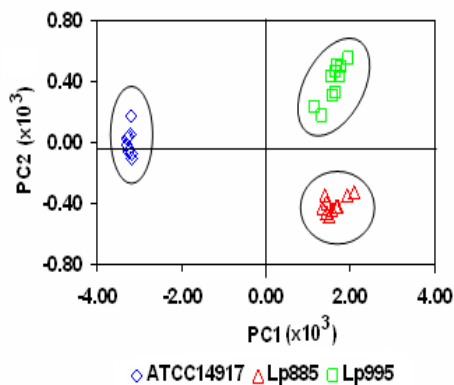


Figure 6.4. Scores plot obtained from the PCA analysis performed on 30 samples (10 of ATCC14917, 10 of Lp885 and 10 of Lp995) of supernatant b.

The score plot obtained by the PCA analysis performed on 20 samples of Lp885 supernatant in absence (a) and in presence (b) of cholesterol is shown in Figure 6.5. The difference between the two sets of samples was obtained along the PC1 that was able to explain 97% of the total variance. A more detailed study is in progress to verify the feasibility of NIR spectroscopy for a rapid determination of not removed cholesterol in supernatants.

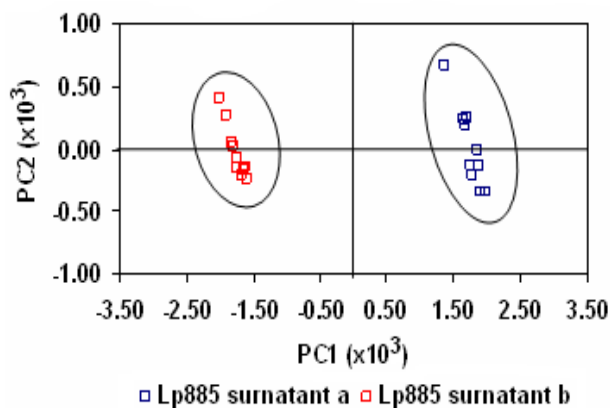


Figure 6.5. Scores plot obtained from the PCA analysis performed on 20 samples of Lp885 supernatant (10 of supernatant a and 10 of supernatant b).

## 6.4 CONCLUSIONS

Preliminary results obtained supported the applicability of NIR and MIR spectroscopy to study the removal of cholesterol by some strains of LAB and suggested that the mechanism that involves cholesterol should be related to an interaction of this molecule with cell walls by hydrogen bonds. Further studies will be addressed to confirm these observations and to develop a fast method, based on NIR spectroscopy, for the determination of the cholesterol.

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7. NEAR INFRARED SPECTROSCOPY AND  
AQUAPHOTOMICS AS TOOLS FOR BACTERIAL  
CLASSIFICATION

## 7.1 INTRODUCTION

Lactic acid bacteria (LAB) are Generally Recognized as Safe (GRAS) microorganisms typically associated with human gastrointestinal tract (GIT) and foods. Certain LAB species from dairy foods are known for their probiotic effects during GIT passage (Georgieva et al., 2008).

Probiotics are live bacteria containing foods, and, upon digestion, they exert beneficial effects to host. The beneficial actions of probiotic bacteria provide aid in lactose digestion, resistance to enteric pathogens, anti-colon cancer effect, small bowel bacterial overgrowth, allergy, and immune system modulation. Microorganisms used in probiotic products generally contain lactobacilli and bifidobacteria.

Within the genus *Lactobacillus*, *Lactobacillus plantarum* is studied for its probiotic characteristics. It is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations (Zago et al., 2011). It is one of the main non-starter LAB species contributing to the final sensorial properties of different kind of cheeses. Strains of *L. plantarum* have proven ability to survive gastric transit and colonize the intestinal tract of humans and other mammals (Georgieva et al., 2009). Various special therapeutic or prophylactic properties have been associated with *L. plantarum*, such as reduced incidence of diarrhea in daycare centers, reduced pain and constipation associated with irritable bowel syndrome, reduced bloating, flatulence, ability to displace enteropathogens from Caco-2 cells, and capacity to exert positive effect on immunity in HIV<sup>+</sup> children (Parvez et al., 2006).

The identification of microorganisms and the study of their probiotic features are based on genotypic and phenotypic tests which give results able to discriminate the strains. These methods are often time consuming and laborious, involve numerous reagents, and may have difficulty in the distinction of closely related organisms. Molecular DNA analysis methods provide a more reliable differentiation of microorganisms at the species level; however, these methods are also reagent- and time-intensive so their applications in industrial settings are not yet widespread (Bhatta et al., 2006). An optics-based method of Fourier transform infrared spectroscopy has been proposed as an alternative in clinical and food industry applications, with reasonable success; in particular there has been a growing interest in the use of near-infrared (NIR) technology as an analytical tool for agricultural and food investigations.

This technique has been shown to be a useful tool for the characterisation of bacteria, fungi, metabolic footprinting and fingerprinting, as well as a number of food-based analyses.

Many authors reported how NIR is able to detect and identify bacterial contamination in liquids studying the overtones and combination bands of fundamental organic molecules.

Within the food industry, at present, no technology exists for a rapid and accurate detection system for microbiologically spoiled or contaminated food. Ellis et al. (2004) developed a method for spoilage detection in beef evaluating the observation of molecules that are excited by an infrared beam, where an infrared absorbance spectrum represents a “fingerprint” characteristic of any (bio)chemical substance.

Lin et al. (2004) evaluated the feasibility of this technique to quantify the microbial loads in chicken meat and to develop a rapid methodology for monitoring the onset of spoilage.

In spectroscopy, water has been considered an obstacle to analytical process of other molecules in biological systems. However, latest reports have begun to acknowledge the importance of studying the behavior of water when exploring biological and aqueous systems. Yet, the development of methodologies for understanding the biological significance of water in living systems is exceedingly difficult. Recently, a new concept, which describes the dynamic spectroscopy of biological and aqueous systems based on the behavior of water called “Aquaphotomics”, has been proposed. Aquaphotomics describes the spectroscopic ways and means of tracing changes of water molecular vibrations in relation to other molecular vibrations

present in a biological specimen, which helps to understand the functions of the whole system (Jinendra et al., 2010).

Therefore the objectives of this study were to evaluate the feasibility of FT-NIR applications in microbiology and to develop methodology for the rapid identification of bacterial strains by combining FT-NIR spectroscopy, Aquaphotomics with multivariate statistical methods.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Strain selection

A total of 42 strains were used in this study. Thirty-five strains of *Lactobacillus plantarum*, belonging to the collection of the CRA-FLC (Fodder and Dairy Productions Research Centre, Lodi), were isolated from different matrixes (from dairy products, from olive brines and from grape marcs); 6 strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1 strain of *Lactobacillus gasseri* isolated from probiotic Japanese yoghurt.

Strains were kept as frozen stocks at -80 °C in MRS broth (Merck, Darmstadt, Germany) added with 20% (v/v) glycerol. Frozen stock cultures were thawed and subcultured twice (2% inoculum) in MRS at 30 °C overnight for *L. plantarum* and at 42°C for *L. delbrueckii* subsp. *bulgaricus* and *L. gasseri* before use.

### 7.2.2 Molecular typing

Bacterial DNA was extracted using a Chelex<sup>®</sup> 100 method described by Walsh et al. 1991. Two-hundred µl of culture were washed twice with 500 µl di TE<sub>0.1</sub> pH 8.0 (TE<sub>0.1</sub>: Tris-HCl 10 mM, EDTA 0.1 mM) and the pellet was harvested and resuspended in 245 µl of the same buffer and 5 µl of lysozyme (stock solution 50 mg/ml). After that, the strains were incubated at 56°C for 45 min.

To each eppendorf were added 218.7 µl of TE<sub>0.1</sub> pH 8.0, 5 µl of DTT 1 M, 10 µl of EDTA 0.5 M pH 8.0, 12.5 µl of 20% SDS and finally 3.8 µl of proteinase K (stock solution 20 mg/ml). The strains were incubated for 1 hour at 37°C and then 500 µl of a 5% Chelex<sup>®</sup> 100 (Sigma – Aldrich) solution were added. This ionic resin allows the DNA purification by precipitation of all impurities generated by bacterial lysis. The extracted DNA was diluted 1:10 and used as a template for PCR reactions.

RAPD-PCR was performed using M13 primer (5' – GAGGGTGGCGGTTCT – 3') as described by Rossetti and Giraffa (2005). Amplification reactions were carried out in 25 µl volume containing 2 mM of M13 primer, 2.5 units/100 µl of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 3 mM MgCl<sub>2</sub>, 2.5 ng/µl of total DNA, and 0.2 mM of each dNTP. PCR amplifications were performed in a Perkin–Elmer thermal cycler (model 9700 Applied Biosystem). The cycling program consisted of an initial denaturation step at 94 °C for 120 s and then 40 cycles of: 94 °C for 60 s, 45 °C for 20 s, and 72 °C for 120 s. The final elongation was performed at 72 °C for 10 min.

PCR products were analyzed by electrophoresis through 1.5% agarose gels at 26 V for 12 h in 1 × Tris-acetate EDTA (TAE) buffer (1 × TAE: 40 mmol/l Tris acetate, 1 mmol/l EDTA, pH 8.0) stained with ethidium bromide, and visualized under UV light.

The fingerprinting profiles were processed using *Bionumerics* software (5.1 Applied Maths, Belgium). Similarity among different profiles was evaluated by applying the Pearson's correlation coefficient.

### 7.2.3 Spectroscopical typing

Cell cultures, obtained as previously described, were centrifuged (7000 rpm x 15 min) and resuspended in the same volume of water to eliminate the interferences due to the presence and to the complexity of the composition of the culture broth in which bacteria were grown. The



cultures were subsequently analyzed directly or diluted prior to analysis.

The spectra of the bacterial cultures were collected at room temperature in triplicate with a NIR system Fantec (Fantec, Japan) in transmittance mode using test tubes with an optical path of 0.5 cm. Each spectrum collected is the result of the average of 32 scans in the spectral range between 600 and 1100 nm.

Before analyzing the data using Pirouette software ver. 3.11 (Infometrix, USA), the spectra were averaged.

Qualitative analysis was performed by applying SIMCA algorithm (soft independent modeling of class analogy) to determine and highlight the differences between the spectra of each sample analyzed. The spectra collected were divided into two data sets of equal size one of which was used for the validation of the model. The SIMCA method is based on the analysis of principal component analysis (PCA) applied to classify objects into groups. It consists of a set of models PCA one for each group present in the data set. The unknown samples are assigned to classes based on the minimum distance (Mahalanobis) PCA models previously calculated.

## 7.3 RESULTS AND DISCUSSION

### 7.3.1 Molecular typing

The genotyping of strains allowed to highlight a good level of intraspecific heterogeneity among tested strains, as shown in Figure 7.1, often linked to the matrix of isolation [6].

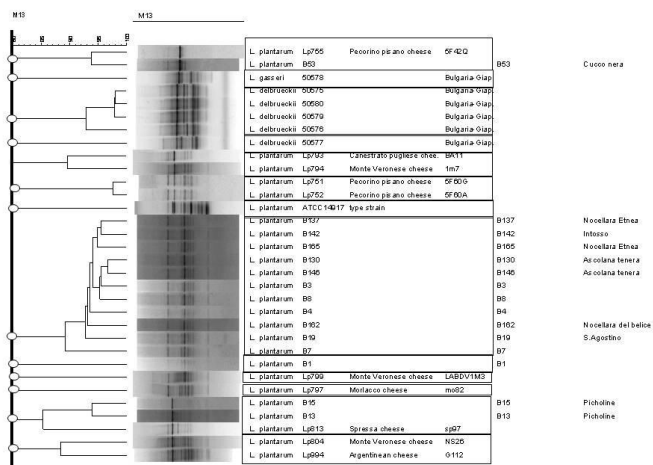


Figure 7.1: Dendrogram generated by tested strains

This biodiversity results in a numerical difference between species, highlighting the different capacity to adapt to the conditions imposed during the transformation process.

Currently, it is increasingly important to distinguish and then identify quickly and accurately, even microbial strains belonging to the same species, but with different technological properties.

Analyzing the profiles for the 41 studied strains, 13 cluster corresponding to a similar number of different biotypes, were obtained (Figure 7.1).

The individual clusters contain from 2 to 11 strains although it is possible to notice some cluster consisting of a single strain.

Most biotypes includes strains having different origins while a limited number of groups contain strains having the same origin: for instance in the cluster 8 all 11 strains that make it up have been isolated from brine for olives.

### 7.3.2 Spectroscopical typing

Preliminary tests have concerned the development of a method of monitoring the growth of strains culture broth during a complete cycle of growth. In Figure 7.2 is shown the result of the SIMCA analysis in which it is evident that with this method it was possible to reproduce the typical microbial growth trend.

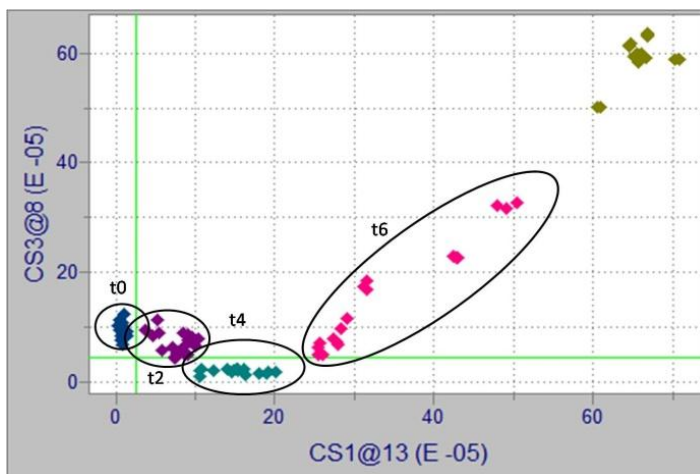


Figure 7.2: Monitoring of microbial growth in culture broth.

The Interclass Distances values (Table 7.1) have corroborated the robustness of the model because all values are greater than 3. According to Morita et al. (2011), the interclass distance of less than 0.8 indicates a small difference between two groups while the distance of more than 3 supports a reliable separation. Therefore, this separation is considered robust.

Tempo	0	2	4	6
0	0	3.40324	7.224778	37.94837
2	3.40324	0	4.56802	18.63801
4	7.224778	4.56802	0	10.33202
6	37.94837	18.63801	10.33202	0

Table 7.1: Interclass distance values related to Figure 7.2

In order to investigate the differences among each strain samples and reflect them in the regression analysis SIMCA was performed for NIR spectral data as qualitative analysis. The bacterial cultures were diluted 1:10 in sterile water to avoid interference due to the culture broth, to highlight how the characteristics of aqueous systems in which the microorganisms are dispersed can be used as fingerprinting of the culture.

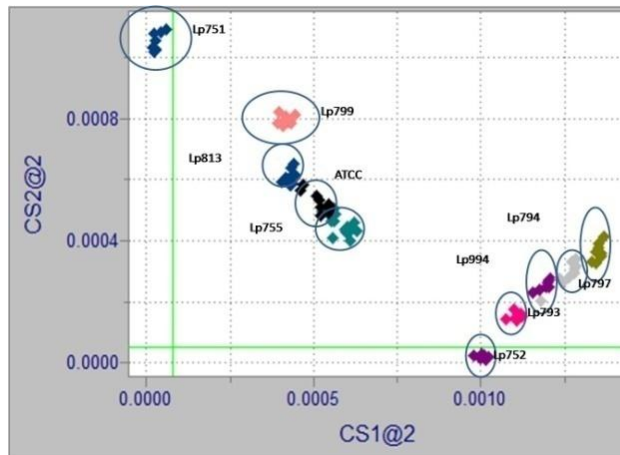


Figure 7.3: Strain classification by SIMCA

Figure 7.3 where it is shown the result of the application of SIMCA model on the validation set, demonstrates how this non-destructive technique is able to separate strains considered part of the same biotype. Comparing this result with that obtained by genotyping the couples of strains Lp751 and Lp752, Lp793 and Lp794 Lp804 and Lp994 that genotypic level are considered to be part of the same cluster, are separate and well differentiated. The separation is corroborated also by the values of interclass Distances greater than 3: 16.26 for the pair Lp751/Lp752, 13.23 for the pair Lp793/Lp794 and 13.15 for the pair Lp804/Lp994 respectively.

Moreover, the predictive power of the model has led to correctly classify the 95.1% of the samples (39/41), indicating that the differences between the bacterial strains can be detected also by the spectral differences related to them.

To identify what are the wavelengths responsible for this classification, an Aquagram has been done (Figure 7.4), where are reported the absorptions characteristic of each strain at different wavelengths.

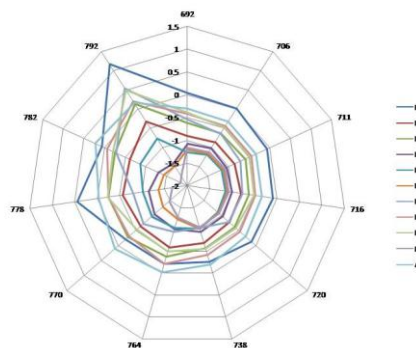


Figure 7.4: Aquagram related to studied strains

The graph shows that the spectrum can be used to describe the differences between the strains. The major differences in intensity of absorption characteristics of the strains studied, it can be noted at the wavelengths 778 and 792 nm.

## 7.4 CONCLUSIONS

The analysis of the spectral data showed that the NIR spectroscopy is able to monitor the bacterial growth in real time without having to resort to the validation of the method with reference analysis.

For the first time, phenotypic and genotypic difference among strains were successfully extracted and visualized by quantitative analysis based only on NIR spectral data of diluted supernatant samples.

In particular the application of the algorithm SIMCA allowed to highlight differences among the strains which were not detected by the methods of genotypic characterization, based on the amplification of DNA.

These results prove that NIR spectroscopy is an effective and high accurate screening method, suitable as high throughput technology for decision making at an industrial level for the selection of strains of technological interest.

## 7.5 REFERENCES

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