In ricordo di nonna Teresa

Nella revisione della memoria
e della dimensione del destino,
tra il vuoto del presente
e il peso di sogni perduti,
lungo un equilibrio
di respiro lento della notte
di contorni sterili di ombre
e di silenzio del tempo,
cadere è stato facile;
sperimentando
il piacere di precipitare
e il bisogno di risalire;
ma camminare sempre
verso orizzonti più lontani
della mente,
dove la forma
del pensiero
diventava libera…

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Host-probiotic interaction: in vitro analyses

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INDEX

ABSTRACT

Functional foods can positively influence functions of the body, by improving the health or reducing the risk of disease. Some functional foods contain 'probiotics', defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. The development and use of *in vitro* and *in vivo* protocols to assess the probiotic efficacy of microorganisms are highly encouraged by FAO and WHO.

In this thesis, the probiotic potential of the lactic acid bacterium *Lactobacillus plantarum*, wild type and derivative mutant strains, was investigated. The distinctive cell surface features exhibited by stress gene mutants prompted us to produce, by gene knockout, other *L. plantarum* defective strains and led us to investigate whether these characteristics could affect host-microbe interaction. The bacterial survival of *L. plantarum* strains and commercial probiotics was evaluated by designing an *in vitro* system simulating the transit along the human oro-gastrointestinal tract. Different carrier matrices were assayed in relation to possible prebiotic effects. The bacterial molecular response to such stresses was monitored by analysing the expression of stress, adhesion and probiosis genes. Interaction with the host was studied *in vitro* by i) assessing bacterial adhesive ability to gut epithelial cells; ii) investigating anti-inflammatory properties and induction of innate immunity genes in human host cells.

L. plantarum strains were resistant to the combined stress at the various steps of the simulated oro-gastrointestinal tract. Major decreases in viability were observed mainly under drastic acidic conditions (pH $\leq$ 2.0) of the gastric compartment. Abiotic stresses associated to the intestinal environment (small intestine) poorly affected bacterial vitality. The protective effect of vehicle matrices correlated with composition and bacterial nutritional needs. A relationship was found between bacterial survival and stress gene pattern. All strains significantly adhered to human intestinal epithelial cells, with the Δ*ctsR L. plantarum* mutant exhibiting the highest adhesion. Colonization ability was improved by addition of prebiotics. Supernatants from all strains of *L. plantarum* reduced proinflammatory cytokine secretion by activated human immune cells. Induction of immune-related genes resulted generally higher upon incubation with heat-inactivated bacteria, rather than with live ones. For specific genes, a differential transcriptional pattern was observed upon stimulation with the different *L. plantarum* strains, pointing to a possible role of the knocked out bacterial

genes in modulation of host cells response. Particularly, cells from Δ*hsp18.55* and Δ*ftsH* mutants strongly triggered immune defence genes.

This study highlights the relevance of the microbial genetic background in host-probiotic interaction and might contribute to: i) define selection criteria and/or conditions for probiotic screening and delivery; ii) identify candidate bacterial genes and/or molecules involved in probiosis, so to tailor probiotics for specific clinical applications.

1. INTRODUCTION

1.1. Probiotics.

Humans live in close association with a large number of microorganisms occurring on the skin, in the mouth and all along the gastrointestinal (GI) mucosa. The highest concentration of commensal microorganisms is found in the GI tract, which has more than 400 m^2 of surface area. The GI tract harbours a rich and complex microbiota of more than 500 different bacterial species, some of which play important health functions on the host, including immune system stimulation, protection from invading bacteria and viruses, and support to digestion of nutrients (Mcfaralane and Mcfarlane, 1997; O'Hara and Shanahan, 2006; Neish, 2009). The normal gut flora, which is essential for human homeostasis, is rapidly acquired after birth and remains relatively stable throughout the life. While the intestinal microbiota is developing, its interaction with the host results in the evolution of a unique and distinct intestinal immune system. The great challenge for the host mucosal immune system is to discriminate between pathogens and benign organisms by stimulating protective immunity without excessive inflammatory response that may disrupt the integrity of the GI mucosa (Mc Ghee et al, 1999)

The use of antibiotics, immunosuppressive therapy and other treatments, may profoundly alter the composition on the commensal GI microbiota. Therefore, the dietary supplementation of beneficial bacterial species may be a very attractive therapeutic alternative to re-establish the microbial equilibrium and prevent disease (Vanderhoof and Young, 1998). In this regard, the helpful bacteria are the so-called 'probiotics'. According to the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), 'probiotics' are live microorganisms that confer a health benefit on the host (FAO/WHO, 2002). Whereas, the related term 'prebiotic' indicates non-digestible food ingredients that improve host health by stimulating growth and activity of beneficial components of the gut microflora (Gibson and Roberfroid, 1995).

1.1.1. History and definition of probiotics.

The Russian scientist and Nobel laureate Eli Metchnikoff, was the first to conceive a positive role of certain bacteria in the human body. At the beginning of the 20th century, he suggested that it might be possible to replace harmful microbes with useful ones. He believed that the aging process was due to toxins such as phenols, indols and ammonia in the large intestine, produced by proteolytic microbes such as Clostridia. He noted that milk

fermented with lactic acid bacteria inhibited the growth of the proteolytic bacteria because of the low pH produced by lactose fermentation. Metchnikoff also observed that some rural peoples in Europe, who used to drink milk fermented by lactic acid bacteria, had a relatively long life. He then introduced the use of fermented sour milk, using a bacterial species that he later called '*Bulgarian bacillus*' (Vaughan, 1965).

The French pediatrician Henry Tissier first isolated a *Bifidobacterium*. He isolated it from a breast-fed infant and called it '*Bacillus bifidus communis*' (later renamed *Bifidobacterium bifidum*). Tissier 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, the German 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. So, at this time, Metchinikoff's theory was disputed and the idea of fermented food died out (Cheplin and Rettger, 1920).

Werner Kollath first introduced the term 'probiotics'. In 1953, he wrote about probiotics as being in contrast to harmful antibiotics, and defined 'Probiotika' those 'active substances that are essential for a healthy development of life'**.** Rosalie Lilly and Daniel Still well coined the term in 1965. They defined it as 'a substance produced by a microorganism stimulating the growth of another microorganism'. That is the opposite of antibiotic (Lilly and Stillwell, 1965). In 1974, R. B. Parker gave a different definition: those 'organisms and substances which contribute to intestinal balance' are probiotics (Parker, 1974). Roy 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).

Over the years, experts have long argued on how to define probiotics. WHO and FAO have recently developed a widely accepted definition: 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002).

1.2. Microorganisms used as probiotics.

Most probiotic microorganisms are Gram-positive bacteria belonging to the genera of *Lactobacillus* and *Bifidobacterium*. However, even *Lactococcus*, *Streptococcus*, and *Enterococcus* genera, as well as some non-pathogenic strains of *Escherichia*, and certain yeast strains are currently used as probiotics (**Table 1.1**) (Ouwehand et al, 2002).

Genus	Species	Strain	Health benefits
Lactobacillus	*acidophilus*	La5	- Reduced antibiotic associated diarrhoea
	casei	Shirota	- Shortening of rotavirus diarrhoea - Reduced recurrence of superficial bladder cancer - Immune modulation
	johnsonii	La1	- Improved oral vaccination - Reduced colonisation by Helicobacter pylori
	plantarum	299v	- Relief of irritable bowel syndrome - Reduction of LDL-cholesterol
	rhamnosus	GG	- Shortening of rotavirus diarrhoea - Immune modulation - Relief of inflammatory bowel disease - Treatment and prevention of allergy
Bifidobacterium	*longum*	BB536	- Reduction of incidence of influenza
	lactis	Bb12	- Treatment of allergy - Shortening of rotavirus diarrhoea - Reduced incidence of travellers diarrhoea - Improved oral vaccination
Escherichia	*coli*	Nissle 1917	- Fewer relapses of inflammatory bowel disease
Enterococcus	*faecium*	SF68	- Fewer relapses of inflammatory bowel disease

Table 1.1. **Microbes used as probiotics and related documented clinical effects**.

Lactic Acid Bacteria (LAB), a heterogeneous group of Gram-positive, lactate-producing bacteria, are commonly used in the formulation of functional probiotic foods (Ljungh and Wadström, 2006; Schroeter and Klaenhammer, 2009; Bron and Kleerebezem, 2011). LAB have been traditionally employed for the preparation of fermented foods (milk, meat, vegetables and beverages); moreover, several species are natural inhabitants of the human oro-gastrointestinal (OGI) tract and vagina.

In the following sections, some of the major bacterial species used as probiotics will be briefly described.

***Lactobacillus plantarum*.** *Lactobacillus plantarum* (**Figure 1.1**) is a Gram-positive, aero-tolerant LAB that produces both isomers (D and L) of lactic acid. *L. plantarum* is extremely widespread as it inhabits foods of plant or animal origin, but also soil and the mammalian gut. It is used for the production and preservation of fermented foods obtained from different raw materials (mostly of plant origin) (**Table 1.2**), in which it is either present as a contaminant or added as a starter to carry out fermentations. *L. plantarum* contributes to specific organoleptic and nutritional properties of the final product (Kleerebezem et al, 2003). *L. plantarum* is among the most common lactobacilli occurring on the human oral and intestinal mucosa (Molin, 2001; de Vries et al, 2006).

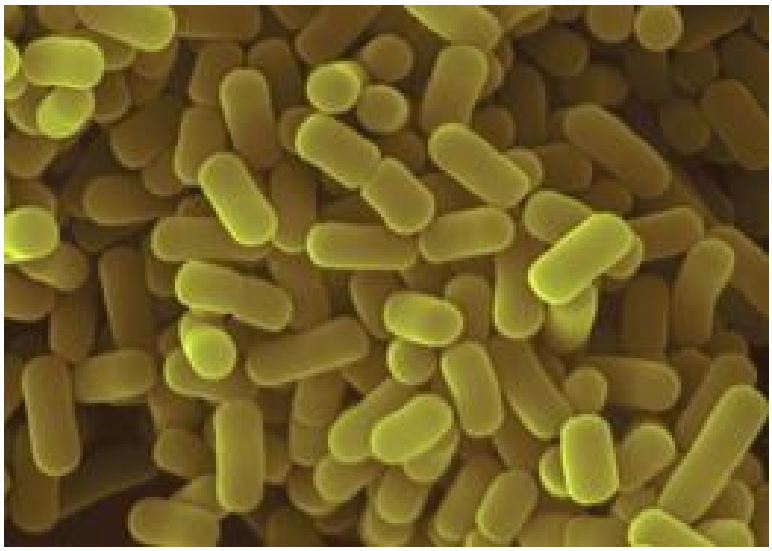


Figure 1.1. **Scanning electron microscopy image of *L. plantarum***.

Raw material	Product
Plant	Brined olives; Cocoa beans; Cassava; Sauerkraut; Togwa; Nigerian *ogi* (from maize or sorghum); Ethiopian *kocho (from starch)*; Wine; Sourdough
Milk	Stilton cheese; ricotta cheese; feta cheese
Meat	Fermented dry sausages; Fermented Italian sausages

Table 1.2. **Foods containing *L. plantarum*** (*adapted from de Vries et al, 2006*).

Diverse *L. plantarum* strains have been ascribed healthy properties; because of its natural occurrence and history of safe use in food product, *L. plantarum* is present in a variety of currently marketed probiotic foods (**Table 1.3**). A well known and broadly used probiotic strain is *L. plantarum* 299v which was originally isolated from the human intestinal mucosa. Several reports, including human clinical studies, document the potential beneficial effects of such a strain (Molin, 2001; de Vries et al, 2006).

The complete genome of *L. plantarum* WCFS1, a single colony of *L. plantarum* NCIMB 8826 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK), isolated from human saliva, was sequenced and annotated in 2003 (Kleerebezem et al, 2003). The availability of such data has prompted the genetic and molecular dissection of this species, also in relation to its probiotic behavior.

Product name	Formulation
IFlora Acidophilus Formula **Probiotic Eleven** **Plantadophilus** **FloraFood** **Living Vitamine C caps** **Udo's Choise** **Super Detox System**	Capsule
Proviva	Fruit drink
Lactovitale	Drink
ProBios	Powder/gel

Table 1.3. **Marketed probiotic food products containing *L. plantarum*** (*adapted from de Vries et al, 2006*).

***Lactobacillus acidophilus*.** *Lactobacillus acidophilus* (**Figure 1.2**) (Latin name meaning: *acid-loving milk-bacterium*) is a homo-fermentative LAB (fermenting sugars into lactic acid), which grows readily at low pH (below 5.0) and has an optimum growth temperature of 37°C. *L. acidophilus* occurs naturally in the animal and human GI tract, mouth, and vagina. Strains of *L. acidophilus* are commercially used in many dairy products, such as yoghurt, sometimes together with *Streptococcus salivarius subsp. thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (Ashraf and Shah, 2011).

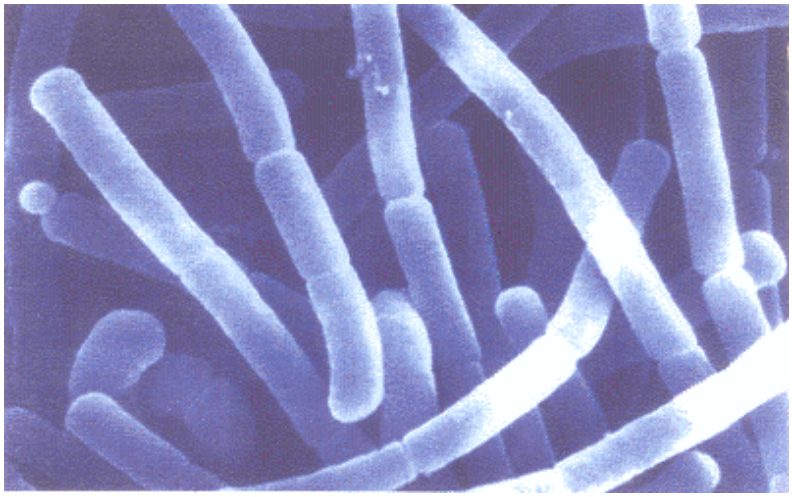


Figure 1.2. **Scanning electron microscopy image of *L. acidophilus***.

L. acidophilus La-14 is a putative probiotic strain usable in therapeutic approaches for humans (Todorov et al, 2011). *L. acidophilus* La-14 showed ability to produce bacteriocins against *Lysteria monocytogens* ScottA and was resistant to drugs used in common antibiotic therapies. Therefore its potential use would be appropriate in parallel to pharmacological therapies.

L. acidophilus LA-5 strain has been attributed documented probiotic properties and is extensively used for the preparation of commercial functional foods, especially those containing milk-derived matrices (Chr. Hansen). A recent study revealed a reduction in salivary Mutans Streptococci and Lactobacilli levels in children, after consumption of 'probiotic ice-cream' prepared with *L. acidophilus* LA-5 in association to *Bifidobacterium animalis subsp. lactis* BB-12 (Singh et al, 2011).

***Bifidobacterium*.** *Bifidobacterium* (**Figure 1.3**) is a non-motile, non-spore-forming, non-gas-producing, Gram-positive, anaerobic, catalase-negative bacterium with a high GC content. Bifidobacteria cells look like irregular V- or Y-shaped rods. The actual reason for the irregular shape of Bifidobacteria is not yet clearly understood. However, studies have revealed that *in vitro* growth media can induce the typical bifid shape (Lee and O'Sullivan, 2010). Bifidobacteria produce water-soluble vitamins in the large intestine, including many of the B group. Moreover, Bifidobacteria restore the constipation in elderly people (Mayo et al, 2008).

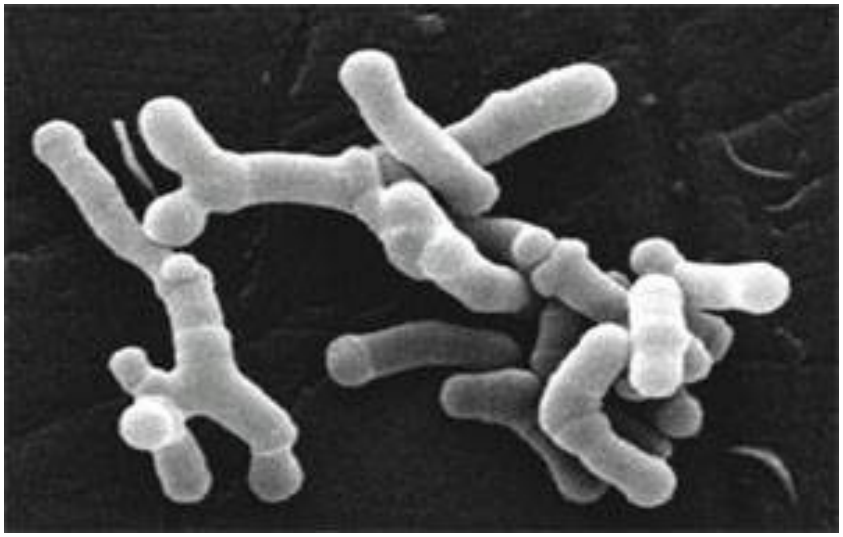


Figure 1.3. **Scanning electron microscopy image of *Bifidobacterium***.

B. animalis subsp. lactis BB-12 is marketed as probiotic. Such strain was given to preterm infants in a double-blind, placebo controlled, randomized clinical study. Feces from infants supplemented with *B. lactis* BB-12 showed lower viable counts of Enterobacteriaceae subsp. and Clostridia subsp., than the placebo group. However, *B. lactis* BB-12 supplementation did not reduce the gut colonization by antibiotic-resistant strains (Mohan et al, 2006).

1.3. Requisites and mechanisms of probiosis.

In order to be defined probiotics, microorganisms have to fulfill specific requisites of (**Table 1.4**). 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).

Requisite of probiosis	Benefit
Documented clinical effects	True health benefits
Safety	No heatlh risk for consumer
Tolerance to gastric acidity, bile salts and pancreatic enzymes	Survival of passage through the intestinal tract
Adhesion to intestinal mucosa	Balancing of intestinal microbiota; strengthening of epithelial barrier; immune modulation
Human origin	Species specific interactions with the host; non-pathogenic;
Good technological properties	Strain stability; resistance to storage and food processing conditions

Table 1.4. **Main requisites of probiotic microorganisms and related advantages**.

1.3.1. Beneficial effects of probiotics: clinical studies.

The rationale of probiotic therapies is to correct and/or prevent imbalances of the indigenous microbiota and gut barrier dysfunctions (Isolauri, 2001; Owehand et al, 2002) (**Table 1.5**).

Effect	Mechanism
Nutritional management of acute diarrhoea	Reduction in the duration of rotavirus shedding, normalization of gut permeability and microbiota
Nutritional management of allergic disease	Degradation/structural modification of enteral antigens, normalization of the properties of aberrant indigenous microbiota and of gut barrier functions, local and systemic inflammatory response, increased expression of mucin
Reducing the risk of infectious disease	Increase in IgA-secreting cells against rotavirus, induced expression of mucins
Reducing the risk of allergic/inflammatory disease	Promotion of gut barrier functions, anti-inflammatory potential, regulation of the secretion of inflammatory mediators, and promotion of development of the immune system

Table 1.5. **Potential clinical targets of probiotic therapy** (*adapted from Isolauri et al, 2004*).

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).

Well-controlled clinical and nutritional studies are necessary to demonstrate the claimed health effects of probiotics. So far, probiotic interventions have been proven to be effective in varied pathologic conditions, such as necrotizing enterocolitis, antibiotic-associated diarrhoea, *Helicobacter pylori* infections, inflammatory bowel disease, cancer and surgical infections (Reid et al, 2003).

Necrotizing enterocolitis. Necrotizing enterocolitis (NE) is a devastating intestinal disorder affecting preterm infants. It is a mortal disease characterized by abdominal distension, bilious vomiting, bloody diarrhoea, lethargy, apnoea, and bradycardia. Preterm infants who survive have intestinal obstruction and multi-organ failure (Caplan and Jilling, 2000).

Low weight preterm infants, delivered by Caesarean section, are often breast fed only after several days from birth. In addition, the normal process by which microorganisms such as *Lactobacillus* species are ingested via vaginal birth and propagated by mother's milk does not take place in these infants. Therefore, these infants are exposed to various pathogenic microbes (*Clostridium, Escherichia, Salmonella, Shigella, Campylobacter, Pseudomonas, Streptococcus, Enterococcus, Staphylococcus* and coagulase negative *Staphylococcus*) which colonize the intestine and increase the risk of NE. Furthermore, pre-term infants, given formula feeding, have fewer *Lactobacillus* and *Bifidobacterium* species in their stool compared to controls. These findings suggest a correlation between NE and *Lactobacillus* species. A human trial with live *L. acidophilus* and *B. infantis* given to newborn resulted in 60% reduction in NE (Gewolb et al, 1999; Hoyos, 1999).

Antibiotic associated diarrhoea. Probiotics have preventive as well as curative effects on several types of diarrhoea of different etiologies. The dietary supplementation of probiotics bacteria (e.g., *L. rhamnosus* GG*, E. coli* strain Nissle 1917, *Enterococcus faecium* SF 68) and yeasts (*Sacchromyces boulardii*) alleviated symptoms of diarrhoea (de Vriese and Marteau, 2007).

Antibiotic associated diarrhoea (AAD) still affects hospitalized patients, although new antibiotics with a broad spectrum of activity and fewer side effects have been developed. The complications of AAD include electrolyte imbalance, dehydration, pseudomembrane colitis and toxic megacolon. A clinical study confirms the efficacy of probiotics in the prevention and treatment of AAD with *S. boulardii* (D'Souza et al, 2002).

***Helicobacter pylori* infections**. *H. pylori* is a major cause of chronic gastritis and peptic ulcer and a risk factor for gastric malignancies. Antibiotics for *H. pylori* eradication are 90% effective, but they are expensive and cause side effects and resistance. Probiotic-based approaches to treat *H. pylori* consequences have been performed. The studies revealed that probiotics had an *in vitro* inhibitory effect on *H. pylori* and reduced *H. pylori* associated gastric inflammation in animals; moreover, probiotic treatment reduced *H. pylori* therapy associated side effects (Lesbros-Pantoflickova, 2007).

Inflammatory bowel disease. Inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease, representing chronic inflammations of the GI tract. Both clinical and experimental observations associate IBD to i) an imbalance in the composition of the intestinal microbiota, with relative predominance of aggressive bacteria and relative paucity of protective bacteria, and to ii) over-stimulation of proinflammatory immunological mechanisms. Preliminary studies suggest a positive response to probiotic interventions in IBD patients. The probiotic mixture VSL#3 provided a support to patients with intestinal mucosa depleted of protective bacteria (Gionchetti et al, 2000; Mitsuyama et al, 2002).

Cancer. In intestinal tumors, Latobacilli prevent or delay the tumor development by metabolizing and/or binding to mutagenic compounds and suppressing the growth of bacteria which convert pro-carcinogens into carcinogens. Moreover, Lactobacilli reduce the levels of β-glucoronidase and other carcinogens (Ling et al, 1994). Recurrences of urinary bladder cancers decreased following internal instillation of probiotics such as *L. casei* Shirota, but this finding needs further confirmation (Aso et al, 1995).

Surgical infections. Before the advent of antiseptics and antibiotics, fermented milk was used for healing wounds and fighting infections. Recent studies show the application of probiotics for treating and preventing surgical infections. *L. fermentum* RC-14 inhibits *Staphylococcus aureus* infection and bacterial adherence to surgical implants. One week

supplementation of the probiotic strain *L. plantarum* 299 with oat fibres reduced episodes of infection and pancreatic abscesses (Gan et al, 2002; Olah et al, 2002). Such clinical studies remind us that the potential use of probiotics is not necessarily restricted to heal gut associated dysfunctions, but has broader perspectives of applications.

1.3.2. Safety.

Because probiotics are supplemented as live microorganisms, they may cause infection to the host. Lactobacilli and Bifidobacteria are simply considered safe in reason of their taxonomic position, and for their long traditional use in food preparation. The human origin of the bacterial isolate and/or its natural occurrence in the OGI tract represent a further guarantee of safety for human consumption. In fact, the first human feeding trial shall also assess the safety of probiotic species.

Systemic infections have been rarely reported with *Bifidobacterium,* although many cases of sepsis with *L. rhamnosus* GG or *L. casei* have been reported (Adlerberth et al, 1991). Episodes of sepsis occur mainly in immune-compromised individuals or infants. But the conclusion, based on different reports, is that the risk of infection with probiotic Lactobacilli or Bifidobacteria is similar to infection with commensal strains, and that consumption of such products presents a negligible threat to consumers, including immune-compromised hosts (Ouwehand and Vesterlund, 2003). However, in order to establish safety guidelines for probiotic organisms, FAO and WHO recommend to characterize probiotic strains with a series of essential tests to assess antibiotic resistance pattern, metabolic activities, toxin production, hemolytic activities, infectivity in immune-compromised animal models, side-effects in humans, and adverse outcome in consumers. In 2002, 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.

1.3.3. Tolerance to oro-gastrointestinal conditions.

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 (**Figure 1.4**).

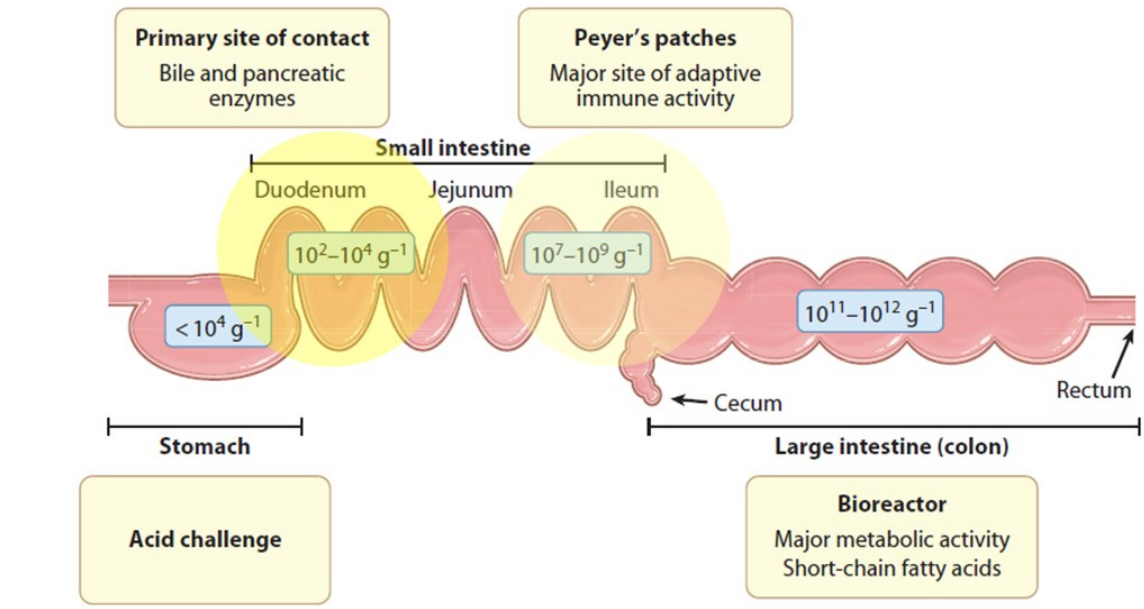


Figure 1.4. **Different regions of the human GI tract and related densities of the residing bacterial population**. Food-borne bacteria face the sequential stress of the acidic environment in the stomach, bile and pancreatin in the small intestine. Dietary supplementation of probiotics can generate a 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*).

Passage of probiotics through the human OGI tract represents a 'hazardous journey', with the initial stages designed to compromise the survival of most pathogenic microorganisms. The principal sources of stresses for the bacteria are: i) pH down-shifting, encountered in the stomach and resulting from gastric acids; ii) presence of bile in the duodenum, a digestive secretion from the hepatic system, which serves to emulsify and solubilize lipids and lipid soluble vitamins. Exposure to acids negatively affects the proton motive force across the bacterial membrane, as a result of the accumulation of protons inside the cell. Exposure to bile disrupts the integrity of the cell membrane, affects DNA and RNA structures as well as protein folding; moreover, it exposes the cell to oxidative stress and low intracellular pH (Leverrier et al, 2003; Begley et al, 2005; Corcoran et al, 2008).

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). 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 (De Angelis and Gobbetti, 2004; Sugimoto et al, 2008).

1.3.4. Adhesion to host epithelial cells and pathogen displacement.

Adhesion to the intestinal mucosa is a desirable feature of probiotic microorganisms, as it ensures persistence in the intestinal tract, which is necessary for probiotics 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 (Apostolou et al, 2001; Isolauri et al, 2004).

Several bacterial cell surface proteins have been identified, which might mediate adhesion to the mucous layer and to the extracellular matrix of intestinal cells. In fact, the bacterial colonization may be improved by specific 'adhesins' which promote a tight interaction with host epithelial cells.

'Mucus-binding protein' (MUB) is a bacterial cell-surface protein which is responsible for the adhesion to the intestinal mucus layer (MacKenzie et al, 2009). MUB proteins were well characterized in *L. reuteri* 1063 (Roos and Jonsson, 2002) and in *L. acidophilus* NCFM (Buck et al, 2005) revealing the typical domain organization of cell-surface proteins of Gram-positive bacteria: a signal peptide targeting the protein to plasma membrane is found at the N-terminus; an anchoring motif (LPXTG) for covalent attachment to the peptidoglycan of the bacterial wall is found at the C-terminus. An internal domain, containing the tandemly arranged mucus-binding repeats (Mub1 and Mub2), is responsible for adhesion to the host (Desvaux et al, 2006).

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).

Myosin cross-reactive antigens (MCRAs) are conserved proteins found across a wide range of bacteria, including LAB. These proteins were discovered initially in *Streptococcus pyogenes* as potential antigens capable to share epitopes with myosin, contributing to blood survival and keratinocytes adherence. In fact MCRA is a FAD-containing enzyme with fatty acid hydratase activity on cis-9 (9Z) - and trans-11 (11E) double bonds of C-16, C-18 non-esterified fatty acids producing 10-hydroxy and 10,13-dihydroxy fatty acids (Volkov et al, 2010). *In silico* analysis of the *L. acidophilus* NCFM genome sequence revealed the presence of a gene showing similarity to N-terminal FAD/NAD(P)-binding domain of MCRA proteins. Deletion of this gene reduced the ability of *L. acidophilus* mutant strains to adhere on Caco-2 layers (O'Flaherty and Todd, 2010).

The ability to bind to host fibronectin (Fn) is a common characteristic among many Gram-positive species. Fn is a large glycoprotein present in soluble or in insoluble form on the cell surface, in the extracellular matrix, and in basal membranes. Therefore, targeting Fn is considered a strategy by which establishing interaction with the host (Papasergi et al, 2010). A 48 kDa putative Fn-binding protein, named alfa-enolase 1 (EnoA1), was recently

identified in *L. plantarum* (Castaldo et al, 2009). Its cell surface localization was demonstrated by immune electron microscopy. Moreover, the role of EnoA1 as a *L. plantarum* Fn-specific adhesion protein was assessed by deletion and functional analysis of the *enoA1* gene: disruption of *enoA1* caused a decreased adherence of mutant strain cells. Enolases are so-called "moonlighting proteins", that is proteins with more than one function within the cell. Indeed, enolases function both as glycolytic enzyme as well as adhesins, once secreted outside the cells.

Thanks to the expression of specific adherence factors, probiotics can successfully compete with pathogens for the same attachment sites, thereby inhibiting their colonization, and possible infection: a phenomenon referred to as 'pathogen exclusion'.

The S-layer protein, which forms a crystal layer structure on the bacterial surface (Boot et al, 1996), 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).

Other mechanisms concurring to pathogen exclusion rely on the synthesis of potent antimicrobial molecules, the so-called 'bacteriocins'. Bacteriocins may allow a strain invasion into an established microbial community, or inhibit the invasion of other strains into an occupied niche (Riley and Gordon, 1999). Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins; they are ubiquitous in the microbial world. In Gram-positive bacteria (including many probiotics), most bacteriocins are small in size (20–70 aminoacids) and cationic, and act by destabilizing the integrity of the inner membrane of target bacterial cells (Diep and Nes, 2002). Gram-positive bacteriocins have also been assigned a role in quorum sensing and signal communication in bacterial biofilms (Gillor, 2007).

In vivo studies have recently highlighted the relevance of bacteriocin production by probiotic strains. *L. salivarius* UCC118, a bacteriocin-producing strain, was effective in protecting mice against invasive *Listeria*, while the corresponding wild type, bacteriocin-negative strain, failed in defending mice from infection (Corr et al, 2007). Dabour et al (2009) showed that intragastric administration of the bacteriocin pediocin PA-1 in ICR mice infected with *Listeria*, reduced pathogenic count and translocation into the liver and spleen, as compared with the control group.

Using *in vitro* tests, *E. coli* L1000, producing microcin B17, was demonstrated to be active against a broad panel of antibiotic-resistant and -sensitive Salmonella strains isolated from patients suffering from salmonellosis (Zihler et al, 2009).

Interestingly, the presence of the *plnEFI* locus (encoding plantaricins, a subgroup of bacteriocins) in *L. plantarum* strains was associated to enhanced anti-inflammatory effects, in terms of lower IL-10/IL-12 ratios observed in bacteria-stimulated human peripheral blood mononuclear cells (PBMC) (van Hemert et al, 2010).

1.4. Probiotic action and interaction with host cells.

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).

A scheme of the different actions supporting probiosis is shown in **Figure 1.5**. Probiotic effects depend on both microbe-microbe and host-microbe interactions. Metabolic interactions with the endogenous microbiota include phenomena of metabolic cooperation and/or competition for nutrient digestion, as well as production of antimicrobial compounds and competitive exclusion (Gueimonde and Salminen, 2006). All these activities may contribute to positively modulate the composition of the intestinal microbiota and inhibit detrimental species. Major metabolic interactions occur also with respect to intestinal host cells. For instance, 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). Moreover, bacteria contribute to ion absorption and can metabolize dietary carcinogens and/or other toxic compounds. Interaction with host epithelial cells strengthens the barrier function by induction of mucin secretion, tight junction reinforcement, cytoskeleton stabilization (via Hsp induction), epithelial apoptosis reduction, and by triggering innate immune system activity (Lebeer et al, 2010). Probiotics can also interface with the mucosal adaptive immune system, thus modulating maturation and cytokine expression of intestinal dendritic cells.

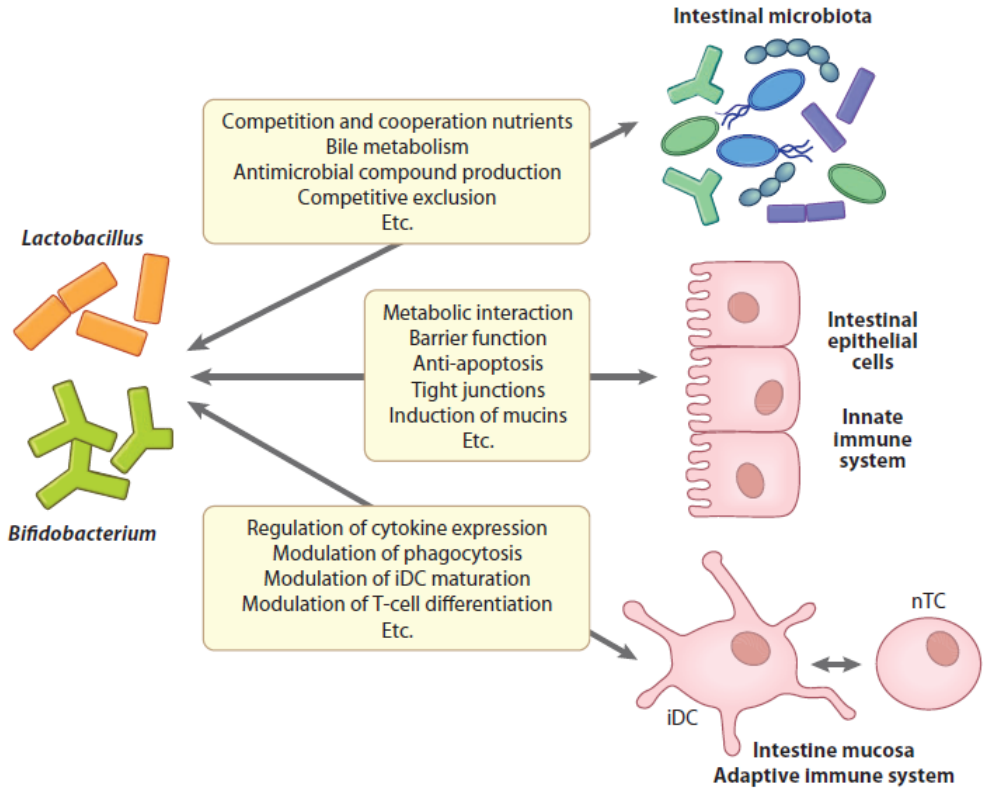


Figure 1.5. **Interactions, and related effects, occurring in the gut between probiotics (*Lactobacillus* and *Bifidobacterium*) and the endogenous microbiota, the intestinal epithelial cells and the mucosa-associated immune cells** (*from Kleerebezem and Vaughan, 2009*).

1.4.1. Molecular interplay between probiotics and host cell.

Because of their close interaction with the intestinal mucosa, probiotics begin a 'sort of molecular dialogue' with the host cells, including both the surface epithelial cells and the underlying gut associated lymphoid tissue (GALT) elements. Intercellular prokaryote-eukaryote communications are switched on, signals cascades are activated and specific biochemical response are raised within the animal cells (Shi and Walker, 2004).

1.4.1.1. Host receptors and signal cascades.

The main elements of cross-talk between bacteria and host cells are the 'Toll-like receptors' (TLRs), the central 'sensing' apparatus of the innate immunity. The innate immunity represents the most archaic part of vertebrate immune defence. In contrast to adaptive immunity, which is restricted to higher vertebrates, the innate immune response is present in the whole animal kingdom. The innate immune system relies on a limited number

of receptors that recognizes a variety of pathogen-associated molecular structures, and on defensive mechanisms that counteract the broadest spectrum of potential pathogens (Medzhitov and Janeway, 1997).

TLRs belong to a larger receptor group named 'Pattern recognition receptors' (PRRs), which play a critical role in initiating and regulating innate responses, by recognizing 'microorganisms-associated molecular patterns' (MAMPs), which are widespread and conserved. PRRs are expressed by a lot of cells including monocytes, dendritic cells, neutrophils, and epithelial cells (Medzhitov and Janeway, 2002). The interaction between MAMP and PRR results in the induction of signal cascades that develops a molecular response against the detected microorganism; this response can include the secretion of immunomodulatory cytokines, chemokines, and antimicrobial agents (**Figure 1.6**).

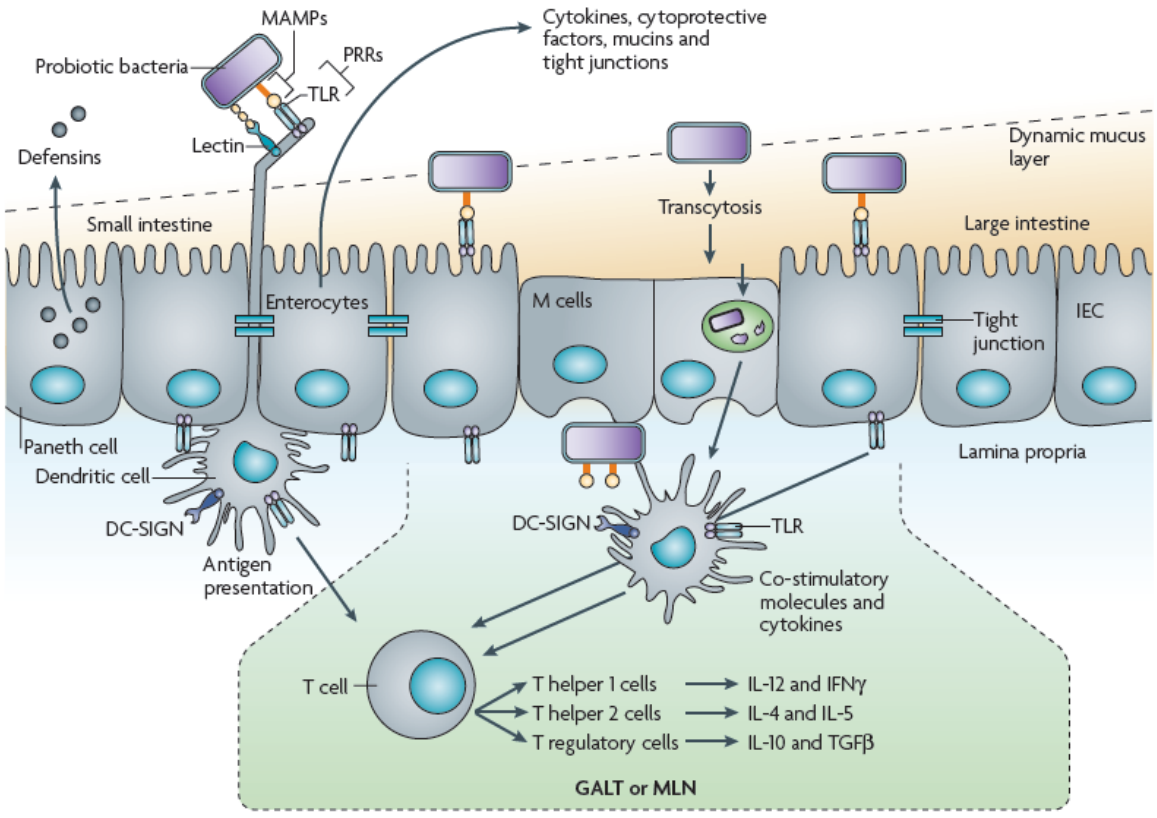


Figure 1.6. **Molecular interaction of probiotic bacteria with intestinal epithelial cells and dendritic cells from the GALT.** Host pattern recognition receptors (PRRs) sense the microorganism by recognizing their associated molecular patterns (MAMPs): this interaction will lead to specific molecular response, depending on the cell type. For example, Paneth cell shall produce defensins, whereas Goblet cells secrete mucins (*from Lebeer et al, 2010*).

The evoked signalling cascades usually involve nuclear factor-kB (NF-kB) and mitogen activated protein kinase (MAPK) systems, which rapidly transmit the signals to the nucleus to trigger transcription of immune-related genes (**Figure 1.7**).

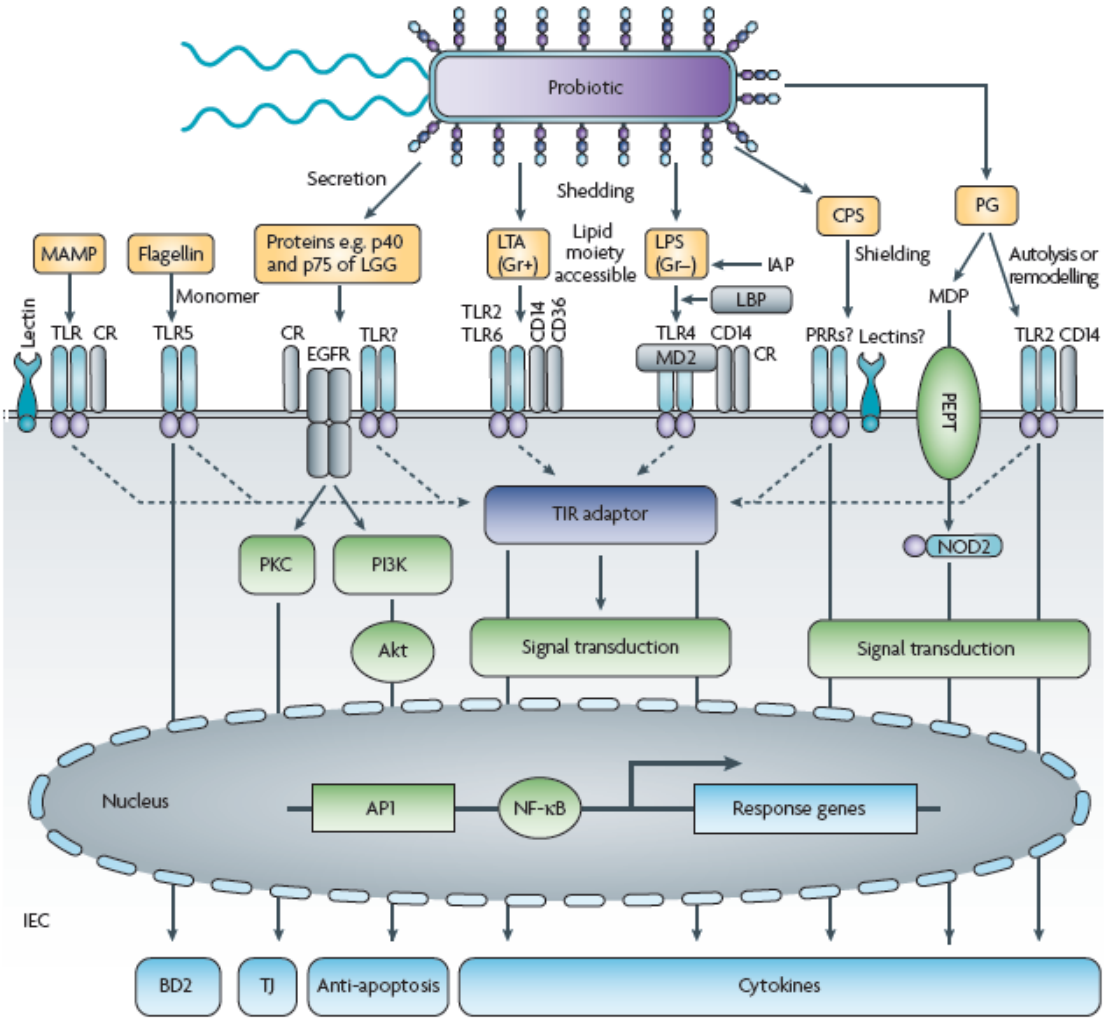


Figure 1.7. **Overview of the probiotic MAMP-PRR interaction and associated signalling events.** Probiotic microorganisms interact with intestinal epithelial cells (IECs) through various surface molecules, including flagellins, cell-wall associated enzymes, lipoteichoic acids, peptidoglycan, etc. After dimerization, TLRs receptors send the signals to 'kinases' (MAPK pathway) through 'adaptors', and activate 'transcription factors' (AP-1, NF-kB) involved in binding specific DNA sequences. Reported IEC response includes induction of β-defensin 2, cytokine secretion, tight junction promotion, anti-apoptotic signals (*from Lebeer et al, 2010*).

The anti-inflammatory properties of some probiotics have been frequently associated to the inhibition of the NF-kB pathways (Petrof et al, 2004; Zhang et al, 2005). By contrast, Schlee et al, (2008) demonstrated that VSL#3 bacterial mixture and probiotic Lactobacilli up-regulate HBD-2 gene via induction of proinflammatory pathways such as NF-kB and AP-1, as well as MAPKs. Recent transcriptome analyses in *in vivo* models confirm that the majority of the host mucosal genes modulated by probiotic supplementation are NF-kB dependent (van Baarlen et al, 2009).

1.4.2. Relevance of the bacterial cell surface features in the interaction with the host.

Microbial cell surface features are expected to be essential in the primary host-microbe interaction. Indeed, the different probiotics and immune-modulating properties of lactic acid bacteria seem closely related to their cell envelope composition and structure (Vinderola et al, 2004; Kleerebezem and Vaughan, 2009; Lebeer et al, 2010). The Gram-positive cell envelope of is made up by numerous characteristic structural components (**Figure 1.8**) that can be recognized by PPRs, induce signaling pathways and thus lead to specific health-promoting effects. MAMPs can be associated to macromolecules such as the peptidoglycan, cell wall- or membrane-associated teichoic acids, exopolysaccharides and various classes of surface proteins.

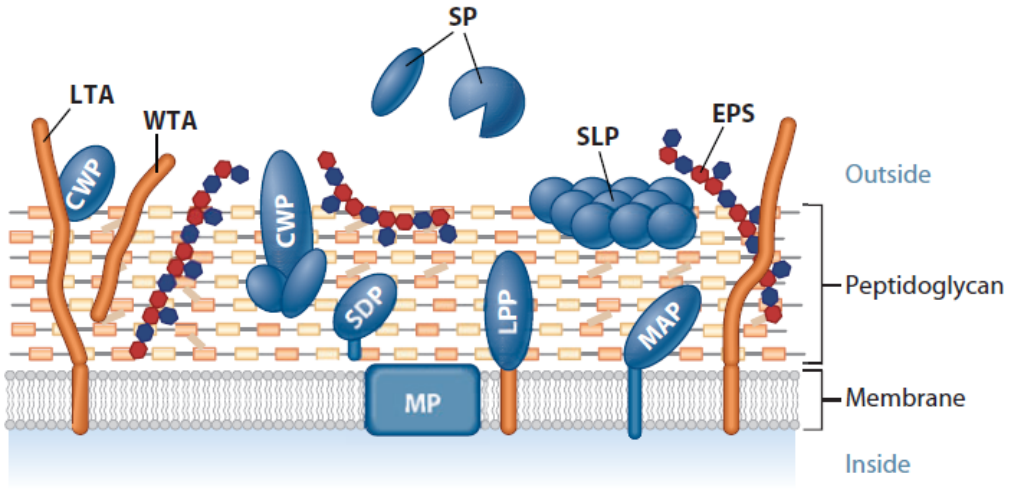


Figure 1.8. **Gram-positive cell wall.** Components of the cell surface macromolecules have been proposed to be directly involved in interaction with host cells. Specific MAMPs, and related host modulation properties, can be associated to: peptidoglycan (PG) layer, the predominant cell wall component; 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*).

Subtle variations in the composition and structure of the cell wall may account for species- and strain-specific interactions with the host. In probiotic clinical application, such as treatment of IBD and allergic disorders, an intriguing goal is just to tailor these interactions, in order to achieve specific therapeutic outcomes. Indeed, by studying a *L. plantarum dlt* cell wall mutant, which synthesized modified teichoic acids, Grangette et al (2005) demonstrated that such specific cell surface biochemical feature might positively affect the interaction between microorganism and host, enhancing its probiotic effect in terms of increased protection from intestinal disorders. Similar results were also observed in *in vitro* and *in vivo* studies on lipoteichoic (LTA)-deficient strains of the probiotic *Lactobacillus acidophilus* (Mohamadzadeh et al, 2011) and *Lactobacillus rhamnosus* GG (Claes et al, 2010). In line with these reports, Schlee and coworkers (Schlee et al, 2007) used a genetic loss of function approach to demonstrate that the induction of human β-defensin 2 was specifically mediated by the flagellins of the probiotic *E. coli* Nissle 1917.

1.4.3. Host cell response - Modulation of innate defense mechanisms.

Modulation of the gut immune function seems one of the main mechanisms through which probiotics provide beneficial effects to the host. Indeed, probiotic and commensal bacteria influence the production of humoral immune factors, such as cytokines and antimicrobial agents, secreted by the gut-associated lymphoid tissue as well as by the intestinal epithelium (Borchers et al, 2009). It is thus clear that a great part of the beneficial effects of probiotics depends on their immunomodulatory abilities, both as immune-enhancing and as well as anti-inflammatory effect. Because antimicrobial peptides, mucous components, microbicidal enzymes and cytokines play key roles in the barrier and immune function of the intestinal mucosa, the expression of genes encoding such molecules has been frequently analyzed when assessing the microbial probiotic potential (Mack et al, 1999; Morita et al, 2002; Wekhamp et al, 2004).

Antimicrobial peptides (AMPs) are key effectors of the innate immune response. The AMPs produced all along the GI tract of the host constitute a front line of chemical defence against dangerous microorganisms. This defence system functions in the airways, gingival epithelium, cornea, and in the reproductive, urinary and GI tracts. AMPs enable the innate immune system to respond in a matter of hours, well before the adaptive immune system can be sufficiently mobilized (Liévin-Le Moal and Servin, 2006).

Cathelicidins and defensins are the two main families of intestinal AMPs. Cathelicidins constitute a unique mammalian gene family. They are structurally organized with an N-terminal signal peptide, a highly conserved prosequence - the cathelin domain - and a variable cationic peptide at the C-terminus. The conservation of the cathelin domain is striking between species and indicates that the diverse members of this family evolved from a common ancestor gene (Bals and Wilson, 2003). LL-37 is the only cathelicidin described in humans. It is synthesized as a precursor, named 'human cationic antimicrobial protein 18' (hCAP18), which is then cleaved to the mature peptide by a serine protease. LL-37 can act alone and in synergy with other antimicrobial proteins (i.e., lysozyme), displaying bactericidal activities against Gram-positive and Gram-negative bacteria. LL-37 was initially found in specific neutrophil granules but is now known to also be expressed by other leukocytes, as well as keratinocytes and epithelial cells in the respiratory, urogenital, and GI tracts (Travis, et al, 2000; Sörensen et al, 2001). Additional studies suggest that expression of *hCAP18* gene by human colon epithelial cells is a marker of epithelial cell differentiation (Hase et al, 2002).

Defensins are arginine-rich cationic peptides characterized by a β-sheet fold and a framework of six disulfide-linked cysteines (Lehrer, 2004). The two main defensin subfamilies are α- and β- defensins; α-defensins comprise the group of human neutrophil peptides (HNP-1 to 4) and human defensins 5 and 6 (HD-5 and HD-6). Four human β-defensins (HBD-1 to 4) have been described. Human β-defensins (HBD) have been isolated from many cell types, mainly epithelial, confirming that, these cells actively participate to host defence. HBD-1 is mainly expressed in the epidermis and in the epithelia of pancreas, kidney and urinary tract; HBD-2 and -3 are found in the skin and in airway epithelia; HBD-4 is expressed in testis, stomach, lung and neutrophils. HBD-2 is expressed by human intestinal epithelial cells (O'Neil et al, 1999; Lievin-Le Moal and Servin, 2006) and its transcription was shown to be activated *in vitro* by probiotic lactobacilli and VSL#3 mixture (Wekhamp et al, 2004; Schlee et al, 2007). These authors first suggested a novel effect of probiotics: the enhancement of the mucosal intestinal defense against pathogens through the up-regulation of defensins. Indeed, increased level of AMPs synthesized by the intestinal epithelial cells counteract adherence and invasion by pathogens.

Lysozyme. Lysozyme is a microbicidal enzyme directed against the β(1→4) glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid residues of peptidoglycan. Lysozyme is expressed by skin, oral mucosa and intestinal epiyhelial cells (Ganz, 2004). In fishes, probiotic water supplementations have been reported to increase lysozyme level in the skin, while dietary supplementation did not induce lysozyme in either serum or skin mucosa (Nayak, 2011).

Mucins. The mucous-gel layer, occurring at the interface between the gut lumen and the epithelial cells, provides a physical barrier against potentially harmful bacteria and molecules, while acting as a lubricant for intestinal motility (Phillipson et al, 2008).

Microorganisms have developed diverse strategies to degrade the mucous layer (i.e., reduction of mucin disulfide bonds by *Helicobacter pylori*, protease activity by *Candida albicans*, and glycosidase activity by both oral and intestinal microbial communities) allowing them easier invasion and/or uptake of mucus-derived nutrients (De Repentigny et al, 2000; Windle et al, 2000). Mucins (i.e., glycosylated proteins located into the endomembrane system of intestinal epithelial cells or secreted into the lumen) are the main constituents of the mucous layer. Mucins are produced by specialized Goblet cells, as well as by intestinal epithelial cells all along the intestine tract (Lievin-Le and Servin, 2006). Some probiotics have been shown to increase mucin expression *in vitro*. Lactobacilli were found to induce mucin in human intestinal cell lines, thus blocking invasion and adherence of pathogenic *E. coli* strains (Mattar et al, 2002; Mack et al, 2003). In this way, probiotics may improve the barrier function of the intestinal mucosa and contribute to reduce gut permeability (Saulnier et al, 2009).

Cytokines. By optimizing the balance of pro- and anti-inflammatory cytokines and other immune modulators, probiotics are thought to contribute to realize a healthy gut homeostasis. Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and haematopoiesis. They are produced in response to an immune stimulus, and generally act over short distances and short time spans, at very low concentration. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules (Foster, 2001). Cytokines 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).

Interleukins are signaling, secreted cytokines that promote the development and differentiation of T, B, and hematopoietic cells. The function of the immune system largely depends on interleukins, and rare deficiencies of a number of them have been described (i.e., autoimmune diseases or immune deficiency). 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; interferon gamma (IFNγ) activates macrophages; IL-3, IL-7 and Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF) stimulate haematopoiesis (Dinarello, 2000).

Tumor necrosis factor (TNF) is a proinflammatory cytokine contributing to recruitment and activation of immune cells, release of cytolytic enzymes and reactive oxygen species (ROS), and exacerbation of tissue damage at inflammation sites. Increased levels of TNF are thought to contribute to the pathology of GI disorders such as Crohn's disease (Kirman et al, 2004).

Chemokines are a large family of small (8–14 kDa) secreted chemotactic cytokines involved in adhesion and directional homing of immune and inflammatory cells. These molecules have been divided into 4 subfamilies based on the arrangement of highly conserved cysteine residues in the amino-terminus: C, C-C, C-X-C and C-X_3-C. (Zimmerman et al, 2008). The C-C chemokines (including the macrophage inflammatory protein MIP-1α and MIP1-β) have 2 adjacent cysteines at the amino-terminal; the C-X-C chemokines (e.g., interleukin IL-8) have amino-terminal cysteines separated by an intervening amino acid (Horuk, 2007). Chemokines recruit leucocytes at the site of immune reaction. The macrophage inflammatory protein 3α (MIP-3α) is a lymphocyte directed C-C chemokine which is predominately 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 this chemokine might play an important role in the pathogenesis of human IBD (Kwon et al, 2002).

When cytokine signaling is unbalanced, serious diseases may occur in humans. The use of probiotics to restore interleukins unbalances of human and/or animals diseases, is largely

documented. Several studies report the probiotic-mediated suppression of human TNF production by host immune cells (Ménard et al, 2004; Lin et al, 2008). *In vitro* studies also demonstrated that probiotic Lactobacilli and Bifidobacteria attenuate IL-8 production by LPS, or IL-1 stimulated human intestinal epithelial cells (Candela et al, 2008). Such documented probiotic effects underlie the potential therapeutic use of selected microbes for the treatment of IBD. Administration of probiotic *L. delbruekii* and *L. fermentum* strains to ulcerative colitis (UC) patients, alleviated the inflammation by decreasing the colonic concentration of IL-6 and the expression of TNF-α and NF-kB p65. Therefore, probiotic supplementation could help in maintaining remission and preventing relapse of UC (Hegazy and El-Bedewy, 2010). Angiogenesis is required for wound healing, but its dysregulation is involved in GI inflammation. *Bacillus polyfermenticus* can promote the angiogenesis of human intestinal microvascular endothelial cells (HIMECs). In fact, the exposure of HIMECs to *B. polyfermenticus* increased the level of proangiogenic C-X-C chemokine IL-8, suggesting that the bacterium-mediated induction of IL-8 contributes to intestinal wound healing (Im et al, 2009).

1.5. Methodologies to study probiotics.

In order to investigate and ascertain the probiosis of microorganisms and in view of their potential therapeutic application, preliminary studies can rely on an array of *in vitro* assays. Indeed, *in vivo* approaches are often too complex and demanding, especially in the initial screenings for probiotics and for suitable food matrices. In this regards, many studies are available from the scientific literature.

1.5.1. Oro-gastrointestinal tract simulators.

Analysis of potential probiotics in *in vitro* multi-compartmental models that simulate the physico-chemical conditions of the human OGI tract is a prerequisite to subsequent *in vivo* experiments. As a result, development and implementation of such systems are highly encouraged by FAO and WHO (2002) and several recent studies have addressed this issue (Mainville et al, 2005; Masco et al, 2007; Fernández de Palencia et al, 2008; Lo Curto et al, 2011).

An ideal simulator should recreate the multi-segmentation of the human OGI tract, mimicking the events of food ingestion and digestion, and allowing for the addition of a food matrix through which the probiotic is delivered. The food matrix should primarily

shield the delivered microbe from the OGI hostile environment, sustaining its growth and activity. Prebiotic ingredients, that is 'non host-digestible constituents which selectively promote growth and activity of the beneficial microbial species' (Gibson and Roberfroid, 1995), may also be added to enhance the overall benefit of the designed functional food. Common food matrices (**Table 1.6)** are skim milk and soy gem powders (Mainville et al, 2005), sometimes enriched by fiber components with prebiotic action.

Food matrix	Commercialized products
Milk	Yogurt, fermented milks, Kefir
	Probiotic ice cream
Carbohydrate polymers	Encapsulated probiotics
Vegetables	Artichokes and olives
Fruit	Fruit juices
Starch	Probiotic bread (future perspective)

Table 1.6. **Food matrices frequently adopted to vehicle probiotics**.

The first steps of the OGI transit are associated to lysozyme and chewing stresses. Then, the models mimic the events of digestion into the stomach, from a situation of complete filling of the gastric pouch to progressive emptying. Immediately after food ingestion (full stomach condition), the matrix embedded bacteria is thought to be at pH values of 5.0 - 6.0; pH lowering (to values of 2.0 - 1.5) simulates the progressive emptying of the stomach and the digestion of food. The nature of the food affects the transit period through the stomach. Normally, food remains in the stomach between 2 and 4 hours; however, liquids empty from the stomach faster than solids, taking only about 20 minutes (Smith, 1995; Huang and Adams, 2004). The adverse conditions of the small intestine include the presence of bile salts and pancreatin. The transit time through the small intestine takes from 1 to 4 hours. In the lumen of the small intestine, pH is around 8.0. A concentration of 0.15 - 0.3% of bile salts has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Smith, 1995; Huang and Adams, 2004).

Several working groups have developed different OGI tract simulators, each presenting advantages and drawbacks. Fernández de Palencia et al (2008) and Masco et al (2007) used static models in which the transit is performed in a single container recreating the entire OGI tract. The idea would be that of a method that divides the OGI tract into all its various

organs (i.e., mouth, stomach, duodenum, ileum, jejunum, colon). Both researches tested the bacterial viability using combined fluorescent probes. The advantage of such detection method is its high reproducibility and rapidity compared with conventional plate counts. However, the colorimetric methods may give non-specific results following the challenge of bacteria cells resuspended in matrix food.

Lo Curto et al (2011) and Mainville et al (2005) used dynamic OGI-simulating systems, automated through a computerized core; parameters of the OGI transit (pH, temperature, stirring, peristalsis, input of matrix) could be monitored by the operator. However these systems did not consider the interaction between intestinal endogenous microflora and the exogenous tested bacterium.

Decroos et al (2006) and De Boever et al (2000) performed the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) consisting of a succession of 5 reactors (i.e., stomach, small intestine, ascending colon, transverse colon and descending colon). An inoculum prepared from human feces, and stabilized over days using a culture medium, was introduced into the SHIME. In the monitoring period, a human microbiota with Enterobacteriaceae, coliforms, *Lactobacillus subsp.*, *Staphylococcus subsp*. and *Clostridium subsp*., was established. Such a system would allow to study potential probiotic strains interacting with the resident flora of the human intestine.

1.5.2. Methodologies to study bacterial adherence to the intestinal mucosa.

The ability to adhere to the intestinal mucosa is one of the more important selection criterion for probiotics because adhesion to the epithelial layer allows colonization. Due to obvious difficulties in performing *in vivo* studies, preliminary studies of potentially adherent strains are mainly based on *in vitro* adhesion assays. The use of at least two different systems is recommended, therefore, adhesion assays are frequently performed on both mucus and epithelial cell monolayers, representing the early and late stage of adhesion, respectively. Tissue cultures of the human colon carcinoma cell lines Caco-2 and HT-29 are the most frequently used (Salminen et al, 1996). Caco-2 cells also provide a valuable system for immunological studies (Ou et al., 2009). Tissue cultures are used not only to evaluate the level of bacterial adhesion, but also to investigate competition for binding sites with other pathogenic species (Candela et al, 2005).

Caco-2 cells (**Figure 1.9**) 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. A brush border develops, length and density of microvilli increase, the surface occupied by each cell decreases and intercellular junctions tighten. After 30 days, the cells have a typical enterocyte-like morphology (Pinto et al, 1983; Vachon and Beaulieu, 1992).

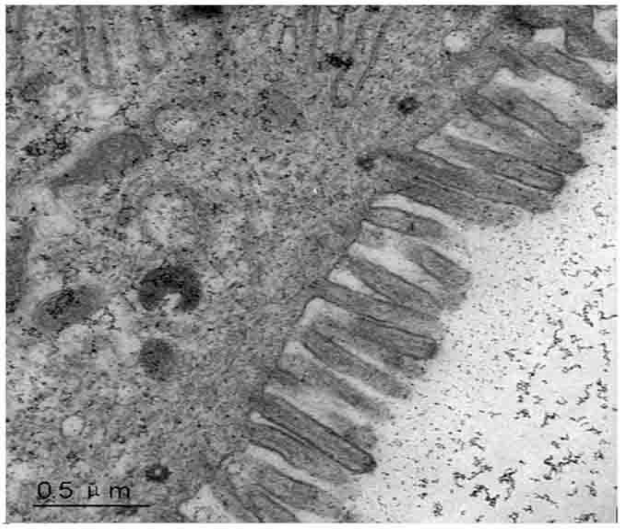


Figure 1.9. **TEM micrograph of Caco-2 cell monolayers: morphological details of the apical side** (*from Yang et al, 2007*).

1.6. Lactic Acid Bacteria.

Lactic acid bacteria (LAB) are Gram-positive, low-GC, acid-tolerant, generally asporigen, rod- or cocci-shaped, catalase-negative, microaerophilic bacteria. The common feature of LAB is the production of lactic acid as the major metabolic end-product of carbohydrate fermentation (Carr et al, 2002). LAB are the most numerous group of bacteria linked to humans. They are naturally associated with the mucosal surfaces, particularly the 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.

The main LAB genera include: *Lactobacillus*, *Leucocostoc*, *Pediococcus* and *Streptococcus* (Schroeter and Klaenhammer, 2009).

1.6.1. *Lactobacillus plantarum*: a model organism to study LAB and probiotics.

The genus *Lactobacillus* includes a considerable number of different species with high degree of diversity (Stiles and Holzapfel, 1997). Among these, *L. plantarum* is a flexible and versatile species, found in a variety of environmental niches, including the human body, especially saliva and GI tract (Ahrne et al, 1998).

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 complete genome sequence of *L. plantarum* WCFS1 has been published and deposited in an open access database (Kleerebezem et al, 2003). The genome has a GC content of 44.5% and 3,052 open reading frames (ORFs). The metabolic versatility of *L. plantarum* is supported by a large number of genes coding for sugars transport systems and for enzymes involved in carbon metabolism. Moreover, the abundance of genes encoding membrane-anchored proteins suggests the ability of *L. plantarum* to grow on various substrates. The environmental flexibility of *L. plantarum* is confirmed by the presence of diverse genes located in a specific chromosome region named 'lifestyle adaptation region' (Kleerebezem et al, 2003).

Thanks to the development of expression plasmids and vector systems for partial or total gene deletion, *L. plantarum* may be genetically modified, thus enabling to study its genetics, physiology and specific biochemical functions (Ferain et al, 1996; Hols et al, 1997). Moreover, its ability to persist in the human OGI tract has stimulated the research into the use of *L. plantarum* as a 'delivery' tool of therapeutic compounds (i.e., oral vaccines) (Pouwels et al, 1998). In an interesting study, *L. plantarum* was used as live oral vehicle of antigens to protect mice from tick-transmitted *Borrelia burgdorferi* infection, inducing both systemic and mucosal immunity (del Rio et al, 2008).

1.7. Stress response in bacteria.

Because bacteria have successfully colonized several niches, they are continuously exposed to a multitude of fluctuating stress factors, including sudden changes in temperature (heat and cold), variation of external pH value (acid and alkaline shock), modification of osmolarity (hypo- and hyperosmotic shock), oxidative stress by reactive oxygen species, starvation and presence of toxins (Storz and Hengge-Aronis, 2000).

Bacteria use different adaptation strategies to thrive in a wide range of niches which may generate stress. Stress factors typically induce cellular responses leading to changes in gene expression pattern. This stress response helps the bacterial cells to protect vital processes, to restore cellular homeostasis and to increase the resistance against subsequent stress challenges. The genetic programs allowing bacteria to manage stressful conditions consist of three different steps: (i) the stress factor is registered either directly or indirectly by a sensor (often through a damaged molecule), (ii) the sensor leads to the induction of a subset of 'stress genes' involved in the adaptation to the new situation, and (iii) in many cases, expression of the stress genes is turned off after adaptation, through feedback inhibition (classical stress response) (Schumann, 2009).

1.7.1. Heat shock proteins.

Historically, stress genes were discovered after exposing *Drosophila melanogaster* larvae to high temperature. The products of these genes were identified as 'heat shock proteins' (HSPs) (Ritossa, 1962; Tissier et al, 1974); then, it was shown that HSPs could be induced by a wide range of stress factors

HSPs include small HSPs (sHSPs), GroEL, DnaK, HtpG and Clp. Overall, they are designated as 'molecular chaperones' to describe the common property of assisting the assembly of other proteins. In fact, not all, but most HSPs function as molecular chaperones to guide critical conformational states in the folding, translocation and assembly of newly synthesized proteins. Although many newly synthesized proteins can fold in the absence of molecular chaperones, a minority requires them (Hightower, 1991; Hartl, 1996).

sHSPs constitute a family of low molecular weight (12 - 43 kDa) proteins that can form large multimeric structures and display a wide range of cellular functions, including the endowment of cells with thermo-tolerance *in vivo* and the ability to act as molecular chaperones *in vitro*. sHSPs co-aggregate with other proteins (**Figure 1.10**), for subsequent

efficient disaggregation. The release of substrate proteins from the transient sHSP reservoirs and their refolding require cooperation with ATP-dependent chaperone systems. sHSPs share a highly conserved alpha-crystallin domain that can behave as a chaperone-like protein by sequestering unfolded proteins, and inhibiting subsequent aggregation and insolubilisation thereby maintaining 'intracellular transparency' (Van Montfort et al, 2001).

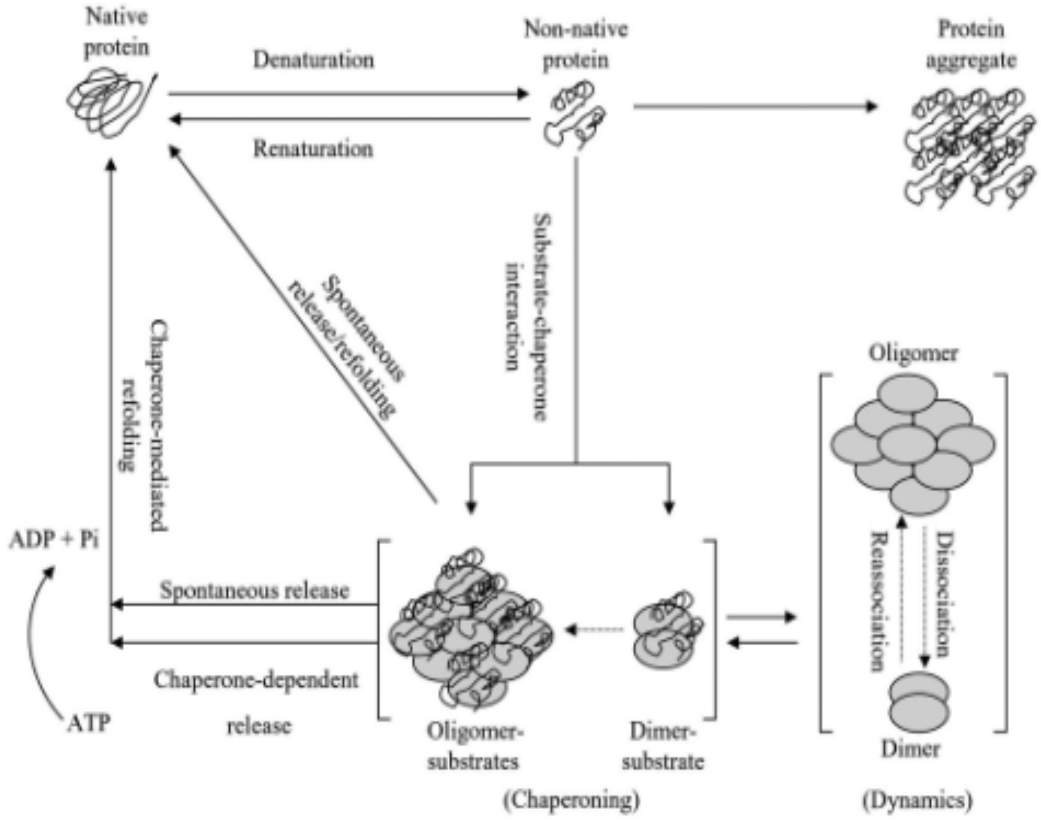


Figure 1.10. **Representation of the process of recognition and refolding of unfolded and non-native proteins by sHSPs**. Unfolded and/or non-native proteins may be bound by large sHSP oligomers or small sHSPs dimers, preventing irreversible aggregation. When intracellular conditions return favorable, unfolded and/or non-native proteins are released and refolded either spontaneously or with the assistance of ATP-dependent chaperones. sHSPs exist as oligomers in dynamic equilibrium with dimers. Proteins which are not recognized by sHSPs, form aggregates and precipitate. *(from Sun and MacRae, 2005*).

GroEL (HSP60 in Eukaryotes) is an ATP-dependent foldase. GroEL forms a complex together with its co-chaperone GroES. GroEL is a double-ring 14mer with a hydrophobic patch at its opening. GroES is a single-ring heptamer that binds to GroEL in the presence of ATP or ADP. GroEL/GroES may not be able to undo previous aggregation, but it does compete in the pathway of misfolding and aggregation (Ranson et al, 2006).

DnaK (HSP70 in Eukaryotes) is perhaps the best-characterized chaperone. DnaKs are assisted by DnaJ proteins (HSP40 in Eukaryotes), which increase their ATPase activity. Although a precise mechanism has not yet been determined, it is known that the DnaKs have a high-affinity for unfolded proteins when bound to ADP, and a low-affinity when bound to ATP (Mayer and Bukau, 2005).

HtpG (HSP90 in Eukaryotes) is expressed at low levels and is non-essential. Each HtpG has an ATP-binding domain, a middle domain and a dimerization domain. It was originally thought to clamp onto its substrate protein (also known as a 'client protein') upon binding ATP. The 3D structures published by Vaughan et al (2006) and Ali et al (2006) indicate that client proteins may bind externally to both the N-terminal and middle domains of HtpG.

Clp proteins (HSP100 in Eukaryotes) have been studied both *in vivo* and *in vitro* for their ability to link and unfold misfolded proteins. Clps form large hexameric structures with ATP-dependent unfoldase activity. These proteins are considered to function as chaperones by recessively threading client proteins through a small pore (20 Å diameter), thereby providing them with a second opportunity to fold (Wawrzynow et al., 1996).

1.7.2. Genetic regulation of bacterial heat shock proteins.

Most of the genes coding for heat shock proteins are expressed at ambient temperatures, at a basal level, and therefore are transiently induced after a challenge. These genes are organized into several regulons, constituting the so-called 'heat shock stimulon'. In *Bacillus subtilis*, the genetic model organism of the Gram-positive bacteria, the heat shock stimulon comprises 6 classes of heat shock genes. However, to date, members and regulation of only 3 classes (i.e., class I, II, and III) have been well elucidated (Schumann, 2003).

Class I heat shock genes consist of 2 operons, the bicistronic *groE* operon and the heptacistronic *dnaK* which are preceded by a σ^A-type promoter presenting inverted repeat sequences of 9 bp, separated by a 9-bp spacer (TTAGCACTC-N_9-GAGTGCTAA). The inverted repeats are called 'CIRCE' (Controlling Inverted Repeat of Chaperone Expression) and function as the binding site for the HrcA repressor (Schmidt et al, 1992; Homuth et al, 1997). The bicistronic *groE* operon codes for the molecular chaperones GroEL and GroES. The heptacistronic *dnaK* operon consists of the *hrcA* gene (coding for the transcriptional repressor of both operons), followed by *grpE*, *dnaK*, and *dnaJ* genes (encoding the

molecular chaperones GrpE, DnaK and DnaJ, respectively) and by 3 open reading frames *orf35* (yqeT), *orf28* (yqeU), and *orf50* (yqeV).

Genes of **Class II** are under the positive control of the alternative sigma factor σ^{β}. They are not only induced by the classical heat shock regimen, but also by salt, oxidation, desiccation, acid stress, as well as starvation for oxygen, glucose and phosphate. The genes make up an octacistronic *sigB* operon which is preceded by a σ^{A}-type promoter (P_A), ensuring the basal expression of all eight genes (*rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB*, *rsbX*). In addition, under stress condition, expression of the last 4 genes initiates at a σ^{β}-dependent promoter (P_B) (Wise and Price, 1995; Derré et al, 1999b).

Class III comprises the tetracistronic *clpC* operon (Krüger et al, 1996) and the 2 monocistronic *clpP* and *clpE* operons. Genes of the *ClpC* operon code for CtsR (class III stress repressor), McsA and McsB (A and B modulators of CtsR), involved in regulating the activity of CtsR, and the ATPase subunit ClpC. *clpE* codes for ATPase subunits, while *clpP* codes for a proteolytic subunit of the ClpCP and potential ClpEP ATP-dependent proteases. *clpC* and *clpP* operons are preceded by an upstream σ^{β}- and a downstream σ^{A}-dependent promoter and clpE operon by 2 σ^{A}-type promoters. All σ^{A}-dependent promoters are under the negative transcriptional control of the CtsR repressor. The operator sequence, which is recognized by CtsR, has been determined as a highly conserved heptanucleotide direct repeat located upstream of the transcriptional units: $A_{/G}GTCAAA_NA_NA_{/G}GTCAAA$ (Wise and Price, 1995; Derré et al, 1999b).

1.7.3. sHSPs in *Lactobacillus plantarum* WCFS1.

In contrast to Lactobacillus species such as *L. acidophilus* (Altermann et al, 2005), *L. delbrueckii subsp. bulgaricus* (van de Guchte et al, 2006) and *Oenococcus oeni* (Delmas et al, 2001), which have a single *shsp* gene copy, three genes coding for sHSPs have been identified in the genome of *L. plantarum* WCFS1 (Kleerebezem et al, 2003; Spano et al, 2004a; Spano et al., 2005). The three genes are annotated as *lp_0129*, *lp_2668* and *lp_3352*, or *hsp18.5*, *hsp18.55*, and *hsp19.3* respectively, in accordance with the predicted molecular weights of the putative proteins.

With regards to this small family of sHPSs, some interesting features may be pointed out: i) basal mRNAs levels during the exponential growth phase suggest a putative housekeeping role of these proteins; ii) significant transcriptional induction by different abiotic stresses

(heat, acidic pH, ethanol, low temperatures); iii) *hsp18.55* is co-transcribed with a preceding unknown function gene, *lp_2669*, both forming a small operon (Spano et al, 2004a; Spano et al, 2005).

Based on *in silico* and functional analyses of the promoter sequences, and according to the heat shock gene classification of *B. Subtilis*, the following regulations have been proposed for *L. plantarum* sHsps:

i. *hsp18.5* is likely to be under a dual negative regulation mediated by both CtsR, which was shown to bind the operator sequence in the promoter (Fiocco et al, 2009), and HrcA, whose CIRCE elements have been found in the upstream region. Therefore, this gene seems to belong to both class I and class III.
ii. *hsp18.55* promoter presents -10 and -35 *cis*-elements which might be recognized by the sigma factor σ^{β}; although this sigma factor is not present in *L. plantarum*, an intriguing regulation hypothesis has been suggested (Bove et al, 2011).
iii. *hsp19.3* promoter possesses inverted repeated sequences highly homologous to CIRCE elements; therefore, like most LAB *shsp*, *hsp19.3* is likely to be under HrcA control (Spano et al, 2005).

Taken together, the stress tolerance behavior of *L. plantarum* strain overproducing the single sHsp, transcriptional patterns, functional and *in silico* analyses of the promoters, suggest that the three *L. plantarum* sHsps might accomplish different tasks in relation to stress response mechanisms.

1.7.4. The FtsH protein of *Lactobacillus plantarum* WCFS1.

FtsH proteins are membrane-bound ATP- and Zn^{2+}-dependent metalloproteases with intrinsic chaperone activity (**Figure 1.11**). Because of their dual chaperone-protease function, FtsH proteins play a key role in the protein quality control network, which not only allows refolding or degradation of denatured and misfolded proteins, but also enables the temporal control of many cellular processes by regulating the stability of specific regulators (Langer, 2000; Ito and Akiyama, 2005).

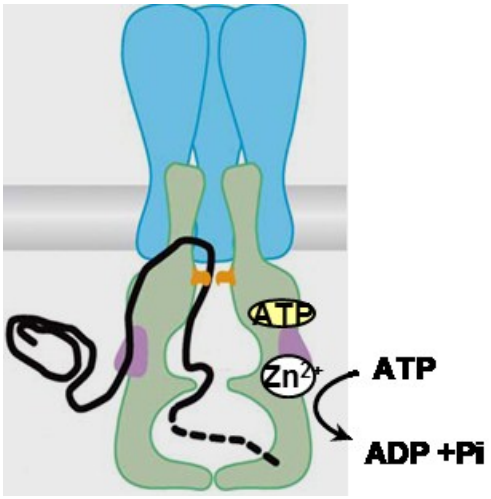


Figure 1.11. **Spatial organization of FtsH protein across the membrane.** A substrate protein (in black) moves along the active site of the FtsH protease and is cleaved parallel to hydrolysis of ATP molecule in ADP and inorganic phosphate (a*dapted from Ito and Akiyama, 2005*).

Studies on *ftsH* and *ctsR* mutant strains of *L. plantarum* WCFS1 have been performed to identify the transcriptional control mechanism on the *ftsH* gene. Sequence analysis and mapping of the *ftsH* transcriptional start site have revealed a potential CtsR operator which was specifically bound by a recombinant CtsR, as determined by electrophoretic mobility shift assay. Moreover, *ftsH* mRNA level was up-regulated in the *ctsR* mutant. All these evidences indicate that *L. plantarum* is a member of the CtsR regulon (Fiocco et al, 2009). Studies on *ftsH* expression in *L. plantarum* WCFS1 revealed mRNA induction by different stress conditions, including heat, bile, hyperosmotic and oxidative stress, as well addition of a membrane-fluidizing agent; conversely, a repression was caused by a carbon-source depletion during the exponential growth (Fiocco et al, 2009; Bove et al, 2011).

1.8. Phenotypical features of *L. plantarum* WCFS1 mutant strains.

One of the main objectives of this thesis was to test the probiotic potential of some *L. plantarum* mutant strains, included in the microbial collection of our laboratory. These mutants have been obtained by deletion of stress-related genes, including *ctsR* and *hsp18.55*, whose function has been described above. The foremost results concerning the phenotypical analyses of these mutants are briefly described below.

Δ*ctsR* mutant. With respect to the wild type, the Δ*ctsR* strain displayed similar growth rates when cultivated under optimal temperature conditions. However, a growth impairment became evident in mutant cultures under suboptimal higher temperature, suggesting the involvement of CtsR in coping with a prolonged heat stress regimen. Investigations on the cell morphology of *L. plantarum* wild type and mutant strain revealed no significant difference under optimal growth temperature conditions; however, after heat shock, mutant cell envelopes appeared stiffer and presented sorts of surface breaks. These intriguing features suggested that the cell wall might be critically damaged in mutant strain, and thus pointed to novel CtsR function for cell wall integrity control in *L. plantarum* (Fiocco et al, 2010).

Δ*hsp18.55* mutant. By comparing the growth rate of wild type *L. plantarum* WCFS1 and its relative Δ*hsp18.55* mutant strain, no relevant difference was observed under either optimal temperature or stress conditions such as heat, low pH and high osmolarity. However, a longer lag phase was observed when the mutant strain was cultivated after exposure to a short intense heat stress. This suggests that the *hsp18.55* gene of *L. plantarum* may be involved in recovery of stressed cells in the early stage of high temperature stress. In addition, morphology of the mutant cells, investigated by scanning electron microscopy, revealed that cells clumped together and had rough surfaces, and that some cells had a shrunken empty appearance, which contrasted with the characteristic rod-shaped, smooth-surface morphology of control wild type *L. plantarum* cells. Indeed, inactivation of the *hsp18.55* gene affected membrane fluidity and physicochemical surface properties of *L. plantarum* as determined by MATS assay and fluorescence anisotropy measurements (Capozzi et al, 2011).

2. AIMS OF THE RESEARCH

The objective of this thesis was to study the interaction between host and probiotic microorganisms at the cellular and molecular level. Our attention was focused on the bacterial response to the stress encountered along the human OGI tract and, on the other hand, on the immunomodulatory effects of the bacteria on the human host. *L. plantarum* was chosen as a model organism for its recognized probiotic use, high versatility and feasible laboratory culture conditions.

Given the relevance of bacterial cell envelope structure in host-microbe interaction and taking into account the distinctive morphological traits observed in some stress gene mutants of *L. plantarum,* we sought to:

- generate other *L. plantarum* knock out strains for stress-related genes and analyse their phenotype especially in relation to cell surface properties;
- study the probiotic potential of the *L. plantarum* mutant strains obtained during this work and from the laboratory collection.

To this aim, an *in vitro* system reproducing the physiological events of ingestion and digestion of the human OGI tract was designed and improved. In such a model, the survival potential of *L. plantarum* wild type and related mutants was analysed and compared with that of commercial probiotic strains. The effect of different vehicle matrices on the bacterial survival to the OGI stress was analysed. Moreover, to shed light on the bacterial response to the OGI stress, the transcriptional pattern of bacterial stress and probiotic marker genes was assessed during the simulated transit. Adhesion and immunomodulatory properties of the *L. plantarum* strains were evaluated on human cells.

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Microorganisms.

The bacterial strains used in experiments were:

***L. 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);

L. plantarum* WCFS1 Δ*ftsH, mutant strain for the *ftsH* gene encoding FtsH chaperone-protease;

***L. plantarum* WCFS1 *ftsH*+**, overproducer strain of FtsH chaperone-protease;

***L. plantarum* WCFS1 pGIZ** control strain harbouring the empty pGIZ906 vector;

L. plantarum* WCFS1 Δ*ctsR, mutant strain for *ctsR* gene coding for CtsR transcriptional regulator;

L. plantarum* WCFS1 Δ*hsp18.55, mutant strain for *hsp18.55* gene coding for the small heat shock protein Hsp18.55;

***L. acidophilus* LA-5** and ***B. animalis subsp. lactis* BB-12**, marketed probiotics (Chr. Hansen, Hörsholm, Denmark);

***Escherichia coli* DH10B** used as intermediate host in molecular cloning.

Bacterial cells were stored at -80°C in 15% glycerol solution.

3.1.2. Animal cells.

Caco-2 cell line: epithelial cells originated from human colonic adenocarcinoma, employed for adhesion and immune-stimulation experiments;

THP-1 cell line: human acute monocytic leukemia-derived cells, differentiated to macrophage-like cells for studying TNF production.

Animal cells stocks were stored under liquid nitrogen, in FBS + DMSO 10% solutions.

3.1.3. Culture media.

MRS (de Man Rogosa Sharpe) broth, is a non-selective medium for growth of LAB, available as lyophilized powder (Oxoid). It was prepared by resuspending 62 g in 1 litre of distilled H_2O. MRS broth was also used as food matrix in experiments of OGI tract transit.

LB (Luria-Bertani) broth is the most widely used medium for the growth of bacteria (e.g., *Escherichia coli*): 10 g/L Trytone; 5 g/L Yeast extract; 10 g/L NaCl.

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

MEM Alpha Medium (Minimal essential medium with alpha modification) and **DMEM Medium** (Dulbecco's Modified Eagle Medium) (GIBCO) were used for Caco-2 cells culture and supplemented with:

- 10% (v/v) Heat-inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich)
- 2mM L-Glutamine (Sigma-Aldrich)
- 50 U/ml Penicillin (GIBCO)
- 50μg/ml Streptomycin (GIBCO)

RPMI medium 1640 (Invitrogen) was used for THP-1 cells culture and supplemented with:

- 10% (v/v) Heat-inactivated FBS (Sigma-Aldrich)
- 100 U/mL Penicillin (GIBCO)
- 100 μg/mL Streptomycin (GIBCO)

3.1.4. Food matrices.

Skim Milk was used in lyophilized form, reconstituted to a concentration of 10% (w/v) in distilled H_2O and autoclaved at 110 °C for 15 minutes.

Control pasta and **barley beta-glucans-enriched pasta** (Granoro – 3% Beta-glucans (w/w)) were used as homogenized formula. Both control and beta-glucans-enriched pasta were boiled in tap water, homogenated by mixer, and resuspended in saline solution to a concentration of about 30 mg/mL, with minimal variations depending on the sizes of pasta. Aliquots of 30 mL were stored at 4 °C. Both types of pasta were diluted in a volume of saline solution, to reach the contraction of 0.1% beta-glucans (w/w) for enriched-pasta. All operations were performed under sterile conditions.

3.1.5. Antibiotics.

Erythromycin and **Chloramphenicol** stock solutions were prepared by dissolving the powder (Sigma-Aldrich) in ethanol at 10 mg/mL or 100 mg/mL. Aliquots were sterilized by filtration and stored at -20 °C.

Ampicillin stock solution was prepared by dissolving the lyophilized antibiotic salt (Sigma-Aldrich) in distilled H_2O at concentration of 1 mg/mL, sterilized by filtration and stored at -20°C.

Stock solutions of **Penicillin-Streptomycin,** concentration: 5,000 U - 5,000 µg/mL (GIBCO).

3.1.6. Buffers and Solutions.

10X Gastric electrolyte solution (GES) was prepared by dissolving the following components in distilled H_2O and autoclaved at 121°C for 15 minutes: 62 g/L NaCl; 22 g/L KCl; 12 g/L $NaHCO_3$; 2.2 g/L $CaCl_2$.

10X Intestinal electrolyte solution (IES) was obtained and autoclaved at 121°C for 15 minutes, by mixing in distilled H_2O: 50 g/L NaCl; 6 g/L KCl; 3 g/L $CaCl_2$. **1X GES** and **1X IES** were used in OGI transit assays.

Saline solution contained NaCl at a concentration of 0.85% (w/v) and autoclaved at 121°C for 20 minutes. It was also used as food matrix.

TE: 20mM Tris-HCl and 1 mM EDTA. Autoclaved at 121°C for 15 minutes and stored at room temperature.

50X TAE Stock Solution: 242.0 g/L Tris Base; 57.1 mL Glacial Acetic Acid; 100 mL of 0.5 mM EDTA, for each litre of solution. Autoclaved at 121°C for 20 minutes. 1X TAE was obtained by dilution of 50X TAE.

10X Phosphate-buffered saline (PBS) was prepared by dissolving the following components in distilled H_2O and adjusting pH to 7.4 with HCl: 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; KH_2PO_4 1mM. Sterilized by filtration. 1X PBS was obtained by dilution of 10X PBS.

Ethidium bromide stock solution (10mg/mL) was stored at 4 °C, protected from light.

Agarose gel loading buffer (6X): 0.25% Bromophenol blue; 0.25% Xylencianol; 30% Glycerol. Stored at room temperature.

Agarose gel for RNA and DNA electrophoresis.

0.8% agarose gel was used routinely for genomic and plasmid DNA electrophoresis; 1% agarose gel was used for RNA electrophoresis, while 1.5 - 2.0% agarose was used for analysis of PCR products. The gel was prepared as follows: 0.8 - 1 - 2 g of Agarose in 100 mL of 1X TAE. 1X TAE was used as running buffer.

3.1.7. Drugs and Supplements.

Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) was dissolved in DMSO. Aliquots stored at -20 °C, protected from light.

***Escherichia coli* O127:B8 LPS** (Sigma-Aldrich) was prepared to a concentration of 100 ng/mL, sterilized by filtration and stored at 4°C.

Porcine Bile Extract (Sigma-Aldrich) was dissolved in 1X IES at the concentration of 60 g/L and pH was adjust to 6.5 by 1 M $NaHCO_3$. Aliquots were used at working concentration of 3 g/L and stored at 4°C.

L-Cysteine hydrochloride (Sigma-Aldrich) was dissolved in deionized H_2O, sterilized by filtration and stored at 4°C, protected from light.

L-Glutamine solution (Sigma-Aldrich). Aliquots of 2 mM L-Glutamine were prepared and stored at -20°C.

Fetal Bovine Serum (FBS) (GIBCO) was heated at 56°C in a water bath for 30 minutes to destroy heat-labile complement proteinsand stored in aliquots at -20°C.

Beta-glucans, extracted from *Pediococcus parvulus* 2.6 (Garai-Ibabe et al, 2010) were used at a concentration of 0.5 % (w/v) in DMEM medium, for adhesion assays.

3.1.8. OGI enzymes.

All OGI enzymes (animal origin) were stored at -20°C.

Both **Lysozyme** and **Pepsin** (Sigma-Aldrich) stock solutions were prepared by dissolving the powder in 1X GES at the concentrations of 4.5 g/L and 120 g/L, respectively. They were used at the final concentrations of 150 mg/L and 3g/L, respectively.

Pancreatin (Sigma-Aldrich) stock solution was prepared by dissolving the powder at the concentration of 20 g/L in 1X IES and adjusting pH to 6.5 by 1 M $NaHCO_3$. Aliquots were used at working concentration of 1 g/L and stored at 4°C.

0.05% Trypsin-EDTA (1X) (GIBCO) was prepared by dilution of 0.5% Trypsin-EDTA stock solution in PBS 1X.

3.1.9. Enzymes and kits for nucleic acids manipulation and analysis.

Extraction kits, Taq polymerases, reverse transcriptase, restriction enzymes, alkaline phosphatase and T4 DNA ligase were purchased from Cabru, Qiagen, Invitrogen, Fermentas and Promega and were used as recommended by the suppliers.

3.1.10. Oligonucleotides.

Sequences of the primers used are reported in **Tables 3.1**, **3.2** and **3.3**.

Oligonucleotide	Sequence 5' → 3'
FB_1HSP_1	AAGAGCTCTGAATCGGAGAATGAGTCGG
RB_1HSP_1	AAGAGCTCAGCCATACTAACAATCCCCT
FB_2HSP_1	AAATTTAAATCCGAGCGCGAATGACGGTCA
RB_2HSP_1	AAATTTAAATGGCCCGCAGTTAACTCCGAC
EcoRI_FB1HSP1	AAGAATTCTGAATCGGAGAATGAGTCG
XbaI_ RB2HSP1	AATCTAGACCCGCAGTTAACTCCGAC
$FB_1HSP_{3\_}$	AACTCGAGGTTGTACTTCGCTGTCCAAG
$RB_1HSP_{3\_}$	AAATTTAAATGTCCCAATTCATCATATCGT
FB_2HSP_3	AAATTTAAATGCAGCTGCGGATACCCATCA
RB_2HSP_3	AAATTTAAATCTTCACGTCCACTGTTTCCG
EcoRI_FB1HSP3	AAGAATTCTTGTACTTCGCTGTCCAAG
XbaI_RB2HSP3	AATCTAGATTCACGTCCACTGTTTCC
ftsHKOF	ATGGTACCGGACTTATTCGAACAAGCTAAG
ftsHKOR	TAGGATCCGTAAGCTGCTTGTTGGTTG
ftsHF	AAAACTGCAGAATCGACGCAATGGAC
ftsHR	GCTCTAGACGCTCATAACCGAATTAACG
CatFor	TCAAATACAGCTTTTAGAACTGG
CatRev	CCAGTAAATGAAGTCCATGGA
pUC_ery_F	CCAGGCTTTACACTTTATGC
pUC_ery_R	TGGAAAGTTACACGTTACTAAAG

Table 3.1. Oligonucleotides used for molecular cloning and gene knockout.

Oligonucleotide	Sequence 5' → 3'
Clp B For	AGTTACCGGGCGTCCATACTG
Clp B Rev	GACTCAAAGCCGTCCTCAAG
Clp E For	TTTACCAACCCCAGCTTCAC
Clp E Rev	GGCAAAATCGATCCAGTGAT
Clp F For	TAGTTGCTAAGCCGGGCAGT
Clp F Rev	ATGTTATCCGGTCCCATTGA
Hsp1_rt_F	AGGTTGATGTCCCTGGTATTG
Hsp1_rt_R	TAAAGACACCGTCAGCTTGG
rthsp18.55F	CGGTGAAGTATGACGAA
rthsp18.55R	TTACCTTCGCTATCCCGCAAC
Hsp_3_rt_F	CGCGAGTGAACGTCAAACTG
Hsp_3_rt_R	ATCCGCAGCTGCCTTCTTT
GroEL For	ACCGGATTGAAGATGCTTTG
GroEL Rev	AACCAGCATTTTCAGCGATT
DnaK For	TCAACCGTGTCACCCAAGTA
DnaK Rev	TCCTTCAGTTGTGGCATTCA
CtsRrtF	AATTTGGTCGATGATGCTGATG
CtsRrtR	TAAGTCCCGGTCCGTTAATCC
ftsHrtF	GCAGCTACCTTCGAAGAATCCA
ftsHrtR	GGGAAACTTGGTTCAGCAACA
MUB for	TGACACGCCAGATAAAGTCG
MUB Rev	ATTGGTTTGCGGTAAAGTCG
MCRA For	AATGCTAGCGAATGGGTCAG
MCRA Rer	TGCACCACGATCGACATATT
MSA for	GACAGCTAACGACACCAGCA
MSA rev	CGCTTAGCCATACCAAGGAG
EnoA1 For	ATGGGCGTTGCTAACTCAAT

EnoA1 Rev	CGGCTCATTGAACCAGTCTT
plnEF for	GTTTTAATCGGGGCGGTTAT
plnEFrev	GGAAAACGCCACCTGAAATA
ldhDF	ACGCCCAAGCTGATGTTATATC
ldhDR	AGTGTCCCACGAGCAAAGTT

Table 3.2. Oligonucleotide sequence of primers used to analyse bacterial gene expression by qRT-PCR.

Oligonucleotide	Sequence 5' → 3'
HBD-2 For	ATCAGCCATGAGGGTCTTGT
HBD-2 Rev	GAGACCACAGGTGCCAATTT
LYZ For	AAAACCCCAGGAGCAGTTAAT
LYZ Rev	CAACCTTGAACATACTGACGGA
MUC-2 For	CCAAGACCGTCCTCATGAAT
MUC-2 Rev	TCGATGTGGGTGTAGGTGTG
IL-6 For	TACCCCCAGGAGAAGATTCC
IL-6 Rev	TTTTCTGCCAGTGCCTCTTT
IL-8 For	TGTGGAGAAGTTTTTGAAGAGGG
IL-8 Rev	CCAGGAATCTTGTATTGCATCTGG
MIP-3α For	CTGGCTGCTTTGATGTCAGTG
MIP-3α Rev	GGATTTGCGCACACAGACAA
GAPDH For	CGACCACTTTGTCAAGCTCA
GAPDH Rev	AGGGGTCTACATGGCAACTG

Table 3.3. Sequences of the primers used for qRT-PCR analysis of human immune related genes.

3.2. METHODS

3.2.1. Bacteria.

3.2.1.1. Bacterial culture conditions.

Lactobacilli were propagated on De Man Rogosa Sharpe broth (pH 6.2). The medium was supplemented with 0.05% L-cysteine for *B. lactis* BB-12, 0.1% Tween and 0.05% L-cysteine for *L. acidophilus* LA-5. All incubations were performed at 28°C, except for *L. acidophilus* LA-5 and *B. lactis* BB-12 which were grown at 37 °C.

To produce the bacterial cell-free medium, *L. plantarum* WCFS1 bacterial culture was grown until the early-stationary phase, centrifuged at 8,000 rpm for 10 minutes and the supernatant was filter-sterilized (0.22 μm). The final pH of supernatant was adjusted to 7.2 - 7.4.

For preparation of cell suspensions challenged with the OGI tract, all strains were grown until they reached the mid-exponential phase (corresponding to a bacterial concentration between 10^9 and 10^{10} colony forming units (CFUs)/mL for *L. plantarum*, and between 10^8 and 10^9 CFUs/mL for *L. acidophilus* and *B. lactis*). Bacterial cells were sedimented by centrifugation (4,000 rpm, 10 minutes) and resuspended in various matrices (i.e., control pasta, beta-glucans-enriched pasta, milk, MRS, saline solution).

For adhesion and immune-stimulation experiments, *L. plantarum* WCFS1 and related mutant strains were sedimented as above and resuspended in the appropriate volume of Dulbecco's modified Eagle medium.

3.2.1.2. Analysis of bacterial stress tolerance by minispot plating and CFUs count.

Heat, cold, salt, bile, oxidative and acidic stress on *L. plantarum* wild type, Δ*ftsH*, pGIZ and *ftsH*+ strains were tested by spotting 10 μL serial dilutions (from 10^{-2}- to 10^{-5}-fold) of exponentially growing cultures on MRS agar plates.

Thermotolerance was investigated by incubating plates at 30°C, 40°C (heat stress) and 15°C (cold stress). Tolerance to high osmolarity, oxidative and acidic stress was monitored by spotting the dilutions on 2% NaCl-enriched, 10 mM diamide-containing, lactate or HCl-acidified (pH 4.0 and pH 5.0) and bile porcine extract-supplemented (0.1% w/v) MRS plates, respectively. Exponential cultures of Δ*ftsH* and wild type strains were serially diluted and plated on MRS + 4.7% NaCl at 30°C or on MRS plates incubated at 40°C. To test for sensitivity to short intense heat shocks, cultures were plated after 15 minutes temperature upshift to 50°C and then incubated at 30°C.

Viability was determined by the number of colony-forming units (CFUs). CFUs were counted and percent survival was calculated by normalizing the data to non-stressed control for each strain.

3.2.1.3. Biofilm assay.

The ability to form stable biofilms was studied, using the following method:

- Prepare an overnight pre-culture in MRS broth at 30 °C, for all strains.
- Dilute the pre-culture in MRS both to an OD_{600nm} of 0.1 and transfer 1 mL of each strain into microplate (Costar24-well Multiple-well): four repetitions for each strain and MRS as negative control.
- Incubate the microplate at 30°C for 24 hours until to 48 hours.
- Remove the content from each well.
- Wash each well twice with saline solution.
- Add 0.5 mL of 0.05% crystal violet in each well and incubate for 45 minutes.
- Wash each well gently three times with saline solution.
- Add 1 mL of 96% ethanol into each well and measure the OD at 595 nm.

3.2.1.4. Microbial adhesion to solvents method (MATS).

The physico-chemical characterization of cells surface properties was estimated following the MATS method (Pelletier et al, 1997):

- Centrifuge bacterial cells in stationary phase at 5,000 rpm for 10 minutes.
- Wash twice in saline solution.
- Resuspend in saline solution to an $OD_{400\ nm}$= 0.8 (A_0).
- Add 0.4 mL of solvent to 2.4 mL of cell suspension.
- Vortex for 40 seconds.
- After 15 minutes, recover the aqueous phase and measure its $OD_{400\ nm}$ (A_1).

The percentage of bacterial adhesion to solvent was calculated as $(1 - A_1/A_0) \times 100$. Adherence was tested on three different solvents: hexadecane, an apolar solvent; chloroform, a monopolar, acidic solvent; and ethyl acetate, a monopolar, basic solvent.

3.2.1.5. Scanning electron microscopy (SEM) analysis.

L. plantarum wild type and Δ*ftsH* strains were grown in MRS broth at 30 °C in flasks containing AISI 304 stainless steel coupons (25 x 25 x 1 mm) (Goodfellow, SARL, Lille, France). Heat shock treatment was performed by transferring mid-exponential phase cultures to a water bath at 42 °C for 30 minutes. SEM experiments were performed as reported before (Chavant et al, 2002) with slight modifications. Steel coupons were washed twice using tryptone water (0.85% NaCl, 1% tryptone); adherent bacteria were fixed for 1 hour at 4°C with a 3% glutaraldehyde solution in phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4) containing 11 g/L NaCl; coupons were washed three times with the same buffer. A series of dehydration steps was performed using graded ethanol bathes (70, 90 and 100% three times, for 10 minutes each). Final dehydration was performed in increasing concentrations of acetone (30, 50, 70 and 100%, for 10 minutes each). After gold-coating, bacterial cells were observed using a Philips XL30 ESEM scanning electron microscope. For each experiment, two replicates resulting from two independent inocula were analyzed.

3.2.1.6. Oro-gastrointestinal transit assay.

The procedure used to mimic the OGI transit is a modified version of a previous system (Fernández de Palencia et al, 2008) and is schematically represented in **Figure 3.1**. The oro-gastrointestinal solutions were prepared fresh daily according to the protocols described by Marteau et al. (1997) and Huang and Adams (2004), and information reported on human physiology literature (Sanseverino, 1996; Rindi and Manni, 1998). All incubation steps were performed at 37 °C and under shaking to replicate chewing and peristaltic contractions. To simulate the *in vivo* dilution of saliva, 3 mL of 1X GES (Marteau et al, 1997), containing 150 mg/L lysozyme (final concentration), was added to 27 mL of food-matrix bacterial suspension (pH 6.5). After incubation for 5 minutes, 1 mL aliquot was withdrawn (sample G1). To simulate the gastric conditions, 3g/L pepsin was added to the bacterial suspension, whose pH value was previously adjusted to 6.0 by HCl addition. After 10 minutes of incubation, 1 mL aliquot was taken (sample G2). Then, the pH curve in the stomach was reproduced by adding 1 M HCl to the cell suspension: so that the initial pH value of 6.0 was progressively reduced to 5.0, 4.0, 3.0, 2.0, and 1.5, and suspension was sequentially incubated for 10, 10, 30, 30 and 10 min at each pH value, respectively. 1 mL samples (G3–G7) were withdrawn for analysis. To mimic the natural gastric emptying (Marteau et al, 1997), 5 mL aliquots were collected from the cell suspension after incubation at the different

pH values. To simulate the intestinal biochemical environment, the pH value of samples G2-G7 was adjusted to 6.5 with 1 M $NaHCO_3$ and 3g/L bile salts and 1g/L pancreatin at pH 8.0, were added in order to reproduce the *in vivo* release of pancreatic juice into the duodenum. After 1 hour incubation, samples I1G2-I1G7 were withdrawn. Subsequently, all residue samples were diluted (1:1) with 1X IES (final concentration) to mimic the dilution and adsorption phenomena occurring in the last tract of the small intestine (jejunum and ileum). After 1 hour further incubation, samples I2G2-I2G7 were recovered for analysis. All samples were subject to immediate analysis. Appropriate dilutions from control and treated suspensions were plated on MRS agar plates and incubated to allow growth. CFU were counted and percent survival was determined with respect to unstressed control.

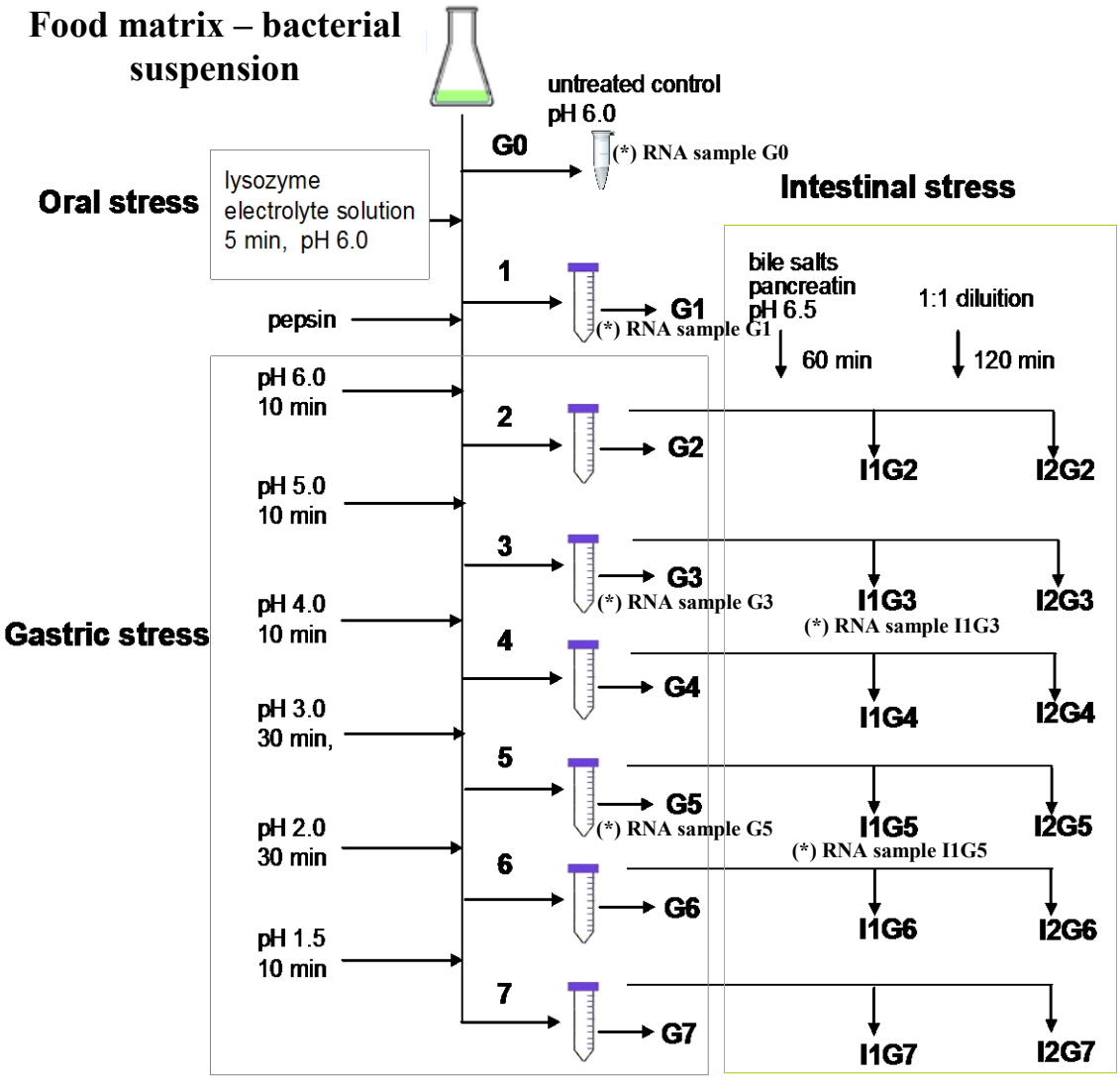


Figure 3.1. **Scheme of the *in vitro* system simulating the human oro-gastrointestinal tract**. Bacterial pellets were resuspended in food-matrix solution and subjected to the sequential conditions indicated in the picture. Oral stress compartment was mimicked by addition of a lysozyme-containing electrolyte solution (step 1, sample G1). Gastric stress was simulated by addition of pepsin and progressive pH reduction (steps 2-7, samples G2-7). Samples of gastric-stressed bacteria (from steps 2-7) were adjusted to pH 6.5 and supplemented with bile salts and pancreatin to simulate gastrointestinal stress (samples I1G2-7 and I2G2-7). Incubations were performed for the time indicated in the scheme, at 37°C and under shaking. Original unstressed bacterial suspension (sample G0) served as internal control.
Samples used for RNA extraction and qRT-PCR analysis are also indicated. RNA sample G0 was extracted from unstressed bacterial cells, resuspended in food-matrix. RNA sample G1 was extracted from bacterial cells exposed to lysozyme; RNA samples G3 and G5 from cells exposed to gastric stresses to pH 5.0 and pH 3.0, respectively, and RNA samples I1G3 and I1G5 from corresponding gastro-intestinal stresses.

3.2.2. Molecular cloning procedure.

3.2.2.1. *L. plantarum* WCFS1 genomic DNA extraction.

Genomic DNA was purified using UltraClean Microbial DNA Isolation Kit (Cabru) following manufacturer instructions. DNA concentration was determined by spectrophotometric (JENWAY 6715) absorption measurements at 260 nm and 280 nm. The integrity and purity was controlled by electrophoresis visualization on 0.8% agarose gel.

3.2.2.2. Polymerase chain reaction (PCR).

Taq DNA polymerase Qiagen was used in routine and colony PCR experiments, by preparing PCR mixtures with the followings components per reaction: 1.5 mM $MgCl_2$, 200 µM dNTP mix, 250 – 300 nM of each primer, 2.5 U of Taq DNA polymerase, 500 ng of template DNA and H_2O-DNase free to a final volume of 50 – 100 µL. PCR experiments were cycled as it follows:

Initial denaturation	3 minutes	94°C
Numbers of cycles: 25-30		
Denaturation	0.5–1 minutes	94°C
Annealing	0.5–1 minutes	50–68°C
Extension	1 minutes	72°C
Final extension	10 minutes	72°C

A proof reading polymerase (Pfu from Promega) was used for high fidelity amplification of genomic DNA fragment to be cloned. 50 ng of genomic from *L. plantarum* WCFS1 (as quantified by spectrophotometer, JENWAY 6715) were amplified with the Pfu DNA Polymerase in a final volume of 50 µL, with 200 µM dNTP mix, 300 nM upstream and downstream primer. The amplification mix was thermal cycled (BIORAD iCycler) using the following profile:

Initial denaturation	2 minutes	95°C
Numbers of cycles: 30		
Denaturation	1 minutes	95°C
Annealing	30 seconds	55°C
Extension	2 minutes	72°C
Final extension	5 minutes	72°C

3.2.2.3. Purification of PCR products.

PCR products were purified before and after the treatment with restriction enzymes. Moreover, plasmid DNA was subjected to purification subsequently the digestion with restriction enzymes and/or alkaline phosphatase. The QIAquick PCR Purification Kit (Qiagen) was used following manufacturer instructions. When required**,** cloning vectors and/or PCR amplicons were extracted from agarose gel using the QIAquick Gel Extraction Kit (Qiagen), as recommended by manufacturer.

3.2.2.4. Restriction enzymes.

Enzymatic digestions were performed on both plasmids pNZ5319 (Lambert et al, 2007) and pUC18Ery to linearize and create compatible ends (blunt- or sticky-ends) with PCR products, which were also subjected to restriction enzymes.

The restriction enzymes used, were: ***Kpn*I**, ***Bam*HI**, ***Eco*RI** and ***Xba*I**, all from Invitrogen; ***Ecl136*II**, ***Swa*I**, and ***Xho*I** all from Fermentas.

Protocol:

- 30-50 µL of PCR product/plasmid;
- 10 µL of reaction buffer (10X) with specific ionic strength and pH;
- 1 µL of restriction enzyme (10 U/µL);
- Sterile Milli Q H_2O to a final volume of 100 µL.

The mixture was incubated at 37°C (or other optimal temperature for the enzyme) for 4 - 5 hours.

3.2.2.5. Dephosphorylation.

Alkaline phosphatase (Promega) catalyses the hydrolysis of 5´-phosphate groups from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. This enzyme was used to prevent recircularization and religation of linearized plasmid (i.e., pNZ5319 and pUC18Ery) by removing phosphate groups from both 5´-termini.

The protocol required:

- 30 µL (about 10 µg) of linearized plasmid;
- 5 µL of 10X reaction buffer;
- 4 µL of SAP enzyme (Shrimp Alkaline Phosphatase) (1 U/µl);
- Sterile Milli Q H_2O to a final volume of 50 µL.

Incubate for 15 minutes at 37°C. Inactivate the phosphatase at 65°C for 15 minutes.

3.2.2.6. Ligation.

Ligase catalyzes the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. T4 DNA ligase (Promega) was used. Ligation was perfomed in final volume of 10 µL, mixing the reagents:

- Vector DNA/Insert DNA using a 1:1, 1:3 or 3:1 molar ratio;
- 1µL of 10X buffer;
- 1 µL of T4 DNA ligase (3U/µL);
- sterile Milli Q H_2O.

Incubate at:

- room temperature for 3 hours or 4°C overnight, for sticky-ends ligation;
- 15°C for 4–18 hours, for blunt-ends ligation.

3.2.2.7. *E. coli* DH10B Ca^{2+}-competent cells and transformation procedure.

E. coli DH10B cells were used as host bacteria in molecular cloning experiments. It was necessary to make *E. coli* cells Ca^{2+}competent, suitable to transformation. The method is described below:

- Select a colony and inoculate 5 mL of LB (no antibiotics) at 37°C with shaking for 12 hours.
- Inoculate with 1 mL of the starter culture, 100 mL of 2xL medium (20g/L Tryptone, 10g/L Yeast Extract, 1g/L NaCl, adjust pH to 7.5).
- Incubate at 30°C with agitation (180 rpm) until an optical density (OD_{600nm}) of 0.45 - 0.50.
- Chill on ice the culture for 2 hours.
- Centrifuge at 3,000 rpm for 15 minutes at 4°C.
- Gently resuspend the pellet in 40 mL of buffer TRAFO (100 mM CaCl2, 70 mM $MnCl_2$, 40 mM CH_3COONa, pH 5.5, sterilized by filtration).
- Chill on ice for 45 minutes.
- Centrifuge at 2,500 rpm for 10 minutes at 4°C.
- Discard the supernatant and resuspend in 6 mL of cold buffer 15% glycerol/TRAFO.
- Dispense in aliquots into fresh tubes and store at -80°C.

The procedure was realized under sterile conditions, using a laminar flow hood. All solutions used were sterile.

3.2.2.8. *E. coli* DH10B transformation by heat shock.

- Mix 100 mL of competent cells to 5 µL of the ligation product and mix gently.
- Chill on ice for 30 minutes.
- Heat for 30 seconds at 42°C.
- Incubate on ice for 2 minutes.
- Add 1mL of LB and incubate at 37°C for 1 hour, with shaking.
- Plate on LB agar with appropriate antibiotic and incubated at 37 °C.

3.2.2.9. Screening of trasformants and recombinant clones by colony PCR.

- Select and take the bacterial colony grown on LB or MRS agar medium, with a sterile tip.
- Transfer the colony in 50 µL of sterile distilled H_2O.
- Incubate the bacterial suspension at 100°C for 10 minutes (the DNA is released following the cell disruption).
- Centrifuge at 10,000 rpm for 1 minute and transfer the supernatant into a new tube.

This DNA may be used in PCR applications. Alternatively, for LAB, it is possible to start from an overnight culture broth. Therefore:

- Centrifuge at 10,000 rpm for 5 minutes.
- Discard the supernatant and resuspend the pellet in 0.5 mL in TE buffer.
- Transfer the cell suspension in a tube with glass-microbeads.
- Vortex by bead beating at maximum speed for 1 minute.

Transfer the supernatant in a new tube and use 5 µL in PCR reaction.

3.2.2.10. Plasmid purification and DNA sequencing.

E. coli DH10B was used as an intermediate host to clone the pNZ5319 and pUC18Ery recombinant vectors. Large amounts of the recombinant plasmids were subsequently purified, following instructions of the QIAprep Spin Midiprep Kit (Qiagen).

In order to confirm correct cloning, selected regions of the recombinant plasmids were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer instructions. The Molecular Biology of CNR-IPSA Institute (Unit of Lecce, Italy) provided the nucleotide sequences using a DNA sequencer ABI Prism 3100 (Applied Biosystems).

3.2.2.11.Electroporation of *L. plantarum* WCFS1.

The following procedure allows *L. plantarum* cells to become elettrocompetents, that is inclined to transformation by exposure to an electric field. The protocol was carried out under sterile conditions, by using laminar flow hood. All solutions used were sterile.

- Allow the growth of an *L. plantarum* culture until an OD_{600nm} of 0.60 - 0.65 is reached and then chill on ice for 15 minutes, shaking by inverting every 2 - 3 minutes.
- Centrifuge at 5,000 rpm for 10 minutes at 10°C.
- Discard the supernatant and wash the pellet with 1 volume of cold 1 mM $MgCl_2$.
- Centrifuge at 5,000 rpm for 10 minutes at 10°C.
- Discard the supernatant and wash the pellet with 1 volume of cold 30% PEG 1500.
- Centrifuge at 5,000 rpm for 10 minutes at 10°C.
- Discard the supernatant and resuspend the pellet with 1/100 volume 30% PEG 1500.
- Subdivide in aliquots of 50 µL.
- Store at -80 ° C.

Electroporation:

The purified recombinant plasmids were transformed into *L. plantarum* by electroporation using a Gene Pulser Xcell with Shock Pod Cuvette Chamber (BIORAD) and the following parameters:

- voltage = 1,500 V,
- resistance = 400 Ω;
- capacitance =25 µF.

The electroporation procedure was:

- Chill on ice a electroporation cuvette.
- Mix gently into the cuvette, 50 µL of electrocompetents cells and 1 - 2 µL of plasmid DNA (500ng - 1µg).
- Place the cuvette in the Gene Pulser and start the electrical impulse.
- Add immediately 500 µL of MRS containing 1 mM $MgCl_2$ + 0.3 M sucrose.
- Incubate at 37°C for 2 hours.
- Plate on MRS + antibiotic
- Incubate at 30°C.

Transformants containing pUCFTSH, were selected on MRS + erythromycin (30 µg/mL); transformants containing the recombinant pNZ5319 plasmids were selected on MRS agar containing chloramphenicol (10 µg/mL) and replica-plated on MRS + erythromycin (30

µg/mL) to check for erythromycin sensitivity and so to select for double cross over recombinants.

3.2.2.12. Gene knockout of *L. plantarum* WCFS1 *shsp* genes.

In order to obtain mutant strains of *L. plantarum* WCFS1 for both *hsp18.5* and *hsp19.3* genes, we opted for a genic deletion system based on a Cre-*lox* mutagenesis vector. This system used the plasmid pNZ5319 (**Figure 3.2**), engineered to replace the target gene by the selectable marker cassette *lox66-P*$_{32}$*-cat-lox71*, through an event of double cross-over. Then, these lox sites can be recombined to give a *lox72* site, by the Cre enzyme which is expressed transiently by a second plasmid (pNZ5348), thus allowing deletion of the *P*$_{32}$*-cat* cassette from bacterial genomic DNA. *lox72* site is poorly recognized by Cre recombinase (Lambert et al, 2007).

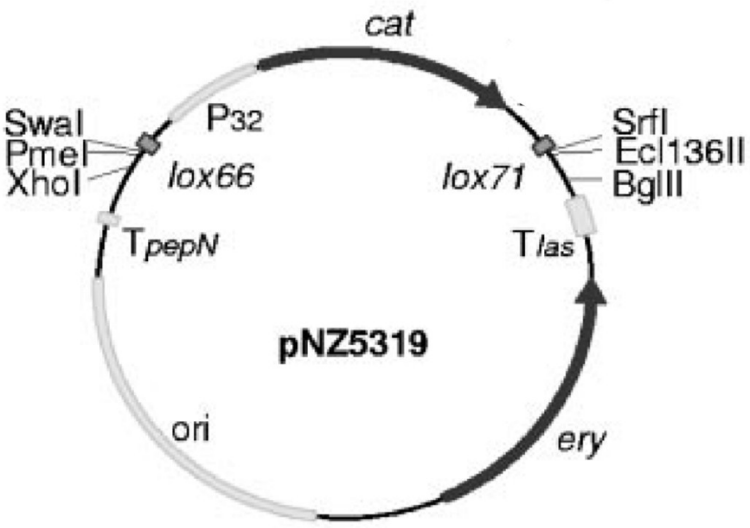


Figure 3.2. **Schematic representation of the mutagenesis vector pNZ5319**. Origin of replication (*ori*), erythromycin resistance gene (*ery*), chloramphenicol resistance gene (*cat*) under the control of the P_{32}promoter (P_{32}-*cat*), flanked by *lox66* and l*ox71* sites, lactococcal T*las* and T*pepN* terminators. Rare-cutting sites are: blunt-end restriction sites *Swa*I, *Pme*I, *Srf*I, and *Ecl*136II, and sticky-end restriction sites *Xho*I and *Bgl*II, respectively. The two selectable-marker gene cassettes (P_{32}-*cat* and *ery*) allows direct selection of double-crossover integrants based on their antibiotic resistance (Cm^r) and sensitivity (Em^s) phenotype. (*from Lambert et al, 2007).*

Figure 3.3 shows the mechanism of the double cross-over event followed by the Cre-mediated recombination. By homologous recombination, the segment of the mutagenesis vector pNZ5319 containing the upstream (UP) and downstream (DOWN) regions of the target gene and the marker gene (*cat*) is transferred onto the genome replacing the target endogenous gene. Subsequently, the Cre recombinase catalyzes the site specific recombination between *lox66* and *lox71* sites, thus allowing the deletion the *cat*-cassette and originating a new lox site (*lox72*).

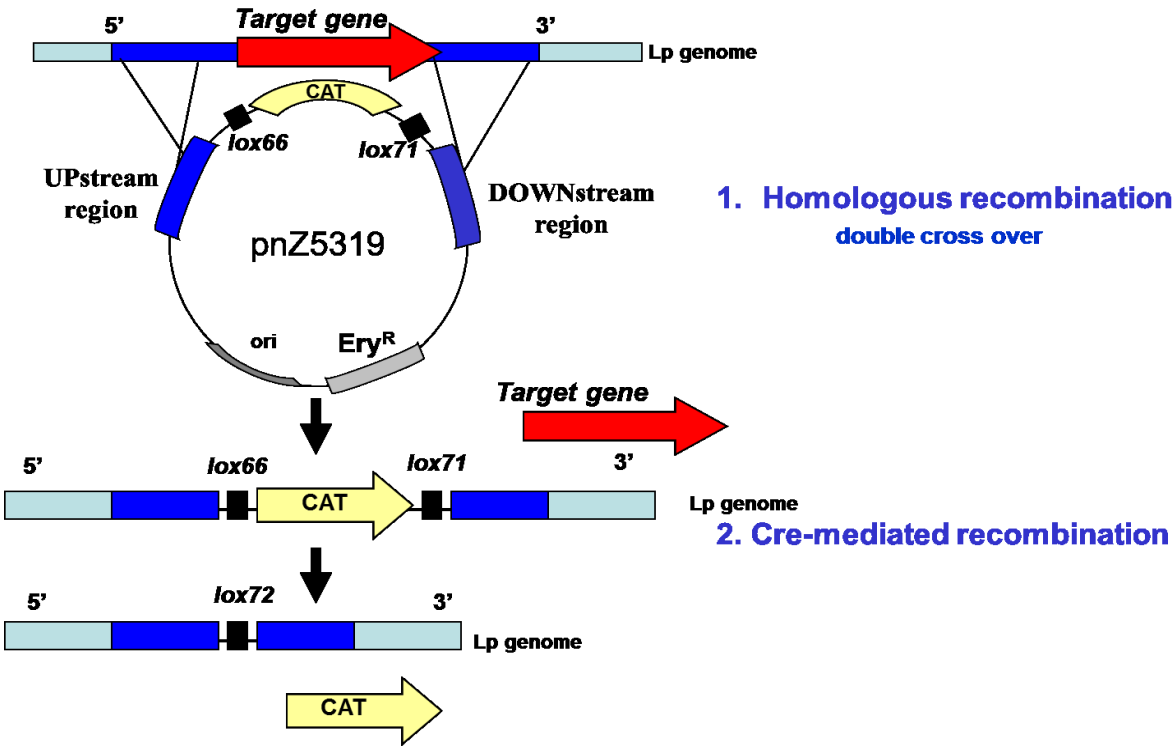


Figure 3.3. **Main steps of the Cre-*lox* mutagenesis system**.

For *hsp18.5* gene, the UP and DOWN regions (850 and 962 bp, respectively) were amplified with primers **FB_1HSP_1** and **RB_1HSP_1**, **FB_2HSP_1** and **RB_2HSP_1** (**Table 3.1**), digested with *Ecl*136II and *Swa*I, and cloned in *Swa*I and *Ecl*136II restriction sites of vector pNZ5139, respectively.

For *hsp19.3* gene, an UP region of 872 bp was amplified with primes **FB_1HSP_3** and **RB_1HSP_3**, digested with *Xho*I enzyme and cloned between the *Xho*I-*Swa*I restriction sites;

while a DOWN region of 910 bp was amplified with primes **FB_2HSP_3** and **RB_2HSP_3** (**Table 3.1**), digested with *Swa*I enzyme and cloned in *Ecl*136II restriction site.

The resulting constructs, *pNZ5319:hsp18.5* and *pNZ5319:hsp19.*3, were transformed into *E. coli* DH10B cells. Transformants were selected on LB agar + ampicillin (100 μg/mL) and recombinants were checked by colony PCR.

A different strategy of gene knockout was used, based on pUC18Ery vector (**Figure 3.4**).

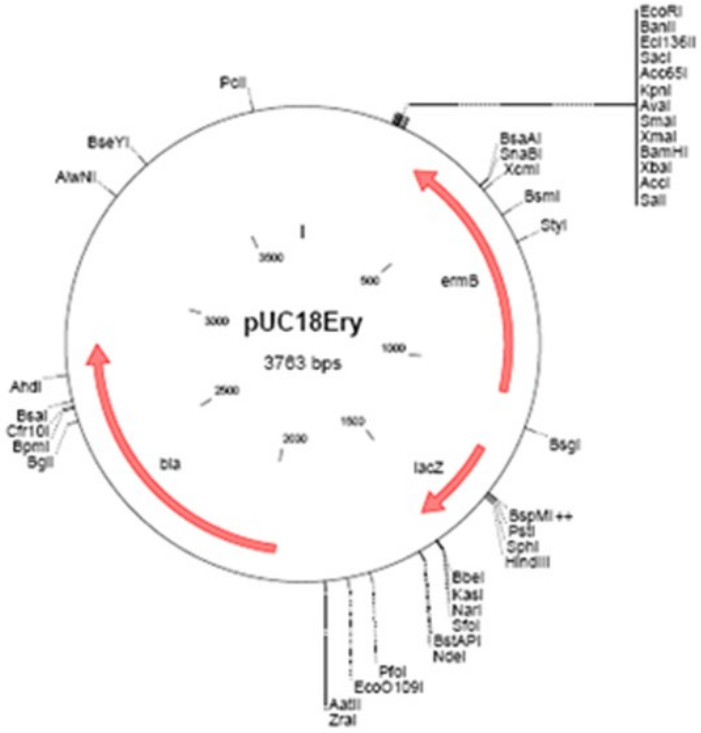


Figure 3.4. **Schematic representation of the pUC18Ery vector**, with beta-galactosidase promoter (*lacZ*), erythromycin resistance gene (*ermB*), ampicillin resistance gene (*bla*) and a polylinker with the major restriction recognition sites useful in molecular cloning (e.g.,: *Eco*RI, *Kpn*I, *Bam*HI, *Xba*I).

The fragments containing UP and DOWN regions of either *hsp18.5* or *hsp19.3*, together with the *cat* gene and *lox* sites, were re-amplified from the recombinant pNZ5319 plasmids (*pNZ5319:hsp18.5* and *pNZ5319:hsp19.*3), using forward (restriction site for *Eco*RI) and reverse (restriction site for *Xba*I) primers (**Table 3.1**) and thus cloned between the *Eco*RI and *Xba*I in pUC18Ery. **EcoRI_FB1HSP1** and **XbaI_ RB2HSP1** primers were used for knockout *hsp18.5*; while **EcoRI_FB1HSP3** and **XbaI_RB2HSP3** primers were used for knockout *hsp19.3*. Amplicons (obtained with Pfu DNA Polymerase) and plasmid pUC18Ery were digested with *Eco*RI and *Xba*I; ligated and transformed into *E. coli* DH10B cells Ca^{2+}-competent. Transformants were selected as above.

3.2.2.13. Disruption of *L. plantarum* WCFS1 *ftsH* gene.

The *ftsH* gene of *L. plantarum* WCFS1 was disrupted by single-crossover plasmid integration, using the vector pUC18Ery. An internal *ftsH* gene fragment of 870 bp was amplified with using primers **ftshKOF** and **ftshKOR** (**Table 3.1**) and cloned into pUC18Ery between the *Kpn*I and *Bam*HI restriction sites. The resulting recombinant plasmid, pUCFTSH, was transformed into *L. plantarum* WCFS1 by electroporation and candidate integrants were selected on MRS agar plates containing 30 µg/mL erythromycin. Correct integration of pUCFTSH in the *ftsH* locus (**Figure 3.5**) was confirmed by PCR analysis using primers annealing to the flanking genomic regions, **ftsHF** and **ftsHR**, combined with vectors specific primers, **pUC_ery_F** and **pUC_ery_R** (**Table 3.1**).

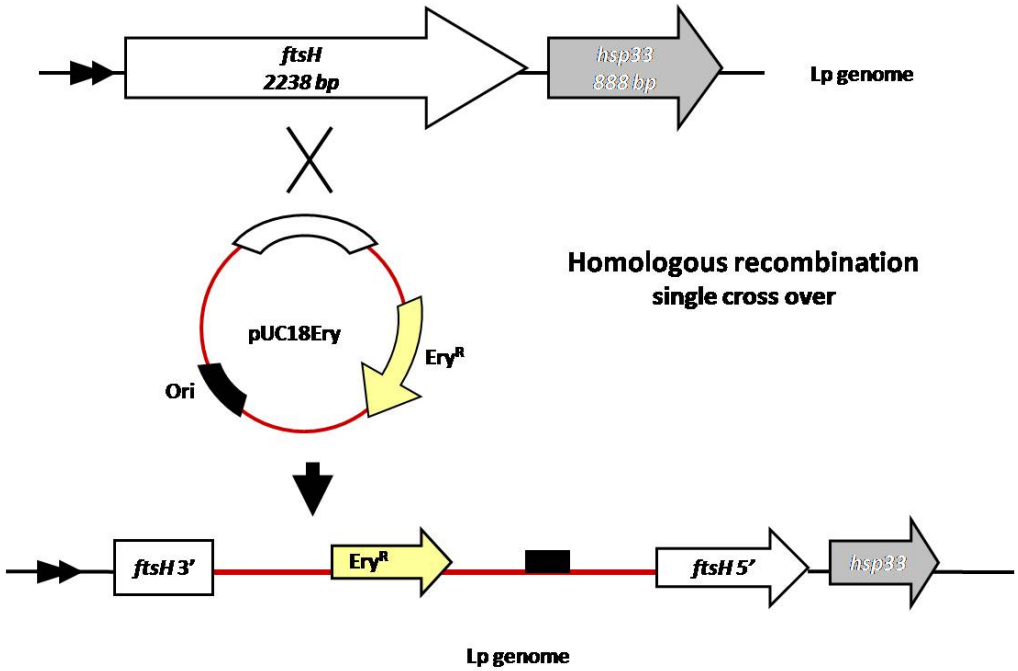


Figure 3.5. **Schematic overview of the single cross over event leading to disruption of *L. plantarum ftsH*.**

3.2.3. Mammalian cells.

3.2.3.1. THP-1 cell culture and ELISA assay.

THP-1 monocytoid cells were maintained in RPMI medium 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL Penicillin and 100 µg/mL Streptomycin. To induce a macrophage-like phenotype, THP-1 monocytoid cells were incubated with 200 nM PMA.

The effect of TNF-α production by *L. plantarum* WCFS1 and related mutant strains on activated human monocytoid cells was evaluated in a co-incubation system. THP-1 cells (5

x 10^4 cells) were co-incubated with 5% (v/v) cell-free supernatant of *L. plantarum* planktonic cells and 100 ng/mL *E. coli* serotype O127:B8 LPS in a 24-well culture cluster plate (Corning) at 37°C in a humidified incubation chamber containing 5% CO_2 for 3.5 hours.

To determine the level of TNF-α secreted into culture supernatants, the human monocytes/macrophages were pelleted (1,500 rpm, 5 minutes, 4°C), and the amount of TNF-α was determined by quantitative enzyme linked immunosorbent assays (ELISAs) (Human TNF-alpha DuoSet Kit, R&D Systems) in a SpectraMax 340PC 384 microtiter plate-based absorbance spectrophotometer (Molecular Devices).

3.2.3.2. Caco-2 cell culture and adhesion test.

The Caco-2 epithelial cell line was employed for the adhesion experiments. These cells were used in their differentiated state to mimic small intestine mature enterocytes.

Caco-2 cells were grown in Mem-Alpha Medium supplemented with 10% (v/v) heat-inactivated FBS, 2mM L-Glutamine, 50U/mL Penicillin and 50µg/mL Streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 96-well tissue culture plates (Falcon Microtest) at 1.25 x 10^4 cells per well and grown as monolayers for 10 to 15 days to obtain differentiation. The medium (0.1 mL/well) was changed every 2 days; 24 hours before an adhesion assay, an antibiotic-free medium was used. In post-confluent cultures **(Figure 3.6**), the viable cell number, as counted in a Burker chamber, was about 4.5 x 10^4 cells per well.

To study the adhesion of each strain, Caco-2 cells were overlaid with bacteria resuspended in DMEM (0.1 mL/well) to a final concentration of approximately 5.0 x 10^8 CFU/mL (ratio ≥ 1000 : 1, bacteria to Caco-2 cells). Preliminary experiments indicated that such bacterial concentration was saturating in terms of adhesion. After 1 hour incubation at 37°C under 5% CO_2 atmosphere, wells were washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove unbound bacteria. Caco-2 cells and adherent bacteria were then detached by trypsin-EDTA 0.05% treatment and resuspended in PBS. Serial dilutions of the samples were plated onto MRS-agar plates to determine the number of cell-associated bacteria (viable counts) expressed as CFUs. CFUs counts from control unwashed wells provided total bacterial load. Experiments were performed in triplicate.

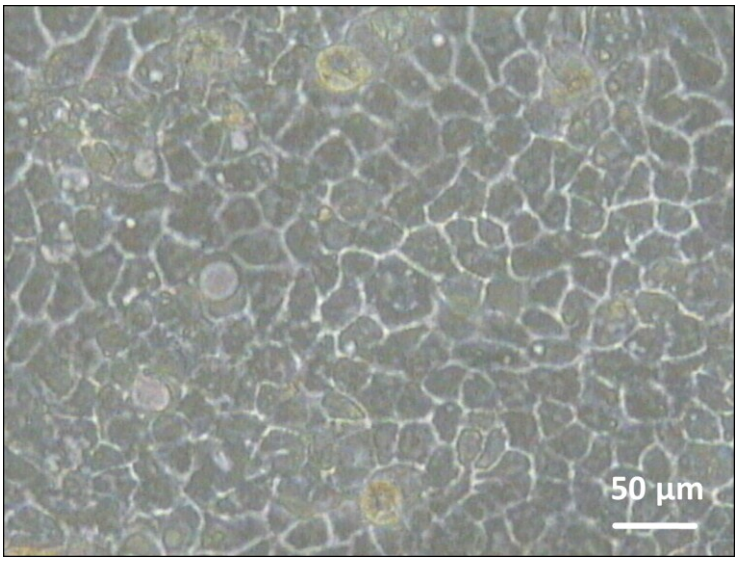


Figure 3.6. **Postconfluent Caco-2 cells as imaged by inverted light microscopy**.

3.2.3.3. Polarization of Caco-2 cells.

Polarizaton of Caco-2 monolayers was assessed by measuring their Trans Eletrical Epithelial Resistance (TEER). Caco-2 cells were seeded on 6.5 mm diameter Snapwell inserts, 0.4 µm pore size (Corning) at the density of 2.5×10^4 per filter. The inserts were located onto Transwell Supports (Corning) and the culture medium (DMEM supplemented as above) was changed every two days, by adding 0.8 mL into the lower compartment and 0.2 mL into the upper compartment, respectively. The TEER of Caco-2 cell layers was measured by a voltohmmeter (Millicell-ERS), every 48 hour, over a culture period of 15-20 days.

3.2.3.4. Caco-2 cell stimulation assay.

For immune stimulation experiments, Caco-2 cells were seeded at a density of 1.4×10^4 cells per well in 24-well tissue-treated culture plates (Iwaki). The culture medium (DMEM supplemented as above; 0.8 mL/well) was changed every two days. Post-confluent cells were incubated with serum- and antibiotic-free medium for at least 12 hours before bacterial stimulation test, in order to avoid any interference with immune gene expression and with bacterial viability. The viable cell number, as counted in a Burker chamber, was about 2×10^5 cells per well. Caco-2 cells were incubated with either live or heat inactivated (1 hour at 65°C) bacteria at a concentration of 5×10^8 CFU/mL (**Figure 3.7**). The concentration of live bacteria was monitored over time by CFUs count analysis.

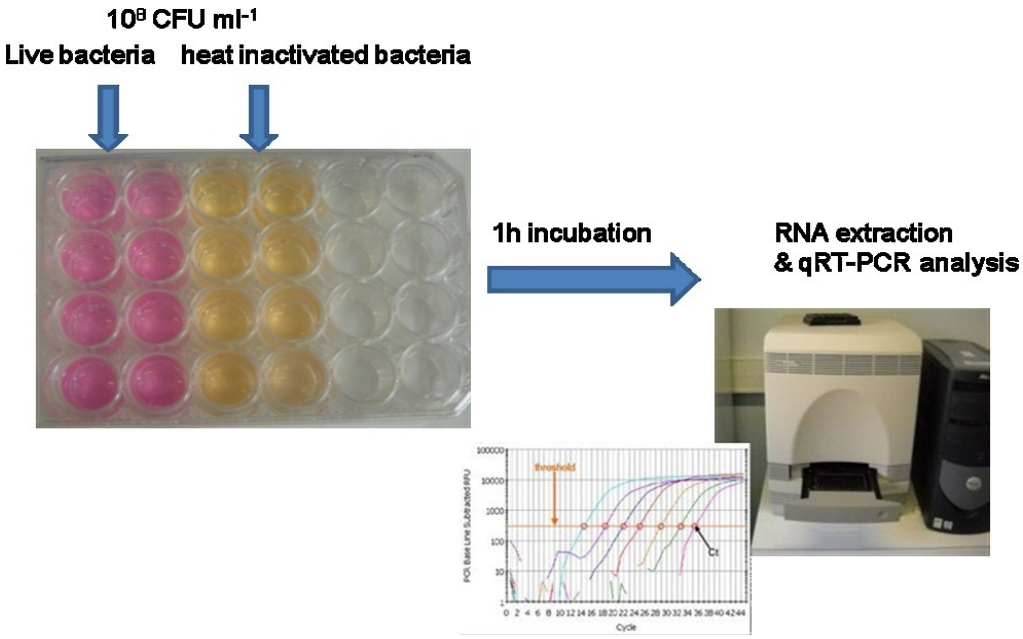


Figure 3.7. **Schematic overview of the Caco-2 cell stimulation assays**. Incubation with live bacteria led to acidification of the cell culture medium.

3.2.3.5. RNA extraction from bacteria and animal cells.

Total RNA was extracted from bacteria (mid log phase cells from the different *L plantarum* strains and OGI stressed *L plantarum* wild type cells) using the UltraClean Microbial RNA Isolation Kit (Cabru), according to manufacturer instructions.

Total RNA was isolated from untreated Caco-2 cells (control) and after 1, 3 and 5 hours of bacterial stimulation. Cells were washed with PBS and harvested with TRIzol reagent (Invitrogen), according to the following procedure:

- Adherent Caco-2 cells (approximately 10^6 cells) are detached and resuspended by pipetting 1 mL of TRIzol reagent.
- Mix well by repeating pipetting.
- Incubate the homogenized samples for 5 minutes at room temperature.
- Add 0.2 mL of chloroform, shake vigorously for 15 seconds and incubate at room temperature for 3 minutes.
- Centrifuge at 13,000 rpm for 15 minutes at 4°C and transfer the upper aqueous phase to a new tube.
- Precipitate by adding 0.5 mL isopropanol (at -20 °C for 45 minutes).

- Centrifuge at 13,000 rpm for 15 minutes at 4 °C.
- Remove the supernatant, wash the pellet with 1 mL ice-cold 75% ethanol, and centrifuge again at 10,000 rpm for 5 minutes at 4°C.
- Discard the supernatant and let the pellet dry for 10 – 15 minutes at room temperature.
- Resuspend the RNA pellet in 20 - 50 µL of RNase-Free H_2O

RNA quantity and quality was determined spectrophotometrically and by electrophoresis on 1.0 % agarose gel. RNA samples are stored at -80 °C.

3.2.3.6. cDNA synthesis.

After extraction, both animal and microbial RNAs were reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) which includes a DNA removal step to avoid genomic contamination. 1µg of total RNA sample was retrotranscribed following the kit instruction. Reaction were incubated at 42°C for 30 minutes. Reverse transcriptase was inactivated for 3 minutes at 95°C.

3.2.3.7. Quantitative Real Time PCR.

The transcriptional level of genes encoding:

- interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage inflammatory protein 3α (MIP-3α), human β-defensin-2 (HBD-2), lysozyme (LYZ) and mucin-2 (MUC-2);
- clp proteases (ClpB, ClpE, ClpP);
- small heat shock proteins (Hsp18.5, Hsp18.55, Hsp19.3);
- stress controlling factors CtsR and FtsH;
- molecular chaperones GroEL and DnaK, and
- enolase A1 (EnoA1), mucin-binding protein (Mub), myosin cross reactive antigen (MCRA), mannose adhesine (Msa) and plantaricin EF (plnEF)

was analysed by quantitative real-time PCR (ABI 7300; Applied Biosystems) using SYBR green I detection.

Each reaction mixture, containing 5 µL of 20-fold-diluted cDNA, 10 µL of QuantiFastSYBR Green PCR Master Mix (Qiagen) and 100 nM of each sense and antisense primer, was subject to amplification. Cycling conditions included initial denaturation-enzyme activation at 95°C for 10 minutes, followed by 35 cycles of 20 seconds at 95°C, 30 seconds at 58°C, and 30 second at 72°C. Fluorescence was monitored during each extension phase, and a melting-curve analysis was performed after each run to confirm the

amplification of specific transcripts. A melt curve was generated after each PCR run, and subsequently analysed to check for specificity of the amplicons.

Data were analysed using AB 7300 software. Each PCR assay included duplicates of each cDNA samples, no-template and RNA controls to check for contamination. The ΔΔCt method was used to assess relative transcriptional level (Livak and Schmittgen, 2001). The expression level of housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase D (*ldhD*) were monitored to normalize expression of target animal and bacterial genes, respectively.

3.2.3.8. Statistics.

Statistical analysis was performed using two-tailed, nonpaired Student's t-test. Any *P*-value <0.05 was considered significant. Possible correlations were assessed by the Pearson's correlation coefficient.

4. RESULTS

4.1. Generation of *L. plantarum* WCFS1 mutant strains.

4.1.1. Strategies to delete *L. plantarum* WCFS1 *hsp18.5* and *hsp19.3* genes.

Following the cloning of UP and DOWN regions of both *hsp18.5* and *hsp19.3* genes, PCR analysis on pNZ5319-derived plasmids confirmed the correct cloning with respect of *cat*-cassette. The pairs of primers FB_1HSP_1 - CatRev for *hsp18.5* UP region and CatFor - RB_2HSP_1 for *hsp18.5* DOWN region, respectively; and the pairs of primers FB_1HSP3_ - CatRev for *hsp19.3* UP region and CatFor - RB_2HSP_3 for *hsp19.3* DOWN region, respectively, were used (**Table 3.1**). By agarose gel elettrophoresis (**Figure 4.1**), the amplicons were visualized at the expected sizes.

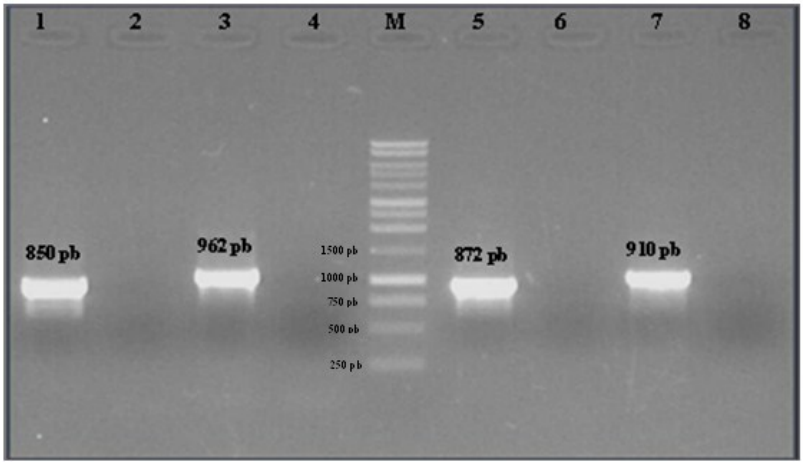


Figure 4.1. **Polymerase chain reaction on pNZ5319-derived plasmids using primers designed on UP/DOWN regions of *shsp* genes and *cat* marker of plasmid, respectively**. Lane 1: UP region of *hsp18.5* gene (FB_1HSP_1/CatRev), lane 3: DOWN region of *hsp18.5* gene(CatFor/RB_2HSP_1); lane5: UP region of *hsp19.3* gene (FB_1HSP3_/CatRev); lane 7: DOWN region of *hsp19.3* gene(CatFor/ RB_2HSP_3); lanes 2, 4, 6, 8: negative controls; lane M: 1Kb DNA ladder.

Sequence analyses on pNZ5319-derived plasmids (*pNZ5319:hsp18.5* and *pNZ5319:hsp19.3*) further confirmed the correct cloning. The sequences proved that the UP and DOWN regions were flanking the *cat*-marker, giving a knockout cassette of about 2,800 bp.

After replication in the intermediate *E. coli* DH10B host, the recombinant mutagenesis vectors, *pNZ5319:hsp18.5* and *pNZ5319:hsp19.3*, were introduced into *L. plantarum* WCFS1 by electroporation. Chloramphenicol-resistant transformants were selected on MRS agar. The transformation provided 109 colonies Cm^r and Em^s for *pNZ5319:hsp18.5* vector

and 73 colonies Cm^r and Em^s for *pNZ5319:hsp19.3* vector, respectively. However, colony-PCR screening using primer annealing in the up- and downstream region of *hsp18.5* gene (FB_1HSP_1/RB_2HSP_1) and in the up- and downstream region of *hsp19.3* gene ($FB_1HSP_3\_/RB_2SPS_3$), respectively (**Table 3.1**), revealed that these colonies were false positives. Indeed the genomic region corresponding to the target gene resulted unchanged with respect to wild type. **Figure 4.2**, shows the electrophoresis of the colony-PCR products from *L. plantarum* cells electroporated with *pNZ5319:hsp18.5* and *pNZ5319:hsp19.3* vectors. In both situations, the amplicons resulted of about 2,200 bp, exactly the size of amplicons obtained from wild type genome. Conversely, amplicons of 2,800 bp were expected if the *cat- lox* cassette had correctly integrated into genome, replacing the target gene.

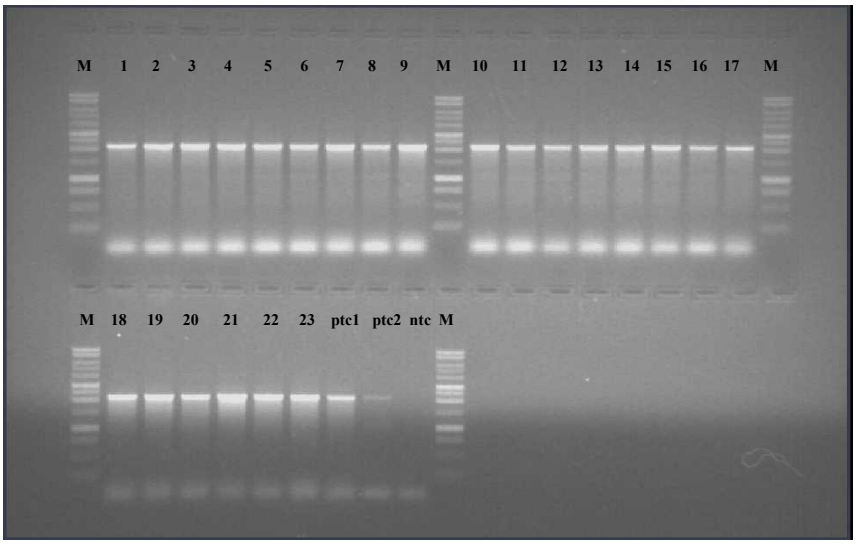


Figure 4.2. **Colony-PCR screening of *L. plantarum* WCFS1 trasformants deriving from electroporation with *pNZ5319:shsp*.** Samples 1 - 13: PCR products from clones *pNZ5319:hsp18.5*-derived, using primer pair FB_1HSP_1/RB_2HSP_1; sample 14 - 23: PCR products from clones *pNZ5319:hsp19.3*-derived, using primer pair $FB_1HSP_3\_/RB_2SPS_3$; ptc1 and ptc2: positive controls on *L. plantarum* WCFS1 genome; ntc: negative control; M: molecular weight marker. Length of all amplicons: 2,200 bp.

After repeating attempts of deletion, the failure in obtaining *L. plantarum* mutants with the Cre-*lox* system prompted us to try another strategy. Therefore, the upstream and downstream regions of *hsp18.5* and *hsp19.3* were cloned into the pUC18Ery vector, flanking the *ery* gene. The recombinant vectors were electroporated into *L. plantarum*, however, no Em^r transformants could be selected so far.

4.1.2. Disruption of the *ftsH* gene.

The *ftsH* gene of *L. plantarum* WCFS1 was disrupted by single-crossover plasmid integration, as reported previously (van Kranenburg et al, 1997). An internal *ftsH* fragment was PCR-amplified and cloned into pUC18ery between the *Kpn*I and *Bam*HI restriction sites (**Figure 4.3**).

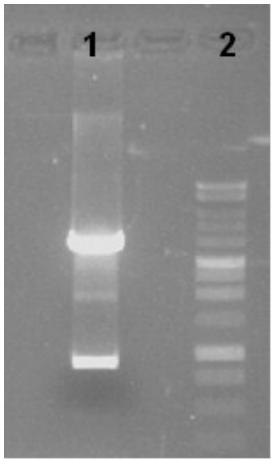


Figure 4.3. ***Kpn*I-*Bam*HI restriction on the recombinant pUCFTSH plasmid sets free the cloned 850 bp internal region of the *ftsH* gene**. 1, digestion products; 2, molecular weight marker.

The resulting recombinant plasmid, pUCFTSH, was transformed into *L. plantarum* by electroporation and candidate integrants were selected on MRS agar plates containing erythromycin (**Figure 4.4**). Correct integration of pUCFTSH in the *ftsH* locus was confirmed by PCR analysis using primers annealing to the flanking genomic regions (ftsHF and ftsHR) combined with vectors specific primers (pUCeryF and pUCeryR) (**Figure 4.5**) (**Table 3.1**). A single *ftsH* disruption mutant was selected and used in subsequent studies. The absence of the *ftsH* transcript in the mutant strain was confirmed by qRT-PCR using primers ftsHrtF and ftsHrtR (data not shown).

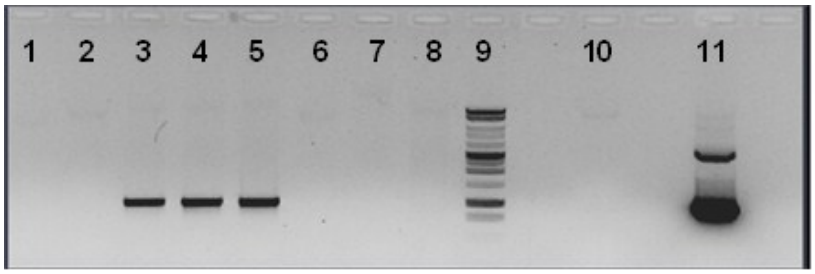


Figure 4.4. **Colony-PCR screening of *L. plantarum* trasformants deriving from electroporation with pUCFTSH.** Primer pair: pUCeryF and pUCeryR. The expected *ftsH* fragment is detected only from PCR on colonies 3, 4, 5, while colonies 1, 6, 7, 8 give negative results. 9, molecular weight marker; PCR products on *L. plantarum* genomic DNA (negative control, 10), pUCFTSH plasmid (positive control, 11)

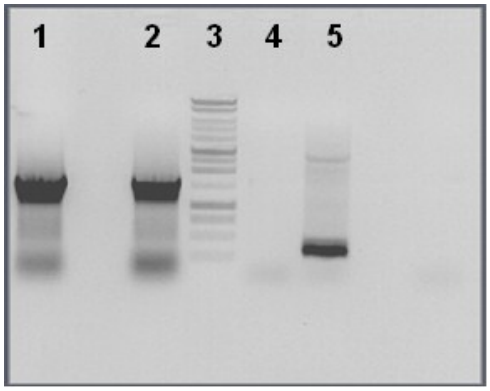


Figure 4.5. **Exemplificative PCR analysis to confirm correct integration of the pUCFTSH plasmid into *L. plantarum* genome (thus disrupting the *ftsH locus*)**. Primers pair: pucEryF and ftsHR. Templates: genomic DNA from two candidate *L. plantarum* mutants (1,2); genomic DNA from *L. plantarum* wild type (4); pUCFTSH plasmid. 3, molecular weight marker. The expected amplicon of ~1300 pb is detected only in the genome of putative integrants/mutants (see details in material and methods).

4.2. Phenotypic analyses of *L. plantarum* mutant strains.

4.2.1. *ftsH* gene deletion affects growth of *L. plantarum* WCFS1.

In order to understand the relevance of *L. plantarum ftsH* gene in stress protection, the phenotype of the Δ*ftsH* strain was analysed and compared with that of wild type and of a *ftsH*-overexpressing strain (containing the pGIZ–*ftsH* plasmid) named *ftsH*+.

The ability to grow under different stress conditions (including heat, cold, hyperosmotic, bile, acid and oxidative environment) was tested by plating serial dilutions of exponentially growing cultures on MRS plates (**Figure 4.6**).

As previously reported, under physiological conditions, the Δ*ftsH* strain displayed a relatively slower growth rate than the wild type strain (**Figure 4.6 A**) (Fiocco et al, 2009). The heat sensitivity of the Δ*ftsH* strain (Fiocco et al, 2009) was confirmed by its inability to form visible colonies when plates were incubated at 40°C (**Figure 4.6 B**). Difference in growth between the wild type and the Δ*ftsH* mutant strains was remarkable also when cells were subject to hyperosmotic stress (2% NaCl, **Figure 4.6 C**); intriguingly, the Δ*ftsH* mutant strain could grow faster than wild type in MRS containing bile salts (0.1% porcine bile, **Figure 4.6 D**).

The *ftsH*+ strain displayed a phenotype which was partially complementary to that of the Δ*ftsH* mutant (**Figure 4.6**). Indeed, overexpression of *ftsH* slightly enhanced the growth rate under physiological-optimal conditions (**Figure 4.6 A'**), and markedly improved growth ability under heat stress (**Figure 4.6 B'**) and in presence of high salt content (**Figure 4.6 C'**) with respect to control strain pGIZ (harbouring the empty overexpression vector). Under bile

stress, *ftsH*+ strain exhibited enhanced survival capacity compared to control strain (**Figure 4.6 D'**).

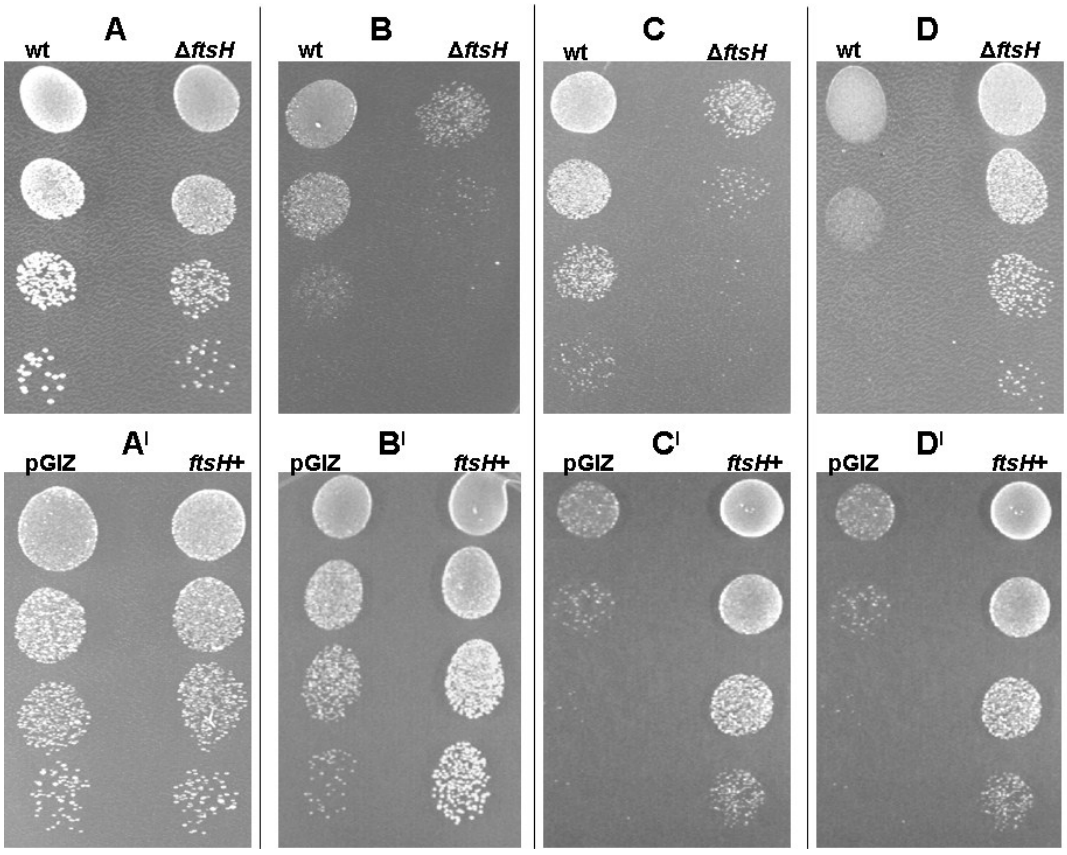


Figure 4.6. **Serial dilutions of cultures of *L. plantarum*** wild type (wt), *ftsH* mutant (Δ*ftsH*), control harbouring the empty pGIZ906 vector (pGIZ), and *ftsH*-overexpressing (*ftsH*+) spotted (10µL) on MRS plates and incubated at the temperatures of 28°C (control temperature, panel A–A'), 40°C (heat stress, B–B'), 30°C on MRS containing either 2% NaCl (osmotic stress, C–C'), and 0.1% porcine bile (acidic bile stress, D–D'). Pictures were taken after 20 h of growth.

4.2.2. Morphological and physico-chemical surface properties of *L. plantarum* Δ*ftsH* and other mutant strains.

The morphological and cell surface features of *L. plantarum* Δ*ftsH* mutant were investigated and compared with those of the wild type strain, as well as with the Δ*ctsR* and Δ*hsp18.55* mutants, belonging to the laboratory microbial collection and previously obtained (Fiocco et al, 2010; Capozzi et al, 2011).

Cell morphology. The cell morphology of *L. plantarum* wild type and mutant strains was analysed before and after heat stress exposure by scanning electron microscopy (SEM) (**Figure 4.7**). Under optimal growth temperature conditions (30°C), wild type cells exhibited the characteristic rod-shaped, smooth-surface morphology of *L. plantarum*, and no relevant differences with Δ*ctsR* and Δ*ftsH* strains could be observed. In contrast, the cell surface of Δ*hsp18.55* strain appeared damaged at both 30°C and 42°C. Mutant cells were clumped together and had rough surfaces. Moreover, some cells had a shrunken appearance resembling that of cells undergoing dehydration or cell wall damage. After heat shock (42°C for 30 minutes), compared to those of the wild type, the Δ*ctsR* cells envelopes looked stiffer and presented somewhat fissured surfaces. These intriguing features suggest that the cell wall might be critically damaged in this mutant strain. Conversely, Δ*ftsH* cells did not reveal any relevant difference in the surface cell morphology with respect to wild type.

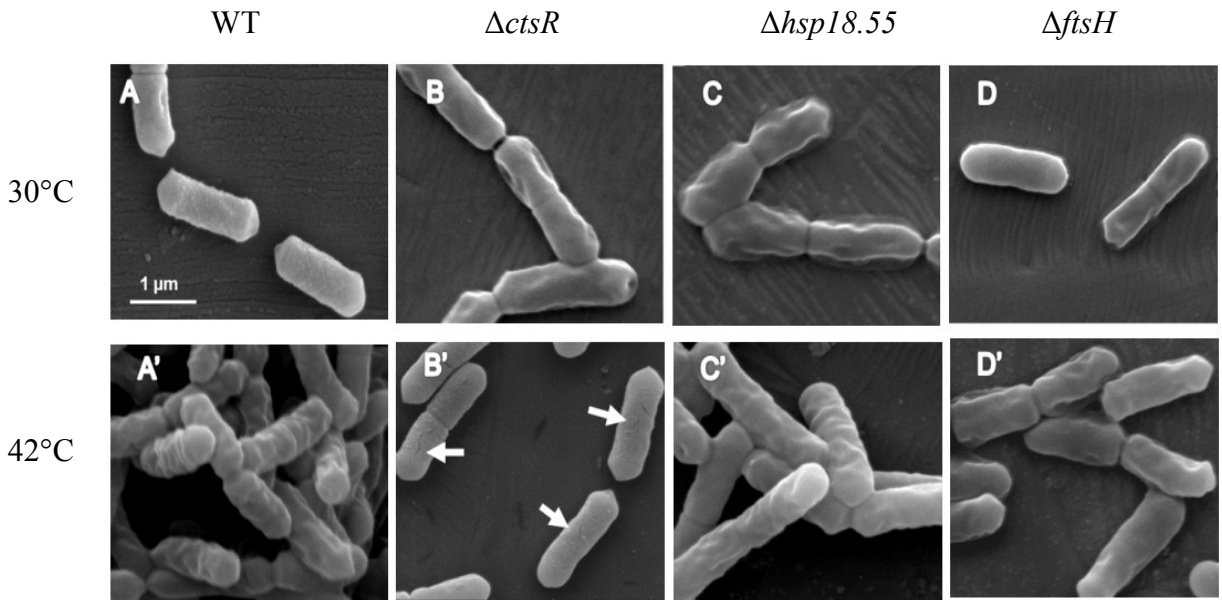


Figure 4.7. **SEM analysis of *L. plantarum* wild type and mutant strains**. Exponentially growing cells (OD_{600nm} = 0.6) were imaged by SEM before (A, B, C and D, respectively) and after (A', B', C' and D', respectively) a 30-minutes temperature upshift to 42°C. Arrows show fissures in Δ*ctsR* mutant cell envelopes.

Biofilm formation. The ability to form biofilms is an important probiotic feature of Lactobacilli (Kubota et al, 2008; Macfarlane, 2008). Because the adherence capacity strictly reflects the cell surface properties, we sought to determine whether biofilm formation capacity might be affected by inactivation of *ctsR*, *hsp18.55* and *ftsH* genes. Bacterial adhesion on hydrophilic-treated polystyrene wells was evaluated by crystal violet staining.

Absorbance at 595 nm of the crystal violet was considered from the 24 hours and 48 hours biofilms, cultured at 30°C (**Figure 4.8**). A slight increase of biofilm formation (~20%) was observed for the Δ*ctsR* strain, while a reduced capacity to form biofilm was observed for the Δ*ftsH* strain, compared to wild type after 24 hours. No significant difference was observed for the Δ*hsp18.55* mutant strain. After 48 hours, biofilm formation by wild type and Δ*hsp18.55* reduced of about 20%; biofilm formation by the Δ*ctsR* strain declined of about 50%, while biofilm formation of Δ*ftsH* strain was stable, although lower than wild type.

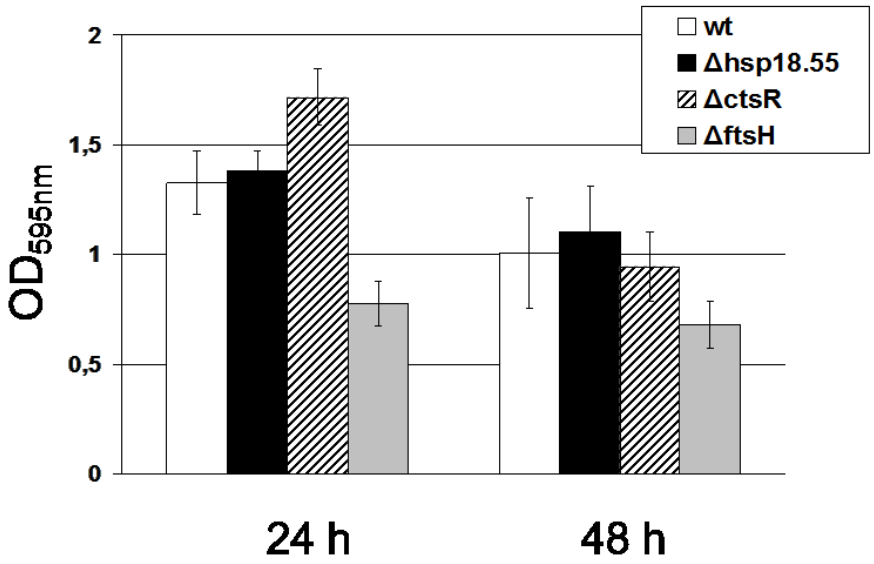


Figure 4.8. **Biofilm formation by *L. plantarum*** WCFS1 wild type (white bars), Δ*hsp18.55* (black bars), Δ*ctsR* (hatched bars) and Δ*ftsH* (grey bars). Cultures were grown in MRS broth in 24-well cell culture plate at 30°C. Absorbance at 595 nm of the crystal violet extracted with ethanol from the 24 hours and 48 hours biofilms is indicated. The graph shows the averages and standard deviations from three independent experiments.

Physico-chemical surface properties. We sought to verify whether inactivation of *ctsR*, *ftsH* and *hsp18.55* genes could alter the physico-chemical properties of *L. plantarum* cell envelope. To this aim, cells were collected at early stationary phase and analysed for their binding affinity towards solvents such as chloroform (monopolar and acidic), hexadecane (apolar), and ethyl acetate (monopolar and basic). As reported in **Figure 4.9**, all strains strongly adhered to the acidic solvent chloroform, except for the Δ*ftsH* mutant strain which showed a lower affinity relative to the other strains. A general low affinity (about 10%) for the basic solvent ethyl acetate was observed for all the strains, confirming the widespread basic character of Lactobacilli cell surfaces (Pelletier et al, 1997). The average adherence to hexadecane ranged 40%, but a much lower affinity for this apolar solvent was evident for the Δ*ftsH* strain compared to the other strains.

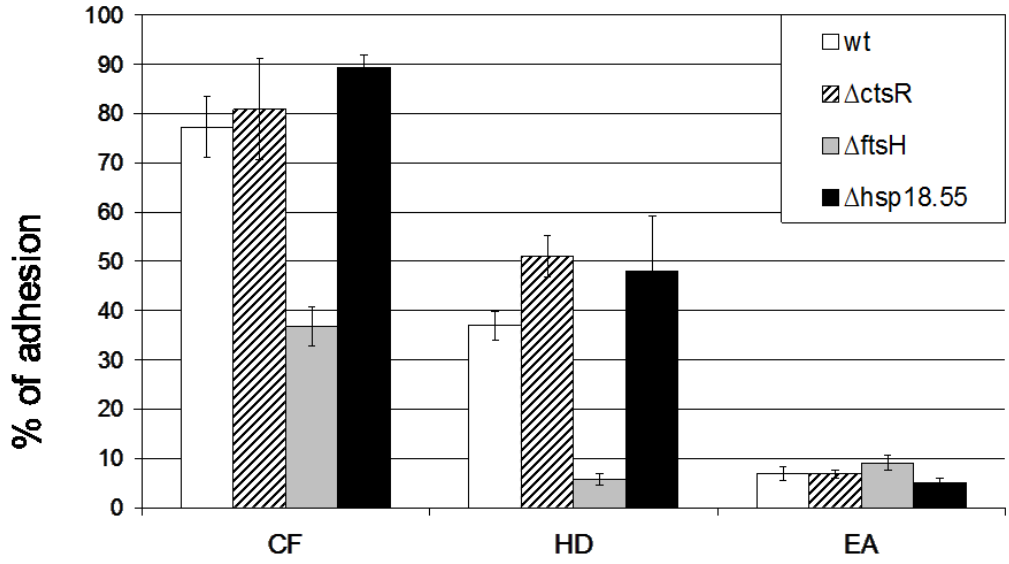


Figure 4.9. **Physico-chemical analysis** of the cell surface of *L. plantarum* wild type (white bars), Δ*ctsR* (hatched bars), Δ*ftsH* (grey bars) and Δ*hsp18.55* (black bars), respectively. Affinity was evaluated towards the organic solvents chloroform (CF), hexadecane (HD), and ethyl acetate (EA) and expressed as adhesion percentage. Values are mean and SE from at least three independent experiments.

4.2.3. Transcript profile of genes associated to probiosis.

In order to characterize the probiotic potential of the different *L. plantarum* strains, the transcriptional level of genes involved in probiotic interactions with the host, including adhesion/surface proteins and plantaricins, was analysed under physiologic conditions, in the different genetic backgrounds of the wild type and mutant strains of *L. plantarum* WCFS1 (**Table 4.1**). Overall, the Δ*ctsR* strain exhibited expression levels similar to those of wild type, differing only for a lower expression of the mannose adhesin gene (*msa*). By contrast, higher levels of *enoA1* were observed in both Δ*ftsH* and Δ*hsp18.55* mutants; in the latter strain a higher level of *mcra* mRNA was detected, too.

	Δ*ctsR*	**Δ*ftsH***	**Δ*hsp18.55***
mub	1.3 ± 0.3	1.1 ± 0.5	0.7 ± 0.1
mcra	1.3 ± 0.3	0.8 ± 0.3	**2.0 ± 0.4**
msa	**0.5 ± 0.1**	1.2 ± 0.5	1.3 ± 0.2
enoA1	0.9 ± 0.2	**3.6 ± 1.4**	**9.4 ± 1.7**
plnEF	0.6 ± 0.3	0.7 ± 0.3	1.5 ± 0.3

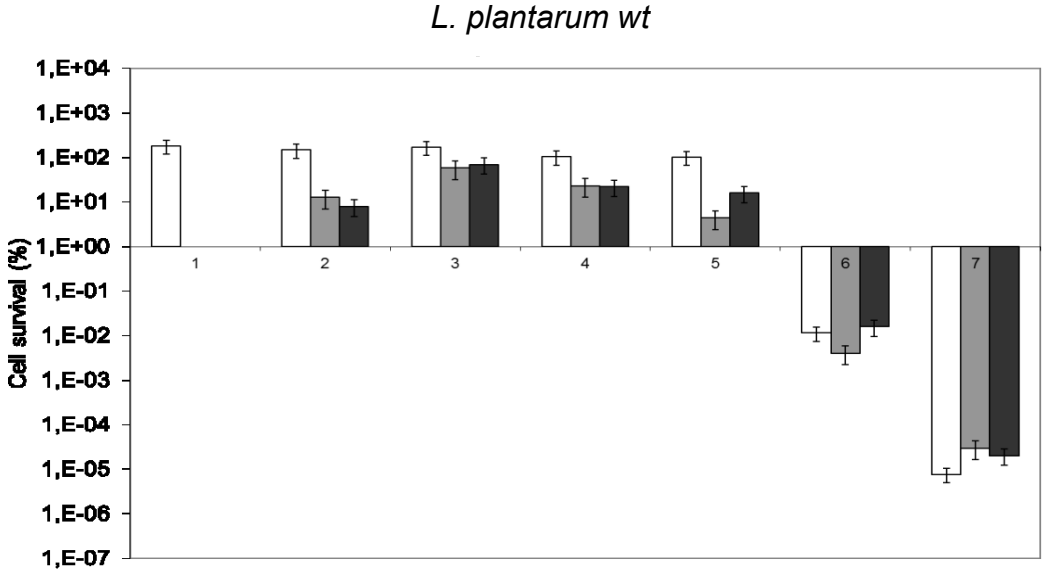
Table 4.1 Transcriptional pattern of probiotic genes in *L. plantarum* mutant strains as determined by qRT-PCR. mRNA levels are relative to those of wild type strain. *ldhD* was used as internal control. Values are mean and SD from 2 different experiments. Differential expression compared to wild type is highlighted in bold.

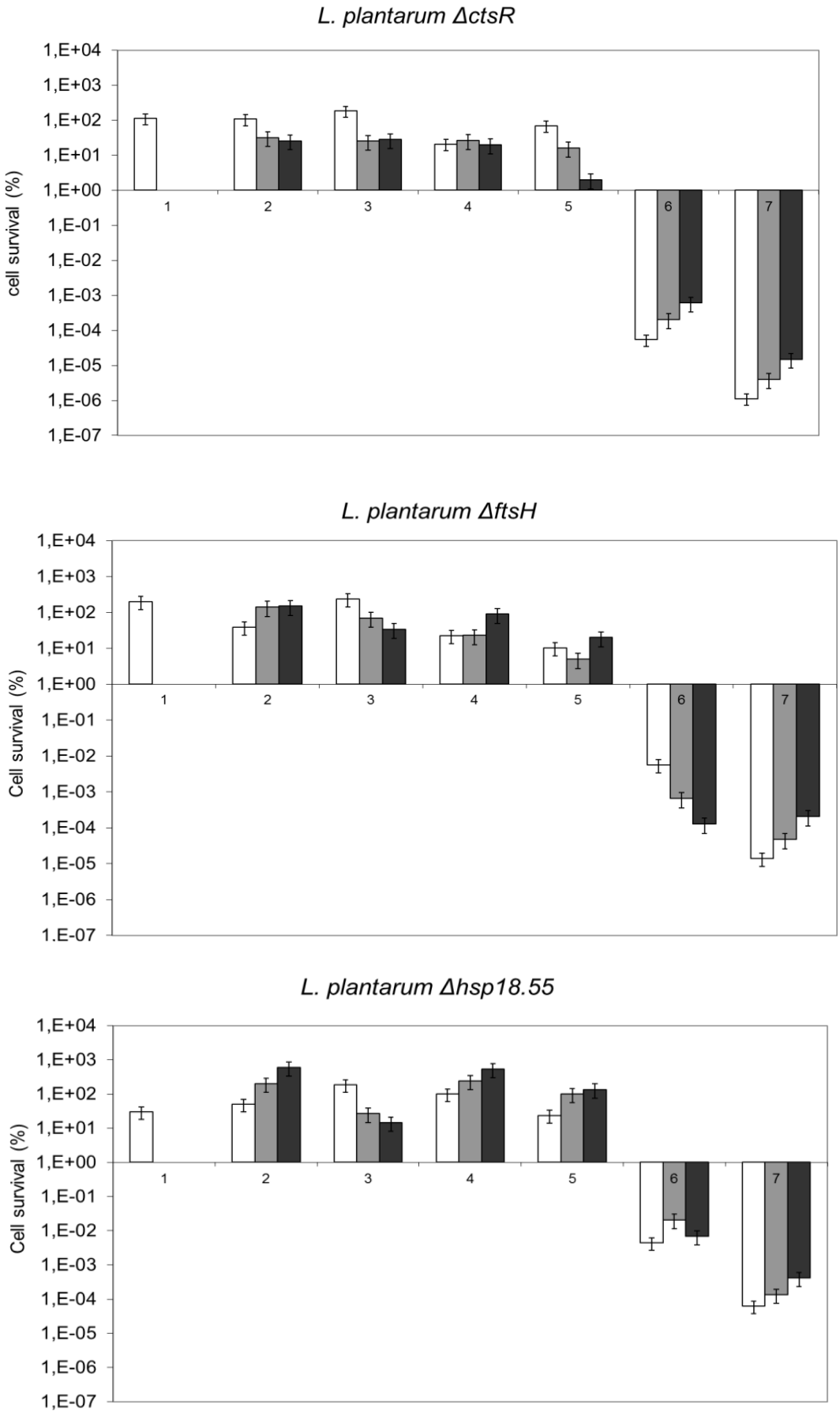
4.3. Development of an oro-gastrointestinal tract simulator.

4.3.1. Survival during the transit through the *in vitro* OGI tract model.

The tolerance of *L. plantarum* wild type and mutant strains, as well as of *L. acidophilus* LA-5 and *B. lactis* BB-12, to the oro-gastrointestinal tract conditions, was investigated by treating mid-log-phase bacteria according to the scheme depicted in **Figure 3.1**. This system reproduces the various steps of the transit of food bolus from the oral cavity into the stomach and small intestine. With respect to the model developed by Fernández de Palencia et al (2008), some major modifications were introduced: i) the oral stress was considered separately from the gastric one; ii) gastric conditions were simulated by progressive acidification with different incubation times to reproduce in detail the various phases of digestion; iii) every gastric step was then subjected to intestinal conditions including a first phase, mimicking the duodenum events, and a second phase simulating the progression of food along the terminal region of the small intestine.

Bacterial cells were included in a milk solution to consider the potential protective effect of an exemplar food matrix which is commonly used to vehicle probiotic microorganisms. The survival of each strain was evaluated by plate counts analysis and percentage survival is reported relative to untreated samples. As shown in **Figure 4.10,** all of the *L. plantarum* strains performed quite well in the first steps of the oro-gastric conditions: addition of lysozyme, pepsin action and progressive pH downshift from 6.5 to 3.0 were generally well tolerated by bacteria (oro-gastric stress, samples G1-G5).




L. plantarum ΔctsR
cell survival (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
1,E-03
1,E-04
1,E-05
1,E-06
1,E-07
1
2
3
4
5
6
7
L. plantarum ΔftsH
Cell survival (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
1,E-03
1,E-04
1,E-05
1,E-06
1.E-07
1
2
3
4
5
6
7
L. plantarum Δhsp18.55
Cell survival (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
1,E-03
1,E-04
1,E-05
1,E-06
1,E-07
1
2
3
4
5
6
7

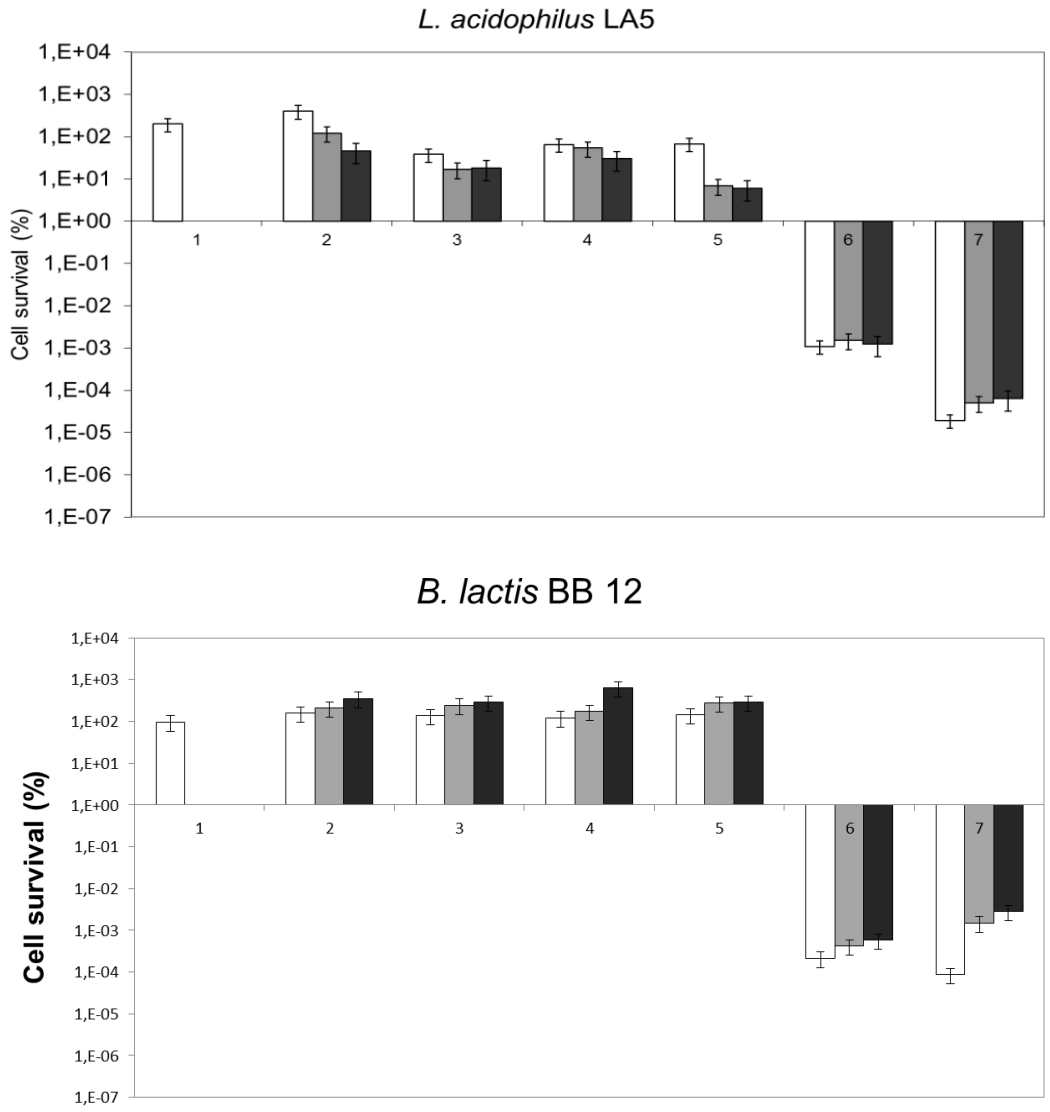


Figure 4.10. **Bacterial cell survival after simulated oro-gastrointestinal stress**. Viability was monitored after oro-gastric stress (samples **G1-7**, open bars) and after corresponding, subsequent 60 minutes (samples **I1G2-7**, grey bars) or 120 minutes (sample **I2G2-7**, black bars) intestinal stress, according to the scheme depicted in Figure 3.1 (see material and methods). Viability is expressed as a percent survival relative to untreated control (time zero, sample G0). The values represent mean and standard deviation of three different experiments.

L. plantarum wild type strain slightly increased (relative to control) in the first 3 samples (G1-G3) and then remained in the same order of magnitude till pH 3.0 was reached (sample G5).

L. plantarum Δ*ctsR* viability resulted stable in the first 3 steps (samples G1-G3), while *L. plantarum* Δ*ftsH* exhibited a tendency to grow in the first and third samples (G1, G3), with a temporary reduction after addition of pepsin (G2); for both mutants, a decreased viability (approximately 1 log reduction for the Δ*ftsH* strain) was observed in samples G4-G5,

relative to control, indicating a major susceptibility than wild type under the same conditions.

Lysozyme and pepsin seemed to negatively affect the growth of Δ*hsp18.55* mutant only (compared to other strains) although a recover was observed during subsequent pH downshift, as well as in the following duodenal conditions. Incubation at pH 3.0 for 30 minutes (sample G5) especially reduced viability of Δ*ftsH* and Δ*hsp18.55* mutants.

L. acidophilus LA-5 population increased between G1 and G2, suggesting that the presence of a rich medium (milk) stimulates growth even in the presence of digestive enzymes (lysozyme and pepsin) but under pH conditions (6.0) which are optimal for members of this bacterial species; a 1 log reduction in *L. acidophilus* LA-5 cell viability was detected in subsequent steps from G3 to G5.

B. lactis BB-12 strain did not seem to suffer the pH downshift from G1 to G5. The bacterial population remained stable without any obvious decrease of initial titre.

A general drastic drop of vitality was observed for all the *L. plantarum* strains, *L. acidophilus* LA-5 and *B. lactis* BB-12 when pH values shifted from 3.0 to 2.0 and then to 1.5 (sample G6, G7), with incubation periods of 30 and 10 minutes, respectively. The capability to form colonies was reduced by 4 and 8 log units for *L. plantarum* wild type, 7 and 8 log units for Δ*ctsR*, 5 and 7 log for Δ*ftsH*, Δ*hsp18.55*, and *L. acidophilus* LA-5, and 6 and 7 log units for *B. lactis* BB-12.

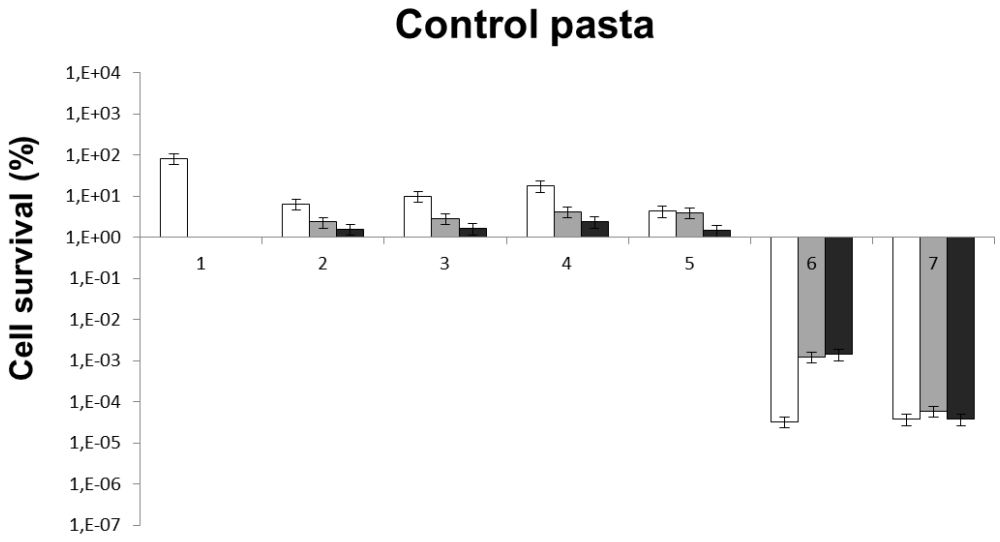
When gastric-treated samples were further incubated in conditions resembling the duodenal environment (pH value up-shifted to 6.5, addition of specific electrolyte solution containing bile and pancreatin: samples I1G2-7), either no or 1-log reduction in cell viability was generally detected for *L. plantarum* wild type, Δ*ctsR* and Δ*ftsH* strains, with respect to corresponding gastric samples. In these conditions, the Δ*hsp18.55* strain was generally more tolerant. However, a tendency to recover viability was noticed for bacterial samples deriving from the last two steps of gastric stress (I1G7 wild type, I1G6-7 Δ*ctsR*, I1G7 Δ*ftsH*, I1G6-7 Δ*hsp18.55*), indicating that neutralization of the strongly acidic pH, although accompanied by pancreatin and bile salts, alleviates bacterial stress and allows for a moderate cell proliferation. *L. acidophilus* LA-5 seemed to moderately recover viability upon duodenal incubation following the most acidic gastric stages (samples I1G6-7 and I2G6-7). Regarding *B. lactis* BB-12, the amount of bacteria recovered from all duodenal samples (I1G2-G7) was almost equal to that resulting from the gastric ones (G2-G7). It was detected only a 1-log reduction at I1G7 point.

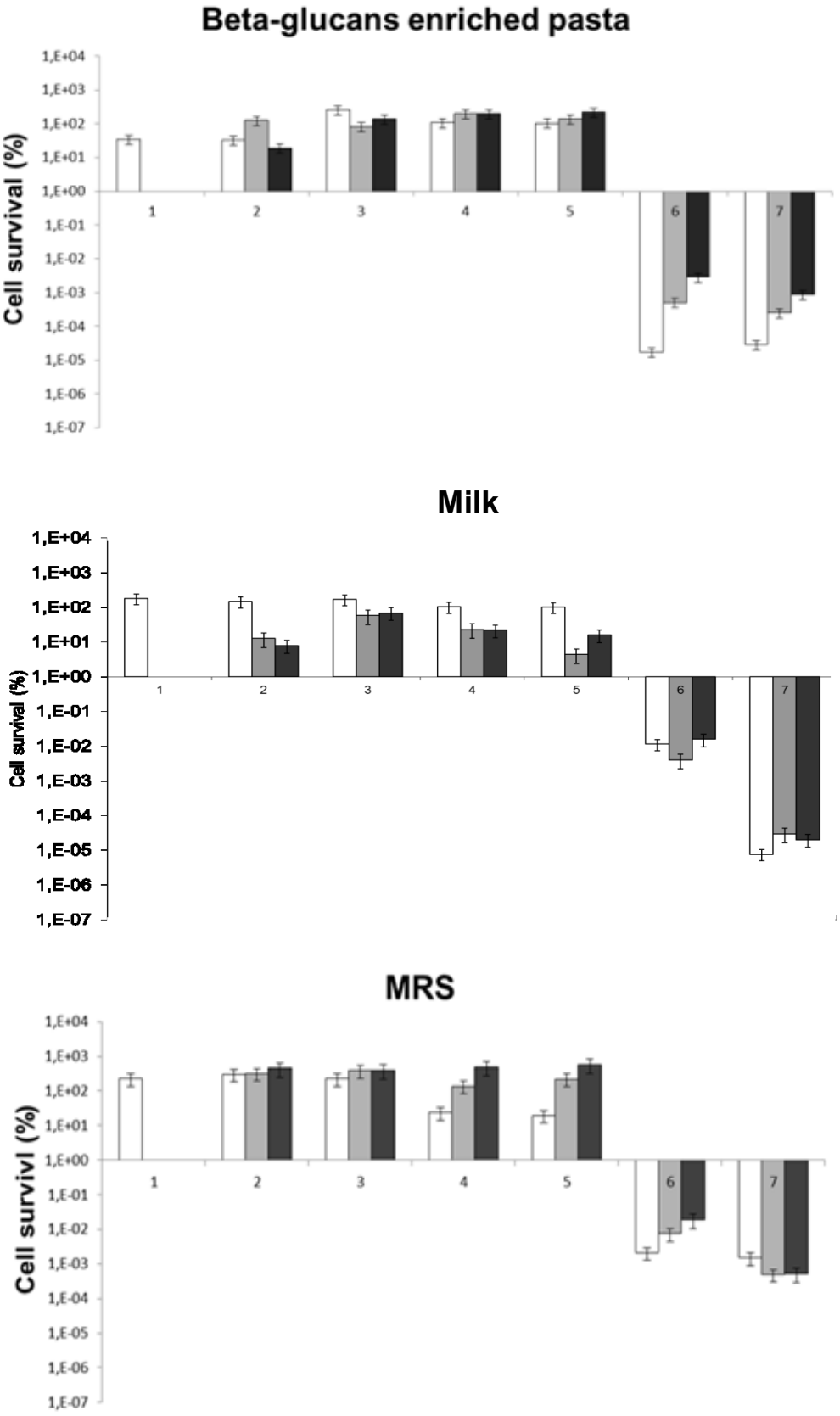
Cell survival was also evaluated after 1 hour further incubation in diluted duodenal secretions (samples I2G2-7) in order to simulate conditions in the final tract of the small intestine. According to our data, these conditions did not affect cell survival with respect to the previous incubation conditions. A tendency to a slight increase in viability was noticed particularly for the Δ*ftsH* (I2G-4,5,7) and Δ*hsp18.55* (samples I2G-2,4,5,7) strains, indicating a good tolerance to the intestinal conditions. The percent survival of *L. acidophilus* LA-5 in response to post-duodenal conditions remained approximately stable. The percent survival of *B. lactis* BB-12 showed a 1-log increase in I2G4 point and only a 1-log reduction in I2G7 point, respectively, compared to relative gastric points (G4 and G7).

4.3.2. Matrix effect on the viability of *L. plantarum* WCFS1 wild type during the transit in the oro-gastrointestinal tract model.

Bacterial cells of *L. plantaru*m wild type were included in different vehicle matrices and subject to the simulated oro-gastrointestinal tract in order to evaluate and compare their protective effects. Milk is not the unique food matrix that can be used for dietary supplementation of probiotics; therefore, other possible food matrices such as ordinary pasta, beta-glucan-enriched pasta, MRS (ordinary LAB culture medium) and saline solution (0.85% NaCl) were considered, too. Bacterial cells from mid-late-log-phase were mixed with matrices and challenged with the OGI transit; cell survival was evaluated by plate counts analysis and percentage survival was reported relative to untreated samples.

Figure 4.11 reports the various transit assays of *L. plantarum* WCFS1 wild type in the simulated oro-gastrointestinal tract, using different matrices.




Beta-glucans enriched pasta
Cell survival (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
1,E-03
1,E-04
1,E-05
1,E-06
1,E-07
1
2
3
4
5
6
7
Milk
Cell survival (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
1,E-03
1,E-04
1,E-05
1,E-06
1,E-07
1
2
3
4
5
6
7
MRS
Cell survivl (%)
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
1,E-02
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2
3
4
5
6
7

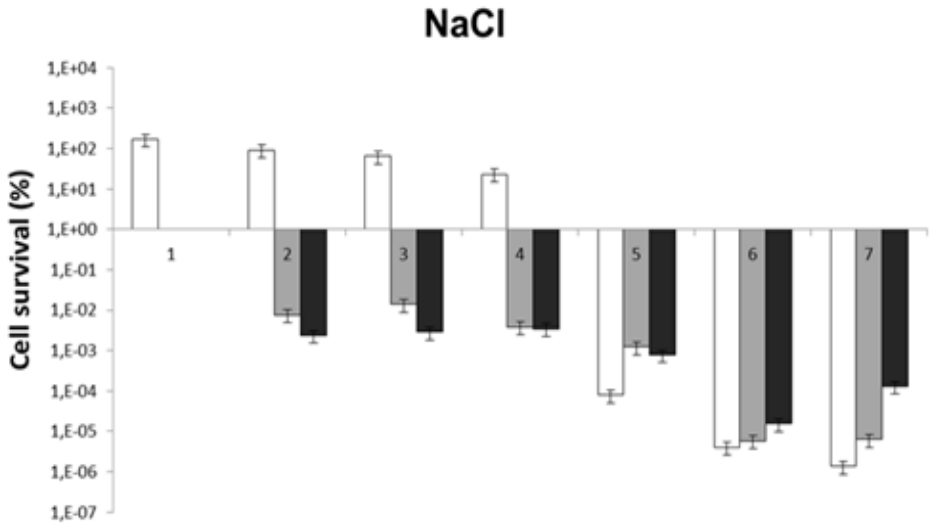


Figure 4.11. Survival of *L. plantarum* WCFS1 wild type along the OGI transit, in different vehicle matrices, including pasta, β-glucans-enriched pasta, milk, MRS and 0.85% NaCl solution.

When comparing the effect of ordinary and beta-glucans-enriched pasta, a major resistance to gastric and intestinal stresses was noticed with the enriched pasta, although *L. plantarum* WCFS1 gave exhibited similar tolerance to lysozyme stress (G1) in both systems. The samples recovered from G2 to G5 gastric points of bacterial cells treated in control pasta showed a higher reduction with respect to samples included in beta-glucan-enriched pasta. For both matrices, the survival was kept on the same order of magnitude in G6 and G7 points. A drastic decrease in cell survival (1-2-log units) was observed among the samples stressed in control pasta, in comparison to those stressed in beta-glucan-enriched pasta.

With regards to survival of *L. plantarum* cells mixed with MRS medium, an appreciable tolerance all along the OGI tract was observed. In the first points, G1-G3, I1G1-G3 and I2G1-G3, the bacteria did not exhibit evident changes in their survival. However, a 1-log unit reduction was displayed in both G4 and G5 steps, improving overall of about 1-log unit in the duodenal shift and in the last intestinal tract, respectively. In addition, the lowering of pH to 2.0 and 1.5 in the stomach, did not seem to affect severely the bacterial viability; also pancreatin and bile salts stresses (I1G6-G7) as well as the long incubation (120 minutes) in the last intestinal tract (I2G6-G7), did not cause a drastic decrease in bacterial population.

The survival in saline solution was also valuated. Saline solution was considered as a negative control in testing the food matrices. In fact, at moderate acid value of pH 3.0 (G5), the cell survival reduced greatly of about 6-log units. It remained constant in the G2, G3 and

G4 points of gastric stress, then gradually dropped in the final part of stomach (G5, G6 and G7), reducing of 8-log units. Furthermore, the survival reduction was more evident in the intestinal sector: in contrast to what observed for other matrices, bacteria were not able to recuperate even after the passage from pH 6.0 of the stomach (G2) to pH 6.5 of the intestine (I1G2 and I2G2). At this point, cell survival lost 4-log unit. In the segments I1G2-G5 and I2G2-G5 the bacterial titer was about 3-log units lower negatively, while dropped 2-log units in I1G6-G7 tracts and recovered 1-log unit in I2G6-G7.

It is apparent that the saline solution does not allow a good tolerance to various stresses which the bacterium manages during the simulated transit. Conversely, the beta-glucans-enriched pasta, the MRS medium and the milk provide to microorganism good resistance in the acid environment; in particular MRS-delivered microrganism tolerates better both pH 2.0 and 1.5, than in pasta and milk. Moreover, in the intestinal segments, MRS-delivered bacteria grow well perhaps because both intestinal and MRS pH are nearly equal. In milk, the microorganisms seem to suffer the stress conditions, mainly in the intestinal tract. This could be supported by potential hydrolysis and emulsification of milk components which might lose their original conformation and inhibit bacterial growth.

Overall, beta-glucans-enriched pasta offers a good level of survival in all areas of the oro-gastrointestinal tract.

4.3.3. Molecular response of the bacteria to the stress conditions of the OGI tract simulator.

When a microorganism manages any environment in which stress events occur, it implements a series of molecular responses necessary to overcome stress and restore physiological conditions. In the specific case of the simulated oro-gastrointestinal tract, we analyzed the probiotic character of *L. plantarum* WCFS1, detecting the expression profile of clp proteases (*clpB*, *clpE*, *clpP*), small heat shock proteins (*hsp18.5*, *hsp18.55*, *hsp19.3*), stress controlling factors *ctsR* and *ftsH*, molecular chaperones (*groEL*, *dnaK*) and probiosis genes (*enoA1*, *mub*, *mcra*, *msa*, *plnEF*) possibly involved in enhancing colonization of the intestinal mucosa and competition events. In particular, as shown in **Figures 4.12**, **4.13**, **4.14**, **4.15**, **4.16**, we considered mRNAs from bacterial samples exposed to lysozyme (point G1), simulated gastric environment pH 5.0 (G3) and relative duodenal stress (point I1G3), gastric compartment pH 3.0 (G5) and relative duodenal stress (point I1G5), respectively

(**Figure 3.1**). Gene expression was evaluated by real time qRT-PCR and the constitutive *ldhD* gene was used as an internal control.

A particularly strong induction of stress-related genes was noticed, especially at the gastric pH 3.0 (point G5). At this level, the gene induction was about 150-fold respect to the sample control. **Figure 4.12** reports the pattern expression of *clp* genes. All genes presented a low induction at points G1, I1G3 and I1G5 of the oro-gastrointestinal simulator, in the range of about 5-fold. In the point G3, gene induction was detected in the range 10-15-fold. Both *clpE* and *clpP* genes shown the highest up-regulation at point G5, 50-fold and 170-fold, respectively.

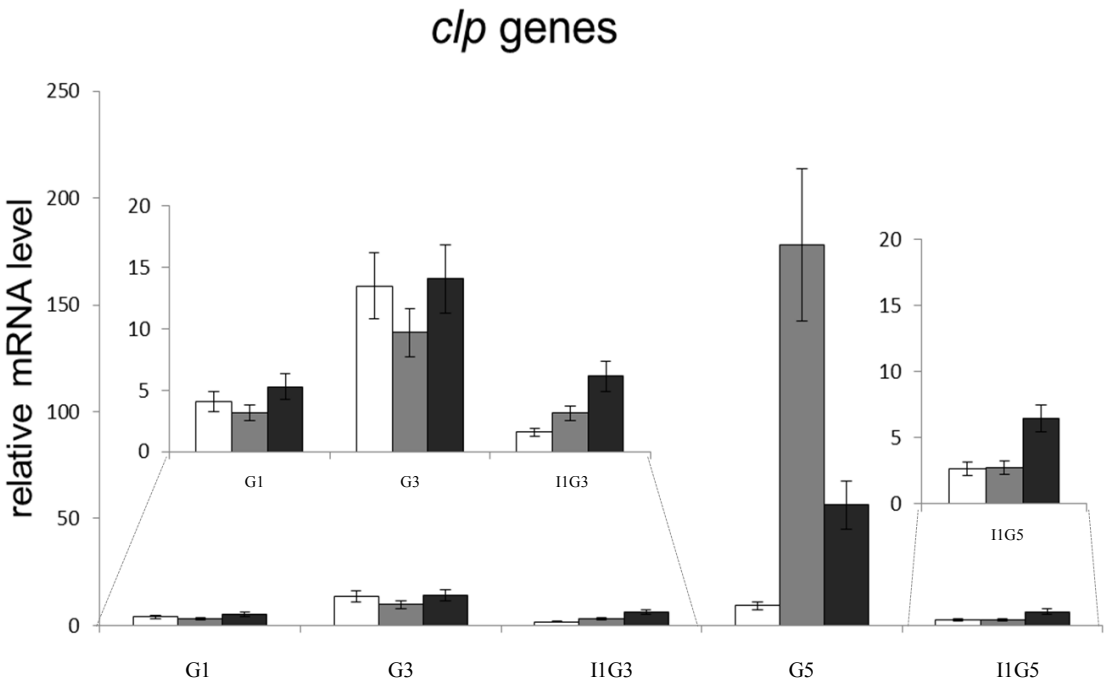


Figure 4.12. **Transcriptional pattern of *clp* genes** in *L. plantarum* WCFS1 cells challenged with lysozyme (G1), gastric pH 5 (G3) and relative duodenal stress (I1G3), gastric pH 3 (G5) and relative duodenal stress (I1G5). Gene expression was analysed by qRT-PCR. mRNA levels are related to that of unstressed bacterial cells and normalized using *ldh* gene as internal control. Genes encoding clpB (white bars), clpE (grey bars) and clpP (black bars). The data are the averages and standard deviations of three independent experiments.

The expression level of *shsp* genes is summarized in **Figure 4.13**. The *shsp* genes were induced slightly at points G1, I1G3 and I1G5: average values of expression level were maintained at around 10-fold, from a maximum of 20-fold to a minimum of 0-fold. An induction (about 20-fold) for *hsp18.5* gene was observed at point I1G3. The expression level of *hsp18.5* increased of about 50-fold at pH 5.0 of stomach (point G3), up to about 150-fold at pH 3.0 (point G5); the *hsp18.55* gene was induced at pH 3.0 (75-fold); the *hsp19.3* gene raised from 25-fold of point G3, to 45-fold of point G5.

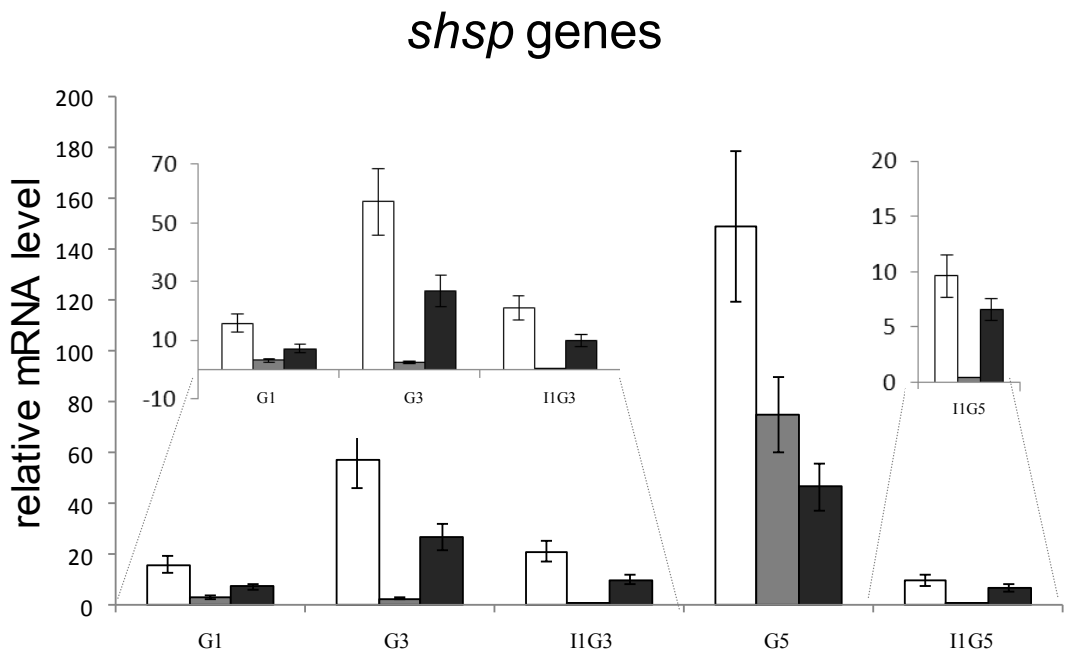


Figure 4.13. **Transcriptional pattern of s*hsp* genes** in *L. plantarum* WCFS1. Genes encoding sHSP18.5 (white bars), sHSP18.55 (grey bars) and sHSP19.3 (black bars). The data are averages and standard deviations of three independent experiments.

The transcriptional pattern of both *groEL* and *dnaK* genes is reported in **Figure 4.14**. Neither the lysozyme stress (point G1), nor the intestinal stresses (points I1G3, I1G5) produced considerable induction for these genes: it was possible to appreciate a weak induction of the order of 5-fold. However, under the gastric stress, these genes were significantly up-regulated. In particular, *groEL* gene was induced of 20-fold at pH 5.0 (point G3) and reached an induction of 80-fold at point G5; whereas *dnaK* gene shown a slight induction (10-fold) and then improved up to 110-fold.

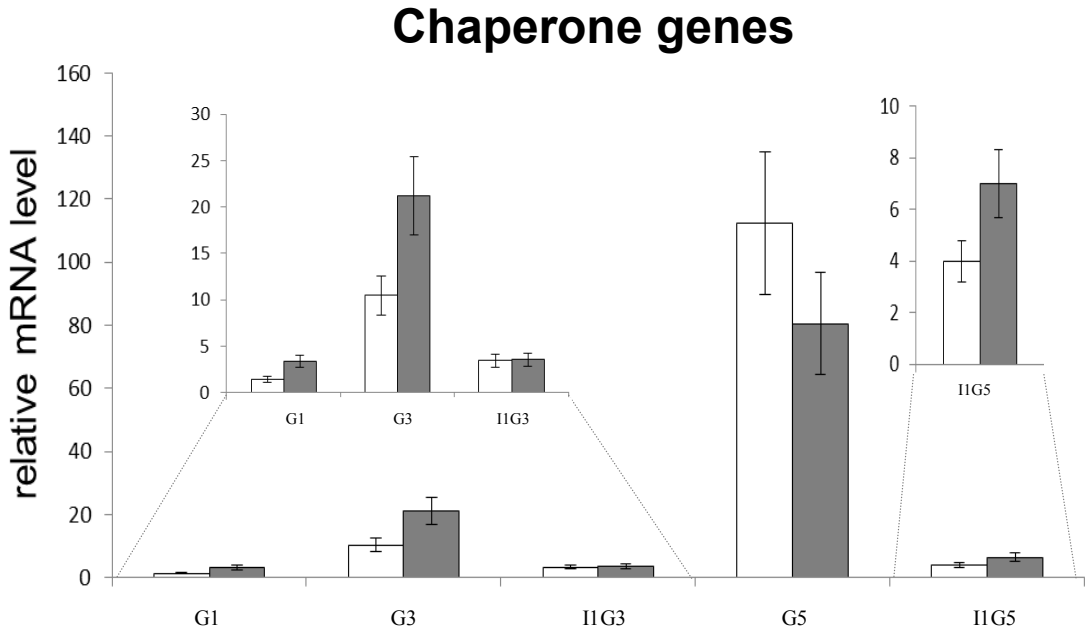


Figure 4.14. **Transcriptional pattern of chaperone genes** in *L. plantarum* WCFS1. Genes encoding DnaK (white bars) and GroEL (grey bars). The data are averages and standard deviations of three independent experiments.

Figure 4.15 shows that the *ctsR* gene was induced about twice with respect to *ftsH* gene, in lysozyme stress (point G1). In the gastric segments (points G3, G5), the *ftsH* gene level was lower compared to that of *ctsR* gene, which was expressed 20-fold and 25-fold at pH 5.0 and 3.0, respectively. Low expression was observed for both genes in the intestinal tracts (I1G3, I1G5). The high level of *ctsR* transcription may indicate the turning off stress response by cell in order to avoid an indiscriminate synthesis of stress proteins.

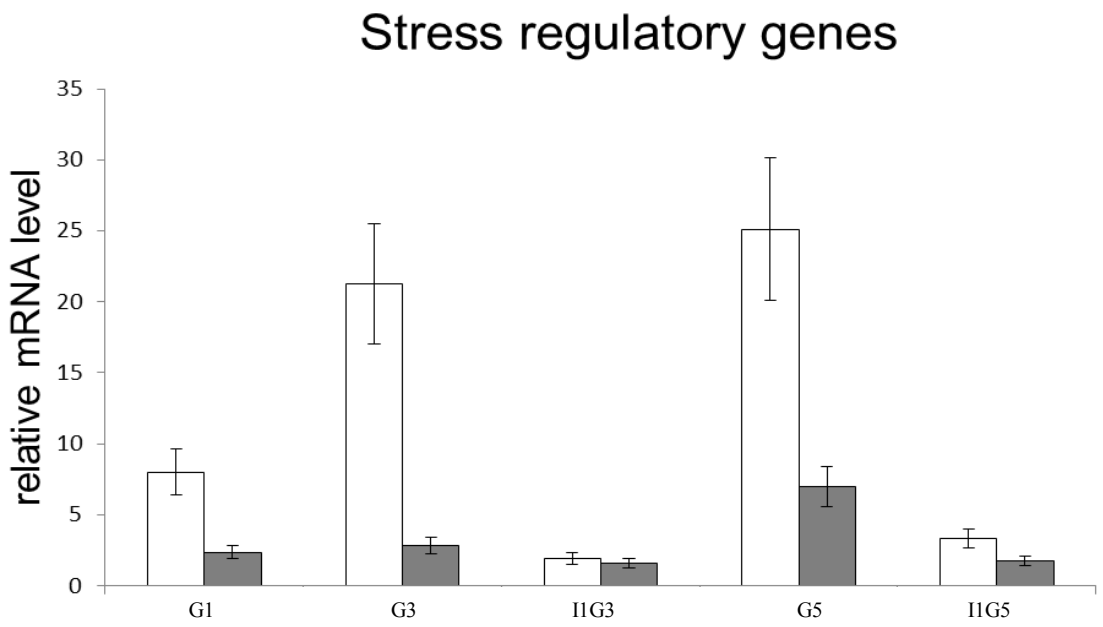


Figure 4.15. Transcriptional pattern of stress regulatory genes in *L. plantarum* WCFS1. Genes encode for CtsR (white bars) and FtsH (grey bars). The data are the averages and standard deviations of three independent experiments.

To analyze the probiotic traits of *L. plantarum* WCFS1 through the oro-gastrointestinal tract, a real time qRT-PCR was performed on genes involved in bacterial binding to host epithelial surface and competition phenomena. The expression level of *enoA1* (enolase), *mub* (mucus binding protein), *mcra* (myosin cross reactive antigen) homologous to an adhesion protein found in *L acidophilus* (O'Flaherty and Todd, 2010), *msa* (mannose adhesin) and *plnEF* (plantaricins EF) genes is represented in **Figure 4.16**. In general, all genes were induced (about 5-fold) by lysozyme stress (point G1), with the exception of *enoA1* gene whose level was about 2.5-fold induced. Both *msa* and *plnEF* were the most expressed genes at point G3 of the oro-gastrointestinal simulator (30-fold); then the

expression level reduced at point G5 (approximately 5-fold). *enoA1* gene displayed the maximum peak of induction at point G5 (about 10-fold). *mub* gene exhibited the same expression level in both gastric conditions, at pH 5.0 and 3.0, with a slight trend to decrease from point G3 to point G5 (from 16- to 12-fold). *mcra* gene also showed the same expression level in both points G3 and G5, as *mub* gene, nevertheless with a slight trend to increase (from 8- to 13-fold). The intestinal stresses (points I1G3 and I1G5) did not give significant induction of either gene.

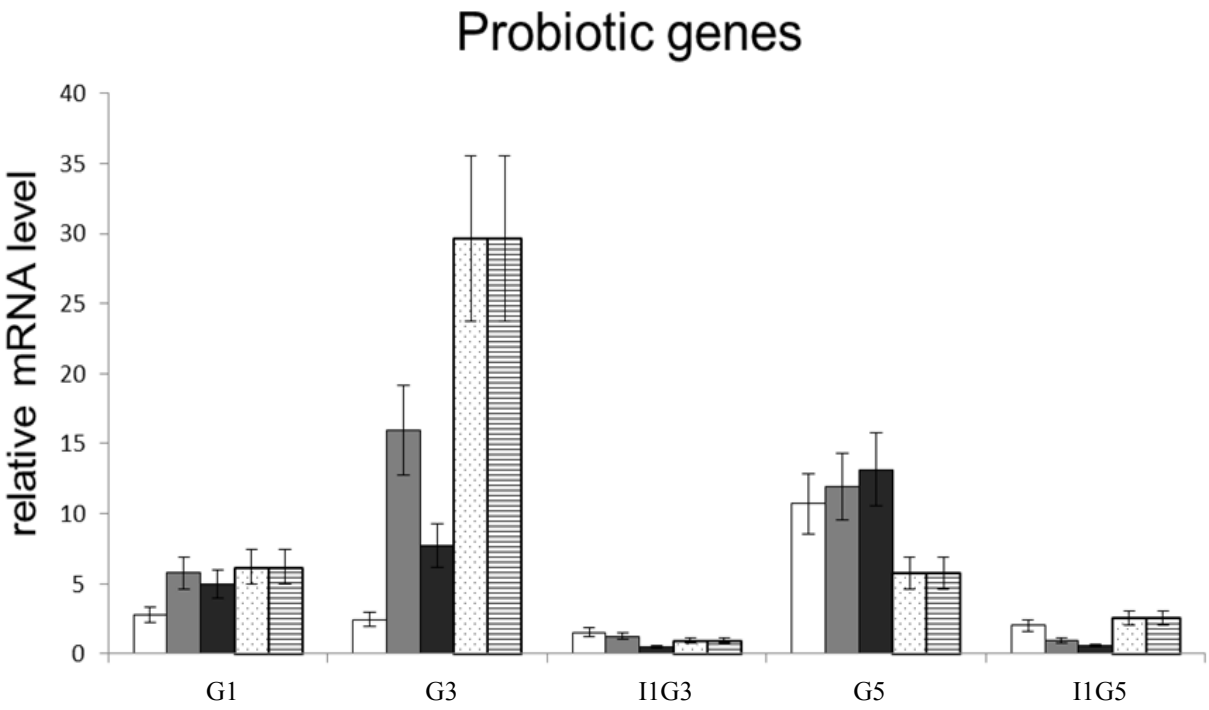


Figure 4.16. **Transcriptional pattern of probiotic genes** in *L. plantarum* WCFS1. Genes encoding EnoA1 (white bars), Mub (grey bars), Mcra (black bars), Msa (dotted bars) and PlnEF (horizontally striped bars). The data are the averages and standard deviations of three independent experiments

4.4. Interaction with the host cells.

4.4.1. Differentiation of Caco-2 cell monolayers.

In order to verify that the adhesion and interaction assays were conducted on effectively polarized and differentiated Caco-2 cells monolayers, the value of Trans Epithelial Electrical Resistance (TEER) was monitored and the expression level of *LL37/hCAMP18* gene was evaluated.

The confluence of the cellular monolayer is quickly recognized by a sharp increase in TEER due to the tightening of intercellular interactions and adhesive junctions (Delie and Rubas, 1997). Starting with the same titer at which the Caco-2 cells were seeded for the adhesion assays, but inoculating the cells in transwell membranes, the TEER value (Ω cm^2) was monitored constantly over a period of 20 days in (**Figure 4.17**). From the fifth day of culture, the TEER value increased linearly to reach a peak level of ~500 Ω cm^2 at day 13 and then kept steady (around values of 400 Ω cm^2) from day 15 to 22.

Normally differentiated, polarized Caco-2 cells have a TEER value of >200 Ω cm^2. Typical TEER readings for a 21 days Caco-2 cell monolayers is about 300-500 Ω cm^2 (MacCallum et al, 2005). Therefore our Caco-2 cell monolayers reached a good degree of differentiation.

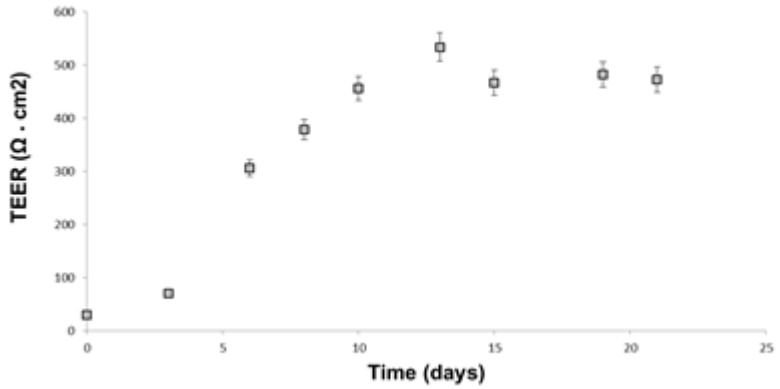


Figure 4.17. **Change of trans epithelial electrical resistance (TEER) of Caco-2 monolayers, grown on Transwell membranes**. Blank electrical resistance value were usually 30 Ω cm^2. Error bars represent standard error of the means.

The expression of *LL-37/hCAP18* by human colon epithelial cells is considered a marker of intestinal epithelial cell differentiation (Hase et al, 2002). Indeed, the expression level of *CAMP* gene, coding the cathelicidin LL-37/hCAP18, was analysed by real time qRT-PCR in 12-15 days old cultures of Caco-2 to confirm their differentiation (data not shown).

4.4.2. Adhesion of bacterial cells to human intestinal epithelial cells.

The adhesion properties of *L. plantarum* wild type and mutant strains were evaluated by CFUs count analysis and compared to that of the probiotics *L. acidophilus* LA-5 and *B. lactis* BB-12 strains, which were previously shown to possess good adhesive characteristics (Fernández de Palencia et al, 2008), and therefore served as positive controls.

Quite a variable adhesion capability was observed (**Figure 4.18**), with the wild type *L. plantarum* strain exhibiting the lowest adhesion (~70 bacterial cells/Caco-2 cell) whereas Δ*ctsR*, Δ*ftsH* and Δ*hsp18.55* mutant strains exhibited higher adhesion level (average values of 420, 120 and 226 bacterial cells/Caco-2 cell, respectively) with respect to the parental strain. Remarkably, Δ*ctsR* adhesion capacity was comparable to that of the probiotic *L. acidophilus* LA-5 (410 bacterial cells/Caco-2 cell). Among all tested bacterial strains, *B. lactis* BB-12 displayed the highest adhesion rate (670 bacterial cells/Caco-2 cell), confirming its well-known probiotic properties. The large size of the intestinal epithelial cells as well as phenomena of inter-bacteria adhesion may account for the relatively high number of adherent bacteria.

Based on previous works (Candela et al, 2005) and according to our data, we can define all the analyzed strains as strongly adherent to Caco-2 cells.

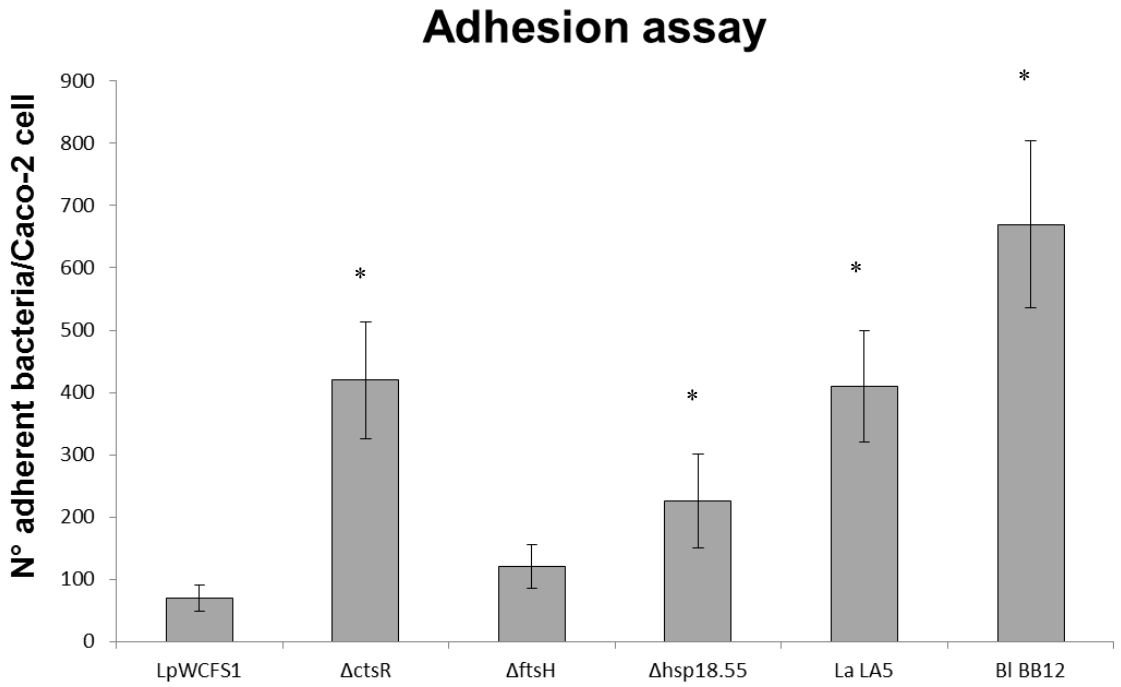


Figure 4.18. **Bacterial adhesion to Caco-2 cell monolayers**. The number of adherent bacterial cells from *L. plantarum* WCFS1, Δ*ctsR,* Δ*ftsH,* Δ*hsp18.55*, *L. acidophilus* LA-5 and *B. lactis* BB-12 strains was determined by CFU analysis. The values represent means and standard deviation of at least two different experiments performed in triplicates. * $P < 0.05$ compared with *L. plantarum* wild type.

4.4.3. Effect of beta-glucans addition on bacterial adhesion to Caco-2 cells.

We further evaluated the adhesion propensity of *L. plantarum* WCFS1 to Caco-2 cells, considering the potential prebiotic activity of exopolysaccharides. In detail, we studied the adhesion propriety of *L. plantarum* WCFS1 cells alone or supplemented with 0.5% (w/v) β-glucans (**Figure 4.19**). In presence of β-glucans, the adhesion level (about 130 bacteria per Caco-2 cell) increased approximately 2-fold with respect to control bacterial cells (about 50 bacteria per Caco-2 cell).

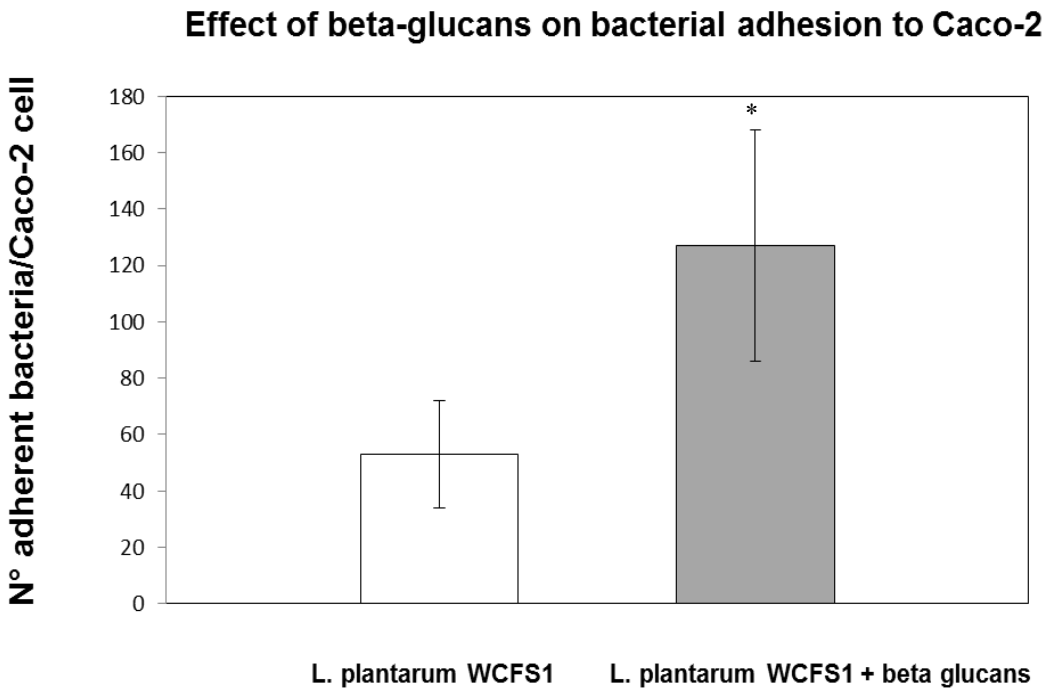


Figure 4.19. ***L. plantarum*** **WCFS1 adhesion to Caco-2 cell monolayers**. The number of adherent control bacterial cells (white bar) and β-glucans-mixed bacteria (grey histogram) was determined by CFUs analysis. The values represent means and standard deviation of at least two different experiments performed in triplicates. * $P < 0.05$ compared with absence of β-glucans .

4.4.4. Expression of immune-related genes in Caco-2 cells upon interaction with cells from different *L. plantarum* strains.

To study the interaction of *L. plantarum* with human enterocytes, and to consider possible strain-dependant differences in such interaction, Caco-2 cell monolayers were co-incubated with either live or heat killed bacterial cells from *L. plantarum* wild type and mutant strains. The growth of co-incubated live bacteria was monitored over time and found to increase by approximately 2 log units (**Figure 4.20**). During the 5-hours incubation with live bacteria, pH conditions turned acidic, as evidenced by the changing colour of the

phenol-red indicator in the growth medium (data not shown). Acidification was observed only in presence of live bacteria.

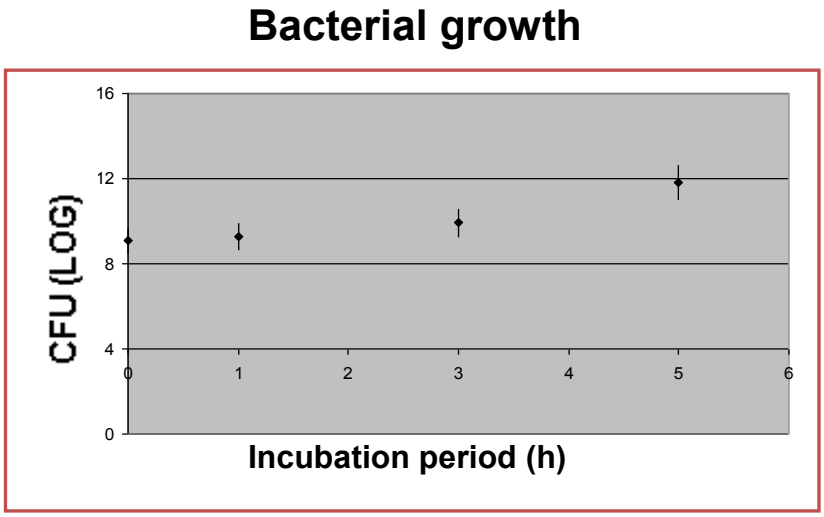
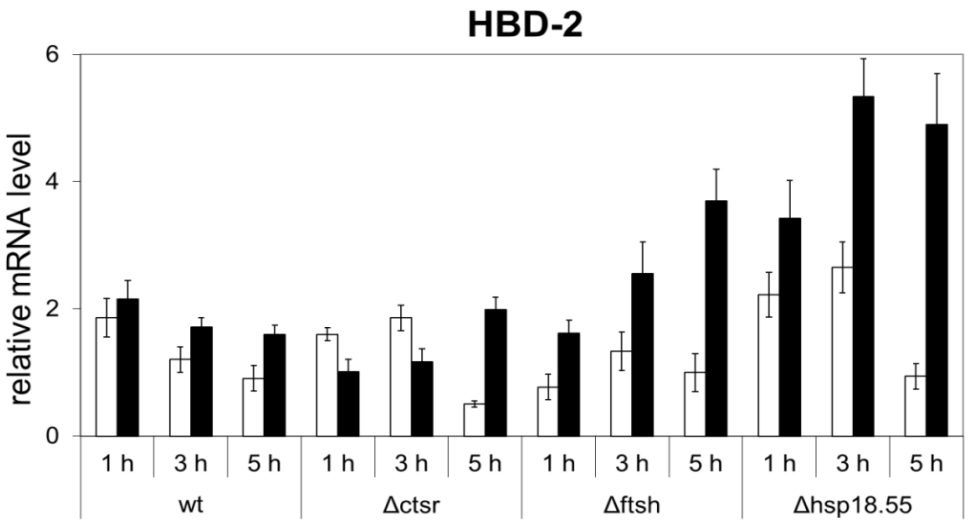
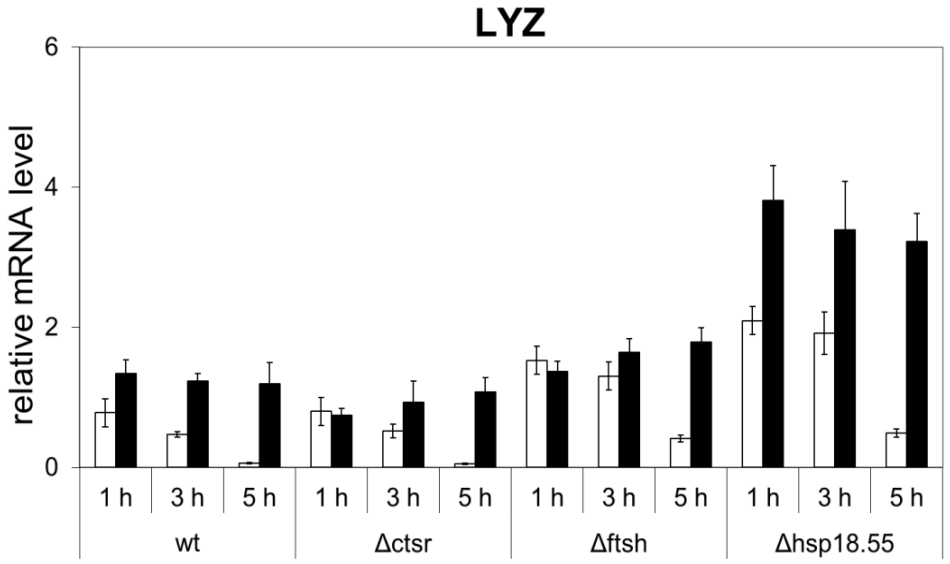
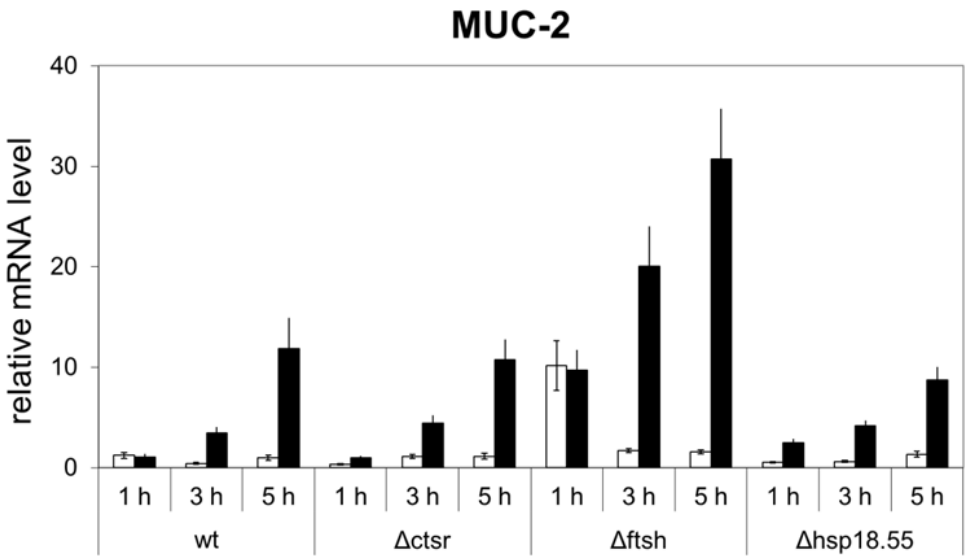


Figure 4.20. **Growth of live bacteria during co-incubation with Caco-2 cells**. Growth was monitored by CFUs analysis at time 0 and after 1, 3 and 5 hours since co-incubation started. Data represent the average titre ± standard deviations of *L. plantarum* wild type, Δ*ctsR*, Δ*ftsH* and *Δhsp.18.55* strains.

Moreover, the effect of such co-incubation was evaluated by investigating the expression of a set of immune-related genes, including those involved in raising the physical-chemical protective barrier of the intestinal mucosa (e.g., genes coding for the antimicrobial peptide human β-defensin-2, for the microbicidal enzyme lysozyme and for the mucous component mucin-2) and in immune modulation (e.g., genes encoding the proinflammatory cytokines IL-6, IL-8 and the C-C chemokine MIP-3α). The time-response of immune genes transcriptional pattern was examined by real time qRT-PCR upon treatment of Caco-2 cells, with defined titre of either live or heat-inactivated cells from *L. plantarum* wild type, Δ*ctsR*, Δ*ftsH* and Δ*hsp18.55* strains, used separately (**Figure 4.21**).




LYZ
relative mRNA level
6
4
2
0
1 h
3 h
5 h
wt
Δctsr
Δftsh
Δhsp18.55


MUC-2
relative mRNA level
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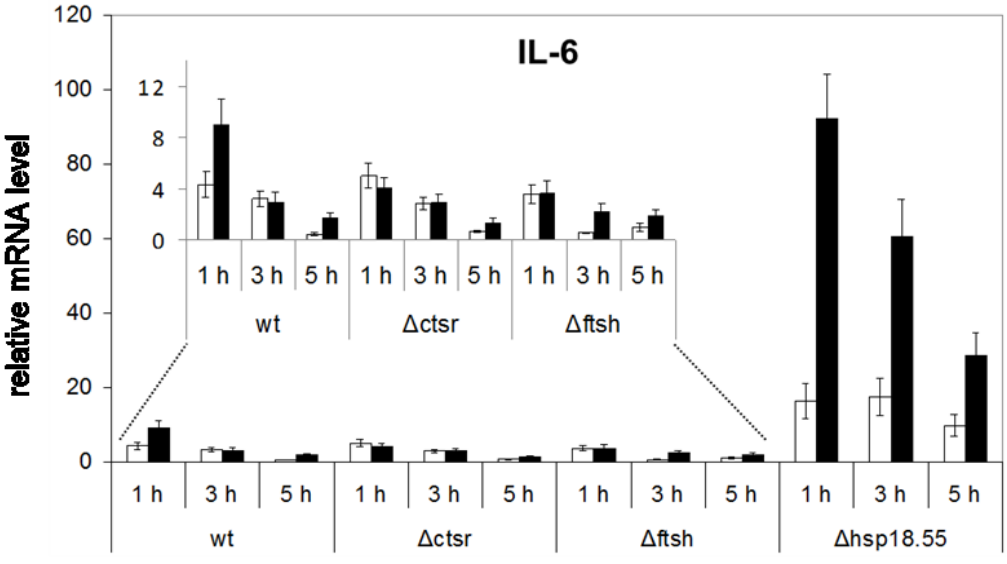
30
20
10
0
1 h
3 h
5 h
wt
Δctsr
Δftsh
Δhsp18.55


IL-6
relative mRNA level
120
100
80
60
40
20
0
12
8
4
0
1 h
3 h
5 h
wt
Δctsr
Δftsh
Δhsp18.55

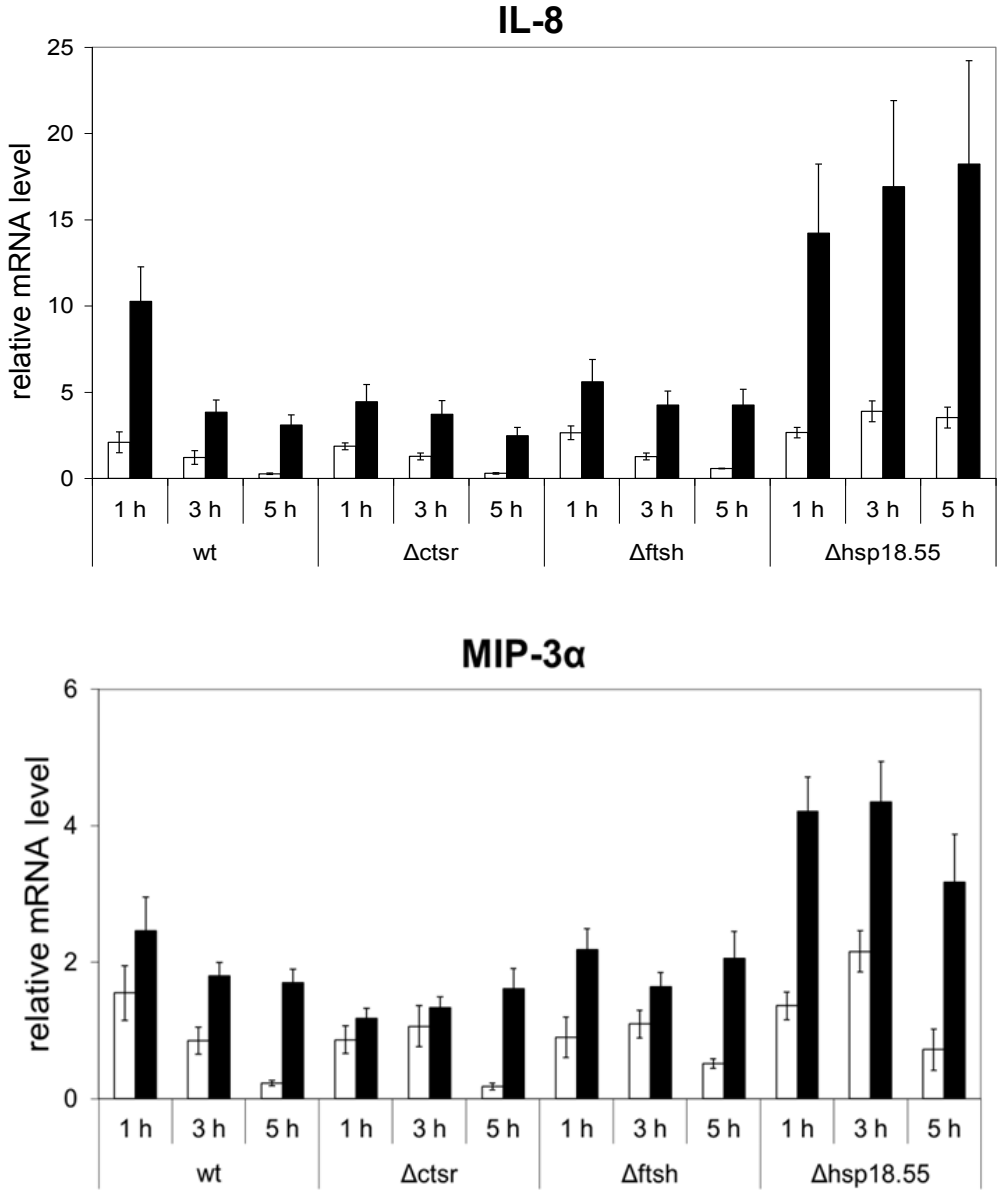


Figure 4.21. **Transcriptional pattern of immune-related genes in Caco-2 cells challenged with bacterial cells from wild type (wt), ΔctsR, ΔftsH, and Δhsp18.55 strains of L. plantarum**. Caco-2 monolayers were stimulated for 1, 3 and 5 hours with either live (white bars) or heat inactivated (black bars) bacterial cells. Gene expression was analysed by real time qRT-PCR. mRNA levels are relative to that of unstimulted Caco-2 cells and normalized using GAPDH as internal control. Genes encoding cytokine IL-6, chemokine IL-8, macrophage inflammatory protein 3α (MIP-3α), human β-defensin-2 (HBD-2), lysozyme (LYZ) and mucin-2 (MUC-2) were analysedAverages and standard deviations of three independent experiments.

Human β-defensin-2 (HBD-2) gene was transcriptionally induced by treatment with *L. plantarum* wild type dead cells, in accordance with previous report (Wehkamp et al, 2004); a weaker and transient induction was observed even upon treatment with live cells. The three *L. plantarum* mutant strains differently induced HBD-2 with respect to wild type.

Indeed, Δ*ctsR* live cells strongly induced HBD-2 gene after 1 and 3 hours incubation; whereas Δ*ftsH* and Δ*hsp18,55* heat killed cells were much more potent inducers of HBD-2 expression with a progressive increase of mRNA level peaking after 5 and 3 hours, respectively. In contrast to wild type, all mutants, in the form of heat inactivated cells, tended to augment HBD-2 expression upon longer incubation.

Expression of lysozyme (LYZ) was progressively down-regulated by live cells of wild type and Δ*ctsR*. Indeed after 5 h incubation, its mRNA level was approximately 20-fold lower than that of control cells. Following an initial increase (~2-fold), LYZ gene down regulation was less evident after 5 h incubation with Δ*ftsH* and Δ*hsp18.55* live cells (~0.5 level relative to control). Treatment with wild type and Δ*ctsR* dead cells had no consistent effect on LYZ transcription; whereas an appreciable increase (more than 3-fold) resulted from incubation with Δ*hsp18.55* dead cells. Also Δ*ftsH* dead cells weakly stimulated LYZ expression.

Mucin-2 (MUC-2) transcription was mainly induced by dead cells challenge and its level was definitely increasing upon longer incubation periods. Up-regulation with respect to untreated control Caco-2 cells was noticeable: after 5 hours incubation MUC-2 mRNA level increased by 12-, 11-, 30- and 9-fold, following challenge with heat inactivated cells of wild type, Δ*ctsR*, Δ*ftsH* and Δ*hsp18.55* strains, respectively. Overall, transcription was more strongly stimulated by the Δ*ftsH* strain. Moreover, while live cells of wild type, Δ*ctsR* and Δ*hsp18.55* did not stimulate mucin expression, incubation with live Δ*ftsH* bacterial cells did lead to increased transcription, particularly after 1 hour treatment (10-fold change). This leads us to hypothesize that some immune-inducing bacterial cell component, which is already present on intact live cell, might become more available after cell death.

IL-6 transcription was significantly up-regulated by all the analysed strains, both as live and heat-killed cells, especially at the beginning of the bacterial treatment (1 hour incubation); as the incubation period increased, IL-6 transcriptional level tended to decrease suggesting a transient induction. Notably, after 1 hour challenge, wild type heat inactivated cells induced IL-6 expression much more than Δ*ctsR* and Δ*ftsH* cells (9-, 4-, and 3.6-fold, for wild type, Δ*ctsR* and Δ*ftsH,* respectively). Intriguingly, treatment with both live and heat inactivated Δ*hsp18.55* remarkably stimulated IL-6 transcription, at levels much higher (~10-fold, using heat killed cells) than those induced by the other strains.

Except for Δ*hsp18.55* treatment, IL-8 was particularly induced by dead bacterial cells, whereas no or little up-regulation was detected upon treatment with live cells and, similarly

to IL-6, also IL-8 transcription was more strongly induced in the initial phase of incubation, then showing a decreasing level. Even for IL-8, the initial transcriptional stimulation by *L. plantarum* wild type cells was approximately 2-fold higher than that by Δ*ftsH* and Δ*ctsR* mutant cells. The outcome of Δ*hsp18.55* treatment appreciably differentiated both for the strongest stimulating effect (by live, as well as dead cells) and for the increasing temporal transcription pattern.

MIP-3α was moderately induced only by dead bacterial cells, especially by Δ*hsp18.55* (~4-fold) and, to a minor extent, by wild type and Δ*ftsH* (approximately 2-fold); interestingly, the presence of live bacteria tended rather to repress its transcriptional level (approximately 5-fold decrease after 5 h incubation with wild type and Δ*ctsR* cells). Overall, MIP-3α transcriptional pattern was rather irregular and variable during incubation.

4.4.5. Modulation of TNF-α production in host immune cells.

The anti-inflammatory properties of *L. plantarum* strains was assessed by evaluating their capacity to modulate human TNF-α production by human immune cells.

Supernatants from planktonic bacterial cultures were added to human monocytoid THP-1 cells in the presence or in the absence of *E. coli* lipopolysaccharides (LPS), which stimulate the production of pro-inflammatory TNF-α. As shown in **Figure 4.22**, the bacterial supernatant from all the *L. plantarum* strains substantially decreased TNF-α production in the presence of LPS, as compared with the control conditions in which either no bacterial supernatant or medium only were added. No significant difference in the suppression of TNF-α secretion was observed among strains.

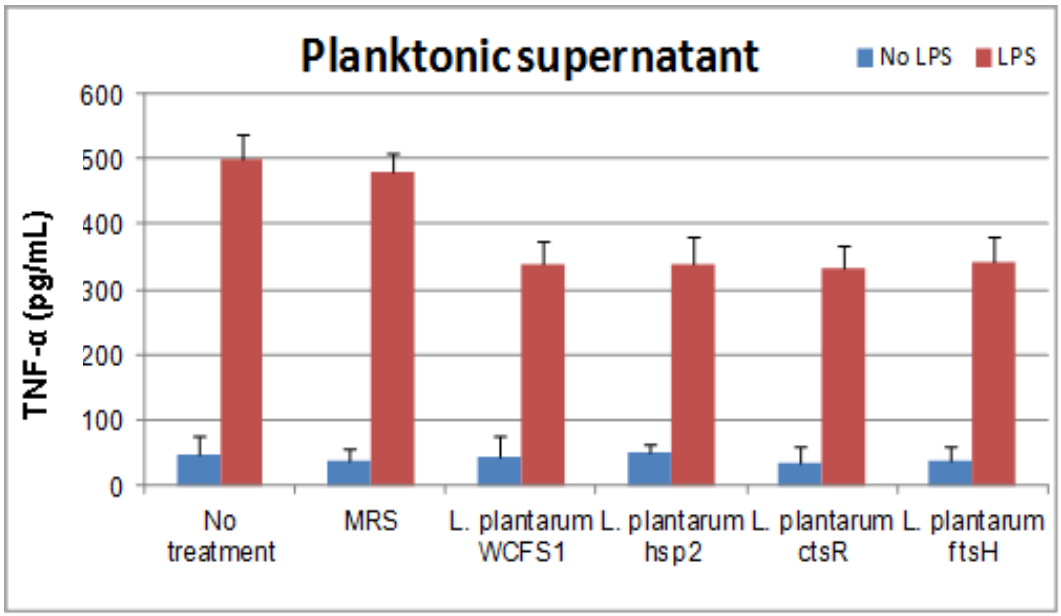


Figure 4.22. Inhibition of TNF-α production by THP-1 cells in the presence of probiotic-derived secreted factors. Cell culture bioassays were performed by co-incubating for 3.5 hours human monocytoid cell line THP-1 with *E. coli*-derived lipopolysaccharides (LPS) and cell-free supernatants from *L. plantarum* cultures of wild type (WCSF1), Δ*hsp18.55* (hsp2), Δ*cts*R and Δ*ftsH* strains. Addition of lactobacilli medium only (MRS) served as control. Human TNF-α cytokine was determined by ELISA in culture supernatant.

5. DISCUSSION

5.1. Mutant strains of *L. plantarum* WCFS1.

Shsp. The nucleotide sequences of *hsp18.5* and *hsp19.3* have been well characterized in *L. plantarum* WCFS1 (Spano et al, 2004a; Spano et al, 2005). These genes code for low molecular weight proteins which confer thermo-tolerance and act as molecular chaperones.

In gene replacement vector, the gene disruption cassette, which consists of a selectable marker gene flanked with DNA fragments homologous to the target gene, is introduced into host genome where it will integrate by either homologous recombination or ectopic recombination (Madyagol et al, 2011). The failure in obtaining knockout mutants for these two genes, even using different KO strategies, suggest that they might code for essential functions (Charusanti et al, 2010; Christen et al, 2011). Alternatively, we hypothesize that *hsp18.5* and *hsp19.3* are located in genomic regions which are not prone to recombination.

PCR analysis on false positive *L. plantarum* transformants, showing resistance to chloramphenicol, but not to erythromycin, never detected disruption of the target genes. These clones might have arisen from random and aspecific integration of the pNZ5319 knockout vector into the *L. plantarum* genome, probably in regions distant from the target genes, thus inducing resistance to chloramphenicol. Either a limited size of the cloned up and downstream regions of the *hsp* genes or a low transformant efficiency of the vectors have determined the failure of our gene knockout procedures. This encourages the implementation of further cloning strategies (Ma et al, 2000; You et al, 2009).

ftsH. Functional studies have revealed an important role for FtsH in bacterial stress responses. Interestingly, the consequences of *ftsH* mutation are rather species specific, ranging from drastic growth impairment (Begg et al, 1992) to milder effects on sporulation, development, and stress response (Deuerling, et al, 1997; Lysenko et al, 1997; Fischer et al, 2002). For some bacteria, such as *E. coli*, *L. lactis*, and *H. pylori*, the apparent impossibility of isolating any viable *ftsH*-null mutant indicates that this protease is essential (Tomoyasu et al, 1993; Akiyama et al, 1994; Nilsson et al, 1994; Ge and Taylor, 1996). By contrast, in species such as *B. subtilis* and *Caulobacter crescentus*, FtsH seems dispensable for growth under physiological conditions (Deuerling, et al, 1997; Fischer et al, 2002). Besides, minor effects on normal growth and the cellular stress response were observed in an *ftsH* mutant strain of *Corynebacterium glutamicum* (Lüdke et al, 2007). Since we could get a viable *ftsH*

mutant strain by insertional inactivation, FtsH is clearly not essential in *L. plantarum*. According to our results, the relevance of *ftsH* function in *L. plantarum* pairs with what observed in bacteria such as *B. subtilis*, *C. crescentus*, and *C. glutamicum* but with a marked contribution to growth under heat shock conditions.

The involvement of *ftsH* in the stress response was also confirmed by phenotypic analysis of the Δ*ftsH* mutant strain, which displayed significant growth defects under heat and hyperosmotic stress. Conversely, a control *L. plantarum* strain overexpressing *ftsH*, showed enhanced growth under heat stress and in medium simulating hyperosmotic or bile stress conditions, indicating that increased intracellular levels of FtsH might promote the ability of *L. plantarum* to resist these conditions. All these findings point to the involvement of FtsH in tolerance to diverse types of stress and are in agreement with the phenotypic characteristics of *ftsH* mutants in other bacterial species (Nilsson et al, 1994; Deuerling et al, 1997; Fischer et al, 2002).

5.2. Surface properties of the Δ*ftsH* mutant and comparison with the other *L. plantarum* strains.

The cell envelope is the first structure to be damaged by physico-chemical stress. The cell membrane itself plays an important role in stress resistance: its composition can change in adaptation to harsh conditions; moreover, membrane sensors, transporters and proteases may be involved in stress resistance (van de Guchte et al, 2002).

The differences in affinity towards solvents between wild type and Δ*ftsH* strains are likely to reflect some changes in the cell envelope chemical composition, suggesting that *ftsH* could either directly or indirectly control the physico-chemical features of the cell surface in *L. plantarum*. Minor differences with respect to wild type were observed for the physico-chemical features of the Δ*hsp18.55* and Δ*ctsR* strains, as revealed by MATS analysis.

The altered physico-chemical properties of the Δ*ftsH* strain might parallels with the decreased biofilm formation capacity of this mutant. Indeed, cell surface physico-chemical properties are recognized to play an important role in the interactions with a support during biofilm development, especially in its initial stages (Branda et al, 2005). The fact that Δ*ctsR* and Δ*hsp18.55* mutant and wild type strains show a major capacity to form biofilm compared with the Δ*ftsH* strain, strongly suggest that FtsH might be involved in such phenomenon. The production of biofilm is often used by bacteria to enhance resistance to

environmental stresses and colonize diverse niches (Kubota et al, 2008), therefore it is not surprising that the stress-related *ftsH* gene might play a role in biofilm development. A link between biofilm formation and stress-related proteases had been previously reported for other bacterial species (Lemos and Burne 2002; Frees et al, 2004; Simionato et al, 2006). Notably, in *E. coli*, FtsH was reported to degrade a membrane-protein which is probably linked to biofilm formation (Beloin et al, 2004). We can speculate that FtsH might control the stability of transcriptional factors directing biofilm development; another hypothesis is that, consistently with its proteolytic activity, FtsH might regulate post-translationally the turnover of some surface-associated adhesion proteins which are necessary for biofilm formation in *L. plantarum*.

Both analysis of the adhesion abilities and physico-chemical characterization of the cell surface provided indirect evidences that FtsH might be involved in the cell envelope architecture. However, in contrast to the other mutants, investigation by scanning electron microscopy (SEM) did not reveal any relevant difference in the surface cell morphology between *L. plantarum* wild type and Δ*ftsH* mutant, even when cells were imaged after heat-stress exposure.

5.3. OGI tract simulator.

International Health and Food Organisms recommend the development and improvement of *in vivo* and *in vitro* procedures to assess functionality and safety of probiotics (FAO/WHO, 2002). Potentially probiotic species that should be incorporated into functional food, need to be selected for their resistance to passage through the human oro-gastrointestinal (OGI) tract. Here we developed and refined an *in vitro* OGI system to test the viability of different LAB strains, incorporated into a milk matrix. *L. plantarum* wild type could survive even the harshest gastric conditions, confirming its common occurrence in the human gut (Ahrnè et al, 1998), and in accordance with several *in vivo* experiments demonstrating both its survival and colonization ability after oral administration to human volunteers (Johansson et al, 1993; de Vries et al, 2006). The reliability of our system was further confirmed by analysing viability of the commercial probiotics *L. acidophilus* LA-5 and *B. lactis* BB-12, which are highly tolerant to acid conditions and commonly employed for the production of functional food (Shah, 2000; Fernández de Palencia et al, 2008).

Notably, starting the OGI transit with an initial titre of approximately 10^9 CFU/mL, the CFU capability remained always detectable, even after the most severe OGI stress

conditions. This was observed for both the wild type and the mutant strains of *L. plantarum*. Some relevant strain-specific differences were observed, including a higher sensitivity of the Δ*hsp18.55* mutant to the initial oro-gastric compartment together with an improved tolerance to intestinal conditions, and greater susceptibility of the Δ*ctsR* strain to the very acidic gastric condition (pH 2.0 - 1.5). However, overall the survival pattern was similar and satisfactory for all the *L. plantarum* strains, suggesting that knockout of the analysed stress genes does not drastically compromise its survival potential under these conditions. Incubation in gastric juice at pH values below 3.0 caused a generally high mortality (between 2- and 8-log units), which is in agreement with previous works (Mainville et al, 2005; Fernández de Palencia et al, 2008; Weiss and Jespersen, 2010). Conversely, subsequent incubations in duodenal digestive fluids and bile salts had usually a minor impact on the survival rate. These data point out that the final stages of gastric emptying may provide very low amounts of viable bacteria to the intestine. By contrast, relatively high bacterial loads (approximately 10^8 CFU/mL) can reach the small and large intestine from the gastric compartment within the first 60 minutes of digestion (pH ≥ 3).

Matrix effect. The OGI system was also used to study the survival of *L. plantarum* wild type into different 'food carriers', in order to evaluate the possible effect of the vehicle matrix on the bacterial resistance to host-derived stress. Among the tested matrices, ordinary pasta and special pasta enriched in barley beta-glucans were used. This latter were investigated as beta-glucans are known to have prebiotic effect (Welman and Maddox, 2003). Beta-glucans, belonging to the wide family of exopolysaccharides (EPSs), are hydrolysed by bacteria into small carbohydrates (e.g., monosaccharides, disaccharides) and short chain fatty acids (SCAFs: butyrate, propionate, acetate) important for their fermentative metabolism as well as for host health (Hosseini et al, 2001; Salazar et al, 2008). Overall, a lower *L. plantarum* survival to the OGI transit was detected for bacteria included in control pasta, with respect to beta-glucan-enriched pasta: it is reasonable that the carbohydrate polymers, also in reason of their viscosity, may form protective shells or shell-like structures with the function of protective film around the bacterium, which shield from the deleterious effects of low pH and enzymes (Stack et al, 2010). There is no previous study analyzing the behavior of microorganisms in a simulated OGI tract, in presence of a beta-glucans-enriched food matrix. However, the treatment of *L. paracasei* NFBC 338 strain with exogenously added gum acacia, a complex polysaccharide, had improved its ability to

survive to heat, bile, H_2O_2, spray drying, and simulated gastric conditions (Desmond et al, 2001). Moreover, during the treatment under both gastric and duonenal secretions (Martinez et al, 2011), in presence of galactooligosaccharide (GOSs), *Lactobacillus amylovorus* DSM 16698 exhibited a good level of survival, compared to control condition. Physiological data demonstrated that the acquisition of bile resistance produces a shift in the catobolism of carbohydrates (Sánchez et al, 2007). In accordance whit this, studies proved the increasing activity of glycosyltansferase enzyme in *B. animalis subspp. lactis* (Ruas-Madiedo et al, 2009). These results demonstrate that the exogenous administration of any polysaccharide contributes to the stability and persistence in formulation of a probiotic strain, in term of shelf-life, storage and stressful challenge. Alternatively, *in situ*-EPSs- and isolation of natural EPSs-producing strains offer many advantages with respect to non-EPSs producers. An EPSs-producing strain improves the organoleptic properties of food, increases cell viabity during processing, enhances the growth of other beneficial microbes in the GI tract and ameliorates the health of host (Ruas-Madiedo et al, 2006; Stack et al, 2010).

When using saline solution as vehicle, both the lack of nutrients and the absence of any shielding effect were confirmed by the high mortality observed. This is in accordance with similar studies comparing the effect of milk and water to vehicle different *Lactobacillus* species through a simulated OGI digestion (Lo Curto et al, 2011). The hypothetical protective effect of MRS (an ordinary culture medium of lactobacilli) was confirmed by the good viability of the microorganisms included in such a vehicle. MRS medium is specifically designed for the growth of LAB; it is likely that the abundance of nutrients (particularly sugars) may implement bacterial growth and assure the survival along the various hostile steps of the OGI tract (Bujalance et al, 2006).

Comparison between the different matrices indicates that milk and EPSs are potential candidates for the development of symbiotic formulations (i.e., probiotic and prebiotic) suitable to human diet. Moreover, considering the positive effect of MRS, the design of matrices that enhances probiotic performances should also take into account the specific nutritional needs of the bacterial species.

The basic principle of the formulation of synbiotic products is to identify a food matrix whit the buffering property against both the acidic condition of the stomach and the enzymatic anctivity of intestine (Ruas-Madiedo et al, 2002). However, it is hard to plan an OGI model, because a microorganism, whose intestinal viability is proven *in vivo*, is commonly found unable to tolerate the harsh conditions of the stomach *in vitro* (Morelli,

2000; Meanville et al, 2004). Indeed, some *in vivo* elements (such as other microbial inhabitants, buffering compounds of the host, etc.,), which cannot be reliably reproduced *in vitro*, are likely to enhance bacterial survival during the OGI transit.

Major emphasis should be given to the protective effect of the matrix. Charteris et al, (1998) stressed Lactobacilli and Bifidobacteria with pepsin (pH 2.0) and pancreatin (pH 8.0); milk proteins enhanced bacterial survival during passage through the stomach. Moreover, the same study showed that the addition of mucin increased tolerance to gastric juice. The mucin scaffold is made up by sulphated oligosaccharide chains and covalently bound fatty acids. This allows mucin to resist protease action and therefore to act as coating surface on the microorganism (Slomiany et al, 1996). A similar study, on *L. acidophilus* M29 (Kos et al, 2000), showed that whey proteins exhibited an excellent protective ability against both strong acidity and bile acids. Therefore, the model we present here, together with data obtained from other investigators, confirm the importance of incorporating the microorganisms into a food matrix (Mainville et al, 2005).

Bacterial gene expression in response to OGI stress. Bacteria have evolved complex networks of stress response pathways to promote their survival to environmental challenges.

The transcriptional level of bacterial stress-related genes, including proteases and chaperones, remarkably paired with the extent of stress during the OGI transit, as revealed by the observed survival rate of bacteria. Indeed, the OGI stress steps of high mortality corresponded to those of major induction of stress genes such as *clp* family, *groEL* and *dnaK*. This finding confirms the involvement of such genes in the mechanisms of response to typical stresses of the OGI tract: thanks to their enhanced expression, bacterial cells may better adapt to the gastric compartment. This result also paves the way to the use of such genes as molecular markers for the screening of strains with possible probiotic applications.

According to our results, and in agreement with previous studies on *L. acidophilus* (Weiss and Jespersen, 2010), the most stressing conditions are those associated to acidic gastric juice. Indeed, a declining gene up-regulation usually accompanied the passage from gastric compartment to corresponding intestinal digestion. This means that, in spite of bile and pancreatin addition, the increasing pH (from 5.0 or 3.0 to 6.5) tends to normalize stress gene expression, so that cellular functions may return physiological.

The molecular chaperones GroEL and DnaK were extremely induced in conditions of strong acidity (pH = 3.0), parallel to proteases ClpE. Under the same conditions, ClpB and

ClpP were relatively less induced. In addition, *hsp18.5* gene had an induction peak at pH 3.0, while the transcriptional repressor CtsR was significantly induced under both conditions of low pH (5.0 and 3.0). We can assume that the low pH stress might increase the intracellular level of the CtsR repressor. This negative repressor might thus keep relatively low the levels of ClpB, ClpP and FtsH proteases (Fiocco et al, 2009; Fiocco et al, 2010). However, the high level of *clpE* mRNA might indicate that this particular protease may respond also to other control signals, besides CtsR, and thus be recruited in responses to low pH stress.

So far, expression of *shsp* under simulated OGI conditions had never been investigated in *L. plantarum*. The three *shsp* genes, especially *hsp18.55*, exhibited differential induction in response to the oro-gastric and duodenal stress, although sharing a considerable induction at pH 3.0. *hsp 18.5* mRNA level showed ampler fluctuations and was the only stress gene to be significantly induced (~15-fold) by oral stress. This finding is in agreement with the different transcriptional regulation demonstrated or suggested to work on the *hsps* genes in *L. plantarum* (Spano et al, 2004a, 2005; Fiocco et al, 2010). Moreover the different gene activation indicates that the three sHsps play distinctive roles in adaptation to stress, consistently with previous studies aiming at their functional characterization (Fiocco et al, 2007; Capozzi et al, 2011).

The *hsp18.5* gene was strongly induced by conditions of acidity (pH 5.0 and 3.0). A dual mechanism of regulation for *hsp18.5* gene has been proposed, based on HrcA and CtsR transcription factors (Spano et al, 2004a; Fiocco et al, 2009). Because our results also revealed a significant activation of *ctsR* by gastric pH values, we suggest that the activation of *hsp18.5* gene may shift towards the regulatory circuit of HrcA. This could also explain the massive stimulation of *dnaK* and *groEL* genes, which are organized in operons controlled by HrcA (Schmidt et al, 1992; Homuth et al, 1997).

By analyzing bacterial genes involved in promoting the colonization of the host (putative adhesion factors) and in probiotic effects (plantaricins), a slight induction was observed in the salivary compartment, while increased transcriptional level was detected in the gastric sector at both pH 5.0 (where especially *msa* and *plnEF* were strongly induced) and pH 3.0. In the simulated intestinal regions, all genes were minimally or not at all induced. In regard to expression of adhesive cell surface proteins, our data diverge from previous similar studies in *L. acidophilus* which reported higher adhesin (mucin- and fibronectin-binding proteins) induction after prolonged incubation under condition resembling the duodenal

tract, rather than the gastric compartment (Weiss and Jespersen, 2010). According to our result, signals arising from the gastric stress may contribute to prompt the up-regulation of adhesion genes that will be necessary for the following stage of the OGI transit. In contrast and unexpectedly, the intestinal environment did not trigger adhesion and *pln* genes; this may be due to the lack of other important signals and stimuli, probably of biological origin, which were not included in our simulated OGI system. Such signals may comprise molecules secreted and/or shed by animal host cells, as well as signaling factors produced by other microbial species of the indigenous microflora. Accordingly, Ramiah et al (2007), found a consistent induction of Mub and other adhesion proteins in a probiotic strain of *L. plantarum*, only, and/or especially, when mucins were added to a media simulating gut conditions.

In our OGI system, we simulated a situation in which the microorganism was mixed with a milk matrix and subject to the action of gastric enzymes and gut physico-chemical parameters. However, during the OGI transit we did not reproduce any direct interaction between the bacterial cells and the host epithelial cells. This consideration represents an incentive to develop and improve our OGI system in order to make it as close as possible to the real host-microorganism interactions. It would be desiderable to observe the expression of the above mentioned bacterial genes, after interaction between bacteria and animal cells. Bron et al (2004) and Marco et al (2007) studied the gene expression profile in *L. plantarum*, orally administered to mice, at different times during the OGI transit. Gene expression was studied and compared in different compartments of mouse GI tract and found to be modulated over time. Similarly to our investigation, in which an induction of Clp protease was observed under low pH conditions, Bron et al (2004) found an induction of *clpC* stress-related gene. The *plnI* gene, encoding a plantaricin immunity protein, was up-regulated in the intestinal compartments of mice (Marco et al 2007); conversely, we did not detect significant induction of *plnEF* in the simulated intestinal regions of our *in vitro* system. Such comparative analyses testify how studying the bacterial gene expression both *in vivo* and *in vitro* may sometimes lead to different results.

We have also to consider the possible pleiotropic functions of the analyzed genes. Indeed, some of the encoded proteins, besides having proven adhesive properties, play also key roles in certain metabolic pathways. For instance, EnoA1 is mainly an enolase (Castaldo et al, 2009), while Msa and Mub act also as hydrolases (Pretzer et al, 2005). Therefore the expression level of these genes may be affected by other metabolic signals and by

components of the milk (sugars, lipids) used as vehicle matrix. This effect might have masked the induction due to stress conditions.

5.4. Interaction with the host.

Adhesion. Adhesion to intestinal epithelial cells is considered a requisite of 'probioticity', as a close interaction between bacteria and host cells enables a transient colonization of the intestinal mucosa, thus allowing both antagonist effects against pathogens and host immune modulation (Bernet et al, 1994; Isolauri et al, 2004). All the analysed *L. plantarum* strains significantly adhered to Caco-2 cells, although the adhesion level was quite variable. The variable adhesive properties confirm the notion that the adhesion to human intestinal epithelium is a strain-dependent trait of lactic acid bacteria. The observed phenotype might somewhat correlate with the different cells surface features, as partially revealed by previous studies (Fiocco et al, 2010; Bove et al, 2011; Capozzi et al, 2011). Interestingly, no evident correlation was found between the adhesion capacity and the extent of immune stimulation of the host colonic epithelial cells. This finding suggests that a direct physical interaction between microbe and host cell is not strictly essential to trigger host response or, alternatively, that this is not necessary when bacterial concentration is above a saturation limit, as is the case in this study.

Interestingly, we could not find any apparent relation between the ability to form biofilm on abiotic surface (biofilm assays on polystyrene) and the level of bacterial adhesion to human intestinal cells. This suggests the involvement of different adhesion mechanisms and surface elements in the two processes.

Exopolysaccharides (EPSs) are branched polymers synthetized by enzymes located in the inner/outer membrane and periplasm of bacteria. These biopolymers are covalently linked to bacterial surface forming a capsule; they can be non-covalently associated with the surface or be totally secreted (Leivers et al, 2011) It has been reported that bile salts induce their synthesis in strains of Bifidobacteria; therefore, it seems that these polymers could have a protective role for the producing bacteria (Ruas-Madiedo et al, 2009). These polymers can be used by lactic acid bacteria to adhere to epithelial cells and also to overcome stressful conditions of OGI tract. Some human oral mucosa bacteria form biofilms because of the presence of EPSs on their membrane (Burns et al, 2010). Adhesion assays showed that addition of beta-glucans significantly increased the number of adherent bacteria. Evidently, the beta-glucans (belonging to the family of EPSs) might have promoted bacterial adhesion

to Caco-2 monolayers both for their gluing effect (Burns et al, 2010) and for their ability to stimulate bacterial growth by providing fermentable substrates. In agreement with this, Fernández de Palencia et al (2009) demonstrated that an EPS-producing microbial strain (i.e., *Pediococcus parvulus* 2.6) exhibited stronger adhesiveness to Caco-2 cells, compared to its relative non-EPS-producer strain. Furthermore, our data partially agree with those of a previous study in which adherence to human intestinal mucus of *L. rhamnonus* GG and *B. longum NB 667* was examined (Raus-Madiedo et al, 2006). By using fraction of the EPSs from different bacterial sources, the adhesion of the two bacterial strains resulted modulated in a EPSs type- and dose-dependent manner. Therefore, the surface characteristics of a probiotic strain, in relation to the type and quantity of EPSs it produces, contribute to adhesion within the GI tract. In addition, EPSs produced by probiotic strain interfere with the bacterial adhesion to human GI tract. Then, EPSs play a role in the gut-intestine environment, related to colonization and pathogen exclusion (Raus-Madiedo et al, 2006).

Immune response of the host. Addition of supernatant from *L. plantarum* cultures attenuated the proinflammatory response of LPS-activated human immune cells, in terms of TNF secretion. Other probiotics have been investigated for this trait and some of them share the same ability. Indeed, several reports demonstrate that LAB strains may down-regulate the production of pro-inflammatory TNF (Ménard et al, 2004; Lin et al, 2008). This is a particularly desirable feature in probiotics, as it represents a condition for their potential therapeutic uses, as for the treatment of inflammatory bowel diseases (IBD). In contrast to what observed for gene transcription analysis in bacteria-treated intestinal epithelial cells, no strain specific effect was evident. This discrepancy might depend on the different type of target host cell (immune vs epithelial cells), on different bacteria-deriving stimuli (soluble vs whole bacterial cell factors), possible differently activated signal cascades, different level of analysis (protein vs mRNA), etc.

Caco-2 cells were used also as *in vitro* model for analysis of innate immunity stimulation by *L. plantarum*. To mimic the action of probiotics on the intestinal mucosa, Caco-2 cell monolayers were exposed to live or dead bacterial cells. *L. plantarum* treatment, especially in the form of dead cells, significantly induced transcription of some genes involved in innate immunity response. It appeared that *L. plantarum* can strengthen the mucosal barrier function by transcriptional activation of HBD-2 and mucin-2, both molecules contributing to inhibit and prevent pathogen colonization. This result is in line with previous studies on

other probiotic Lactobacilli and *L. plantarum* strains (Mack et al, 1999; Wekhamp et al, 2004). The gene encoding lysozyme, another innate immunity key effector, resulted to be the least modulated and, depending on the strain, sometimes down-regulated.

Induction of proinflammatory signals. A significant induction of the proinflammatory cytokines IL-6 and IL-8 was detected for all strains, especially in the form of dead cells; while transcription of the chemokine MIP-3α, which was investigated in reason of its association with the onset of inflammatory bowel diseases (Kwon et al, 2002), was much less stimulated compared to the other cytokine genes. In accordance with previous works (Ruiz et al, 2004), and as expected for non-pathogenic species, the temporal transcription pattern we observed suggests rather a transient induction of proinflammatory cytokines (except for IL-8 induction by Δ*hsp18.55*).

Induction of proinflammatory modulators is considered a hallmark of the intestinal inflammatory response and thus might be detrimental for probiotics, which, in fact, are positively selected for their anti-inflammatory effects, especially in view of potential therapeutic applications (Petrof et al, 2004; Grangette et al, 2005; Mohamadzadeh et al, 2011). Our data contrast with studies by Morita et al (2002), finding no or scanty induction of IL-6 and IL-8, at the protein level, in Caco-2 cells upon treatment with a wide range of LAB species. Nonetheless, our results pair with more recent works demonstrating that some probiotics do stimulate IL-6 and IL-8 secretion, as well as a transient activation of proinflammatory signals in human intestinal epithelial cells (Wekhamp et al, 2004; Wong and Ustunol, 2006; Ruiz et al, 2005; Kim et al, 2006; Schlee et al, 2008). Indeed, it seems that different probiotics - even closely related species or strains - may lead to diverse, sometimes opposite effects with respect to triggering proinflammatory signalling pathways (Jijon et al, 2004; van Baarlen et al, 2009). The classical proinflammatory cascades, (e.g., NF-kB) induce the synthesis of innate immunity defence effectors (such as antimicrobial peptides and mucus components), as well as the expression of proinflammatory cytokines including IL-6 and IL-8 (Pahl, 1999; Akira et al, 2006). Proinflammatory cytokines function as a danger signal to alert immune cells. However, the transience of proinflammatory stimulation and the complexity of cytokine signalling network might account for the development of mucosal immune tolerance (i.e., hyporesponsiveness) to commensal or probiotic microbiota, while protective humoral response is maintened against enteropathogens.

Strain-specific immune gene modulation. The transcriptional pattern of some genes was differently modulated in response to treatment with the different *L. plantarum* strains. Particularly, the effect of Δ*ftsH* and Δ*hsp18.55* mutants clearly differentiated from that of the wild type and Δ*ctsR* strains. Indeed, Δ*ftsH* cells induced higher up-regulation of mucin-2 and antimicrobial peptide HBD-2. Moreover, induction of IL-6 gene by Δ*ftsH* was overall lower than wild type. Conversely, Δ*hsp18.55* treatment was a far more potent stimulator of proinflammatory mediators, especially IL-6, with respect to the other strains. Besides, Δ*hsp18.55* triggered more strongly transcription of HBD-2 and LYZ, which can be considered protective effectors of the intestinal mucosa. Based on our data, some *L. plantarum* mutants (e.g., Δ*ftsH* and Δ*hsp18.55*) possess distinctive immunomodulatory features, associated to a higher enhancement of the chemical-physical barrier of the intestinal mucosa. Hence, these strains deserve to be further analysed, *in vitro* and *in vivo*, for perspective probiotic use. We can speculate that deletion of *ftsH* and *hsp18.55* genes may have affected structure or concentration of some microbe-associated molecular patterns (MAMPs) recognised by pathogen recognition receptors (PRRs) of the host. However, since these genes code for regulatory functions (i.e., protease and chaperone activities, respectively), their mutation is expected to generate pleiotropic effects, therefore making difficult the identification of altered bacterial components, directly involved in interaction with the host. Some specific probiotic cell components/determinants which are critical for interaction with host cell receptors, have been recently identified (Jijon et al, 2004; Grangette et al, 2005; Yan et al, 2007; Konstantinov et al 2008; Mohamadzadeh et al, 2011), most of them referring to cell surface biochemical features. A series of *L. plantarum* genetic *loci* linked to specific immunomodulatory effects, has also been suggested (Meijerink et al, 2010). Here we analysed *L. plantarum* mutants that exhibit differential cell surface properties, including peculiar cell envelope morphology under stress, altered membrane fluidity and cell surface hydrophobicity (Fiocco et al, 2010; Bove et al, 2011; Capozzi et al, 2011). It is likely that these differences might somehow correlate with the observed variable adhesive properties as well as with the differential immune stimulation, but of course further detailed researches are needed to find out specific bacterial determinants. In this regard, we hypothesize that lack of FtsH or Hsp18.55 may lead to an accumulation of misfolded proteins (in the respective mutant strains), thus causing unmasking of several more potential

epitopes with respect to wild type. This might partially account for the higher immune stimulation by *ftsH* and *hsp18.55* mutants.

Effect of viable vs heat killed bacteria. Viable and heat killed bacteria were used in order to compare potential difference in the type of immune stimulation. Indeed, clear differences in the degree of transcriptional activation were observed, with a more powerful inducing effect exerted by the heat-inactivated bacterial cells. Incubation with live bacteria might better mimic the natural host-microbe interaction, however it also leads to alteration of the media (e.g., acidification) which might interfere with epithelial cell response. By contrast, the use of heat-inactivated cells allows to keep stable experimental conditions, although a narrower range of immune response is likely to be expected. As previously demonstrated (Wekhamp et al, 2004; Schlee et al, 2007), dead bacterial cells are potent stimulator of host innate defence mechanism, in agreement with a broader definition of probiotics, that considers also inactivated microorganisms and single cellular components as elements endowed with health-promoting properties *per se* (Lammers et al, 2003; Kataria et al, 2009). Differences in immune modulation upon treatment with either live or killed bacteria, has been documented for *L. plantarum* (Bloksma et al, 1979; van Baarlen et al, 2009). Sometimes, also the bacterial growth phase or the type of bacterial inactivation may cause different effects (Wong and Ustunol, 2006; van Baarlen et al, 2009). Indeed we noticed that, for some genes (e.g., MUC-2, LYZ) the expression pattern significantly diverged depending on the bacterial strain viability. Heat inactivation neutralizes the activity of most enzymes and may cause the release of some bacterial cell envelope components or cytosolic molecules, including DNA fragments, which may promote specific immune response pathways. Moreover, some antigenic determinants may become more accessible on heat-denatured proteins, thereby enhancing immune stimulation of Caco-2 cells.

6. CONCLUDING REMARKS

- The developed OGI *in vitro* system represents a helpful tool for the initial screening of potential probiotic species and for assessing their survival performance in the human OGI tract. This system is easy, handy, quite reliable and cost-effective; however, it deserves to be further improved, especially taking into accounts major drawbacks such as its static character, lack of automation and feedback effects, absence of biological components and competition events.

- Data obtained with the different matrices, also in relation to adhesion ability, may be valuable for the design of protective and host/bacteria-friendly carrier foods.

- Analysis of bacterial gene expression gives a better understanding of *L. plantarum* functional adaptation during passage through the human gut; moreover, it may contribute to define selection criteria for probiotic candidates.

- Our observations highlight the relevance of the genetic background in the interaction with the host, confirming that probiotic properties, such as adhesion capacity and immune modulation, are strongly strain-dependant, rather than species-specific, and indicating that they are also tuneable by subtle genetic variations that can affect bacterial cell components involved in probiosis. Understanding the mechanisms of probiosis by specific strains may be functional to tailor probiotics for specific clinical applications. Indeed, such studies contribute to assess the role of specific bacterial genes in the interaction with the host, and pave the way to the design and development of *L. plantarum* mutant strains with desired probiotic attributes, thus representing an intriguing opportunity for targeted therapeutic interventions.

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8. PUBLICATIONS AND CONFERENCES

Publications:

P. Bove, V. Capozzi, D. Fiocco, G. Spano. 2011. Involvement of the sigma factor *sigma H* in the regulation of a small heat shock protein gene in *Lactobacillus plantarum* WCFS1. *Annals of Microbiology* 61: 973-977.

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Oral Communications:

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