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Polyphasic characterization of exopolysaccharides produced by *Lactobacillus plantarum* Lp90 strain

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ABSTRACT

Introduction

Lactic acid bacteria (LAB) occur in a variety range of fermented foods. *Lactobacillus plantarum* is a widespread LAB species which is encountered in diverse niches and some *L. plantarum* strains have been recognized as probiotics.

Several LAB are able to secrete exopolysaccharides (EPS), which can be either highly adherent or loosely bound to the microbial cell surface, thus distinguished into capsular and secreted forms, respectively; they are thought to provide protection against adverse environment.

The ability to produce EPS by LAB has been reported to be strictly correlated to the presence of specific *eps*/*cps* gene clusters.

EPS and EPS-producing LAB have been investigated in relation to their application in food industry and in bacteria-host interaction. Moreover, the prebiotic and pro-technologicals functions of exopolysaccharides produced by LAB are topics of growing interest.

Aims of the research

In this study, a polyphasic characterization of exopolysaccharides (EPS) produced by a *Lactobacillus plantarum* strain, named Lp90, was performed. The strain was previously isolated from wine and selected for a typical ropy phenotype.

Materials and methods

EPS produced by *L. plantarum* Lp90 were purified and quantified by phenol-sulfuric acid method. Furthermore, chemical characterization was performed by gas-liquid chromatography (GLC).

The genome of *L. plantarum* Lp90 was sequenced using the Illumina GAIIx platform and annotated by RAST (Rapid Annotation using Subsystem Technology) server, allowing a comparative genome analysis with *L. plantarum* strains already sequenced.

Knockout of genes responsible for the ropy phenotype was performed and *L. plantarum* Lp90 non-ropy mutant strains obtained.

Host-lactobacilli (EPS producing) interaction was performed in order to understand the probiotic potential of *L. plantarum* Lp90 and the possible prebiotic actions of exopolysaccharides produced by this strain. Bacterial survival during the simulation of the gastro-intestinal tract was assayed. The ability of *L. plantarum* strain Lp90 to adhere and compete for adhesion sites with *E. coli* O157: H7 on Caco-2 cells, and the colonization of *L. plantarum* strain Lp90 fluorescently labeled on enterocytic cells of zebrafish larvae, was performed. The potential immune-modulation effects of Lp90 on Caco-2 cells as well as on macrophage-differentiated THP-1 cells with digested yogurt containing this bacterial strain were also evaluated. Moreover, the affinity with abiotic surfaces was observed by the biofilms formation on glass tubes.

The potential role of exopolysaccharides produced by *L. plantarum* Lp90 in relation to its original habitat (wine) was analysed during microvinification assays and in presence of typical wine stresses, such as ethanol, pH and sulfur dioxide.

Results

Transmission Electron Microscopy (TEM) images clearly showed the presence of exopolysaccharides around the cell wall of *Lactobacillus plantarum* Lp90. Moreover, the chemical analysis suggested that they are hetero-polysaccharides, composed by rhamnose, glucose, galactose, glucosamine and galactosamine.

L. plantarum Lp90 genome is about 3,324,076 bps long with a total of 3,273 predicted genes. Four different *cps/eps* gene clusters involved in exopolysaccharides biosynthesis were identified; in particular the *cps2* gene cluster presented three glycosyltransferase genes apparently unique in Lp90 but homologous to *Lactobacillus fabifermentans* T30PCM01.

Following the entire or partial *cps*2 cluster deletion, we obtained two non-ropy mutant strains, (Lp90Δcps2 and Lp90Δcps2.5 respectively), thus suggesting that ropy phenotype of *L. plantarum* Lp90 is inherent to the cluster *cps2*.

EPS produced by *L. plantarum* Lp90 do not seem to promote *in vitro* and *in vivo* bacterial adhesion on intestinal epithelium, as well as the immune-modulation after the interaction of Caco-2 cells, while their inhibitory effect on *E. coli* adhesion on Caco-2 was observed. Furthemore, *L. plantarum* Lp90 showed a moderate survival during *in vitro* models of the gastro-intestinal tract, which is an added value for this strain considering its origin habitat.

Exopolysaccharides produced by *L. plantarum* strain Lp90 mask the ability of this strain to form biofilm on glass surface.

Exopolysaccharides produced by *L. plantarum* strain Lp90 confer increased tolerance to certain stressful conditions (ethanol, low pH, sulfur dioxide, lysozyme) usually encountered during winemaking.

Finally, preliminary analysis of yogurt produced with *L. plantarum* strain Lp90, showed a positive technological features and immune-modulation of cytokine-mediating genes.

Keywords: exopolysaccharides, ropy phenotype, *Lactobacillus plantarum,* probiotic, prebiotic.

SOMMARIO

Introduzione

I batteri lattici sono presenti in un'ampia gamma di alimenti fermentati. *Lactobacillus plantarum* è una specie diffusa di batteri lattici, riscontrabile in diverse nicchie e alcuni di essi sono stati riconosciuti come probiotici.

Diversi batteri lattici sono in grado di secernere esopolisaccaridi (EPS), che possono essere molto aderenti o debolmente legati alla superficie della cellula microbica, rispettivamente classificati come capsulari o dispersi; inoltre si ritiene che essi forniscano protezione contro ambienti avversi.

È stato dimostrato che la capacità dei batteri lattici di produrre EPS è strettamente correlata a specifici "cluster" genetici (*eps/cps*).

Gli esopolisaccaridi e i batteri lattici che li producono sono stati studiati per le loro applicazioni nel settore alimentare e nell'interazione con l'organismo ospite. Inoltre, le funzioni prebiotiche e protecnologiche degli esopolisaccaridi prodotti dai batteri lattici sono argomenti di crescente interesse.

Obiettivi della ricerca

In questo studio, è stata effettuata una caratterizzazione polifasica di esopolisaccaridi (EPS) prodotti da un ceppo di *Lactobacillus plantarum,* nominato Lp90. In precedenza, questo ceppo è stato isolato da vino e selezionato per il suo tipico fenotipo "ropy" (viscoso).

Materiali e metodi

Gli esopolisaccaridi prodotti da *L. plantarum* Lp90 sono stati purificati e quantificati con il metodo felono-acido solforico. Inoltre, la caratterizzazione chimica è stata eseguita mediante cromatografia gas-liquido (GLC).

Il genoma di *L. plantarum* Lp90 è stato sequenziato mediante "Illumina GAIIx platform" e annotato utilizzando RAST (Rapid Annotation using Subsystem Technology) server, permettendo così l'analisi comparativa con il genoma di altri *L. plantarum*.

È stata effettuata la delezione dei geni responsabili del fenotipo "ropy", al fine di ottenere ceppi mutanti di *L. plantarum* Lp90 cosiddetti "non-ropy".

Sono stati compiuti alcuni esperimenti sull'interazione organismo ospite-lattobacilli (produttore di EPS) al fine di comprendere le potenzialità probiotiche di *L. plantarum* Lp90 e le possibili azioni prebiotiche degli esopolisaccaridi prodotti da questo ceppo. È stata inoltra analizzata la sopravvivenza batterica mediante un sistema che simula il tratto gastro-intestinale. Inoltre, è stata studiata la capacità di Lp90 di aderire e competere per i siti di adesione con *E. coli* O157: H7 su cellule Caco-2 *in vitro*, come anche la colonizzazione *in vivo* di Lp90 marcato con una proteina fluorescente su enterociti di larve di "zebrafish". Sono stati valutati i potenziali effetti immuno-

modulatori di Lp90 su cellule Caco-2, nonché sui macrofagi THP-1 differenziati in seguito all'esposizione con yogurt digerito contenente questo ceppo batterico. È stata analizzata l'affinità con le superfici abiotiche mediante formazione di biofilm su tubi di vetro.

Sono state effettuate analisi per comprendere il ruolo degli esopolisaccaridi prodotti da *L. plantarum* Lp90 in relazione al suo habitat di origine (vino). A tal proposito, sono stati eseguiti esprimenti di microvinificazione e studi sulla resistenza a diversi stress tipici dell'ambiente vino (etanolo, pH bassi, anidride solforosa, lisozima).

Risultati

Le immagini ottenute dalla microscopia elettronica in trasmissione hanno mostrato chiaramente la presenza di esopolisaccaridi intorno alla parete cellulare di *Lactobacillus plantarum* Lp90. Inoltre, le analisi chimiche hanno suggerito che sono etero-polisaccaridi, composti da ramnosio, glucosio, galattosio, glucosamina e galattosamina.

Il genoma di *L. plantarum* Lp90 ha una lunghezza di 3.324.076 bps con un totale di 3.273 geni predetti. Sono stati identificati quattro diversi "cluster" di geni *cps/eps* coinvolti nella biosintesi esopolisaccaridi; in particolare il cluster *cps2* ha presentato tre glicosiltransferasi apparentemente uniche in Lp90 ma omologhe in *Lactobacillus fabifermentans* T30PCM01.

In seguito alla delezione intera o parziale del cluster *cps2*, sono stati ottenuti due ceppi mutanti "non-ropy" (rispettivamente Lp90Δcps2 e Lp90Δcps2.5), suggerendo così che il fenotipo "ropy" di *L. plantarum* Lp90 è inerente al "cluster" *cps2*.

Gli esopolisaccaridi prodotti da *L. plantarum* Lp90 non sembrano promuovere l'adesione batterica *in vitro* o *in vivo* sull'epitelio intestinale, così come l'immuno-modulazione dopo l'interazione con cellule Caco-2, invece è stato osservato un loro effetto inibitorio sull'adesione di *E. coli* su cellule Caco-2. Inoltre, *L. plantarum* Lp90 ha mostrato una modesta sopravvivenza durante i modelli *in vitro* del tratto gastro-intestinale, il che rappresenta un valore aggiunto per questo ceppo considerando il suo habitat originale.

Gli esopolisaccaridi di Lp90 mascherano l'abilità di questo ceppo nella formazione di biofilm su superficie di vetro.

Gli EPS prodotti da *L. plantarum* Lp90 conferiscono una maggiore resistenza a determinate condizioni di stress (etanolo, pH basso, anidride solforosa, lisozima) tipiche del processo di vinificazione.

Infine, le analisi preliminari sullo yogurt prodotto con *L. plantarum* Lp90, hanno mostrato risultati positivi sulle proprietà tecnologiche e sull'immuno-modulazione di geni coinvolti nella mediazione di citochine.

Parole chiave: esopolisaccaridi, fenotipo "ropy", *Lactobacillus plantarum,* probiotico, prebiotico.

1. INTRODUCTION

1.1 Probiotics

1.1.1 The origins of probiotis

The first scientist who realized a positive function played by bacteria that colonize the human body, was the Nobel laureate Eli Metchnikoff. Considering that the aging process is caused by toxic substances such as phenols, indoles and ammonia produced by proteolytic microbes in the large intestine, he suggested the possibility to replace the harmful bacteria with beneficial ones. He also observed that some rural peoples in Europe, who used to drink milk fermented, had a relatively long life, and that milk fermented by lactic acid bacteria (LAB), inhibited the growth of proteolytic bacteria due to the low pH value. Subsequently, Metchnikoff introduced the use of fermented sour milk, using a bacterial species that he later called '*Bulgarian bacillus*' (Vaughan, 1965). Tissier (1900) first isolated a *Bifidobacterium* from a breast-fed infant, at first called *Bacillus bifidus communi* and later renamed *Bifidobacterium bifidum*. He concluded that this species was predominant in the microflora of breast-fed infants and recommended it for feeding babies suffering from diarrhea (Tissier, 1900).

In 1917, professor Alfred Nissle isolated the bacterium *Escherichia coli* from the feces of a World War I soldier who did not develop enterocolitis during a severe outbreak of shigellosis. He successfully used this strain to treat intestinal diseases such as shigellosis and salmonellosis (Nissle, 1918). At that time antibiotics were not discovered yet. The probiotic *E. coli* Nissle 1917 is still in use today and recent studies have demonstrated its direct interaction with the host adaptive immune system (Molin, 2001).

In 1920, professor Leo F. Rettger showed that '*Bulgarian Bacillus*', later known as *Lactobacillus delbruekii* subsp. *bulgaricus*, could not live in the human intestine. Therefore, at this time,

Metchinikoff's theory was disputed and the idea of fermented food died out (Cheplin and Rettger, 1920).

The word "probiotic" was coined from the Greek, "προ" plus "βιοτος" meaning literally "for life".

Probably the first author that used the term "probiotic" has been Kollath in 1953, describing it as organic and inorganic supplement necessary to restore health to patients suffering a form of malnutrition resulting from eating too much highly refined food (Kollath, 1953). In 1954 Vergin suggested that antibiotics can upset the microbial balance of the body, and that this can be restored by a proper diet of probiotics, including fermentation products (Vergin, 1954). Lilly and Stillwell in 1965 following their observations give a more limited use of this word, giving the name probiotics to 'growth promoting factors produced by microorganisms' (Lilly and Stillwell, 1965). While, in 1973 Fujii and Cook defined as "compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms *in vitro"*, referring to synthetic chemicals that protected mice against infection with *Staphylococcus aureus* (Fujii and Cook, 1973). Instead, Parker in 1974 seems to have been the first to use the word in relation to the interactions of micro-organisms with the whole animal or human host (Parker, 1974). Subsequently, Fuller in 1989 defined as probiotic 'a live microbial food supplement which beneficially affects the animal host by improving its intestinal microbial balance' (Fuller, 1989).

1.1.2 Probiotics nowadays

Currently, the probiotics are defined as 'live microorganisms which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002).

In order to be defined probiotics, microorganisms have to fulfill specific requisites. These characteristics include documented clinical efficacy, safety for human consumption, ability to reach, survive and colonize, at least transiently, the human gut, where probiotics exert their beneficial effects (Owehand *et al.,* 2002).

Deterrence and reversion of intestinal dysbioses, enhancement of immune defenses, prevention of food allergies and infections, reinforcement of the gut barrier are among the beneficial effects ascribed to probiotics (Deshpande *et al.,* 2011).

Specific probiotic strains are known to (i) normalize altered gut microecology and intestinal permeability; (ii) attenuate mucosal hypersensitivity and inflammatory reactions; (iii) stimulate non-specific host resistance to microbial pathogens and favour their eradication (Isolauri *et al.*, 2004).

The positive impact of *Lactobacillus plantarum* and others probiotic LAB is thought to be mediated by various mechanisms including enhancement of the epithelial barrier, increased adhesion to intestinal mucosa and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microbial substances and modulation of the immune system (Marco *et al.,* 2006; Bermudez-Brito *et al.,* 2012).

FAO/WHO developed Operating Standards establishing guidelines for all companies producing probiotic products (FAO/WHO, 2002; Reid, 2005).

These guidelines include:

- guidelines for the use of probiotics;
- phase I, II and III of clinical trials to prove health benefits;
- good manufacturing practice and production of high quality products;
- studies to identify mechanism of action *in vivo*;
- informative labelling;
- development of probiotic organisms that can deliver vaccines to hosts;
- expansion of proven strains to benefit the oral cavity, nasopharynx, respiratory tract, stomach, vagina, bladder and skin as well as for cancer, allergies and recovery from surgery or injury.

Resistance to the extreme conditions of the oro-gastrointestinal (OGI) tract, including highly acidic gastric juices and pancreatic bile salt secretions, is an essential criterion for the selection of orally delivered (food-borne) probiotics. The viability of probiotics is extremely important in

order to guarantee high bacterial loads into the main site of action (e.g., the intestine) and their optimal functionality.

All along the different OGI sections, bacteria are challenged also by the action of diverse digestive enzymes, including lysozyme (in the oral cavity); pepsin (stomach), pancreatin, chimotrypsin, and carboxypeptidases (intestine). These enzymes can remarkably compromise bacterial cell structures, by attacking and degrading surface-exposed macromolecules (Frenhani and Burini, 1999).

Bacterial cells are naturally equipped with various defence mechanisms to enhance survival in hostile environments (Van de Guchte *et al.,* 2002; Spano and Massa 2006; Fiocco *et al.,* 2007; Fiocco *et al.,* 2010). These include chaperone proteins, which assist the folding of misfolded proteins, proteases which degrade irreversibly damaged proteins, transport systems to maintain correct osmolarity, catalases and superoxide dismutases to tackle reactive oxygen species, as well as proton pumps, decarboxylases and transporters to counteract intracellular pH decreases (Sugimoto *et al.,* 2008).

1.2 Lactic acid bacteria

The denomination of "lactic acid bacteria" refers to bacteria involved in milk fermentation and capable to produce lactic acid from lactose. The family name Lactobacteriacea was applied by Orla-Jensen (1919). Today the main LAB genera include: *Lactobacillus*, *Leucocostoc*, *Pediococcus* and *Streptococcus* (Schroeter and Klaenhammer, 2009).

Lactic acid bacteria are heterogeneous group of Gram-positive, low-GC, acid-tolerant, generally asporigen, rod- or cocci-shaped, catalase-negative, microaerophilic bacteria. Being a gram-positive bacterium, the cell envelope is a multilayered structure, which is mainly composed of peptidoglycan with embedded teichoic acids, proteins, and polysaccharides and which is essential to for the cellular integrity and shape (Silhavy *et al.,* 2010). The common feature of

LAB is the production of lactic acid as the major metabolic end-product of carbohydrate fermentation (Carr *et al.,* 2002). Several species are inhabitants of the human oro-gastrointestinal (OGI) tract. They are naturally associated with the mucosal surfaces, particularly the Gastro-Intestinal (GI) tract, mouth and vagina of mammals, and are also indigenous to food-related habitats, including plants (fruits, vegetables, and cereal grains), wine, milk, and meat. LAB are important in food industry: they are used as microbial starters to drive several fermentation processes, contributing to determine texture, organoleptic properties, and shelf-life of the final products. Moreover, LAB commonly used in the formulation of functional probiotic foods (Bron and Kleerebezem, 2011) and specific LAB are marketed as health-promoting organisms or probiotics (FAO/WHO 2002).

1.2.1 Carbohydrates metabolism of lactic acid bacteria

The main pathways in lactic acid bacteria for the metabolism of glucose are:

- Glycolysis/Embden-Meyerhof-Parnas (EMP) pathway;
- 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (phosphoketolase- or pentose phosphate pathway) (Fugelsang and Edwards, 1997).

Before that glucose enters into one of these two pathways is transported into the cell where it is phosphorylated by hexokinase, an ATPdependant reaction.

The final products of EMP pathway are lactic acid and CO_2. This pathway is also known as homolactic fermentation in LAB and it is divided into two steps: glycolysis, whereby pyruvate is produced from glucose, followed by the conversion of pyruvate to produce lactic acid (Ribéreau-Gayon *et al.,* 2006).

The final products of 6-PG/PK pathway, also known as heterolactic fermentation, consist in lactic acid, CO_2, ethanol and acetate. *Leuconostoc* some *Lactobacillus* species and *Oenococcus oeni*, use this pathway.

Other *Lactobacillus* species are facultative heterofermentors, including *L. plantarum* and *L. casei*. These LAB make use of the EMP pathway for hexose metabolism and the 6-PG/PK pathway for the metabolism of pentose sugars and other substrates.

Many LAB are able to ferment pentose sugars, which are phosphorylated, converted by epimerases or isomerases to phosphate derivatives ribulose-5-phosphate or xylulose-5-phosphate, subsequently they are metabolised via the bottom half of the 6-PG/PK pathway. The final products of pentoses metabolism are lactic acid, acetic acid and CO_2 (Lerm *et al.,* 2010).

1.2.2 Lactic acid bacteria in wine

Oenological lactic acid bacteria have a wide variability due to region, cultivar and vinification procedures. There is a successional growth of several species of LAB during vinification (Wibowo *et al.,* 1985; Fugelsang and Edwards, 1997; Lerm *et al.,* 2010). *Oenococcus oeni* is the main LAB species associated with MLF in wine. However, several species belong to *Pediococcus* and *Lactobacillus* genera occur during or after MLF is completed (Wibowo *et al.,* 1985; Powell *et al.,* 2006, Lerm *et al.,* 2010).

The LAB population in the grape must generally range from 10^3 to 10^4 CFU/mL after crushing and the start of alcoholic fermentation. The major species of LAB present at this stage include *Lactobacillus plantarum*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Pediococcus damnosus* and *O. oeni,* which largely decline at the end of alcoholic fermentation. This could be due to ethanol concentrations, high SO_2 concentrations, low pH, low temperatures, the nutritional status and competitive interactions with the yeast culture (Lerm *et al.,* 2010).

1.2.3 The malolactic fermentation (MLF)

Malolactic fermentation (MLF) is a secondary fermentation process that normally takes places after the alcoholic fermentation by yeasts. It is carried out by one or more species of lactic acid bacteria (Arthurs and Lloyd, 1999), including bacteria from the genera *Oenococcus*,

Lactobacillus, *Pediococcus* and *Leuconostoc,* among these, *O. oeni* is best adapted to the harsh wine environment, such as high alcohol, low pH and sulphur dioxide (SO_2) (Wibowo *et al.,* 1985; Spano and Massa 2006).

MLF plays an important role in the winemaking process as it contributes to the deacidification of wine, microbial stability and has an influence on the aroma profile, mainly in red wines (Lerm *et al.,* 2010).

The MLF reaction consists in the conversion of L-malic acid, which is a dicarboxylic acid, to L-lactic acid, a monocarboxylic acid, and the production of CO_2.

Lactic acid bacteria have three possible enzymatic pathways for the conversion of L-malic acid to L-lactic acid and CO_2 (Lerm *et al.,* 2010):

1. Direct conversion of malic acid to lactic acid via malate decarboxylase, also known as the malolactic enzyme (MLE).
2. Pathway employing the malic enzyme to convert L-malic acid to pyruvic acid, which is reduced by L-lactate dehydrogenase to lactic acid.
3. Reduction of malate by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and reduction to lactic acid.

The physiological function of the malate fermentation pathway is mainly involved to generate a proton motive force (PMF) as a means to acquire energy to drive essential cellular processes (Konings, 2002).

At the end of MLF, the remaining LAB can still metabolise residual sugar, which could result in spoilage including volatile acidity (Fugelsang and Edwards, 1997). For this reason, it is essential to check the potential impact of residual LAB populations.

1.2.4 *Lactobacillus plantarum*

The genus *Lactobacillus* which belong to the phylum *Firmicutes* includes a considerable number of different species with high degree of diversity (Stiles and Holzapfel, 1997). Among these,

Lactobacillus plantarum is a widespread LAB species which is found in diverse niches associated to food matrices, vegetables, soil, human body and it is among the most common lactobacilli occurring on the human oral and intestinal mucosa (Ahrne *et al.,* 1998; de Vries *et al.,* 2006; Siezen *et al.,* 2010), and it contributes to specific organoleptic and nutritional properties of the final product (Kleerebezem *et al.,* 2003). *L. plantarum* is a facultative heterofermentative organism closely related to *Lactobacillus paraplantarum, Lactobacillus pentosus* and *Lactobacillus fabifermentans* (Siezen and van Hylckama Vlieg, 2011). Cells are Gram positive, rod shaped, non-spore-forming and non-motile.

Lactobacillus plantarum produces both isomers (D and L) of lactic acid and it is used for the production and preservation of fermented foods obtained from different raw materials, in which it is either present as a contaminant or added as a starter to carry out fermentations. Recently, it has been considered as the next generation starter culture for malolactic fermentation in wine, because it is one of the most dominant species of lactobacilli occurring throughout the winemaking process (du Toit *et al.,* 2011; Capozzi *et al.,* 2012).

The genus *Lactobacillus* has GRAS (Generally Recognized as Safe) status (De Angelis and Gobbetti, 2004), due to its natural occurrence and long history of safe use in food production. Several *L. plantarum* strains have been investigated for healthy properties and have been recognized as probiotics. Several reports, including human clinical studies, document the potential beneficial effects of lactobacilli, including *L. plantarum* (de Vries *et al.,* 2006; van Baarlen *et al.,* 2013) and some *L. plantarum* strains can be found in a variety of marketed probiotic functional foods.

The *Lactobacillus plantarum* WCFS1, a single colony of *L. plantarum* NCIMB 8826 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK), isolated from human saliva, has been the first *L. plantarum* complete genome sequenced and annotated in 2003 (Kleerebezem *et al.,* 2003). In 2012 the complete genome of WCFS1 was sequenced and annotated again, and 116 nucleotide corrections were identified, improving the function prediction for nearly 1,200

proteins (Siezen *et al.,* 2012). *L. plantarum* WCFS1 has become one of the model strains in LAB research since the initial genome publication. Different bioinformatics tools have been used to predict the function of its genes, reconstruct metabolic pathways and gene regulatory networks, and compare its genome with genomes of other LAB. The genomic, phenotypic, and metabolic diversity of *L. plantarum* has been previously described. Moreover, *L. plantarum* has been employed as a model for LAB interactions with mammalian gut tissues in studies that provided insights into the microbial adaptation to that habitat and identified candidate probiotic genes (Siezen *et al.,* 2012).

The ecological flexibility of *L. plantarum* is confirmed by the observation that this species has one of the largest genomes (approximately 3.3 Mb) known among LAB.

The availability of such data has prompted the genetic and molecular dissection of this species, also in relation to its probiotic behavior.

1.2.5 *Lactobacillus plantarum* Lp90

Lactobacillus plantarum Lp90 is a strain previously isolated from fermented wine in a winery located nearby Foggia, Italy. This strain is characterized by a distinctive ropy phenotype (**Figure 1.1**), which was ascribed to its capacity to over-produce exopolysaccharides (EPS) (Caggianiello *et al.,* 2013). This strain was already analysed for different features (Spano *et al.,* 2004; Spano *et al.,* 2005; Fiocco *et al.,* 2007; Siezen *et al.,* 2010). The complete genome of this strain has been sequenced and it is the first *L. plantarum* genome coming from a strain of wine origin (Lamontanara *et al.,* 2015).

Property	**Term**
Current classification	Domain *Bacteria* Phylum *Firmicutes* Class *Bacilli* Order *Lactobacillales* Family *Lactobacillaceae* Genus *Lactobacillus* Species *Lactobacillus plantarum* subspecies *plantarum*
Gram stain	Positive
Cell shape	Rod
Motility	Non motile (flagella not present)
Sporulation	Non sporulating
Temperature range	Mesophilic (15-45 °C)
Optimum temperature	30 – 40 °C
Carbon source	Sugars (hexoses, pentoses)
Energy source	Various compounds (e.g., glucose, citrate)
Terminal electron receptor	Various compounds (e.g., pyruvate, citrate, malate)
Habitat	*Apulian wine*
Salinity	Growth is usually observed at 4% w/v NaCl and not at 6.5% w/v
Oxygen	Microaerophilic
Biotic relationship	Commensal
Pathogenicity	Non pathogenic
Geographic location	Foggia, Italy
Sample collection time	2003
Latitude Longitude	41.45 N15.533333 E
Depth	Surface
Altitude	76 m above sea level

Table 1.1 - Classification and general features of *Lactobacillus plantarum* Lp90 (*adapted from Lamontanara, et al., 2015*).

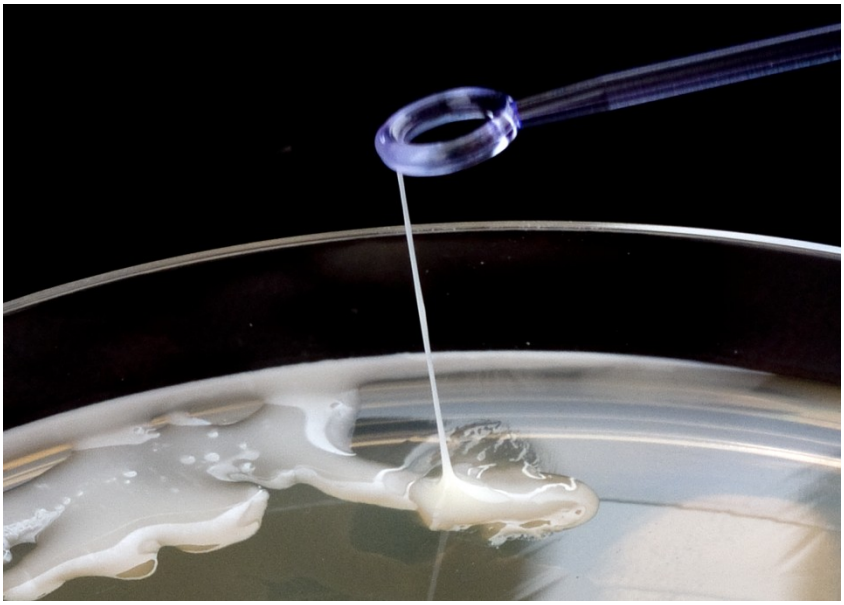

Figure 1.1 - Ropy phenotype of *Lactobacillus plantarum* Lp90 on MRS plate.

1.3 Prebiotics

A first definition of prebiotic was given by Gibson and Roberfroid (1995) as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". It has been proposed to refine the original definition to "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host wellbeing and health" (Gibson *et al.,* 2004).

Prebiotics can be incorporated into many foodstuffs and they act on the intestinal flora and improve the balance of the flora by enhancing the growth of beneficial intestinal bacteria and/or inhibiting the growth of harmful ones, resulting in scavenging in the intestinal environment. The candidate prebiotics include oligosaccharides, dietary fiber, resistant starch, (Mitsuoka; 2014).

The demonstration of a prebiotic effect must be carried out *in vivo* by validated methodologies to produce sound scientific data (Roberfroid *et al.,* 2007).

To determine whether a dietary carbohydrate could be considered a potential prebiotic, need to evaluate several factors: (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (Gibson *et al.,* 2004).

The first two ingredients that fulfill these criteria were inulin and trans-galactooligosaccharides (TOS).

Although still insufficient, promising data on prebiotic activity have been reported for glucooligosaccharides, isomaltooligosaccharides, lactosucrose, polydextrose, soybean oligosaccharides, and xylooligosaccharides. Moreover, there are still many substances for which are being evaluated the possible prebiotic effects, including the microbial exopolysaccharides.

1.4 Microbial exopolysaccharides (EPS)

1.4.1 Exopolysaccharides produced by LAB

The term "exopolysaccharides" (EPS) as proposed by Sutherland (1972) provides a general name for all forms of bacterial polysaccharides found outside the cell wall. Several LAB are able to secrete long-chains of homo- or hetero-polysaccharides, consisting of branched, repeating units of sugars or sugar derivatives (Ruas-Madiedo *et al.,* 2002). Such exopolysaccharides (EPS) can be either highly adherent or loosely bound to the microbial cell surface and are thus distinguished into capsular and secreted forms. EPS-producing LAB could be responsible for a ropy phenotype characterized by a viscous and texture observed in spoiled alcoholic beverages, such as wine especially with a pH > 3.8 (Coulon *et al.,* 2012). This phenomenon has been already observed by Pasteur in 1860. The ropy appearance in wines is due to the presence of exopolysaccharides produced by some lactic acid bacteria, such as *Pediococcus parvulus* found in Bordeaux wines (Dols-Lafargue and Lonvaud-Funel, 2009), but mainly by *Pediococcus*

damnosus. Moreover, it was found that some ropy strains are more tolerant to ethanol and SO_2 stress conditions (Lonvaud-Funel, 1999; Dols-Lafargue *et al.,* 2008).

The ability to produce EPS by LAB has been reported to be strictly correlated to the presence of specific gene clusters (*eps*/*cps*), located either on plasmids (Van Kranenburg, *et al.,* 1997) or on the main chromosome (Stingele *et al.,* 1996; De Vuyst *et al.,* 1999) (**Figure 1.2**).

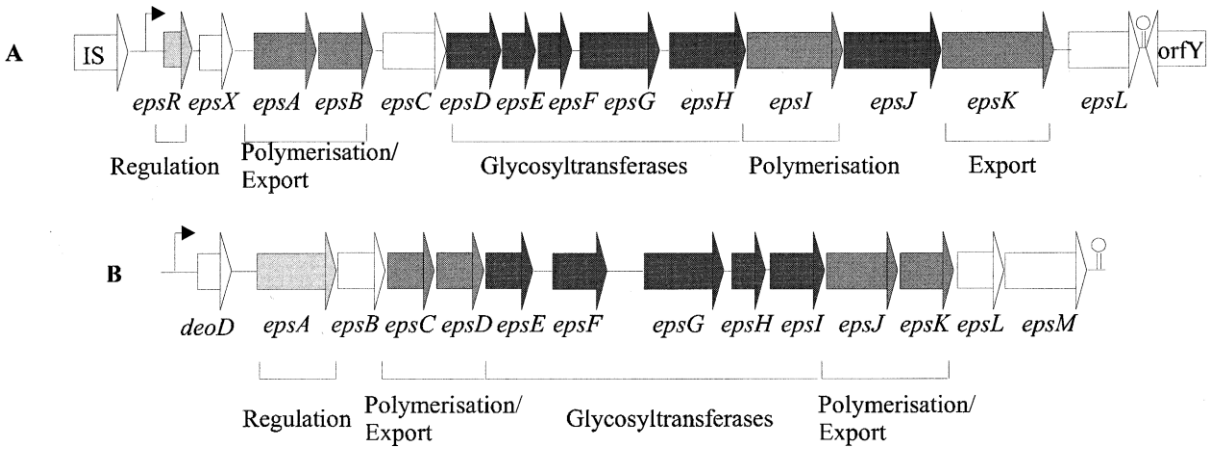

Figure 1.2 - Eps genes cluster organization (A) Eps gene cluster involved in the exopolysaccharides biosynthesis in *L. lactis* subsp. *cremoris* NIZO B40 (plasmid-localized) (Van Kranenburg, *et al.,* 1997); (B) *S. thermophilus* Sfi6 (chromosomally encoded) (Stingele *et al.,* 1996).

In the chromosomal genome of *L. plantarum* WCFS1, 4 *cps* genes clusters are associated with surface polysaccharide production (Remus *et al.,* 2012) (**Figure 1.3**). The *cps1, cps2, cps3* clusters are separated by transposase genes and fragments, encoding proteins involved in biosynthesis and export of extracellular or capsular polysaccharides (Siezen *et al.,* 2011).

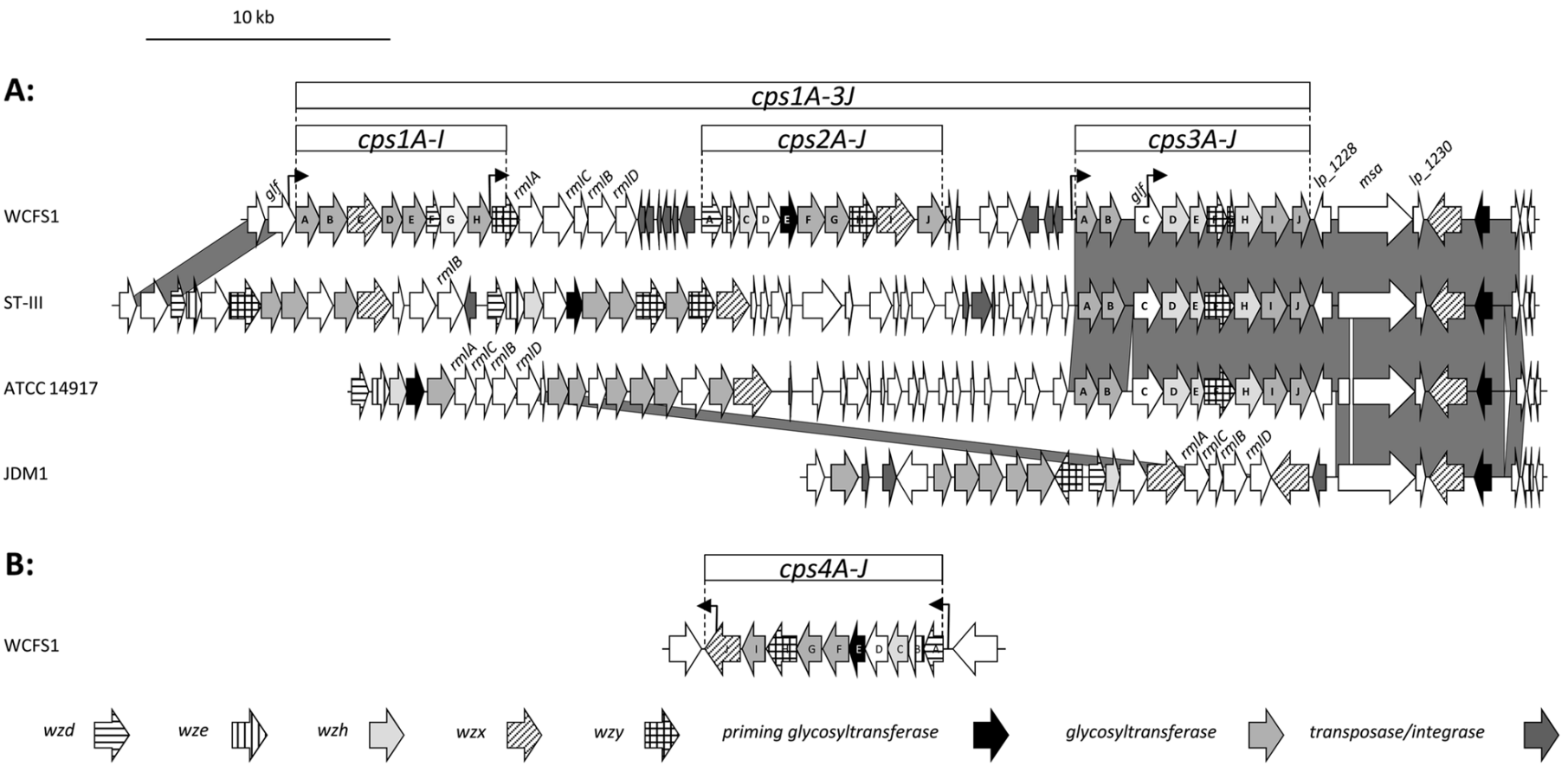

Figure 1.3 - Schematic representation of polysaccharide biosynthesis gene clusters in *L. plantarum* strains *(from Remus et al., 2012).*
(A) *Cps1, Cps2* and *Cps3* gene clusters of *Lactobacillus plantarum* WCFS1 involved in polysaccharide biosynthesis and comparison with the corresponding clusters of *L. plantarum* strains ST-III, JDM-1 and ATCC 14917. Dark-grey colored connecting blocks indicate regions of high sequence conservation between *L. plantarum* genomes. (B) *Cps4* cluster of *L. plantarum* WCFS1 involved in polysaccharide biosynthesis.

The *eps/cps* clusters exhibit a conserved modular organization and include genes encoding both regulatory factors and enzymes involved in EPS biosynthesis, polymerization and secretion, including glycosyl-transferases, which are responsible for the assembly of the characteristic EPS-repeating unit (De Vuyst *et al.,* 1999; Welman and Maddox, 2003; Lebeer *et al.,* 2009). Polymer length depends on a tyrosine kinase phosphoregulatory system, whose genes are located in the initial part of the cluster (**Figure 1.2**).

The repeating units are synthesized in the cytoplasm and assembled on the lipid carrier undecaprenyl phosphate by sequential transfer of monosaccharides from nucleotide sugars by specific glycosyltransferases (De Vuyst *et al.,* 1999).

The polymerization mechanism of the repeating unit and export from the cell in LAB, is not entirely known. A model has been proposed for *Lactococcus lactis* based on the action of "flippase" which move the lipid-bound repeating units from the cytoplasmic face of the membrane to the periplasmic face (Laws *et al.,* 2001). Other mechanisms have been proposed for *Streptococcus pneumoniae* (Bentley *et al.,* 2006) and *Lactobacillus rhamnosus* (Lebeer *et al.,* 2009) (**Figure 1.4**). A polymerase could catalyse the linking of the repeating units and an enzyme could uncouple the lipid-bound polymer and control chain length (Welman and Maddox *et al.,* 2003).

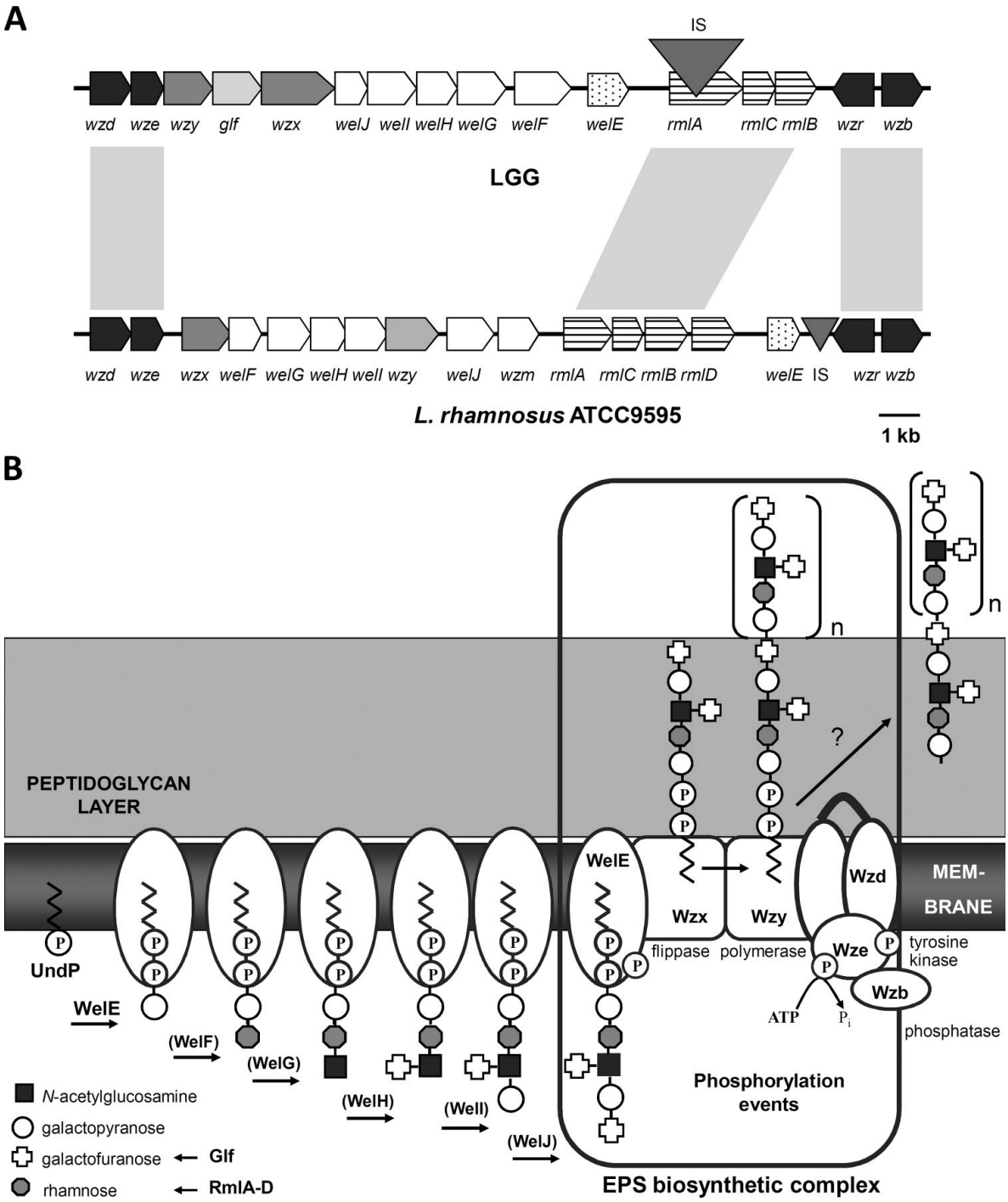

Figure – 1.4 (A) Schematic representation of the EPS gene cluster of *L. rhamnosus* GG and comparison with the corresponding gene cluster in *L. rhamnosus* ATCC 9595. The arrows with the same color of gray indicate genes encoding similar functions in EPS biosynthesis. The dark gray arrows indicate the genes encoding proteins putatively involved in the regulation of EPS production and polymerization. The light gray arrows indicate the gene putatively encoding the polysaccharide transporter and polymerase. The white arrows indicate the genes encoding the putative glycosyltransferases. The long stripes arrows indicate genes encoding the proteins involved in the biosynthesis of the dTDP-rhamnose precursor. The lightest gray arrows indicate the *glf* gene, which encode the UDP-galactopyranose mutase. The triangles indicate insertion sequence elements (IS). Gray boxes indicate the genes with high homology. **(B) Putative representation of the EPS biosynthesis in *L. rhamnosus* GG.** The membrane-associated priming glycosyltransferase *WelE* allows the transfer of a phosphogalactosyl residue from an activated nucleotide sugar to the undecaprenyl phosphate (UndP)-lipid carrier on the cytoplasmic face of the membrane. Consequently, unique glycosyltransferases *WelF* to *WelJ* add the remaining sugars in a sugar and glycosidic linkage-dependent manner. A *Wzx* flippase allows translocation across the cytoplasmic membrane of a complete subunit EPS, followed by linkage of the repeating units into long polysaccharides by a specific *Wzy* polymerase. *Wze* autophosphorylating tyrosine kinase and a *Wzb* phosphotyrosine protein phosphatase forming a phosphorylation complex could be involved in the regulation of EPS biosynthesis *(from Lebeer et al., 2009).*

EPS biosynthesis can be divided into three main steps: (i) assimilation of a carbon substrate; (ii) intracellular synthesis of the polysaccharides; (iii) EPS exudation out of the cell (Vandamme *et al.,* 2002).

The physiological role that exopolysaccharides play in the bacterial ecology of probiotics lactic acid bacteria is not yet entirely clear. EPS are thought to protect against biotic stress, like competition, and abiotic stresses that might include temperature, light intensity, pH or osmotic stress. In the cases of acidophilic or thermophilic species, EPS aid in adapting to extreme conditions. It has been suggested that EPS from other bacteria can act as protective agents against desiccation, antimicrobial compounds, bacteriophage attack, and to permit adhesion to solid surfaces (De Vuyst and Degeest, 1999; Forde *et al.*, 1999; Looijesteijn *et al.,* 2001; López *et al.,* 2004). They can also be involved in adhesion to surfaces and biofilm formation and to cell adhesion/recognition mechanisms (Ruas-Madiedo *et al.,* 2002; Broadbent *et al.,* 2003; Rozen *et al.,* 2004), however, the involvement of these biopolymers in bacterial adhesion to the intestinal epithelium *in vivo* has not yet been validated (Ruas-Madiedo *et al.,* 2008).

Despite the wide diversity of microbial EPS with physicochemical properties that are industrially promising, only two EPSs are authorised for use as additives in the food industry in the United States and Europe: xanthan (30 000 tons/year) and gellan (Donot *et al.,* 2012).

EPS from microbial sources can be classified into two groups: homopolysaccharides (e.g. cellulose, dextran, mutan, alternan, pullulan, levan and curdlan) and heteropolysaccharides (e.g. gellan and xanthan). Homopolysaccharides consist of repeating units of only one type of monosaccharide (D-glucose or D-fructose) and can be divided into two major groups: glucans and fructans. By contrast, heteropolysaccharides from LAB have repeating units that demonstrate little structural similarity to one another. The molecular mass of these polymers ranges between 4.0×10^4 and 6.0×10^6 Da (Welman and Maddox, 2003).

The structural diversity of EPS among lactobacilli may determine strain-specific properties important for probiotic action and technological applications (adhesion, stress resistance).

EPS have been reported to possess a number of health benefits, such as immune-stimulatory (Vinderola *et al.,* 2006; Matsuzaki *et al.,* 2014), and antitumoral effects (Kitazawa *et al.,* 1991) lowering blood cholesterol (Nakajima *et al.,* 1992; Maeda *et al.,* 2004b) and prebiotic effects (Dal Bello *et al.,* 2001; O'Connor *et al.,* 2005). Surface polysaccharides may also contribute to protection against intestinal innate immune factors such as the antimicrobial peptide LL-37 (Lebeer *et al.,* 2011). Exopolysaccharides produced by LAB can regulate inflammatory responses in the intestinal lumen (Notararigo *et al.,* 2014). The cell surface-associated exopolysaccharide of the probiotic *Bifidobacterium brevis* reduces the production of pro-inflammatory cytokines and suppresses the generation of *B. brevis*-specific antibodies, thus allowing this probiotic to be tolerated in the gut Fanning *et al.,* 2012).

1.4.2 The potential prebiotics properties of exopolysaccharides

Currently, little is known about the prebiotic properties of EPS produced by lactic acid bacteria, although they have received increasing attention in relation to health benefits (i.e., immune stimulation, antimutagenicity, and antitumor activity (Kitazawa *et al.,* 1998; Ruas-Madiedo *et al.,* 2002; Salazar *et al.,* 2014).

A potential prebiotic effect has been reported for exopolysaccharides produced in whey by *L. plantarum*. The EPS produced can be used by the probiotic parent strain, thus suggesting that it could possess enzymes capable to degrade the EPS (Tsuda and Miyamoto, 2010). An α-d-glucan produced by *Lactobacillus plantarum* exhibited lowest digestibility by artificial gastric juice and *in vitro* prebiotic activities showed increased growth of probiotic bacteria such as *Bifidobacterium infantis* and *Lactobacillus acidophilus*, but did not support the growth of non-probiotic bacteria such as *Escherichia coli* and *Enterobacter aerogenes* indicating their potential use as prebiotic additive for food products (Das *et al.,* 2014). The prebiotic properties levan-type EPS from *Lactobacillus sanfranciscensis* were studied and the bifidogenic effect of the EPS was observed (Dal Bello *et al.,* 2001).

EPS from *Weissiella cibaria, W. confusa, L. plantarum* and *P. pentosaceus* exhibited high resistance to gastric and intestinal digestions, selective enhancement of beneficial gut bacteria (particularly bifidobacteria group) suggesting their prebiotic potentials (Hongpattarakere *et al.,* 2012). The ingestion of exopolysaccharide-producing lactobacilli improve lipid metabolism, associated with changes in the gut microbiota (London *et al.,* 2014).

A positive effect of the β-D-glucan produced *by P. parvulus* was observed on the growth of both *L. plantarum* and *L. acidophilus* strains, suggesting that its use as a prebiotic may positively modulate the growth of probiotic organisms (Russo *et al.,* 2012). Conversely, purified EPS from *P. parvulus* did not show prebiotic effect in the mouse model, although ingestion of live EPS-producing bacterium antagonized *Enterobacteriaceae* without disturbing the homeostasis of the microbiota (Lindström *et al.,* 2013).

1.4.3 Exopolysaccharides in food industry

Since several decades exopolysaccharides produced by lactic acid bacteria has received increasing interest, regarding their potential use in industrial field (Cerning, 1995). In the food industry, EPS produced by LAB and other microorganisms are used as viscosifiers, stabilizers, emulsifiers, or gelling agents to modify the rheological properties, texture and 'mouthfeel' of fermented dairy and non-dairy products (Hassan, 2008; Galle *et al.,* 2012). Most of the strains used in the production of functional dairy food synthesize heteropolysaccharides (Welman and Maddox, 2003; Mende *et al.,* 2012).

Several authors evaluated the affect of EPS produced by LAB on rheological and sensorial properties in yogurt (Hassan *et al.,* 2003; Doleyres *et al.,* 2005; Folkenberg *et al.,* 2006; Yang *et al.,* 2014), the product of fermentation of milk led by starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in ratio 1:1. Both bacteria produce EPS from 30 to 890 mg/L for *S. thermophilus* and from 60 to 150 mg/L for *L. delbrueckii* subsp. *bulgaricus* (Bouzar *et al.,* 1997; Marshall and Rawson, 1999). It has been found that

exopolysaccharides in yogurt contribute to improve the viscosity and texture and they do not alter the flavor of the final product (Jolly *et al.,* 2002; Badel *et al.,* 2011).

Several species of lactobacilli are described to produce exopolysaccharide. The best documented species are *L. casei, L. acidophilus, L. brevis, L. curvatus, L. delbrueckii bulgaricus, L. helveticus, L. rhamnosus, L. plantarum* and *L. johnsonii*. *L. reuteri* 121 has been found to synthesize several HoPSs in the same culture conditions (van Geel-Schutten *et al.,* 1999) and it is capable to secrete β-(2,1) fructans (inulin like polysaccharide) recognized as prebiotic (van Hijum *et al.,* 2002). The soluble reuteran has been found opportunities in baking industry in association with levan synthesized by *L. reuteri* and *L. sanfranciscensis,* as their polysaccharides provides beneficial effect on bread flavour, texture and shelf-life of products derived from sourdough fermentation (Tieking *et al.,* 2005; Badel *et al.,* 2011).

The use of LAB starter cultures which produce EPS *in situ* during fermentation could be a valid alternative for products whose polysaccharides addition requires the specification of food additives, which is a condition not much appreciated by consumer.

1.5 Bacterial resistance to the oro-gastro-intestinal transit

The human gastrointestinal tract (GIT) is colonized by an enormous and diverse community of microbes which are essential to its proper functioning. These microbes have evolved in concert with their host to occupy specific regions and niches in the GIT. A balanced, complex microflora is necessary for normal digestion and to maintain the homeostasis of intestinal ecosystem (Simon and Gorbach, 1986).

Tolerance to the harsh conditions of the oro-gastro-intestinal transit (OGI), which comprises highly acidic gastric juices and pancreatic bile salt secretions, is a fundamental criterion for the selection of orally delivered probiotics. For this reason, the analysis of potential probiotics *in vitro* multi-compartmental models simulating the physico-chemical conditions of the human OGI

tract is a prerequisite to subsequent *in vivo* experiments. Development and implementation of such systems are highly encouraged by FAO and WHO (2002) and several recent studies have addressed this issue (Fernández de Palencia *et al.,* 2008; Lo Curto *et al.,* 2011).

The lysozyme and chewing stress represent the first obstacle of the oral tract. The various proposed models, simulate the phenomena that occur during the digestion, from filling to the gradual emptying of the stomach. In the condition of full stomach, bacteria ingested together with the food matrix are subjected to pH values of 5.0-6.0, then undergo more drastic acidic conditions, as there is a lowering of pH at values of 2.0 - 1.5. Bacteria exposure to acids environments, disturb the proton motive force across the membrane, causing an accumulation of protons inside the cell (Corcoran *et al.,* 2008). The emptying of the gastric pouch is an event that takes place gradually, in tandem with the digestion of food. The liquids empty from the stomach is faster than solids and in general food remains in the stomach between 2 and 4 hours, while the transit time through the small intestine takes from 1 to 4 hours. The adverse conditions of the small intestine include the presence of bile and pancreatin in the lumen of the small intestine, pH is around 8.0. Bile salts secreted in the duodenum emulsifies and solubilize lipids and lipid soluble vitamins (Begley *et al.,* 2005). A concentration of 0.15 - 0.3% of bile salts has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin and Gorbach, 1992; Huang and Adams, 2004).

Bacterial cells have various defense mechanisms to resist the hostile environments (Van de Guchte *et al.,* 2002; Mills *et al.,* 2011). The chaperone proteins assist the folding of misfolded proteins, proteases which degrade irreversibly damaged proteins, transport systems to maintain correct osmolarity, catalases and superoxide dismutases to tackle reactive oxygen species, as well as proton pumps, decarboxylases and transporters to counteract intracellular pH decreases (De Angelis and Gobbetti, 2004; Sugimoto *et al.,* 2008).

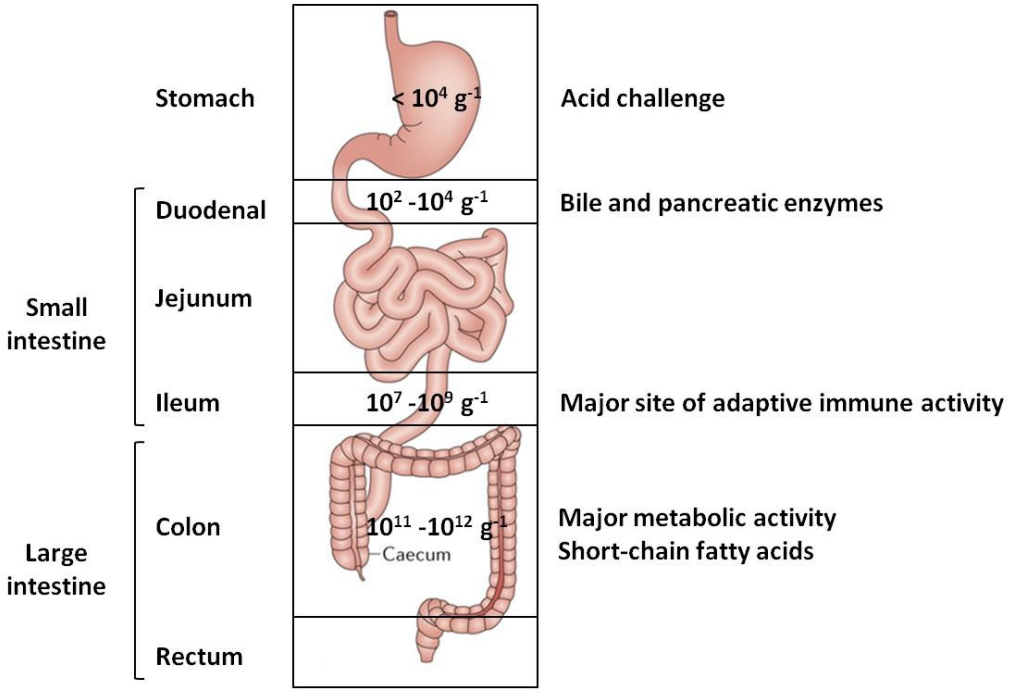

Figure 1.5 - Compartments of the human GI tract and related densities of the residing bacterial population. Food-borne bacteria stress sequential in the acidic environment of the stomach and subsequently pancreatin and bile into the small intestine. Dietary supplementation of probiotics can generate a relative high abundance of these species in the first tract of the small intestine, where their metabolic activity can be relevant. The ileum, where the probiotic loads tend to decrease with respect to the indigenous microbiota, is the major site of probiotic immune activity. In the large intestine, commensal bifidobacteria and probiotic supplements contribute to catabolize diet- and host-derived glycans, generating a variety of short chain fatty acids that are used as important energy source by the colonic mucosa (a*dapted from: Kleerebezem and Vaughan, 2009; Mowat and Agace, 2014)*.

1.5.1 The role of exopolysaccharides during the *in vitro* gastro-intestinal transit

An important aspect related to the potential prebiotic effect of microbial exopolysaccharides, is the behavior that they have during the gastro-intestinal transit, considering that the low pH stress is usually the hardest obstacle for survival of probiotic bacteria (Both *et al.,* 2010; Bove *et al.,* 2013). Fernández de Palencia *et al.* (2009) reported that a ropy strain of *Pediococcus parvulus* and its relative non ropy strain subjected to an *in vitro* gastric or gastro-intestinal stress, have the same pattern of resistance to stress, indicating that the presence of EPS did not confer to bacterial cells an advantage for survival in the human digestive tract. By contrasty, synthesis of the *P. parvulus* β-glucan confers to *Lactobacillus paracasei* higher survival during gastrointestinal passage or technological process (Stack *et al.,* 2010). Arena *et al.* (2014a) reported that

exogenous polysaccharides such as food matrices containing β-glucans, enhanced the oro-gastrointestinal stress tolerance of lactobacillus probiotic strains.

1.6 Bacterial adhesion to the intestinal mucosa and displacement of pathogen bacteria

The ability to adhere to the intestinal mucosa is an advantageous feature of probiotic microorganisms, as it ensures persistence in the intestinal tract, which is necessary for them to come in close contact with host epithelial cells, to control the balance of the intestinal microflora, to antagonize pathogen growth, and to exert immune modulation on the host (Isolauri *et al.,* 2004).

Adhesion to the surface of host epithelial cells is a key pathogenic factor of intestinal pathogens (Scaletsky *et al.,* 2002). Enterohemorrhagic *Escherichia coli* (EHEC) is a human pathogen that enters the intestinal tract as a result of food contamination and causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Kim *et al.,* 2009). Lactobacilli have been shown to possess surface adhesins similar to those on bacterial pathogens (Neeser *et al.,* 2000) and thus they may interfere with pathogen adhesion on the intestinal mucosa. The ability of probiotic bacteria to adhere on the intestinal surface, is an important factor in the displacement of pathogens (Lee and Puong, 2002; Gueimonde *et al.,* 2006). A probiotic should be able to compete with a pathogen for the binding sites, nutrients, production of antimicrobial substances and immune-stimulating compounds.

A first physical barrier to host-cell stimulation by bacteria in the gut, is represented by the mucus layer bound to gastro-intestinal epithelia. This is composed of a continuous gel matrix, which is formed primarily of complex glycoproteins that acts as a protective barrier for the host against harmful antigens and promote luminal motility. The adhesion to mucus layer is therefore the first requirement for probiotic organisms to interact with host cells. The thickness of the human intestinal tract mucus layer is variable. Generally it is greater starting from the small intestine,

where the intestinal flora is more abundant, and it is thinner in the rectum (Van Tassell and Miller, 2011).

The polymers that compose intestinal mucin are considered nutritive glycans for commensal bacteria in the promotion of their residence and associated benefits (Carrington *et al.,* 2009).

Probiotic persistence and colonization do not permanently exist in the GI tract and they provide host benefits only for brief periods, once finished the administration (Tannock *et al.,* 2000; Garrido *et al.,* 2005). Bacteria at first adhere to gastro-intestinal surfaces by nonspecific physical interactions, which are reversible attachments. Many lactobacilli have large surface proteins with highly repetitive structures that are involved in mucus adhesion (Van Tassell and Miller, 2011).

Mucus-binding proteins showing lectin-like interactions have been isolated; they may be conserved in numerous *Lactobacillus* species, and some of them showed to promote mucus adhesion. Mucus-targeting bacterial adhesins is the mucus-binding protein (MUB), produced by *L. reuteri* (Tannock *et al.,* 2000; Roos and Jonsson, 2002), and in *L. acidophilus* NCFM (Buck *et al.,* 2005) have been identified.

Probably, carbohydrate-protein interactions play a key role in the adhesion of these proteins to mucin-bound oligosaccharides. Numerous MUB homologues and MucBP domain containing proteins have been found, almost exclusively in lactic acid bacteria and mainly in lactobacilli found naturally in intestinal niches (Van Tassell and Miller, 2011). This suggests that MucBP domain containing proteins play an important role in establishing host-microbial interactions in the gut and promoted the evolution of the species as primarily GI organisms (Boekhorst *et al.,* 2006; Dam and Brewer, 2010).

S-layer proteins and glycoproteins can form a monolayer coating the surface of bacterial cells (Boot *et al.,* 1996; Sleytr *et al.,* 1997), they are present in only some *Lactobacillus* species, and has been ascribed a role in adhesion to host cell and inhibition of pathogen adhesion to the same surface. In *Lactobacillus crispatus* ZJ001, S-layer proteins are responsible for adhesion to epithelial cells and competitive exclusion of pathogens such as *E. coli* O157:H7 and *Salmonella*

typhimurium (Chen *et al.,* 2007). Ramiah *et al.* (2007), found a consistent induction of Mub and other adhesion proteins in a probiotic strain of *L. plantarum*, especially when mucins were added to a media simulating gut conditions.

Another mechanism of bacterial adhesion is based on the binding to mannose-containing receptors on epithelial cells. Among probiotic bacteria, *L. plantarum* is able to recognize mannose-residues. By *in silico* studies, the predictive sequence of a *L. plantarum* WCFS1 adhesin gene (*lp_1229*) was identified. Knockout of this gene resulted in a complete loss of yeast agglutination ability, while its overexpression enhanced this phenotype. Moreover, analysis of the protein showed putative carbohydrate-binding domains, supporting its role in binding mannose residues. Therefore, this gene was designated to encode the mannose-specific adhesin (*msa*), probably involved in the interaction of *L. plantarum* with the host along the intestinal tract (Pretzer *et al.,* 2005).

The EPS produced by probiotic strains could be able to adhere to intestinal mucus, the effect being dose and EPS type dependent. This could reflect the adaptation of probiotics to their natural environment. Thereby, EPS could act as adherence factor that may play a role in the transitory colonization of the intestinal mucosa by probiotics. The ubiquity of EPS gene clusters on probiotic genomes suggest that a number of strains from the intestinal microbiota may produce extracellular polymers in this environment and that high EPS concentrations could be locally reached in the gastrointestinal tract (Ruas-Madiedo *et al.,* 2006).

1.6.1 Caco-2 cell *in vitro* model adhesion

The molecular mechanisms underlying probiotic activities are being disclosed more and more by *in vitro* and *in vivo* studies focused on the interaction between probiotic bacteria and host intestinal epithelial or immune cells (Marco *et al.,* 2006). Due to obvious difficulties in performing *in vivo* studies, preliminary studies of potentially adherent strains are mainly based

on *in vitro* adhesion assays. Currently there is not an *in vitro* adhesion standard protocol, in fact for this reason the results are highly variable (Laparra and Sanz, 2009).

Tissue cultures of the human colon carcinoma cell lines Caco-2 are the most frequently used, and their applications are well documented in the literature. They are considered one of the best representations of the *in vivo* environment and they can be grown in culture to form a homogeneous polar monolayer of mature enterocytes resembling the tissue of the small intestine (Pinto *et al.,* 1983). Caco-2 cells represent a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells, developed by the Sloan-Kettering Institute for Cancer Research. Caco-2 cells are capable to initiate spontaneous differentiation and reach confluence under normal culture conditions (e.g., presence of glucose and serum) (Fossati *et al.,* 2008). Over a period of 20 - 30 days of post-confluent culture, Caco-2 cells gradually acquire a morphological polarity comparable with those of mature intestinal absorbing cells. Caco-2 cells also provide a valuable system for immunological studies (Ou *et al.,* 2009).

Moreover, some microorganisms provide essential vitamins (e.g., folate, biotin, vitamin K) and produce short chain fatty acids that are used as energy source by colon cells (Saulnier *et al.,* 2009).

1.6.2 Zebrafish *in vivo* model adhesion

In recent years, zebrafish (*Danio rerio*) has been found as an interesting model to study vertebrate development, immunity and disease because of their small size, high fecundity, rapid development, optical transparency of the embryos, amenability to genetic screens, and structural similarities to mammals (Meeker and Trede 2008; Sullivan and Kim 2008). Scientific studies on this model were several: host immune response under a number of microbial infections (van der Sar *et al.,* 2004; Rojo *et al.,* 2007); interactions between the host and its natural gut microbiota (Milligan-Myhre *et al.,* 2011); host-probiotics interactions (Gioacchini *et al.,* 2012; Rendueles *et al.,* 2012; Carnevali *et al.,* 2013; Rieu *et al.,* 2014).

The use of gnotobiotic models, i.e. models whose microbiota is unknown or absent, may allow a better understanding of host-probiotics interaction. This could be a real problem in animal models due to the diversity of microorganisms that reside in the host gut. The use of zebrafish is very advantageous because the generation of gnotobiotic organisms is less complex with respect to mammalian models. Moreover, zebrafish eggs are fertilized externally and the development of embryos occurs within their protective chorions, and the axenic conditions can be easily conserved.

1.7 Host cells and probiotics interaction

The human gastrointestinal microbiota is essential to human health, because it contributes to the digestion of food and development and the proper functioning of the immune system. Some microorganisms provide essential vitamins (e.g., folate, biotin, vitamin K) and produce short chain fatty acids that are used as energy source by colon cells (Saulnier *et al.,* 2009). In the years have been selected bacterial species with capacities for improving the host health, defined as probiotics. These microorganisms mainly belong to the genera *Lactobacillus* and *Bifidobacterium* (Marco *et al.,* 2006).

Several applications of probiotics have been observed following clinical trials, including the prevention of the gastrointestinal infections, inflammatory bowel diseases, allergic diseases, and as adjuvants in vaccinations (Borchers *et al.,* 2009).

Improvement of the intestinal epithelial barrier by modulation of immune responses is one of the mechanisms by which probiotics are thought to contribute to human health (Lebeer *et al.,* 2010).

The immune system is divided in two interconnected systems of immunity: innate and adaptive. Innate immunity is more primitive, and it prevents infection or quickly eliminates invaders such as viruses, bacteria, fungi or parasites. It includes physical and chemical barriers against infection, as well as cellular responses. By contrast, adaptive immunity is based on the B and T

lymphocytes, it requires a longer reaction time because few cells have the appropriate receptor to thwart a dangerous agent but it is more specific than the innate immune system (Owen *et al.,* 2013).

Currently, lactic acid bacteria are widely studied for probiosis. The cell wall molecules (i.e. peptidoglycan, proteins, teichoic acids and polysaccharides) are fundamental in the interact mechanisms between probiotic and host receptors, inducing signaling pathways (Lebeer *et al.,* 2010).

Once ingested and after crossing of the mucus layer, probiotics can to interact with intestinal epithelial cells (IECs) or with dendritic cells (DCs) residing in the lamina propria sample luminal bacterial antigens by passing their dendrites between IECs into the gut lumen (Rescigno *et al.,* 2001). DCs can also interact with bacteria that have gained access to the dome region of the gut-associated lymphoid tissue (GALT) through specialized epithelial M cells (Artis, 2008).

These cells can interact with and respond to gut microorganisms by means of their pattern recognition receptors (PRRs), which detect microorganism associated molecular patterns (MAMPs).

The main elements of PRRs are the 'Toll-like receptors' (TLRs). The interaction between a MAMPs and a PRRs results in the induction of signalling cascades that develops a molecular response against the detected microorganisms; this response can include the secretion of immunomodulatory cytokines, chemokines, and antimicrobial agents.

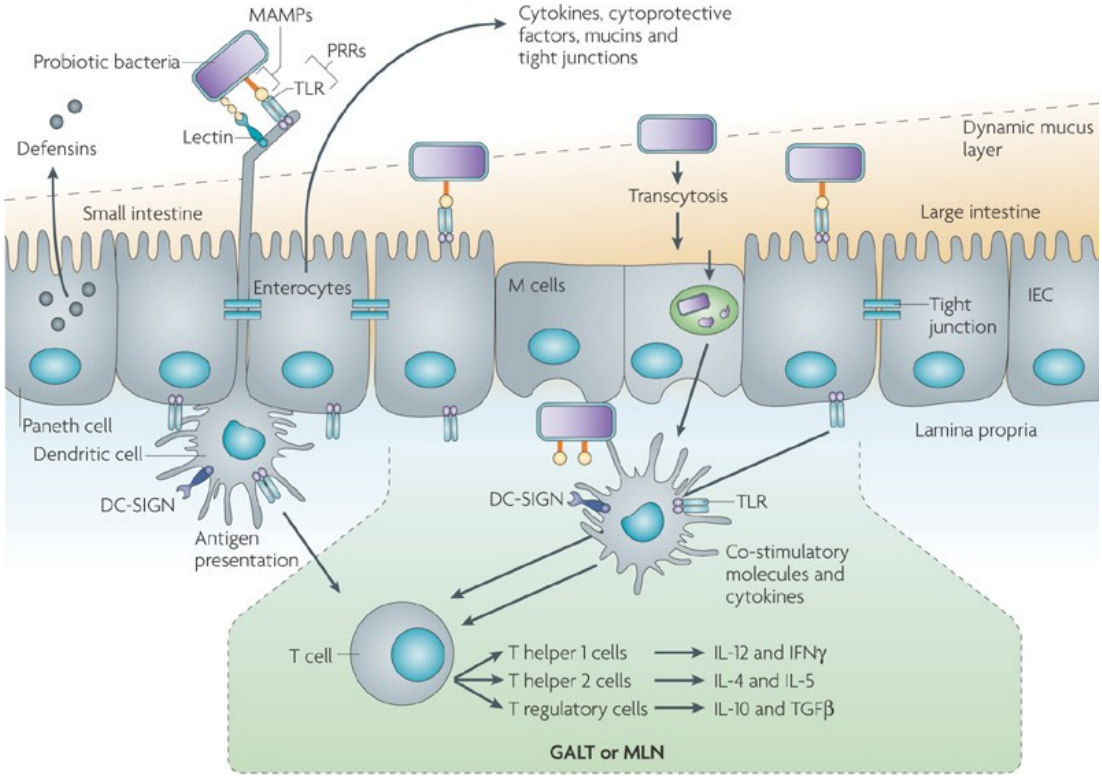

Figure 1.6 - Molecular interaction of probiotic bacteria with intestinal epithelial cells (IECs) and dendritic cells from the GALT. Host pattern recognition receptors (PRR) recognize the organism through the associated molecular patterns (MAMPs): Depending on the type of cell, this interaction leads to a specific molecular response (from Lebeer et al., 2010).

The cell wall of probiotics has had considerable attention to its surface properties because underlie recognition with host cells, and provides species- and strain-specific properties that are probable involved in specific host interactions. The Gram positive bacteria wall contains several structural components (**Figure 1.7**), which are recognized by PPRs, inducing signaling pathways. MAMPs are attributable to macromolecules such as the peptidoglycan, cell wall- or membrane-associated teichoic acids, exopolysaccharides and various classes of surface proteins (Kleerebezem and Vaughan, 2009). *L. plantarum dlt* cell wall mutant, which synthesized modified teichoic acids, demonstrated that such specific cell surface biochemical feature might positively affect the interaction between microorganism and host (Grangette *et al.,* 2005).

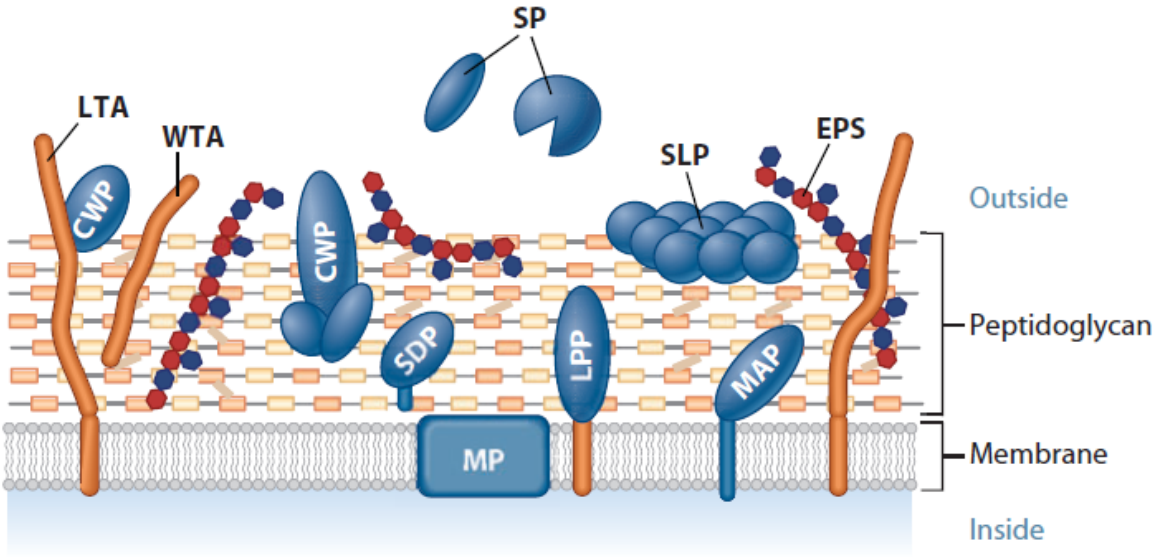

Figure 1.7 - Representation of Gram-positive cell wall. Several components of the cell surface macromolecules have been proposed to be directly involved in interaction with host cells. Specific MAMPs, and related host modulation properties, can be associated to: peptidoglycan (PG) layer, the predominant cell wall component; wall- and lipotheicoic acids (WTA, LTA); exopolysaccharides (EPS); and various types of surface associated proteins: secreted proteins (SP), membrane proteins (MP), cell-wall-associated proteins (CWP), sortase-dependent proteins (SDP), lipoproteins (LPP), membrane-anchored proteins (MAP), and surface layer proteins (SLP) (*from Kleerebezem and Vaughan, 2009*).

Although CPS (capsular polysaccharides) molecules are key virulence factors in pathogens agents (Kasper, 1986), the role in host–microorganism interactions of CPS and EPS in probiotic bacteria are not well documented (Welman and Maddox, 2003). The main role of the CPS in pathogens is to shield other molecules on the cell surface and prevent them from interacting with host PRRs. Lebeer *et al.* (2009) reported that the CPS in *L. rhamnosus* GG shield fimbriae. Wang *et al.* (2006) found that polysaccharide A is able to activate NF-κB signaling and cytokine production in DCs by TLR2-dependent mechanisms, modulating antigen presentation and CD4+ T cell activation. CPS-producing *L. rhamnosus*, decreased flagellin-induced IL-8 production in Caco-2 cells (Lopez *et al.,* 2008). Lebeer *et al.* (2011) reported that exopolysaccharides produced by *Lactobacillus rhamnosus* GG may protect, by shielding effect, against intestinal innate factors such as the antimicrobial peptide LL-37. Remus *et al.* (2012) suggested a shielding role of surface polysaccharides *L. plantarum* cell envelope (MAMPs). Fanning *et al.* (2012) reported that EPS in bifidobacteria can facilitate colonization of the host through evasion of potentially

damaging immune responses, and can provide direct health-promoting benefits, reducing pathogen colonization. In *Bifidobacterium longum* the EPS might be a mild immune modulator for macrophages, and also it might increase the capacity of the host to fight against gastrointestinal infections, and has potential application as a natural preservative against food-spoilage bacteria (Wu *et al.,* 2010).

1.7.1 Host cell response and immune-modulation

Currently, is well known that the beneficial effects of probiotics are also due to their ability to stimulate the immune system (immune-enhancing effect and anti-inflammatory). Probiotics can modulate the expression of genes such as mucin (MUC), toll-like receptors (TLR), pro-inflammatory transcription factors, cytokines, antimicrobial agents. The interaction lactobacilli and host cells has mainly been studied in *in vitro* models, which simulate simplified gastrointestinal tract conditions (Dicks and Botes, 2010). In this models are used different types of human cell lines, including epithelial cell lines such as Caco-2, HT-29 and T-84, or immune cell lines like peripheral blood mononuclear cell, dendritic cells, or macrophages. Several studies have been conducted on the immunomodulatory activity by bacterial species belonging to the *Lactobacillus* genus.

Among these there are cytokines such as interleukin-10 (IL-10) which is a marker for an anti-inflammatory response, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) which are markers for pro-inflammatory response (Christensen *et al.,* 2002; Remus *et al.,* 2011).

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and haematopoiesis. They include “lymphokines” (released by lymphocytes), “monokines” (released by monocytes), “chemokines” (cytokines with chemotactic activities), and “interleukins” (cytokines released by one leukocyte and acting on other leukocytes). Cytokines are produced in response to an immune stimulus, and generally act over short distances and short time spans, at

very low concentration. Probiotics contribute to realize a healthy gut homeostasis, as optimize the balance of pro- and anti-inflammatory cytokines and other immune modulators. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules (Foster, 2001).

Chemokines are a wide family of small chemoattractive cytokines, and their receptors play an important role in the regulation of the immune response and homeostasis. Chemokines produced within the gastrointestinal mucosa, head the balance between physiological and pathophysiological inflammation in health, inflammatory bowel disease and the progression to colon cancer (Zimmerman *et al.,* 2008). The macrophage inflammatory protein 3α (MIP-3α) is a chemokine mainly expressed by colonic epithelial cells. Its expression level was found to be up-regulated in patients with Crohn's disease or ulcerative colitis, suggesting that it might play an important role in the pathogenesis of human IBD (Kwon *et al.,* 2002).

Interleukins are a class of immunomodulatory cytokines, which are involved in the regulation of immune responses, they may have pro- or anti-inflammatory functions in chronic liver diseases, and some interleukins even both, dependent on the inflammatory stimulus, the producing and the responding cell type. Interleukins promote the development and differentiation of T, B, and hematopoietic cells. They are produced in large amounts by various cell types during inflammatory reactions. The majority of interleukins are synthesized by helper CD4+ T lymphocytes, as well as by monocytes, macrophages, and endothelial cells. Interleukin 1 (IL-1) activates T cells; IL-2 stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6 stimulate proliferation and differentiation of B cells (Dinarello, 2000). IL-12 balances T-helper (Th)-1 and Th2 cell responses in infectious disease models. IL-10 is the prototypic anti-inflammatory interleukin with tissue-protective functions during chronic liver injury and fibrogenesis. Despite its critical role for inducing the acute-phase response in the liver, IL-6 signaling is protective during fibrosis progression, but promotes hepatocellular carcinoma (Hammerich and Tacke, 2014).

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine contributing to activation of immune cells, release of cytolytic enzymes and reactive oxygen species (ROS), and exacerbation of tissue damage at inflammation sites. Tumor necrosis factor alpha (TNF-α) is a potent inflammatory cytokine secreted upon cellular stress as well as immunological stimuli and is implicated in the pathology of inflammatory diseases and cancer (Nickles *et al.,* 2012). Has been report that probiotic lactobacilli can suppress human TNF production by host immune cells (Lin *et al.,* 2008)

Antimicrobial peptides (AMPs) are cationic amphiphilic peptides, which are the first line of defense to protect organisms from microbial infection. Naturally occurring or synthetic AMPs are considered a new functional class of antibiotics. AMPs are antimicrobial agents based on their activity against the prokaryotic membrane (Méndez-Samperio, 2014). AMPs allow the innate immune system to respond faster than the adaptive immune system can be sufficiently mobilized (Liévin-Le Moal and Servin, 2006). The main families of AMP intestinal are cathelicidins and defensins. LL-37 is the only cathelicidin described in humans, it can act alone and in synergy with other antimicrobial proteins such as lysozyme (Singh *et al.,* 2000), displaying bactericidal activities against Gram-positive and Gram-negative bacteria.

Some β-defensins (HBD) human defensins (HBD-1, HBD-2 and HBD-3) are shown to play a role in the defense of epithelial sites. HBD-2 and HBD-3 is increased during infection and inflammation (Bevins *et al.,* 1999; Zaalouk *et al.,* 2004).

Mucin is a mucous-gel layer provides a physical barrier against dangerous bacteria and molecules, acting as a lubricant for intestinal motility (Phillipson *et al.,* 2008). Lactobacilli were found to induce mucin in human intestinal cell lines, hindering adhesion of pathogenic *E. coli* strains (Mattar *et al.,* 2002; Mack *et al.,* 2003).

The tight junctions (zonula occludens) are proteins able to keep connected the adjacent cell membranes allowing occlusion of the intercellular space at variable distances (Farquhar and Palade, 1963). They represent a dynamic complex constantly affected by external stimuli,

including commensal bacteria. They play an important role in the regulation of nutrients, water, ions and pathogens entry. Tight junction structure is composed by several proteins; among these the claudins represent the structural basis (Furuse *et al.,* 1998) and claudin-1, -3, -4, -5, and -8 are thought decrease paracellular permeability, while claudin-2 forms charge-selective paracellular pores (Bücker *et al.,* 2010). Occludins are another type of proteins belonging to the tight junctions and they regulate the diffusion of small molecules into intermembrane diffusion and paracellular (Balda *et al.,* 1996; Ulluwishewa *et al.,* 2011).

It is reported that *L. plantarum* is able to modulate human epithelial tight junction, increasing occludins levels, thus enhancing intestinal barrier function (Karczewski *et al.,* 2010; Anderson *et al.,* 2010).

Toll-like receptors (TLRs) are family of cellular receptors of the innate immunity response, which recognize molecules unique to microbes. Toll-like receptor 4 (TLR4) is the specific receptor for lipopolysaccharide (LPS) and molecules of the membrane of Gram-negative bacteria. Lipopeptides and other components of Gram-positive bacteria are able to activate TLR2 in conjunction with either TLR1 or TLR6 (Moresco *et al.,* 2011).

Nuclear factor-kappa B (NF-kB) is a transcription factor which regulate the gene expression of several cytokines, growth factors, adhesion molecules, and enzymes involved in cellular processes such as inflammatory responses and oxidative stress (Hayden and Ghosh, 2008).

Hegazy and El-Bedewy (2010) suggested that *L. delbruekii* and *L. fermentum* strains administered to ulcerative colitis (UC) patients, decreased the level of IL-6 and the expression of TNF-α and NF-kB p65, thus alleviating the inflammation.

Thymic stromal lymphopoietin (TSLP) is a gene which encodes a hemopoietic cytokine. The protein promotes T helper type 2 (TH2) cell responses that are associated with immunity in various inflammatory diseases, including allergic response, such as bowel ulcerative colitis (UC) and Crohn's disease but also in asthma and dermatitis events (Taylor *et al.,* 2009).

1.8 Lactic acid bacteria and stress tolerance

The ability to resist stressful conditions is an important feature for survival of lactic acid bacteria that possess probiotics and pro-technologicals properties.

Due to high ethanol concentration, low acidity, sulfur dioxide and temperature, the wine represents a hostile environment for bacterial survival (Cecconi *et al.,* 2009). *Lactobacillus, Oenococcus* and *Pediococcus* are the main species that occur in wine as they lead the malolactic fermentation (Fugelsang and Edwards, 1997; Lonvaud-Funel, 1999). *L. plantarum* is widely used in fermented food production and has been proposed as a functional strain to improve winemaking conditions (Lerm *et al.,* 2011) and currently it is used as commercial starter culture, to ensure malolactic fermentation in musts or wines. The acclimation of enological lactic acid bacteria to high concentrations of ethanol and low pH is necessary to increase their ethanol resistance during winemaking (Lerm *et al.,* 2010).

Although probiotic properties are rarely studied in enological lactic acid bacteria, recent study has been reported on probiotic features of *Lactobacillus* spp., *Pediococcus* spp., and *Oenococcus oeni*, concerning saliva and acid resistance, bile tolerance and exopolysaccharides production (García-Ruiz *et al.,* 2014).

1.8.1 Tolerance to ethanol

Ethanol is the main yeast metabolite formed during alcoholic fermentation in wine; it is a toxic compound for bacterial cell, because it compromises the membrane integrity, for this reason, seems that the change of membrane fluidity is a defense mechanism. In fact, it has been found that *O. oeni* is capable to modify the membrane phospholipid content (Teixeira *et al.,* 2002; Grandvalet *et al.,* 2008), but also in *L. plantarum* has been observed an increase of saturated fatty acids content at the expense of the membrane fluidity (van Bokhorst-van de Veen *et al.,* 2011). The ability of *Lactobacillus plantarum* and *Oenococcus oeni* to tolerate ethanol was

already documented (G-Alegría *et al.,* 2004). High ethanol concentrations hinder malolactic fermentation (Zapparoli *et al.,* 2009) and Bravo-Ferrada *et al.* (2013) reported that the oenological properties of *L. plantarum* after acclimation in the presence of 6 and 10% v/v, improves the cultivability and L-malic acid consumption in synthetic wine and increases the resistance of cytoplasmic membrane. This is an important requirement considering that malate decarboxylation by LAB requires an intact membrane for the transport of protons inside the cell (da Silveira *et al.,* 2002).

Currently, very little is known about the specific role of exopolysaccharides produced by lactic acid bacteria present in ropy and non-ropy wine. In this regard, Velasco *et al.* (2006) suggested that concentration of ethanol up to 4.9% had positive impact on EPS production by *Pediococcus parvulus* 2.6. Lonvaud-Funel and Joyeux (1988) noticed that pediococci isolated from ropy wine exhibited a strong resistance to conditions in wine including ethanol stress; Dols-Lafargue *et al.* (2008) showed that wild or recombinant oenological bacterial strains, harboring a functional *gtf* (glycosyltransferase) gene are more resistant to several stresses occurring in wine such as alcohol, but also pH, and SO_2. By contrast, Walling *et al.* (2005) reported that EPS produced by *P. damnosus* is not a response to ethanol stress;

On the other hand, the expression of genes involved in stress defense mechanisms has been studied. During ethanol shock *O. oeni* produces large amounts of the sHsp Lo18 (Coucheney *et al.,* 2005). Maitre *et al.* (2014) found that the abundance of *hsp18*, the gene encoding the Hsps Lo18, was much higher in *O. oeni* after 30 min of ethanol shock than in bacteria grown under optimal conditions.

Van Bokhorst-van de Veen *et al.* (2011), reported that during exposure of *L. plantarum* to 8% ethanol in MRS 57 genes significantly differentially expressed were identified. Capsular polysaccharide biosynthesis cluster (*cps1*, *cps3*, and *cps4*) were downregulated after exposure to ethanol for 30 min or 24 h, and the genes associated with the fatty acid biosynthesis pathways

were affected resulting in an increase of saturated fatty acids (van Bokhorst-van de Veen *et al.* (2011).

A potential role of small heat shock proteins (Hsps) in tolerance to ethanol stress has been observed in *L. plantarum*, the exposure to 12% of ethanol resulted in an overproduction of Hsp 19.3 and Hsp 18:55 (Fiocco *et al.,* 2007). Moreover the inactivation of the *hsp18.55* gene affected membrane fluidity and physicochemical surface properties of *L. plantarum* (Capozzi *et al.,* 2011).

1.8.2 Tolerance to acid

Generally lactic acid bacteria are consider neutrophiles, because its optimal pH for growth varies from 5 to 9 (van de Guchte *et al.,* 2002).

Stress acid is a condition that normally occurs due to the organic acids production during the fermentation of foods and beverage operated by LAB. Moreover, probiotics lactobacilli are exposed to extreme acid stress when they reach the stomach, which is a strongly acid environment (De Angelis and Gobbetti, 2004). The proton-translocating ATPase is the most important mechanisms that regulate the homeostasis of internal pH in lactic acid bacteria (Hutkins and Nannen, 1993). The arginine deiminase (ADI) pathway is another acid-stress response mechanism (Sanders *et al.,* 1999). Heterofermentative lactobacilli, which are generally less acid-tolerant, derive energy and ammonia from arginine catabolism, thus becoming more competitive in the acid and alcoholic stressful environment of wine (Liu and Pilone, 1998).

The ability of *L. plantarum* to both lactic acid and pH stresses have been studied, by continuous culture followed by transcriptome profiling or by the measurement of intracellular pH (Pieterse *et al.,* 2005). Ingham *et al.* (2008) reported that a multiple levels of heterogeneity exist within pH-stressed cultures of *L. plantarum,* with subpopulations of cells able to grow in acidic conditions. Two new *Lactobacillus plantarum* strains isolated from sour turnip and traditional dried fresh cheese, showed excellent survival rates at pH 2.4 and during 3 hours of incubation at

pH 2.0 (Šeme *et al.,* 2014). *Lactobacillus* and *Pediococcus* strains isolated from wine showed the ability to survive at pH 1.8 (García-Ruiz *et al.,* 2014) and G-Alegria *et al.* (2004) reported the ability of *L. plantarum* strains to grow under acid stress conditions at pH range from 3.2 to 3.6. Still much remains to be understood about the role of exopolysaccharides in resistance of lactic acid bacteria to acid stress. In this regard, Fanning *et al.* (2012) reported that EPS layer produced by *Bifidobacterium breve* has a protective effect under low pH. In addition, high EPS production of *Bifidobacteria* may be important in the selection of probiotic strains for resistance to low pH (Alp *et al.,* 2010). The high EPS producing strains from yogurt starter showed a protective effect against low pH (Boke *et al.,* 2010).

1.8.3 Tolerance to sulfur dioxide

Sulfur dioxide is commonly used in winemaking in order to protect the wine from undesirable bacteria development (Ribéreau-Gayon *et al.,* 2006), and it been subjected to maximum authorized levels and the European Commission has recognized the health issues raised by its use in the wine industry (Commission Regulation (EC) N° 606/2009; OIV, 2011). Sulfur dioxide may be present in equilibrium in wine as bound SO_2, molecular or free SO_2, bisulphite (HSO_3^{-1}) and sulphite (SO_3^{-2}) ions (Fugelsang and Edwards, 1997). The equilibrium of sulfure dioxide is correlated to the acidity of the medium and the antimicrobial effect of SO_2 decreases with increasing pH. At low pH free SO_2 predominates, consisting mainly of bisulphite and a small fraction of molecular SO_2 and sulphite anions (Usseglio-Tomasset, 1992; Bauer and Dicks, 2004).

Molecular SO_2 has the major inhibitory effect, especially at lower pH values, inside the cells, the molecular SO_2 is transformed into bisulphite that may react with several cell components, like proteins and as result affect the growth of LAB (Bauer and Dicks, 2004). The inhibitory action of SO_2 on LAB is mainly due to rupturing of disulphide bridges in proteins as well as reacting with cofactors like NAD^+ and FAD, thereby affecting the growth of LAB (Carreté *et al.,* 2002).

The antimicrobial activity of SO_2 can also influence the malolactic fermentation (Lonvaud-Funel, 1999). Because, also low amount of molecular SO_2, (0.1-0.15 mg/L), may be inhibitory to the bacterial growth, a concentration of total SO_2 and bound SO_2 less than 100 mg/L and 50 mg/L respectively are recommended to ensure successful malolactic fermentation (Powell *et al.,* 2006).

Tolerance to SO_2 is a characteristic species-dependent; in fact, Larsen *et al*. (2003) reported that *Oenococcus oeni* strains were less tolerant to high total SO_2 concentrations than *Pediococcus* strains.

Still much remains to understand about a possible action offered by exopolysaccharides producing- LAB, against the cellular damage caused by SO_2 in wine. In this regard, Dols-Lafargue *et al.* (2008) showed that ropy Pediococci generally displayed high levels of resistance to SO_2.

1.8.4 Tolerance to lysozyme

Lysozyme is an enzyme which is normally present in saliva at concentrations up to 180 μg/ml (Koh *et al.,* 2004), it been proposed as an alternative to SO_2 in wine for the control of LAB and to delay malolactic fermentation. This enzyme acts by splitting the *β*-(1-4) linkage between N-acetyl muramic and N-acetyl-glucosamine, components of the peptidoglycan in the bacterial cell wall, leading to cell lysis and death (Lerm *et al.,* 2010).

Sever study on *Lactobacillus* showed a high resistance after treatment with lysozyme (Delfini *et al.,* 2004; Zago *et al.,* 2011; Turchi *et al.,* 2013; García-Ruiz *et al.,* 2014).

Although data in the literature on the role of exopolysaccharides to lysozyme resistance are limited, a *Pediococcus parvulus* strain able to synthesize a β-glucan, which confers a ropy texture to the wine, has been found to be resistant to lysozyme. This feature is ascribed to the presence of the β-glucan around the cell offering a protective barrier against stressor agents (Coulon *et al.,* 2012).

Nowadays, lysozyme represent an alternative to prevent bacterial spoilage in wine, especially those with a high pH value, in fact its use has been authorized in EU since October 2001 (OIV, 2011). Moreover, an advantage given by the use of lysozyme is that it does not alter the sensory properties (Lerm *et al.,* 2010).

1.8.5 Tolerance to bile

The bile salts are stored in the gall bladder during fasting conditions, after fat intake they are released into the duodenum, allowing the dispersion and absorption of fats, including bacterial phospholipids and cell membranes (Tannock *et al.,* 1994). The physiological concentrations of human bile are from 0.3% to 0.5% (Dunne *et al.,* 1999; Zavaglia *et al.,* 1998). In recent study, bile concentrations ranging from 0.15% to 0.5% were used (Vizoso-Pinto *et al.,* 2006), and a limit of 0.3% bile to select strains considered to have good resistance to bile has been established (Mathara *et al.,* 2008).

Such enological strains showed good resistance to bile (García-Ruiz *et al.,* 2014) in accordance with other results reported for *Bifidobacterium, Lactobacillus* strains and *P. pentosaceus* (Delgado *et al.,* 2008; Turchi *et al.,* 2013; Jensen *et al.,* 2012).

Bron *et al.* (2004) reported that following an increased concentration of bile a gradual decrease of the growth rate of *L. plantarum* is observed. Furthermore, this phenomenon coincided with a greater variation in the morphology of the cell surface, which may cause the loss of intracellular material, disturbing the energy balance. Moreover, the transcriptional profiling showed bile-responsive genes encoding proteins involved in the cell envelope and in tolerance against oxidative and acid stress (Bron *et al.,* 2006).

Exopolysaccharides layer produced by *Bifidobacterium breve* has a protective effect under bile conditions (Fanning *et al.,* 2012), and high EPS production of Bifidobacteria may be important in the selection of probiotic strains for resistance to bile salts (Alp *et al.,* 2010). *Streptococcus thermophilus* strains and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains from yogurt starter

showed a higher tolerance to bile salts, due to protective effect offered by high exopolysaccharides production (Boke *et al.,* 2010). Burns *et al.* (2010) identified 9 different proteins regulated after bile exposure of two *Lactobacillus delbrueckii* subsp. *lactis* strains, and 17 proteins that showed differences in their levels between the parental and the bile resistant derivative, including general stress response chaperones, proteins involved in transcription and translation, in peptidoglycan/exopolysaccharide biosynthesis, in the lipid and nucleotide metabolism and several glycolytic and pyruvate catabolism enzymes. The synthesis of EPS is favored following bile exposure in *B. animalis* subsp. *lactis* (Ruas-Madiedo *et al.,* 2009), and Lebeer *et al.* (2007) reported that bile stimulates biofilm formation in *Lactobacillus rhamnosus*. On the other hand, bile exposure of *L. acidophilus* caused a repression of exopolysaccharides biosynthesis gene expression (Pfeiler *et al.,* 2007). Koskenniemi *et al.* (2011) suggested that the presence of bile could serve as a signal of gut entrance, resulting in removal of EPS and parallel increased adhesion of *L. rhamnosus* cells to gut. In fact, exopolysaccharides biosynthesis decreased under bile stress condition.

2. AIMS OF THE RESEARCH

The aim of this PhD thesis is a polyphasic characterization of exopolysaccharides (EPS) produced by *Lactobacillus plantarum* strain, named Lp90, previously isolated from Apulian wine (Spano *et al.,* 2004), which exhibits a characteristic ropy phenotype ascribed to its capacity to produce EPS.

In particular, this study focused on:

- Genome sequencing of *L. plantarum* Lp90 and comparative genome analysis.
- Identification and comparative analysis of gene clusters involved in the EPS biosynthesis.
- Deletion of genes considered responsible for the ropy phenotype of *L. plantarum* Lp90, thus obtaining not-ropy mutant strains.
- Purification and preliminary characterization of exopolysaccharides produced by *L. plantarum* Lp90.
- Host-lactobacilli (EPS producing) interaction to evaluate the probiotic activities of *L. plantarum* Lp90 and the prebiotic properties of the exopolysaccharides produced.
- Role of exopolysaccharides in tolerance to stress of *L. plantarum* Lp90 and in relation to its original habitat (wine).
- Preliminary analysis using Lp90 ropy strain for yogurt production and immune-stimulation after yogurt digestion.

3. MATERIALS AND METHODS

3.1 Bacteria

3.1.1Bacterial strains

The bacterial strains used in this work are listed below:

- *Lactobacillus plantarum* Lp90 strain, which exhibit a characteristic ropy phenotype, ascribed to its capacity to produce exopolysaccharides. This strain was previously isolated from Apulian wine musts (Spano *et al.,* 2004). The complete genome of Lp90 strain has been recently sequenced (Accession number JIBX00000000) (Lamontanara *et al.,* 2015).
- *Lactobacillus plantarum* Lp90Δcps2, non-ropy mutant strain, deficient in *cps2* cluster involved in the exopolysaccharides biosynthesis (This study).
- *Lactobacillus plantarum* Lp90Δcps2.5, non-ropy mutant strain, deficient in part of *cps2* cluster (genes from Lp90_1073 to Lp90_1077) involved in the exopolysaccharides biosynthesis (This study).
- *Lactobacillus plantarum* WCFS1, a single colony isolate from *L. plantarum* NCIMB8826 (National Collection of Industrial and Marine Bacteria, Aberdeen, U.K.). Recently, its genome sequence has been re-annotated and deposited in MBL/GenBank at AL935263.2 (Kleerebezem *et al.,* 2003; Siezen *et al.,* 2012)
- *Lactobacillus plantarum* WCFS1Δcps2 (*Lactobacillus plantarum* WCFS1 NZ5333ACm), deficient in *cps2* cluster involved in the capsular exopolysaccharides biosynthesis (Remus *et al.,* 2012).
- *Lactobacillus plantarum* SF2A35B, a ropy strain previously isolated from sour cassava, (South America), (Figueroa *et al.,* 1995).

- *Lactobacillus plantarum* SF2A35BΔcps2, non-ropy mutant strain (provided by NIZO food research).
- *Lactobacillus plantarum* Lp90/pRCR12 (Russo *et al.,* 2015), recombinant Lp90 strain containing pRCR12 plasmid, encoding the monomeric red fluorescence protein "mCherry".
- *Lactobacillus plantarum* B2/pRCR12 (Russo *et al*., 2015), recombinant B2 strain (Arena *et al.,* 2014b) containing pRCR12 plasmid, encoding the monomeric red fluorescence protein "mCherry".
- *Escherichia coli* O157:H7 CECT 4267, used as intermediate cloning host for replication of pRCR12 plasmid.
- *Escherichia coli* strain TOP-10 (Invitrogen, Carlsbad, USA), used as intermediate cloning host for replication of mutagenesis plasmid employed in deletion of cps2 cluster.

3.1.2. Bacterial culture conditions

Lactobacilli were propagated on De Man Rogosa Sharpe broth (pH 6.2), which is a non-selective medium used for the growth of lactic acid bacteria, available as lyophilized powder (Oxoid, UK). It was prepared by resuspending 52 g in 1 litre of distilled H_2O.

Escherichia coli strains were grown on Tryptic Soy broth (Oxoid).

Solid MRS and Tryptic Soy were prepared by adding 15 g/L agar. All media were autoclaved at 121 °C for 15 minutes.

All lactobacilli were incubated anaerobically at 30 °C, while *Escherichia coli* strains were incubated aerobically and with shaking at 37 °C.

To obtain the bacterial cell pellet without medium, *L. plantarum* strains were centrifuged at 5,000 × *g* for 10 minutes at room temperature.

In order to eliminate the EPS, the bacterial cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) at 5,000 × *g* for 10 min. Washed bacteria were used in several assays, as control strain without exopolysaccharides.

3.2 Transmission Electron Microscopy

The EPS production was analyzed by negative staining TEM. 300 µL of cell culture (OD_{600nm}=1.2) were centrifuged at 12,000 × g for 2 min at 21 °C. Bacteria were resuspended in 100 µl of 0.1 M ammonium acetate buffer at pH 7.0 before being analyzed.

For microscopy analysis, commercial electron microscopy grids copper coated Formvar 300 holes and coal (Electron Microscopy Sciences, Hatfield, PA, USA) were used. Prior use, grids were subjected to a minute ionic discharge, in order to make hydrophilic the carbon film thus favoring the sample adsorption. 10 µl of the sample were placed below the grid for 1 min, subsequently the excess solution was removed by filter paper. Furthermore, to identify the negative staining, the grid was placed on a drop of uranyl acetate at 2% for 40 s. (Maeyama *et al.,* 2004). Finally, the excess staining agent was withdrawn and the grid was allowed to air dry before microscopic observation in a JEOL 1230 transmission electron stabilized at 100 kV. Images were digitalized using an Epson Perfection 4870 Photo scanner at 1200 dpi final resolution.

3.3 Exopolysaccharides produced by *L. plantarum* Lp90

3.3.1 Exopolysaccharides isolation

For the isolation of exopolysaccharides produced by *L. plantarum* Lp90, bacteria were grown in SMD (Semi-Defined Media) buffered at pH 6.0, (glucose 20 g/L; casamino acids 5 g/L; bacteria yeast nitrogen base 6,7 g/L; Tween 80 1 g/L; diammonium citrate 2 g/L; $MnSO_4 \cdot 4H_2O$ 0,05 g/L;

K_2HPO_4 2 g/L; sodium acetate 5 g/L; adenine 0,005 g/L; guanine 0,005 g/L; xanthine 0,005 g/L; uracil 0,005 g/L; L-malic acid 4 g/L) (Dueñas-Chasco *et al.,* 1997). An overnight culture was sedimented by centrifugation at 14,000 × *g* for 30 min at 20 °C. Three volumes of cold absolute ethanol were added to the supernatant and stored overnight at -20 °C. The precipitate was recovered by centrifugation, resuspended in deionized water and treated with trichloroacetic acid 12% (w/v) for 30 min at 4 °C. The pellet was removed by centrifugation and the supernatant was neutralized with NaOH to pH 5-6, before adding three volumes of cold ethanol at -20 °C overnight. EPS were recovered by centrifugation, dialyzed on membrane of 12-14 kDa (Medicell International Ltd, London), and freeze-dried.

3.3.2 EPS quantification by phenol-sulfuric acid method

EPS quantification was determined by the phenol-sulfuric acid method (Dubois *et al.,* 1956) using D-glucose to achieve a standard curve. The freeze-dried EPS were resuspended in one volume of deionized water and a volume of phenol solution 5% (v/v) and 5 volumes of concentrated sulfuric acid were added. Immediately the samples were incubated at 100 °C for 5 min. The reaction was stopped in an ice bath and absorbance at 490 nm was measured.

3.3.3 Determination of monosaccharide composition

Freeze-dried EPS was dissolved in ultrapure water and dialyzed with two different membranes Amicon Ultra Centrifugal Filter Devices (Millipore, Billerico, MA, USA) having a cut-off from 3 to 100 kDa. After dialysis, the solution was frozen at -80 °C and lyophilized (Telstar Cryodos equipment, Spain).

For analysis of neutral sugars, the polysaccharides (approximately 1 mg) were first hydrolyzed with 3 M TFA (121 °C, 1 h). The monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ and subsequent acetylation (Laine *et al.,* 1972). Identification and quantification were performed by gas-liquid chromatography (GLC) on a

6890A instrument (Agilent) equipped with a flame-ionization detector, using a HP5 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with He as the carrier gas. Injector and detector were set at 250 °C. Samples (1 μL) were injected with a split ratio of 1:50, with a temperature program: 160 °C for 5 min, then 3.5 °C min^{-1} to 205 °C and finally 210 °C for 0.5 min. Identification was performed on the basis of the coincidence of the retention time of sample components with those previously measured for standards analyzed in identical conditions, using inositol as internal standard. Phosphate content was deduced from inorganic phosphate determination on a 5500 Inductively Coupled Plasma instrument (Perkin Elmer).

3.4 Genome sequencings and annotation of *Lactobacillus plantarum* Lp90

3.4.1 Genomic DNA isolation

Genomic DNA of *L. plantraum* Lp90 was isolated using the extraction kit PowerMicrobial Midi DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to manufacturer's procedure.

3.4.2 Genome sequencing and assembly

For genome sequencing and assembly 2 μg of Genomic DNA was subjected to library preparation using the "TruSeq DNA Sample Prep Kit FC-121-1001" according to the manufacturer's instructions. Whole genome sequencing of Lp90 was performed using the Illumina GAIIx platform.

Before assembly, the raw reads were subjected to a filtering, using PRINSEQ v0.20.3 software (Schmieder and Edwards, 2011), with the aim to remove: the 3' ends showing a quality score below 25 (Q<25); the reads shorter than 75 bp; the reads with an average quality score below 25 (Q<25); the reads containing a percentage of unknown bases equal or greater than 10%; the duplicated reads. After filtering a total of 16,574,199 paired end reads ranging from 75 to 115 bp lengths were obtained corresponding to coverage of about 1,000×. The genome sequences were

assembled again by Ray v2.2.0 assembly program (Boisvert *et al.,* 2010) with default parameters and using a Kmer size of 71. The assembly resulted in 33 contigs with an N50 length of 207,479 bp. The size of the shortest contig was 354 bp while the length of the longest contig was 489,345 bp.

3.4.3 Genome annotation

Genome annotation (ORF calling, gene function prediction) of the assembled genome was performed using the RAST (Rapid Annotation using Subsystem Technology) server (Aziz *et al.,* 2008). Start codons of the predicted ORFs were verified manually, aligning the Lp90 ORFs with homologous ORFs from other sequenced *L. plantarum* strains, exactly: WCFS1, ZJ316, STIII, P8, JDM1 and 16. ORF functional annotations were refined by aligning ORF nucleotide sequences to the Cluster of Orthologous Groups (COG) database (Tatusov *et al.,* 2003) using BlastP and by using the functionality of InterProScan v5.0 in Blast2GO (Conesa *et al.,* 2005) searching for matches against the PRINTS (v42.0), Pfam (v27.0) and TIGRFAMs (v13.0) databases. The TMHMM (v2.0) and Phobius (v1.01) prediction search tools were used respectively to predict transmembrane domains and the presence of signal peptides.

3.5 Construction of genes-deletion *Lactobacillus plantarum* Lp90 mutant strain

3.5.1 Generation of mutagenesis plasmids

For the construction of two *cps*2 non-ropy mutants strain of *Lactobacillus plantarum* Lp90 (Lp90Δcps2 and Lp90Δcps2.5), the Cre-*lox*-based system for multiple gene deletions in *Lactobacillus plantarum* were used (Lambert *et al.,* 2007). In Lp90Δcps2 mutant strain were deleted all the genes of the cluster *cps2*, exactly from Lp90_1067 to Lp90_1077 while in Lp90Δcps2.5 mutant strain were deleted genes from Lp90_1073 to Lp90_1077 (**Figure 3.1**).

Figure 3.1 - Organization of *cps2* cluster in *Lactobacillus plantarum* Lp90.

The knockout mutants were generated by a homologous recombination-based double cross over strategy. To generate two mutagenesis plasmids pNZ8220 and pNZ8221, (**Figure 3.3**) for the construction of Lp90Δcps2 and Lp90Δcps2.5 respectively, was employed a mutagenesis vector plasmid which is unable to replicate in Gram positive bacteria (pNZ5319) (**Figure 3.4**) (Lambert *et al.,* 2007). pNZ5319 contained the upstream (*LF*) and downstream (*RF*) flanking homologous regions of the target genes to delete; precisely *LF1* and *RF* for pNZ8220, and *LF2* and *RF* for the pNZ8220. Target genes were deleted and replaced by the chloramphenicol (*cat*) marker in the event of a double cross-over recombination, by the splicing overlap extension (*SOE*) method (Horton, 1993). The left flanking region (*LF*) and the right flanking region (*RF*) were amplified by PCR using primers (**Table 3.1**) containing an overhang region homologous to the ultimate 5' and 3' regions of the *cat* amplicon (**Figure 3.2, step 1**). PCR was performed using KOD Hot Start DNA polymerase (EMD Bioscience, Gibbstown, USA) according to manufacturer's procedure. The PCR products were analyzed by electrophoresis on 1% agarose gel and purified by Invisorb MSB Spin PCRapace purification kits (Invitek Stratec Molecular GmbH, Germany). Subsequently, for *SOE* product construction the *LF* and *RF* fragments were combined with the *cat* amplicon as template in a second PCR reaction using the 5' primer of the upstream homologous region and the 3' primer of the downstream homologous region, resulting in one amplicon containing all three initial PCR products (**Figure 3.2, step 4**). Each mutant was combined with the *cat* fragment containing a unique DNA-tag (Bron *et al.,* unpublished data) (tag10.3 and tag10.4 for Lp90Δcps2 and Lp90Δcps2.5 respectively). The DNA-tag is a randomly generated sequence that can be used to discriminate the different mutant strains by sequencing methods. The resulting *SOE* product were analyzed on 1% agarose gel and the 3.2 kb band were

purified from the gel using Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, USA).

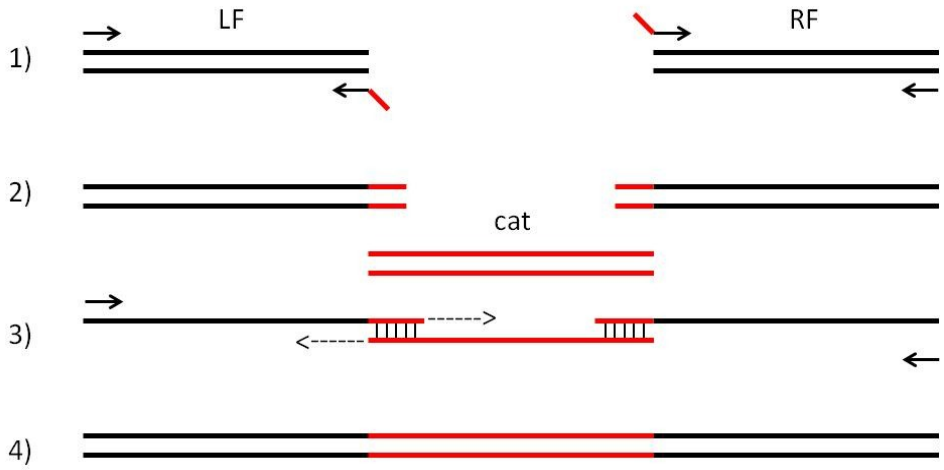

Figure 3.2 - Splicing by overlap extension (*SOE*) method. Upstream (*LF*) and downstream (*RF*) homologous regions flanking the target gene are amplified by primers designated with overhang regions (red) consisting of a *cat* sequences (step 1). The amplified fragments of *LF* and *RF* are combined with *cat* fragment to act as template in a second PCR reaction using the 5' primer of *LF* and 3' primer of *RF* to perform the *SOE* (step 2 and 3), resulting in a *SOE* product containing the *LF*, *cat* and *RF* fragment (step 4). *(from Van Bokhorst-Van De Veen, H. et al., 2012b).*

Oligonucleotide	Sequence (5'- 3')	References
Out1 For	GCCATAGCTGTACGCTAAAAGG	*[1]
LF1 For	AGTATCGGGTGCGACCGATG	*[1]
LF1 Rev	GCATACATTATACGAACGGTAGATTTTGCTTGATCCATCATTCACTCTCC	*[1]
Out2 For	GTGCTGACAGAGGAGTTTAG	*[1]
LF2 For	GAAGATTATTCAGGACTGATG	*[1]
LF2 Rev	GCATACATTATACGAACGGTAGATTTAATCATTGTCCCCCATATAAC	*[1]
RF For	CGGTTACAGCCCGGGCATGAGTGCACAGTGTTTCCGACTGAG	*[1]
RF Rev	GCTATCGCCGCTTTACATGC	*[1]
Out Rev	CGGCTTACCATATCTCATCG	*[1]
R20 For	AATAGTTATCTATTATTTAACGGGAGG	*[2]
R87 For	GCCGACTGTACTTTCGGATCC	*[2]
R120 Rev	AGAACAATCAAAGCGAGAATAAGG	*[2]
Is169 Rev	TTATCATATCCCGAGGACCG	*[2]
Is6 For	CGATACCGTTTACGAAATTGG	*[2]
Is7 Rev	CTTGCTCATAAGTAACGGTAC	*[2]
Is8 For	TCAAATACAGCTTTTAGAACTGG	*[2]
Is9 Rev	ATCACAAACAGAATGATGTACC	*[2]

*[1] This study
*[2] Van Bokhorst-Van De Veen, H. *et al.,* 2012b.

Table 3.1 - Sequences of the primers used for the amplification fragments related to *LF*, *RF*, *SOE*, *cat* and *ery* genes.

To prepare the mutagenesis backbone, pNZ5319 vector was digested by 10U of each *Swa*I (New England BioLabs, United Kingdom) and *Ecl*136II (Fermentas UAB, Vilnius, Lithuania). The restriction enzyme reactions were conducted in the condition recommended by the commercial supplier. The digested pNZ5319 was separated by 1% agarose gel elettrophoresis. The backbone 2.7kb fragment was excise and eluted from the gel using Wizard® SV Gel and PCR Clean-Up System kit (Promega). Mutagenesis plasmids pNZ8220 and pNZ8221 were made by blunt-ends ligation between 2.7kb fragment from pNZ5319 and 3.2kb *SOE* products. The ligations were catalyzed by T4 DNA ligase (Invitrogen Carlsbad, CA, USA).

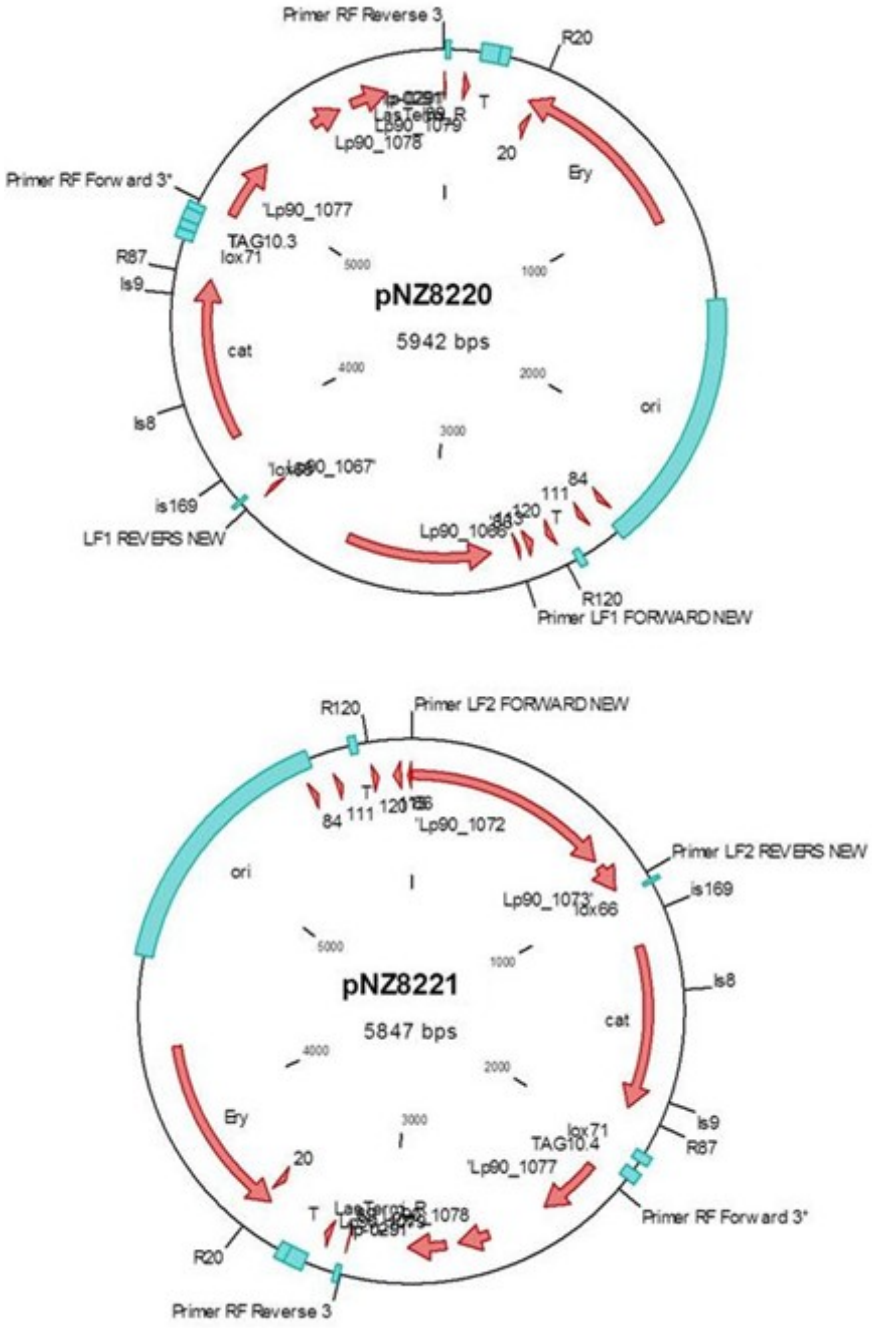

Figure 3.3 - Mutagenesis plasmids pNZ8220 and pNZ8221. The plasmids were constructed by blunt-ends ligation between 2.7kb fragment from pNZ5319 and 3.2kb *SOE* products**.**

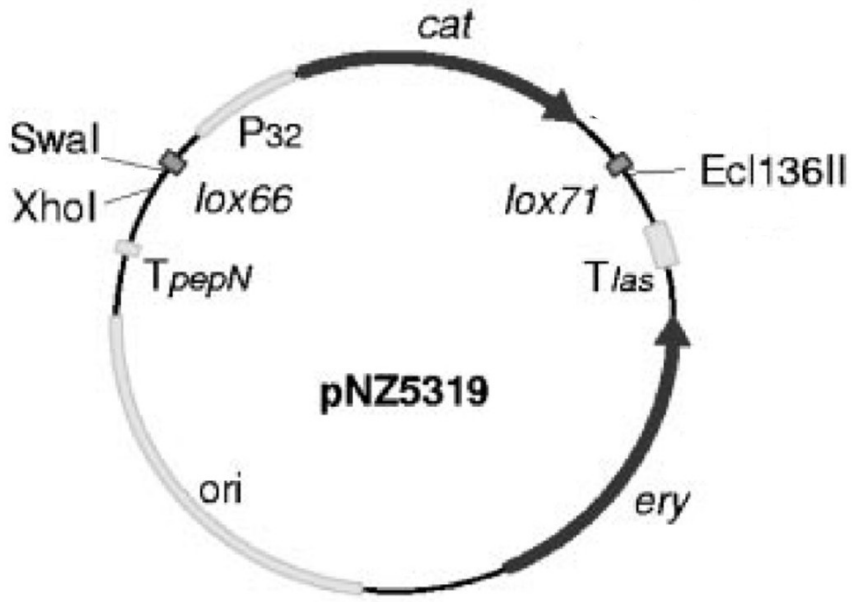

Figure 3.4 - Representation of the mutagenesis vector pNZ5319. Origin of replication (*ori*), erythromycin resistance gene (*ery*), chloramphenicol resistance gene (*cat*) under the control of the P32promoter (P32-*cat*), flanked by *lox66* and l*ox71* sites, lactococcal T*las* and T*pepN* terminators. Rare-cutting sites are: blunt-end restriction sites *Swa*I, and *Ecl*136II, and sticky-end restriction sites *Xho*I, respectively. The two selectable-marker gene cassettes (P32-*cat* and *ery*) allows direct selection of double-crossover integrants based on their antibiotic resistance (Cm^r) and sensitivity (Em^s) phenotype. (adepted *from Lambert et al, 2007).*

3.5.2 *Esherichia coli* transformation procedure

The ligation mixture was chemical transformed into One Shot TOP10 cells according to manufacturer's procedure. The transformed *E. coli* cells were grown on TYA containing 5µg/mL chloramphenicol plates at 37 °C for 2 days.

Colony PCR (Sandhu *et al.,* 1989) was performed to screen the colonies containing correct mutagenesis plasmids with corresponding *SOE* products. To eliminate false positive, the colonies from the transformation were transferred to new TYA + 5µg/mL chloramphenicol plates (Dallas-Yang *et al.,* 1998) and the newly grown colonies were used for screening. The presence of *SOE* products were confirmed by using the forward primer of *LF* (LF1For for Lp90Δcps2 and LF2For for Lp90Δcps2.5), and the reverse primer ls169 that is complement to *cat* fragment (**Table 3.1**). PCR was initiated with 10 min at 95 °C, followed by 35 cycle of amplification (30 sec at 95 °C; 30 sec at 50 °C; 1 min at 72 °C) and finished with 5 min at 72 °C. The PCR mixture was prepared from 2x PCR Master Mix (Promega).

Restriction enzyme digestion patterns were used to reconfirm the presence of *SOE* insert. The plasmids were isolated from the colony-PCR positive colonies and then subjected to *Xho*I (Invitrogen) digestion. The plasmids were confirmed by DNA sequencing by using 4 primers (R20, R87, R120 and ls169) (**Table 3.1**).

Plasmid DNA was isolated from *E. coli* using Jetstar columns as recommended by the supplier (Genomed GmbH, Bad Oberhausen, Germany) and DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands).

3.5.3 Electrocompetent cells and electroporation of *L. plantarum* Lp90

For the preparation of electrocompetent cells of *L. plantarum* Lp90 was grown until OD_{600nm} of 0.60 - 0.65 and then chill on ice for 15 minutes, shaking by inverting every 2 - 3 minutes. The cells were recovered by centrifugation at 5,000 × g for 10 minutes at 10 °C and the pellet was washed with 1 volume of cold 1 mM $MgCl_2$, subsequently washed with 1 volume of cold 30% PEG 1450 and finally was resuspended in 1/100 volume 30% PEG 1450.

10 µL (1µg) of mutagenesis plasmids were mixed gently with 40 µL of competent cells of *L. plantarum* in an electroporation cuvette previously cooled on ice. The electroporation was carried out using a Gene Pulser Xcell with Shock Pod Cuvette Chamber (BIORAD) and the parameters were 1.5 kV, 400 Ω and 25 µF. After the electrical impulse were added 500 µL of MRS containing 0.1M $MgCl_2$ + 0.5 M sucrose, incubated at 37 °C for 2 hours, plated on MRS supplemented with chloramphenicol (10 µg/mL) and incubate at 37 °C for 2 days. Candidate double-crossover clones (Cm^r Em^s) were selected on MRS agar containing chloramphenicol (10 µg/mL) and replica-plated on MRS agar supplemented with erythromycin (30 µg/mL) to check for erythromycin sensitivity. Subsequently, genomic DNA was extracted from Cm^r and Em^s colonies using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) and analyzed by PCR amplification of the *LF1* and *LF2* (Out1 For and Out2 For respectively; Is169 Rev), *RF* (R87

For; Out Rev), *SOE1* and *SOE2* (Out1 For and Out2 For respectively; Out Rev), *cat* (Is8 For; Is9 Rev), *ery* (Is6 For; Is7 Rev) fragments using primer listed in **table 3.1**.

All primers were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands).

3.6 Caco-2 cells: *in vitro* assays

3.6.1 Caco-2 cells growing condition

Caco-2 cells, from human colon carcinoma, were grown in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) with the addition of 10% (vol/vol) heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U/mL penicillin and 50 µg/mL streptomycin (GIBCO). Cells were incubated at 37 °C in humidified atmosphere containing 5% CO_2.

3.6.2 Caco-2 cell culture, adhesion and competition assays for adhesion between *E. coli* and *Lactobacillus plantarum*

For adhesion tests, Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest, Becton Dickinson, NJ, USA) at a concentration of 1.6×10^4 cells per well and cultured for 12-15 days, as previously described (Bove *et al.,* 2012), to obtain monolayers of differentiated cells that mimic small intestine mature enterocytes (Pinto *et al.,* 1983; Fernández de Palencia *et al.,* 2008).

In competition assay for adhesion between the pathogen and *Lactobacillus plantarum,* Caco-2 monolayers (about 5.0×10^4 cells/well, as counted in a Bürker chamber) were overlaid with exponentially growing *E. coli* O157: H7 cells and *L. plantarum* cells (about 5.0×10^7 CFU/well) from exponentially growing phase cultures (OD_{600} 0.6). For simple adhesion tests of *L. plantarum,* Caco-2 cells monolayers (about 5.0×10^4 cells/well) were overlaid with exponentially growing (OD_{600} 0.6) or (late) stationary phase cultures of lactobacilli (OD_{600} 5.0). In both

adhesion and competition assay the multiplicity of exposure (MOE) was 1:1,000, Caco-2 cells to bacteria.

After 1 h of incubation at 37 °C under 5% CO_2 atmosphere, test wells were washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove unbound bacteria. No washing was performed on control wells, with the aim to recover both adherent and not adherent bacteria. Caco-2 cells and adherent bacteria were then detached by trypsin-EDTA 0.05% (GIBCO) treatment (10 min, 37 °C) and resuspended in sterile PBS (GIBCO). Serial dilutions of *L. plantarum* and *E. coli* O157: H7 samples were plated onto MRS and LB agar plates, respectively, to determine the number of cell-bound bacteria (viable counts) expressed as CFUs. CFU counts from unwashed control wells provided total bacterial load (i.e. both adherent and not adherent bacteria). The adhesion rate of *E. coli* was determined by quantitative real time PCR (qPCR) analysis on suspensions obtained from test and control wells (see below). All adhesion experiments were performed in triplicate.

The ability of expolysaccharides produced by *L. plantarum* Lp90 to inhibit *E. coli* O157:H7 adhesion on Caco-2 cells was analysed by (i) competitive adhesion: *E. coli* O157: H7 and *L. plantarum* strains were simultaneously added to Caco-2 cells and co-incubated for 1 h; (ii) displacement of adhesion: *E. coli* O157: H7 was added first to Caco-2 cells and incubated for 1 h, then *L. plantarum* was added and further incubated for 1 h; (iii) inhibition of adhesion: *L. plantarum* was added first and incubated for 1 h, then *E. coli* O157: H7 was added and further incubated for 1 h (Arena *et al.,* 2014b; Koo *et al.,* 2012). Assays of competitive, displacement and inhibition of adhesion of *E. coli* on Caco-2 monolayers were also performed with purified EPS produced by Lp90 at concentrations of 0.1 and 1.0 mg/mL.

After incubation at 37 °C, enumeration of adherent *E. coli* cells was determined by qPCR on samples recovered from test and control (i.e. unwashed) wells (Arena *et al.,* 2014b).

With the aim to understand the influence of EPS on adhesion of *L. plantarum*, adherence assays were performed with either (i) native bacterial cells or (ii) bacterial cells that were washed with

phosphate-buffered saline (PBS, pH 7.4) prior to resuspension in DMEM, in order to remove the EPS attached to the bacterial cell surface (Garai-Ibabe *et al.,* 2010).

To quantify adherent bacterial (*E. coli* O157:H7) cells by real time PCR, cell suspensions from adhesion assays were heat treated (10 min, 95 °C) and then chilled on ice. Aliquots (3µl) were then mixed with 1x iTaq supermix (Bio-Rad), O antigen specific gene (*fliC* H7) TaqMan probe (200nM) and primers (500 nM each) (Perelle *et al.,* 2004).

Reactions were cycled in an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA) as it follows: initial denaturation at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and fluorescence acquisition (FAM) at 72° C for 30 s. Each PCR assay included duplicate reactions on DNA (cell suspension) samples, on no template (negative) control and on (internal standards, i.e.) serial dilutions of *E. coli* suspension (corresponding to a concentration) ranging from 1×10^4 to 1×10^8, to generate a standard curve which was used for quantification.

3.6.3 Caco-2 cells immune stimulation assay

Immune stimulation of Caco-2 cells was performed as previously described by Bove *et al.,* (2012). Briefly, cells were seeded in 24-well tissue-treated culture plates (Iwaki) at a concentration of about 1.8×10^4 cell/mL. The culture medium was changed every 2 days and 24 hours before bacterial addition, an antibiotic and serum-free medium was used in order to avoid any interference with bacterial viability and with immune gene expression. Post-confluent Caco-2 cells, about 6×10^5 cells per well, were incubated with *L. plantarum* cells at a concentration of 5×10^8 CFU/mL (1 mL/well).

3.6.4 RNA isolation and cDNA synthesis transcript profiling

Total RNA was extracted from untreated Caco-2 cells (control) after 1 and 3 h of bacterial stimulation. Cells were washed with PBS and harvested with TRIzol reagent (Invitrogen,

Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration and integrity were determined by spectrophotometry (Biotek Instruments, Winooski, VT, USA) and gel electrophoresis. One microgram of total RNA was reverse-transcribed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA) which includes a genomic DNA elimination reaction. Absence of DNA contamination was confirmed by real-time PCR on DNase I-treated non-retrotranscribed RNAs.

3.6.5 Quantitative Real Time (PCR) and transcriptional profiling

The transcriptional level of immune-related genes (**Table 3.**2) was analysed by quantitative real-time PCR (ABI 7300; Applied Biosystems, Foster City, CA, USA) using SYBR green I detection. Each reaction mixture, containing 5 μL of 40-fold diluted cDNA, 10 μL of 2× QuantiFast SYBR Green PCR Master Mix (Qiagen) and 250 nM of primers was subject to amplification as previously described (Fiocco *et al.,* 2010; Bove *et al.,* 2012). All PCR assay was performed in duplicates of each cDNA samples, were also incuse reactions without cDNA template and RNA controls in order to check contamination. Fluorescence data were analysed by applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Untreated Caco-2 cells corresponded to the calibrator condition. Three potential housekeeping genes, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and hypoxanthine phosphoribosyl transferase 1 (HPRT1), were monitored. Their average level was used to normalize the expression of target genes.

Oligonucleotide	Sequence (5'- 3')	References
GAPDH For	CGACCACTTTGTCAAGCTCA	Bove *et al.,* 2012
GAPDH Rev	AGGGGTCTACATGGCAACTG	Bove *et al.,* 2012
β-Actin For	AAAGACCTGTACGCCAACAC	Bove *et al.,* 2012
β-Actin Rev	CATACTCCTGCTTGCTGATCC	Bove *et al.,* 2012
MIP-3α For	CTGGCTGCTTTGATGTCAGTG	Bove *et al.,* 2012
MIP-3α Rev	GGATTTGCGCACACAGACAA	Bove *et al.,* 2012
HBD-2 For	ATCAGCCATGAGGGTCTTGT	Bove *et al.,* 2012
HBD-2 Rev	GAGACCACAGGTGCCAATTT	Bove *et al.,* 2012
LYZ For	AAAACCCCAGGAGCAGTTAAT	Bove *et al.,* 2012
LYZ Rev	CAACCTTGAACATACTGACGGA	Bove *et al.,* 2012
MUC-2 For	CCAAGACCGTCCTCATGAAT	Bove *et al.,* 2012
MUC-2 Rev	TCGATGTGGGTGTAGGTGTG	Bove *et al.,* 2012
IL-6 For	TACCCCCAGGAGAAGATTCC	Bove *et al.,* 2012
IL-6 Rev	TTTTCTGCCAGTGCCTCTTT	Bove *et al.,* 2012
IL-8 For	TGTGGAGAAGTTTTTGAAGAGGG	Bove *et al.,* 2012
IL-8 Rev	CCAGGAATCTTGTATTGCATCTGG	Bove *et al.,* 2012
hIL10-For	CCAAGCTGAGAACCAAGACC	This study
hIL10-Rev	ATAGAGTCGCCACCCTGATG	This study
IL12A-For	GATGGCCCTGTGCCTTAGTA	This study
IL12A-Rev	TCAAGGGAGGATTTTTGTGG	This study
hHPRT1-For	TGCTCGAGATGTGATGAAGG	This study
hHPRT1-Rev	TCCCCTGTTGACTGGTCATT	This study
hIKBa-For	GCAAAATCCTGACCTGGTGT	This study
hIKBa-Rev	GCTCGTCCTCTGTGAACTCC	This study
hTLR2-For	GGCCAGCAAATTACCTGTGT	This study
hTLR2-Rev	TTCTCCACCCAGTAGGCATC	This study
hTNFα-For	AACCTCCTCTCTGCCATCAA	This study
hTNFα-Rev	ATGTTCGTCCTCCTCACAGG	This study
hZO2-For	GCCAAAACCCAGAACAAAGA	This study
hZO2-Rev	CAGGACTGATTTGGGAGCAT	This study
hCLDN4-For	TTGTCACCTCGCAGACCATC	This study
hCLDN4-Rev	CAGCGAGTCGTACACCTTG	This study
LL37-For	CATCATTGCCCAGGTCCTCA	This study
LL37-Rev	GGCACACTGTCTCCTTCACT	This study

Table 3.2 - Sequences of the primers used for qRT-PCR analysis of human genes.

3.7 Zebrafish *in vivo* model

3.7.1 Transfer of pRCR12 to *L. plantarum* strains

The pRCR12 plasmid, which encodes a monomeric mCherry fluorescent protein, was previously constructed by Russo *et al.,* (2015).

For the preparation of electrocompetent bacterial cells, *L. plantarum* strains Lp90 and B2 were grown until OD_{600nm} of 0.60 - 0.65 and then chill on ice for 15 minutes, shaking by inverting every 2 - 3 minutes. The cells were recovered by centrifugation at 3,000 × g for 10 minutes at 10 °C and the pellet was washed with 1 volume of cold 1 mM MgCl2, subsequently washed with 1 volume of cold 30% PEG 1500 and finally was resuspended in 1/100 volume 30% PEG 1500.

For the electroporation procedure, 50 µL of the cell suspension and 5 µL of pRCR12 plasmid DNA (100 ng/µL) were mixed in a previously cooled Gene Pulser 0.1 cm cuvette (Bio-Rad). The electroporation was performed using a Gene Pulser Xcell with Shock Pod Cuvette Chamber (Bio-Rad) using the following parameters: voltage 1,500 V; resistance 400 Ω; capacitance 25 µF. After the electrical pulse 500 µL of MRS supplemented with 1 mM $MgCl_2$ and 0.3 M sucrose were immediately added and cells were incubated at 37 °C for 2 h. Transformed cells of *L. plantarum* strains with pRCR12 plasmid, were selected on MRS agar containing chloramphenicol at concentration of 10 µg/mL and incubated at 37 °C for 48 hours until the appearance of pink colonies.

3.7.2 mCherry protein fluorescence determination during bacterial growth

The transformed culture of *L. plantarum* Lp90/pRCR12 and B2/pRCR12 were diluted in MRS containing 10 µg/mL of chloramphenicol to OD_{600nm} of 0.05, and 200 µL of each culture were placed in triplicate in Costar 96 - Well EIA/RIA Plate stripwells (ImmunoChemistry Technologies, Bloomington, MN). The levels of fluorescence of the mCherry protein and bacterial growth were measured at the same time using Varioskan Flash system (Thermo Fisher

Scientific, Waltham, MA). The instrument provided quantitative data of cell density by measuring the OD at 600 nm and mCherry expression at an excitation wavelength of 587 nm and an emission wavelength of 612 nm.

3.7.3 Determination of pRCR12 plasmid copy number

Genomic DNA (chromosomal and plasmidic) was extracted from *L. plantarum* strains containing pRCR12 plasmid as described by (Russo *et al.,* 2015). Briefly, bacterial cells were harvested to OD_{600} 1, by centrifugation at 14,000 × *g* for 10 min at 4 ºC and washed with PBS. The pellets were resuspended in 100 µL lysis buffer (20% sucrose, 10 mM EDTA, RNAse at 8 mg mL^{-1}, mutanolysin at 240 U μL^{-1} and lysozyme at 30 mg mL^{-1}) and incubation for 15 min at 37 °C. Subsequently, SDS was added to a final concentration of 1% and crude extracts were passed through a needle to reduce their viscosity. Samples were deproteinated by two extractions with 100 µL of phenol:chloroform (1:1) containing 4% isoamyl alcohol; DNA was precipitated adding 0.3 M sodium acetate pH 7.0 and 2.5 volumes of absolute ethanol and finally resuspended in 100 µL of 10 mM Tris-HCl pH 8.0. The genomic DNA preparations were analyzed on 1.2% agarose gel.

The plasmid copy number (average of three determinations) was calculated using the equation developed by Projan *et al.* (1983):

$$N = \frac{(1.36Dp1) \times Mc}{Dc \times Mp}$$

Dp1 is the intensity value determined for the covalently closed plasmid forms. Mc is the molecular weight of the *L. plantarum* WCFS1 chromosome (3,308,274 bps) and Dc is the intensity value for the chromosomal DNA. Mp is the molecular weight of the plasmid pRCR12 (4,600 bps). The coefficient 1.36 is introduced to correct for the differences in fluorescence due to the efficiency of ethidium bromide to intercalate with linear and supercoiled DNA.

3.7.4 Zebrafish processing

Zebrafish embryos were obtained from wild type adult zebrafish (*Danio rerio*, Hamilton 1822) bred in the AZTI Zebrafish Facility (REGA Number ES489010006105; Derio, Spain) following standard conditions and zebrafish were maintained according to standard protocols (Nüsslein-Volhard and Dahm 2002), and they were fed as described by Russo *et al.* (2015).

Zebrafish embryos were disinfected and the axenity was tested (Russo *et al.,* 2015).

3.7.5 Challenge test and enumeration of *L. plantarum* strains transformed with pRCR12 in infected zebrafish larvae

L. plantarum Lp90/pRCR12 and B2/pRCR12 were grown in MRS broth supplemented with chloramphenicol at 10 μg mL^{-1} and 0.05% cysteine. Overnight cultures were washed three times in PBS (pH 7.0) at room temperature and then diluted to 10^7 CFU/mL. 10 mL of each dilution were poured into Petri dishes (5.5 cm diameter × 1.0 cm). About 10 or 15 gnotobiotic zebrafish larvae of 4 days post fertilization (dpf) were placed into each Petri dishes and incubated at 27 ºC with shaking (90 rpm). After 18 h, the medium was removed and the larvae were washed three times with PBS. After a period of 6, 24, 48 and 72 h, individual larvae were examined visually by Leica MZFL III stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany), (Russo *et al.,* 2015).

For the enumeration of *L. plantarum* Lp90/pRCR12 and B2/pRCR12, zebrafish larvae were euthanized by 200 mg/mL tricaine (MS-222) (Sigma Aldrich) and washed with sterile PBS-0.1% (v/v) and Tween 20 to remove the bacteria loosely attached to the skin. 15 larvae were homogenized with a Pellet Pestle Cordless Motor (Kimble Chase, Vineland, NJ) in 500 μL of PBS. Serial dilutions of the recovered suspension were plated on MRS agar containing 10 μg/mL of chloramphenicol, to determine colony forming units (CFU) (Russo *et al.,* 2015).

3.8 Biofilm formation

The ability of *L. plantarum* strains to adherence on glass surface, thus forming a biofilm was assayed according to Vergara-Irigaray *et al.* (2009), with modifications. Briefly, 5 ml of MRS broth were inoculated with 2% (v/v) of (over-night) cultures of *L. plantarum* strains and incubated for 1, 2 and 7 days at 37 °C, in an orbital shaker at 200 rpm. Residues were washed twice with distilled water, air-dried and then it was stained with crystal violet solution (5 g/L, 0.5% w/v). The biofilm (ring) was solubilized with acetic acid (30% v/v) and optical density was measured at 570 nm. Each experiment was carried in triplicate.

3.9 *Lactobacillus plantarum* strains during *in vitro* gastro-intestinal tract condition

Bacterial cells culture, both in late exponential (OD_{600nm} 1) and stationary (25 hours cells after inoculation diluted to OD_{600nm} 1) (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK) growth phases, were subjected to gastro-intestinal (GI) assay as described previously for *L. plantarum* WCFS1 (van Bokhorst-van de Veen *et al.,* 2012a).

In detail, all *L. plantarum* strains were recovered by centrifugation at 10,000 rpm for 2 min and washed with preheated PBS at 37 °C. Bacterial cells were resuspended in gastric juice (GJ) (freshly added pepsin and lipase) and incubated at 37 °C for 1 hour with head-tailed rotation at 10 rpm. Gastric juice contained: lipase 0.1 g/L (Fluka 62301-G-F from *Aspergillus niger*); pepsin 1.2 g/L (Sigma P-7125 prom porcine stomach); NaCl 3.1 g/L; KCl 1.1 g/L; Na_2CO_3 0.6 g/L; $CaCl_2$ 0.11 g/L. Gastric juice was adjusted at pH 2.4 by adding of HCl 5 M and used for exponential growth phase cells. Gastric juice adjusted at pH 2.3 was used for stationary growth phase of *L. plantarum*. Subsequently, the samples were pH-neutralized adding preheated $NaHCO_3$ 10.3 mM and pancreatic juice (PJ) was added and further incubated for 1 hour as above. Pancreatic juice consisted in: pancreatin 30 g/L (Sigma P7545 form porcine stomach); Bile (sodium gluycocholate hydrate G7132 7.32 g/L; sodium glycodeoxycholate G9910 3.04

g/L; sodium glycochenodeoxycholate G0759 5.59 g/L; taurocholic acid sodium salt hydrate T4009 2.74 g/L; sodium taurodeoxycholate hydrate T0557 0.94 g/L sodium taurochenodeoxycholate T6260 2.58 g/L); NaCl 5.0 g/L; KH_2PO_4 0.68 g/L; Na_2HPO_4 0.30 g/L; $NaHCO_3$ 0.84 g/L). Samples were taken before incubation and after GJ and PJ addition to determine relative survival rates on basis of colony forming units (CFUs) by spot plating of serial dilutions followed by incubation at 30 °C for 48 hours.

3.10 *L. plantarum* Lp90 (EPS producing) in yogurt: oro-gastro-intestinal an immune-stimulation *in vitro* assays

3.10.1 Yogurt production

For yogurt homemade production, three different milk fermentations were performed:

(i) *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (positive control);

(ii) *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum* Lp90;

(iii) *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum* WCFS1 (*L. plantarum* non-EPS producing control strain).

Streptococcus thermophilus UNIFG24 and *Lactobacillus delbrueckii* subsp. *bulgaricus* UNIFG23 were isolated from a homemade yogurt and identified by 16S ribosomal DNA amplification (data not shown).

For yogurt production, cow milk was treated according to Rosburg *et al.* (2010). Briefly, the milk was heat-treated at 85 °C for 30 minutes, with the aim of breaking down the native flora. The absence of microorganism was confirmed by counting of CFU/mL on MRS agar plates. Overnight bacterial cultures were centrifuged (2,000 × g, 10 min), and the pellets were resuspended in milk at final concentration of 1×10^9 CFU/mL. All samples were then incubated at 42 °C until a pH<4.6 (approximately 6 hours), and then stored at 4 °C for 28 days. All trials were performed in duplicate.

3.10.2 Chemical analysis

Fat, protein, casein content and lactose of milk samples were analysed by MilkoScanTM, FT 120; Foss Electric, Hillerød, Denmark.

Yogurt samples were analyzed after 1, 14 and 28 days of storage for lactose, protein, casein and nitrogen fractions by the Kjeldhal method according to the AOAC method (1995), and for fat by the Gerber method according to the British Standards institution (1989). The lactic acid content was detected by enzymatic kits according to manufacturer's instructions (Biogamma s.r.l, Roma, Italy). For monitoring the hydrolysis of protein during storage of yogurt at 4 °C, the pH 4.6-water-soluble extracts (WSEs) of the samples were prepared according to the method proposed by Kuchroo and Fox (1982).

The peptide profiles of the pH 4.6-soluble fractions were determined by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using Agilent 1260 Infinity (Agilent Technologies, Santa Clara, USA). The column used was a ZORBAX 300 SB-C18 (250mm × 4.6mm × 5 μ) (Agilent). The mobile phase was water (solvent A) and acetonitrile (solvent B), both containing 0.1% trifluoroacetic acid, and the solvent flow rate was 1mL/min. The eluate was monitored at 220 nm; all solvents were of chromatography grade (Baker, Inch., Phillisburg, NJ, USA).

3.10.3 Microbiological analysis

The cell viability of *L. plantarum* Lp90 and *L. plantarum* WCFS1 was monitored prior fermentation (T0), and following the fermentation of milk after 1, 7 14, 21, and 28 days by real-time PCR (see below).

3.10.4 Lactobacilli oro-gastro-intestinal tolerance *in vitro* assay in yogurt matrix

Yogurt samples after 14 days storage (approximately middle shelf-life) were exposed to a simulated oro-gastrointestinal transit as described by Arena *et al.* (2014b) (**Figure 3.5**). Briefly,

yogurt samples were subjected to an oral stress step incubating for 5 min with 150 mg/L lysozyme (Sigma-Aldrich) at pH 6.5. Subsequently, pepsin (3 g/L) (Sigma-Aldrich) was added and the pH value was reduced first to 3.0 and then, after 30 min incubation, to 2.0, in order to mimic the gastric compartments. Intestinal stress was performed by increasing the pH value to 6.5, by addition of bile salts (3 g/L) and pancreatin (1 g/L) (all from Sigma-Aldrich) and by eventual dilution of the samples in order to reproduce large intestine conditions.

Aliquots taken prior to oro-gastro-intestinal assay and after the oral, gastric (pH 2.0 and 3.0), and intestinal (small and large intestine sectors) stresses were used for the evaluation of bacterial survival monitoring by Real-Time PCR (see below). The percentage of survival was determined with respect to unstressed control. The samples, after the large intestinal compartment of OGI transit were used for the stimulation of THP-1 cells assay (see below).

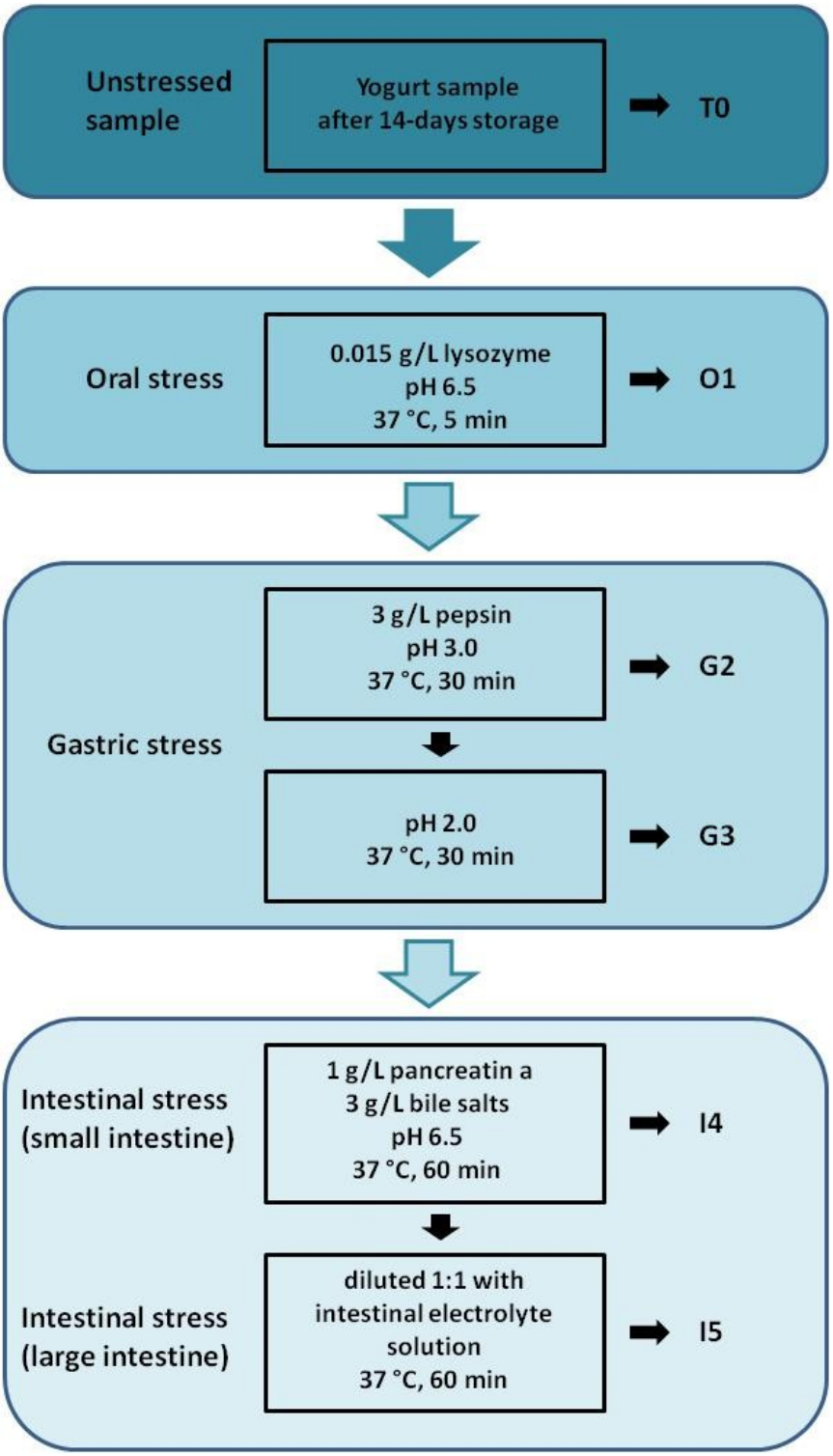

Figure 3.5 - Schematic representation of the *in vitro* system simulating the human oro-gastro-intestinal tract. Yogurt sample was subject to the sequential conditions. Oral stress was mimicked by addition of a lysozyme-containing electrolyte solution (oral sample O1). Gastric stress was simulated by addition of pepsin and progressive pH reduction from 6.5 to 3.0 and 2.0 (gastric samples G2 and G3). Sample of gastric-stressed bacteria was adjusted to pH 6.5 and supplemented with bile salts and pancreatin to simulate intestinal stress (intestinal samples I4 and I5). Incubations were performed for the time indicated, at 37 °C and under shaking. Unstressed yogurt sample (T0) was considered as internal control.

3.10.5 Human monocytoid leukemia-derived cells (THP-1) growth conditions

Human monocytoid leukemia-derived cells (THP-1) (Sigma-Aldrich, St. Louis, MO, USA) were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 10% (v/v) FBS, 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, in atmosphere containing 5% CO_2 at 37 °C. THP-1 cells were used between passage 12 and 25. Then, THP-1 cells were seeded at the concentration of 5×10^5 cells/well in 24-wells plates, resuspended in RPMI 1640 medium without any supplements and induced to differentiate into mature macrophages-like state by treating for 48h with 100 ng/mL phorbol 12-myristate 13 acetate (PMA, Sigma-Aldrich).

3.10.6 Immune-stimulation of THP-1 cells with lactobacilli

The immune-stimulation assay were performed on both untreated (yogurt sample not *in vitro* digested) and *in vitro* digested yogurt samples.

Macrophage-differentiated THP-1 cells were treated as elsewhere described (Grimoud *et al.,* 2010). Briefly, THP-1 cells were exposed to 100 ng/mL of lipopolysaccharide (LPS) from *E. coli* O127:B8 (Sigma), untreated samples and *in vitro* digested samples opportunely diluted were added and incubated for 1 and 4h at 37 °C with 5% CO_2. Positive and negative controls were macrophage-differentiated THP-1 cells incubated with and without LPS, respectively. Human cells were harvested and transcriptional analysis was performed for genes coding immune-related genes (see below).

The ratio bacteria:macrophage-differentiated THP-1 used, were decided considering two aspects. Firstly, the percentage of cells survival after the entire *in vitro* digestion assay, which was approximately 1×10^6 CFU/mL for *L. plantarum* WCFS1 and around 1×10^7 CFU/mL for *L. plantarum* Lp90. Secondly, the samples need to be diluted in a ratio 1:3 (sample:medium), because the digestion solutions and enzymes used in the *in vitro* digestion can affect the human cell viability (Vreeburg *et al.,* 2011). Therefore, bacteria pellets were harvested by centrifugation (2,000 × g for 10 min) and resuspended in RPMI 1640 medium. The final concentration for *L.*

plantarum WCFS1 was 3×10^5 CFU/mL (both for untreated and *in vitro* digested samples) and 3×10^6 CFU/mL for *L. plantarum* Lp90 (both for untreated and *in vitro* digested samples).

3.10.7 Propidium monoazide (PMA) treatment and microbial DNA extraction

In order to discriminate live and dead bacterial cells allowing cells quantification by qPCR method, the yogurt samples were treated with propidium monoazide (PMA) as previously described by Àlvarez *et al.* (2013). Briefly, 100 μM of PMA (Biotium, Inc., Hayward, CA, USA) dissolved in 20% of dimethylsulfoxide (DMSO) (Sigma) were added to 1 mL of yogurt samples and kept in light-transparent 1.5 mL microcentrifuge-tubes. The tubes were incubated in the dark conditions for 10 min and then exposed to halogen lamp (650W, 230V, GY9.5, 3050K; Philips, Japan).

Subsequently, the genomic DNA of each strain was extracted from the fermented samples by a lytic method as described by Quigley *et al.* (2012). Exactly, 1 mL of each sample containing the target microorganism was added to 0.5 mL of breaking buffer for enzymatic lysis and incubated at 37 °C for 1 h. The samples were treated with proteolytic enzyme by adding 250 μg/mL of proteinase K and incubating at 55 °C for 1 h. The suspension was transferred in new tube containing zirconium beads, shaken twice for 90 s and centrifuged at 12,000 × g for 10 min. The supernatant was added to an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), mixed gently and centrifuged at 12,000 × g for 2 min. The upper aqueous phase was transferred into clean tube; sodium acetate 3 M (one-tenth the volume) and 100% ice-cold ethanol (2 volumes) were added. The samples were mixed, stored at -20 °C overnight, and then centrifuged at 14,000 × g for 10 min in order to harvest the pellet that was washed with 70% ice-cold ethanol followed by centrifugation at 12,000 × g for 5 min and dried. The final pellet was resuspended in 100 μL TE buffer and used in qPCR detection.

3.10.8 THP-1 RNA extraction and cDNA synthesis

RNA extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR) were performed as described by Bove *et al.* (2012). Briefly, THP-1 macrophages were harvested using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) the total RNA was extracted according to manufacturer's instructions. The RNA concentration and integrity were determined by spectrophotometry (Biotek Instruments, Winooski, VT, USA) and gel electrophoresis. One microgram of total RNA was reverse-transcribed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA), which includes a genomic DNA elimination reaction. Absence of DNA contamination was confirmed by real-time PCR on DNase I-treated non-retrotranscribed RNAs.

3.10.9 qPCR analysis

The extracted microbial DNA were diluted (1:20) and 5µL were used to perform the q-PCR analysis (ABI 7300, Applied Biosystems, Foster City, CA, USA) in a reaction mixture containing 15 µL of PCR mix (Power SYBR Green PCR Master Mix; Applied Biosystems) and 100 nM of forward and reverse primers for gyrA amplification specific for *L. plantarum* specie (Fiocco *et al.,* 2009). Serial dilutions of known *L. plantarum* WCFS1 DNA amount ranging from 1×10^4 to 1×10^8 CFU/mL were carried out to generate a reference standard curve which was used for the relative quantification.

The human cDNA samples were also diluted (1:20), 5µL were used to perform the q-PCR analysis and different primers genes were tested. Primers related to glyceraldehyde phosphate dehydrogenase (GAPDH), β-actin (β-actin), interlukine 8 (IL-8) and interlukine 6 (IL-6) genes were previously reported by Bove *et al.* (2012) (**Table 3.2**), while the primers for the gene tumor necrosis factor α (TNF-α); interleukin 10 (IL-10) and hypoxanthine phosphoribosyl transferase 1 (HPRT1) were designed (**Table 3.2**). Thymic stromal lymphopoietin primer (TSLP forward: ATGTTCGCCATGAAAACTAAGGC; TSLP reverse:

GCGACGCCACAATCCTTGTA); interleukine 1β primer (IL1β forward: ATGATGGCTTATTACAGTGGCAA; IL1β reverse: GTCGGAGATTCGTAGCTGGA); nuclear factor kappa B primer (NF-κB1 forward: GGTGCGGCTCATGTTTACAG; NF-κB1 reverse: GATGGCGTCTGATACCACGG) were all selected from PrimerBank (http://pga.mgh.harvard.edu/primerbank) (Spandidos *et al.,* 2010). GAPDH, β-actin and HPRT1 genes (**Table 3.2**) were used to normalize the expression of target genes by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each primer was used at concentration of 100 nM.

The thermal conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s, 72 °C for 30 s. Each PCR assay included duplicate reactions.

3.11 Tolerance of *Lactobacillus plantarum* strains to ethanol, acid, sulfur dioxide, lysozyme, and bile stress

Tolerance of *Lactobacillus plantarum* strains to different stress was evaluated on overnight bacterial cells cultures (OD_{600nm} 5), subjected to:

- ethanol stress (13%);
- acid stress (pH 2.5);
- SO_2 stress (70 mg/L);
- lysozyme stress (200 µg/mL);
- bile stress (3 g/L).

In ethanol and acid stress the bacterial culture were directly supplemented with an appropriate volume of ethanol and HCl 1M, at final concentration of 13% ethanol and a pH of 2.5.

In SO_2, lysozyme and bile stress, the bacterial culture were previously recovered by centrifugation at 5,000 × *g* for 10 min, and resuspended in an equal volume of saline solution (0.85% NaCl), containing the various stressors to different concentrations mentioned above.

Subsequently, they were incubated at 37 °C for 30 min, serial dilutions were plated on MRS agar for the enumeration CFU/mL and incubated at 37 °C for 48 hours.

The bacterial survival expressed as relative survival (log10 CFU/mL) was determined with respect to unstressed samples controls.

3.12 Microvinification assays

Microvinification assays were performed using *L. plantarum* Lp90 and *L. plantarum* Lp90Δcps2 (control strain) in grape must of "Nero di Troia" without SO_2 and with SO_2 (70 mg/L).

Microvinifications were carried out in (i) co-inoculation and (ii) sequential inoculation:

(i) *L. plantarum* strains and commercial *Saccharomices cerevisiae* EP2 (Maurivin, Sydney, Australia) were co-inoculated to induce simultaneous alcoholic and malolactic fermentation. Before co-inoculation lactobacilli were previously cultivated in MRS broth for 16 h at 30 °C.

(ii) Commercial *Saccharomices cerevisiae* EP2 was inoculated first and then *L. plantarum* strains were inoculated at the end of alcoholic fermentation, inducing sequential alcoholic and malolactic fermentation. Before sequential inoculation bacterial cells were grown in MRS at pH 6.5 for 16 h at 30 °C.

All microvinifications were performed in a total volume of 50 mL of grape must under magnetic stirrers at 25 °C for 14 days.

In both co-inoculation and sequential inoculation methods, *Saccharomices cerevisiae* EP2 was inoculated at final concentration of 2×10^6 CFU/mL, while *L. plantarum* strains were inoculated at final concentration of 1×10^9 CFU/mL.

The viability of *L. plantarum* strains was monitored after 1, 2, 7, 14 days post inoculation. Serial dilution were plated on MRS agar supplemented with cicloeximide (10 mg/L), and incubated for 48 h at 30° C.

MLF was monitored by measuring the consumption of malic acid and the production of lactic acid, with enzymatic kit for L-lactic and L-malic acid (BioGamma) as recommended by the supplier.

3.13 Statistical analysis

Data represents the mean±SD of two biological experiments and three technical replicate. One-way analysis of variance (ANOVA) was performed using the statistical software PAST version 2.17C (Hammer *et al.,* 2001). P values <0.05 were considered as statistically significant.

4. RESULTS AND DISCUSSION

4.1 *L. plantarum* Lp90 cells: Transmission Electron Microscopy imaging

Cells of *Lactobacillus plantarum* Lp90, imaged by TEM, displayed a thick matrix of electron-dense extracellular (**Figure 4.1**). Such extracellular matrix appears both to partially cover the cell and to be released into the medium, thus forming a reticulate structure. We presume that this typical aspect depends on the ability of *L. plantarum* to overproduce EPS. When cells were imaged after phosphate buffer solution (PBS) washing, the extracellular dense matrix almost completely disappeared, thus suggesting that EPS are either weakly bound to the cell wall or secreted into the extracellular medium.

Since PBS was effective in removing putative EPS matrix, this treatment was adopted to generate wild-type Lp90 cells without EPS. It was used as control strain for the experiments carried out before obtaining Lp90Δcps2 non-ropy mutant strain.

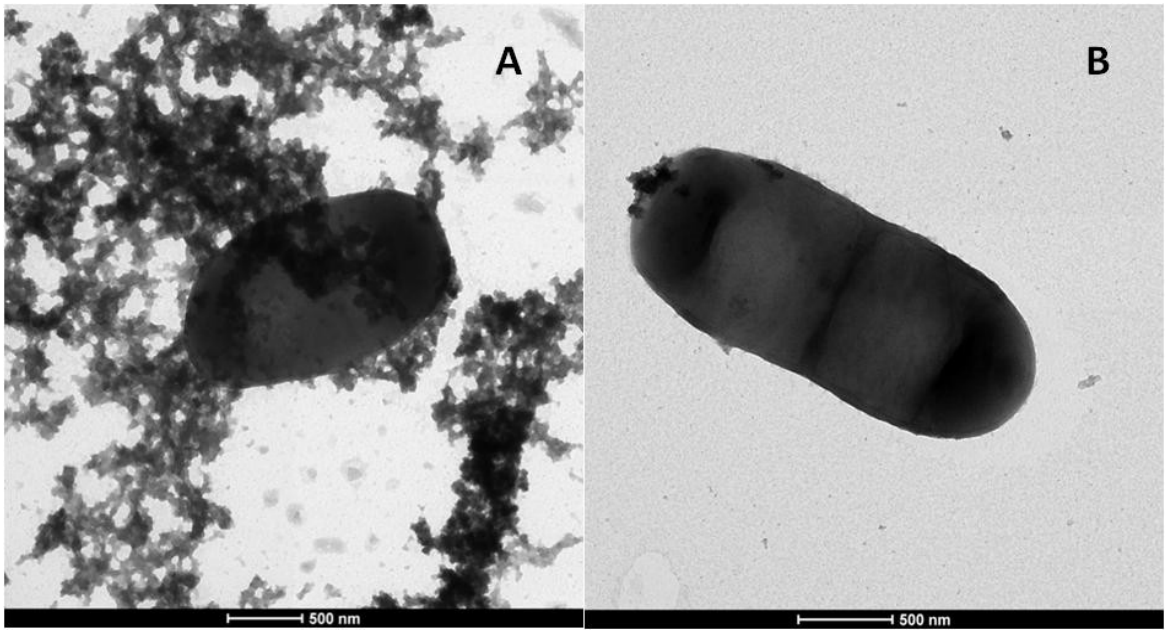

Figure 4.1 - Transmission Electron Micrograph of *L. plantarum* Lp90 cells before (A) and after (B) PBS was.

4.2 Exopolisaccharides of *L. plantarum* Lp90

4.2.1 Exopolisaccharides yield

When *L. plantarum* Lp90 was cultivated in a semi-defined medium (SMD) at 30 °C for 15 hours (OD_{600nm} 3) the ropy phenotype was clearly visible and the yield of EPS, determined by phenol-sulfuric acid method (Dubois *et al.,* 1956) was 43.04±3.46 mg/L (dry weight). However, the production of EPS of this strain is lower in comparison with other EPS-(over)producing LAB, although different chemical composition was observed. Indeed, *L. lactis* NZ9000 and *L. mesenteroides* RTF10 were able to produce 561±18 mg/L and 1870±180 mg/L respectively (Notararigo *et al.,* 2013)*;* Tsuda (2013) reported that the highest yields of hetero-polysaccharides produced by lactic acid bacteria are 2775 mg/L for *Lactobacillus rhamnosus* RW-9595M (Macedo *et al.,* 2002) and 2500 mg/L for *L. kefiranofaciens* WT-2B (Maeda *et al.,* 2004a).

It is well known that composition and yield of the EPS produced by LAB is very influenced by culture and fermentation conditions (i.e. pH, temperature, and medium composition) (Dueñas *et al.,* 2003). Although complexes growth media consent to obtain a higher exopolysaccharides production, this leads to a higher concentration of contaminants (Ruas-Madiedo *et al.,* 2005).

4.2.2 Chemical characterization of exopolysaccharides produced *L. plantarum* Lp90

Chromatographic analysis of EPS isolated from *L. plantarum* Lp90 revealed the presence of two peaks, with a high (10^6 Da) and low (10^4 Da) molecular weight.

The exopolysaccharides composition (**Table 4.1**), as revealed by Size Exclusion Chromatography (SEC) analysis of the neutral sugar content of dialyzed EPS, suggested that they are heteropolysaccharides (HePS), as the fraction analyzed have three different sugars (rhamnose, glucose and galactose) in different percentages. Rhamnose and glucose are mainly present in the soluble fraction (C), glucose has a low percentage in fraction (A), while the galactose is mainly present in the pellet (A). However, regardless to the fractions, galactose was

the monosaccharide with the lowest concentration. In the chemical composition of EPS two additional amino sugars were observed: glucosamine ($GlcNH_2$) and galactosamine ($GalNH_2$), both present in greater proportion in the pellet (A).

FRACTION	Rhamnose (%)	Glucose (%)	Galactose (%)	$GlcNH_2$ (%)	$GalNH_2$ (%)	Recovery (%)
(A) pellet	14,7	0,8	11,5	7,8	8,9	43,7
(B) EPS > 100 kDa	14,5	17,0	7,1	3,3	2,7	44,6
(C) 100 kDa > EPS > 3 kDa	43,6	33,7	8,9	2,5	0,0	88,7

Table 4.1 - Sugar and amino sugar composition of the EPS produced by *L. plantarum* Lp90.

4.3 Non contiguous-finished genome sequence of *Lactobacillus plantarum* strain Lp90

4.3.1 *L. plantarum* Lp90 genome properties

The genome of *L. plantarum* Lp90 strain was subjected to shotgun DNA sequencing using the Illumina sequencing technology (Sequencing platforms: Illumina GAIIx). It has been released in Genbank on July 21, 2014, with JIBX00000000 as Genbank ID. A total of 16,574,199 paired end reads of 2 x 115 bps length were de novo assembled into 33 contigs with an N50 length of 207,479 bps. The size of the shortest contig was 354 bps while the length of the longest contig was 489,345 bps. The genome of *L. plantarum* Lp90 is 3,324,076 bps long with a CG content of 44.32% (**Table 4.2**). The genome size and the CG content is comparable to the published *Lactobacillus plantarum* genome sizes (http://www.ncbi.nlm.nih.gov/genome/genomes/1108). After genome annotation we identified 3,273 predicted genes among this 3,155 were protein coding genes, 34 were identified to be pseudo-genes while 84 were RNA coding genes, divided in 70 tRNAs and 14 rRNAs. The presence of a signal peptide was predicted for 311 proteins. Transmembrane region analysis revealed 869 proteins containing transmembrane helices. The Lp90 proteins were searched against the COG database to identify the functional group of

belonging. 2,256 genes were assigned to a COG functional category and the distribution is shown in **Table 4.3**.

Attribute	Genome (total)	
	Value	% of total*
Size (bps)	3,324,076	100.00
G+C content (bps)	1,473,261	44.32
Coding region (bps)	2,784,484	83.76
Number of contigs	33	
Contig N50	207,459	
Total genes	3,273	100.00
Protein-coding genes	3,155	96.39
RNA genes	84	2.56
Pseudo-genes	34	1.03
Genes assigned to COGs	2,256	68.92
Genes with signal peptides	311	9.50
Genes with transmembrane helices	869	26.55

* The total is based on either the size of the genome in base pairs or the total number of genes in the annotated genome.

Table 4.2 - Genome statistics *(from Lamontanara et al., 2015).*

Code	Value	% of total*	Description
J	153	4.84	Translation
A	0	0.00	RNA processing and modification
K	276	8.64	Transcription
L	149	4.72	Replication, recombination and repair
B	0	0.00	Chromatin structure and dynamics
D	26	0.82	Cell cycle control, mitosis and meiosis
Y	0	0.00	Nuclear structure
V	57	1.80	Defense mechanisms
T	95	3.01	Signal transduction mechanisms
M	140	4.43	Cell wall/membrane biogenesis
N	5	0.15	Cell motility
Z	0	0.00	Cytoskeleton
W	0	0.00	Extracellular structures
U	22	0.69	Intracellular trafficking and secretion
O	56	1.77	Posttranslational modification, protein turnover, chaperones
C	107	3.39	Energy production and conversion
G	314	9.95	Carbohydrate transport and metabolism
E	251	7.95	Amino acid transport and metabolism
F	86	2.72	Nucleotide transport and metabolism
H	81	2.56	Coenzyme transport and metabolism
I	71	2.25	Lipid transport and metabolism
P	158	5.00	Inorganic ion transport and metabolism
Q	39	1.23	Secondary metabolites biosynthesis, transport and catabolism
R	386	12.23	General function prediction only
S	215	6.81	Function unknown
-	899	28.49	Not in COGs

***** The "% of total" is based on the total number of protein coding genes in the annotated genome.

Table 4.3 - Number of genes associated with the 25 general COG functional categories
(from Lamontanara et al., 2015).

4.3.2 Comparison with other *L. plantarum* genomes

OrthoMCL (Li *et al.,* 2003) was used with the default parameters to generate groups of orthologous genes (OGs) among the protein sequences of 11 *L. plantarum* genomes obtained from GenBank (**Table 4.4**) and the Lp90 protein sequences. An *L. plantarum* pan-genome of 4,726 OGs was identified. The core genome, represented by the genes shared by all the strains,

consisted of 2,207 OGs while the total variable (or accessory) genome was represented by 2,519 OGs Lp90, among the considered strains, contained one of the most variable OGs (858 OGs) bested by the WJL, the ATCC-14917 and the ZJ316 strains (1,105, 877 and 870 orthologous groups, respectively) (**Table 4.4**).

Strain	Core genome	Variable genome	Total	Strain specific	Genome size (Mb)	GenBank ID
16	2207	515	2722	48	3.36	CP006033.1
JDM1	2207	682	2889	63	3.2	CP001617.1
P8	2207	594	2801	45	3.23	CP005942.1
ST_III	2207	731	2938	18	3.31	CP002222.1
WCFS1	2207	764	2971	105	3.35	AL935263.2
ZJ316	2207	870	3077	138	3.3	CP004082.1
Lp90	**2207**	**858**	**3065**	**114**	**3.32**	**JIBX00000000.1**
ATCC 14917	2207	877	3084	97	3.21	ACGZ00000000.2
IPLA88	2207	834	3041	137	3.25	ASJE00000000.1
WJL	2207	1105	3312	182	3.48	AUTE00000000.1
NC8	2207	614	2821	19	3.21	AGRI00000000.1
UCMA 3037	2207	665	2872	104	3.11	APHP00000000.1
ALL	2207	2519	4726	1070	-	

Table 4.4 - Number of orthologous groups in the core and in the variable genome of different *L. plantarum* strains. The orthologous groups found and the genome sizes were obtained from NCBI-GenBank *(from Lamontanara et al., 2015).*

114 orthologous were found to be unique to Lp90. Most of these genes were hypothetical genes of unknow function and prophage associated genes. However three genes, (Glycosyltransferase, Polysaccharide pyruvyl transferase and mannosyltransferase) involved in EPS biosynthesis not found in the other *L. plantarum* strains were identified.

The relatedness with the genomes of 11 *L. plantarum* strains present in GenBank, was established drawing a phylogenetic tree using the core orthologous protein sequences conserved within the analyzed *L. plantarum* genomes (**Figure 4.2**). Phylogenetic analysis showed that *L. plantarum* Lp90 is more closely related with the NC8 (Axelsson *et al.,* 2012) and the ATCC strains which are two strain isolated from samples of vegetable origin. NC8 was isolated from

grass silage and the ATCC-14917 strain was isolated from pickled cabbage. Lp90, furthermore, is related with the cluster formed by the WJL (Kim *et al.,* 2013) and ST-III (Wang *et al.,* 2011) strains which were isolated, respectively, from fruit fly and kimchi, a traditional fermented korean vegetables. Lp90 showed the higher distance with WCFS1 (Kleerebezem *et al.,* 2003) and ZJ316 (Li *et al.,* 2013) both isolated from human samples and the IPLA 88 (Ladero *et al.,* 2013) strain isolated from sourdough. UCMA (Naz *et al.,* 2013) and P8, which are two strains isolated from dairy products have a single separated cluster together with the *L. plantarum* 16 (Crowley *et al.,* 2013) a malt production steep water isolate.

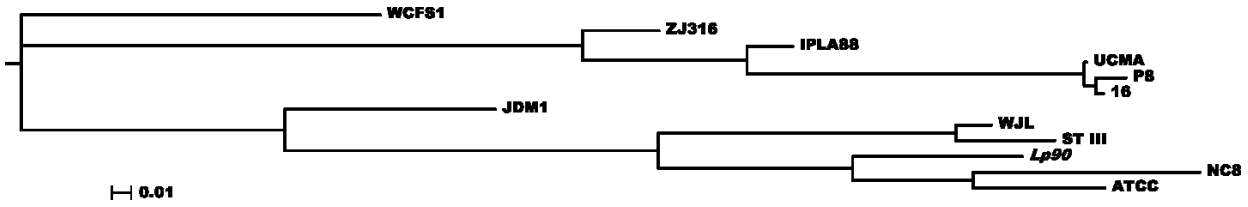

Figure 4.2 - Phylogenetic tree highlighting the position of *Lactobacillus plantarum* strain Lp90 relative to other sequenced strains of *L. plantarum*. The tree was built comparing the concatenated amino acid sequences of 2207 orthologs genes conserved in all strains. Multiple sequences alignments were performed by using MUSCLE (Edgar, 2004). The approximately-maximum-likelihood phylogenetic tree was constructed by using FastTree 2 program (Prince *et al.,* 2010) using the Jones-Taylor-Thorton (JTT) model of amino acid evolution. *(from Lamontanara et al., 2015).*

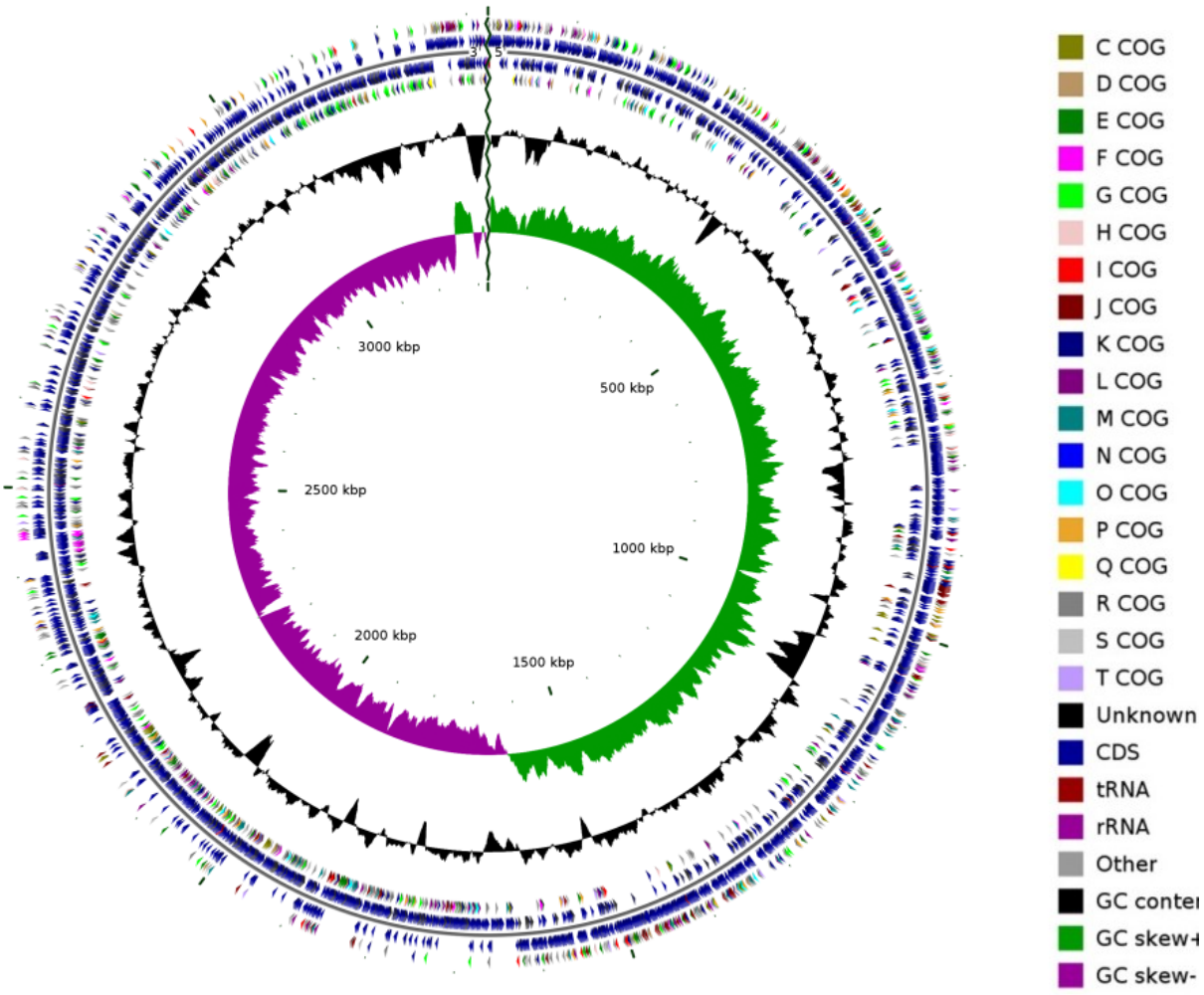

Figure 4.3 - Graphical circular map of the *Lactobacillus plantarum* Lp90 genome. The outermost circle indicates positions of CDSs on the forward and reverse strands. The CDSs are showed as arrow. The genes associated with the COGs categories are colored according to the legend on the right. Moving toward the center, the second circle shows C+G content. The third circle shows C+G skew in green (+) and purple (-). The scale (in kbp) is indicated in the innermost circle. The map was generated with the CGviewer software (Stothard and Wishart, 2005) *(from Lamontanara et al., 2015).*

4.3.3 Comparison of Lp90 *cps* clusters and homologous clusters in *L. plantarum* species

Following the genome sequencing of *L. plantarum* Lp90 (Lamontanara *et al.,* 2015), it has been possible to investigate the genetic basis of EPS production, by identification and comparative analysis of genes clusters involved in EPS biosynthesis with other *L. plantarum* genomes.

The comparative analysis of the *cps* clusters of *L. plantarum* Lp90 shown that the genes from Lp90_1067 to Lp90_1071 of *cps2* cluster (14; 15; 16; 17; 18 as shown in **Figure 4.5**) are homologous to the *cps2A-E* genes of *L. plantarum* WJL, ST-III, NC8, WCFS1, ZJ316 strains. The *cps4* is found to be the most conserved cluster in species. The first three genes in *cps2* and

cps4 clusters (14; 15; 16; and 45; 44; 43 respectively), which are a capsular polysaccharide biosynthesis protein; exopolysaccharide synthesis protein; capsular polysaccharide biosynthesis protein or lipopolysaccharide biosynthesis are homologous to the *cps2ABC* and *cps4ABC* in WCFS1 (Remus *et al.,* 2012), presenting the typical components of the tyrosine kinase phosphoregulatory circuit involved in control of capsule synthesis (Yother, 2011). The fourth genes (17 and 42 in *cps2* and *cps4* respectively) indicated as nucleoside-diphosphate-sugar epimerase are homologous to UDP N-acetyl glucosamine 4-epimerase (*cps2D*) and an UDP-N-acetyl-D-galactosamine (*cps4D*) of WCFS1 strain (Remus *et al.,* 2012). The fifth genes (18 and 41 in *cps2* and *cps4* respectively) reported as exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase are homologous to a priming glycosyltransferase, polyprenyl glycosylphosphotransferase (*cps2E*) and a polysaccharide biosynthesis polyprenyl glycosylphosphotransferase, priming glycosyltransferase (*cps4E*) of WCFS1 strain (Remus *et al.,* 2012). The remaining genes in the *cps4* are homologous in the other *L. plantarum*, with respect to the relative clusters. Interesting differences were found in *L. plantarum* Lp90 for the remaining part of *cps2* cluster in comparison with other *L. plantarum*, indeed, WCFS1 encode glycosyltransferase proteins, flippase and polymerase (Remus *et al.,* 2012). Conversely, Lp90_1074, Lp90_1075 and Lp90_1077 genes (20; 21; 23 **in Figure 4.5**), which are a glycosyltransferase family 2, a polysaccharide pyruvyl transferase and a mannosyltransferase respectively, were not found in the species. Otherwise they are homologous (58.59; 57.18; 57.81 of homology percentages) to two hypothetical proteins and a glycosyltransferase of *Lactobacillus fabifermentans* T30PCM01, (**Figure 4.8**), a strain isolated from fermenting grape marc (Treu *et al*., 2014). The similitude in organism's lifestyle (wine environment) and the similarity levels detected, led us to suggest a possible intra-genus horizontal transfer event. *Lactobacillus fabifermentans* species was previously described by De Bruyne *et al.* (2009) and found to be closely related to *Lactobacillus plantarum*. The presence of these three unique genes

would indicate that the ropy phenotype of *L. plantarum* Lp90 could be due to specific glycosyltransferase.

The organization of *cps1* cluster is similar to the corresponding cluster in JDM1 and it has partial homology with the corresponding cluster of the others *L. plantarum* genomes. Moreover, in *cps1* cluster the first five genes are predicted to be glycosyltransferase and it seems deficient in priming glycosyltransferase and flippase (**Figure 4.4**). Conversely, cluster 3 presents high homology with *cps3* of WJL, ST-III, NC8, WCFS1, ZJ316 and ATCC 14917 *L. plantarum* strains, while IPLA88 strain has homology in clusters *cps3* only for the Lp90_1089, Lp90_1090 and Lp90_1092 genes (26, 27 and 29 as indicated in **Figure 4.6**). The *cps2A-J* and *cps4A-J* clusters seem to encode all functions required for capsular polysaccharide formation, while the *cps1A-I* and *cps3A-J* clusters lack genes encoding chain-length control functions and a priming glycosyltransferase. However, Lp90_1096 and Lp90_1097 (a polysaccharide biosynthesis protein and sugar transferase) have homology with lp_1231 and lp_1233 of WCFS1 (a flippase and a priming glycosyltransferase), these genes could complete the polysaccharide synthesis machinery of *cps3* (Remus *et al.,* 2012).

We speculate that the ropy phenotype of *L. plantarum* Lp90 is intrinsic to the cluster *cps2*, in particular for the three genes mentioned above, which are apparently unique in Lp90 compared to other sequenced bacteria of the same species. Indeed, after the deletion in *L. plantarum* Lp90 of the entire *cps2* cluster (Lp90Δcps2), as well as the genes from Lp_1073 to Lp90_1077 (Lp90Δcps2.5) the lack of ropy phenotype was observed (see below).

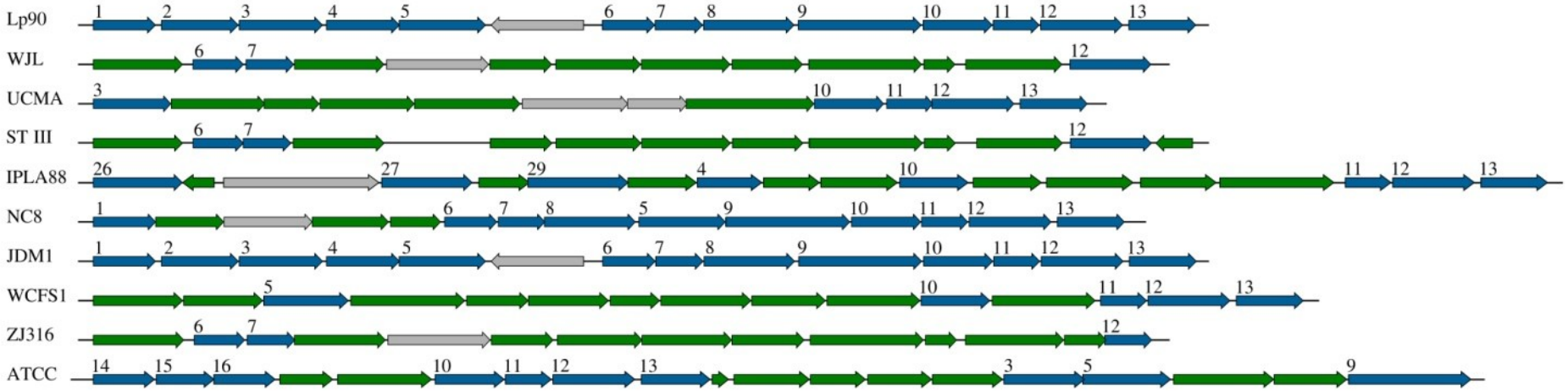

Figure 4.4 - Organization of the *cps1* genes cluster involved in the EPS biosynthesis of *Lactobacillus plantarum* Lp90 and comparison with other *L. plantarum* genomes. Blue arrows represent the genes found in the Lp90 cluster (homologous genes). Green arrows represent the genes found in the other *L. plantarum* strains but absent in Lp90 (non-homologous genes). Gray arrows represent gene apparently not involved in EPS production (membrane protein or hypothetical protein), which are not numbered. Genes 14; 15;16 in ATCC are homolougus to the gens of the *cps2* cluster in Lp90.

Gene 1: (Lp90_1049) Glycosyltransferase.
Gene 2: (Lp90_1050) sugar phosphotransferase.
Gene 3: (Lp90_1051) Glycosyltransferase, group 2.
Gene 4: (Lp90_1052) glycosyltransferase.
Gene 5: (Lp90_1053) glycosyltransferase.
Gene not numbered: (Lp90_1054) Membrane protein.
Gene 6: (Lp90_1055) Capsular polysaccharide biosynthesis protein.
Gene 7: (Lp90_1056) polysaccharide biosynthesis protein.
Gene 8: (Lp90_1057) Beta-lactamase class C, penicillin binding protein.
Gene 9: (Lp90_1058) polysaccharide biosynthesis protein.
Gene 10: (Lp90_1059) Glucose-1-phosphate thymidylyltransferase.
Gene 11: (Lp90_1060) dTDP-4-dehydrorhamnose 3,5-epimerase.
Gene 12: (Lp90_1061) dTDP-glucose 4,6-dehydratase.
Gene 13: (Lp90_1062) dTDP-4-dehydrorhamnose reductase.

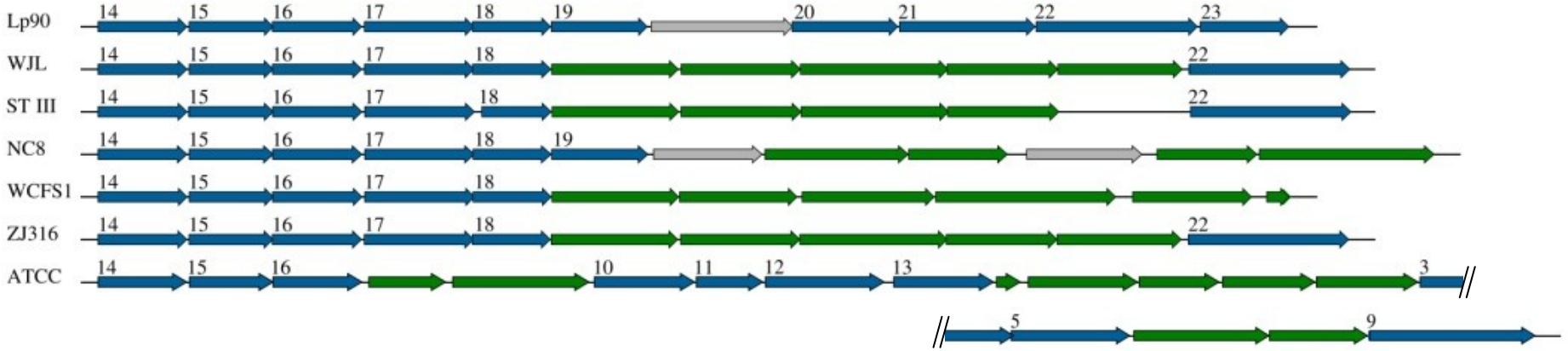

Figure 4.5 - Organization of the *cps2* genes cluster involved in the EPS biosynthesis of *Lactobacillus plantarum* Lp90 and comparison with other *L. plantarum* genomes. Blue arrows represent the genes found in the Lp90 cluster (homologous genes). Green arrows represent the genes found in the other *L. plantarum* strains but absent in Lp90 (non-homologous genes). Gray arrows represent gene apparently not involved in EPS production (membrane protein or hypothetical protein), which are not numbered. The genes numbered as 20,21 and 22 were found to be unique in Lp90.
Gene 14: (Lp90_1067) Capsular polysaccharide biosynthesis protein
Gene 15: (Lp90_1068) Exopolysaccharide biosynthesis protein
Gene 16: (Lp90_1069) Capsular polysaccharide biosynthesis protein, *CpsB/CapC*
Gene 17: (Lp90_1070) Nucleoside-diphosphate-sugar epimerase
Gene 18: (Lp90_1071) Exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase
Gene 19: (Lp90_1072) glycosyltransferase
Gene not numbered: (Lp90_1073) Membrane protein
Gene 20: (Lp90_1074) Glycosyltransferase, family 2
Gene 21: (Lp90_1075) Polysaccharide pyruvyl transferase
Gene 22: (Lp90_1076) Polysaccharide biosynthesis protein
Gene 23: (Lp90_1077) mannosyltransferase

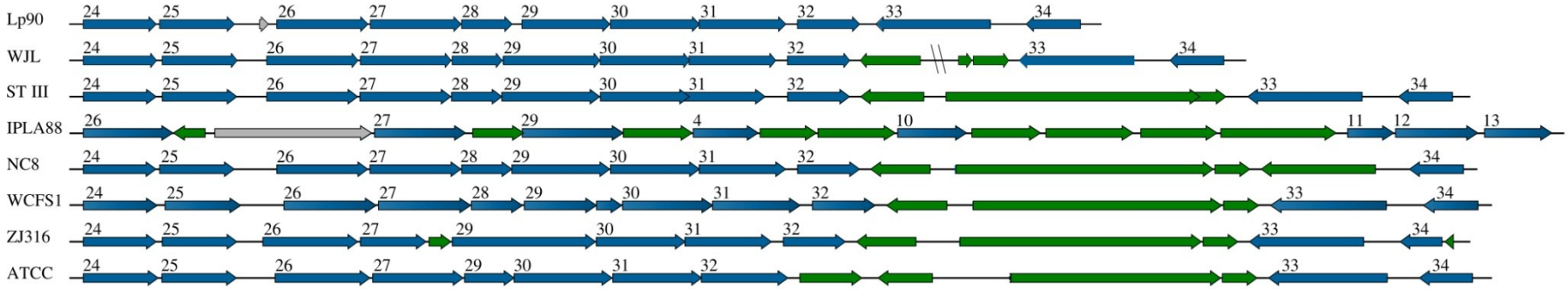

Figure 4.6 - Organization of the *cps3* genes cluster involved in the EPS biosynthesis of *Lactobacillus plantarum* Lp90 and comparison with other *L. plantarum* genomes. Blue arrows represent the genes found in the Lp90 cluster (homologous genes). Green arrows represent the genes found in the other *L. plantarum* strains but absent in Lp90 (non-homologous genes). Gray arrows represent gene apparently not involved in EPS production which are not numbered.
Gene 24: (Lp90_1086) glycosyltransferase
Gene 25: (Lp90_1087) Glycosyltransferase, family 2
Gene not numbered: (Lp90_1088) Hypothetical protein
Gene 26: (Lp90_1089) UDP-galactopyranose mutase
Gene 27: (Lp90_1090) polysaccharide biosynthesis protein
Gene 28: (Lp90_1091) polysaccharide biosynthesis protein (putative)
Gene 29: (Lp90_1092) Membrane protein
Gene 30: (Lp90_1093) polysaccharide biosynthesis protein
Gene 31: (Lp90_1094) Acyltransferase 3
Gene 32: (Lp90_1095) exopolysaccharide biosynthesis protein
Gene 33: (Lp90_1096) Polysaccharide biosynthesis protein
Gene 34: (Lp90_1097) sugar transferase

.

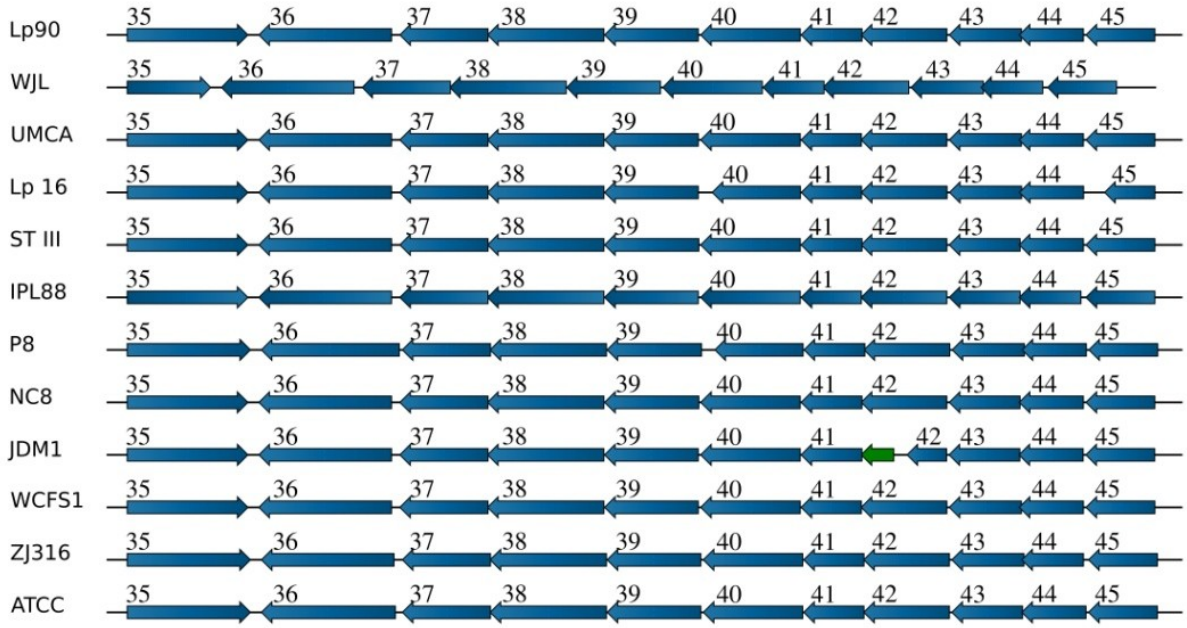

Figure 4.7 - Organization of the *cps4* genes cluster involved in the EPS biosynthesis of *Lactobacillus plantarum* Lp90 and comparison with other *L. plantarum* genomes. Blue arrows represent the genes found in the Lp90 cluster (homologous genes). Green arrows represent the genes found in the other *L. plantarum* strains but absent in Lp90 (non-homologous genes).
Gene 35: (Lp90_1834) Phosphoesterase
Gene 36: (Lp90_1835) Polysaccharide biosynthesis protein
Gene 37: (Lp90_1836) Glycosyltransferase, family 2
Gene 38: (Lp90_1837) polysaccharide polymerase
Gene 39: (Lp90_1838) Glycosyltransferase, family 1
Gene 40: (Lp90_1839) Glycosyltransferase
Gene 41: (Lp90_1840) Exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase
Gene 42: (Lp90_1841) Nucleoside-diphosphate-sugar epimerase
Gene 43: (Lp90_1842) Capsular polysaccharide biosynthesis protein
Gene 44: (Lp90_1843) Exopolysaccharide synthesis protein
Gene 45: (Lp90_1844) Lipopolysaccharide biosynthesis

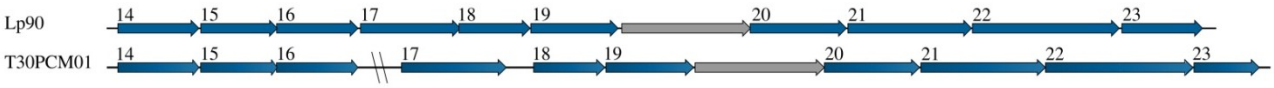

Figure 4.8 - Organization of the *cps2* genes cluster involved in the EPS biosynthesis of *Lactobacillus plantarum* Lp90 and comparison with *Lactobacillus fabifermentans* T30PCM01 genomes. Blue arrows represent the genes found in the Lp90 cluster (homologous genes). Gray arrows represent gene apparently not involved in EPS production (membrane protein or hypothetical protein), which are not numbered. The genes numbered as 20,21 and 22 were found to be unique in Lp90 with respect to the species, but they are homologous to the genes of *Lactobacillus fabifermentans* T30PCM01.
Gene 14: (Lp90_1067) Capsular polysaccharide biosynthesis protein
Gene 15: (Lp90_1068) Exopolysaccharide biosynthesis protein
Gene 16: (Lp90_1069) Capsular polysaccharide biosynthesis protein, *CpsB/CapC*
Gene 17: (Lp90_1070) Nucleoside-diphosphate-sugar epimerase
Gene 18: (Lp90_1071) Exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase
Gene 19: (Lp90_1072) glycosyltransferase
Gene not numbered: (Lp90_1073) Membrane protein
Gene 20: (Lp90_1074) Glycosyltransferase, family 2
Gene 21: (Lp90_1075) Polysaccharide pyruvyl transferase
Gene 22: (Lp90_1076) Polysaccharide biosynthesis protein
Gene 23: (Lp90_1077) mannosyltransferase

4.4 Genes-deletion of *Lactobacillus plantarum* Lp90: Lp90Δcps2 and Lp90Δcps2.5 two non-ropy mutant strains

4.4.1 pNZ8220 and pNZ8221 mutagenesis plasmids and *E. coli* transformation

In order to generate *L. plantarum* Lp90Δcps2 and Lp90 Δcps2.5, non-ropy mutants strains of parental Lp90, two mutagenesis plasmids (pNZ8220 and pNZ8221) were previously created using the mutagenesis vector plasmid pNZ5319 (Lambert *et al.,* 2007). The cloning of the upstream (*LF1* and *LF2,* for pNZ8220 and pNZ8221 respectively*)* and downstream (*RF,* in common for the two mutagenesis plasmids) flanking homologous regions of the target genes were performed using the genomic DNA of Lp90 as template and the pairs of primers LF1, LF2 and RF, listed in **table 3.1.** Electrophoretic analysis on 1% agarose gel (**Figure 4.9**) clearly confirmed the size of amplification fragments of about 1 kbps.

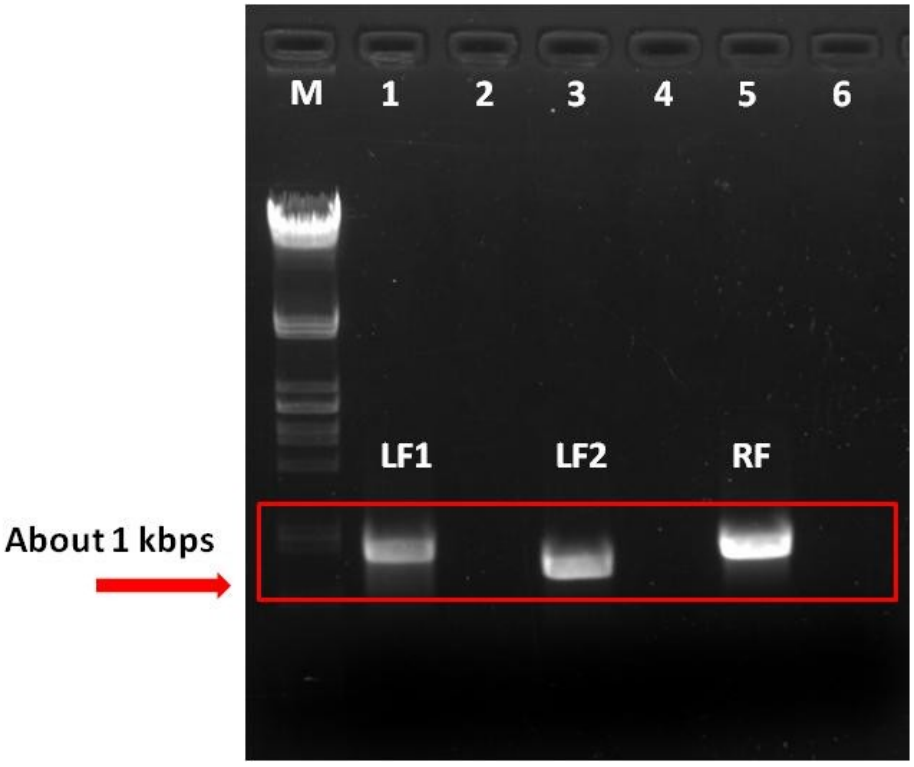

Figure 4.9 - PCR fragments of the upstream and downstream flanking regions. Lane 1: *LF1* left flanking region. Lane 3: *LF2* left flanking region. Lane 5: *RF* right flanking region. All the amplified products were about 1 kbps. Lane M: DNA ladder. Lanes 2, 4, 6: negative controls.

Subsequently, *SOE* products were constructed in order the replace the target genes with chloramphenicol (*cat*) marker, by the splicing overlap extension (*SOE*) method (Horton, 1993). Therefore, the purified *LF*1, *LF2* and *RF* fragments were combined with the *cat* amplicon, and the PCR products were loaded in three parts on 1% agarose gel. As expected, the size of each *SOE* product (*LF1-cat-RF* and *LF2-cat-RF*) was about 3.2 kbps and the amplified products were recovered and purified from the gel (**Figure 4.10**).

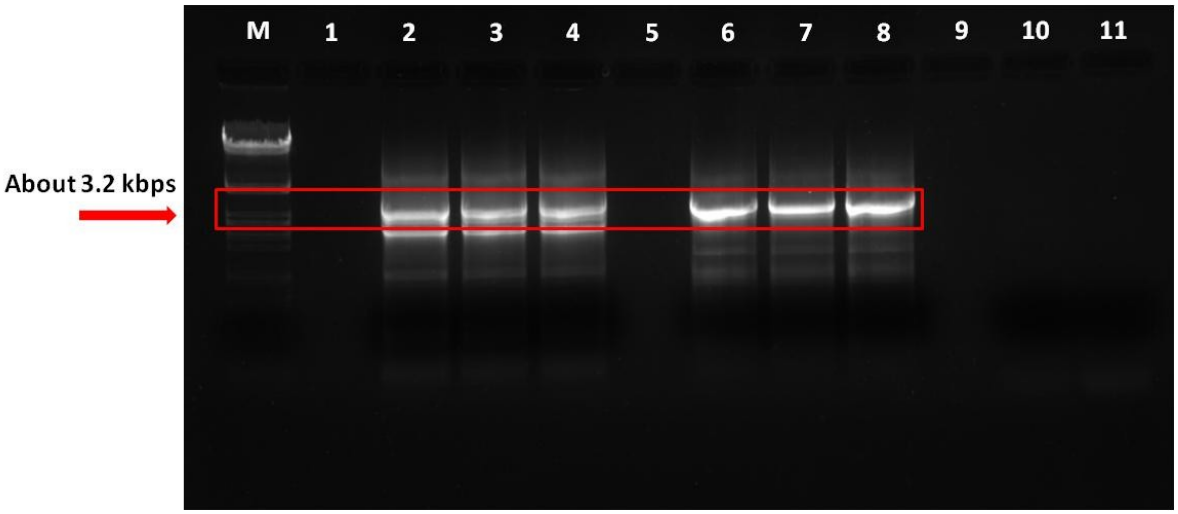

Figure 4.10 – *SOE* products (*LF1-cat-RF* and *LF2-cat-RF*). Lanes 2, 3, 4: *LF1-cat-RF SOE* product. Lanes 6, 7, 8: *LF2-cat-RF SOE* product. Lane M: DNA ladder. Lanes 1, 5: empty. Lanes 10, 11: negative controls. All the amplified products were about 3.2 kbps.Each *SOE* product was loaded in three parts, to better recover the fragments from the gel.

The vector plasmid pNZ5319 was digested by the restriction enzymes *Swa*I and *Ecl*136II and separated on 1% agarose gel. The backbone 2.7 kbps fragment was recovered from the gel and purified (**Figure 4.11**). Finally, the mutagenesis plasmids pNZ8220 and pNZ8221 were obtained by blunt-ends ligation between fragment from pNZ5319 of 2.7 kbps and *SOE* products of 3.2 kbps (*LF1-cat-RF* and *LF2-cat-RF,* for the respective plasmids mutagenesis).

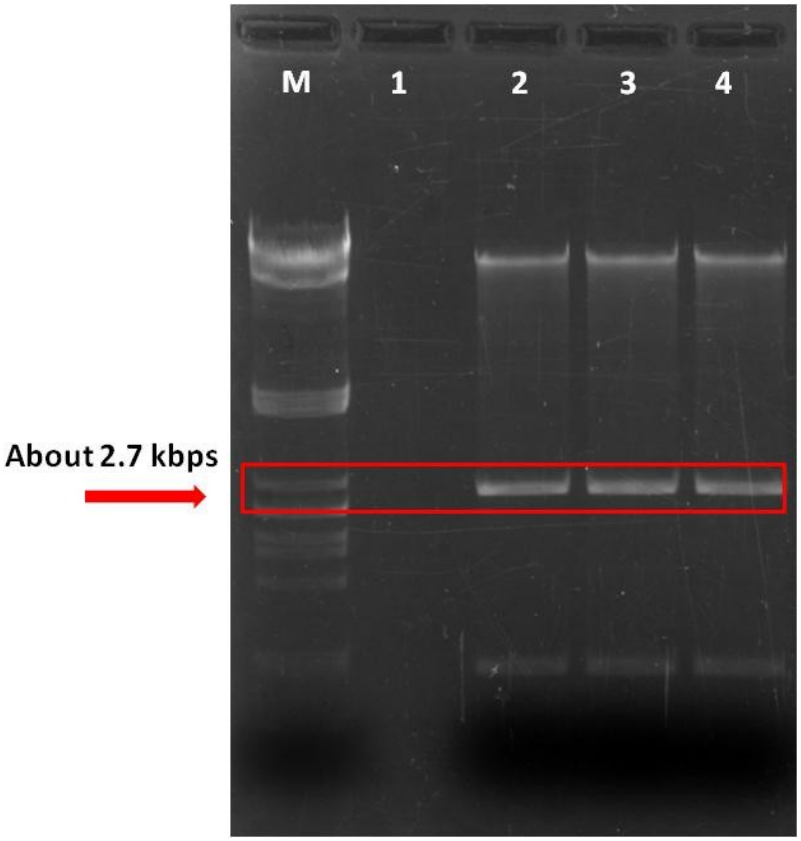

Figure 4.11 – Digested vector plasmid pNZ5319. Lanes 2, 3, 4: backbone 2.7 kbps fragment. Lane M: DNA ladder. Lanes 1: empty. Digested product was loaded in three parts, to better recover the fragment from the gel.

Colony PCR performed on chemicals transformed *E. coli* with pNZ8220 and pNZ8221 mutagenesis plasmids, confirmed the presence of *SOE* products. Subsequently, the mutagenesis plasmids were extracted from the colony-PCR positive colonies and digested with *Xho*I. Electrophoretic analysis on 1% agarose gel, showed the expected size of the digested plasmids (5.9 and 5.8 kbps for pNZ8220 and pNZ8221 respectively). Moreover, plasmid DNA sequencing confirmed the correct cloning of the mutagenesis plasmids (data not shown).

4.4.2 *L. plantarum* Lp90 transformation with pNZ8220 and pNZ8221 mutagenesis plasmids

The purified pNZ8220 and pNZ8221 mutagenesis plasmids were electroporated in electrocompetent cells of *L. plantarum* Lp90. Each colony, obtained from both transformed Lp90 (Lp90/pNZ8220 and Lp90/pNZ8221) was streaked on two kinds of plates with different antibiotics: MRS agar + chloramphenicol (10 µg/mL); MRS agar + erythromycin (30 µg/mL).

The transformed bacterial colonies which were resistant to chloramphenicol (Cm^r) and sensitive to erythromycin (Em^s) allowed us to distinguish the deletion mutants generated by homologous recombination in double crossover-based strategy. Furthermore, *L. plantarum* Lp90Δcps2 and Lp90 Δcps2.5 (non-ropy mutants strains) were confirmed by PCR analysis, which clearly showed the presence of *LF*, *RF*, *SOE*, *cat* and the absence of *ery (erythromycin)* amplification fragments, on 1% agarose gel (**Figures 4.12 and 4.13**).

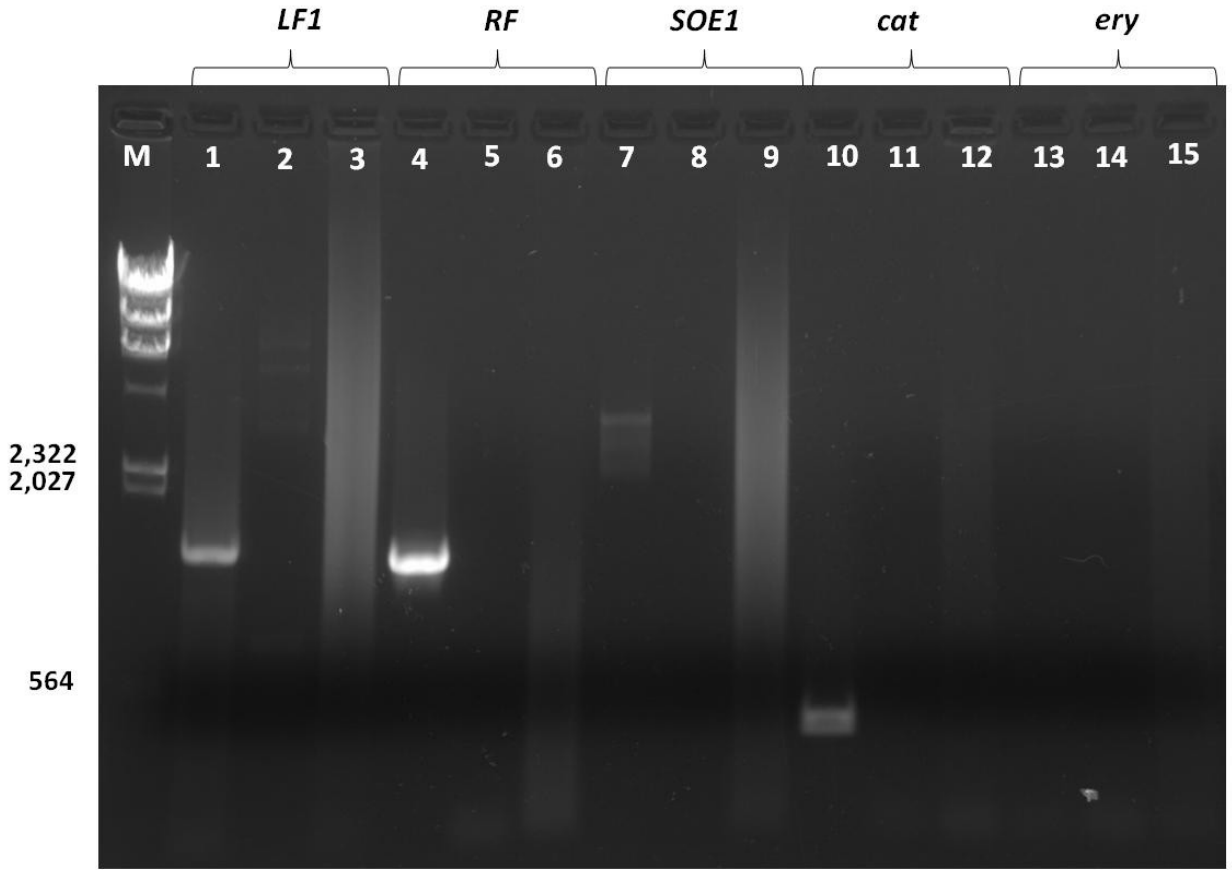

Figure 4.12 – PCR screening of *L. plantarum* Lp90Δcps2 mutant strain. Lane1: *LF1* fragment of Lp90Δcps2. Lane 2: *LF1*negative control of Lp90. Lane 3: *LF1*negative control H_2O. Lane 4: *RF* fragment of Lp90Δcps2. Lane 5: *RF* negative control of Lp90. Lane 6: *RF* negative control H_2O. Lane 7: *SOE1* fragment of Lp90Δcps2. Lane 8: *SOE1* negative control of Lp90. Lane 9: *SOE1*. negative control H_2O. Lane 10: *cat* fragment of Lp90Δcps2. Lane 11: *cat* negative control of Lp90. Lane 12: *cat* negative control H_2O. Lane 13: *ery* fragment absence of Lp90Δcps2. Lane 14: *ery* negative control of Lp90. Lane 15: *ery* negative control H_2O. Lane M: DNA ladder.

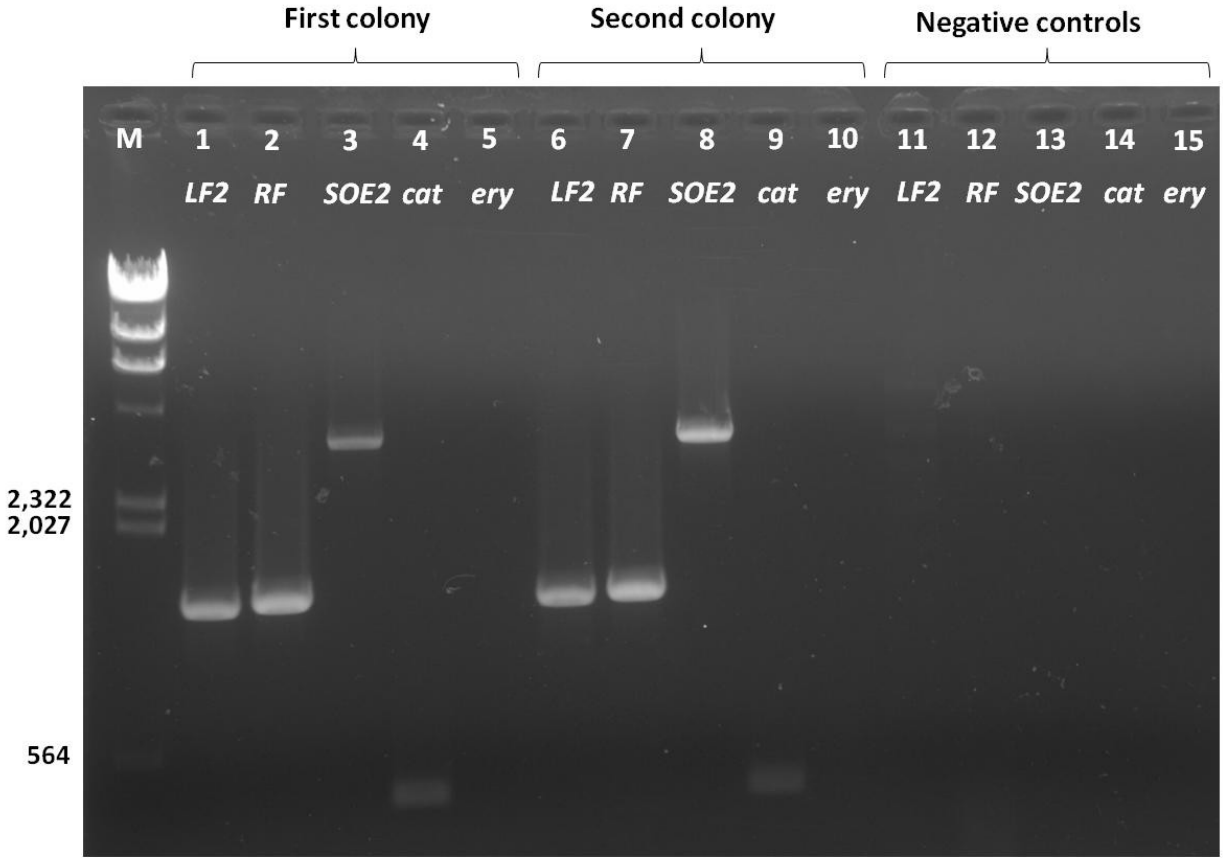

Figure 4.13 – PCR screening of *L. plantarum* Lp90Δcps2.5 mutant strain. Lanes 1, 2, 3, 4, 5: respectively *LF2, RF, SOE2, cat, ery* fragments of a first colony of Lp90Δcps2.5. Lanes 6, 7, 8, 9, 10: respectively *LF2, RF, SOE2, cat, ery* fragments of a second colony of Lp90Δcps2.5. Lanes 11, 12, 13, 14, 15: respectively *LF2, RF, SOE2, cat, ery* negative control H_2O. Lane M: DNA ladder.

Following the entire *cps2* cluster deletion (genes from Lp90_1067 to Lp90_1077) as well as the partial *cps2* deletion (genes from Lp90_1073 to Lp90_1077) (**Figure 3.1**) of *L. plantarum* Lp90, the respective Lp90Δcps2 and Lp90Δcps2.5 mutants strains lost the typical ropy phenotype.

This phenomenon was clearly visible in MRS broth cultures, as shown in **Figure 4.14 A and B.** Moreover, Transmission Electron Microscope (TEM) analysis confirmed the lack of extracellular polysaccharides around the bacterial cell wall in non-ropy mutant strains compared to parental Lp90 (**Figure 4.15 A and B.**).

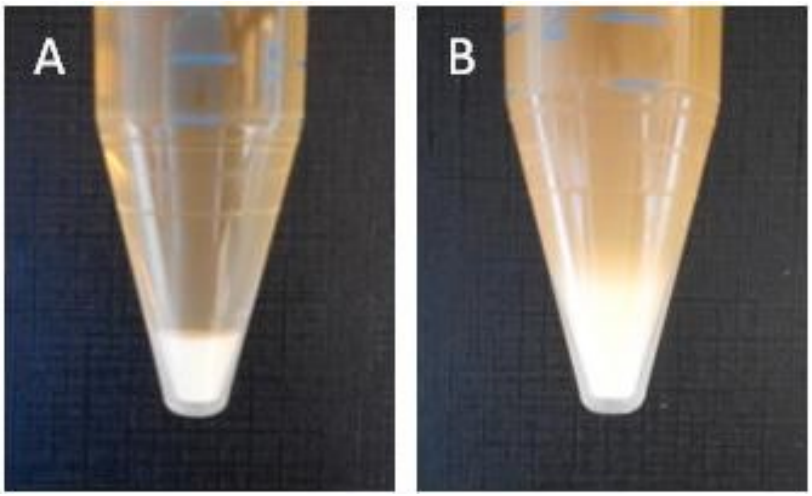

Figure 4.14 - *Lactobacillus plantarum* Lp90Δcps2 mutant strain growth in MRS broth (A). Ropy phenotype of *Lactobacillus plantarum* Lp90 EPS-producing strain in MRS broth (B).

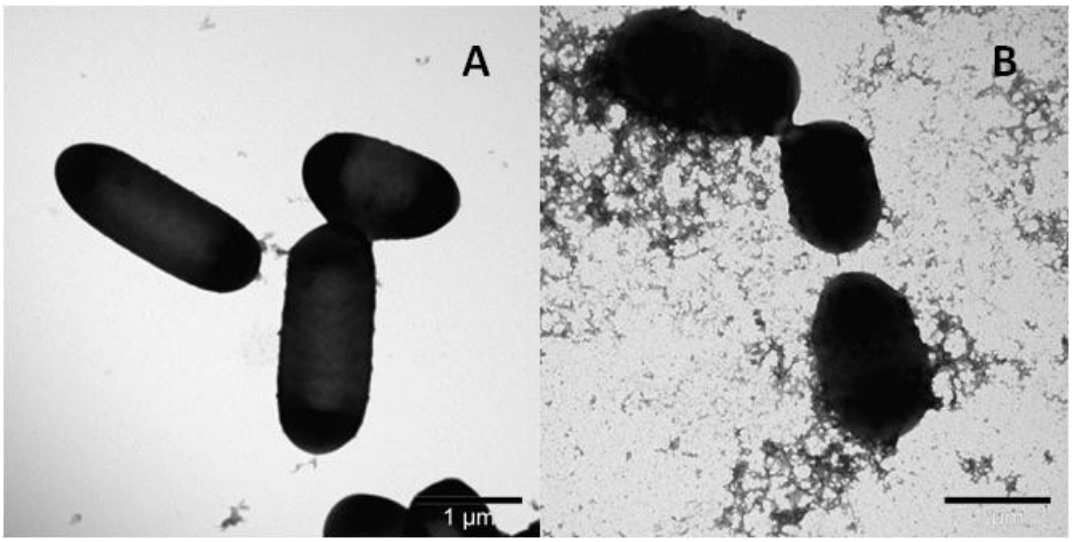

Figure 4.15 - Transmission Electron Micrograph of *L. plantarum* Lp90Δcps2 non-ropy mutant strain (A) and Lp90 wild type cells (B).

4.5 Lactobacilli and Caco-2 cells *in vitro* interactions

4.5.1 Lactobacilli adhesion on Caco-2 cells

In order to evaluate the influence of EPS on lactobacilli ability to adhere on Caco-2 cell monolayer, *L. plantarum* Lp90, WCFS1, SF2A35B and their respective Δcps2 mutant strains were used. The bacterial cells were harvested in a stationary growth phase, since in this stage the ropy phenotype of Lp90 is more pronounced, presumably due to a greater accumulation of EPS. The percentage of bacterial adhesion was determined by CFUs count considering the total concentration of added bacteria (i.e. both adherent and not adherent bacteria) (**Figure 4.16**).

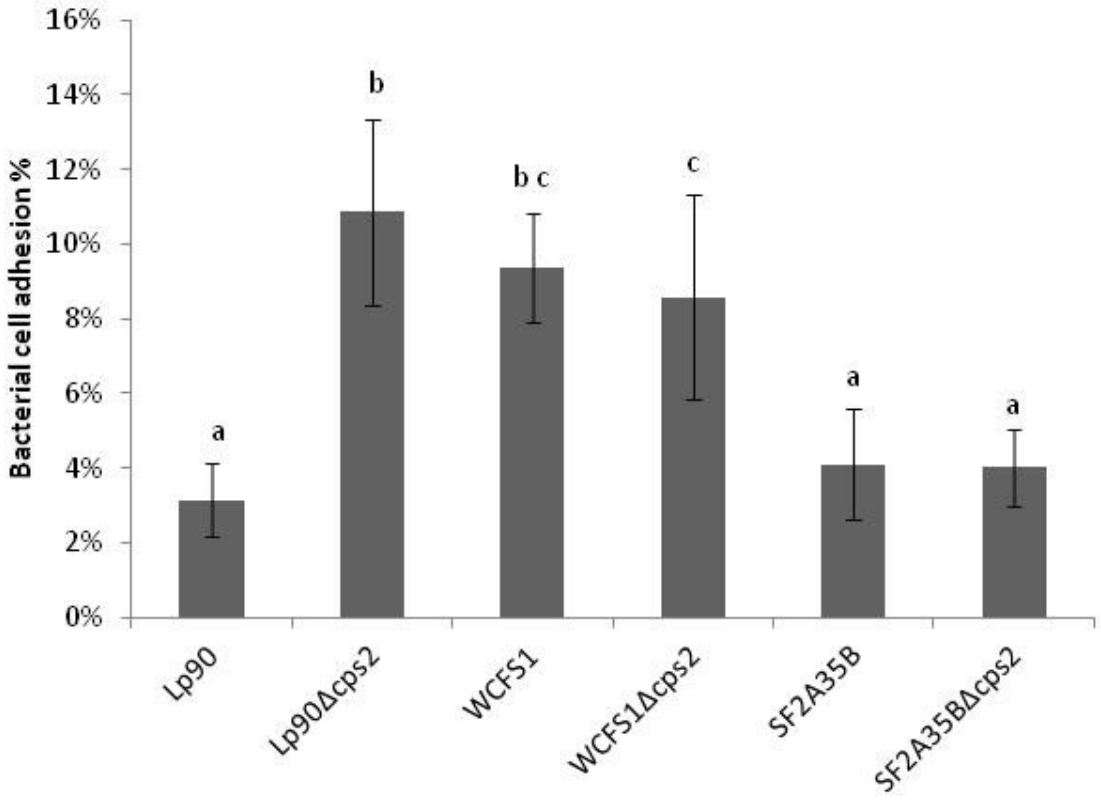

Figure 4.16 - Adhesion of *L. plantarum* strain to Caco-2 cells. Adhesion levels are expressed as the percentage of the adhered CFUs relative to the total number of added bacteria (1,000: 1, bacteria to Caco-2 cells). Values represent mean ± standard deviation of three different experiments. Different superscript letters indicate statistically significant differences ($p<0.05$) in adhesion as assessed by one-way ANOVA test.

L. plantarum Lp90 showed a statistically significant lower percentage of adhesion than Lp90Δcps2, WCFS1, WCFS1Δcps2, suggesting that it attached more weakly to Caco-2 cells. Otherwise, the bacterial cells adhesion of Lp90 is comparable with adhesion level of both ropy and non-ropy *L. plantarum* SF2A35B and SF2A35BΔcps2 respectively.

This result suggests that removing EPS might enhance bacterial attachment, therefore the deficiency of extracellular polysaccharides in Lp90Δcps2, WCFS1, WCFS1Δcps2 non-ropy strains improving bacterial attachment. This effect is more evident in Lp90 cells from stationary phase, when an increased amount of EPS is accumulated outside the cells compared to log phase (data not shown).

As suggested by previous studies, and in accordance with our findings, the EPS removal might unmask adhesins and/or other cell surface factors which enable the process of bacterial adherence (Ruas-Madiedo *et al.,* 2006). Moreover, EPS could interfere with adhesion to intestinal cells by a competitive inhibition mechanism (Ruas-Madiedo et al 2006).

Noticeably, a reduced ability of Lp90 to attach Caco-2 cells compared to Lp90Δcps2, WCFS1 and WCFS1Δcps2, reflects a lower potential probiotic activity. Again this feature could be ascribed to the different original niches of the strains; considering that WCSF1 has been isolated from human saliva, it might be more prone to adhesion on human cells.

As indicated by other authors, the EPS layer might shield specific adhesion factors on the bacterial cell surface, and/or electrostatically interfere with the binding to receptors of mucosal surface, thus hindering the adhesion process and the recognition mechanisms which are required for stable adherence on animal cells (Leeber *et al.,* 2009; Denou *et al.,* 2008). Nikolic *et al.* (2012) reported that three non-ropy derivatives improved *in vitro* adhesion with respect to the parental strains. A negative impact on adhesion has been reported also for capsule polysaccharides of gram-negative bacteria, (Schembri *et al.,* 2004). Nevertheless, some authors have also observed opposite effects. For instance, the β-glucans secreted by *Pediococcus parvulus*, apparently increase the adhesion abilities of the producing-microorganism (Fernández

de Palencia *et al.,* 2009; Garai-Ibabe *et al.,* 2010), as well as exopolysaccharides produced by certain lactic acid bacteria from wine (García Ruíz *et al.,* 2014) and when exogenous β-glucans were added to *L. plantarum* (Russo *et al.,* 2012). In this regard, the ambivalent effect of EPS might depend on their specific chemical nature (Fernández de Palencia *et al.,* 2009).

4.5.2 Competition against *Escherichia coli* O157: H7 in adhesion assays on Caco-2 cells

In order to assess the potential of *L. plantarum* Lp90 in preventing the intestinal colonization by microbial pathogen, we studied its ability to compete with, displace or inhibit the adhesion of the enteropathogen *E.coli* O157: H7 on Caco-2 cells (**Figure 4.17**). In order to understand the possible contribute of the EPS to the behavior of Lp90, WCFS1 was also used as a control strain and bacterial cells were used either before or after PBS wash; moreover, EPS isolated from Lp90 were also investigated in the adhesion tests.

(i) In competitive adhesion assay, *L. plantarum* Lp90 seemed to favor adhesion by *E. coli* (relative adhesion of 2.8±0.4 - 2.7±0.5); isolated EPS also increased *E. coli* adhesion, in a concentration-dependent fashion (3.0±0.9 - 4.1±1.2 with 0.1 and 1.0 mg/mL EPS, respectively). Conversely, *L. plantarum* WCFS1 did not significantly change *E. coli* relative adhesion level. For both strains, the effect on adhesion was not influenced by the PBS wash.

The different competitive abilities of Lp90 and WCFS1 confirms findings by Lee and Puong (2002) who evidenced a strain-dependent degree of competition, which was probably determined by the affinity of adhesins on respective bacterial surfaces for the stereo-specific receptors that they are competing for, or their relative positions in the case of steric hindrance.

The increased adhesion of the pathogen observed in competition assays with both the EPS-producing Lp90 unwashed cells and isolated EPS is in agreement with results from Ruas-Madiedo *et al*. (2006), who have hypothesized that components of the bacterial pathogen surface could bind specific EPS and then, such bound EPS would adhere to cellular mucus, thus favoring pathogen attachment. On the other hand, pathogen adhesion could have been favored in the

presence of washed Lp90 cells possibly in reason of a lower lactobacilli adhesion, which might depend on altered bacterial cell surface, due to the PBS wash. Indeed, in such situation, more binding sites on intestinal cells would be available for the pathogens.

(ii) In displacement of adhesion assay, both *L. plantarum* strains favored the adhesion of *E. coli*, with observed relative adhesion values ranging from 2.02±0.60 to 2.62±0.38. These results suggest the inability of *L. plantarum* strains to displace the pathogen once it has colonized the cell monolayer. Even in this case, no significant difference could be ascribed to bacterial wash, i.e. PBS treatment. These results are in accordance with previous findings about the displacement ability exhibited by lactobacilli. Indeed, as already observed, lactobacilli seem rather able to compete efficiently for adhesion with pathogenic gastrointestinal (GI) bacteria when they are co-incubated, while their ability to displace already attached pathogenic bacteria is generally much lower than the capacity to inhibit their adhesion either by direct competition (e. g., coincubation) or by exclusion (e.g. preincubation) (Lee *et al.,* 2003). Interestingly, it is reported that many GI bacteria could not be displaced within 1 h incubation; however, when the incubation time was extended (to 2 h), higher degrees of displacement were observed suggesting that displacement of GI bacteria is a very slow process (Lee *et al.,* 2003). Likewise, Bernet *et al*. (1994) found that when *L. acidophilus* LA 1 was incubated on Caco-2 cells before or together with *E. coli* (ETEC) H1040, an identical inhibition of pathogen-cell association was seen. By contrast, a significant decrease of efficacy was seen when pathogens were incubated with Caco-2 cells before adding LA 1. Conversely, the addition of purified EPS resulted in a significant decrease of *E. coli* adhesion. This could be due to the capacity of EPS to bind *E. coli* eroding the adhesion to Caco-2 cells.

(iii) In inhibition of adhesion assay, both strains strongly inhibited adhesion of the pathogen. Pre-treatment with naïve Lp90 and WCFS1 cells, as well as with PBS-washed Lp90 cells, resulted in appreciable inhibition of pathogen adhesion (relative adhesion values of 0.15±0.08; 0.21±0.06; 0.22±0.06, respectively). A lower percentage of inhibition (i.e., pathogen relative

adhesion of 0.50±0.16) was observed when using PBS-washed *L. plantarum* WCFS1. We hypothesize that, during the first hour of incubation, WCFS1 and Lp90 might have adhered to the majority of accessible sites on Caco-2 cell surface, making them no more available for the pathogen. Such findings confirm that lactobacilli have a discrete potential to prevent intestinal colonization by pathogens (Reid and Burton, 2002); Arena *et al.* (2014b) reported that when lactobacilli adhere in a stable manner on the epithelial layer they are able to contrast more strongly the *E. coli* adhesion.

Moreover, we speculate that the washing of WCFS1 cells may have compromised their adhesion capacity, probably by altering surface structures or molecules involved in the mechanisms of cellular adhesion, and hence reduce their inhibitory effect towards the pathogen.

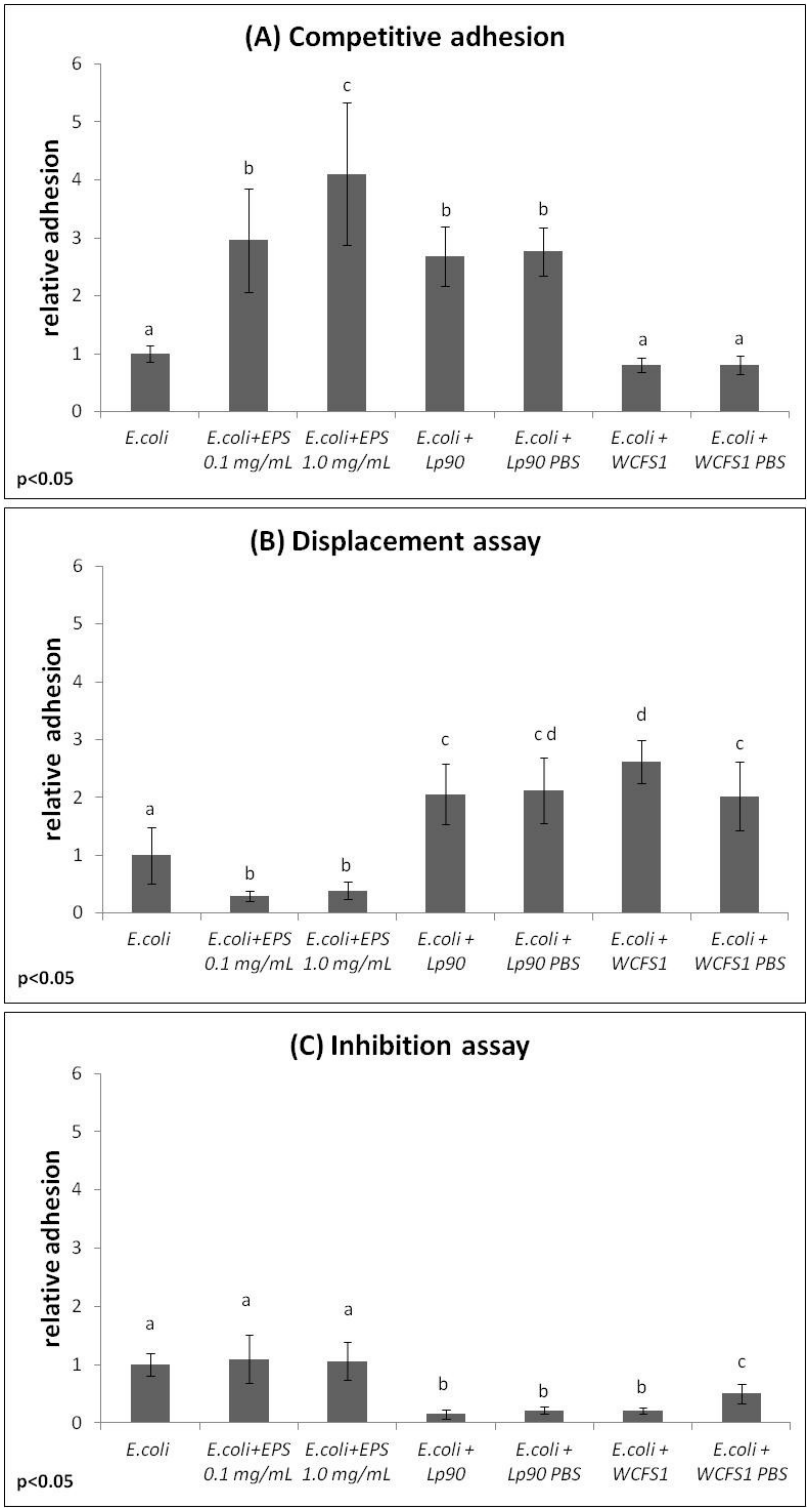

Figure 4.17 - Influence of isolated Lp90 EPS and *L. plantarum* on the adhesion of *E. coli* O157:H7 on Caco-2 cell monolayers. A) Competitive adhesion assay: EPS or *L. plantarum* and *E. coli* cells were co-incubated with Caco-2 cells; B) displacement assay: *E. coli* was pre-incubated with Caco-2 cells, then EPS or *L. plantarum* were added; C) inhibition assay: EPS or *L. plantarum* were pre-incubated with Caco-2 cells, then *E. coli* was added. The inhibition of pathogen adhesion was determined by a quantitative PCR-based method, and expressed as a relative adhesion level with respect to the adhesion observed when *E. coli* was tested alone (control sample). Values represent mean ± standard deviation of three different experiments. Different superscript letters indicate statistically significant differences ($p<0.05$) in adhesion as assessed by one-way ANOVA test. EPS isolated from Lp90 were used at concentrations of 0.1 and 1.0 mg/mL. *L. plantarum* cells from Lp90 or WCFS1 strains were used, with or without PBS wash.

4.5.3 Immune gene expression after co-incubation of Caco-2 cells and lactobacilli

The potential immune-modulation effects were evaluated by co-incubating Caco-2 cells and *L. plantarum* strains and by subsequent monitoring the transcriptional pattern of genes involved in immune modulation and signal transduction (IL6, IL12a, IL-8, IL-10, MIP3alpha; IKBalpha), in antimicrobial activity (HBD2, LL37, lysozyme) in physical barrier reinforcement of the mucosal surface (CLDN4, ZO2, MUC2) and receptors of the innate immunity response (TLRs). Gene expression were determined by quantitative real-time PCR, and mRNA levels were calibrated on untreated Caco-2 cells and normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and hypoxanthine phosphoribosyl transferase 1 (HPRT1) as internal controls. Stationary phase bacteria cultures were used as in this growth stage accumulation of EPS is expected to be higher. The immune-modulation effect of Lp90 was compared with that of the non-ropy strain WCFS1, moreover stimulation was performed with either washed (WCFS1 PBS and Lp90 PBS) or not washed bacterial cells (WCFS1 and Lp90) to understand the possible contribute of EPS and or other cells surface weakly bound molecules (**Figure 4.18**).

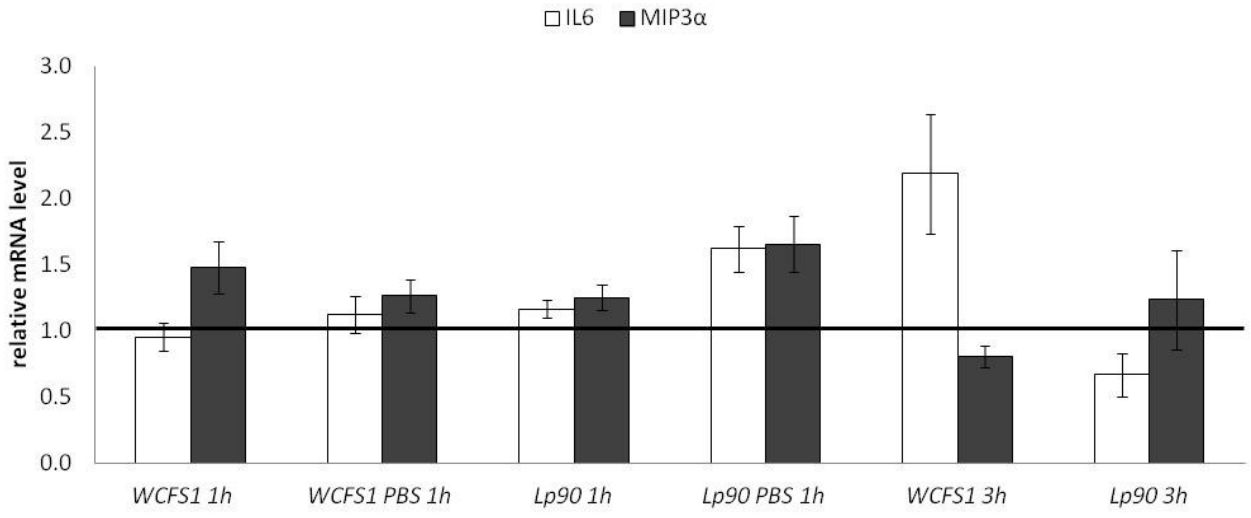

Figure 4.18 - Immune modulation analysis of *L. plantarum* strains on Caco-2 cells. The expression of immune related IL-6 gene (open bar) and MIP3α gene (full bar) were determined by quantitative real-time PCR. Gene expression analysis of *L. plantarum* strains WCFS1 and Lp90 was performed from stationary phase washed or unwashed collected cells. PBS indicate the washed bacterila cells treatment with phosphate saline solution.

Among the different genes analyzed, only minor differences in expression patterns were found by comparing the effect of Lp90 and WCFS1.

IL-6 showed a higher transcriptional level, after 3 hours of stimulation treatment of Caco-2 with WCFS1, as well as after 1 hour of incubation with Lp90 PBS washed strain, although in a lower level. By contrast, following the stimulation with EPS producing Lp90, IL-6 transcriptional level decreased. These results suggest that EPS hinder the stimulation of this gene, in agreement with Fanning *et al*. (2012) whom reported that the cell surface-associated exopolysaccharide of *Bifidobacterium breve* decrease the production of pro-inflammatory cytokines. Conversely, the high-molecular-mass polysaccharides of the *L. casei* Shirota cell wall induced the production of various cytokines by macrophages, including IL-6 (Yasuda *et al.,* 2008); EPS from *Lactobacillus plantarum* strongly induced of the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Liu *et al.,* 2011).

MIP3α expression level showed a higher relative mRNA level in cells treated with *L. plantarum* WCFS1 and Lp90 PBS washed after 1 hour of Caco-2 co-incubation, confirming the previous hypothesis, i.e. that the extracellular polysaccharides are disadvantageous for the bacterial cells-host interaction. However, as previously mentioned by Bove *et al.* (2012) MIP-3α is only moderately induced by dead *L. plantarum* rather than live bacterial cells.

For the other investigated genes neither repression nor upregulation at significant level were observed with either Lp90 or WCFS1 strain (data not shown). Moreover, no relevant difference in transcriptional pattern could be also ascribed to the washing treatment of bacterial cells aiming at removing the EPS component from their surface (data not shown). Such data confirm that *L. plantarum* has a low immune-stimulating action when used in the form of intact live cells, while higher immune induction could be observed when treating Caco-2 cells with dead bacteria (Bove *et al.,* 2012).

Taken togheter, these results suggest that EPS do not contribute to the immune modulation as no significant difference in transcript levels were observed between ropy and non-ropy strains, nor

between PBS-washed and native cells of both tested *L. plantarum* strains. We assume that exopolysaccharides may mask the molecules responsible for the recognition between the bacterial cell wall and that of the eukaryotic cell. This effect has been already observed for exopolysaccharides produced by *Lactobacillus rhamnosus* GG which protect by shielding, against intestinal innate factors (Lebeer *et al.,* 2011). Moreover, these data are also consistent with the results obtained in the adhesion test between *L. plantarum* EPS producer and the Caco-2 cells, where EPS seem counter the adhesion/ recognition by a cellular shielding effect.

4.6 Zebrafish gut *in vivo* colonization by mCherry-labelled *L. planatrum* strains

4.6.1 Fluorescent labeling of *Lactobacillus* strains with pRCR12 and detection of the mCherry protein

Probiotic potential of oenological LAB has been analyzed only in few cases, and the EPS-producing phenotype was one of the more attractive features (García Ruíz *et al.,* 2014). EPS from LAB was thought regulate inflammatory responses in the intestinal lumen (Notararigo *et al.,* 2014), and they could exert some prebiotic activity (Russo *et al.,* 2012). In this regard, the ability of *L. plantarum* Lp90 to adhere *in vivo* on enterocytic cells of zebrafish larvae, relatively to its exopolysaccharides production, was investigated by fluorescent labeling of lactobacilli. *L. plantarum* B2 was analyzed as an additional non-EPS producer strain. Furthermore, this microorganism was previously shown to adhere *in vitro* to human intestinal epithelial cells and to be able to synthesize vitamin B2 in co-culture systems with Caco-2 cells (Arena *et al.,* 2014b). The labeling of lactobacilli was realized by pRCR12 plasmid insertion in *L. plantarum* strains, which was easily confirmed by the typical pink color conferred by mCherry protein to the colonies of Lp90/pRCR12 and *L. plantarum* B2/pRCR12 (**Figura 4.19**); this is in accordance with previous findings reported by García-Cayuela *et al.* (2012). Fluorescence was observed 11

days post plating on MRS agar + 10 µg/mL of chloramphenicol (**Figure 4.20**), thus suggesting the high stability of the plasmid and the mCherry protein in the bacterial cells.

L. plantarum Lp90/pRCR12 colonies showed a more intense color than *L. plantarum* B2/pRCR12, in fact the fluorescence detected in exponential bacterial cultures analyzed by fluorescent microscopy was higher (data not shown). This could be attributed to the different pRCR12 plasmid copy number of Lp90/pRCR12 and B2/pRCR12 (respectively 62±2 and 54±3 pRCR12 plasmid DNA molecules per bacterial genome) (**Figure 4.21**).

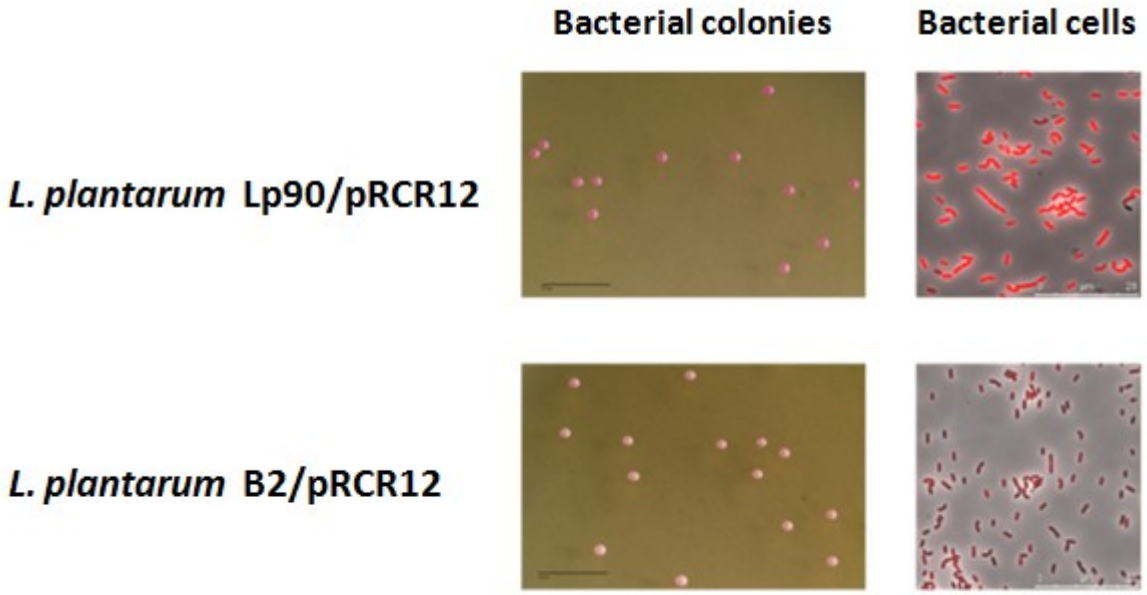

Figure 4.19 - mCherry protein fluorescence in *L. plantarum* Lp90/pRCR12 and B2/pRCR12. Bacterial colonies grown on MRS agar plates containing 10 µg/mL of chloramphenicol and bacterial cells in exponential growth phase under exposure to fluorescence microscope.

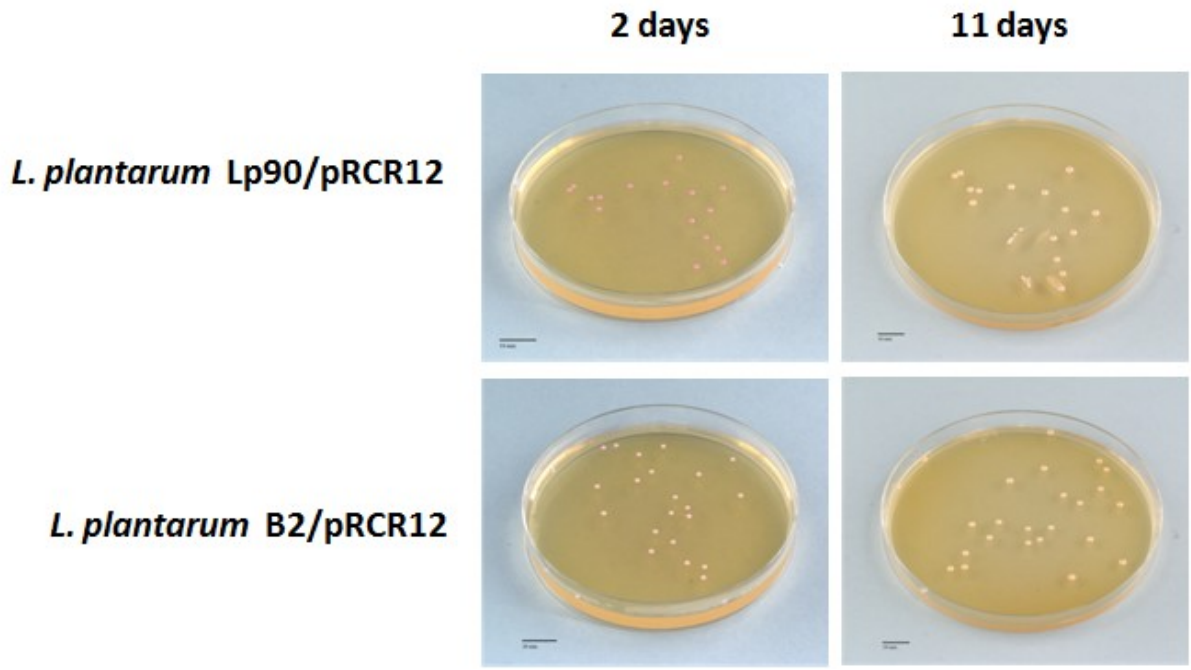

Figure 4.20 - Colonies of *L. plantarum* Lp90 and B2 carrying pRCR12 on MRS agar plates containing 10 µg/mL of chloramphenicol after 2 and 11 days of incubation at 37 °C.

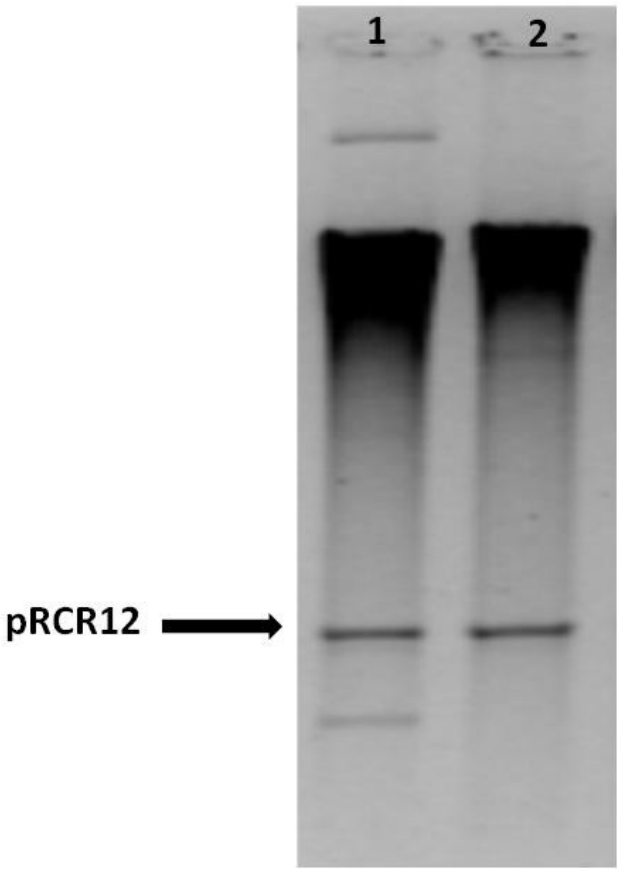

Figure 4.21 - Analysis of pRCR12 plasmid. Total DNA extracts from *L. plantarum* Lp90/pRCR12 (lane 1) and B2/pRCR12 (lane 2); the pRCR12 plasmid was separated by electrophoretic analysis on agarose gel.

The levels of fluorescence allowed the measurement of mCherry active protein in real time during bacterial growth. Therefore fluorescence and optical density were analyzed during the different growth phases of bacterial cultures grown in MRS containing chloramphenicol (10 μg/mL) (**Figure 4.22**).

In both *L. plantarum* strains the fluorescence increased during the bacterial exponential growth phase, reaching different levels of intensity. Lp90/pRCR12 showed an additional increase during the stationary growth phase (**Figure 4.22**), this could indicate that mCherry protein is more stable in this strain.

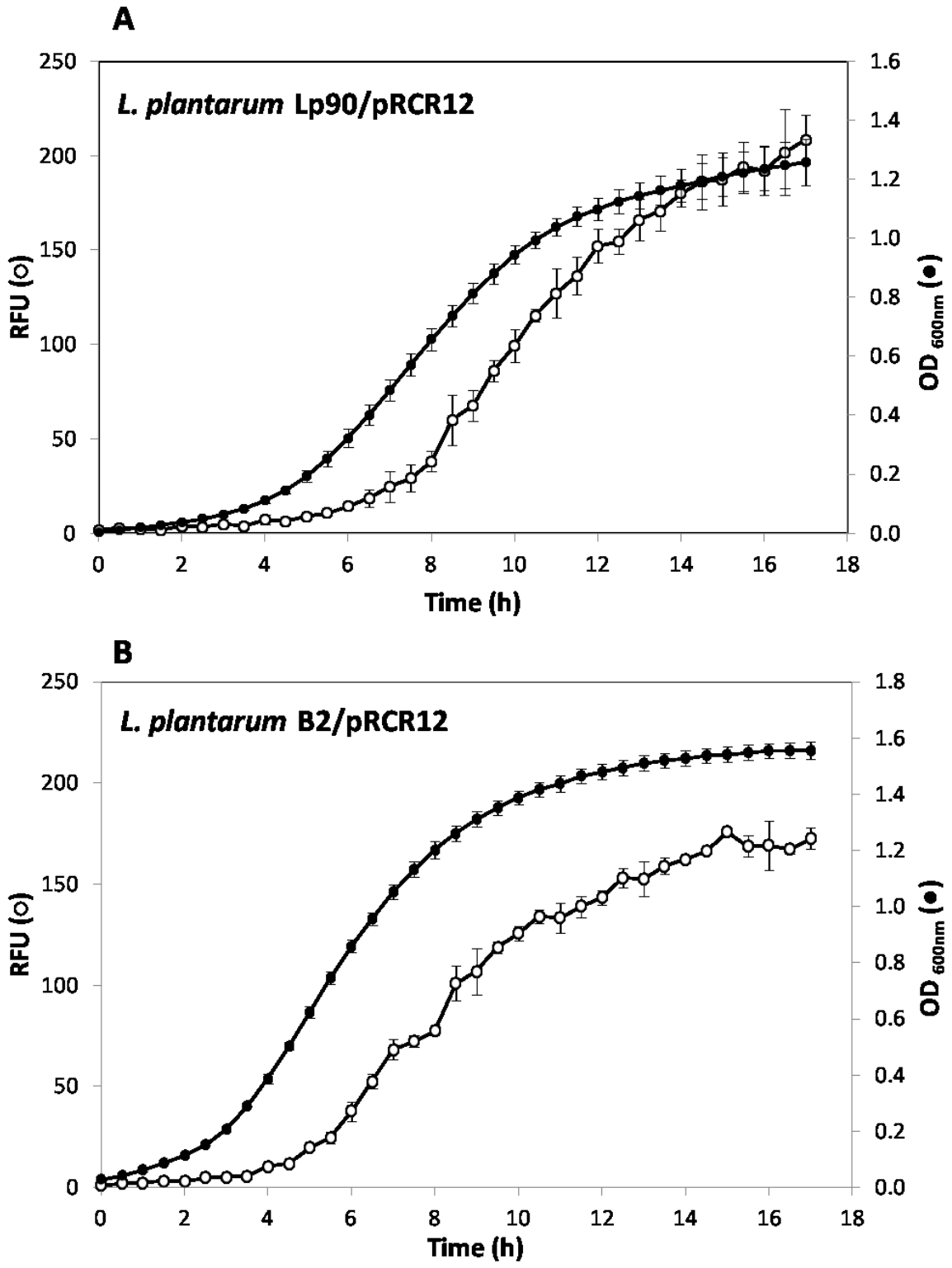

Figure 4.22 - Spectrophotometric detection of *L. plantarum* Lp90/pRCR12 and B2/pRCR12 strains. Optical density (OD_{600nm}) (●) and mCherry fluorescence levels (○) of *L. plantarum* Lp90/pRCR12 (A) and *L. plantarum* B2/pRCR12 (B) bacterial cultures were monitored in real time during 17 hours.

Currently, fluorescent labelling methods are increasingly being used to obtain real-time and *in vivo* evidence of a wide range of biological phenomena (Chudakov *et al.,* 2005). For instance, tagged strains with reporter genes were used to monitor the localization of *Bifidobacterium* species in complex ecosystems like food and faecal microbiota (Landete *et al.,* 2014), or within the mouse gastrointestinal tract (Cronin *et al.,* 2008). However, tracking of fluorescence or luminescence in biological environments is mainly based on the detection of green fluorescence proteins (GFP) or on luciferase-based systems. The use of GFP-producing *Vibrio cholera* cells allowed an easy visualization of the gut infection in zebrafish larvae (Runft *et al.,* 2014). A similar approach for the first time as an *in vivo* screening system to detect probiotic strains with anti-inflammatory properties Rieu *et al.* (2014) infected zebrafish larvae with a strain of *Lactobacillus casei* using GFP-expression to visualize their location in the gut.

Analogously, our findings showed that pRCR12 plasmid could be a valid fluorescent tag of lactobacilli; moreover it does not affect the growth of bacterial host as no relevant difference in growth rates between growth curves of parental strains and pRCR12 transformed strains were observed (data not shown).

4.6.2 Zebrafish larvae colonization by *L. plantarum* strains tagged with mCherry

The mCherry labeling allowed high resolution monitoring of the *in vivo* colonization ability of *L. plantarum* strains in the intestinal tract of the zebrafish larvae and detection of their adhesion to enterocytic cells (**Figure 4.23**). At 6 hours post infection (hpi), abundant red fluorescence was visible in larvae exposed to both *L. plantarum* strains (**Figure 4.24**) and Lp90/pRCR12 emitted a statistically significant higher percentage of fluorescence than B2/pRCR12 (**Figure 4.25 A**).

Interestingly, in larvae inoculated with both *L. plantarum* strains, a spatial displacement of bacteria from the medium to the posterior intestinal tract was observed during the time, suggesting a transient colonization by these bacterial strains (**Figure 4.24 and 4.25 B**). In particular, after 6 hpi few larvae showed red fluorescence in the posterior intestine when they

were exposed to either B2/pRCR12 or Lp90/pRCR12 strains; this percentage increased after 24 hpi ranging from 20 to 30 % of the total (**Figure 4.25 B**).

In order to confirm that fluorescence was related to labeled lactobacilli adhered on gastrointestinal tract, zebrafish larvae were euthanized and the number of viable bacteria at each time of analysis was determined by plate count. At 6 hpi, Lp90/pRCR12 viable cells were significantly higher, although the number decreased over time. Conversely, at 24 hpi, B2/pRCR12 exhibited significantly higher CFU than Lp90/pRCR12. After 48 hpi, the bacterial CFU per larva was very low and most larvae did not show any detectable bacteria (**Figure 4.27**).

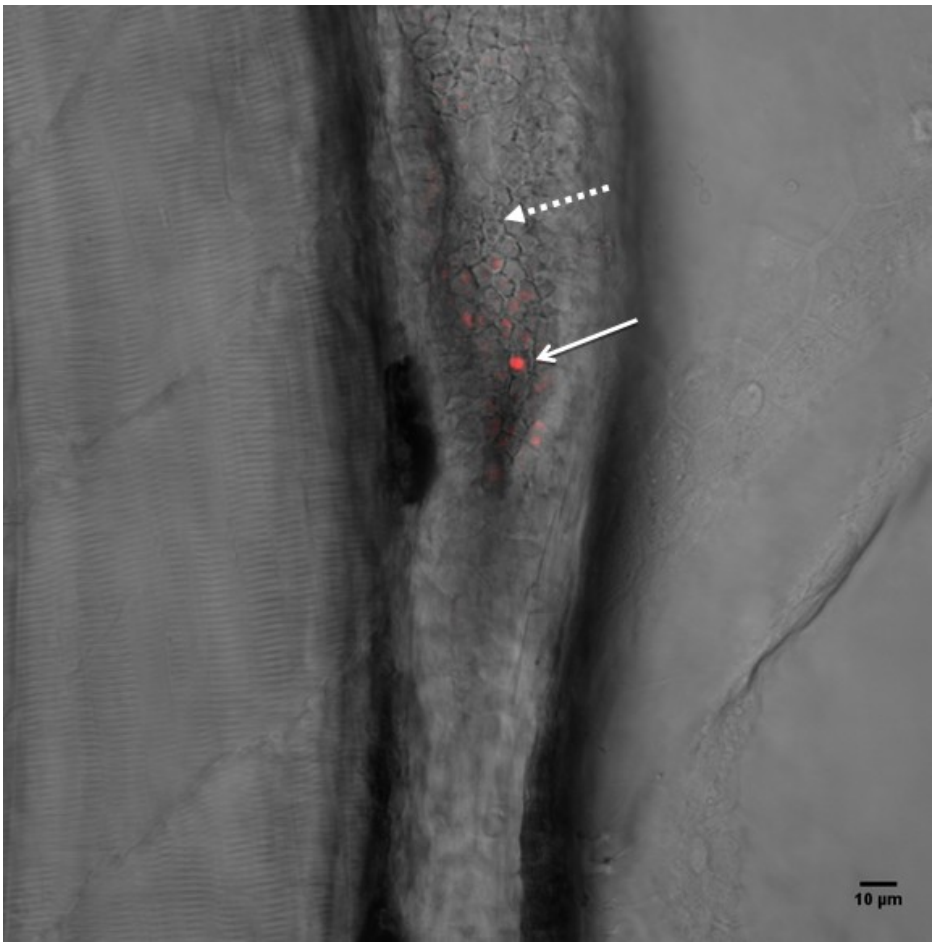

Figure 4.23 – Adhesion of *L. plantarum* Lp90/pRCR12 to zebrafish larvae enterocytes. Images were captured at 48 hpi using a confocal microscope. Full and dashed white arrows mark the localization of Lp90/pRCR12 and enterocytes, respectively.

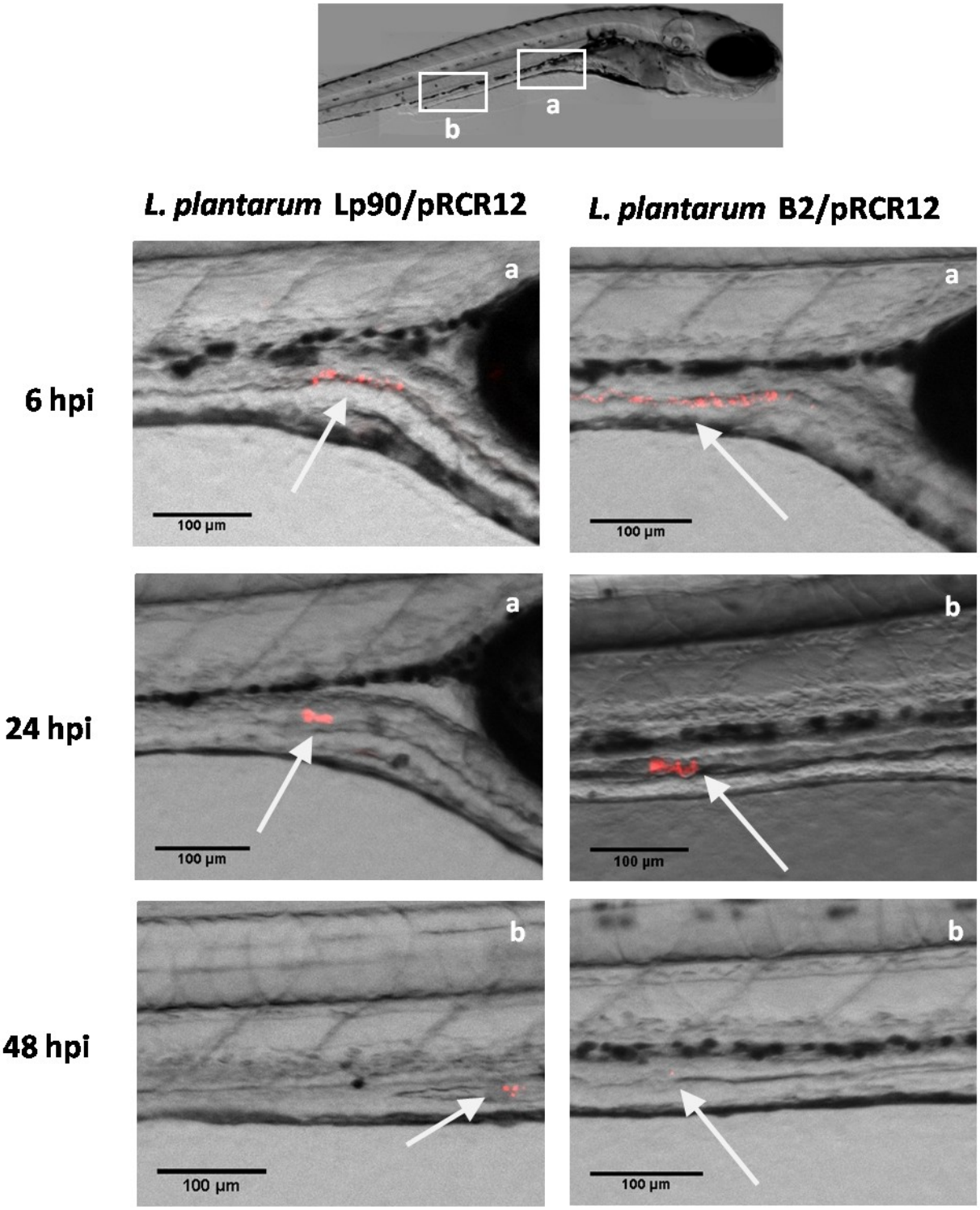

Figure 4.24 - Intestinal distribution of *L. plantarum* strains tagged with pRCR12. Zebrafish larvae infected with either *L. plantarum* Lp90/pRCR12 or *L. plantarum* B2/pRCR12 observed under a fluorescence stereomicroscope at 6, 24 and 48 hpi. White arrows mark the localization of lactobacilli in the medium (a) or posterior (b) intestinal tract.

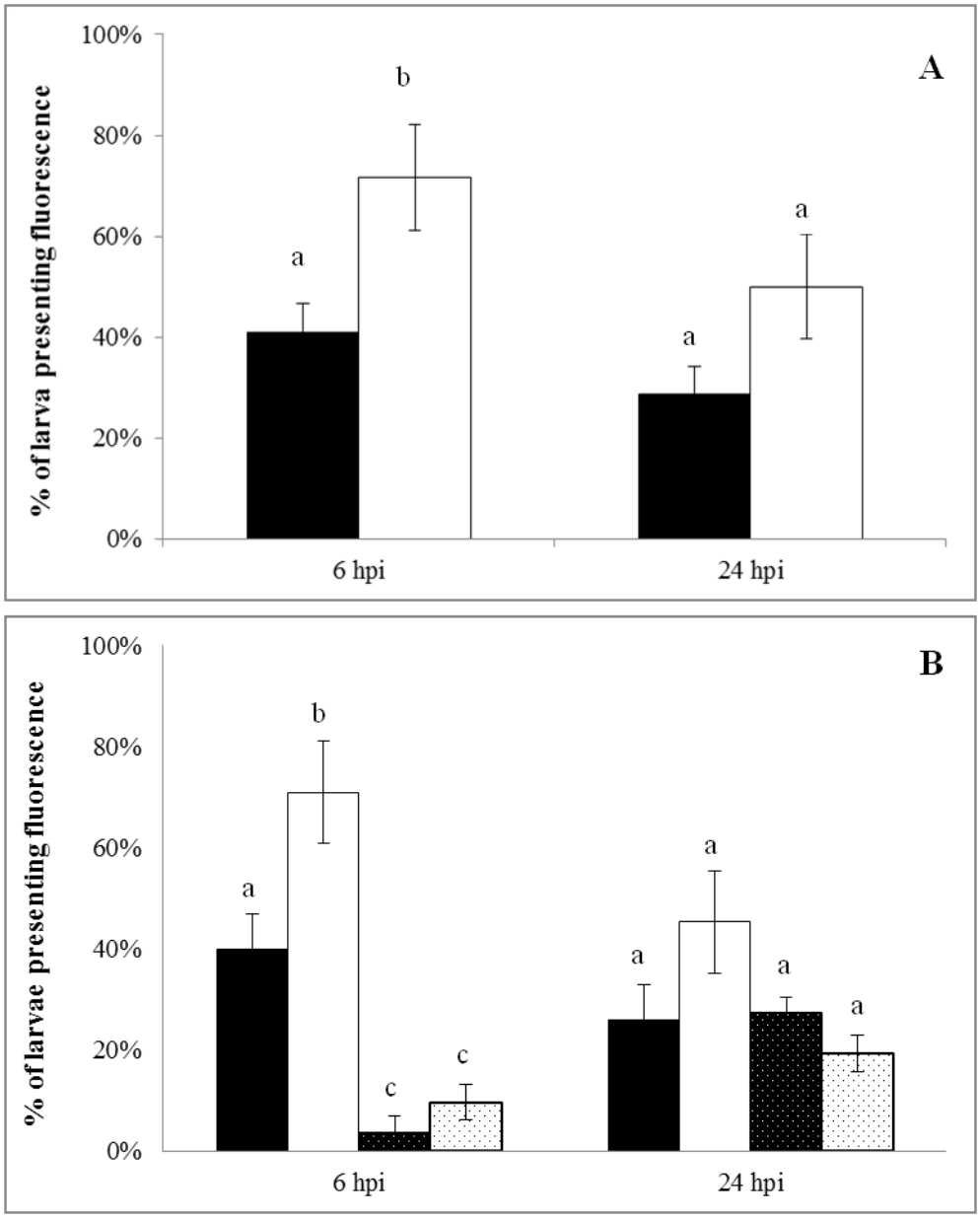

Figure 4.25 - Quantification of *L. plantarum* strains prevalence in zebrafish larvae digestive tract by mCherry fluorescence measurement. The percentage of the total zebrafish larvae presenting fluorescence (A) and occurrence of the fluorescence in the medium (filled bars) or in the posterior (dotted bars) intestine (B) at 6 and 24 hpi with *L. plantarum* Lp90/pRCR12 (white bars) or *L. plantarum* B2/pRCR12 (black bars). Values represent mean ± standard deviation of three replicates of 15 larvae each. Statistically significant differences were determined by t-student test, $p<0.05$.

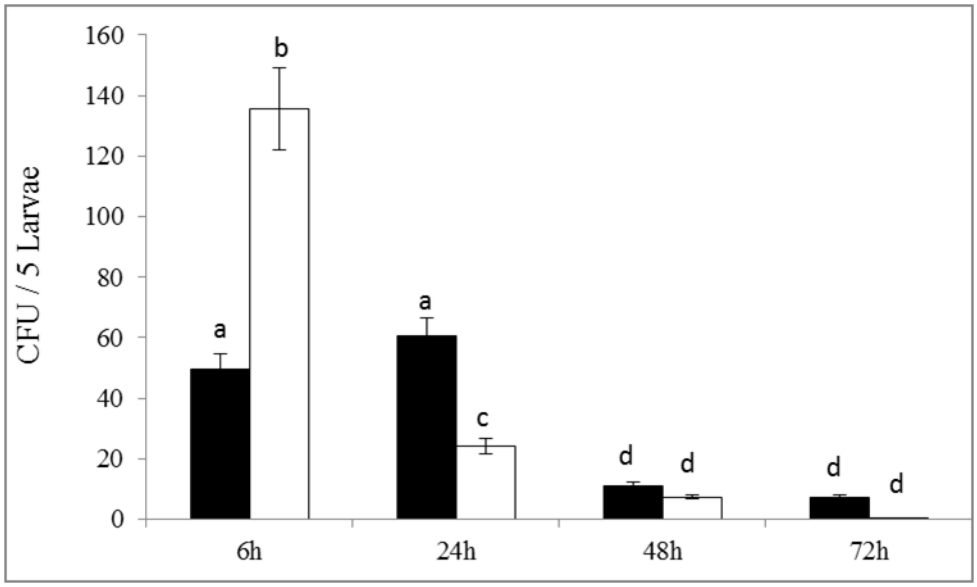

Figure 4.27 - Quantification of *L. plantarum* strains prevalence in zebrafish larvae digestive tract by plate count. Colonization of zebrafish larvae intestines by *L. plantarum* Lp90/pRCR12 (white bars) and *L. plantarum* B2/pRCR12 (black bars) was determined by plate count at 6, 24, 48, 72 hpi. Values represent mean ± standard deviation of three replicates of 15 larvae each. Statistically significant differences were determined by t-student test, $p<0.05$.

Based on these results, *L. plantarum* Lp90 showed a good potential colonization *in vivo* especially in the first hours post infection (6 hpi) and then decreased after 24 hpi, which could be correlated to its ability to synthesize EPS. For this reason, we hypothesized that the washings performed before the zebrafish larvae infection may have partially washed off exopolysaccharides, thus favoring the adhesion during the early observation phases; while after 24 hpi Lp90 would produce *in situ* other EPS which hindered the adhesion on enterocytic cells. This effect is consistent with our results of the *in vitro* adhesion on Caco-2 cells previously described, where EPS seem to hinder the adhesion of lactobacilli in stationary phase, due to an increased accumulation of EPS around the bacterial cells. However, in the scientific literature the role of EPS on microbial adhesiveness is controversial for both *in vitro* and *in vivo* studies: a positive correlation between EPS production and the percentage of binding to Caco-2 cells was reported for strains isolated from cider and wine (Fernández de Palencia *et al.,* 2009; Garai-Ibabe *et al.,* 2010; García Ruíz *et al.,* 2014). Conversely, Nikolic *et al.* (2012) found that three non-ropy derivatives improved *in vitro* adhesion compared to the parental phenotypes, suggesting

that the presence of a surrounding EPS layer could hinder the attachment to different cell lines. Similar, opposing results were also reported for *in vivo* models. For instance, the inability to permanently colonize the intestine of germ-free mice was attributed to the EPS-producing properties of *Lactobacillus kefiranofaciens* (Chen and Chen, 2013). By contrast, Lebeer *et al.* (2011) found a higher persistence of *Lactobacillus rhamnosus* GG than its isogenic derivative EPS-mutant when using a murine model. Indeed, it is presumable that different levels of adhesion are detected between the *in vitro* and *in vivo* binding phenotypes of the same strain (Turpin *et al.,* 2013).

In a previous study, *L. plantarum* was identified as highly-adhesive when zebrafish adults were fed with a probiotic diet supplemented with ten *Lactobacillus* strains (Zhou *et al.,* 2012).

Overall, fluorescence data suggest that both *L. plantarum* strains share a similar adhesion capacity and they were able to adhere to the posterior intestine of larvae after 24 hpi, although they seem to prefer different site of adhesion and/or different gut transition kinetics because *L. plantarum* B2/pRCR12 seemed to be displaced with time to the distal gut.

Nevertheless, the microbial count analysis indicates that *L. plantarum* B2 has the ability to persist longer in zebrafish gut.

Finally, the approach here used allows us to affirm that mCherry protein could be successfully employed as a strategy to track in real-time the localization of potential probiotic strains within the gut of transparent gnotobiotic zebrafish larvae. In addition, this system avoids the need to sacrifice the animal, thus ensuring that experiments are both scientifically and ethically justified (Dothel *et al.,* 2013).

4.7 Biofilm formation on abiotic surface

The capacity to form biofilm on glass surface was investigated to ascertain the influence of EPS producing Lp90 on biofilm development, which was monitored over a 7 days period and

compared with that realized by Lp90Δcps2, WCFS1, WCFS1Δcps2, SF2A35BΔcps2 (non-ropy strains) and SF2A35B (ropy strain). As reported in **Figures 4.28 and 4.29**, for all *L. plantarum* strains the biofilm increased proportionally to the observation period, except SF2A35BΔcps2. In particular, Lp90 exhibits lower ability to form biofilm, especially after two days post inoculation and then showed a greater production of biofilm after 7 days post inoculation. By contrast, its relative mutant strain Lp90Δcps2 produced high amounts of biofilm since 1 day post inoculation. Therefore we assume that the absence of EPS could favor the capacity of lactobacilli to adher on abiotic surfaces. These findings are in agreement with our results concerning the adhesion *L. plantarum* strains on Caco-2 cells, again suggesting that the exopolysaccharides might not have chemical affinity with surfaces such as glass, rather they could cover some molecules of the bacterial cell wall which have major binding properties.

A negative effect on biofilm formation was evidenced for the galactose-rich cell wall associated EPS produced by the well documented probiotic *L. rhamnosus* GG (Leeber *et al.,* 2009); by contrast, the beta-glucan containing capsules of *P. parvulus* and *O. oeni* enhanced their adhesion capacities on abiotic surface (Dols-Lafargue *et al.,* 2008).

The role of EPS in biofilm formation could be affect by the chemical structure, relative quantity and charge, properties of the abiotic surface and surrounding environment (Van Houdt and Michiels, 2010).

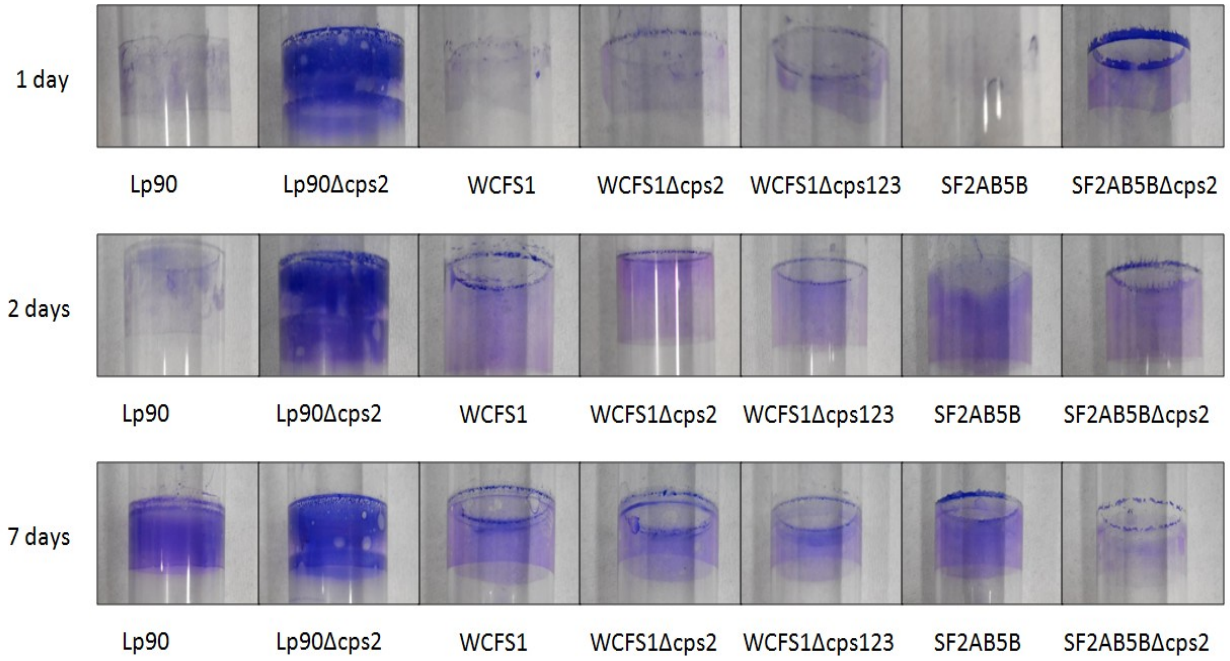

Figure 4.28 – Biofilm rings on glass surface, stained by crystal violet. The biofilm was monitored over a 7 days period.

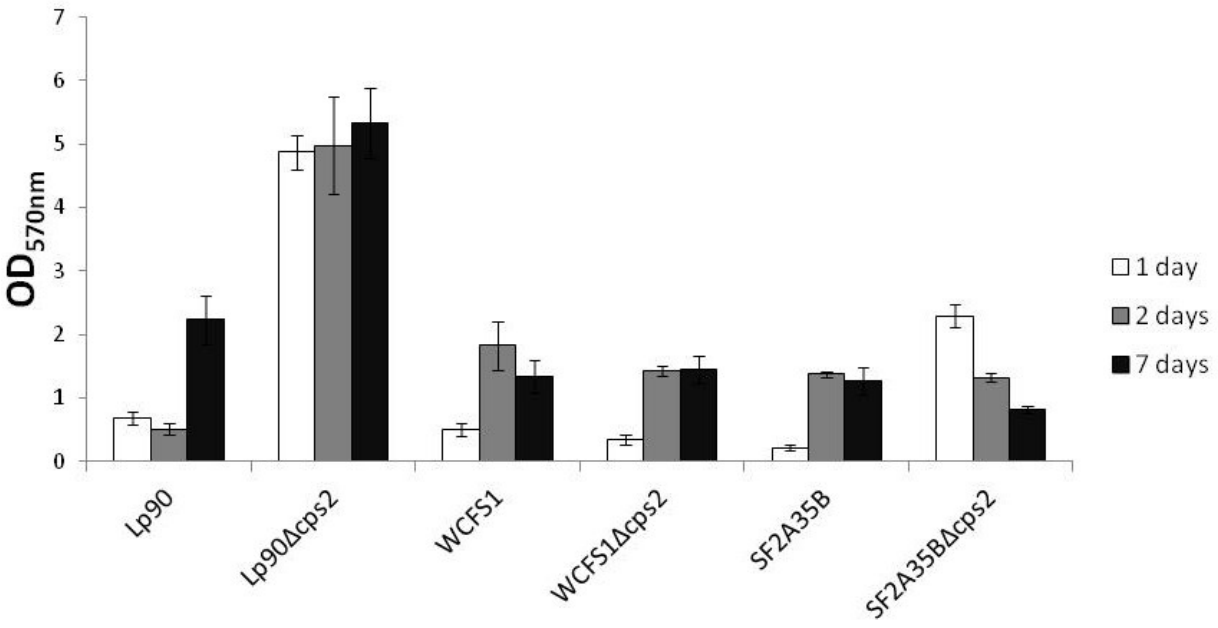

Figure 4.29 - Quantification of the biofilm rings formed on glass surface. The graphs report the absorbance at 570 nm of the biofilm rings after crystal violet staining and dissolution in acetic acid. The values represent the averages and standard deviations of three independent experiments. The biofilm was monitored after 1, 2, and 7 days post inoculation, represented as white, gray and black bars respectively.

4.8 Lactobacilli survival during *in vitro* gastro-intestinal (GI) tract condition

The ability of *L. plantarum* Lp90, WCFS1, SF2A35B and their respective Δcps2 mutant strains to tolerate the gastric-intestinal tract conditions was investigated in accordance with a previous model described by van Bokhorst-van de Veen *et al.* (2012a). Survival abilities were tested on bacterial cells from both exponential (**Figure 4.30**) and stationary phases.

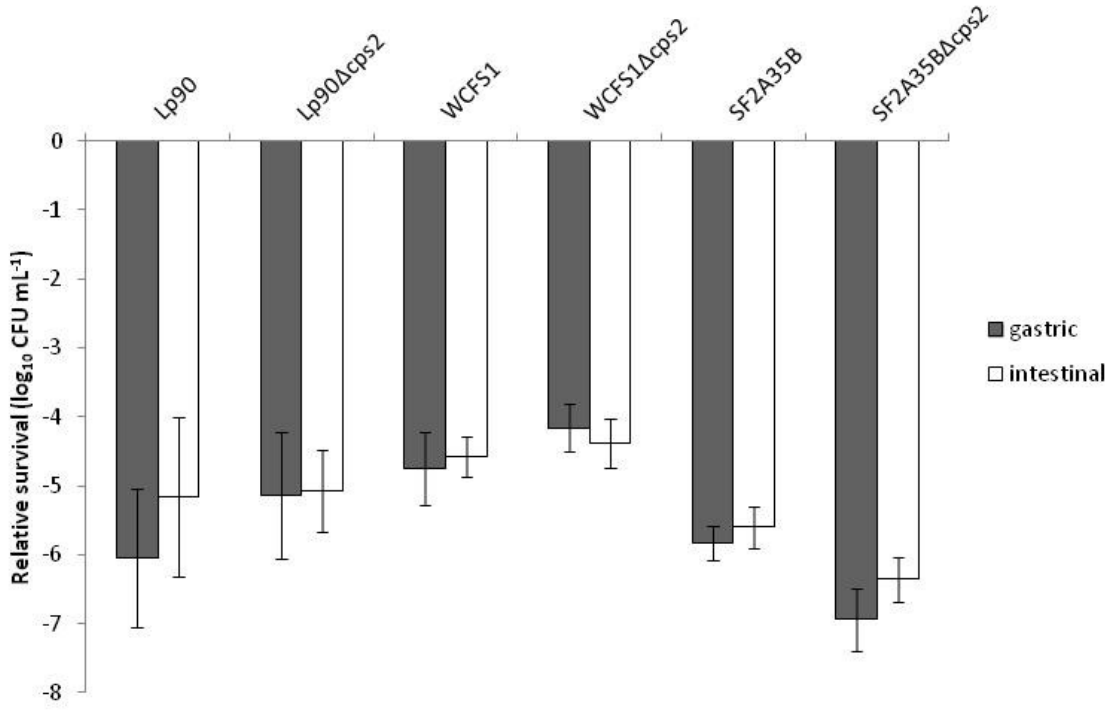

Figura 4.30 - Relative survival of *L. plantarum* strains after an *in vitro* Gastro-Intestinal tract assay, as previously described by Bokhorst-van de Veen *et al.*, (2012a). Bacterial cells recovered in exponential phase and subject to a gastric stress (full bars) and intestinal stress (open bars). The results were obtained from the averages and standard deviations from three independent experiments.

L. plantarum Lp90 in exponential growth phase showed a higher sensitivity (-6 $\log_{10}$ CFU mL^{-1}), following the simulation of the *in vitro* gastric tract compared to WCFS1 and WCFS1Δcps2 (-5 and -4 $\log_{10}$ CFU mL^{-1}), while the relative survival was comparable with Lp90Δcps2 and with the other ropy strain of SFA352B and its mutant SFA352BΔcps2. Contrary, there were no clear differences between strains of *L. plantarum* subjected to the simulated intestinal stress.

In the simulation of the gastro intestinal tract of stationary phase cells, were not observed differences in relative survival between Lp90, Lp90Δcps2, WCFS1 and WCFS1Δcps2. By contrast, a strong log reduction was noted for both SFA352B and SFA352BΔcps2 (data not shown).

Considering that the EPS matrix is the only difference between Lp90 and Lp90Δcps2, while it is one of the main differences with respect the other analyzed strains, EPS matrix does not seem to offer protection to bacterial cells from the stressful conditions of the *in vitro* gastro-intestinal system. In this regard, Fernández de Palencia *et al.,* (2009) reported that EPS produced by *P. parvulus* do not confer advantage for survival to GI tract conditions. Conversely, other studies have reported that the presence of endogenous EPS confer greater resistance to both simulated gastric juice and acid (HCl) stress (Stack *et al.,* 2010). Moreover, the endogenous production or addition of microbial glucans has been proven to enhance growth, stress tolerance and probiotic potential of lactobacilli (Stack *et al.,* 2010; Russo *et al.,* 2012). Addition of plant polysaccharides led to different effects, as it either (substantially) improved probiotic tolerance to simulated GI conditions (Desmond *et al.*, 2002; Bove *et al.,* 2013), or had no influence on stress resistance even though ameliorating the subsequent microbial recovery, once the source of stress was removed (Arena *et al.,* 2014b).

Finally, increased tolerance to gastric stress of WCFS1 and WCFS1Δcps2 could be due to the different origins of the investigated strains. We have to consider that WCFS1 strain has been isolated from human saliva, and thus it has been naturally selected to withstand the typical stresses of its original habitat. Moreover, genome association analysis of the transcriptome and survival data revealed 13 genes potentially involved in GI-survival (van Bokhorst-van de Veen *et al.,* 2012a). By contrast, in Lp90, which was isolated from wine, the ability to resist these specific conditions may represent an added feature relative to its original ecological niche.

4.9 Immune-stimulation of macrophage-differentiated THP-1 cells with *in vitro* oro-gastro-intestinal digested yogurt containing *L. plantarum* Lp90

4.9.1 Preliminary chemical analysis of yogurt

The chemical composition of milk used in all experiments was determined prior to fermentation processes and resulted as following: fat 3.6±0.1%, protein 3.3±0.2%, lactose 4.7±0.1%, and casein 2.5±0.1%. Subsequently, the yogurt samples were analysed for their pH, lactic acid, protein, casein, nitrogen fractions, fat content and peptide profile in order to investigate the influence by different strains of *L. plantarum* on yogurt fermentation over 1, 14 and 28 days of storage at 4 °C (**Table 4.5**).

Time / Strain	pH	Prot (%)	Casein (%)	WSEs (%)	Fat g/100g	Lactic acid (g/L)
1 day						
CNT	4.19±0.01	3.28±0.11	2.45±0.11	0.15±0.01	2.68±0.17	3.98±0.35
Lp90	*4.10±0.01	3.27±0.11	2.66±0.12	0.17±0.01	3.00±0.23	4.60±0.39
WCFS1	*4.07±0.01	3.43±0.11	2.41±0.11	0.19±0.01	*3.75±0.39	4.43±0.65
14 days						
CNT	4.25±0.01	3.03±0.00	2.42±0.00	0.11±0.01	4.43±0.10	4.90±0.81
Lp90	*4.13±0.01	3.03±0.00	**2.62±0.00	0.10±0.00	4.38±0.10	4.45±0.81
WCFS1	*4.18±0.01	2.95±0.11	2.34±0.11	0.10±0.00	4.48±0.10	5.05±0.04
28 days						
CNT	4.22±0.01	1.99±0.11	1.61±0.07	0.10±0.00	4.30±0.18	5.14±0.23
Lp90	4.22±0.01	*2.71±0.00	*2.30±0.00	0.08±0.00	4.48±0.15	4.58±1.73
WCFS1	*4.17±0.01	*2.87±0.00	*2.24±0.02	0.10±0.00	4.43±0.05	5.46±0.63

Table 4.5 - Chemical composition of yogurt fermented with i) *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (CNT, positive control); ii) *S. thermophilus* and *L.delbrueckii* subsp. *bulgaricus* and *L. plantarum* Lp90; iii) *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* WCFS1. Values represent mean ± standard deviation of two different experiments. Statistical analyses were carried out by Student's t test and significant differences are relative to control sample (*$p<0.05$ and **$p<0.005$).

The results showed that the pH values of the control yogurt (fermented only by starter strains *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) were 4.19, 4.25 and 4.22 after 1, 14, and 28 days of storage respectively. The yogurt samples inoculated with *L. plantarum* Lp90 and *L.*

plantarum WCFS1 presented pH values after 1 and 14 days of storage significantly different from the control. However, these differences disappeared after 28 days of storage for *L. plantarum* Lp90, while for the yogurt inoculated with *L. plantarum* WCFS1 the pH values remained significantly lower (pH 4.17). Frequently, pH of yogurt decreases during the storage, which may cause a loss of organoleptic quality. Commonly, the consumers prefer yogurts presenting mild acidity (pH 4.2-4.4), thus microbial cultures with mild acid production ability are usually selected in order to obtain yogurts with mild acidity and pH stability during shelf-life (Chandan *et al.,* 2013; Mollet, 1999). Interestingly in our case, both *L. plantarum* strains, once they carried out the fermentation during the yogurt production, did not determine further lowering of pH in yogurt samples over the entire storage time.

The protein fraction was also quantified and, as an average, it contents was around 3.35 and 3.03% after 1 day and 14 days) with no significant differences among collected samples. Protein contents of all yogurts inoculated with *L. plantarum* strains after 28 days were significantly higher (2.71, 2.87% for Lp90 and WCFS1, respectively) than amount measured in the control sample (1.99%).

The percentage of casein degreased over time in all cases, although the reduction was higher for the control sample, (from 2.45 to 1.61%), whereas for the yogurts inoculated with the target lactobacilli strains the total amount of casein after 28 days was significantly higher.

The water-soluble extracts (WSEs) decreased during the shelf-life, without any significant differences between the trials. Similarly, no significant variances were observed for fat amount, except for the yogurts inoculated with WCFS1 after 1 day.

The lactic acid values were also analyzed and not significant differences were detected among the samples. As an average, lactic acid content was 4.51, 5.01 and 5.1 g/L after 1-, 14- and 28-days respectively.

Overall, the results demonstrated that the yogurts fermented with *L. plantarum* strains co-inoculated with the two starter strains *S. thermophilus*, *L.delbrueckii* subsp. *bulgaricus* lead to

obtain final product showing a different pH value over small and medium but not long term of storage. Moreover, the samples presented higher protein and casein content respect to the control. Conversely, the percentage of water-soluble extracts (WSEs) and fat, and the lactic acid amount were globally similar to the control.

During the milk fermentation, lactic acid bacteria are involved in caseins proteolysis in order to provide to amino acids and peptides needed for their growth. Thus, the molecules accumulation in the final fermented product depends on the hydrolase pathways possessed by selected strains of bacteria. Consecutively, the peptides profile may influence the nutrition quality of fermented product and may condition the growth of other co-inoculated microorganisms (Papadimitriou *et al.,* 2007). For instance, it is known that the gradual degradation of peptides by the yogurt starter *L. bulgaricus* cultures promotes the growth of *S. thermophilus* that more rapidly produce lactic acid (Bautista *et al.,* 1966; Rajagopal and Sandine, 1990). Here, we analysed the WSEs during the storage of yogurt fermented with different lactobacilli strains co-inoculated with the two yogurt starter cultures *S. thermophilus* and *L. bulgaricus* subsp. *delbrueckii*. The RP-HPLC profiles of the water-soluble extracts (WSEs) showed a basically similar peptide profiles for all treatments with quantitative differences of the peptide content of the water-soluble extracts over the storage time, increasing in time-dependent manner (data not shown).

4.9.2 Viability of *Lactobacillus plantarum* strains in yogurt

The viability of *L. plantarum* strains was investigated using qPCR-PMA methodology. The PMA associated to qPCR has been shown valuable to discriminate between live and dead microorganisms because it penetrates selectively the membranes of dead cells and links the dsDNA (Àlvarez *et al.,* 2013). The dsDNA-PMA complex can be activated by light and bind cellular hydrocarbon moiety to form highly stable compounds. The dsDNA-PMA-hydrocarbon complex is not amplified during qPCR, therefore the DNA of dead cells is not detected.

The viability of *L. plantarum* strains of each yogurt sample was also analyzed at time 0 (initial inoculation prior to start the fermentation), 1, 14, 21 and 28 days of storage.

As shown in **Figure 4.31**, the CFU/mL of lactobacilli decreased in a time-dependent manner with no significant differences after 1, 7 and 14 days.

Overall, the culture counts during the storage were higher than 10^8 CFU/mL, according to the probiotic recommended threshold to adduce beneficial effects on human (Shortt, 1999).

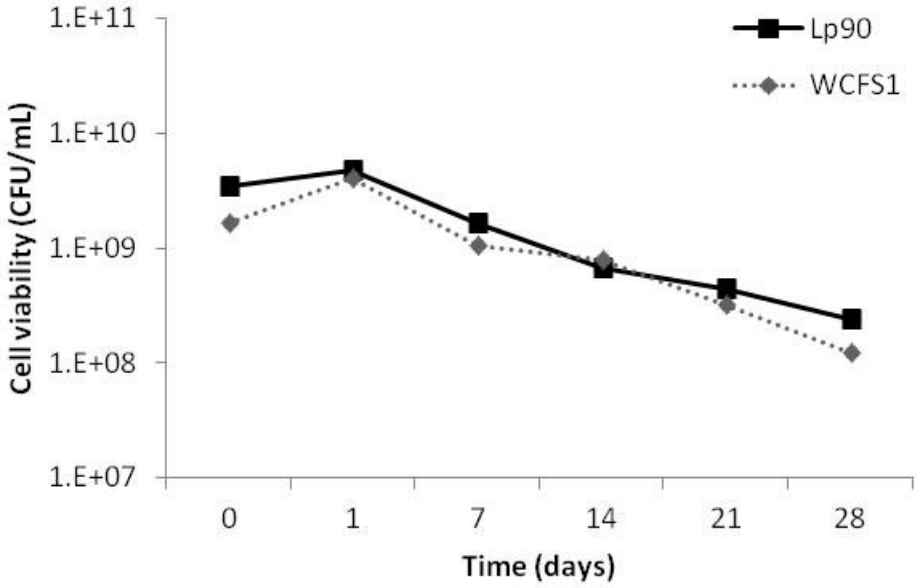

Figure 4.31 - Cell viability of *L. plantarum* Lp90 (continuous line) and WCFS1 (dashed line) used to produce yogurt at time 0 (initial inoculation prior to start the fermentation), 1, 14, 21 and 28 days of storage at 4°C. Values represent mean±standard deviation of two different experiments.

4.9.3 Tolerance of *L. plantarum* strains inoculated in yogurt during an *in vitro* oro-gastro-intestinal assay.

The ability of *L. plantarum* Lp90 and WCFS1 inoculated in yogurt to tolerate the human digestion was investigated by an *in vitro* simulation of the oral, gastric and intestinal conditions (Arena *et al.,* 2014b).

The results showed variable survival percentages depending on strains and gastrointestinal stress steps (**Figure 4.32**).

The bacterial percentage of survival with respect to untreated samples was not influenced by oral stress. On the contrary, the persistence of lactobacilli strains was mainly affected under gastric conditions in a pH-depending manner similarly to results obtained by other authors (Arena *et al.,* 2014b; Bove *et al.,* 2013). These findings are correlated to the greater difficulty of bacteria to resist to low pH and underlined the necessity to select probiotic bacteria with a strong ability to tolerate the acid environments in order to overcome the gastric sector and reach the intestine.

In both *L. plantarum* strains, the cell survival after the exposure to gastric stress at pH 3.0 decreased of about 1 Log unit and it drastically dropped following the gastric stress at pH 2.0 (about 4 Log units). Furthermore, under small and large intestinal simulation, the cell viability increased for both lactobacilli strains.

Overall, *L. plantarum* Lp90 and WCFS1 showed a higher ability of to tolerate gastric conditions at pH 3.0; however, in agreement with our previous findings (see paragraph 4.8), the exopolysaccharides produced by Lp90 do not seem to offer greater tolerance to gastric stress.

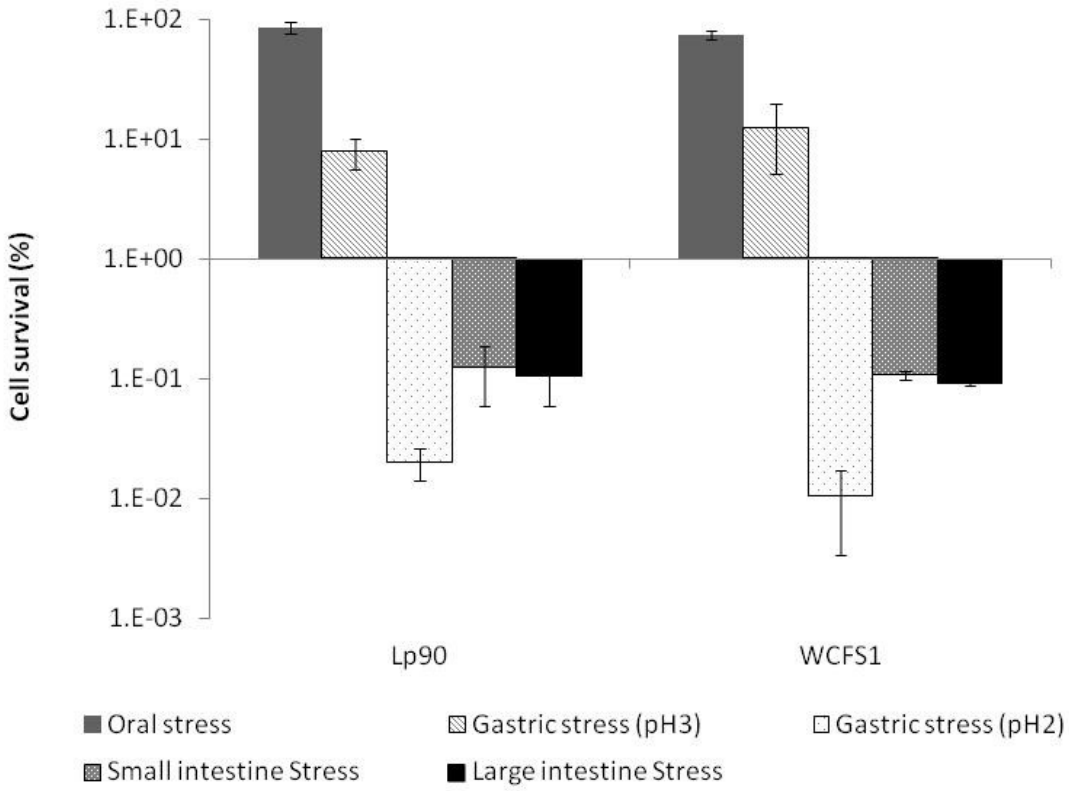

Figure 4.32 - Survival of *L. plantarum* Lp90 and WCFS1 inoculated in yogurt during the exposure to an *in vitro* oro-gastro-intestinal model (oral, gastric pH 3.0 and pH 2.0, and small and large intestine stresses). Viability was expressed as survival percentage relative to untreated control (i.e., unstressed bacteria). Values represent mean±standard deviation of three different experiments.

4.9.4 Stimulation of THP-1 cells with lactobacilli and expression of cytokine-related genes

The potential ability of probiotic strains to exhibit an influence on the expression level of genes involved in immune modulation was investigated. Since food assumed by diet is exposed to several digestive steps to be metabolized, we carried out the assays exposing THP-1 cells to both untreated and *in vitro* digested yogurt samples containing *L. plantarum* Lp90 (ropy strain) and WCFS1 (non-ropy strain), in order to understand whether the *in vitro* digestion could affect the immune-modulation properties as well as a possible role of EPS. In fact, several authors reported that the microbial exopolysaccharides are involved in immune-response mechanisms (Vinderola *et al.,* 2006; Fanning *et al.,* 2012; Matsuzaki *et al.,* 2014; Notararigo *et al.,* 2014). Components of bacteria cell wall, peptidoglycan (PG), occurred in both gram-positive and gram-negative

bacteria, and lipopolysaccharide (LPS), showed in gram-negative microorganism, may stimulate the human cells in a receptor-dependent process activating the release of several immune mediators. For instance, LPS-activated macrophages can produce cytokines, such as interleukins (IL-8, IL1β, IL-6) and/or tumor necrosis factor-α (TNF-α) involved in the immune response (Erickson and Hubbard, 2000). In this regard, we exposed the differentiated THP-1 cells to only LPS (positive control) and LPS with lactobacilli in order to compare the transcriptional level of several genes involved in the regulation of immune-response, such as IL-8, TNF-α, IL1β, TSLP, IL-6, NF-κB1 and IL-10.

Cytokines play a central role in the inflammatory process, as they are able to coordinate the initiation, amplification and interruption of immune-response (Wichers, 2009). In our study, the transcriptional levels of IL-8 were significantly reduced by all lactobacilli treatments, both undigested and *in vitro* digested samples. A slightly higher ability of undigested samples to moderate the transcriptional level of IL-8 gene after 1 h of incubation was observed, compared to the *in vitro* digested samples. On the contrary, this trend was not noted after 4 h of exposure, where even the *in vitro* digested lactobacilli were mostly able to moderate the expression of this gene. There were no clear differences between *L. plantarum* EPS-producing (Lp90) compared to the control WCFS1 strain (**Figure 4.33**). High levels of the cytokine IL-8 are associated to inflammatory diseases and conditions as asthma, inflammatory bowel disease (IBD), and in response to LPS exposed to the wall surface of gram-negative bacteria (Roebuck, 1999). Overall, regardless the exopolysaccharides production, the transcriptional analysis showed that *L. plantarum* strains were able to down regulate the gene expression of IL-8.

The strongest activators of IL-8 gene are the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) (Roebuck, 1999). TNF-α is able to coordinate the enhancement of inflammatory response by activating neutrophils, mononuclear phagocytes (Danis *et al.,* 1991; DeNichilo *et al.,* 1991; Ferrante, 1992). IL-1β plays an important role in the cascade response of innate immune system incrementing the cytokine production in dendritic

cells, stimulating the phagocytosis in macrophages and promoting the differentiation and the proliferation of T cells (Sims and Smith, 2010). The results of TNF-α gene analysis, showed a significant reduction of expression both after 1 h and 4 h of exposure for all treatments with lactobacilli without significant differences between undigested and *in vitro* digested samples. Similarly, we found a down regulation of the transcriptional levels of gene after 1h and 4 h of incubation with *L. plantarum* strains. Interestingly, both TNF-α and IL-1β transcriptional analysis were observed higher levels of expression ($p \bullet < 0.05$) after 1 h exposure of THP-1 with undigested yogurt containing *L. plantarum* Lp90. These results suggest that the extracellular polysaccharides do not down regulate these genes; rather they could mask other molecules of the bacterial cell wall which favor this type of immune-response. This phenomenon would be less noticeable after digested sample and higher human cells-lactobacilli exposure times that would reduce the shielding effect of EPS around the bacterial cells.

IL-6 is a multifunctional interleukin implicated in both pro- and anti-inflammatory processes, produced in response to pathogens infections, as well as after LPS-induction (Vinderola *et al.,* 2005). In our case, the IL-6 gene expression was significantly reduced by exposure to all lactobacilli treatments with respect to the positive control LPS, but no significant differences were observed for Lp90 not digested and both Lp90 and WCFS1 digested samples after 4 h of exposure. However, we observed that the transcription level of IL-6 was more quickly decreased after shorter time of exposure (1 h) respect to longer incubation with lactobacilli (4 h). Moreover, were not found significant differences attributable to the presence of EPS.

Some authors reported that several probiotic microorganisms induced a dramatic reduction of secretion of IL-8 protein in HT-29 cells highlighting their anti-inflammatory effects (Grimoud *et al.,* 2010). Furthermore, strains belonging to *Kluyveromyces*, *Lactobacillus* and *Bifidobacterium* genera showed to decrease the level of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α (Maccaferri *et al.,* 2011; Candela *et al.,* 2008). Moreover, reduction of IL-6 levels was observed

when probiotic lactobacilli were co-cultured with pathogenic *Escherichia coli* (Vinderola *et al.,* 2005).

The nuclear factor κB (NF-κB) proteins family includes several genes (e.g. NF-κB1 and NF-κB2) which can be activated by LPS through toll-like receptors 4 (TLR4). NF-κB is the most important transcription factors of cytokine-mediated pro-inflammatory genes (IL-8, TNF-α, IL-1β, IL-6). The expression of TLR4 and then NF-κB genes are aberrant in chronic intestinal disease such inflammatory bowel disease (IBD). In fact, in health patients the TLR4 and NF-κB expression is at very low levels (Vinderola *et al.,* 2005). As shown in **Figure 4.33**, the inoculation of *L. plantarum* strains with LPS-stimulated THP-1 cells indicated that tested lactobacilli inhibited the activation of NF-κB1 gene, except for some treatments after 1 h of exposure (undigested *L. plantarum* Lp90 and digeste WCFS1). We speculate that longer time of incubation could be necessary to determine a higher reduction of this gene expression. In fact, the increment of transcriptional level was also increased by LPS mainly after 4 h (around 9-fold time) respect to shorter incubation of 1 h (around 2-fold time). No significant differences were observed between *L. plantarum* Lp90 (ropy strain) compared to WCFS1 (non-ropy control strain).

Whereas during an inflammatory process the immune system provides to contrast the origin of injury or the infection rousing the production of many pro-inflammatory molecules, the complex immune network affords also to restore the immune homeostasis inducing the anti-inflammatory cytokines production. The gene coding for the interleukin 10 (IL-10) can mediate the down regulation of inflammatory progression (Jung *et al.,* 1995).

We observed a relevant increasing of IL-10 transcriptional level for all *in vitro* digested samples after 1 h and for all treatments after 4 h of exposure and a slight reduction of expression after 1 h of incubation by undigested WCFS1. Noticeably, Lp90 digested sample (1 h of exposure) showed a significant ($p \bullet < 0.05$) higher expression level compared to WCFS1 in the same condition, presumably attributable to EPS. In this regards, a similar results was observed by

Bleau *et al.* (2010) exopolysaccharides from *Lactobacillus rhamnosus*. Cui *et al.* (2004) reported the effects of probiotic on intestinal mucosa of patients with ulcerative colitis (UC) demonstrated that they were able to enhance the expression of the anti-inflammatory cytokine IL-10 and to decrease the activation of NF-κB, reducing the expressions of TNF-α and IL-1β genes.

Lastly, we investigated the ability of *L. plantarum* strains to modulate the expression level of the thymic stromal lymphopoietin (TSLP) gene. This is involved in the allergic response, i.e. bowel ulcerative colitis (UC) and Crohn's disease but also in asthma and dermatitis events. During those inflammatory processes the transcriptional level of TSLP are up-regulated (Taylor *et al.,* 2009; Li *et al.,* 2011). TNF-α and IL-1β are able control the induction of TSLP expression (Comeau and Ziegler, 2010), but it may be alternatively increased via other pathways (Li *et al.,* 2011). TSLP have been also shown to have a role in the tumor development of intestinal cells (Takai, 2012).

With respect to TSLP gene, we found that the exposure of LPS-stimulated THP-1 cells to lactobacilli resulted in a decrease in the gene transcription occurring within 4 h, showing none differences in occurrence of microbial exopolysaccharides.

Plausibly, the fact that *L. plantarum* strains may modulate the TSLP gene expression promotes a suitable application of these beneficial microbes as immune-modulator.

Comprehensively, the transcriptional analysis of cytokine-mediating genes showed that lactic acid bacteria used in this study have a favorable influence on immune modulation. Overall, we did not found significant differences between undigested and *in vitro* digested treatments, thus we concluded that the effects on immune stimulation were did not correlated to digestive processes.

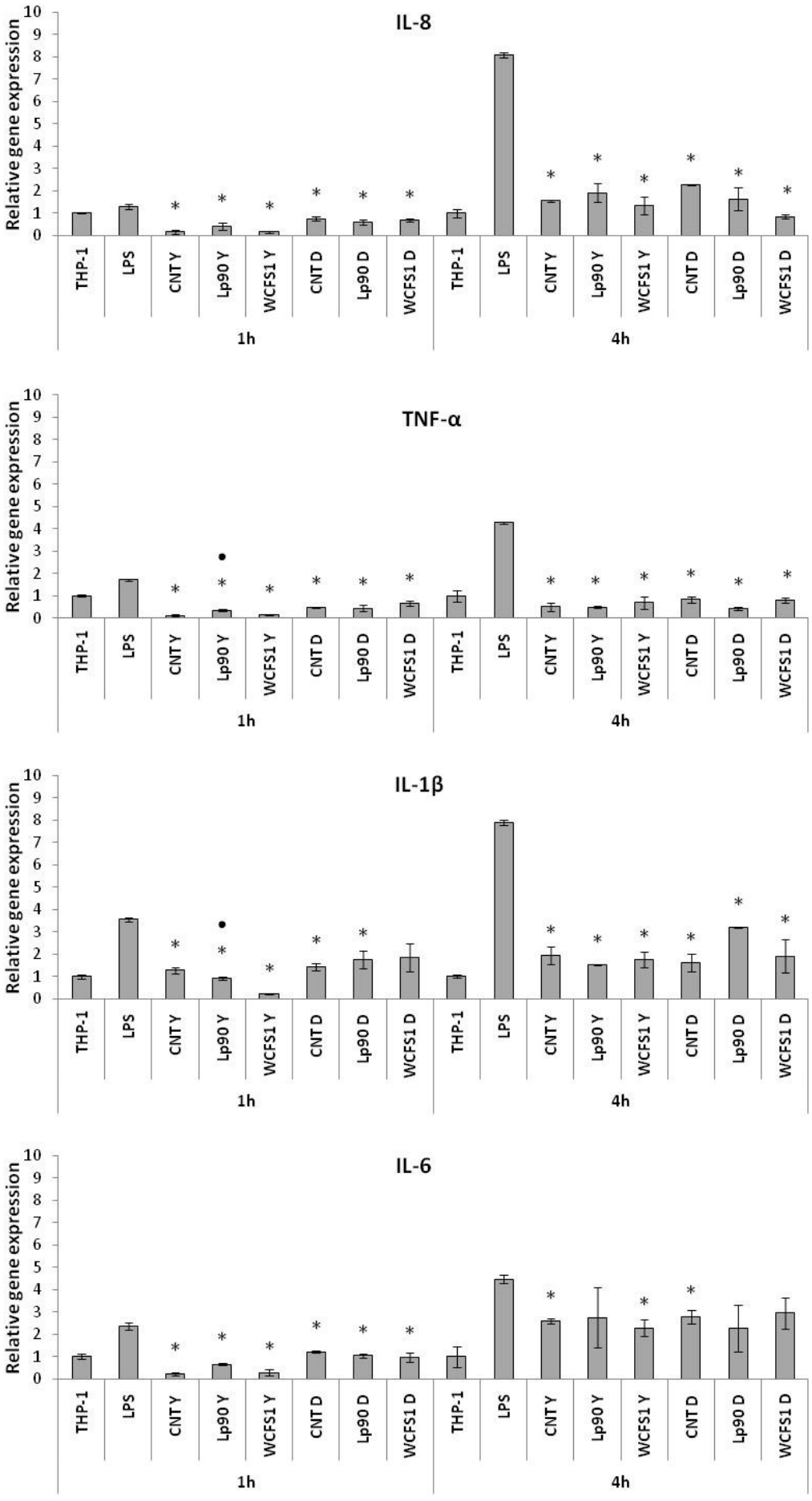

Figure 4.33 - ***(continue)***

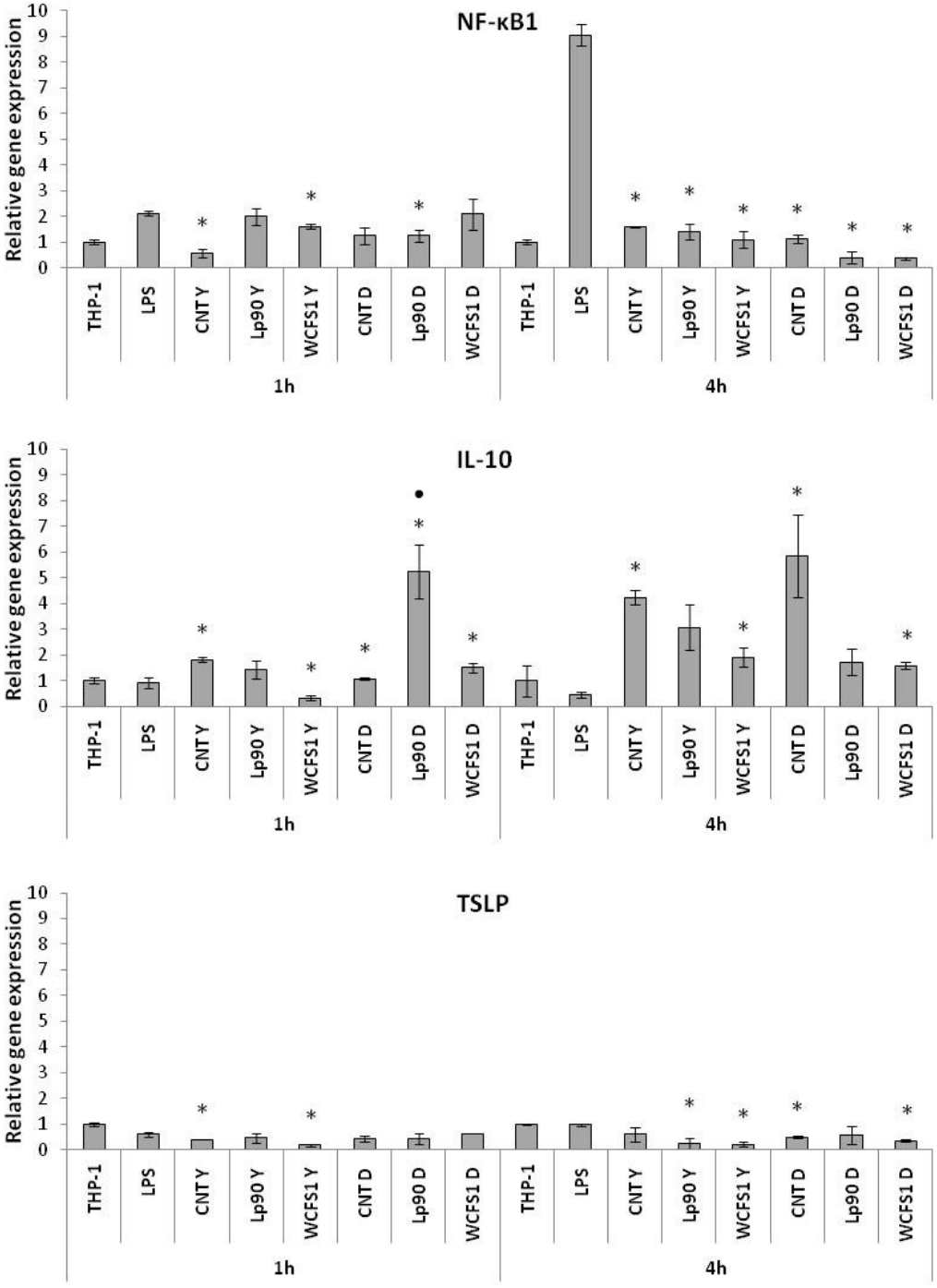

Figure 4.33 - Relative expression of cytokine-related genes after the exposure to undigested (Y) and *in vitro* digested (D) samples over 1 h and 4 h of treatments. Values represent mean ± standard deviation of two different experiments. Statistically significant differences were determined by t-student test, p*<0.05 is the difference between *L. plantarum* strains with respect to LPS-stimulated THP-1 cells without *L. plantarum* strains (LPS, positive control), p•<0.05 is the difference between Lp90 (ropy strain) and WCFS1 (non-ropy control strain) upon equivalent treatment. No LPS-stimulate THP-1 cells were used as negative control.

4.10 Tolerance to stress

4.10.1 Tolerance of *L. plantarum* strains to ethanol stress

The function of EPS produced by *L. plantarum* Lp90 in ethanol stress resistance was study considering that this is one of the major stressors for bacterial cells in wine environment (the original habitat of Lp90). In this regard, for the assay we chose to use 13% of ethanol, which corresponds to a typical alcoholic strength of red wine.

Interestingly, among the different *Lactobacillus plantarum* strains, Lp90 was significantly found to be the most resistant to alcohol stress with a relative survival of -0.08±0.03. Instead, Lp90Δcps2 non-ropy mutant strain showed a lower relative survival (-0.26±0.03), comparable with that of WCFS1, WCFS1Δcps2, and SF2A35BΔcps2 (**Figure 4.34**). Remarkably, SF2A35B ropy strain showed a drastic decrease of survival although ethanol is not a typical component of its original environment.

Taken together this results suggest that exopolysaccharides might assist the native bacterial cells to survive in an alcoholic medium such as wine, following the accumulation of ethanol produced during alcoholic fermentation operated by yeasts. Some authors reported that such oenological bacterial strains, harboring a functional *gtf* (glycosyltransferase) gene are more resistant to alcohol stresses (Dols-Lafargue *et al.,* 2008). Conversely, EPS produced by *P. damnosus* was found not to be involved in ethanol resistance (Walling *et al.,* 2005). Nevertheless, the ability of bacterial cells to resist to ethanol stress depends also on other factors. For instance *L. plantarum* strains have been described as able to grow in wine as they develop some mechanisms of ethanol resistance, such as changes in membrane lipid composition (G-Alegría *et al.,* 2004; van Bokhorst-van de Veen *et al.,* 2011). Fiocco *et al.* (2007) observed a potential role of small heat shock proteins (Hsp) in tolerance to ethanol stress in *L. plantarum*. Furthermore, in *O. oeni* under ethanol condition the transcriptional level of *hsp18* was higher, and Lo18 protein is thought to be involved in the adaptation to ethanol, modifying the fatty acid content of the bacterial membrane

Maitre *et al.* (2014). Therefore, we suppose that the greater survival shown by Lp90 compared to its Lp90Δcps2 mutant strain, can be interpreted as an additive effect offered by EPS in the ethanol resistance, in addition to other mechanisms of tolerance developed in relation to its original habitat.

Moreover, the strong reduction of viability of SF2A35B ropy strain let us to assume that the protection given by exopolysaccharides might depend also on their physico-chemical properties.

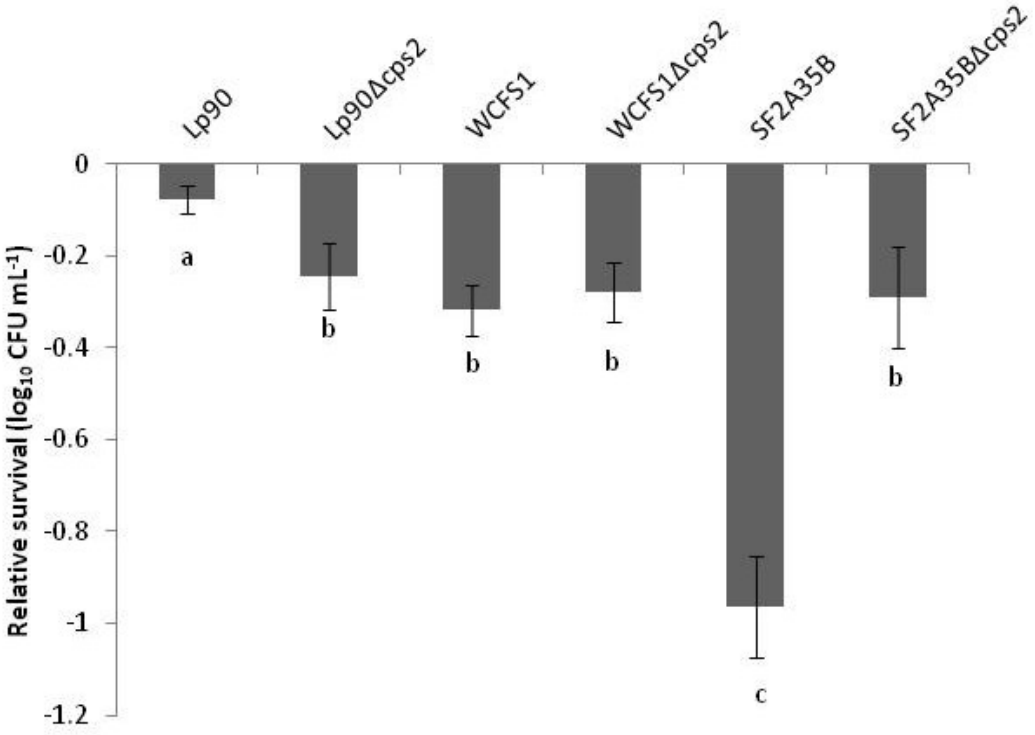

Figure 4.34 - Relative survival (log_{10} CFU mL^{-1}) of *L. plantarum* strains after 30 min of incubation with 13% of ethanol. The results were obtained from the averages and standard deviations from three independent experiments. Different superscript letters indicate statistically significant differences in adhesion ($p<0.05$) as assessed by one-way ANOVA test.

4.10.2 Tolerance of *L. plantarum* strains to acidic stress

The viability of lactic acid bacteria in an acidic environment is a key factor for those with potential probiotic properties, as to perform their beneficial effect in the gut they must survive to the harsh conditions of the stomach. Tolerance to the typical pH of the wine represents an added value for the oenological LAB, as it allows a better development of malolactic fermentation led by these microorganisms. For this purpose the ability of *L. plantarum* Lp90 to tolerate low pH

values (pH 2.5) was investigated in relation to EPS production and compared with that of WCFS1, WCFS1Δcps2, SF2A35B and SF2A35BΔcps2. The relative survival of Lp90 (-0.09±0.06) was significantly higher than Lp90Δcps2 (-0.25±0.06), and similar to that of WCFS1, WCFS1Δcps2. While a greater reduction of viability for SF2A35B and SF2A35BΔcps2 was observed (**Figure 4.35**).

On the basis of these data, it can be inferred that exopolysaccharides produced by Lp90 minimize the exposure of the bacterial cells to acid environments. Although not statistically significant, this trend was also observed for SF2A35B and SF2A35BΔcps2. The ability of *L. plantarum* WCFS1 harvested in stationary growth phase, to tolerate pH values of 2.3 in simulated gastric condition was attributed to specific genes, some of these could contribute to the pH homeostasis and others on the thickness of the capsular polysaccharides (van Bokhorst-van de Veen *et al.,* 2012a). Several *L. plantarum* strains able to tolerate low pH have been documented (G-Alegria *et al.*, 2004; Pieterse *et al.,* 2005; Šeme *et al.,* 2014; García-Ruiz *et al.,* 2014).

The proton-translocating ATPase represents the main defense mechanism implemented by the lactic acid bacteria after concern (Hutkins and Nannen, 1993), as well as the arginine deiminase (ADI) pathway (Sanders *et al.,* 1999). Nevertheless, several authors suggest the rule played by microbial exopolysaccharides in assisting the bacterial cell against stress acid. For instance, the EPS produced by *Bifidobacteria* would protect the cells under the action of low pH, improving probiotics properties (Fanning *et al.,* 2012; Alp *et al.,* 2010). However, this phenomenon should be considered as further defense mechanisms with respect to those mentioned above, to counteract the acid stress.

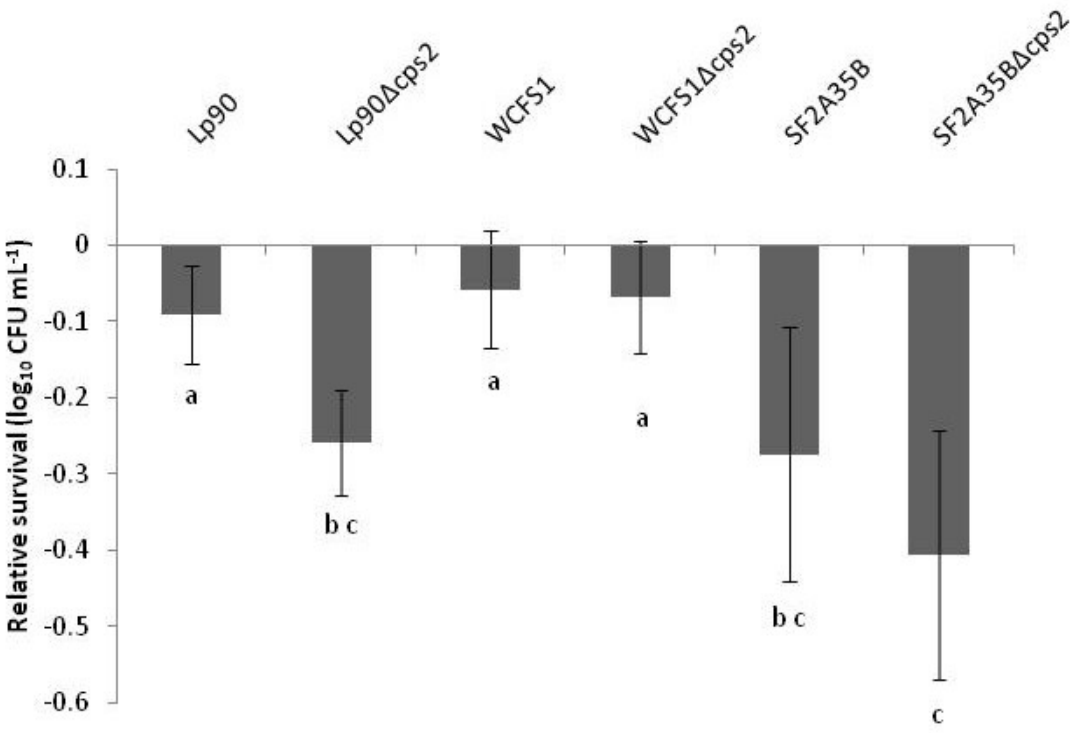

Figure 4.35 - Relative survival (log_{10} CFU mL^{-1}) of *L. plantarum* strains after 30 min at pH 2.5. The results were obtained from the averages and standard deviations from three independent experiments. Different superscript letters indicate statistically significant differences in adhesion ($p<0.05$) as assessed by one-way ANOVA test.

4.10.3 Tolerance of *L. plantarum* strains to sulfur dioxide stress

Lactobacillus plantarum viability was tested in presence of sulfur dioxide (SO_2), with the aim to evaluate a possible bacterial protection offered by exopolysaccharides originated by Lp90 against this antimicrobial substance. This represents a typical wine stress, as sulfur dioxide is routinely used in winemaking in order to prevent the development of spoilage bacteria.

The results reported in **Figure 4.36** indicate that the viability of Lp90 significantly decreased less than non-ropy mutant Lp90Δcps2 with relative survival values of -2.47±0.11 and -3.38±0.17 respectively; a drastic decrease of survival occurred for SF2A35B and it was undetectable for SF2A35BΔcps2. Noticeable, WCFS1Δcps2 was least affected by the action of sulfur dioxide stress, followed by parental strain WCFS1.

Overall, these results suggest that exopolysaccharides of Lp90 could allow the bacterial cell to better tolerate sulfur dioxide stress; this phenomenon seems to be confirmed by the total

mortality of SF2A35BΔcps2 caused by such stress. Contrariwise, the deletion of capsular polysaccharides WCFS1 resulted in increased resistance to sulfite stress.

The inhibitory action of SO_2 on LAB is principally due to the splitting of disulfide bonds in proteins, as well as reacting with cofactors like NAD^+ and FAD (Carreté *et al.,* 2002). We hypothesize that exopolysaccharides, which envelop the bacterial cell wall, could slow the lytic action of sulfites towards membrane protein by masking effect; even sulfites might react with exopolysaccharides, thus resulting less aggressive against the other membrane molecules. In this regard, was noticed that ropy Pediococci generally displayed high tolerance to SO_2 (Dols-Lafargue *et al.*, 2008).

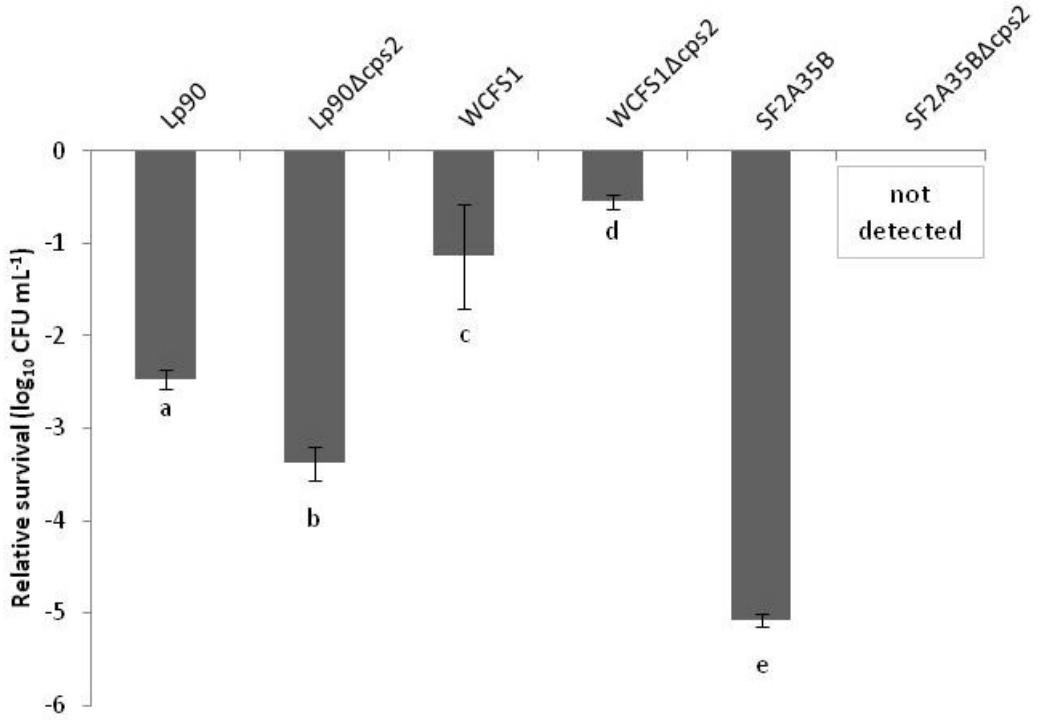

Figure 4.36 - Relative survival (log_{10} CFU mL^{-1}) of *L. plantarum* strains after 30 min of incubation with 70 mg/L of sulfur dioxide. The results were obtained from the averages and standard deviations from three independent experiments. Different superscript letters indicate statistically significant differences in adhesion ($p<0.05$) as assessed by one-way ANOVA test.

4.10.4 Tolerance of *L. plantarum* strains to lysozyme stress

Lysozyme is an enzyme capable to split the β-(1-4) linkage between N-acetyl muramic and N-acetyl-glucosamine, which are components of the peptidoglycan in the bacterial cell wall. It is present in saliva, thus representing one of the first obstacles encountered by lactic acid bacteria

during chewing of food; the high tolerance to this enzyme is a fundamental requirement for probiotics. Therefore, the resistance of the *L. plantarum* to the action of lysozyme was studied.

After the bacterial cultures treatment of 30 minutes with 200 μg/mL of lysozyme, *Lactobacillus plantarum* Lp90 resulted sensitive to this enzyme with a relative survival value of -0.14±0.03, however a significant decrease (-0.26±0.03) was observed in Lp90Δcps2 non-ropy mutant strain. By contrast, WCFS1 and SF2A35B wild type strains showed a higher resistance to lysozyme than WCFS1Δcps2, SF2A35BΔcps2 mutant strains, as well as, in comparison with Lp90 and Lp90Δcps2 (**Figure 4.37**). The greater viability of the WCFS1 after this treatment could be attributed to its original habitat, as it has been isolated from human saliva. The resistance of WCFS1 to lysozyme treatment has been already observed for the cells permeabilization study, which required a more intense and longer processing; in fact an exposure of 10 minutes has been insufficient to permeabilise *L. plantarum* (de Vries *et al.,* 2004).

Overall, our result showed that *L. plantarum* wild type strains possess a better adaptability to lysozyme treatment. We suppose that the presence of exopolysaccharides (both capsular and secreted form) around the cell wall, could offer protection to the bacterial cell wall, preventing the splitting of the β-(1-4) bonds of the peptidoglycan layer. As reported by Coulon *et al.,* (2012), β-glucans produced by a ropy strain of *P. parvulus* surrounding the bacterial cell wall could protect it from the enzymatic activity of lysozyme, while a strong sensitivity has been observed for the relative non-ropy *P. parvulus* mutant strain, this phenomenon has been accentuated for cell cultures in stationary phase. Similarly, in our case the lysozyme stress was applied on bacterial cells from a growth cultures stationary phase, where we presume there is a greater EPS accumulation (data not shown), thus protecting the bacterial cells from a direct attack of the stressor.

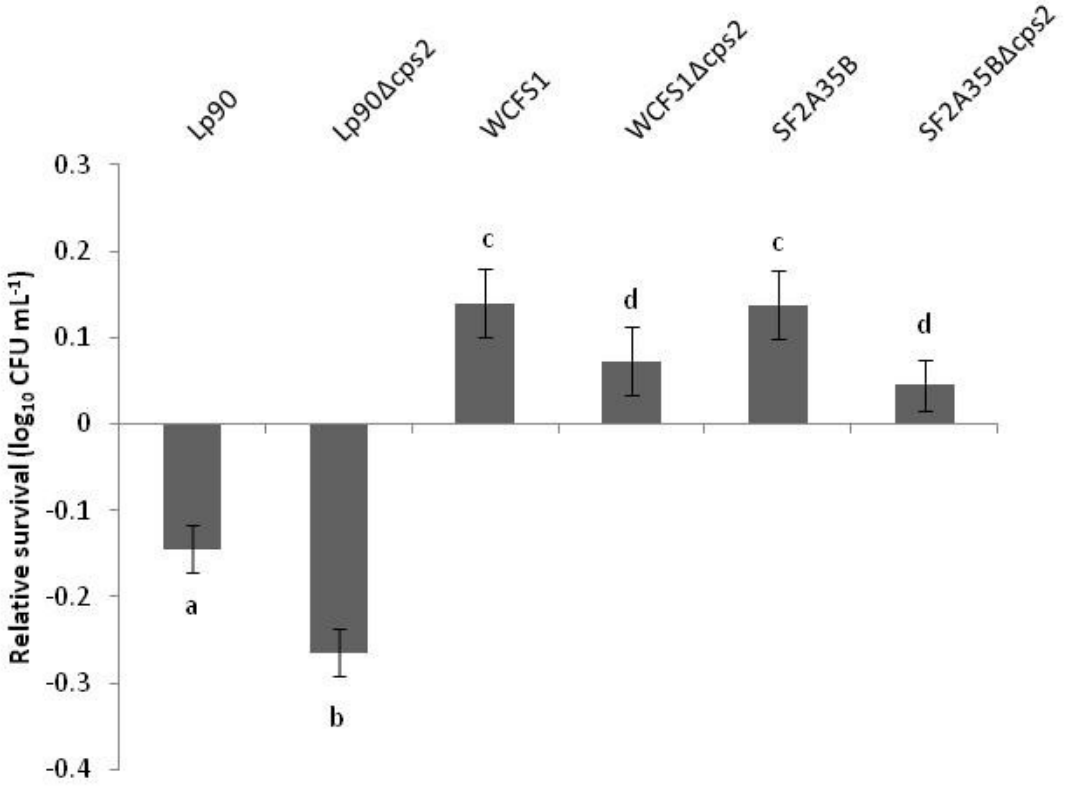

Figure 4.37 - Relative survival (log_{10} CFU mL^{-1}) of *L. plantarum* strains after 30 min of incubation with 200µg/mL of lysozyme. The results were obtained from the averages and standard deviations from three independent experiments. Different superscript letters indicate statistically significant differences in adhesion ($p<0.05$) as assessed by one-way ANOVA test.

4.10.5 Tolerance of *L. plantarum* strains to bile stress

The bacterial survival in the presence of porcine bile at a concentration of 3 g/L was evaluated. The bile salts allow the dispersion and absorption of fats, including phospholipids thus affecting the integrity of the bacterial cell membrane. Among the different *L. plantarum* strains analyzed, no statistically significant differences were observed for Lp90 compared to Lp90Δcps2 non-extracellular polysaccharides producer, as well as, between SF2A35B and SF2A35BΔcps2. Conversely, the relative survival of WCFS1 was significantly higher than WCFS1Δcps2 (**Figure 4.38**).

In *L. plantarum* WCFS1 was found a bile salt hydrolase (*bsh1)* involved in tolerance to specific bile salts, such as glycocholic acid (Lambert *et al.,* 2008), this is in agreement with the higher bile tolerance of WCFS1 reported in our results.

Overall, these data suggest that exopolysaccharides in dispersed form do not seem to offer more protection to the bacterial cell against the action of bile salts. Interestingly, bile exposure of other *Lactobacillus* species resulted in a decreased EPS biosynthesis gene expression and removal of exopolysaccharides (Pfeiler *et al.,* 2007; Koskenniemi *et al.,* 2011). Instead, some authors observed that exopolysaccharides protect bacterial cells under bile conditions (Fanning *et al.,* 2012; Boke *et al.,* 2010). Moreover, *Bifidobacterium animalis* subsp. *lactis* bile exposure promotes exopolysaccharides biosynthesis (Ruas-Madiedo *et al.,* 2009).

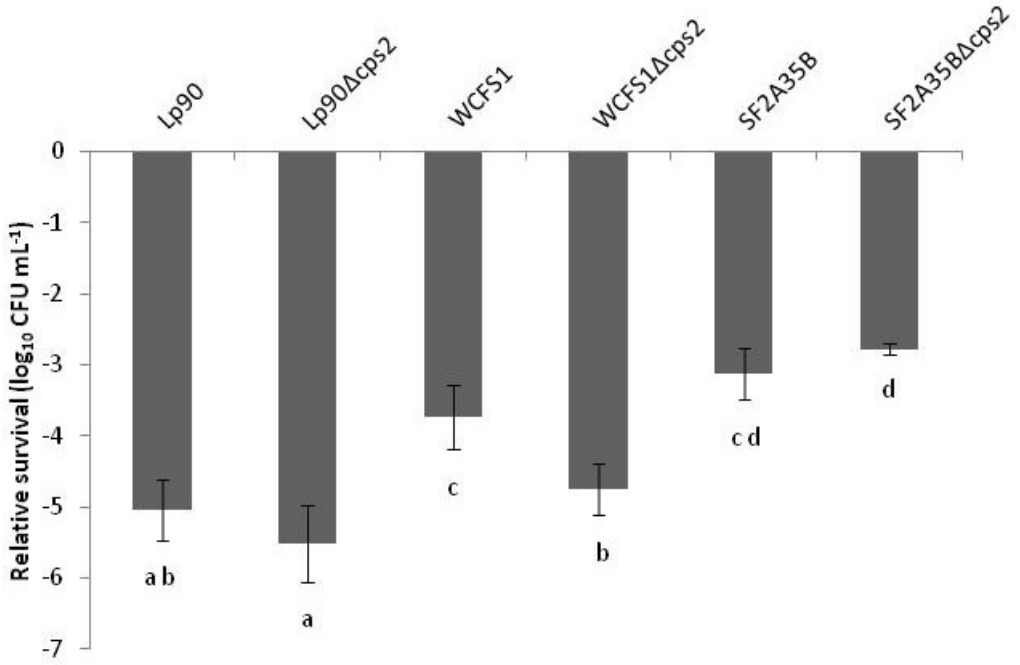

Figure 4.38 - Relative survival (log_{10} CFU mL^{-1}) of *L. plantarum* strains after 30 min of incubation with 3 g/L of porcine bile. The results were obtained from the averages and standard deviations from three independent experiments. Different superscript letters indicate statistically significant differences in adhesion ($p<0.05$) as assessed by one-way ANOVA test.

4.11 Bacterial survival and malolactic fermentation in microvinification assays

Microvinification assays were performed in grape must of "Nero di Troia", with the aim to understand the behavior of *L. plantarum* Lp90 in its original habitat, according to exopolysaccharides production.

Bacterial survival and malolactic fermentation were studied in:

- grape must co-inoculated with *L. plantarum* and *S. cerevisiae* EP2 (co-inoculation method);
- grape must after alcoholic fermentation by *S. cerevisiae* EP2 and then inoculated with *L. plantarum* (sequential inoculation method).

In both methods grape must was supplemented or not with SO_2 (70 mg/L), in order to evaluate the influence of sulfur dioxide on bacterial viability and malolactic fermentation led by *L. plantarum* strains.

After co-inoculation of grape must with *L. plantarum* and *S. cerevisiae,* Lp90 showed a higher survival compared to Lp90Δcps2 non-ropy mutant strain, especially in the first two days post inoculation in both grape musts (with or without SO_2) (**Figure 4.39 A and B**). After 7 days, there was a lower survival of Lp90 than Lp90Δcps2 in must without SO_2 (**Figure 4.39 A**). On the contrary, the viability of Lp90 was greater in the presence of SO_2 (**Figure 4.39 B**). In all cases, the viability was undetected after 14 days from the co-inoculation. Furthermore, as reported in **Figure 4.40 A,** Lp90 showed an increased production of L-lactic acid than Lp90Δcps2, which was greater in must without SO_2 addition; to lesser extent the same trend was observed in the presence of SO_2. Concomitantly, at the end of malolactic fermentation a higher residue of L-malic acid was observed for Lp90Δcps2 compared to Lp90, especially in the grape must containing sulfur dioxide; however, this difference was not significant in the absence of SO_2 (**Figure 4.40 B).**

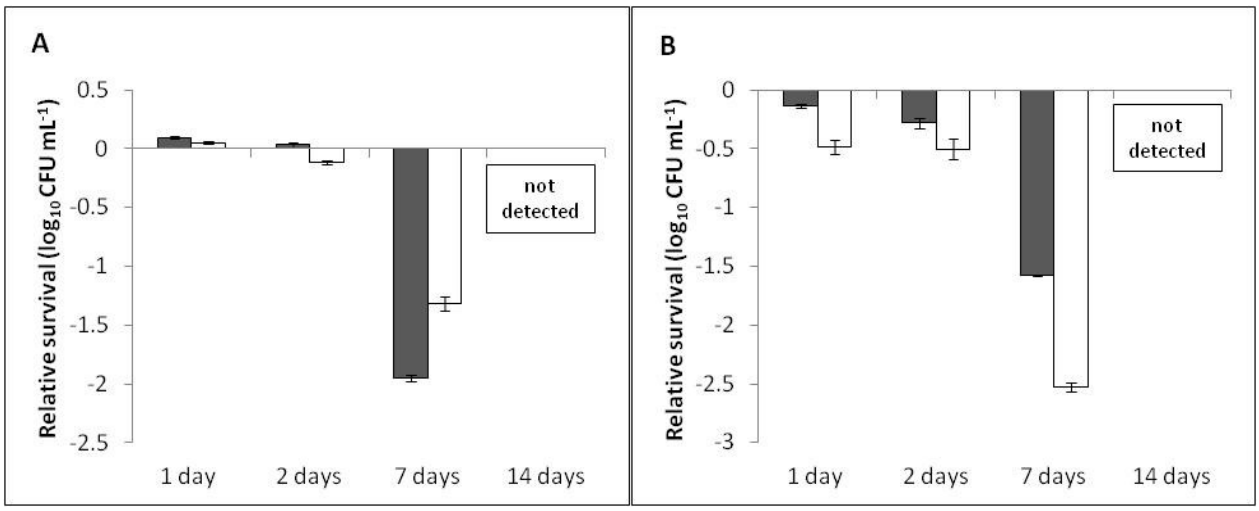

Figure 4.39 – Relative survival (log10 CFU mL^{-1}) of *L. plantarum* Lp90 (full bars) and *L. plantarum* Lp90Δcps2 (open bars) co-inoculated with *S. cerevisiae*, in grape must without SO_2 (A) and with SO_2 (B). The survival was monitored over a 14 days period. The results were obtained from the averages and standard deviations from three independent experiments.

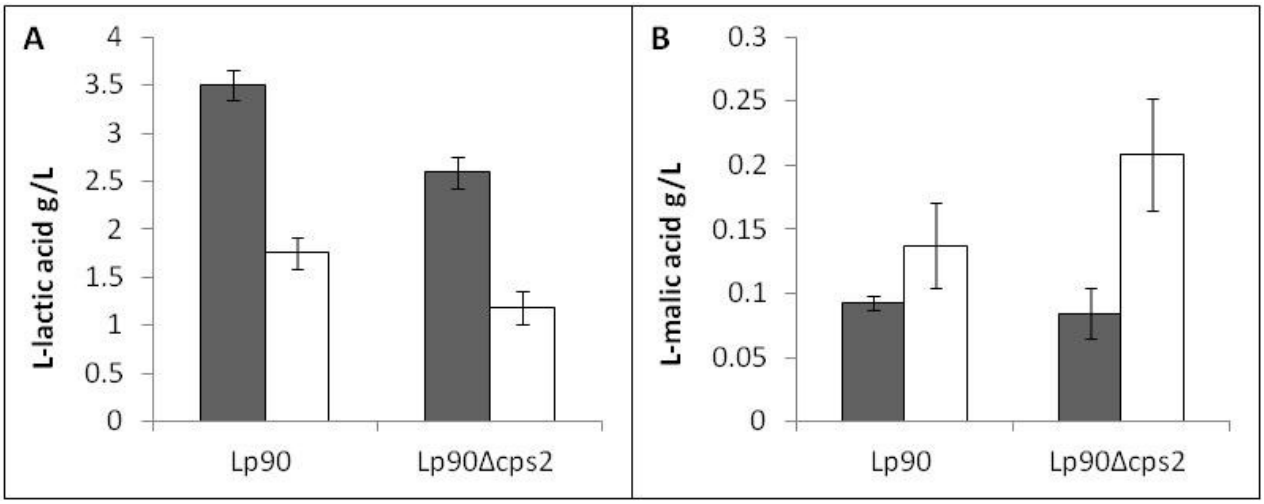

Figure 4.40 – Final concentration of malolactic fermentation products. L-lactic acid (A) and L-malic acid (B) concentrations were assayed after 14 days after co-inoculation with *L. plantarum* stranis and *S. cerevisiae,* in grape must without SO_2 (full bars) and with SO_2 (open bars). The results were obtained from the averages and standard deviations from three independent experiments.

Following the sequential inoculation of *L. plantarum* strain in grape must previously fermented by *S. cerevisiae,* the relative survival of Lp90 decreased as a function of time, but less than Lp90Δcps2 (**Figure 4.41 A and B**). Also in this case was not detected CFU 14 days post-inoculation. Moreover, in absence of SO_2 Lp90 showed a greater final concentration of L-lactic acid compared to Lp90Δcps2; no significant differences were observed between *L. plantarum* strains as regards the residual concentration of L-malic acid (**Figure 4.42**).

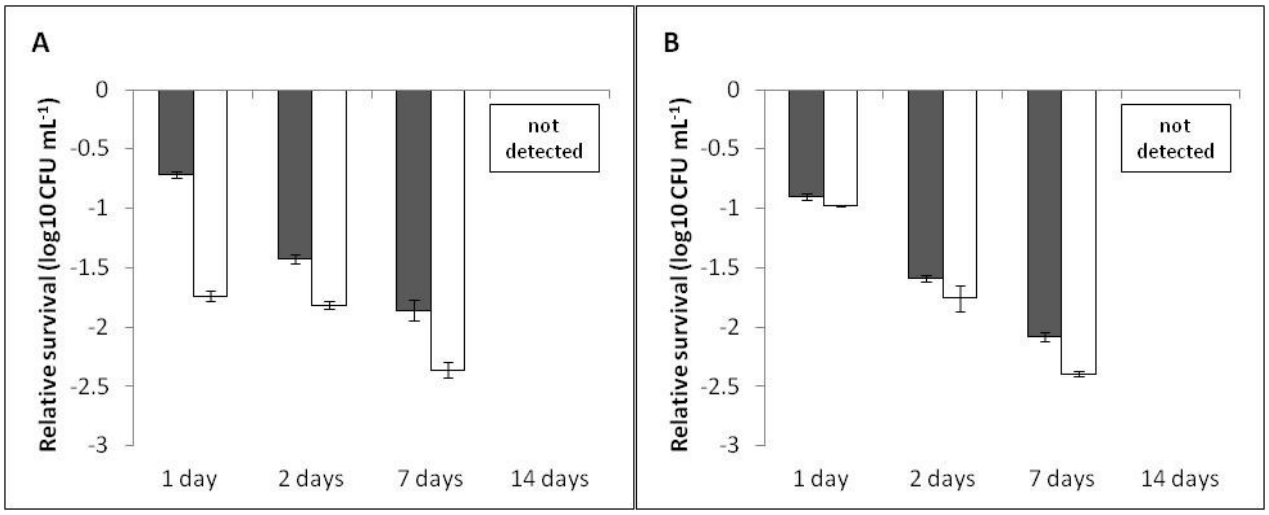

Figure 4.41 – Relative survival (log10 CFU mL^{-1}) of *L. plantarum* Lp90 (full bars) and *L. plantarum* Lp90Δcps2 (open bars) sequentially inoculated to *S. cerevisiae*, in grape must without SO_2 (A) and with SO_2 (B). The survival was monitored over a 14 days period. The results were obtained from the averages and standard deviations from three independent experiments.

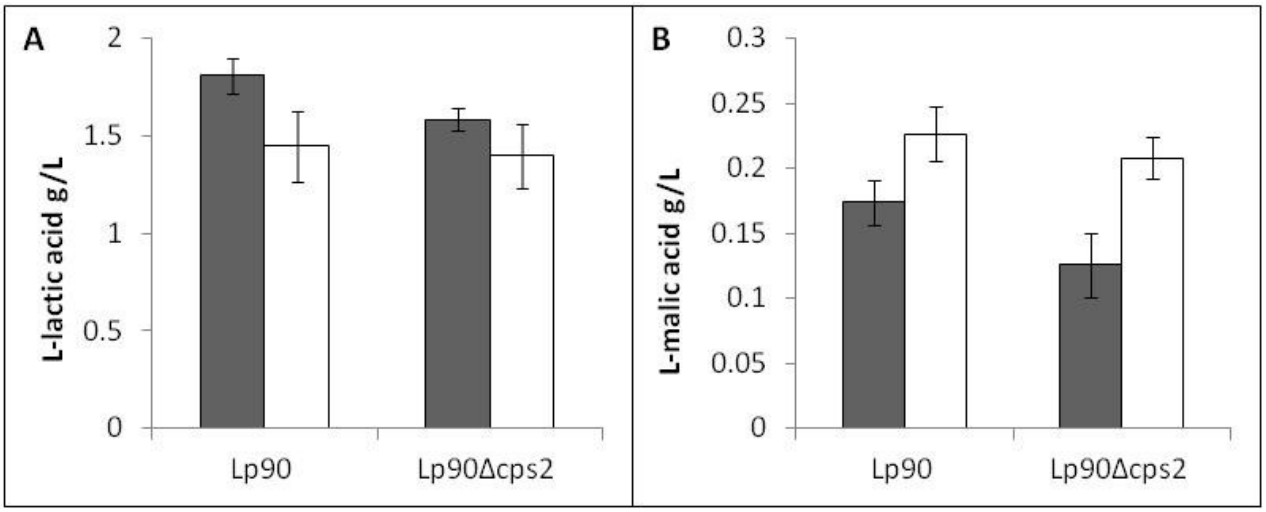

Figure 4.42 – Final concentration of malolactic fermentation products. L-lactic acid (A) and L-malic acid (B) concentrations were assayed after 14 days after sequential inoculation with *L. plantarum* strains. Grape must previously inoculated with *S. cerevisiae* for alcoholic fermentation were: without SO_2 (full bars); with SO_2 (open bars). The results were obtained from the averages and standard deviations from three independent experiments.

Taken together, the results obtained from microvinification assays suggest that exopolysaccharides produced by *L. plantarum* Lp90 might confer higher survival to the bacterial cells, protecting them from hostile conditions of wine. In this regards, some authors reported that such oenological ropy strains are more tolerant to ethanol, pH and SO_2 stress conditions (Lonvaud-Funel, 1999; Dols-Lafargue *et al.,* 2008). In our case, the possible protective effect of

EPS was clearly observed when *L. plantarum* strains were simultaneously inoculated with *S. cerevisiae* in grape juice sulfur dioxide free, showing a slight bacterial growth in the two days after co-inoculation. Furthermore, this finding is supported by the higher ability of Lp90 to lead malolactic fermentation in co-inoculation with yeast. Obviously, this was favored by the greater availability of sugar in grape must, only partially fermented by yeast and thus still available for lactic acid bacteria. Furthermore, since the fermentation started after co-inoculation of *S. cerevisiae* and *L. plantarum* strains, the low ethanol concentration and the modest acidic conditions resulted less stressful for lactobacilli.

On the other hand, when lactobacilli were inoculated at the end of alcoholic fermentation, Lp90 showed a lower viability, although greater than its relative not-ropy mutant strain, again suggesting a possible protective role of EPS.

In general, in both inoculation methods (simultaneous and sequential), the sulfur dioxide addition negatively affected the bacterial survival, mainly for Lp90Δcps2; noticeably, these data are consistent with our findings related to sulfur dioxide stress.

5. CONCLUSIONS

Lactobacillus plantarum Lp90 strain has a distinctive ropy phenotype attributable to the exopolysaccharides (EPS) production. The images of Transmission Electron Microscopy, displayed an extracellular matrix partially around the cell wall as well as in dispersed form into the medium.

Lp90 produces hetero-polysaccharides composed by three different sugars (rhamnose, glucose and galactose) and two amino sugars (glucosamine and galactosamine) in different percentages.

Lp90 strain is the first sequenced genome of *L. plantarum* from wine origin. The genome resulted 3,324,076 bps long with a total of 3,273 predicted genes. By comparative analysis based on protein orthology among 12 sequenced *L. plantarum* strains, we identified 4,726 orthologous genes (OGs) which represent the pan-genome of this specie, while the core genome consisted of 2,207 OGs. Lp90 genome contained one of the most variable OGs preceded by WJL, ATCC-14917 and ZJ316 strains. The complete *L. plantarum* Lp90 genome sequence allowed the characterization of the gene clusters responsible for exopolysaccharides biosynthesis and four different *cps/eps* gene clusters were found. Furthermore, into the *cps2* gene cluster of Lp90 we identified three genes (glycosyltransferase) apparently unique to this strain, which are homologous to two hypothetical proteins and a glycosyltransferase of *Lactobacillus fabifermentans* T30PCM01, a strain isolated from fermenting grape marc (Treu *et al*., 2014).

The *cps*2 cluster deletion as well as the partial *cps2* deletion (including unique genes) of *L. plantarum* Lp90 allowed obtaining two mutant strains, Lp90Δcps2 and Lp90Δcps2.5 respectively. These deletion strains evidently lost their ropy phenotype, further confirmed by the Transmission Electron Microscope analysis, which showed the loss of the extracellular matrix around the bacterial cell wall, compared to the parental strain. Based on these results we

concluded that the ropy phenotype of *L. plantarum* Lp90 is intrinsic to the cluster *cps2*, in particular for the three unique genes.

Adhesion to intestinal mucosa, inhibition of pathogen adhesion and modulation of the immune-system represent necessary features for a potential probiotic microorganism (Bermudez-Brito *et al.,* 2012). In our case, the exopolysaccharides deficiency improved bacterial adhesion on Caco-2 cells; therefore in agreement with other authors, we supposed that the EPS might mask the surface molecules involved in bacterial adherence (Ruas-Madiedo *et al.,* 2006; Denou *et al.,* 2008; Leeber *et al.,* 2009). Furthermore, *L. plantarum* Lp90 was able to inhibit the *E. coli* adhesion of on Caco-2 cells when lactobacilli were previously added to the human cells monolayer, rather than simultaneously or successively inoculated to the pathogen. This suggests the inability of Lp90 to displace *E. coli* once it has colonized the cell monolayer, while when lactobacilli adhere in a stable manner on the epithelial layer they are able to contrast more strongly the *E. coli* adhesion (Arena *et al.,* 2014b). Noticeably, we observed an inhibitory effect on pathogen adhesion after the addition of EPS isolated from Lp90.

Moreover, EPS produced by *L. plantarum* Lp90 do not seem to modulate the immune response following the stimulation of Caco-2 cells. In fact, no significant transcriptional levels were observed for some genes related to anti- and pro-inflammatory response, antimicrobial activity, reinforcement of mucosal surface and receptors of the innate immune-response. This appears to be in line with the results of lactobacilli adhesion on Caco-2 cells monolayer. We assume that exopolysaccharides may mask the molecules responsible for the recognition between the bacterial cell wall and that of the eukaryotic cell, thus hindering the immune-response, as reported by Lebeer *et al.* (2011) for exopolysaccharides produced by *Lactobacillus rhamnosus* GG.

In our study, we observed that pRCR12 plasmid (Russo *et al.,* 2015) is an effective vector to express the fluorescent mCherry protein in lactic acid bacteria, useful to monitor in real time their colonization and persistence within zebrafish larvae intestinal tract. *L. plantarum* Lp90

showed a higher *in vivo* colonization especially in the first hours post infection and then decreased over time, probably due to *in situ* production of exopolysaccharides. This result is in agreement with our results of the *in vitro* adhesion on Caco-2 cells, where we reported that EPS seem to hinder the adhesion of lactobacilli in stationary phase, where there is a greater accumulation of exopolysaccharides around the bacterial cells.

Regarding the ability of *L. plantarum* Lp90 to adhere on abiotic surfaces forming a biofilm layer on glass tubes, it showed a lower affinity for this substrate, in contrast to its non-ropy mutant strain Lp90Δcps2. A similar effect was observed for *L. rhamnosus* GG EPS-producing (Leeber *et al.,* 2009). Therefore exopolysaccharides might not have chemical affinity with glass surfaces; they could even mask some molecules of the bacterial cell wall which have major binding properties. Van Houdt and Michiels (2010) reported that chemical structure, relative quantity and charge, properties of the abiotic surface and surrounding environment could affect the EPS in biofilm formation.

L. plantarum Lp90 did not show an improved resistance to *in vitro* gastro-intestinal (GI) tract, thus suggesting that EPS produced by Lp90 do not confer advantage for survival to these conditions, as previously observed by Fernández de Palencia *et al.* (2009) for *P. parvulus* ESP-producing. However, *L. plantarum* Lp90 showed a modest survival after these stressful conditions, thus presenting an added value although this strain is of oenological origin.

In this study, we reported the production of yogurt obtained with conventional starter cultures, (*S. thermophilus* and *L.delbrueckii* subsp. *bulgaricus*) co-inoculated with *L. plantarum* Lp90. Compared to control yogurt, the final products obtained with Lp90 showed: a similar chemical profile in term of lactic acid, nitrogen fractions and fat content; a higher casein and protein content; a different peptide profile; a good quality stability over 28 days of storage. Moreover, the viability of Lp90 was persistent over the shelf-life and it was moderately able to tolerate the oral, gastric and intestinal conditions into yogurt matrix. Overall, the transcriptional analysis of cytokine-mediating genes involved in the immune-response showed that *L.*

plantarum Lp90 has a positive effect on immune-homeostasis following the treatment of differentiated THP-1 cells with LPS. No significant differences were found between undigested and *in vitro* digested samples, thus suggesting that the effect on immune-stimulation was not correlated to digestive processes.

Further investigations on the rheological and sensorial properties will provide information about the ability of exopolysaccharides produced by Lp90 *in situ* during yogurt fermentation to influence the characteristics of the final product. For instance, some authors found that exopolysaccharides contribute to improve the viscosity, texture and they do not alter the flavor of yogurt (Jolly *et al.,* 2002; Badel *et al.,* 2011).

The tolerance to stressful conditions is an essential feature for probiotics and pro-technologicals lactic acid bacteria properties. For this reason, resistance to the most representative stress of wine environment (i.e. ethanol, pH, sulfur dioxide, lysozyme) were also investigated. Taken together, exopolysaccharides produced by Lp90 seem to support the bacterial cells resistance to some stress, such as ethanol, low pH, sulfur dioxide and lysozyme. Likewise our results, Dols-Lafargue *et al.* (2008) observed increased resistant to alcohol and sulfur dioxide stresses for such oenological bacterial strains harboring a functional glycosyltransferase gene. Other authors suggested that microbial exopolysaccharides assist the bacterial cell against acid stress (Fanning *et al.,* 2012; Alp *et al.,* 2010) and β-glucans produced by a ropy strain of *P. parvulus* surrounding the bacterial cell wall could protect it from the enzymatic activity of lysozyme (Coulon *et al.,* 2012).

Furthermore, our microvinification experiments showed that exopolysaccharides of *L. plantarum* Lp90 might confer higher survival to the bacterial cells, against hostile conditions of winemaking. This result was most evident in grape must without sulfur dioxide simultaneously inoculated with Lp90 and *S. cerevisiae,* where increased malolactic fermentation was also observed.

Finally, based on findings regarding the bacterial resistance to various stresses as well as the microvinifications assays here reported, we conclude that ropy phenotype, associated to exopolysaccharides produced by *L. plantarum* Lp90 represent a defense mechanism developed by this strain to counter the harsh conditions of the wine environment.

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7. APPENDIX

7.1 List of scientific publications

Lamontanara, A., **Caggianiello, G.,** Orrù, L., Capozzi, V., Michelotti, V., Bayjanov, J.R., Renckens, B., van Hijum, S.A.F.T., Cattivelli, L., and Spano G. (2015). Draft genome sequence of *Lactobacillus plantarum* Lp90 isolated from wine. *Genome Announcements*. 3(2):e00097-15. doi:10.1128/genomeA.00097-15.

Arena M.P.*, **Caggianiello, G.*,** Russo, P., Albenzio, M., Massa, S., Fiocco, D., Capozzi, V. and Spano, G. (2015). Functional starters for functional yogurt. *Foods*, *4,* 15-33; DOI:10.3390/foods4010015. * These authors contributed equally to this work.

Russo, P., Iturria, I., Mohedano, M.L., **Caggianiello, G.,** Ranieri, S., Fiocco, D., Pardo, M.A., López, P., and Spano, G. (2015). Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. *Applied Microbiology and Biotechnology*. DOI: 10.1007/s00253-014-6351-x.

Arena, M.P., **Caggianiello, G.,** Fiocco, D., Russo, P., Torelli, M., Spano, G., and Capozzi, V. (2014). Barley β-Glucans-Containing Food Enhances Probiotic Performances of Beneficial Bacteria. *International Journal of Molecular Sciences*, *15*(2), 3025-3039.

7.2 Participation to national and international congresses

Spano, G., **Caggianiello, G.,** Fiocco, D., Arena, M.P., Russo, P., Orrù, L., Lamontanara, A., van Hijum, S.A.F.T., Capozzi, V. (2014). Comparative genome analysis reveals strains specific gene clusters involved in exopolysaccharides biosynthesis in *Lactobacillus plantarum. 11th International Symposium on Lactic Acid Bacteria. Egmond aan Zee, the Netherlands, from August 31 to September 4, 2014.*

Spano, G., **Caggianiello, G.,** Lamontanara, A., Orrù, L., Capozzi, V., Michelotti, V., Renckens, B., Bayjanov, J.R., van Hijum, S.A.F.T., Cattivelli, L., (2014). Draft genome sequence and annotation of *Lactobacillus plantarum* strain Lp90 isolated from Apulian (Italy) wine. *11th International Symposium on Lactic Acid Bacteria. Egmond aan Zee, the Netherlands, from August 31 to September 4, 2014.*

Spano, G., **Caggianiello, G.,** Fiocco, D., Arena, M.P., Russo, P., Parisi, F., Capozzi, V. (2014). Probiotic survival in symbiotic yogurt-like cereal-based beverage. *11th International Symposium on Lactic Acid Bacteria. Egmond aan Zee, the Netherlands, from August 31 to September 4, 2014.*

Spano, G., Arena, M.P., **Caggianiello, G.,** Capozzi, V., Russo, P., Fiocco, D. (2014). Antimicrobial activity of *Lactobacillus plantarum* strains and antimicrobial properties of *L. plantarum* small heat shock proteins. *11th International Symposium on Lactic Acid Bacteria. Egmond aan Zee, the Netherlands, from August 31 to September 4, 2014.*

Caggianiello, G., Puertas, A., Capozzi, V., Russo, P., Peña, N., Spano, G., Dueñas, M.T., López, P., Fiocco, D. (2013). Section: Competition, dominance and evenness: how microorganisms manifest their supremacy. Ropy phenotype of *Lactobacillus plantarum* confers higher tolerance

to acidic and bile stress. International conference *Microbial Interactions in Complex Ecosystems.* Torino, October 23-25, 2013.

Iturria, I., Russo, P., Mohedano, M.L., Ranieri, S., **Caggianiello, G.,** Fiocco, D., López, P., Spano, G., Pardo, M.A. Exploring the colonization ability of probiotic lactic acid bacteria strains in zebrafish: an *in vivo* model. Session VI: Symbiosis of microbes with humans, animals and plants. International conference *Microbial Interactions in Complex Ecosystems.* Torino, October 23-25, 2013.

Caggianiello, G., Russo, P., Puertas, A., Capozzi, V., Peña, N., Spano, G., Dueñas, M.T., López, P., Fiocco, D. (2013). Exopolysaccharides increase tolerance to acidic and bile stress in *Lactobacillus plantarum. 19ème colloque du Clus des Bactéries Lactiques. Bordeaux, France, 16, 17 et 18 octobre, 2013.*

Iturria, I., Russo, P., Mohedano, M.L., Ranieri, S., Spano, G., **Caggianiello, G.,** López, P., Pardo, M.A. Explorando la capacidad colonizadora de bacterias lácticas probióticas en el modelo *in vivo* pez cebra. VII REUNIÓN DE LA RED ESPAÑOLA DE BACTERIAS LÁCTICAS. *Participación de las bacterias lácticas en la salud humana y en la calidad alimentaria.* Centro de Investigaciones Biológicas (Madrid, Spain), 4-5 July 2013.

Caggianiello, G. Polyphasic characterization of exopolysaccharides produced by *Lactobacillus plantarum.* XVII Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Bologna, Cesena, 19-21 September, 2012.

Capozzi, V., Fiocco, D., **Caggianiello, G.,** Russo, P., Lopez, P., Fernández-de Palencia, P., Dueñas, M.T., Spano, G. (2012). Beta-glucans improve growth, viability and colonization of probiotic microorganisms. *III CONVEGNO NAZIONALE Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), BARI, 26-28 GIUGNO 2012.*

7.3 Experiences in other research centers

NIZO Food Research BV (Health Department) - P.O. Box 20 6710 BA Ede, The Netherlands. Supervisor: professor Michiel Kleerebezem (Scientist Functional Genomics & Bacterial Metagenomics).
May 2014 – July 2014 (Three months).

"Centro de Investigaciones Biológicas" (C.I.B) - Ramiro de Maeztu 9, 28040 Madrid, Spain. Department of "Microbiología Molecular y Biología de las Infecciones" Lab: "Biología Molecular de Bacterias Gram-positivas". Supervisor: Dr. Paloma López Garcia.
May 2013 – July 2013 (Three months).

Genomics Research Centre, "Consiglio per la Ricerca e Sperimentazione in Agricoltura" (CRA), Via S. Protaso 302, Fiorenzuola D'Arda, Piacenza, Italy.
June 2012.

7.4 University workshops

Statistics. Dr. Antonio Bevilacqua.

English. Professor Sarah Christopher.

Epistemologia e metodologia nella ricerca. Professor Giacomo Zanni.

Glocal Oppotunities Biotech workshop – Giusy Cannone. Servizio innovazione intesa San Paolo. Mario Bonaccorso. Assobiotec. Foggia, 9 Settembre 2013.

Glocal Cheese Biotech workshop – Fergal P. Rattray. Christian Hansen; Catherine Donnelly, Universityof Vermont-Institute for Artisan Cheese. Foggia, 17 Settembre 2013.

Glocal Wine & Bread Biotech workshop – Luc De Vuyst, Vrije Universiteit Brussel; Sibylle Krieger –Weber; Marina Bely, Institut des sciences de la vigne et du vin, University of Bordeaux. Foggia, 20 Settembre 2013.

Corso sulla sicurezza nei laboratori. Dr. Roberto Di Caterina. Università degli studi di Foggia. Marzo- Aprile 2013.

SEMINAR/STAR AgroEnergy Scientific σ Technological Advancement in Research on Agro-Energy. "The fate of tar after biomass pyrolysis: a microbiological point of view". Dr. Lorenzo Brusetti, Faculty of Science and Technology Free University of Bozen, Bolzano. Auditorium Università degli Studi di Foggia, Via Gramsci 79, Foggia, 5 APRILE 2013.