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Final dissertation for the achievement of the degree of Doctor (Ph. D.) in "**Food Biotechnology**"

INTERACTION BETWEEN *LACTOBACILLUS PLANTARUM* AND FOOD RELATED MICROORGANISMS BY PROTEOMICS AND BIOINFORMATICS

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SUMMARY

Lactobacillus plantarum is a versatile and widespread microorganism found in materials and environments ranging from vegetable, dairy products and meat fermentations to the human gastrointestinal (GI) tract. Some strains are marketed as probiotics that are claimed to provide a health benefit for the consumer. Furthermore, certain strains of *Lb. plantarum* are known for their ability to produce several natural antimicrobial substances. The production of these metabolites could represent stress conditions that strongly affects the development of undesirable microbial species. There are many scientific reports that highlight antimicrobial effects of *Lb. plantarum* strains on undesirable bacteria. Several strains of *Lb. plantarum* showed a broad spectrum of antibacterial activity (including *Bacillus cereus, S. aureus, Listeria monocytogenes, Salmonella enterica, E. coli,* and *Enterobacter aerogenes*) and carries several plantaricin genes of the *pln* locus. Moreover various other bacteriocins produced by *Lb. plantarum* species isolated from fermented food are well known.

Nevertheless, the effectiveness of bacteriocin-producing strains in foods can be limited by several factors including narrow activity spectrum, limited, diffusion in solid matrices, inactivation through proteolytic enzymes or binding to food ingredients such as lipids, low production level and the emergence of bacteriocin-resistant bacteria. Although, the use of class IIa bacteriocins or bacteriocins-producing strains represent a promising alternative for the control of spoilage or pathogenic microorganisms in foods, their efficacy could be compromised by onset of bacteriocins resistant strains and cross-

resistance between bacteriocins. However, the use of bacteriocins in combination with other hurdles (e.g. salt, acid, other natural substances etc.) may result extremely effective for inhibit *L. monocytogenes* and reduce its resistance. A number of experiments have been showing the anti-listeria effect due to production of acid organics, including lactic acid by *Lb. plantarum* and 3-Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid, PLA).

The genomic architecture and the induced metabolic consequences are central to the success of *Lb. plantarum* in industrial applications. Moreover, the most of *Lb. plantarum* selected for their antimicrobial activity has been isolated from fresh or fermented food.

Therefore, the characterization and selection of food-borne *Lb. plantarum* strains remains a topic of great interest for applied research. On the basis of this last finding, the first part of this PhD study (**Chapter II**) was devoted to isolate and identify food borne *Lb. plantarum* as well as to evaluate their antimicrobial range. Thirty-two samples from three type of traditional fermented food were subjected to microbiological analyses in order to isolate and select *Lb. plantarum* strains to be used as antagonistic strains (producers) against undesirable food-stuff microorganisms. To identify LAB isolates, several approaches were used, consisting of the DGGE analysis and 16S rRNA gene sequencing. While the antimicrobial activity, exerted by cells or cell-free supernatants of *Lb. plantarum* strains, were evaluated by spot on the lawn test and by agar well diffusion assay test. The results evidenced that *Lb. plantarum* represents the prevailing lactobacilli species in sourdough andred wine, while this species were detected only in few sample of fermented sausages. In detail 60 *Lb. plantarum* strains were isolated from red wines, 36 strains from sourdoughs, and 10 from fermented

sausages. Out 106 *Lb. plantarum* strains, seven strains evidenced the ability to inhibit Gram negative and Gram positive bacteria as well as moulds strains. These inhibitory effect was not attributable to pH decrease, since in the presence of neutralized CFS of producer strains were also detected with a strong antimicrobial activity. Noticeable was the data that evidenced a strong antimicrobial activity produced by *Lb. plantarum* RTB strain against *L. innocua* ATCC 33090. Since *L. innocua* has been deemed a suitable biological indicator for *L. monocytogenes* and it revealed a similar sensitivity to different stress condition.

In the last years, great attention was focused on the inhibitory action against *Listeria monocytogenes* exerted by *Lb. plantarum* strains. The interest towards this topic is due to diseases caused by *L. monocytogenes* and which are known as "listeriosis" (causative agent of abortions, gastrointestinal diseases or septicaemia, thatoften lead to the death of infected individuals). This pathogen bacterium, growing at low pH, at refrigeration temperature, and at very high salt concentrations, is isolable from several food products, albeit in low numbers. Several studies reported the characterization of antimicrobial substances produced by certain *Lb. plantarum* strains. However it is well known that the knowledge of the undesirable strains response to these antimicrobial substances (stress conditions) represents a crucial step for the definition of an effective bio-control tool. Several mechanisms, can be developed by *Listeria* spp. in order to resist the injuries caused by stress conditions (temperature, acidity, NaCl). In detail, the stress seems to induce variations in the synthesis of certain cell components, especially proteins. Nevertheless the literature is very poor in studies focusing on the mechanisms of response, in terms of susceptibility or resistance, expressed by *L. monocytogenes* against antimicrobial substances produced by *Lb. plantarum*. Therefore, the second part

of the present PhD activity focused the attention on the stress response of *Listeria* to the presence of *Lb. plantarum* (**Chapter III** e **Chapter IV**). In detail, a commercial *L. innocua* strain was used as a pathogen surrogate throughout this study. For this purpose a multiple technique approach was adopted in the study, consisting of microbiological (dynamic model to predict the growth, cell counts) and proteomics (SDS-PAGE and 2D-E) approaches

Results showed that both cell and cell free supernatant of *Lb. plantarum* strain RTB represent a strong stress factor for *L. innocua* ATCC 33090, expressed through its growth inhibition. In detail, the inhibition was not attributable to organic acids produced by *Lb. plantarum,* since *L. innocua* ATCC 33090 expressed a series of new protein including Universal Stress Protein (USP) in the presence of lactic acid alone, that allowed to react to the acidic environment. On the other hand, the presence of *Lb. plantarum* RTB produced on *L. innocua* not only the expression of USP, but also the degradation or non-expression of other proteins. This phenomenon could be due to several antimicrobial substances and mechanisms carried out by the producer strain, and they could be responsible for the inhibition exerted by *Lb. plantarum* RTB against *L. innocua* ATCC 33090.

Particular attention was focused on the neo-expressed USP, a group of proteins induced by different stress conditions and which are found in numerous prokaryotic as well as eukaryotic organisms. The majority of UPS genes are monocistronically expressed, and different transcription factors, promote transcription of USPs. The significance of USPs in the resistance or susceptibility model of *L. monocytogenes* is presently unknown. Moreover few information are available in literature about the biochemical function and 3D-structures of USPs in bacteria and there are no 3D-structures for USP of *Listeria*.

available. Bioinformatics approach can help to get more information about the structure of USPs and the function of these proteins. Therefore in the 3rd phase of this PhD study (**Chapter V**), the three-dimensional (3D) structure of a USP (EHN60729.1) belonging to *L. innocua* was predicted on the basis of the available template (PDB code:3S3T ; structure deposited by Osipiuk et al., 2011) homologues from Protein Data Bank. The Comparative Homology Modeling procedure uses the structure of proteins experimentally determined (template) to predict the 3D structure of a protein that has a similar amino acid sequence (target). The Comparative Homology Modeling approach can be used when the template and target possess at least 30% identity. In the present study the hypothetical USP of *L. innocua* shares 31% amino acid with the template 3S3T which corresponds to about 85% of the C-α with 3.5 Å from the correct position. The accuracy of the model is confirmed by the values of the torsion angles *phi* and *psi* showed in the Ramachandran plot as well as the QMEAN Z-score. The RMSD (0.3 Å) of the final refined model confirms the evolutionary relationship between the model and the template.

Of interest are the results regarding the analyses of the interfaces carried out with both PISA WebServer and with the multiple structural alignment (MUSTANG). The surface of the interfaces ($\Delta G < 0$, see Figure 5.6) is amongst the average values of expected for homologous proteins, but even more interesting is the presence of highly conserved residues in the region involved in the formation of the dimer and of residues that represent the ATP-binding motif, which is fundamental in the formation of the tetramer. This work suggests that *L. innocua* possesses a UspFG-Type and that this protein can assemble in a tetrameric structure. These results, although to be confirmed experimentally, provide important information about a poorly studied protein and may

stimulate experimental investigations.

Overall, the results obtained in this study improved the knowledge both in stress response of *L. innocua* and in develop of bio-control (anti-listeria adjunct starter or protective cultures) useful in food bio-preservation.

INTRODUCTION

1.1 UNDESIRABLE MICROORGANISMS IN FOOD PRODUCTS

1.1.1 SPOILAGE BACTERIA

Food spoilage is a complex of changes that renders a food product unacceptable to the consumers from a sensory point of view. Physical damage, chemical changes (oxidation, colour changes) and microbiological metabolism may be responsible of food spoilage. Microbial spoilage is the most common cause of food decomposition; textural changes (degradation of polymers), off-odours, off-flavours, due of metabolites production, are the main changed sensory observed. The growth and metabolism of spoilage microorganisms is strictly correlated to intrinsic characteristics of raw material (chemical composition, pH, a_w), and extrinsic characteristics, such as temperature, atmosphere etc. Furthermore, the interactions between microorganisms, processing, preservation and storage conditions, are also crucial for the development of a specific specie(s) into a product.

At the point of sensory, the spoilage micro flora is so-called specific spoilage organism (SSO) of the product (Gram et al., 2002).

In general, several of microorganisms are able to produce spoilage of a food product, but the spoilage ability of an organism is related to the level of growth, kind and quantity of metabolites produced.

Meat and fish spoilage

Pseudomonas spp. (*Ps. fragi, Ps. fluorescencs, Ps. lundensis*) are the principal spoilages of the meat stored aerobically at different temperatures (-1 to 25°C) (Koutsoumanis et al., 2006). Population levels of 10^{7-8} CFU/g of pseudomonads have been attributed to slime and sulphydryl off-odour by methionine metabolism (Nychas et al., 2008; Segal & Starkey, 1969).

Cold-tolerant *Enterobacteriaceae* are also correlated to spoilage of chilled meat both stored aerobically and anaerobically; they are responsible of sulphydryl and cheesey off-odour (Dainty & Mackey, 1992; Segal & Starkey, 1969).

Lactic acid bacteria (LAB) and *Brochothrix thermosphacta* are also present among the anaerobic spoilage bacteria of chilled meat, but their contribute in meat spoilage is not relevant. Instead, these bacteria are the most important cause of spoilage of meat under vacuum or modified atmospheres (Skandamis & Nychas, 2002). In particular lactic acid bacteria are responsible of greening (Nychas et al., 2007) and souring of meat (acetic acid, L,D-lactic acid) by glucose, ribose and others CHO compounds degradation (Nassos et al., 1983).

Fish spoilage is frequently due to *Pseudomonas* spp. that is responsible both fruity off-odour and sulphydryl off-odour (Miller et al., 1973). Furthermore, sulphidy off-odour, and fishy off-odour (e.g trimethylamine; TMA) are ascribable to *Shewanella putrefaciens* (Gram & Melchiorsen, 1996). Instead, ammonia putrid may be due to lactic acid bacteria, *Enterobacteriaceae* and *Photobacterium phosphoreum* (Jorgensen et al., 2000).

Milk and dairy spoilage

Members of genus *Pseudomonas* are common in milk, and several species are frequently isolated from raw milk (Ternstrom et al., 1993; Jayarao & Wang, 1999). Species of *Pseudomonas* are been found in pasteurised milk originate from post-process contamination together psychrotrophic *Bacillus* (Ternstrom et al., 1993; Eneroth et al., 2000). Several researches, showed that *Pseudomonas* species produces rancid and bitter off-flavours in milk and in dairy products because have a high lipolytic and proteolytic activity (Wiedmann et al., 2000; Dufour et al., 2008; Boran & Ugur, 2010; De Jonghe et al., 2011). Furthermore, *Ps. fluorescens* is the causative agent of blue coloration in fresh and low-acid cheese (Martin et al., 2011). Other important psychrotrophs associated with raw milk include members of the genera, *Micrococcus, Aerococcus, Lactococcus* and *Enterobacteriaceae* family (Ledenbach & Marshall, 2009).

In processed dairy foods, there are several microorganisms able to contaminate the cheese during manufacturing. Coliforms and yeasts are the most frequent spoilage of short ripening cheeses, and the early blowing is the typical index of their contamination. Instead, the late blowing in hard and semi-hard cheeses is a typical spoilage of *Propionibacterium* spp. and *Clostridium* spp. spores of clostridia may survive during the manufacture of cheese and are able to germinate and grow in the product (Le Bourhis et al., 2005). In addition to abundant gas production, clostridial species (e.g. *C. beijerinckii, C. sporogenes, C. tyrobutyricum*) produce off-odours by release of large quantities of butyric and acetic acid (Le Bourhis et al., 2007).

Alcoholic beverages spoilage

Alcoholic beverages, such as beer and wine, are characterized by alcohol presence which has an antimicrobial effect against several organisms.

Beer has been recognized as a stable beverage from microbiologically point of view because in addition of ethanol presence (0.5-10% w/w), there are many other compounds with antimicrobial activity. Bitter compounds (17-55 ppm of iso-α-acids) carbon dioxide content (0.5%), low pH (3.8-4.7) and reduced concentration of oxygen (less than 0.3 ppm) makes beer an hostile environment for survival of many microorganisms (Sakamoto & Konings, 2003; Suzuki, 2011). Foodborne pathogens cannot survive in beer, but a restrict number of yeasts and bacteria species are able to grow and produce several defects into final product.

The Gram-positive bacteria *Lactobacillus brevis, Lactobacillus lindneri* and *Pediococcus damnosus* are considerate as the major beer spoilers (Back, 2005).

Strains of *Lb. brevis* isolated from beer are capable to produce haze (turbidity), sediment and acidification, but no diacetyl off-flour. *Lactobacillus lindneri* is also responsible of faint haze (turbidity) and sediment with no off-flavours formation. *Pediococcus damnosus* may produce exopolysaccharides, making the beer ropy and gelatinous. Furthermore, beer spoilage caused by *P. damnosus* is characterized by acid and diacetyl off-flavour (Back, 2005).

The Gram-negative bacteria *Pectinatus* and *Megasphaera* are more sensible to alcohol, low pH and hops acids than lactic acid bacteria, therefore, their presence into beer is infrequent. In contrast, beers spoiled by *Pectinatus* and *Megasphaera* are characterized by unpleasant taste and odours, such to make it undrinkable. Furthermore, Megasphaera

slightly turbid beer, instead *Pectinatus* exhibits heavy sediments, haze and small clots too (Back, 2005).

Beer spoilage may be also due to wild yeasts, such as *Saccharomyces cerevisiae*, *Pichia* spp., *Candida* spp. and *Dekkera* spp. (van der Aa Kuhle & Jespersen, 1998).

Wine is another alcoholic beverage characterized by high ethanol concentration, low pH, high acidity, and limited nutrients. For these reasons, a narrow number of bacteria and yeasts species are capable to grow in the wine. Lactic acid bacteria (*Lactobacillus*, *Oenococcus* and *Pediococcus*), and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) are the only groups of bacteria found in grape must and wine (Bartowsky, 2009). All acetic acid bacteria are considered spoilage, they produce many quantities of acetic acid and acetaldehyde from the oxidative metabolism of ethanol (Adachi et al., 1978). Furthermore, acetic acid bacteria can form ethyl acetate from acetic acid, which has a nail polish remover aroma (Bartowsky & Henschke, 2008).

LAB produce several volatile secondary metabolites, which have a strong effect on wine sensory qualities. Members of heterofermentative LAB, such as *Oenococcus oeni* and same *Lactobacillus* species, may be responsible of mousy off-flour and nitrogen-heterocylic compounds [2-acetyl- tetrahydropyridine (ACTPY), 2-acetyl-1-pyrroline (ACPY) and 2-ethyltetrahydropyridine (ETPY)] produced from ornithine and lysine metabolism (Snowdon et al., 2006; Grbin et al., 2007). Mousy wines are been also associated to *Dekkera* and *Brettanomyces* (Grbin et al., 2007).

Others LAB metabolism related compounds are acrolein, exopolysaccharide , mannitol and diacetyl, responsible of bitterness, slimy, viscous and buttery respectively (Bartowsky, 2009).

Bakery products spoilage

Post-process contaminations with moulds and yeasts are quite frequent in bakery products spoilage. The most common moulds correlated to bakery products spoilage are *Aspergillus, Fusarium*, and *Penicillium* (Gerez et al., 2009). Moulds typically produce off-odours, but they are also potential mycotoxin producers that may cause public health problem.

Yeast spoilage may be divided into two types: (a) visible growth on surface of products (white, cream or pink spots) and (b) fermentative spoilage of products and ingredients characterized by alcohol, ester and other odours production (Legan & Voysey, 1991).

Pichia, Candida and *Zygosaccharomyces* are common of visible spoilage instead *Saccharomyces* and *Hansenula* are more frequent in fermentative spoilage.

Sporogenous bacteria also have a potential to deteriorate baked products, in particular *Bacillus subtilis* causes rope in bread. Ropey bread is characterized by fruit odour (similar to pineapple) and by discoloration from brown to black (Rosenkvist & Hansen, 1995).

Truffle spoilage (Tuber spp.)

Tuber spp. (e.g. *T. aestivum* and *T. melanosporum*), better know as truffles, are hypogea ascocarps particularly appreciated for their culinary properties. *Tuber aestivum* and *T. melanosporum* are distributed in Mediterranean countries: France, Italy and Spain (Rivera et al., 2010). Fresh truffles are characterized for a short shelf life and a rapid decay of odour and taste due in part to high presence of microorganisms (10^{4-7} cfu/g) (Nazzaro et al., 2007; Reale et al., 2009). Bacteria associated with truffles are mainly represented by *Pseudomonas* spp., sporogenous bacteria, *Actinomycetes*, *Rhyzobacteria*

and *Enterobacteriaceae* (Sbrana et al., 2002; Barbieri et al., 2007; Rivera et al., 2010). Both yeasts and moulds (e.g. *Debaryomyces hansenii*, *Saccharomyces dairensis*; *Aspergillus*, *Penicillium*) are been also isolated from *T. aestivum* and *T. melanosporum* and associated to decay of product (Buzzini et al., 2005; Rivera et al., 2010).

1.1.2 PATHOGENIC BACTERIA (FOODBORNE PATHOGENS)

Foodborne diseases encompass acute and chronic syndromes of different duration and severity caused by several pathogen microorganisms. The proportion of disease transmitted by food, differs both pathogen (species/strain, inoculum, etc) and host (age, sex, immunity etc.).

Over the last years, several factors such as, genetic factors, antimicrobial resistance, aging of population, increase of internationalization travel and globalization of food trade, have contributed to change the epidemiology of foodborne illness.

In the United States, foodborne diseases caused 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths each year. The most (58%) of illnesses are caused by norovirus, nontyphoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10%) and *Campylobacter* spp. (9%). Nontyphoidal *Salmonella* spp., norovirus, *Campylobacter* spp. and *Toxoplasma gondii* caused the most hospitalizations instead nontyphoidal *Salmonella* spp., *T. gondii*, *Listeria monocytogenes* and norovirus caused the most deaths (Scallan et al., 2011).

In Australia, 32 % (5.4 million) of gastroenteritis are foodborne, and the most of them are caused by norovirus, enteropathogenic *E. coli*, *Campylobacter* spp. and *Salmonella* spp. (Hall et al., 2005).

Likewise, in the European Union (EU) most of foodborne diseases, are caused by *Campylobacter* spp., *Salmonella* spp., virus and bacterial toxins.

Thermophilic Campylobacter spp. is the etiologic agent of campylobacteriosis in humans. *Campylobacter jejuni* followed by *C. coli and C. lari* are the most commonly associated bacteria with human infections. The infective dose of these bacteria is very low (500 cfu) and the symptoms of illness are generally mild (e.g. diarrhoea, abdominal pain, nausea and fever) (Black et al., 1988). Rarely, extra-intestinal infections such as neurological dysfunctions and respiratory paralysis (Guillain-Barré syndrome) occur (Allos, 1997).

Since 2005, *Campylobacter* is considered the most commonly reported gastrointestinal bacteria pathogen in humans in EU. In 2009, 198,252 confirmed human campylobacteriosis cases were reported in EU and an increase of 4.0 % was also recorded, relative to previous year. Furthermore, confirmed cases of campylobacteriosis showed a slightly fluctuating between 2005 and 2009 (EFSA, 2011). The most of cases were reported during the summer (from June to August) and children under the age of five had the highest notification of rate.

The principal reservoirs of *Campylobacter* are the alimentary tracts of domesticated birds and mammals, such as poultry, cattle, pigs and sheep. Therefore, the bacteria can readily contaminate several foodstuffs, including meat, raw milk and dairy products, and less frequently fish and vegetables.

EFSA (2011), in according with also other studies (Sahin et al., 2002; Hermans et al., 2012), reported that poultry meat still appears to be the main foodborne source of *Campylobacter*, because bacteria remains at a high level in fresh poultry meat.

Meats, including beef, pork and lamb have been implicated in infection, but with a reported prevalence lower than those reported on poultry (Kramer et al., 2000).

A significant presence of *Campylobacter* has been detected also on other foods, such as raw milk, dairy foods, fresh and packaged fruit and vegetables (Hussain et al., 2007; Verhoeff-Bakkenes et al., 2011; Giacometti et al., 2012).

Furthermore, *Campylobacter* spp. have been reported to survive in water, at low temperature for up four months, during processing and in the environment generally (Rollins & Colwell, 1986; Buswell et al., 1998; Hazeleger et al., 1998; Park, 2002; Cools et al., 2005). The wide spreading of *Campylobacter* in foodstuff could be due to environmental survival mechanisms, that paradoxically appears to be very limited compared with other bacteria (Park, 2002). Several key regulators of stress response, such as those involved in oxidative stress (SoxRS, OxyR, SodB and KatG), osmotic stress (e.g. BetAB, GbsAB), cold stress (CspA) and heat stress (RpoH), are present in enteric bacteria *Salmonella* spp. and *E. coli* and absent in *Campylobacter* spp. Interestingly, *Campylobacter* contains two negative regulators of heat-shock response (HspR and HrcA) and several two component regulatory systems that are not generally found in other bacteria and could be involved in stress defence (Murphy et al., 2006). More information about the role of these systems, could help to understanding *Campylobacter* strategy adopted for survives in several environments.

Human salmonellosis is the second reported zoonotic disease in humans in EU, following campylobacteriosis. In 2009, 31 % (5,550 outbreaks) of foodborne outbreaks were due to *Salmonella* with a high frequency of *S. enteridis* and *S. typhimurium* serovars. The confirmed cases of human salmonellosis were 108,614 (23.7 cases per 100,000) of which 4,156 in Italy, corresponding to 7.5 cases per 100,000 of population (EFSA, 2011).

Noteworthy, is the decreasing trend of salmonellosis, EFSA (EFSA, 2011) reported an average reduction of 12.0 % per year between 2005 and 2009. This decline is mainly due to reduction of *S. enteridis* in the principal resource of infection, such as eggs and flocks of laying hens as well as other control measures along the food chain.

Human salmonellosis is usually characterised by the acute onset of fever, abdominal pain, nausea, and sometimes vomiting. The most infections are self-limiting but also severe diseases may be occurring, such as bloodstream infection.

The principal reservoir of *Salmonella* is the intestinal tract of domestic and wild animals, therefore this bacteria may be transmitted both food of animal and plant origin.

In EU, eggs and poultry meat represents the food with a highest risk of contamination and the main vehicle of *S. enteridis*. Instead, pig, poultry and bovine meat are the most common foodstuff contaminated by *S. typhimurium* serovar.

Fruit and vegetables irrigated with faecal contaminated water may also represent a risk of infection by *Salmonella*.

Furthermore, many other foodstuff may be contaminated by *Salmonella* and consequently to cause illnesses. For example in the United States a sever outbreak was caused by peanut butter contaminated with *Salmonella* serotype Tennessee (Sheth et al., 2011).

Escherichia coli is another bacteria responsible of numerous diseases in the world. Members of these bacteria are widespread among vertebrate gut microbiota and are considered to be an indicator of fecal pollution in water. Fecal decomposition of *E. coli* into soil represents an intermediate step in a host-soil-water cycle that is one mechanism by which *E. coli* may colonize new hosts (Collins et al., 2005). Human infection with *E. coli* occurs through ingestion of food or water contaminated with faecal matter. The

most of strains of *E. coli* are harmless but certain serotypes are able to cause several infections, with manifestations ranging from mild diarrhoea to severe haemorrhagic colitis and Haemolytic Uremic Syndrome (HUS).

Based on the virulence factor, *E. coli* have been divided into several pathotypes (Nataro & Kaper, 1998; Kaper et al., 2004). Shiga toxin-producing *E. coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), is the pathotype that cause haemorrhagic colitis and haemolytic uremic syndrome named also entero-haemorrhagic *E. coli* (EHEC) (Nataro & Kaper, 1998).

The serotype 0157:H7 is the major STEC associated in human disease, therefore is common to distinguish Shiga toxin-producing *E. coli* in O157 and non-O157 serotype.

Ruminants, such as, cattle and sheep, are the major reservoir of STEC and the consumption of their meat, especially undercooked beef hamburgers, represent one of main cause of Shiga toxin-producing *E. coli* transmission. However, also other kinds of foods have been reported to carry STEC, including pork, poultry, fish, punch and iceberg lettuce (Mathusa et al., 2010).

Infections *E. coli* shiga toxin-producing are less frequent than those of other bacterial zoonoses but are generally more severe. In this regard industrialized countries increased resources and measures for to control and reduce the STEC rate infections associated.

In Connecticut State, both O157 and non-O157 STEC infections incidence decreased from 2000 through 2009 (Hadler et al., 2011), instead STEC infections in Australia have remained fairly steady but low over the past 11 years with a annual rate of 0.4 cases per 100,000 (Vally et al., 2012).Unfortunately, in other industrialized countries, STEC infections have been an opposite and worrying trend. For example, in New

Mexico, shiga toxin–producing *E. coli* (STEC) increased from 0.9 cases per 100,000 population in 2004 to 1.7 in 2007 (Lathrop et al., 2009).

Similar, in EU, also if not statistical significantly, STEC increased from 3,269 to 3,573 (0.75 per 100,000) confirmed cases between 2005 and 2009 and more of 50 % of them were attributed to O157 serotype. In Austria, Belgium, Ireland, and the Netherlands a significant increasing five-year trend was recorded while the five-year trend was significantly decreasing in Estonia, Germany, Hungary, and Malta (EFSA, 2011).

In the last years more attention was also focused for non-O157 STEC serotypes because although less frequent in human diseases, may cause sever infections (Gyles, 2007).

In 2011, a large outbreak of bloody diarrhoea and haemolytic uremic syndrome was recorded in Germany. Shiga toxin-producing *E. coli* serotype 0104:04 associated to consumption of contaminated bean and seed sprouts, caused 3,802 infection cases, 2,938 (77.3%) involved cases of enterohaemorrhagic *E. coli* (EHEC) infection and 864 (22.7%) cases of HUS (Frank et al., 2011).

Listeria monocytogenes is another foodborne pathogen that attracted great attention in the last years by scientists and experts. This bacterium causes human listeriosis, that although relatively rare, it is a disease generally serious characterized by a high morbidity and mortality in vulnerable population. In 2009, 1,645 confirmed human cases were reported in EU, the rate of fatality was 17 % and a significant increasing trend was recorded in Austria, Denmark, Hungary, Italy, Spain and Sweden. Furthermore, on the base of reported confirmed cases, it is estimated that in 2009 there were approximately 270 human deaths due to listeriosis, 90 deaths due to salmonellosis and 40 deaths due to campylobacteriosis in the European Union (EFSA, 2011). The number and the severity of foodborne diseases recorded during the years, help to

understand the strong impact of these illnesses both on the health public and annual costs of countries.

1.2 *LISTERIA MONOCYTOGENES*, A FOODBORNE PATHOGEN BACTERIA

1.2.1 EPIDEMIOLOGY AND INFECTION PROCESS

The genus *Listeria* consists of a number of species including *L. monocytogenes*, *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii*, the two newly identified species, *L. marthii, L. rocourtiae* (Graves et al., 2010; Leclercq et al., 2010) and *L. grayi*, which is distantly related to the other *Listeria* spp. *Listeria monocytogenes* represents the specie most commonly associated with diseases in both animal and humans. This bacteria is a facultative pathogenic saprotroph, can live in soil and decaying vegetation, but once it enters an animal or human host can cause severe diseases. *L. monocytogenes* is the etiological agent of listeriosis, clinically defined when the organism is isolated from blood or cerebrospinal fluid. The most severe clinical manifestations of invasive human listeriosis include septicemia, encephalitis, meningitis, and spontaneous late-term abortion. Subclinical manifestations include a mild influenza-like illness, sometimes combined with gastroenteritis as well as ocular and cutaneous listeriosis (McLauchlin et al., 2004). Healthy human individuals rarely contract invasive listeriosis, while groups at high risk for contracting listeriosis are immunocompromised individuals such as HIV patients and the elderly, infants, and pregnant women (Schlech, 2000). The majority (99%) of the infections caused by *L. monocytogenes* are thought to be foodborne (Swaminathan & Gerner-Smidt, 2007). The first documented outbreak of foodborne listeriosis occurred in 1979 in a Boston hospital (Gellin & Broome, 1989). Twenty three

patients were involved because ate contaminated vegetables prepared within the hospital. In 1981 another outbreak due to consumption of contaminated coleslaw occurred in the Maritime Provinces, Canada (Schlech et al., 1983). During the years, several listeriosis outbreaks have been associated to different foods, such as vegetable products in the early 1980s and dairy products in the early 1990s (Farber & Peterkin, 1991). In the last years, ready-to-eat meat and poultry products have been associated with the outbreak of epidemic listeriosis (Donnelly, 2001).

(Vázquez-Boland et al., 2001) have reported that the incidence of human listeriosis vary from 0.2 to 0.8 sporadic cases/100,000 people per year in Europe and in the US.

In Europe, the incidence of listeriosis outbreaks have increased since 2000 (Allerberger & Wagner, 2010); in particular the incidence among those >65 years old appears to have increased over the two last years.

The pathophysiology of listeriosis has been largely studied: pathogenic *Listeria* enters the host primarily through the intestine. After the intestinal translocation the pathogen reaches the liver where actively multiplies until the infection is controlled by a cell-mediated immune response (Werbrouck et al., 2006). In immunocompromised patients, the proliferation of *Listeriae* in the livery is unrestricted and after a prolonged time of low-level of bacteremia the pathogen may invade secondary target organs such as the brain and the gravid uterus (Longhi, 2004). *L. monocytogenes* is able to survive in macrophages and to invade a variety of cells such as epithelial cell, endothelial cells, hepatocytes and fibroblast. The internalins proteins (e.g. InlA and InlB) are important for internalization of *L. monocytogenes* in to cells.

1.2.2 *LISTERIA* FOOD CONTAMINATION

Listeria monocytogenes is a ubiquitous bacterium and is widespread in nature, it can survive and grow under several environmental conditions. The presence of *L. monocytogenes* has been detected in several foods. Minced beef, minced pork, minced chicken and minced pork–beef mixture samples have been found contaminated by *L. monocytogenes*. The organism was also isolated from cold-smoked fish and from ready-to-eat raw seafood samples (Autio et al., 1999; Inoue et al., 2000; Kovacevic et al., 2012). *L. monocytogenes* has been isolated by raw milk (Aygun & Pehlivanlar, 2006) cheeses, dairy products (Lyytikainen et al., 2000), ice cream and chocolate milk (Dalton et al., 1997; Miettinen et al., 1999).

L. monocytogenes does not form endospores but is quite resistant to the effects of freezing, drying and heating. It can survive for long time in refrigerated, frozen and dried foods, can grow between 0 and 45 °C and show high tolerance to acid conditions and high salt concentrations.

The bacterium usually enters the food through raw materials, water, workers and through a large variety of food-processing equipment (Tompkin, 2002). It can persist in environment working for a long time (Miettinen et al., 1999). Usually, the applications of good sanitization practices are able to kill *L. monocytogenes*, but if the sanitization procedures are inappropriate this micro-organism may be able to establish themselves, multiply and become resident (Kumar & Anand, 1998; Gram et al., 2007). *L. monocytogenes* is able to form biofilms (Møretrø & Langsrud, 2004), an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix (Donlan, 2002). This bacteria can form biofilm on material used in packaging and foods processing. Several studies showed the presence of biofilms on rubber, glass, stainless steel and plastics (Sinde & Carballo, 2000; Stepanovic et al.,

2004; Chae et al., 2006; Poimenidou et al., 2009).

1.2.3 GENETIC STUDIES

In the last years great attention was focused for the study of genomic and comparative sequence in Listeria spp. In a recent study (Bakker et al., 2010) of comparative genomic among genus *Listeria*, has been shown that genome evolution is characterized by limited gene acquisition and limited gene loss. Consequently, it is possible to observe a conserved genome size i between 2.8 and 3.2 Mb.

Extensive phylogenetic research based on nucleotide polymorphism showed that *L. monocytogenes* consist of at least four evolutionary lineages, designed lineages I, II, III and IV (Nightingale et al., 2005; Orsi et al., 2008; Ward et al., 2008). Most serotype of *L. monocytogenes* associated with human clinical cases seem to belong to lineages I (serotypes 1/2b and 4b). Lineage II strains (e.g serotypes 1/2a), common in foods and widespread in the environment, are commonly isolated from animal listeriosis cases and sporadically isolated from human clinical cases (Orsi et al., 2011). Lineage III and IV isolates are not widespread, they are predominantly isolated from animals source and sporadically are involved in human listeriosis (Bakker et al., 2012).

Several studies have shown that *L. monocytogenes* strains have a highly syntenic genome (Hain et al., 2007; Bakker et al., 2010) with same difference (presence/absence) of genes involved in transport and associated with the cell wall. The most clear differences between gene presence in lineages I and II isolates occurr at cell surface (Zhang et al., 2003; Doumith et al., 2004). The virulence genes and/or virulence-related genes are usually present in all *L. monocytogenes* strains (Doumith et al., 2004) but several studies showed that there are the same differences among and within the lineages isolates.

Using microarray analyses, a siderophore was found present in lineages I isolates but absent in the lineage II isolates (Borucki et al., 2003; Call et al., 2003). Siderophores are virulence factors involved with ferrous transport, they are also present in other pathogen bacteria such as *Staphylococcus aureus* and *Bacillus anthracis* (Dale et al., 2004; Abergel et al., 2006). The absence of siderophores among the lineages II isolates could be related to lower virulence in comparison to lineage I isolates (Orsi et al., 2011).

Recently, (Cotter et al., 2008) have described in the listeriolysin S, a new peptide hemolysin, carried in about half of the isolates of lineage I. This virulence factor is encoded in the *Listeria* pathogeniticy island 3 (LIPI-3) by gene lls.

Internalins are proteins characterized by the presence of a leucine-rich repeat (LRR) and are involved in adhesion and internalization of pathogen into host cell. The two members InlA and InlB of internalins family are well studied and are present in all *L. monocytogenes*, while a third member of the family, encoded by inlC , is absent from the same lineage III isolates (Jia et al., 2007). The inlC2, inlD, inlE and inlJ are present in both lineage I and III isolates while inlG, inlH and inlF have been found among lineage II only (Jia et al., 2007; Sabet et al., 2005; Tsai et al., 2006).

Other studies have shown constitution differences of teichoic acids among serotypes 1/2, 3, and 4b (Promadej et al., 1999). The wall teichoic acids plays an important role both antigenicity and phage specificity. Serotype-specific phages are able to recognize the wall teichoic acid of the bacterium.

Same genes involved in stress response (sigC, lmo0421 and lstR) have been only identified in lineage II isolates but not among lineage I isolates (Zhang et al., 2003). These gene who encode for an alternative sigma factor (σ^c), a putative member of the RodA-FtsW family and a PadR-like protein are induced under heat shock stress (Zhang

et al., 2005). Chen et al., (2009) have shown that the gene lmo0038, involved in heat and acid stress response, was present only in lineage I and II isolates whilst absent in lineage III and IV isolates. This aspect could to help to explain the low prevalence of lineage III isolates in food and food-related environment (Orsi et al., 2011).

1.2.4 *LISTERIA INNOCUA* AS SURROGATE BACTERIA OF *L. MONOCYTOGENES*

Ecological and genomic comparative studies in *Listeria* spp. showed a high similarity between the pathogenic *L. momocytogenes* and the non-pathogenic *L. innocua* (Glaser et al., 2001; Girardin et al., 2005).

An extensive study on *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262 showed that the two species had a similar size chromosomes and similar number of protein-coding genes (Glaser et al., 2001). Moreover, both genomes encoded many putative surface proteins. Also internalins family proteins were found in both bacteria species except protein-gene containing LPXTG sorting motif (inlA, inlE, inlF, inlG and inlH) and protein-gene containing a GW repeat anchoring motif (inlB), present only in *L. monocytogenes* EGD-e. Several secreted proteins important for virulence, including PlcA and PlcB, were found in *L. monocytogenes* whilst were not found in *L. innocua*.

Different genes encoding transport proteins, were found in in both species and many of them were devoted to carbohydrate transport, mediated by phosphoenolpyruvate-dependent phosphotransferase systems (PTS). This characteristic would seem to be correlated to the ability of *Listeria* spp. to colonize and grow in a broad range of ecosystems (Glaser et al., 2001). A specific β-glucosides permeases II was found only in *L. monocytogenes* not in *L. innocua*. The carbohydrate β-glucosides has a significant impact on the virulence of *L. monocytogenes* (Kreft & Vázquez-Boland, 2001). Many transcriptional regulators genes were observed in both species but the virulence

regulatory factor PrfA was absent in *L. innocua*. Moreover, four classes of stress proteins (HrcA, sigmaB-dependent, Clp and class IV genes) and genes involved in acid resistance [(e.g., genes encoding glutamic acid decarboxylase (gad)] were identified in both *Listeria* species. Only one of three gad paralogs of *L. monocytogenes* (lmo0447) was missing from *L. innocua*. Also three genes (lmo2067, lmo0446 and lmo0754) involved in bile salt degradation were present in *L. monocutogenes* and absent in *L. innocua*.

The high genomic similarity and the environmental niches shared by the two species, have induced many researchers to use the non-pathogen *L. innocua* as surrogate of *L. monocytogens* for a better understanding of the behaviour of pathogen specie.

L. innocua has been used for study a new thermal process to kill *L. monocytogenes* in hamburger patties (Friedly et al., 2008). The effect on thermally processed was also investigated for growth control of *L. innocua* in orange juice (Char et al., 2009). In other studies, *L. innocua* was used as *L. monocytogenes* surrogate for understanding the grothh ability onto ready-to-eat (RTE) meat surface after ionizing radiation, ultraviolet light-C (UV-C) and flash pasteurization (FP) (Sommers et al., 2002; Sommers et al., 2008).

Furthermore, (Karaibrahimoglu et al., 2004) have studied the effect of pH on the survival of *L. innocua* in presence of calcium ascorbate in fresh-cut apples.

1.3 BIOPRESERVATION OF FOOD

1.3.1 LACTIC ACID BACTERIA AS PROTECTIVE CULTURES

Classical preservation methods, such as chemical (nitrates, nitrites, sulphites), and physical treatments (pasteurization, freezing, high pressure, ionizing rays, etc) are traditionally applied both to extend the shelf-life and to protect the quality of foods.

Although these methods are enough to assure food safety, they often compromise other quality features including taste and nutritional properties.

Furthermore, the concept of food quality is evolved in the last decades; consumers are being more and more inclined to research convenience foods, safe, taste and with high nutritional properties, and are interested in the consume of minimally processed foods with "naturalness" characteristics.

The use of classical preservation methods, especially chemical preservatives, is clearly not compatible with these naturalness characteristics of food products.

This fact has encouraged the research of alternative preservative methods, based on the biopreservation concept. Biopreservation delas with the extension of shelf-life and the enhancing of safety of foods by the use of natural compounds or controlled microflora and/or their antimicrobial products (Stiles, 1996).

The use of natural compounds (e.g. vegetable extracts) and biological agents (protective cultures) in food processing is also in accordance with the contemporaneous food trends that promote new categories of food such as the "bio-products".

Similar to starter cultures and probiotic, protective cultures (PCs) are food-grade bacteria, which may be strains naturally present in the food. PCs are selected for their ability to inhibit undesirable microorganisms rather than to deliver a desired flavour profile. Lactic acid bacteria (LAB) are interesting candidates for biopreservation, they have obtained the GRAS (Generally Recognized as Safe) status (Adams, 1999) and are well known for their ability in antimicrobial compounds production. LAB represent a microbial group largely widespread in nature, they may be isolated from several sources including plants, animals and guts of humans as well as from food such as meat, dairy products, wine and vegetables.

Lactic acid bacteria are extensively used as microbial starter in fermented foods because of their high production of organic acids (e.g. lactic and acetic acid) able to improve sensorial characteristics and to inhibit acid sensible bacteria. Furthermore, many other antimicrobial compounds are produced, including carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, bacteriocin-like inhibitory substances (BLIS) and reuterin (Caplice & Fitzgerald, 1999)

Although, the antimicrobial ability of LAB is well known in fermented food few studies are available for unfermented food. Selected microorganisms that have given good results in fermented food or in a model medium may be not efficient in unfermented food (Wessels & Huss, 1996; Bello et al., 2012).

Successful studies were obtained in seafood products, where LAB species were able to inhibit *Listeria* spp. with bacteriocin production or competition mechanisms (Nilsson et al., 1999; Yamazaki et al., 2003; Nilsson et al., 2005; Vescovo et al., 2006).

Lactobacillus spp. and *Pediococcus acidilactici* isolated from chicken carcasses showed psychrotrophic characteristics and were able to inhibit *Salmonella* spp. and *Listeria monocytogenes* (Sakaridis et al., 2012). These pathogens, were also inhibited in raw chicken meat by two bacteriocinogenic strains of *Enterococcus faecium* and *Lactobacillus fermentum* (Maragkoudakis et al., 2009).

Lactobacillus sakei displayed antimicrobial activity against *L. monocytogenes, B. thermosphacta* and *Leuc. mesenteroides* in cooked meat stored for 34 days in vacuum packaged at 7 °C without change in the sensorial properties (Vermeiren et al., 2004). The BLIS producer *Lb. sakei* CECT 4808, besides to improve the shelf life of sliced beef during the cold storage showed also limited antioxidative ability (Katikou et al., 2005).

Interesting results were obtained also in ready-to-eat fresh fruit and vegetable products biopreservation by the use of lactic acid bacteria. In particular, cell count of *S. typhimurium* and *E. coli* was reduced by 1 to 2 log cfu when apples and lettuce were treated with *Leuconostoc* spp. and *Lb. plantarum*, while the growth of *L. monocytogenes* was completely inhibited. Organic acids, hydrogen peroxide and bacteriocins produced by *Leuc. mesenteroides* were detected as main inhibition mechanisms against *L. monocytogenes* (Trias, Badosa, et al., 2008a).

Lactic acid bacteria, especially the genus *Lactobacillus*, are frequently involved in the antifungal activity (Magnusson et al., 2003; Sjogren et al., 2003; Hassan & Bullerman, 2008; Dalié et al., 2010).

Studies conducted in vitro showed the antifungal activity of *Lactobacillus* by producing several compounds, such as acetic, caproic, formic, propionic, butyric, n-valeric and phenyllactic acid (Corsetti et al., 1998; Lavermicocca et al., 2003). *Lb. plantarum* VTTD-78076 showed the ability to suppress the growth of *Fusarium* VTTD-80147 by the medium of benzoic acid, an imidazolidinedione derivate and a piperazinedione derivate (Niku-Paavola et al., 1999)

Applications in food processing of antifungal activity by some members of *Lactobacillus* spp. were also reported. *Lb. plantarum* 21B isolated from sourdough possessed antifungal activity against the common spoiling fungi of bakery products belonging to the genera *Apergillus, Penicillium* and *Fusarium*. Lavermicocca et al. (2000) hypothesize that the antifungal activity of *Lb. plantarum* 21B against some mould species (*Aspergillus niger, A. flavus, Fusarium graminearum, Penicillium corylophilum, P. roqueforti* and *P. expansum*), is attributable to the production of phenyllactic and 4-hydroxy-phenillactic acids. Sourdough and bread produced with *Lb.*

plantarum FST 1.7 showed consistent ability to retard the growth of *Fusarium* species (Dal Bello et al., 2007). These authors reported that *Lb. plantarum* FST 1.7 produced two cyclic dipeptides [Cyclo (I-Leu-I-Pro) and Cyclo (I-Phe-I-Pro)] with antifungal properties as well as lactic and phenyllactic acid. Furthermore, the bread manufactured with acetic and phenyllactic acid *Lactobacillus* producer and with addition of 0.2 % of conventional antifungal calcium propionate, has shown to have the same shelf life of bread produced with only 0.4 % of calcium propionate (Gerez et al., 2009). Good results of antifungal effect, were also obtained when *Lb. plantarum* 16 was added both to yoghurt and to orange juice, as the yeast *Rhodotorula mucilaginosa* growth was retarded (Crowley et al., 2012).

Although, several studies showed the potential use of PCs and/or their metabolites such as bacteriocins, in food preserving, the commercial applications of these products are still limited for different reasons.

Regarding the application of bacteriocin and/or bacteriocin-producing LAB, the major problem is related to the in situ antimicrobial efficacy, that as reported by Settanni and Corsetti (2008), may to depend by various factors such as proteases, food additives, food components and other inhibitors. Furthermore, other limitations in applying bacteriocin or bacteriocin-producing LAB cultures rest in the, possible development of resistance (Embarek et al., 1994) and ineffectiveness against Gram-negative bacteria (Helander et al., 1997).

Among the various bacteriocin isolated and characterized, nisin (e.g. marketed as Nisaplin® and Natamax®) is that best known, it is produced by Lactococcus lactic and has been approved for use in about 50 countries. Pediocin PA-1 is a bacteriocin produced by Pediococcus acidilactici and is commercialized as ALTA^{TM} 2431 by Kerry

Bioscience.

Regarding the application of protective cultures, is important to understand the nature of inhibition and factors affecting it for to debug an efficient PCs. Inhibition ability by PCs may be due by several mechanisms, such as competition for nutrients, production on inhibitors and parasitism. Moreover, many other factors affecting PCs performance, including temperature effect, inoculum size and food composition.

Currently, some commercial products are available; for example, the Danisco HOLDBAC^{TM} is a commercial preparation of protective cultures with capability to control the growth of fungi and bacteria, including *L. monocytogenes*, in dairy and meat products. Micocin® is another commercial preparation of LAB with anti-listeria activity in meat products.

The extensive application of PCs in food processing is still limited but in the future PCs could be used possibly in synergetic relationship with other hurdles such as the low temperature.

1.3.2 *LACTOBACILLUS PLANTARUM* IN FERMENTED FOOD

Lactobacillus plantarum is one of about 180 species recognized among the genus *Lactobacillus*: http://www.bacterio.cict.fr/l/lactobacillus.html. Many *Lactobacillus* species are highly specialized and are found in a restricte number of niches. For example, *Lb. delbrueckii* spp. *bulgaricus* is adapted to the dairy environment and is widely applied in yoghurt manufacture (van de Guchte et al., 2006). Whereas other species such as, *Lb. rhamnousus, Lb. reuteri, Lb. gasseri and Lb. acidophilus* are found in the mammalian gastro-intestinal tract (Russell & Klaenhammer, 2001; Siezen & G. Wilson, 2010) and are used as probiotic cultures. The specie *Lb. iners* is considered the predominant member of the vaginal microbiota (Macklaim et al., 2011).

In contrast, the ubiquitous *Lactobacillus plantarum* is able to colonize several ecological niches such as meat, fish, vegetables, diary products (Gardner et al., 2001; Ercolini et al., 2003; Aymerich et al., 2003; Bringel, 2005) and mammalian gastro-intestinal tract (Ahrné et al., 1998).

The ability to colonize several environments by *Lb. plantarum* could be attributed to large and organised genome of this bacterium.

Recent researches such as the complete genome sequencing of *Lb. plantarum* strains WCFS1 (Kleerebezem et al., 2003; Siezen et al., 2012), JDM1 (Zhang et al., 2009) and ST-III (Wang et al., 2011) as well as comparative genomic studies (Siezen & van Hylckama Vlieg, 2011) have shown interesting data about the genomic architecture of this bacterium. There is a high phenotypic and genotypic diversity as well as a high metabolic versatility and flexibility among *Lb. plantarum* group (Bringel, 2005; Molenaar et al., 2005; Siezen et al., 2010; Siezen & van Hylckama Vlieg, 2011). The large set of genes involved in sugar uptake and utilization as well as the large number of surface bound extracellular proteins is also likely to contribute to the large versatility with its environment (Kleerebezem et al., 2003). Furthermore, it is hypothesized that *Lb. plantarum* chromosome has specific regions, designated life-style adaptation regions, dedicated to the interaction with the environment (Kleerebezem et al., 2003).

In line with the ability of growing in many ecological niches, *Lb. plantarum* may be used in different food and health applications.

Some studies reported that use of *Lb. plantarum* in sourdough fermentation represent a valid means to improve texture, flavours and shelf-life of bakery products. *Lb. plantarum* 20B showed a good sourdough acidification and acid acetic production when pentosans and L-arabinofuranosidase where also added to the dough (Gobbetti et al.,

2000). Lactic acid produced by LAB (including *Lb. plantarum*) carbohydrate metabolism, is the main compound responsible of sourdough acidification and also have a little effect on bread flavour. Acetic acid is also positive because does improve the sensory properties of the final product. However, for a pleasant flavour perception it is necessary an optimal (2.0-2.7) molar ration lactate/acetate (the fermentation quotient, FQ). Other studies reported that the time of leavening and the acidification degree detected during dough manufacture were improved by the presence of *Lb. plantarum* strains (Pepe et al., 2004). A good sourdough acidification (pH lower than 5.0) also prevents the growth of undesirable microorganisms, such as, *Enterobacteriaceae* and rope-forming bacteria. Growth inhibition of typical bread spoilage rope-forming (*Bacillus subtilis* and *Bacillus licheniformis*) was observed, when 20–30 % of sourdough fermented with *Lb. plantarum* VTT E-78076, was addend to the wheat dough (Katina et al., 2002). Furthermore the *Lb. plantarum* antifungal properties in bakery products of were also observed. The phenyllactic acid and its 4-hydroxy derivate produced by *Lb. plantarum* 21B possessed antifungal activity against the common spoiling fungi of bakery products belong to genera *Apergillus, Penicillium* and *Fusarium* (Lavermicocca et al., 2000; Valerio et al., 2004). Recent studies, showed the potential application of *Lb. plantarum* CRL 778 for improving the nutritional quality and the shelf life of bread made with quinoa sourdough (Dallagnol et al., 2012). A good lactic acid production and protein hydrolysis were observed during fermentation, and an abundant production of phenyllactic and hydroxyphenyllactic acids were also obtained.

Lactobacillus plantarum is considered one of more important lactic acid bacteria used in table olives fermentation (Sabatini et al., 2008; Corsetti et al., 2012). This microorganism has the potentiality to improve the lactic acid yield, control the

microbiological process during green olives fermentation and improve the organoleptic characteristics of the final product (Panagou et al., 2008; Ruiz-Barba et al., 2010; Hurtado et al., 2012). The bacteriocinogetic strain *Lb. plantarum* LPCO10, isolated from Spanish-style fermented green olives, has been successfully used in olive fermentation; both fermentation process and growth of undesirable competitors were well controlled (Jimenez-Diaz et al., 1993; Ruiz-Barba et al., 1994; Leal-Sanchez et al., 2003).

Other vegetable products, such as sauerkrauts, have been also produced with *Lb. plantarum*. For example, the application of *Lb. plantarum* L4 as starter culture during cabbage heads, allowed to use a more low concentration of NaCl (from 4.0 to 2.5%) and reduce the time of fermentation, as well as to improve the product quality (Beganović et al., 2011).

In the last years, is becoming the use of *Lactobacillus* species in oenological field, especially in red wine production. Among *Lactobacillus* species, *Lb. plantarum* is the best candidate for winemaking process because in addition to being able to survive and growth in stress condition (pH 2.8-3.4, alcohol 11-15%) of wine it may drive the malolatcic fermentation (MLF) (Miller et al., 2011; Toit et al., 2011). MLF consist in malic acid decarboxylation with production of lactic acid. During MLF wine became less acid and the aroma compounds envelope. Other than malic acid degradation and production of aroma compounds, several advantages may be obtained when *Lb. plantarum* is employed. For example, antimicrobial effect and biogenic amines degradation were showed by *Lb. plantarum* (Toit et al., 2011; Capozzi et al., 2012).

Although, *Lb. plantarum* is not known as the principal bacterium involved in meat products fermentation, some evidence is reported of its potential use in fermented

sausages. Of interest is the anti-listeria activity showed by bacteriocinogenic strains of *Lb. plantarum* when used as sausage starter cultures (Campanini et al., 1993; Dicks et al., 2004).

Other important properties of *Lb. plantarum* such as probiotic characteristics are been even reported (Kaushik et al., 2009; Čokášová et al., 2012; Martín et al., 2012; Kakisu et al., 2012). An interesting study has shown the potential application of *Lb. plantarum* Lp299 as component of oral care in Intensive Care Unit (ICU) patients (Klarin et al., 2008). This probiotic strain was able to counteract pathogenic bacteria for 24 hours offering a valid alternative to use of antiseptics, such as chlorhexidine (CHX) or antibiotics.

1.3.3 *LACTOBACILLUS PLANTARUM* AS ANTI-LISTERIA CULTURE

Some strains of *Lactobacillus plantarum* are known for their antimicrobial properties against foodborne pathogenic bacteria including Listeria monocytogenes (Diep et al., 2009; Sip et al., 2012). The antimicrobial properties may be ascribable to several reasons: production of antimicrobial compounds such as bacteriocins, bacteriocine-like substances (BLIS), phenyllactic acid, organic acids (mainly lactic and acetic acid), hydrogen peroxide and competition for nutrients (Todorov et al., 2011; Reis et al., 2012).

Bacteriocins represent an heterogeneous group of peptides produced or modified through the ribosomal synthesis, released into the extracellular environment and active against taxonomically related bacteria (Jack et al., 1995). They have been classified into three classes on the basis of common characteristics: i) lantibiotics (class I, e.g. Nisin), ii) small heat-stable peptides (class II, e.g. pediocin) and iii) large heat-labile proteins (class III, e.g. helveticin) (Nes et al., 1996). Among bacteriocins classes, bacteriocins

belonging to either class I and II are active against *Listeria* spp., especially the subclass IIa exert the highest inhibitory effect on *L. monocytogenes* (Fimland et al., 2005). Classe IIa bacteriocins, generally named pediocin-like (pediocin PA-1 has been the first member characterized), have a high level of homology (40-60%) and causes the membrane permeabilization of target cells and a subsequent arrest of transmembrane electrical potential (ΔpH and Δψ) (Chung et al., 2000; Ennahar et al., 2000; Drider et al., 2006). Several studies have shown that *Lb. plantarum* strains may produce class IIa anti-listeria bacteriocins, such as: plantaricin 423 (van Reenen et al., 1998), plantaricin WHE92 (Ennahar et al., 1999), plantaricin C19 (Atrih et al., 2001) and plantaricin AMA-K (Todorov et al., 2008).

Recently, Mills et al. (2011a) reported the anti-listeria effect produced by *Lb. plantarum* LMG P-26358 isolated by artisanal soft cheese. This strain produce a class IIa bacteriocin stable at 100 °C and pH range 1-10 with 100% homology to plantaricin 423. The potential class IIa bacteriocin-producing *Lb. plantarum* Lab572, isolated from golka cheese, were able to inhibit *L. monocytogenes* (Sip et al., 2012). These authors have also observed that the bacteriocin was produced both in logarithmic growth phase and stationary phase of growth, but the maximum level of activity was detected at the beginning of the stationary phase. Other authors have also reported that *Lb. plantarum* LB-B1, isolated from fermented dairy product, synthetized a pediocin LB-B1 (a identical pediocin PA-1) with the maximum production at the early of stationary phase of growth (Xie et al., 2011). Although, the use of class IIa bacteriocins or bacteriocins-producing strains represent a promising alternative for to control the growth of *L. monocytogenes* in a foodstuff, their efficacy could be compromised by onset of bacteriocins resistant strains and cross-resistance between bacteriocins (Naghmouchi et

al., 2007; Tessema et al., 2009).

For these reasons an in-depth understanding of mechanisms of survival, adaptation and resistance to bacteiocins by *L. monocytogenes* is important. However, the use of bacteriocins in combination with other hurdles (e.g. salt, acid, other natural substances etc.) may result extremely effective for inhibit *L. monocytogenes* and reduce its resistance (Mills et al., 2011a; Mills et al., 2011b).

Some experiments showing the anti-listeria effect due to production of acid organics by *Lb. plantarum* (Bernbom et al., 2006; Kaushik et al., 2009). *Lb. plantarum* SK1 showed anti-listeria activity through lactic acid production (Wilson et al., 2005).

As mentioned before in recent times attention has been drawn to the antifungal and antimicrobial compounds such as, phenyllactic acid.

3-Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid, PLA) has been reported as an antibacterial compound with broad-spectrum activity against Gram-negative and Gram-positive bacteria including *L. monocytogenes* (Dieuleveux et al., 1998). The effect of PLA on *L. monocytogenes* caused aggregate formation and complete cells disintegration, moreover anti-listeria activity was observed in culture medium, milk and cheese (Dieuleveux & Gueguen, 1998). *Lb. plantarum* is able to produce PLA similarly tosome LAB members, such as *Lactobacillus, Enterococcus, Weissella* and *Leuconostoc*, but the quantity produced by *Lb. plantarum* seems to be greater than that produced by other species (Mu et al., 2012). Moreover, several studies have shown that PLA production may vary among *Lb. plantarum* strains (Gerez et al., 2010; Rodríguez et al., 2012). In LAB strains the PLA is synthetized by transamination of phenylalanine in phenylpyruvic acid (PPA) and a further reduction of it (Li et al., 2007). A direct correlation between PLA production and phenylalanine content in medium has been

widely reported (Valerio et al., 2004; Li et al., 2007; Rodríguez et al., 2012).

In conclusion, the wide ability of *Lb. plantarum* to survive and adapt in several environmental conditions as well as to produce anti-listrial compounds, could make it a suitable candidate for control the growth of *L. monocytogenes* in the food.

1.4 STRESS RESPONSE IN *LISTERIA MONOCYTOGENES*

1.4.1 SPECIFIC AND GENERAL STRESS RESPONSE

Listeria monocytogenes is recognised as a bacteria able to grow in several environmental conditions such as: temperature ranging -1.5 and + 45 °C, salt concentration up to 12%, pH values of 4.5 and a_w of about 0.92 (Gandhi & Chikindas, 2007). Different stress protection systems are involved and efficiently coordinated to protect cells in response to environmental changing. Generally, these systems involvedifferent changes in gene expression and the induction of proteins linked to particular stress (specific stress response) or involved in several stress conditions (general stress response).

Survival at low temperatures

The low temperature, known to reduce metabolic capacity, causes structural changes in nucleic acids (DNA and RNA), macromolecular assemblies such as ribosomes and alter membrane fluidity (Beales, 2004; Schumann, 2009). To maintain an optimal membrane fluidity, bacteria tend to increase both unsaturated and short fatty acids (Beales, 2004).

Cold stress proteins are involved in response to low temperature: cold shock proteins (Csps) are expressed in response to temperature downshock, instead cold acclimation proteins (Caps) are synthetized during growth at low temperatures (Bayles et al., 1996).

The cold shock proteins family consist on small and highly conserved proteins that seemto serve as nucleic acid (DNA and RNA) chaperones-like (Ermolenko & Makhatadze, 2002; Horn et al., 2007). Four proteins (7 KDa), determined by 2D-electophoresis and immunoblotting techniques, were designated as Csp1, Csp2, Csp3 and Csp4 and associated with cold stress in *L. monocytogenes* LO28 (Wemekamp-Kamphuis et al., 2002). Recently, two of Csp proteins, CspA (CspL) and CspD have been confirmed to be vital for cold growth in *L. monocytogenes* (Schmid et al., 2009).

Microbial growth at low temperature is characterised by cold acclimation that cause several changes in microbial gene expression. The overexpression of mRNA related to general stress chaperone and protease, such as GroEL, ClpP and ClpB was observed at 10 °C in comparison to 37 °C (Liu et al., 2002). In a recent proteomic study, it has been showed that 57 proteins were overexpressed whilst 8 proteins were repressed in *L. monocytogenes* when grown for 14 days at 4 °C. In the same work, the proteome changes were characterised by synthesis of proteins related to energy metabolism, oxidative stress, nutrient uptake and protein folding. The molecular chaperon GroEL and DnaK were overexpressed at 4 °C of about 13-16 fold compared to cells grown at 37 °C (Cacace et al., 2010).

The cold stress associated proteins GroEL, DnaK and Ctc, induced during *L. monocytogenes* cold adaptation, have been also associated to other stress condition. In particular Ctc has been associated to cold and osmotic stress, whilst GroEL and DnaK have been associated to cold and heat stress (Duche et al., 2002a; Duche et al., 2002b; Gardan et al., 2003).

The ability of *L. monocytogenes* to accumulate compatible solutes such as, glycine, betaine and carnitine is another strategy adopted for growthat refrigeration

temperatures. Angelidis & Smith (2003) reported that three transporters are involved in the accumulation of these substances: glycine betaine porter I (BetL), glycine betaine porter II (Gbu) and carnitine transporter (OpuC). The deletion of these osmolyte transporters genes reduced the growth of *L. monocytogenes* at low temperatures (Wemekamp-Kamphuis et al., 2004). LtrC and Lmo1078 (UDP-glucose pyrophosphorylase) proteins have been also proposed as cold adaptation proteins (Chan et al., 2007; Chassaing & Auvray, 2007). In particular Lmo1078 protein is involved in UDP glucose production, an essential substrate of lipoteichoic acids synthesis known for their contribute to the maintenance of cellular membrane integrity (Chassaing & Auvray, 2007).

Survival under heat stress

Upon exposure to high temperatures (>45 °C), *L. monocytogenes* induces the activation of several protection mechanisms (heat-shock response), including specific stress mechanisms and general stress mechanisms (van der Veen et al., 2007). These mechanisms, common also to many other microorganisms, may be distinguished, in a specific heat-stress response (Class I and Class III) and in a general heat-stress response (Class II) (Benson & Haldenwang, 1993; Schulz & Schumann, 1996; Kruger & Hecker, 1998).

Class I heat-stress response involve the induction of Heat shock proteins (Hsps) such as molecular chaperones (DnaK, DnaJ, GroES and GroEL) and the HrcA repressor, instead, Class III heat-stress response induce the overexpression both ATP-dependent Clp proteases and CtsR repressor (van der Veen et al., 2007). Chaperones and proteases are proteins highly conserved and are involved in refolding and degradation on

damaged proteins. Class I heat-shock genes (dnaK, dnaJ, groES and groEL) are controlled by the HrcA repressor, which binds the CIRCE operator sequence (TTAGCACTC-N_9-GAGTGCTAA). Whilst the expression of Class III heat-shock genes is regulated by CtsR repressor.

Class II heat-stress response, involve the overexpression or regression of general stress proteins whose genic expression is regulated by the alternative sigma factor SigB.

Several studies showed that GroEL and DnaK constituted the main Hsps observed in *L. monocytogenes* under heat-stress conditions (Hanawa et al., 2000; Gahan et al., 2001).

Many other mechanisms are involved in heat-stress response, for example the SOS response was observed when *L. monocytogenes* EGD-e was exposed to 48 °C (van der Veen et al., 2007). The SOS response consists of a conserved pathway involved for DNA repair and restart of stalled or collapsed replication forks (Lusetti & Cox, 2002). Usually, proteins such as excinucleases, helicases and recombinase are involved in SOS response and are regulated by repressor LexA and by activator RecA (Cox, 2007; Butala et al., 2009). SOS response has been observed is several bacteria as result of different stress conditions (van der Veen & Abee, 2011). *L. monocytogenes* produced SOS response over the heat stress, also when exposed to acid stress, oxidative stress and mitomycin C (van der Veen et al., 2010).

Ferritin-like protein (Fri) with a molecular weight of 18 KDa and pI of 5.1 was overexpressed (50.6-fold) when *L. monocytogenes* was exposed to 49 °C for 15 min (Phan-Thanh & Gormon, 1995). The importance of this protein both in heat-stress response and in cold-stress response in *L. monocytogenes* has been highlighted by several studies (Hebraud & Guzzo, 2000; Dussurget et al., 2005; van der Veen et al., 2007).

Survival under acid stress

L. monocytogenes is able to survive and grow in environment with low-pH, such as acid foods, gastric trait and in the phagosome of the macrophage (Cotter & Hill, 2003). The exposure of *L. monocytogenes* to mild acid pH of 5.5 (1 M lactic acid) for 1 hour induces acid tolerance resistance (ATR), which is capable of protecting cells from severe acid stress (pH 3.5) (O'Driscoll et al., 1996). Several molecular mechanisms are involved by exposing cells to a lethal acid pH (acid stress) and a non-lethal acid pH (acid adaptation). Acid adapted cells showed an increment of resistance to heat shock (52 °C), osmotic shock (25-30% NaCl) and alcohol stress (cross-protection) (Phan-Thanh et al., 2000). The production of several proteins during acid conditions, including GroEL (produced in response to heat- and cold-stress), could explain the ability *of L. monocytogenes* to increment the resistance to other stress conditions (Phan-Thanh & Mahouin, 1999).

To maintain an optimal level of intracytoplasmic pH, microorganisms use the mechanism of pH homeostasis. In aerobic bacteria the active transport of H^+ is coupled with electronic transport of respiratory chain, whilst in anaerobic organisms, the transport of H^+ is coupled with the F_0F_1-ATPase molecules using energy from ATP hydrolysis. The facultative anaerobic *L. mo*nocytogenes is able to use both systems pH homeostasis (Shabala et al., 2002). The essential role of this enzyme in ATR has been extensively studied. The inhibition of F_0F_1-ATPase with N,N'-dicyclohexylcarbodiimide (DCCD) caused the reduction of three-log respect during acid adaptation of *Listeria* (Cotter et al., 2000).

Other mechanisms, such as the decreasing of the membrane permeability to protons have been observed under mild acid conditions (pH 5.0 to 6.0) *L. monocytogenes*

10403S increased straight chain fatty acids production and decreased the concentration of branched chain fatty acids (Giotis et al., 2007).

The synthesis induction of proteins involved in red-ox reactions such as dehydrogenases (GuaB, PduQ and lmo0560) and reductases (YcgT) together with respiratory enzymes could be important to maintain pH homeostasis (Phan-Thanh & Jänsch, 2006).

The glutamate decarboxylase (GAD) system is another strategy adopted by *L. monocytogenes* to survive acid stress (Cotter et al., 2001). According to the current model, an extracellular glutamate (Glt_e) is imported inside the cell through an antiporter system (Glt/GABA) that exchange for an intracellular ϒ-aminobutyrate ($GABA_i$). The Glt is decarboxylated, and a proton (H^+) is incorporated in GABA. Subsequently the $GABA_i$ is exported out of the cell through the antiporter system that exchange for an other Glt_e (O'Byrne & Karatzas, 2008). The most of *L. monocytogenes* strains include five genes (gadT1, gadT2, gadD1, gadD2 and gadD3) that encodes for GAD system: the genes gadT1 and gadT2 encodes antiporters, whilst the genes gadD1, gadD2 and gadD3 encodes the decarboxylases (Cotter et al., 2005). Cotter et al. (2005) have also shown that the five genes are organized in three loci: gadD1T1, gadD2T2 and gadD3. The gadD1T1 locus function is to enhance the growth under mild acid condition, whilst the gadD2T2 play an important role under extreme acidic conditions (Cotter et al., 2001; Cotter et al., 2005). Recently (see Figure 1.1) it has been shown that GAD system can utilize intracellular Glt (Glt_i) to produce $GABA_i$ independently of the antiport, for this reason it has been proposed to divide the GAD system into extracellular (GAD_e) and intracellular (GAD_i) components (Karatzas et al., 2010; Karatzas et al., 2012). The gadD3 gene together the gadD2T2 loci are important in the GAD_i system. Studies, showed that the GAD_i system is activated firstly (pH 4.5 to 5.0) than GAD_e (pH 4.0 to

4.5) system in response to acidic conditions (Karatzas et al., 2012).

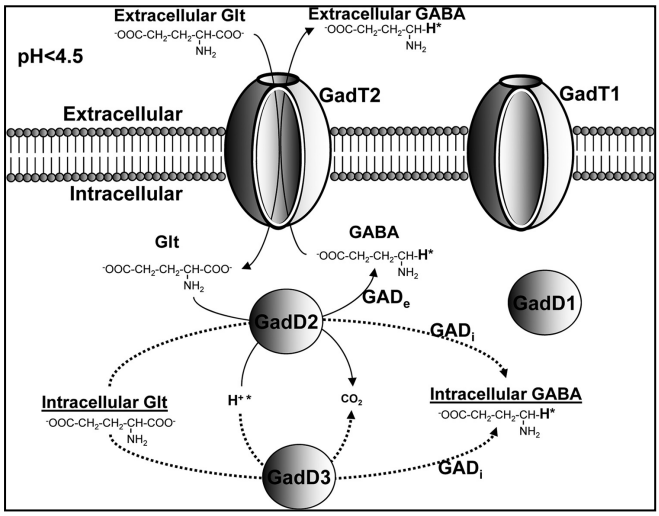

Figure 1 Model for the function of the GAD system under severe acid conditions (pH < 4.5) (Karatzas et al., 2012).

The arginine deiminase (ADI) system is another mechanism used by *L. monocytogenes* against acid stress conditions. In this process, several proteins (ArcA, ArcB, ArcC and ArcD) are involved for the conversion and transfer of arginine into ornithine and production of NH_3. Recent studies showed that expression of ADI genes at low pH and in the presence of arginine were increased (Ryan et al., 2009). The alternative stress sigma factor (σ^B) and a dedicated transcriptional regulator, ArgR were involved in ADI regulation (Ryan et al., 2008; Ryan et al., 2009).

Acid stress response in *L. monocytogenes* may be regulated by several mechanisms, including a two-component regulatory system, consisting of lisR and lisK (Cotter et al., 1999) and a general stress sigma factor (σ^B) (Wiedmann et al., 1998).

Survival under osmotic stress

During osmotic stress, *L. monocytogenes* increase or decrease the synthesis of various proteins. Similarly to the response to cold stress, two groups of proteins were detected in response of osmotic stress: salt shock proteins (Ssps) induced for a short period and

salt acclimation proteins (Saps) overexpressed for several hours Duche et al. (2002a) identified DnaK and Ctc proteins among Ssps proteins, instead with Saps was detected the osmoprotectantGbuA, transporter of glycine betaine. Intracellular accumulation of compatible solutes that have no net charge at physiological pH, play an important role during osmotic stress. The osmoprotectant function of glycine betaine, proline betaine, carnitine and acetyl carnitine in *L. monocytogenes* has been shown (Bayles & Wilkinson, 2000). The authors showed that the presence of these compounds in external environment resulted in an increase of salt resistance in *L. monocytogenes* compared with cell cultivated without the presence osmoprotectant. Expression of transporter genes betL, gbu and opuC, involved in uptake of osmoprotectant glycine betaine and carnitine is regulated by the general stress sigma factor σ^B (Becker et al., 1998).

1.4.2 COMPARATIVE PROTEOMIC APPROACHES TO STUDY STRESS RESPONSE

Contrary to genome, the proteome continually changes in response to environmental events. Microorganisms are able to change their protein expression for adapt oneself to several conditions that characterize a food process. Proteomic analysis can help to understand gene function during the change of the bacterial physiological state. Scientific progress has contributed to improve and diversify the proteomic techniques. Currently, proteomics encompass three main areas: i) structural proteomics - proteins characterization including their post-translational modifications, ii) comparative proteomics - comparison of protein expression in two physiological states, iii) protein-protein interaction using techniques such as mass spectrometry (Pandey & Mann, 2000; Florens et al., 2002; Huber, 2003).

Comparative proteomic studies, based on extensive protein separation, are used to obtain information about proteins expressed or repressed from different cellular

biological states, including cells in normal physiological state versus stressed cells. Two-dimensional electrophoresis (2D-E) is a powerful technique able to separate a complex mixture of proteins. This technique has been developed by O'Farrel and Klose about 40 years ago, and now represents a tool largely applied in the field of proteomics (Klose, 1975; O'Farrell, 1975).

Proteins separation consists of two steps: in the first phase (iso-electrofocalization, IEF) proteins are separated according to their isoelectrical point (pI), whilst in the second step proteins are separated trough SDS-PAGE and subsequently are stained with a colorant (comassie blue, silver stain etc.). Results of 2D-E analysis consist in a gel, in which proteins are represented as spots, and their coloration intensity is strictly related to the protein concentration.

The two-dimensional electrophoretic technique has been successfully applied to detect and quantify microbial stress response. Stress response to different stressors, such as salt, acid, heat and cold, have been studied in *L. monocytogenes* as well as other bacteria, through 2D-E approach (Phan-Thanh & Gormon, 1995; Phan-Thanh & Gormon, 1997; Phan-Thanh & Mahouin, 1999; Phan-Thanh et al., 2000; Duche et al., 2002b; Hecker, 2003; Cacace et al., 2010).

Separated proteins by 2D-E may be subsequently identified with other proteomic techniques. Usually spots detected are digested with a sequence-specific protease and identified through Matrix-Assisted Laser Desorption Ionization (MALDI)- Time-Of-Flight (TOF) mass spectrometers (Jungblut & Thiede, 1997).

Although 2D-E offers numerous advantages in the study of proteomic, it is important tohighlight the limits linked to reproducibility and the difficulty to solubilize and separate hydrophobic and membrane proteins.

The intrinsic gel-to-gel variability of 2D-E can mask the biological differences between the samples and compromise the quantitative protein comparison between the different samples. New equipment and technologies may be adopted to improve the reproducibility of 2D-E and for quantitative protein comparison between more samples. Simultaneous protein samples separation during the first and the second dimension through equipment such as Manifold (for IEF), EttanDALT six or EttanDALT twelve (for SDS-PAGE) can to help to reduce the intrinsic gel-to-gel variability.

Furthermore the new technology, named fluorescent two-dimensional difference gel electrophoresis (2D-DIGE), seems to overcome the limitations of 2D-E reproducibility. In fact, proteins separation with 2D-DIGE consistsin the use of a single gel for the simultaneous separation of multiple protein samples. Fluorescent compounds such as cyanine dyes Cy2, Cy3 and Cy5 are usually used for proteins detections and comparison.

It is known, that certain classes of proteins such as basic proteins or hydrophobic proteins associated to membrane are poorly soluble therefore hardly separable. In general, only proteins with a molecular weight of 10-100 kDa and a pI of 4-8 migrate well within 2D-E gels (Renzone et al., 2005). For this reason may be necessary to modify the experimental conditions for improve protein solubilisation and consequently obtain a better resolution and detection of hydrophobic proteins (Molloy et al., 1998; Herbert, 1999; Weiss & Gorg, 2009; Westermeier & Gorg, 2011).

Although many limits regarding the use of 2D-E and new technologies are emerging, including MudPIT, ICAT, or protein arrays, 2-DE is still the proteomic technique mostused for routine parallel expression profiling of large sets of complex protein mixtures (Weiss & Gorg, 2009).

1.4.3 BIO-INFORMATIC APPROACHES TO STUDY PROTEINS INVOLVED IN STRESS RESPONSE

In the post-genomic era, the development and the application of numerous proteomics techniques including mass spectrometry and two-dimensional electrophoresis have produced a large set of data. Therefore, data analysis through bioinformatics approaches becomes an essential part of this research. For example, in the last years the image analysis of two-dimensional gels has been improved through the implementation of new algorithms. Several commercial software (Delta 2D, Melanie, PDQuest) that differ by the kind of algorithm used, are now available to perform spot detection and spot matching (Blueggel et al., 2004; Palagi et al., 2005). Webservers such as, Flicker, (http://www-lecb.ncifcrf.gov/flicker/) (Lemkin, 1999), WebGel (http://www-lecb.ncifcrf.gov/webgel/) (Lemkin et al., 1999) and free software (ImageJ), offer also useful and flexible tools for the analyses of 2D-E (Bucci et al., 2011). Furthermore, a large number of 2D-E databases are also available for compare gel images, share and exchange information (Appel et al., 1996). A list of database may to be visualised on the ExPASy portal (http://world-2dpage.expasy.org/list/).

Although, 2D-E gel comparison helps to obtain interesting information about protein expression or repression of a biological system, it may be not enough to understand the role of a particular protein. For this reason proteomics techniques such as mass spectrometry (MS) over those applied for protein structure determination (X-ray crystallography, high-resolution microscopy and nuclear magnetic resonance spectroscopy) are used to identify and obtain more information about the detected proteins. Also in these cases, bioinformatics represent a powerful means to process the large amount of data obtained. For example experimental data achieved by MS may be matched with those stored in database for proteins identification. Furthermore, protein

spatial atom coordinates obtained by X-ray or NMR techniques, are stored and available in a central database named Protein Data Bank (PDB).

Knowledge of the three-dimensional (3D) structures of proteins is essential to understand the molecular basis of their functions. The great attention focused on the proteins 3D structure, and their atomistic details produced a large number of crystal and solution structure determination. Currently, about 80,000 experimental protein structures have been released by PDB. Although, several protein structures are now available, this number is small compared to the 500,000 annotated and curated protein sequences in the Swiss-Prot (UniProtKB/Swiss-Prot) and even smaller when compared to the 30 million known protein sequences (UniProtKB/TrEMBL) (Consortium, 2012).

The gap in structural knowledge may be filled by bioinformatics approaches including computational structural biology. Different types of approaches are commonly used, such as homology modeling, *de novo* methods and hybrid method.

Homology or "comparative" modeling is the most accurate method that uses experimentally structure of related protein as templates to model the structure of the protein of interest (target). This technique is based on the observation that evolutionary protein sequences generally have a similar 3D structure.

Homology modeling procedures involve four main steps: 1) identification of template(s) structure and sequences alignment among the target and template; 2) modeling of the structurally conserved regions and prediction of structurally variable regions; 3) refinement of the model; 4) evaluation of the model(s) generated (Schwede et al., 2008).

Identifying and the accurate alignment between template and target is a crucial step to obtain a good model. It is important to consider the target-sequence identity level during the selection of structure template. Generally, from sequence alignments between target

and template with an identity of 50% or higher, are obtained models with “high accuracy” that tend to have about 1 Å root mean square deviation (RMSD) (Marti-Renom et al., 2000). The accuracy of these models may be compared with those of a medium-resolution NMR-derived structure or a low-resolution X-ray structure (Baker & Sali, 2001; Read & Chavali, 2007). Instead, models based on 30-50% sequence identity may be considered “medium accuracy model” whilst comparative model with a sequence identity below 30% are considered “low accuracy model”.

Several sensitive methods such as, Blast, PSI-Blast, Hidden, FFAS03, based on interactive profile searches, are now available for sequence homology detection.

Model building based on alignment between template-target and sequences may be performed by the use of several approaches including methods based on rigid fragment assembly and methods based on maximization of the satisfaction of spatial restraints (Blundell et al., 1987; Peitsch & Jongeneel, 1993; Sali & Blundell, 1993). Furthermore, several specialized protocols are also available to improve the accuracy of non-conserved regions such as loops or side chains (Lovell et al., 2000; Canutescu et al., 2003; Jacobson et al., 2004; Soto et al., 2008).

Once models are built, these may be refined to improve model geometry and to remove unfavourable contact. For this aim molecular mechanics force fields and other methods such as molecular dynamics can be applied (Bordner, 2012).

At the end of homology modeling procedure, it is important to evaluate the structure geometrical accuracy. Methods based on atoms spatial geometrical location and/or molecular energetic state may be used for this purpose. Several software and interactive web application such as MolProbity and QMEAN server sare often used for models validation. The accuracy of a model is important for its applications. There are several

applications for comparative models, such as protein engineering, designing experiments for site-directed mutagenesis, predicting ligand binding sites, docking small molecules in structure-based drug discovery, effect of mutations (Hillisch et al., 2004; Poole & Ranganathan, 2006; Feyfant et al., 2007). These applications are particularly useful in designing new experiments and in the cases for which a qualitative investigation of the structural disposition of specific residues in the proteins needs to be assessed/evaluated.

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INHIBITORY ACTION EXPRESSED BY *LACTOBACILLUS PLANTARUM* STRAINS AGAINST SPOILAGE MICROORGANISMS

2.1 INTRODUCTION

Lactobacillus plantarum is a versatile and widespread microorganism found in materials and environments ranging from vegetable, dairy and meat fermentations to the human gastrointestinal (GI) tract (Kleerebezem et al., 2003). Some strains are marketed as probiotics (Shah, 2007) that are claimed to provide a health benefit for the consumer through interactions with the human GI system (de Vries et al., 2006). Furthermore, same strains of *Lb. plantarum* spp. are known for their ability to produce several natural antimicrobial substances (Ennahar et al., 1996; Todorov et al., 2007; Diep et al., 2009). The production of these metabolites could represent stress conditions that strongly affects the development of undesirable microbial species. There are many scientific reports that highlight antimicrobial effects of many *Lb. plantarum* strains on undesirable bacteria. Ben Omar et al., (2008) evidenced that several strains of *Lb plantarum* showed a broad spectrum of antibacterial activity (including *Bacillus cereus, S. aureus, L. monocytogenes, S. enterica, E. coli,* and *Enterobacter aerogenes*) and carries several plantaricin genes of the *pln* locus. In recent years, various bacteriocins produced by *Lb. plantarum* species isolated from fermented food have been reported (Atrih et al., 2001; Todorov et al., 2010; Xie et al., 2011). Up to now, five different types of the Mosaic plantaricin (*pln*) loci have been identified from several strains of *Lb plantarum* (Maldonado et al., 2003; Rojo-Bezares et al., 2007; Navarro et al., 2008; Diep et al.,

2009; Li et al., 2009). Aguilar et al., (2010) evidenced that a specific *Lb. plantarum* strain exhibited a strong inhibitory effect against the Gram positive *Listeria monocytogenes*. The Authors, asserted that this activity could be due to BLIS action additively to lactic acid accumulation. Moreover *Lb. plantarum* strains have also been described as being active against different plant pathogenic, toxigenic and gushing-active *Fusarium* fungi (Laitila et al. 2000). Siezen et al., (2011) illustrated that the natural genomic architecture and the metabolic consequences here of are central to the success of *Lb. plantarum* in industrial applications. Moreover, as widely reported in literature (Gerez et al., 2010; Dalié et al., 2010) the most of *Lb. plantarum* selected for their antimicrobial activity has been isolated from fresh or fermented food.

Therefore, the characterization and the selection of food-borne *Lb. plantarum* strains remains a topic of great interest for applied research. On the basis of this last finding, the first part of this PhD study was addressed to isolate and to identify food borne *Lb. plantarum* as well as to evaluate their antimicrobial range. Thirty two samples from three type of traditional fermented food were subjected to microbiological analyses in order to identify predominant lactobacilli species and to select *Lactobacillus plantarum* strains to be used as antagonistic strains (producers) against undesirable food-related microorganisms. To identify LAB isolates, several approaches were used, consisting of the DGGE analysis and 16S rRNA gene sequencing. While the antimicrobial activity exerted by cells or cell-free supernatants, *Lb. plantarum* strains were evaluated by spot-on-the-lown test and agar well diffusion assay test. The results evidenced that *Lactobacillus plantarum* represents the prevailing lactobacilli specie in the sourdoughs, red wine, while this specie were detected only in few sample of fermented sausages. In detail 60 *Lactobacillus plantarum* strains were founded in red wines, 36 strains in

sourdoughs, and 10 in fermented sausages. Moreover the results evidenced that DGGE and 16S rRNA gene sequencing allowed to obtain a reliable identification of strains.

Regarding the evaluation of antimicrobial activity, out 106 *Lb. plantarum* strains, seven strains evidenced the ability to inhibit Gram negative and Gram positive bacteria as well as moulds strains. These inhibitory effects was not attributable to organic acids, since in the presence of neutralized cell-free supernatant (CFS) of producer strains were also detected a strong antimicrobial activity. Noticeable was the data that evidenced a strong antimicrobial activity produced by *Lb. plantarum* RTB strain against *L. innocua* ATCC 33090.

2.2 MATERIALS AND METHODS

2.2.1 *LACTOBACILLUS PLANTARUM* FROM TRADITIONAL FERMENTED FOOD OF SOUTHERN ITALY

2.2.1.1 THE SAMPLES

Thirty-two samples from 3 type of traditional fermented food (10 sourdoughs from Campania region, 12 red wine from Campania and Molise region, 10 fermented sausages –*Ventricina type*- from Abruzzo and Molise regions) were collected from different artisanal factories located in various areas of Southern Italy as showed in Table 2.1.

Table 2.1 Type and geographical origin of the assayed fermented foods.

Samples	Type	Geographical Origin
SD AV1	Sourdough	Avellino
SD AV2	Sourdough	Avellino
SD AV3	Sourdough	Avellino
SD AV4	Sourdough	Avellino
SD AV5	Sourdough	Avellino
SD AV6	Sourdough	Avellino
SD AV7	Sourdough	Avellino
SD AV8	Sourdough	Avellino
SD AV9	Sourdough	Avellino
SD AV10	Sourdough	Avellino
VT CB 1	Fermented sausage (Ventricina)	Campobasso
VT CB 2	Fermented sausage (Ventricina)	Campobasso
VT CB 3	Fermented sausage (Ventricina)	Campobasso
VT CB 4	Fermented sausage (Ventricina)	Campobasso
VT CB 5	Fermented sausage (Ventricina)	Campobasso
VT CH 1	Fermented sausage (Ventricina)	Chieti
VT CH 2	Fermented sausage (Ventricina)	Chieti
VT CH 3	Fermented sausage (Ventricina)	Chieti
VT CH 4	Fermented sausage (Ventricina)	Chieti
VT CH 5	Fermented sausage (Ventricina)	Chieti
RW 1	Red wine (Taurasi)	Avellino
RW 2	Red wine (Taurasi)	Avellino
RW 3	Red wine (Taurasi)	Avellino
RW 4	Red wine (Taurasi)	Avellino
RW 5	Red wine (Piedirosso)	Salerno
RW 6	Red wine (Piedirosso)	Salerno
RW 7	Red wine (Pentro d'Isernia)	Isernia
RW 8	Red wine (Pentro d'Isernia)	Isernia
RW 9	Red wine (Tintilia)	Campobasso
RW 10	Red wine (Tintilia)	Campobasso
RW 11	Red wine (Tintilia)	Campobasso
RW 12	Red wine (Tintilia)	Campobasso

2.2.1.2 MICROBIOLOGICAL ANALYSIS

For food in solid form (sourdoughs, cheeses, sausages, fermented olive) about ten grams of each sample were diluted 1:10 (w:v) in physiological sterile solution (9 g/L NaCl) and homogenized in a Stomacker 400 Lab Blender (PBI International, Milan, Italy) (1 min agitation, 1 min pause, 1 min agitation). For wine 10 mL were diluted in 90 mL of physiological sterile solution. Subsequent serial dilutions were prepared and inoculated into appropriate media.

Lactic Acid Bacteria were enumerated and isolated by plating serial decimal dilutions on MRS agar medium (Oxoid, Milan, Italy) adding 40 mg/L actidione. Plates were incubated at 28 °C for 72 h under anaerobic conditions using an anaerobic system (Anaerogen, Oxoid, Milan, Italy). Five colonies randomly picked from plates with the highest dilution having positive growth, following the procedure described by Valmorri et al. (2006). Isolates were than purified by streaking on MRS agar. After morphological examination, presumptive lactobacilli were maintained frozen at -80 °C in MRS medium with 15% glycerol.

2.2.1.3 IDENTIFICATION OF PRESUMPTIVE LACTOBACILLI

Gram staining, catalase testing, microscope observation, study of metabolism assimilation of carbon sources by the API 50 CHL test and APILAB plus software, were used to screen the isolates as described by López et al. (2008) and to presumptively identify those belonging to the *Lactobacillus* genus. Lactobacilli were then identified by PCR-DGGE and 16S rRNA gene sequencing and biotyped by RAPD-PCR. DNA extraction and purification from pure culture. Two milliliters of each overnight culture was centrifuged at 14,000g for 10 min at 4°C to pellet the cells and

the pellet was subjected to DNA extraction according to Querol et al. (1992) with the addition of lysozyme (25 mg/mL, Sigma) and mutanolysin (10 U/mL, Sigma) for bacterial cell-wall digestion. Quantity and purity of the DNA were assessed by optical reading at 260 and 280 nm, as described by Sambrook et al. (1989).

DGGE analysis

The DNA from each strain was prepared for DGGE by amplifying the V1 region of 16S rRNA using the following primers: P1V1 (5'-GCG GCG TGC CTA ATA CAT GC-3) (Cocolin et al., 2001) and P2V1 (5'-TTC CCC ACG CGT TAC TCA CC-3') (Rantsiou et al., 2005). A GC clamp (5 'CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') (Sheffield et al., 1989) was attached to the 5' end of the P1V1 primer. PCR was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany). The reaction mixture (50 μl) consisted of 10 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 200 μmol/L of each dATP, dGTP, dCTP and dTTP, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L of each primer, 200 ng DNA and 1.25 U Taq-DNA polymerase (Finnzymes, Finland). The amplification program consisted of a 1 min denaturation step at 95 °C, a 1 min annealing step at 45 °C and a 1 min extension step at 72 °C. The first cycle was preceded by an initial step at 95 °C for 5 min. After 35 cycles, there was a final 7 min extension step at 72 °C. Negative controls without DNA template were included in parallel. PCR products were separated in 1.5% (w/v) agarose gel (Sigma) by electrophoresis for 45 min at 120 V in TBE 0.59 (Sigma) and were subsequently visualised by UV illumination after ethidium bromide (50 μg/ml) staining (Sigma). PCR products obtained from amplification of V1 region of 16S rRNA were subjected to DGGE analysis, using a DCode Universal Mutation Detection System (BioRad, Hercules, CA, USA). Electrophoresis was performed in a 0.8-mm polyacrylamide gel

(8% [w/v] acrylamide-bisacrylamide [37.5:1]) by using two different ranges of denaturant to optimise separation of the products. Two denaturant gradients, from 40 to 60% (100% denaturant was 7 M urea plus 40% [w/v] formamide) increasing in the direction of electrophoresis run, were used. The gels were subjected to a constant voltage of 120 V for 5 h at 60 °C, and after electrophoresis they were stained for 20 min in 1.259 TAE containing 50 μg/ml ethidium bromide and visualised under UV illumination. DGGE gels were digitally captured by GEL DOC XR System (Bio-Rad, Hercules, CA, USA) using the software Quantity One Analysis (Bio-Rad) and analysed with the pattern analysis software package, Gel Compare II Version 2.0 (Applied Maths, Kortrijk, Belgium). Calculation of similarities in the profile of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by mean of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992).

2.2.1.4 SEQUENCE ANALYSIS

One to four representative strains of each cluster obtained by DGGE analysis were amplified with primers P1 and P4, as described by Klijn et al. (1991), targeting 700 bp of the V1–V3 region of the 16S rRNA gene. After purification, (QIAquick PCR purification kit, QIAGEN GmbH, Hilden), products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany). Sequences were aligned with those in GeneBank with the Blast program (Altschul et al., 1997) to determine the closest known relatives, based on the partial 16S rRNA gene homology.

2.2.2 INHIBITION OF UNDESIRABLE MICROORGANISMS

The antimicrobial activity exerted by *Lb. plantarum* strains (producers) against undesirable microorganisms (indicators), including both Gram positive and Gram negative bacteria, was detected.

2.2.2.1 PRODUCER STRAINS OF *LB. PLANTARUM*

Sixty-four Lb. plantarum strains isolated from traditional fermented food (sourdoughs, red wines and fermented sausage) and previously identified by PCR-DGGE were tested for antimicrobial activity against several spoilage bacteria strains. Producer strains were maintained in MRS soft agar (Oxoid, Basingstoke, UK) at 4°C. Before use, strains were propagated twice for 16 h at 28°C in MRS broth.

2.2.2.2 INDICATOR STRAINS

36 strains belonging to undesirable food-related microorganisms were used as indicator strains. In details the indicators microorganisms strains used and relative conditions of cultivations are listed in Table 2.2.

Table 2.2 Microorganisms used as indicator strains.

N°	Strains	Species	Origin	Collection	Cultivation
1	SL	*Lb. brevis*	wine	DIAAA	MRS, 28 °C
1	SL1	*Lb. brevis*	Ferm. sausage	DIAAA	MRS, 28 °C
2	SERB108, SERB69	*Lb. casei*	wine	DIAAA	MRS, 28 °C
1	ATCC33090	*L. innocua*	type strain	ATCC	BHI, 37 °C
1	DSM 20171T	*B. thermosphacta*	type strain	DSMZ	Corin, 28 °C
1	DSM 50090^{T}	*P. fluorescens*	type strain	DSMZ	Nutrient, 28 °C
1	DSM 795T	*C. sporogenes*	type strain	DSMZ	RCM, 28 °C
1	DSM 3508^{T}	*A. aceti*	type strain	DSMZ	MYP, 28 °C
4	111, 111E, ASRT, ASC	*A. aceti*	type strain	DIAAA	MYP, 28 °C
1	DSM 3509T	*A. pasteurianus*	type strain	DSMZ	MYP, 28 °C
1	DSM 15551T	*A. tropicalis*	type strain	DSMZ	MYP, 28 °C
6	194BV, ASAC4, ASR, ARLA, AC1, 141A	*Ga. hansenii*	wine	DIAAA	MYP, 28 °C
1	203B1	*Ga. hansenii*	fruit	DIAAA	MYP, 28 °C
1	DSM 5602^{T}	*Ga. hansenii*	type strain	DSMZ	MYP, 28 °C
1	DSM 5003^{T}	*Ga. liquefaciens*	type strain	DSMZ	MYP, 28 °C
2	146B and AC6	*G. oxydans*	wine	DIAAA	MYP, 28 °C
5	F1, F2, F4, F5, F6	*Frateuria* spp.	wine	DIAAA	MYP, 28 °C
5	T1, T2, T3, T4, T5	*Penicillium digitatum*	truffle	DIAAA	PDA, 28 °C

2.2.2.3 DETECTION OF ANTIMICROBIAL ACTIVITY

Antimicrobial activity of 106 *Lb. plantarum* strains versus the 31 undesirable bacteria was carried out using the following procedures: the "spot-on-the-lawn" test and agar well diffusion assay using cells or cell free supernatant of *Lb. plantarum* strains as producers.

The spot-on-the-lawn technique was performed as described by Moraes et al. (2010). An aliquot of 2 ml of each *Lb. pantarum* culture previously amplified in MRS broth (incubation at 28 °C for 24 h) was spotted on plates containing 10 ml of MRS and was incubated at 28 °C for 24 h. For this step, 9 cm diameter plates were used and 4 *Lb plantarum* cultures were tested per plate, spotted in equal distances. After incubation,

the plates were overlayed with 8 ml of appropriate media (Table 2.2) semi-solid agar (0.8 g/ 100 ml of bacteriologic agar) inoculated with 10^5 CFU/mL of each culture of indicator strains. The plates were then incubated at 28 °C 24 h. The presence of a distinct inhibition zone around the spots was considered a positive antagonistic effect. Calibrated-densitometer GS-800 was used for plate imaging acquisition and Adobe Photoshop CS4 Extended software was used for measurement of clearing zone. Streptomicine was used as positive control of antagonism activity.

The well diffusion assay was also used to evaluate the activity of cell-free supernatants: overnight cultures, obtained as described before, were centrifuged (12000 rpm for 15 min at 4°C; Centrifuge 5415 R; Eppendorf, Hamburg, Germany) and supernatant was filter-sterilized (Filter Unit Red 0.22-µm pore size; Schleider & Schuell, Dassel, Germany) before its use. Assessment in using the well diffusion assay involved BHI plates overlaid with 7 ml of soft BHI (0.7% agar) inoculated with an overnight culture of each indicator strain (final concentration of about 10^5 CFU ml). In the agar well diffusion assay, wells of 3.0 mm in diameter were bored into BHI plates and 75 µl of an cell-free supernatants of *Lb. plantarum* producers strains were placed into each well. Prior to incubation, plates were refrigerated at 4°C for 4 h (Tremonte et al., 2007). After 24–48 h of incubation at 28 °C, plates were investigated for zones of inhibition as described before. Plates inoculated with each indicator strain and with any supernatant were used as control. Each experiment was carried out in duplicate.

2.2.2.4 ASSESSMENT OF INHIBITORY SUBSTANCES

The presence of acids or proteins with inhibitory effect produced by *Lb. plantarum* strains was evaluated against indicators by the agar-well diffusion test by using soft BHI agar as follows:

(A) Inhibition through acids. Wells were filled with producer culture or its cell-free supernatant. Cell-free supernatant neutralized with NaOH, H_2O or MRS broth acidified with lactic acid (same pH of producer cell-free supernatant) were used as control. Plates were incubated as described above.

(B) Inhibition through proteins. Plates were prepared as described in (A) but wells were filled with cell-free supernatant and cell-free supernatant plus α-chymotrypsin, proteinase K and trypsin, (Rojo-Bezares et al., 2007).

2.2.2.5 STATISTICAL ANALYSIS

Mean values, medians, and standard deviations as well as the occurrence of statistically significant differences were determined with the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA)

2.3 RESULTS AND DISCUSSION

2.3.1 *LACTOBACILLUS PLANTARUM* FROM TRADITIONAL FERMENTED FOOD OF SOUTHERN ITALY

The LAB levels of the samples and the number of isolates recovered from the different samples are reported in Table 2.3.

The level counts founded in the assayed samples closely respected those of tradition sourdoughs, red wines as well as of fermented sausage from Southern (R. Coppola et al., 1998; Spano et al., 2007; Reale et al., 2011) .

Table 2.3 Lactobacilli microbial load and number of isolates in each assayed sample.

	Count levels (log CFU/g or log CFU/mL	Number of isolates
SD AV1	6.4	7
SD AV2	5.2	6
SD AV3	7.1	7
SD AV4	4.4	6
SD AV5	5.5	6
SD AV6	6.7	7
SD AV7	5.7	6
SD AV8	4.6	6
SD AV9	6.3	7
SD AV10	6.4	6
VT CB 1	7.5	8
VT CB 2	7.0	8
VT CB 3	6.5	6
VT CB 4	6.3	7
VT CB 5	7.4	8
VT CH 1	7.9	7
VT CH 2	7.5	7
VT CH 3	6.9	8
VT CH 4	6.8	7
VT CH 5	7.4	8
RW 1	4.3	5
RW 2	3.8	5
RW 3	4.4	5
RW 4	4.6	5
RW 5	4.8	5
RW 6	5.2	5
RW 7	3.9	5
RW 8	4.6	5
RW 9	4.1	5
RW 10	5.2	5
RW 11	3.6	5
RW 12	4.8	5

2.3.1.1 LACTIC ACID BACTERIA FROM SOURDOUGHS

64 Gram positive and catalase-negative bacteria were presumptively identified as presumptive lactobacilli lactic acid bacteria (data not shown) and were subjected to DGGE identification.

DGGE analysis allowed to obtain the dendrogram shown in Figure 2.1. The strains were grouped, according to the migration profile, in 11 clusters and, for each cluster, one to four strains were subjected to sequencing for identification purpose. The results of the sequencing analysis (data not shown) allowed the identification of 21 out of 22 selected strains (marked with an asterisk in Figure 2.1). Combining these results with those obtained from DGGE cluster analysis, it was possible to identify 62 out of 64 strains. Only the two strains from cluster M resulted not identified. In detail, 36 strains were identified as *Lb. plantarum* (clusters A, B, E, G, N), 15 as *Weissella confusa* (clusters C and F) 5 as *Lb. sanfranciscensis* (cluster D) 3 as *Ln. citrium* (cluster H) and 2 as *Ln. pseudomesenteroides* (cluster I). The results evidenced that the main bacterial species isolated from the sourdoughs were *Lb. plantarum*, detected in all the samples. These results are in agreement with other studies (Randazzo et al., 2005; Ricciardi et al., 2005; Catzeddu et al., 2006; Reale et al., 2011). Interesting was the occurrence of *W. confusa* species. In fact 15 out 64 strains were identified as *W. confusa*. This species, as reported by several studies has been frequently isolated from diverse fermented food, such as sourdough, kimchi, and fermented soya (Corsetti et al., 2001; J.-S. Lee et al., 2005; Malik et al., 2009; Robert et al., 2009) as well as from the human intestines (Walter et al., 2001; K. W. Lee et al., 2012). Moreover, as ascertained by Amari et al. (2012), Weissella could be used in various technological applications for its ability to produce dextran enzymes. Noticeable was the result that evidenced the identification of

five strains as *Lb. sanfranciscensis*. This species was described as an important bacterial species often isolates in traditional baked products (Gobbetti et al., 1994; Vogel et al., 1994; Foschino et al., 2001).

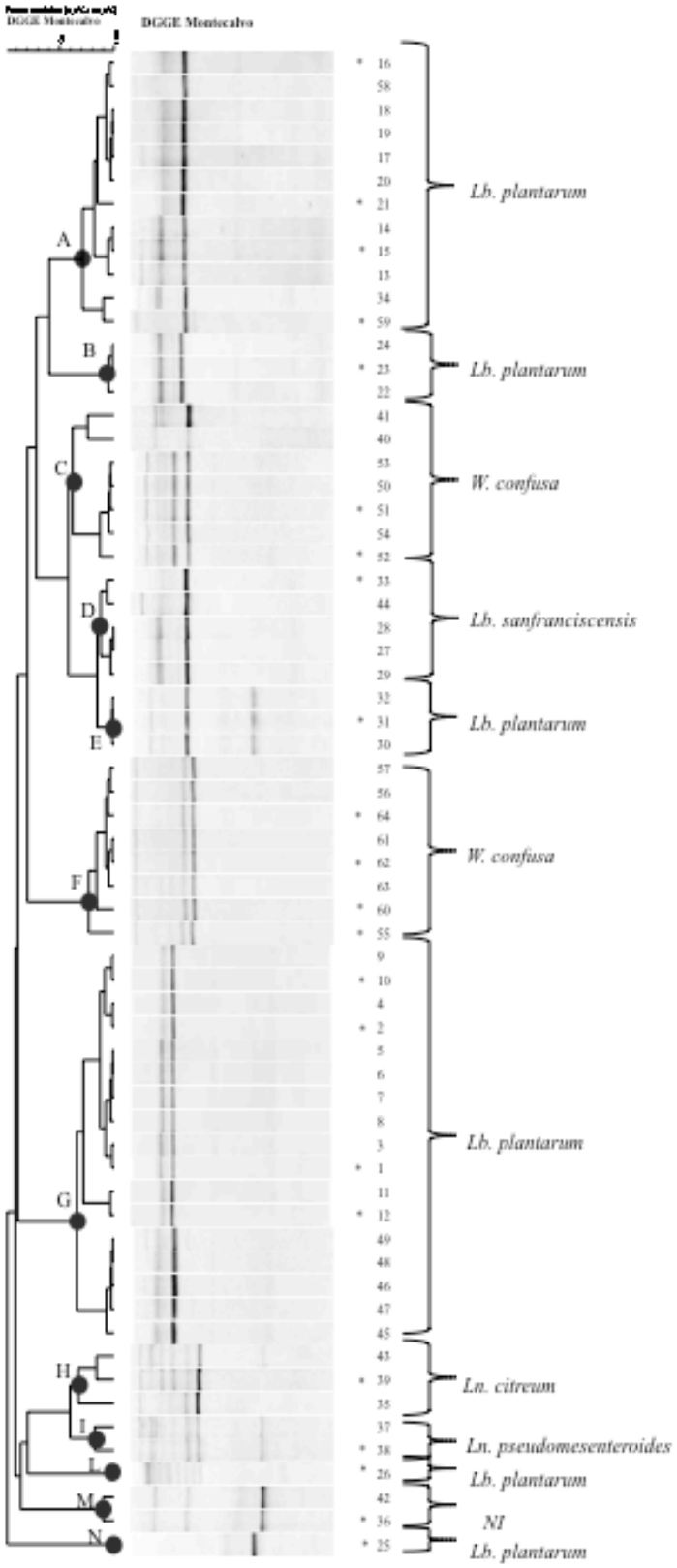

Figure 2.1 Dendrogram showing the similarity among DGGE profiles of 64 lactic acid bacteria isolated from traditional sourdoughs of Campania region. The Asterisks indicate the strains identified by sequencing.

2.3.1.2 *LACTOBACILLUS* FROM FERMENTED SAUSAGES

Out of all isolates, 72 Gram-positive, catalase-negative and presumptive lactic acid bacteria were subjected to DGGE identification.

DGGE analysis allowed to obtain the dendrogram shown in Figure 2.2. The strains were grouped, according to the migration profile, in 16 clusters and, for each cluster, one to four strains were subjected to sequencing for identification purpose. The results of the sequencing analysis (data not shown) allowed the identification of 16 out of 17 all selected strains (marked with an asterisk in Figure 2.2). Combining these results with those obtained from DGGE cluster analysis, it was possible to identify 71 out of 72 strains. In fact, the sole strain from cluster G resulted an uncultured bacterium clone obtained by sequencing analysis and clustered alone in the DGGE dendrogram (Figure 2.2). In detail, 46 strains were identified as *Lb. sakei* (cluster A, B, C, E ed H), 3 as *Lb. coryneformys* (cluster D), 10 as *Lb. plantarum* (cluster F, L, M, N ed O) and 12 as Lb. *curvatus* (cluster P, Q ed R).

Among the studies reported in literature and considering the LAB populations in fermented meats, *Lb. sakei* and or *Lb. curvatus* represent the predominant species. In fact these species were mainly isolated in fermented sausage from several geographic areas (Rantsiou & Cocolin, 2006).

As a matter of fact, only Samelis et al. (1998), R. Coppola et al. (1998) and S. Coppola et al. (2000) did not isolate *L. curvatus*. Results showed in this study evidenced that *L. sakei,* represent the most adapted species of *Lactobacillus* spp. in meat fermentations, as also reported by other authors (Samelis et al., 1998; Papamanoli et al., 2003; Rantsiou & Cocolin, 2006). Moreover other species of *Lactobacillus* were also identified. In detail, the results evidenced that *Lb. plantarum* and *Lb. curvatus* were other important

species that participate to sausage fermentations. However, as reported by other studies (Parente et al., 2001; Papamanoli et al., 2003; Rantsiou & Cocolin, 2006) their frequency of isolation is not as high as for the *Lb. sakei* species.

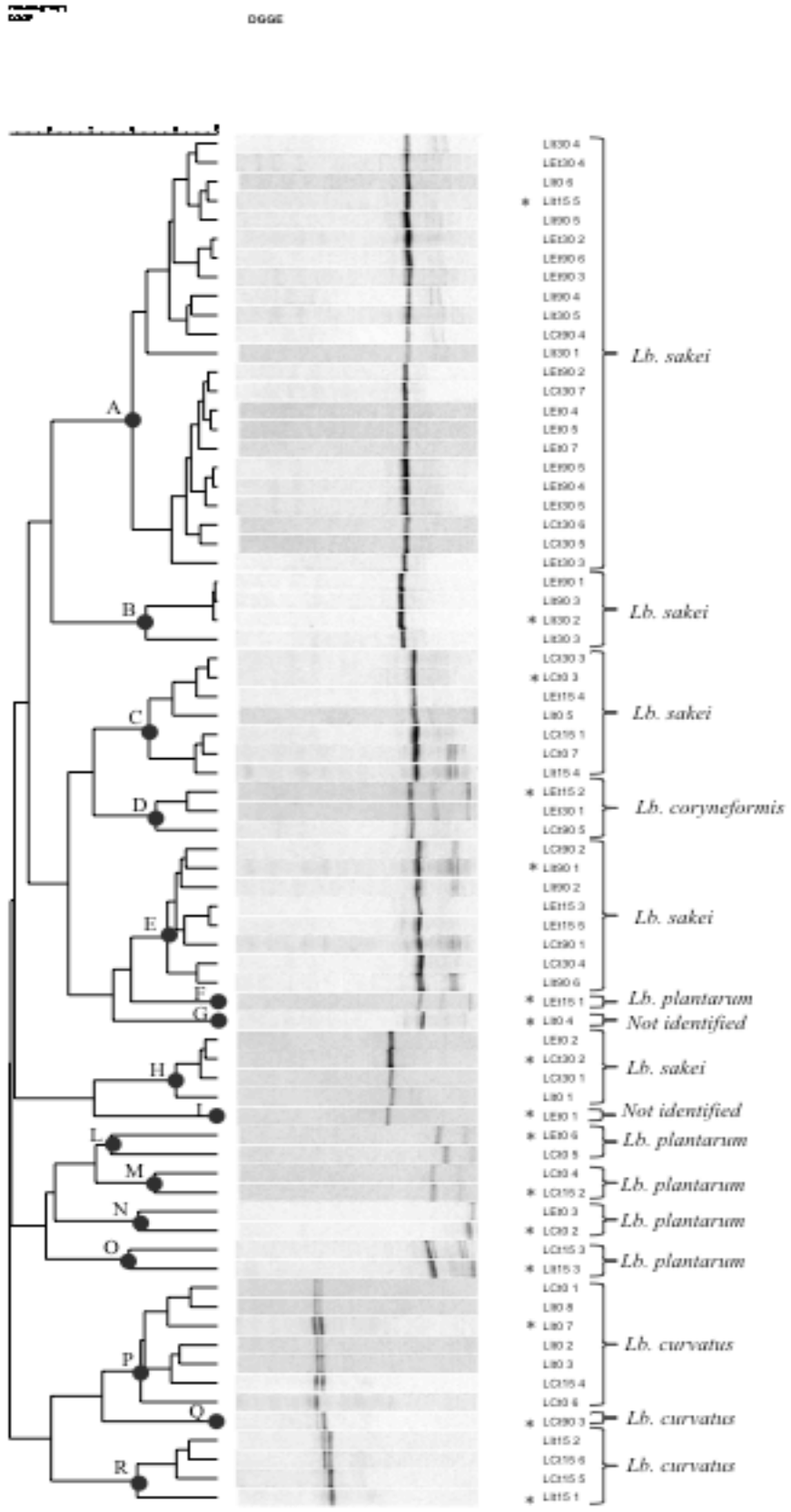

Figure 2.2 Dendrogram showing the similarity among DGGE profiles of 64 lactobacilli isolated from Ventricina, a traditional fermented sausage of Molise and Abruzzo regions. The Asterisks indicate the strains identified by sequencing.

2.3.1.3 *LACTOBACILLUS* FROM RED WINE

60 Gram-positive, catalase-negative, rood-sheep bacteria and presumptive lactobacilli were subjected to DGGE identification.

DGGE analysis allowed to obtain the dendrogram shown in Figure 2.3. The strains were grouped according to the migration profile in four clusters. For each cluster, two strains were subjected to sequencing for sub-species identification purposes. The results of the sequencing analysis (data not shown) allowed the identification of all 8 selected strains. Combining these results with those obtained from DGGE cluster analysis, it was possible to identify the 60 strains as *Lb. plantarum*. This specie, occurring singly or in association with other lactic acid bacteria, are the main *Lactobacillus* specie found in red wine undergoing malolactic fermentation (MFL) and sterilized with sulphite (Spano et al., 2002; Beneduce et al., 2004). In fact as reported by several authors (G-Alegria et al., 2004) *Lactobacillus plantarum* species have displayed the ability to survive the harsh wine conditions. Moreover, they possess many favourable characteristics that would make them suitable MLF starter cultures in the future (Toit et al., 2011). It was shown by several Authors that many wine-associated lactobacilli have the genes that encode for the enzymes involved in the malolactic fermentation and that some of the enzymes are active under winemaking conditions (Vaquero et al., 2004; Grimaldi et al., 2005; Matthews et al., 2007; las Rivas et al., 2009). Spano et al. (2007) evidenced that *Lb. plantarum* was the predominant population at the beginning of MFL in our samples. *Lb. plantarum* species was already observed 10 days after the start of alcoholic fermentation in sample treated with or without $K_2S_2O_5$ and was still detectable at 16 and 22 days. It did not disappear at 28 days even in wine supplemented with $K_2S_2O_5$. The studies (Toit et al., 2011) that have screened oenological lactobacilli for their potential

as MLF starter cultures identified *Lb. plantarum* and *Lb. hilgardi* as having the best potential. This species of *Lactobacillus* also shows a more diverse enzymatic profile than *O. oeni,* which could play an important role in the modification of the wine aroma profile (Matthews et al., 2007). High interest by a commercial culture has recently been released by Lallemand as *Lactobacillus plantarum* V22®. In fact, *Lactobacillus plantarum* has shown the most potential as a starter culture.

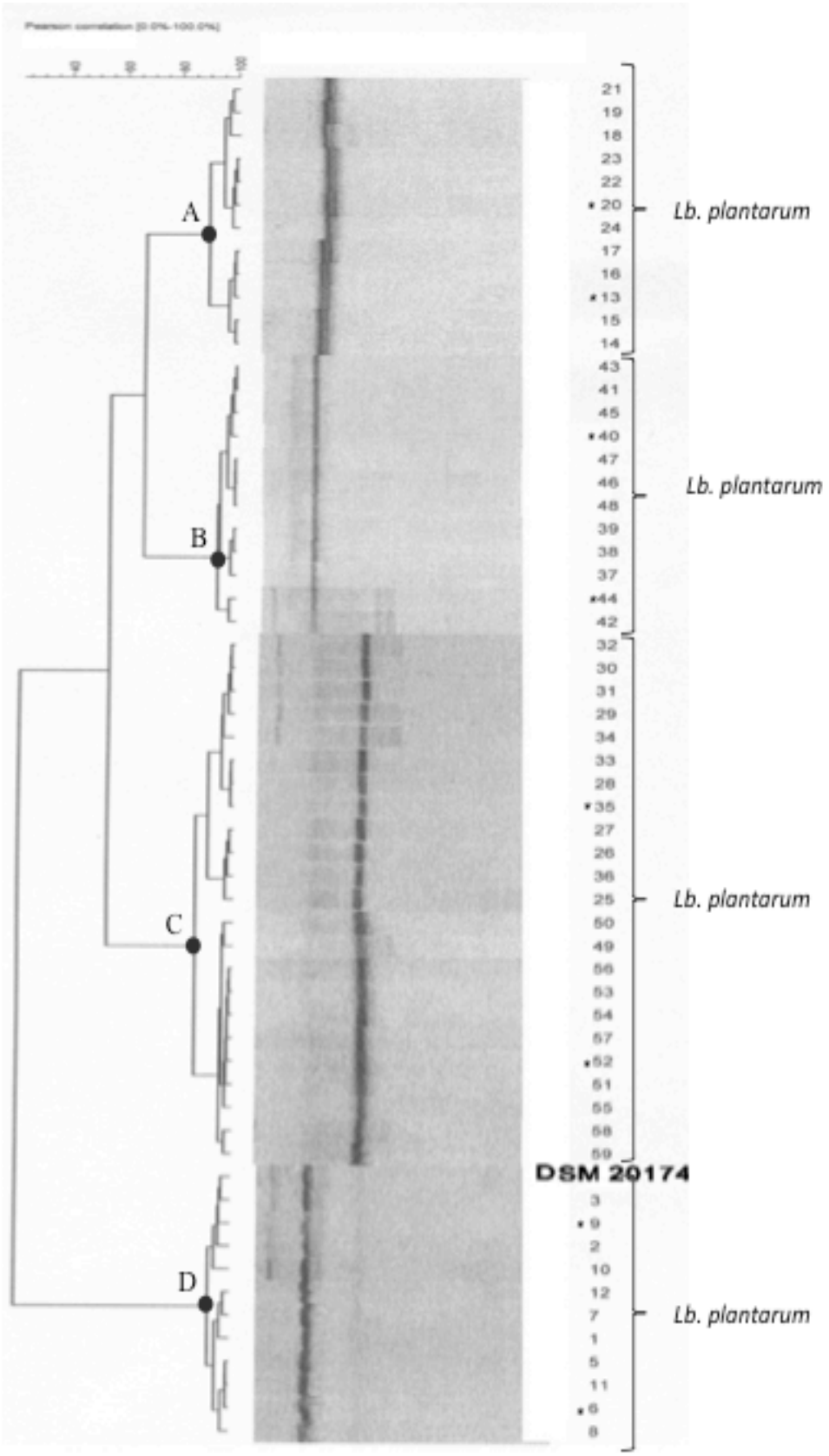

Figure 2.3 Dendrogram showing the similarity among DGGE profiles of 64 lactobacilli isolated from red wine of Molise and Campania regions. The Asterisks indicate the strains identified by sequencing.

Results highlighted also that DGGE analysis and 16S rRNA gene sequencing constist of a suitable multiple approach to identify lactobacilli from wine. Analysis of the amplified variable V3 region of the 16S rDNA has been applied to differentiate lactobacilli species by several authors (Ercolini et al., 2001). In details, the primers P1V1 (Klijn et al., 1991) have been used to study bacteria communities from several food (Reale et al., 2011). As pointed out already in 2001 by Ercolini DGGE analysis, even if is not always suitable for the identification of all species, can be used for screening and grouping the isolates and reducing the number of cultures to identify by other molecular methods, such as 16S rRNA gene sequencing.

2.3.2 INHIBITION OF UNDESIRABLE MICROORGANISMS

Different effects of growing cells of *Lb. plantarum* strains on undesirable microorganisms were assessed by agar spot test (Table 2.4). In detail, Gram negative bacteria, except for *A. pasteurianus*, were inhibited by all the assayed producer strains with a different intensity ($P < 0.05$). Among Gram negative, some species, such as *A. aceti*, *A. tropicalis*, *Ga. hansenii* and *Ga. liquefaciens* showed a higher ($P<0.05$) sensitivity than that exhibited by *Ga. oxydans* and *Frateuria* spp.

Table 2.4 Different effects of growing cells of *Lb. plantarum* strains on undesirable microorganisms assessed by agar spot test.

Indicator strains		Cells of *Lb. plantarum* producer strains				
Species	**Strains**	++++	+++	++	+	-
Lb. brevis	SL, SL1	2	5	9	21	69
Lb. casei	SERB108, SERB69	0	0	7	16	83
L. innocua	ATCC33090	2	15	16	41	32
B. thermosphacta	DSM 20171T	82	17	7	0	0
P. fluorescens	DSM 50090^{T}	21	25	28	23	9
C. sporogenes	DSM 795T	74	23	7	2	0
A. aceti	DSM 3508^{T}	69	19	18	0	0
A. aceti	ASRT, ASC	20	67	16	2	1
A. aceti	111, 111E	39	28	28	11	0
A. pasteurianus	DSM 3509T	0	0	0	2	104
A. tropicalis	DSM 15551T	16	87	3	0	0
Ga. hansenii	194BV, ASAC4, ASR, ARLA, AC1, 141A	38	47	17	4	0
Ga. hansenii	203B1	30	48	25	3	0
Ga. hansenii	DSM 5602^{T}	32	47	23	4	0
Ga. liquefaciens	DSM 5003^{T}	88	14	4	0	0
G. oxydans	146B and AC6	10	18	57	21	0
Frateuria spp.	F4	0	0	26	80	0
Frateuria spp.	F1, F2, F5, F6	4	100	2	0	0
P. digitatum	P1, P2, P3, P4, P5	2	4	6	25	69

A different sensibility was evidenced in Gram positive bacteria. The most of the *Lb brevis* and *Lb. casei* strains were not sensitive to *Lb. plantarum* producer strains. Regarding the inhibition against *Lb. casei* strains, 23 producer strains showed a low (16) or moderate (7) activity, whilst as regard for *Lb. brevis*, 30 producer strains showed a low or moderate inhibition and 7 producer strains expressed a high or very high inhibitory activity. A stronger ($P<0.05$) inhibition was detected against the other Gram positive species. In detail, *B. thermosphacta* and in *C. sporogenes* showed the highest sensitivity; instead a lower ($P<0.05\%$) sensitivity was observed in *L. innocua*. However, noticeable were the data that evidenced a high or very high inhibition exerted by 17 producers strains versus *L. innocua* ATCC 33090.

The highest activity recorded against Gram negative bacteria is unusual, and has thus far only been reported for a few bacteriocins of lactic acid bacteria (De Kwaadsteniet et al., 2005). As reported by other authors (Maragkoudakis et al., 2009) Gram-negative

bacteria are not sensitive to the antibacterial action exerted by lactic acid bacteria. Althoughsome studies (Bernet-Camard et al., 1997; van de Guchte et al., 2001) have demonstrated antibacterial activity of LAB against Gram negative bacteria the nature of the inhibitory effect was not attributed to a bacteriocin and has not been completely elucidated (Bernet-Camard et al., 1997; van de Guchte et al., 2001; Zoumpopoulou et al., 2008) and only in few cases a clear bacteriocinogenic effect against Gram negatives has been reported (Zamfir et al., 1999; Kim et al., 2003; De Kwaadsteniet et al., 2005).

As regarding for the moulds, the majority of lactobacilli strains was unable to inhibit the *P. digitatum* strains or showed a very low inhibition ability. Only six strains of lactobacilli showed the high inhibitory features. In particular the strains *Lb. plantarum* RTB and 14 showed the highest antimicrobial activity. Previous study studies have reported that antimicrobial mulecules produced by LAB are inactive against Gram-negative bacteria and eucaryotic microorganisms such as yeasts or moulds (Batish et al., 1997). Moreover, as evidenced by Dalié et al. (2010) in a recent review, the action of the antifungal properties of LAB on some moulds have also been reported by a few authors (Corsetti et al., 1998; Hassan & Bullerman, 2008).

The cell-free supernatants of *Lb. plantarm* strains produced, against both bacteria and moulds, exerted a lower antimicrobial activity than that exhibited by growing cells of producers strains (Table 2.5). This datum evidenced that the stress conditions exerted by producer strains against indicator microorganisms could be due to several hurdles including the presence of live cells.

Table 2.5 Different effects of cell free supernatants of *Lb. plantarum* strains on undesirable microorganisms assessed by agar well diffusion.

Indicator strains		Cell free supernatants of *Lb. plantarum* producer strains				
Species	**Strains**	++++	+++	++	+	-
Lb. brevis	SL, SL1	0	0	1	4	101
Lb. casei	SERB108, SERB69	0	0	0	1	105
L. innocua	ATCC33090	0	2	23	49	32
B. thermosphacta	DSM 20171T	4	25	46	18	13
P. fluorescens	DSM 50090^{T}	1	23	49	22	11
C. sporogenes	DSM 795T	51	26	21	7	1
A. aceti	DSM 3508^{T}	54	19	33	0	0
A. aceti	ASRT, ASC	0	17	82	4	3
A. aceti	111, 111E	1	21	79	4	1
A. pasteurianus	DSM 3509T	0	0	0	2	104
A. tropicalis	DSM 15551T	0	64	32	10	0
Ga. hansenii	194BV, ASAC4, ASR, ARLA, AC1, 141A	2	31	60	11	2
Ga. hansenii	203B1	1	21	44	31	9
Ga. hansenii	DSM 5602^{T}	1	22	44	29	10
Ga. liquefaciens	DSM 5003^{T}	12	16	57	21	0
G. oxydans	146B and AC6	4	21	53	27	1
Frateuria spp.	F4	0	0	24	28	54
Frateuria spp.	F1, F2, F5, F6	0	76	16	14	0
P. digitatum	P1, P2, P3, P4, P5	0	0	5	30	71

However, the results also highlighted that the cells free supernatant of a considerable number of producer strains exerted a remarkable antimicrobial activity versus the indicators strains. Therefore, as described by others authors (Deegan et al., 2006; Albano et al., 2007; Maragkoudakis et al., 2009) the antimicrobial activity of these producers strains could be due to the synthesis of many compounds such as organic acids, hydrogen peroxide, peptides, as well as antimicrobial proteins (known as bacteriocins).

The inhibition actions produced by the neutralized cell free supernatants of producer strains are reported in Table 2.6. It is clear that most of the neutralized cell-free supernatants produced no inhibitory action against indicators strains. Therefore, it is possible to assert that the antimicrobial activity of most of *Lb. plantarum* strains was mainly due to the production of lactic acid and to the presence of their live cells.

Moreover these strains are not able to produce extracellular proteins or metabolites with antimicrobial activity.

Table 2.6 Inhibitory action produced by the neutralized cell free supernatants of producer strains against the indicators.

Indicator strains		**Neutralized cell free supernatants of *Lb. plantarum* producer strains**				
Species	**Strains**	++++	+++	++	+	-
Lb. brevis	SL, SL1	0	0	1 (RTB)	4 (BB, SP, BP, LCT30)	101
Lb. casei	SERB108, SERB69	0	0	0	1 (RTB)	105
L. innocua	ATCC33090	0	1 (RTB)	1 (BB, BP)	1 (SP)	102
B. thermosphacta	DSM 20171T	0	0	0	3 (RTB, BB, SP)	103
P. fluorescens	DSM 50090^{T}	0	0	0	1 (RTB)	105
C. sporogenes	DSM 795T	0	0	1 (RTB)	1 (BB)	104
A. aceti	DSM 3508^{T}	0	0	1 (BB)	3 (RTB, BP, SP)	102
A. aceti	ASRT, ASC	0	0	2 (RTB, BB)	4 (RTB, BP, SP, LCT30)	102
A. aceti	111, 111E	0	0	1 (RTB,)	5 (RTB, BB, SP, BP, LCT30)	102
A. pasteurianus	DSM 3509T	0	0	0	0	106
A. tropicalis	DSM 15551T	0	0	0	2 (RTB, BB)	104
Ga. hansenii	194BV, ASAC4, ASR, ARLA, AC1, 141A	0	0	0	1 (RTB)	105
Ga. hansenii	203B1	0	0	0	2 (RTB, BB)	104
Ga. hansenii	DSM 5602^{T}	0	0	1 (RTB)	1 (BP)	104
Ga. liquefaciens	DSM 5003^{T}	0	0	1 (RTB)	2 (BB, BP)	103
G. oxydans	146B and AC6	0	0	0	1 (RTB)	105
Frateuria spp.	F4	0	0	2 (RTB, BB)	1 (BP)	103
Frateuria spp.	F1, F2, F5, F6	0	0	2 (RTB, BB)	1 (BP)	103
P. digitatum	P1, P2, P3, P4, P5				5 (RTB, BB, LCT30, 21, 14)	101

Only seven strains of *Lb. plantarum* evidenced the ability to inhibit one or more indicator strains when used as neutralized cell-free supernatants. In detail, the inhibitory action exhibited by these strains is not due to the mere production of lactic acid but to metabolites of other nature.

As reported in Table 2.7, the inhibitory action expressed by the seven producer strains is different for their spectrum of action. In detail, the producers strains *Lb. plantarum* 14 and *Lb. plantarum* 21, both isolated from sourdoughs, showed the lowest spectrum of action inhibiting only the indicator strains belonging to *P. digitatum* species. Also other

authors (Gerez et al., 2009; Garofalo et al., 2012) have evidenced the ability of *Lactobacillus* strains to prevent the mould growth. A moderate spectrum of action was evidenced by *Lb. plantarum* LTC30, *Lb. plantarum* SP and *Lb. plantarum* BP that inhibit respectively 3, 4 and 5 different species. A broad range of action was evidenced by *Lb. plantarum* BB that inhibited several strains belonging to 10 different species. *Lb. plantarum* RTB showed the highest inhibition range producing an antimicrobial action against all the bacteria and moulds strains assayed. Moreover, statistical analyses (TCA) showed a behaviour of these last strains different from all other producer strains (data not shown). Noticeable was also the strong inhibitory action exerted by the neutralized CFS of this last strains versus *L. innocua*.

Table 2.7 Producer strains that exert inhibitory action against the indicators when used as neutralized cell-free supernatants.

***Lb. plantarum* strains**	**Origin**	**Inhibited microorganisms**
BB	red wine (Taursi)	*Lb. brevis, L. innocua, B. thermosphacta, C. sporogenes, A. aceti, A tropicalis, Ga. hansenii, Ga. liquefaciens, Frateuria spp., P. digitatum*
BP	red wine (Taurasi)	*Lb. brevis, L. innocua, A. aceti, Ga. hansenii, Frateuria spp.*
RTB	red wine (Tintilia)	*Lb. brevis, L. casei, L. innocua, B. thermosphacta, P. fluorescens, C. sporogenes, A. aceti, A tropicalis, Ga. hansenii, Ga. liquefaciens, G. oxydans, Frateuria spp., P. digitatum*
SP	red wine (Piedirosso)	*Lb. brevis, L. innocua, B. thermosphacta, A. aceti,*
LTC30	fermented sausage (Ventricina)	*Lb. brevis, A. aceti, P. digitatum*
14	sourdough	*P. digitatum*
21	sourdough	*P. digitatum*

The results related to the effects expressed by supernatants in presence of proteinases could be useful to understand the nature of the compounds responsible of antimicrobial action. The antimicrobial activity was expressed also when the CFS of producer strains was exposed to the proteinases. Therefore, one could suppose that the inhibition expressed by these strains was not due to compounds of proteinaceous nature, such as bacteriocines.

2.4 CONCLUSIONS

The results showed that *Lactobacillus plantarum* is harbored in a variety of environmental niches, including fermented beverages, fermented meats, and sponge doughs. Its largest genome, among other lactic acid bacteria, reflects the ecological flexibility of this bacterium. Therefore, we can state that *Lb. plantarum* is characterized by a high ability to adapt itself to many kinds of foods, as well as by a recognized antimicrobial capacity. Both these features make *Lb. plantarum* a species of remarkable interest in the development of protective culture to be used in the food production.

Interestingly, a specific strain of *Lb. plantarum*, such as *Lb. plantarum* RTB, showed a remarkable antimicrobial activity versus *L. innocua*. However, it is well known that the knowledge of the undesirable strains response to these antimicrobial substances (stress conditions) represents a crucial step for the definition of an effective bio-control tool. Therefore, a further investigation on this strains for their antilisterial ablity are desirable.

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ANTI-LISTERIA ACTION EXPRESSED BY *LACTOBACILLUS PLANTARUM*

3.1 INTRODUCTION

Lactobacillus plantarum represents a common species in microbial communities of several fermented and fresh foods. Its high metabolic plasticity (Molenaar et al., 2005) and the production of several natural antimicrobial substances can cause the inhibition of undesirable microorganisms including *L. monocytogenes*. The anti-listeria properties may be ascribable to production of antimicrobial compounds such as bacteriocins, bacteriocine-like substances (BLIS), phenyllactic acid, organic acids (mainly lactic and acetic acid), hydrogen peroxide as well as competition for nutrients or by effect of their combination (Todorov et al., 2011; Reis et al., 2012)

Lb. plantarum strains may produce class IIa anti-listeria bacteriocins, such as: plantaricin 423 (van Reenen et al., 1998), plantaricin WHE92 (Ennahar et al., 1999), plantaricin C19 (Atrih et al., 2001) and plantaricin AMA-K (Todorov et al., 2005).

Nevertheless, the effectiveness of bacteriocin-producing strains in foods can be limited by several factors including narrow activity spectrum, limited, diffusion in solid matrices, inactivation through proteolytic enzymes or binding to food ingredients such as lipids, low production level and the emergence of bacteriocin-resistant bacteria (Rodriguez et al., 2003). Although, the use of class IIa bacteriocins or bacteriocins-producing strains represent a promising alternative for the control the growth of *L.*

monocytogenes in a foodstuff, their efficacy could be compromised by the onset of bacteriocins resistant strains and cross-resistance between bacteriocins (Naghmouchi et al., 2007; Tessema et al., 2009). However, the use of bacteriocins in combination with other hurdles (e.g. salt, acid, other natural substances etc.) may result extremely effective for inhibition of *L. monocytogenes* and reduce its resistance (Mills, Serrano, et al., 2011a; Mills, Stanton, et al., 2011b). Some authors (Devlieghere et al., 2005) suggested that an alternative to overcome the disadvantages of bacteriocinogenic cultures is the use of non-bacteriocinogenic but nevertheless very competitive cultures. Certain experiments showing the anti-listeria effect due to production of acid organics, including lactic acid by *Lb. plantarum* (Bernbom et al., 2006; Kaushik et al., 2009). *Lb. plantarum* SK1 showed anti-listeria activity through lactic acid production (Wilson et al., 2005). 3-Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid, PLA) has been also reported as an anti-listeria compound (Dieuleveux & Gueguen, 1998).

In this study the anti-listeria activity of *Lb. plantarum* RTB was investigated and a commercial *L. innocua* strain was used as a pathogen surrogate. This is justifiable since *L. innocua* has been deemed a suitable biological indicator for *L. monocytogenes* (Kamat and Nair, 1996) and it revealed a similar sensitivity to different stress condition.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS USED AND EXPERIMENTAL DESIGN

The strain *Lactobacillus plantarum* RTB (producer) previously screened (see Chapter II), was used in this study to detect its anti-listeria activity. The type strain *Listeria innocua* 33090 was utilized as indicator. Both strains were propagated in MRS broth (Oxoid) at 28 °C.

To detect the anti-listeria effect of *Lb. plantarum* RTB several experiments were

performed.

In detail, four batches of MRS (450 mL) were inoculated with *L. innocua* ATCC 33090 at concentration of 10^7 CFU/mL and then added respectively with 50 mL of MRS (as Control) and with 50 mL of MRS containing three stressors as show below:

2stressRTB: culture of *Lb. plantarum* RTB (10^8 CFU/mL);

3stressCFS-RTB: cell free supernatant of culture containing *Lb. plantarum* RTB at 10^8 CFU/mL;

4stressLA: MRS acidified with lactic acid up to pH 4.0 (the same of stress RTB and stress CFS-RTB pH);

The samples were incubated at 28 °C for 24 hours during which the optical density (OD) was detected (several time intervals). The vital count of *L. innocua* was also performed in exponential phase and at the beginning of the stationary phase. In this respect a selective medium (ALOA, Biolife) was used.

3.2.2 DETECTION OF ANTI-LISTERIA ACTIVITY

Two approaches were used to investigate the anti-listeria action expressed by the three stressors (see par. 3.2.1)

The first approach regarded the use of a dynamic model (Baranyi & Roberts, 1994) to predict the growth of *L. innocua* in presence of stressors. The OD values recorded during the growth of *L. innocua* were fitted using the program DMFit v. 3 (Barany and Le Marc 1996). The growth curve parameters (lag time, growth rate and asymptote) of *L. innocua* were estimated when the indicator was cultivated in MRS broth with the stressRTB, stressCFS-RTB and stressLA. The kinetics parameters of the *L. innocua* growth were also evaluated when the indicator was cultivated in MRS broth without

stressors (control).

In the second approaches, the anti-listeria action of stressors was investigated as vital count of *L. innocua* in three different times: just after the inoculum of indicator (time zero), in the exponential phase (log-phase) and at the beginning of the stationary phase.

The anti-listeria activity of different stressors against *L. innocua* was calculated according to (Charernjiratragul et al., 2010)

$$\%\ \text{inhibition} = \frac{(\text{CFU/mL control}) - (\text{CFU/mL stress condition})}{\text{CFU/mL control}}\ \text{X}\ 100$$

where: "CFU/mL control" is *L. innocua* concentration in the log-phase or in the stationary phase in MRS broth whilst "CFU/mL in stress condition" is *L. innocua* concentration in the log-phase or in the stationary phase cultivated in presence of stressors.

3.3 RESULTS AND DISCUSSION

3.3.1 QUANTIFICATION OF *L. INNOCUA* INHIBITION

The application of a dynamic model (Baranyi and Roberts, 1994) allowed to predict the growth of *L. innocua* in presence of different stressors represented by stressLA or stress CFS-RTB (Figure 3.1). The estimated parameters obtained from the fits are shown in Table 3.1. As evidenced by values of mean corrected R-square (min. 0.990 and max. 0.996), the goodness of fit Baranyi model is very high. The growth parameters highlighted significant differences among the several cultural conditions. In details, the *L. innocua* strains cultivated wit-out stressors showed a growth rate of about 0.4 h^{-1} (Table 3.1). This value respected those evidenced also by others authors (Houtsma et al., 1996; Le Marc et al., 2002) In fact, these authors evidenced that *L. innocua* strains

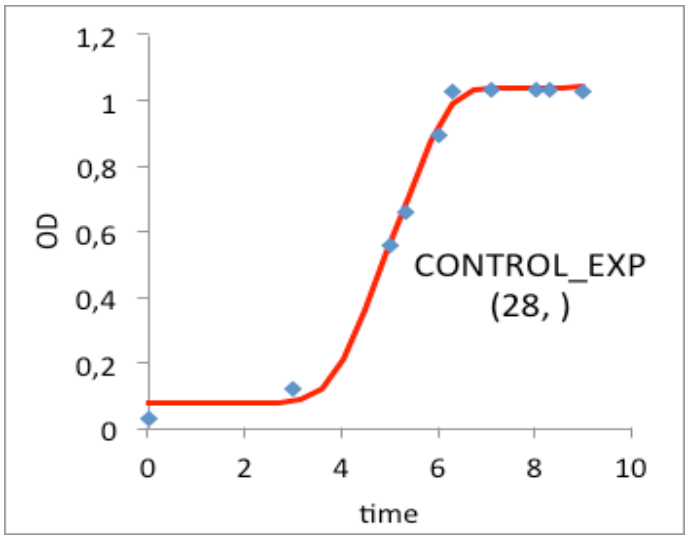
when cultivated with-out stress and at optimal temperature condition exhibited ⌈max values similar to those appreciated in the present study. As also evidenced by others (Le Marc et al., 2002; Kaushik et al., 2009), the results of this study showed that lactic acid was able to produce an injury, even whit low intensity, on the growth of *Listeria innocua*. In fact, the assayed strains when cultivated in presence of stressLA showed growth rate and *yend* values (Table 3.1) significantly lower ($P<0.05$) than those exhibited by the *Listeria* cultivated with-out stress. In agreement whit several studies reposted in literature (Perry & Donnelly, 1990; Wilson et al., 2005) these evidences confirm that the addition of weak acid could represents an approach to enhance foods preservation. Presence of weak acid lowers the pH which causes an increase of the amount of undissociated acid. These features could inhibit the development of *Listeria* and ensure food safety.

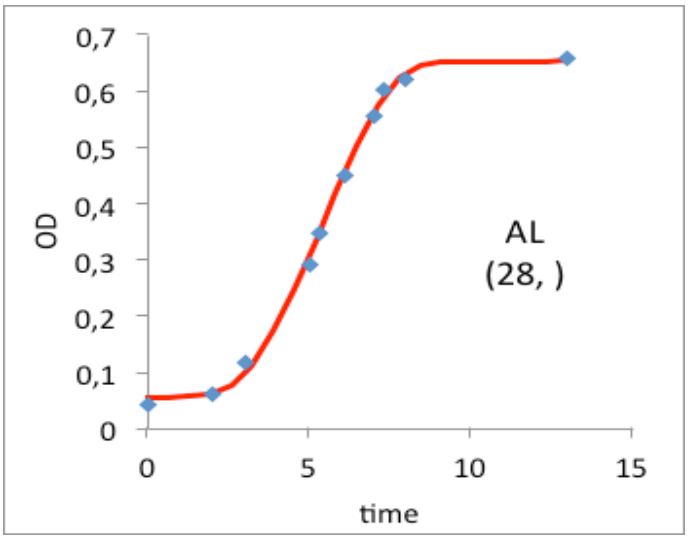
Table 3.1 Growth parameters of *L. innocua* ATCC 33090 cultivated in presence of different stress conditions.

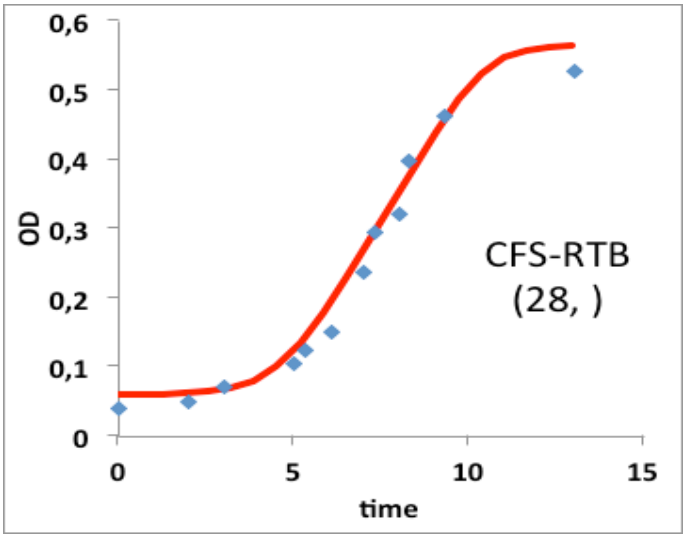
Curve growth	rate	lag	y0	yEnd	R^2_stat
CONTROL	0.40	3.80	0.08	1.04	0.993
stressLA	0.13	3.10	0.06	0.70	0.996
stressCFS-RTB	0.08	4.53	0.06	0.53	0.990

However, in the present study the most noticeable results were represented by the predicted growth of *L. innocua* in presence of stress-CFS-RTB. This last stress conditions produced a highest decrease in growth rate and *yend* values (Table 3.1). Regarding stressCFS-RTB, these values resulted significantly lower than those registered both in control condition and in presence of stressLA. Therefore, one could confirm that the inhibitory action produced by *Lb. plantarum* RTB against *L. innocua* ATCC 33090 is to be due to the production of extracellular compounds having neither acidic nor proteinaceous nature. Also other authors (van de Guchte et al., 2001;

Zoumpopoulou et al., 2008) reported that the antimicrobial effect expressed by some lactobacilli was not attributed to a bacteriocins and has not been completely elucidated.







Fi**gure 3.1** Predicted growths of *L. innocua* ATCC33090 in presence with-out or with stress conditions.

The results relate to the count levels evidenced that *L. innocua* ATCC 33090 in absence or in presence of stresses (stressRTB, stressCFS-RTB, stressLA) showed a different behaviour. In details, without stresses (control) *L. innocua*, from initial concentration of 7 log CFU/mL, reached levels of about 9.6 CFU/mL in the stationary phase. Inhibitory effect was appreciated when the strains was cultivated in presence of stresses. However, the intensity and the behaviour of inhibition effect was different in several stress conditions. There was a strongly decrease of concentration, recorded in the log-phase and in the stationary phase when *L. innocua* ATCC 33090 was cultured with *Lb. plantarum* RTB. Growth inhibition of *L. innocua* ATCC 33090 was also observed in the log-phase in presence of stressLA, stressCFS-RTB, but an adaptation indicator was observed in the stationary phase of indicator in presence of stressLA.

As evidenced by results reported in Figure 3.2, *L. innocua* was inhibited by 100% (both in log-phase and stationary phase) when cultivated in stressRTB. Instead in presence of stress CFS-RTB the indicator strain was inhibited by 97.7% in the log-phase and by 99.8% in the stationary-phase. Interesting *L. innocua* ATCC 33090 was inhibited by 96.7% (in the log-phase) when cultivated with stressLA but it was inhibited only by 92.7% when reached the stationary phase, showing an adaptation to the acid.

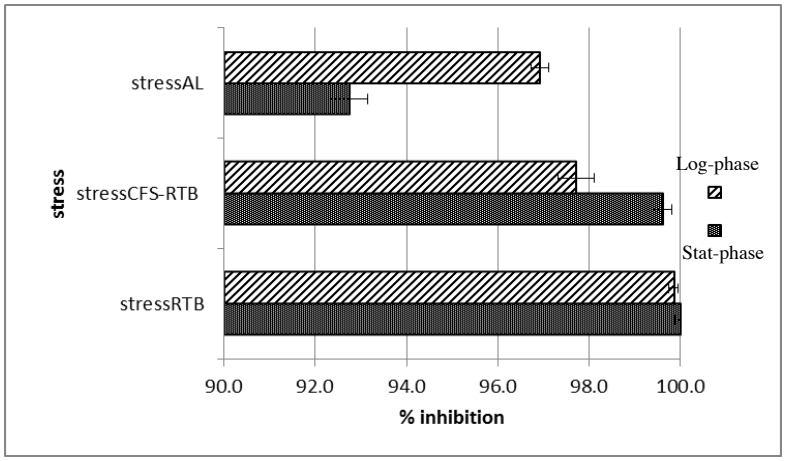


Figure 3.2 Inhibition of *L. innocua* ATCC 33090 cultivated whit several stressors and expressed as percentage.

Therefore, it is possible to assert that the presence of the acid induces a reversible stress condition against the indicator strain, which after a period adaptation is still able to proliferate albeit in a more limited manner than for the control. The indicator resistance could be due to a complex molecular mechanism known as the ATR (Acid Tolerance Response) and described for the first time in 1996 by O'Driscoll et al. It is known that this mechanism results in the activation of various defence mechanisms, such as changes in the composition of the membrane, increasing the proton flux, increased catabolism of amino acids, formation of enzymes that repair DNA (Beales, 2004).

3.4 CONCLUSIONS

The anti-listeria action expressed by *Lb. plantarum* RTB cannot be based solely on the production of lactic acid or protean compounds such as bacteriocin. The exhaustive explanation of the inhibition is more complex and could be due to several factors such as production of other antimicrobial compounds for competition or depletion of specific nutrients.

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STRESS RESPONSE OF *LISTERIA INNOCUA* TO *LACTOBACILLUS PLANTARUM* RTB

4.1 INTRODUCTION

Ecological and genomic comparative studies in *Listeria* spp. showed a high similarity between the pathogenic *L. monocytogenes* and the non-pathogenic *L. innocua* (Girardin et al., 2005; Glaser et al., 2001). The high genomic similarity and the environmental niches shared by the two species have driven many researchers to use the non-pathogenic *L. innocua* as surrogate of *L. monocytogenes* to better understand the behaviour of the latter. *Listeria monocytogenes* is recognised as a bacterium able to grow in several environmental conditions such as temperature ranging from -1.5° to + 45°C, salt concentration up to 12%, a_w of about 0.92 and pH values of 4.5 (Gandhi & Chikindas, 2007). Different stress protection systems are involved and efficiently coordinated to protect the cell in response to environmental changing. Generally, these systems involve different changes in gene expression and the induction of protein expression linked to a particular stress (specific stress response) or involved in several stress conditions (general stress response).

Pathogenic *L. monocytogenes* and non-pathogenic *L. innocua* species share several genes encoding transport proteins involved in the carbohydrate transport and mediated by Phosphoenolpyruvate-dependent Phosphotransferase systems (PTS). This character would seem to be correlated to the ability of *Listeria* spp. to colonize and grow in a broad range of ecosystems (Glaser et al., 2001). Many transcriptional regulator genes

have been observed in both species including four classes of stress proteins (HrcA, sigmaB-dependent, Clp and class IV genes) and genes involved in acid resistance, *e.g*. genes encoding glutamic acid decarboxylase (*gad*) were identified in both *Listeria* species. Only one of the three *gad* paralogs of *L. monocytogenes* (*lmo0447*) was absent in *L. innocua*.

Some authors reported that several mechanisms can be developed by *L. monocytogenes* and/or by *L. innocua* in order to resist to injury processes caused by stress conditions such as temperature, acidity and NaCl (Bergholz et al., 2012; Bowen et al., 2012; Milillo et al., 2012). In detail, the stress seems to induce variations in the synthesis of certain cell components, especially proteins. Nevertheless the literature is very poor in studies focusing on the mechanisms of response, in terms of susceptibility or resistance, expressed by *L. monocytogenes* against antimicrobial substances produced by *Lb. plantarum*. Therefore, the present chapter focused the attention on the stress response of *Listeria* to the presence of *Lb. plantarum*. In detail, a commercial *L. innocua* strain was used as a pathogen surrogate.

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS, GROWTH CONDITIONS, AND STRESS CONDITIONS

As reported previously (Chapter 3), *Listeria innocua* ATCC 33090 was evaluated for its ability to grow in MRS broth (Oxoid). Then, the indicator strain was cultivated in the same medium at 28 °C with or without two stressors. In detail, three batches of MRS (450 mL) were inoculated with *L. innocua* ATCC 33090 at a concentration of 10^7 CFU/mL and then added with 50 mL of MRS (control, without stressors) or with 50 mL of MRS containing the two stressors (stressCFS and stressLA, respectively) as detailed below:

- stressCFS-RTB: cell free supernatant of a culture containing *Lb. plantarum* RTB at 10^8 CFU/mL;
- stressLA: MRS acidified with lactic acid up to pH 4.0 (the same pH registered in the MRS broth added with the cell free supernatant of a culture containing *Lb. plantarum* RTB at 10^8 CFU/mL (stressCFS-RTB));

Batches were incubated at 28 °C under continuous stirring. Cultures of *L. innocua* were recovered during the log phase and the stationary phase. The proteome of *L. innocua* during log and stationary phase was studied: (i) 28 °C in log phase (log phase control), (ii) 28 °C in stationary phase (stationary phase control), (iii) stressLA in log phase, (iv) stressLA in stationary phase, (v), stressCFS-RTB in log phase, and (vi) stressCFS-RTB in stationary phase.

4.2.2 TOTAL PROTEIN EXTRACTION

The six conditions were performed in triplicates. The cells of *L. innocua* were harvested by centrifugation for 15 minutes at 8000 rpm and washed three times with Tris-HCl 50 mM pH 7.5. Pellets were re-suspended in a lysis buffer (Tris-HCl 100 mM, pH 9.5; SDS 1% p/v), added of glass beads, and sonicated (LABSONIC M) for 12 min with the following setting: 100% Amplitude, 0.8 Cycle, pulse 5 s. Samples were then vortexed for 3 min and centrifuged for 15 min at 13200 rpm. The supernatants (in lysis buffer) containing protein extract were recovered and the pellet was discarded. Protein concentrations were measured using the Bradford protein assay kit (Quick Start™ Bradford Protein Assay, Bio-Rad).

4.2.3 SDS-PAGE

About 40 µg of proteins in a Laemmli buffer were charged in polyacrilamide gel, 1.5 mm thickness and 4% T, 2.6% C (stacking gel) and separated in a gel 12.5% T, 2.6% C (running gel). Two wells per gel were charged with molecular standards (Prestained SDS-PAGE Standards, Bio-Rad). Electrophoresis was performed for 180 min at 220V in a Hoefer SE600 apparatus (Amersham, Bio-sciences). Proteins were stained with Coomassie blue G-250 and unstained with a solution of acetic acid/ethanol/water (1/4/5). Gel images were acquired with a Densitometer Calibrate GS – 800 (Bio-Rad) at 42 microns resolution. Background subtraction, lines and bands number were carried out by Quantity – One software (Bio-Rad).

4.2.4 Two-dimensional gel electrophoresis (2D-E)

Proteins were collected by lysis buffer using the Methanol/Chloroform protocol (Wessel and Fugge, 1984). For Isoelectrofocusing (IEF), precast immobilized pH gradient (IPG) strips with a pH 4 to 7 linear gradient were passively rehydrated for 18 h in a IPGBox with 450 µL of rehydration buffer (Urea 8M, CHAPS 2%, DTT 50 mM, Anfoline 3/10 2%, bromophenol blue 0.002%) containing about 250 µg of proteins. IEF was performed at 65,000Vh using the Ettan IPGphor apparatus (GE Healthcare Bio). Strips were equilibrated for 25 minutes in equilibration buffer (Tris-HCl pH 8.8 50mM, urea 6M, glycerol 30%, SDS 2%, bromophenol blue 0.002%) with DTT 65 mM and 25 min in equilibration buffer with iodoacetamide (IAA) 70 mM. 2D-E was performed in gels of 1.5 mm thick (12.5% T, 2.6% C) using the Ettan DALTsix Electrophoresis System apparatus (Amersham, Bio-sciences). Protein spots were visualized by staining with Coomassie Brillante blue G-250.

4.2.5 DATA ANALYSIS

The proteins from the six conditions were performed in triplicates, and electrophoresis run was conducted on 24-cm IPG strips. Each replicate was obtained from three independent electrophoresis runs. 2D-E protein patterns were recorded as digitalized images using the Densitometer Calibrate GS – 800 (Bio-Rad). Spot detection, quantification and analysis were performed using the PDQuest™ 2-D gel analysis software, Version-8 (Bio-Rad, Hercules, CA).

The proteome of *L. innocua* ATCC 33090 grown in MRS broth at 28 °C (Control) was compared with those obtained when the indicator was cultivated in presence of stress conditions. In detail, the protein expression pattern observed at 28 °C in log phase (i) was compared with those expressed in stress conditions: stressLA in log phase (iii) and stressCFS-RTB in log phase (v). Whilst the protein expression pattern observed at 28 °C in stationary phase (ii) was compared with the protein expression pattern in presence of stressLA in stationary phase (iv), and in presence of stressCFS-RTB in stationary phase (vi).

4.3 RESULTS AND DISCUSSION

4.3.1 ANALYSIS OF CELL PROTEIN BY SDS-PAGE

Changes in protein patterns of *L. innocua* ATCC 33090 cultivated in presence or in absence of stressors were analyzed by SDS-PAGE. This trial allowed the ascertainment of the effect on *L. innocua* not only of lactic acid, used as stressor in the batch stressLA, but also that of other metabolites possibly produced by *Lb. plantarum* and present in the cell free supernatant used as stressor in the batch stressCFS-RTB. Figure 4.1 shows the cell protein patterns of *L. innocua* at the beginning of the exponential phase (lane 2), at

the second half of the exponential phase (lane 3), at the second half of the exponential phase in presence of lactic acid (lane 4), and in presence of cell free supernatant of *Lb. plantarum* (lane 5).

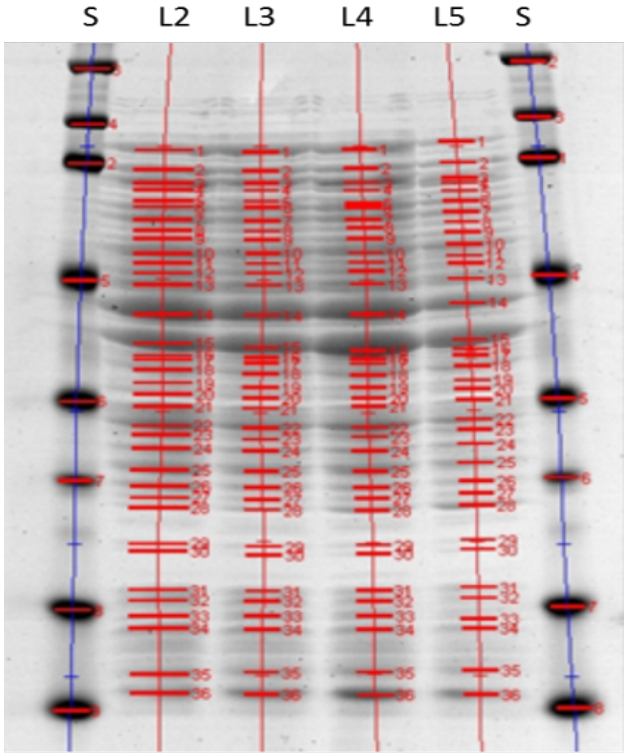

Figure 4.1 Cell protein patterns of *L. innocua* ATCC 33090 at the beginning of the exponential phase (L2), at the second half of the exponential phase (L3), at the second half of the exponential phase in presence of stressLA (L4), and stress CFS-RTB (L5)

In all patterns the presence of 36 bands, with molecular weight between 100 kDa and 8 kDa, was detected (software Quantity-One, Bio-Rad). The intensity of each detected band was first expressed as percentage of the total intensity in lane. Moreover, band intensities of each lane were standardized by dividing them by the intensity of the band at 16.5 kDa, which was present in all patterns with the same intensity. This parameter (PSInt) is useful to compare the bands of different patterns with the same molecular weight. To know the effect produced exclusively by the presence of non-acidic metabolites (stressCFS-RTB) on the protein expression, the effect due to the growth

phase (E Gphase) and to the acidic condition (stressLA) was firstly calculated.

The effects due to the evolution of the exponential growth phase was calculated as follows:

$$\mathbf{E\ Gphase} = \frac{\mathrm{PSInt\ band_{lane3}} - \mathrm{PSInt\ band_{lane2}}}{\mathrm{PSInt_{lane2}}}\,\%$$

The results showed that the evolution of the exponential growth phase did not cause significant changes in protein expression.

The effect of acidic condition (E StressLA) on the protein expression of *L. innocua* ATCC 33090 was calculated as follows:

$$\mathbf{E\ StressAL} = \frac{\mathrm{PSInt\ band_{lane4}} - \mathrm{PSInt\ band_{lane2}}}{\mathrm{PSInt_{lane2}}}\,\% - \mathrm{E\ Gphase}$$

The acidic stress determined the over-expression of seven protein bands. In detail, a strong expression was appreciated for the protein band 11, with molecular weight of 58.2 kDa, for the band 14, with molecular weight of 48.2 kDa, and for the bands 28 and 30, with molecular weight of 26.5 and 23.6 kDa, respectively. Furthermore, an over-expression was also appreciated for three protein bands (16, 17 and 19) ranging between 37.2 and 42.2 kDa.

The effect on protein changes due to non-acidic metabolites present in the CFS of *Lb. plantarum* RTB was estimated as follows:

$$\mathbf{E\ StressCFS - RTB} = \frac{\mathrm{PSInt\ band_{lane5}} - \mathrm{PSInt\ band_{lane2}}}{\mathrm{PSInt_{lane2}}}\,\% - (\mathrm{EGphase} + \mathrm{E\ StressLA})$$

This stress caused a significant change in the expression of nine protein bands. In detail, it was possible to appreciate the over-expression of three protein bands, one with molecular weight of 83.4 kDa and another two with a molecular weight of 26.5 and 23.6

kDa, respectively. These two bands were also over-expressed in the protein pattern of *L. innocua* in stressLA. On the other hand, stressCFS-RTB caused the significant repression of 6 protein bands. In detail, it was appreciated the repression of two bands with molecular weight of 78 and 18.5 kDa. In addition, repression was also observed for 4 protein bands (11, 16, 17 and 19) between 58.2 and 37.2 kDa, which were over-expressed in stressLA.

4.3.2 ANALYSIS OF CELL PROTEIN BY 2D-E

The cell protein 2D-E map of *L. innocua* cultivated without stresses or in presence of stressLA or stressCFS-RTB evidenced significant differences both in exponential and in stationary growth phase.

4.3.2.1 CELL PROTEIN IN EXPONENTIAL PHASE

The map (Figure 4.2) relative to the proteins of *L. innocua* cultivated in stressLA (exponential phase) showed the neo-formation of 35 spots and the over-expression of 30 spots. In Table 4.1 are reported the main proteins induced in *L. innocua* (exponential phase) by stressLA.

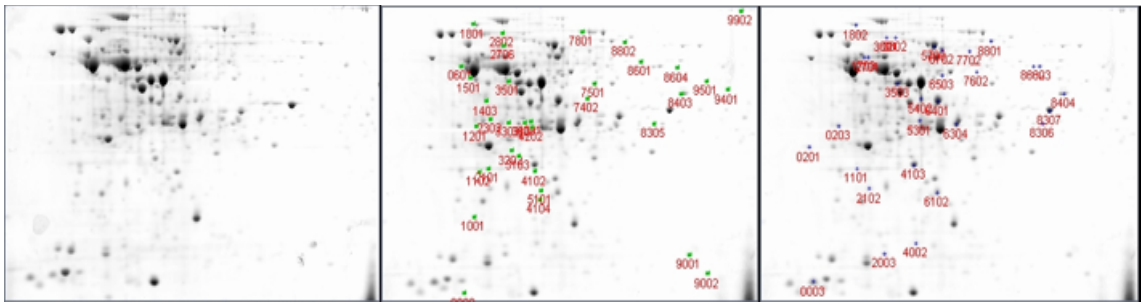

Figure 4.2 2D-E gels of cellular proteome of *L. innocua* cultivated without stress condition (gel left) and in presence of stressLA (gel central and right). The proteins of neo-formation (gel central) and over-expressed (gel right) by the stressLA are indicated by a dot and an identification number. The pI and molecular weight (MW) of the main proteins neo- and over-expressed are reported in the Table 4.1.

Table 4.1 Main proteins induced in *L. innocua* (log phase) by stressLA.

Identification Number	Induction Ratio[a]	Molecular Mass (Kda)	Isoelectric Point (pI)	Putative Protein[b]
9	Novel	10.76	4.45	GroES
601	Novel	59.43	4.63	GroEL
1001	Novel	19.99	4.73	Ycel like family protein
1403	Novel	46.14	4.82	Clpx
2101	Novel	28,35	4,84	Serine/Treonine Fosfatase stp
3307	Novel	38,67	5.11	Phosphoserine amminotransferase
3501	Novel	53.16	4.99	Probable glutamate decarboxylase gamma o GadB
4102	Novel	27.77	5.18	Oxidoreduttase Short chain dehydrogenase
4104	Novel	22.69	5.22	TetR family transcriptional regulator
4202	Novel	38.43	5.16	GroES like protein
7402	Novel	46.97	5.58	PTS system
7501	Novel	52.47	5.63	Ribonuclease Y
7801	Novel	77.57	5.54	PTS system
8601	Novel	61.44	5.98	Probable ABC transporter
9401	Novel	50.12	6.63	Transketolase
5402	3.02	47.04	5.24	Arginine deaminase

[a] The induction ratios given are means of the values from several gels.
[b] putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL

The Mw and the pI of each spot carried out by 2D-E analysis were compared to those obtained by databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL trough the TagIdent tool (http://web.expasy.org/tagident/). Putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL are showed in the Table 4.1.

The comparison evidenced that some spots neo-expressed in stressLA, such as 9, 601, 4202 and 1403, are compatible with putative chaperonine (GroES, GroEL and GroES like protein) and protease (Clpx). According to previous studies (Phan-Thanh & Mahouin, 1999) these proteins are of high interest in *Listeria* stress response. Molecular chaperonins are involved in refolding of proteins damaged by stressors (e.g. acid, osmotic, heat stress), instead proteases degrade proteins misfolded.

Noticeable is the neo-formation of spot 3501, compatible with a protein of the glutamate decarboxylase (GAD) system. The GAD system is a complex of decarboxylases and transporters involved during the decarboxylation of glutamate. This mechanism is adopted by *L. monocytogenes* to survive acidic stress (Cotter et al., 2001). According to the current model, an extracellular glutamate (Glt_e) is imported inside the cell through an antiporter system (Glt/GABA) that exchanges for an intracellular ϒ-aminobutyrate ($GABA_i$). The Glt is decarboxylated, and a proton (H^+) is incorporated in GABA. Subsequently, the $GABA_i$ is exported out of cell through the antiporter system that exchanges for another Glt_e (O'Byrne & Karatzas, 2008). Recently, it has been shown that the GAD system can utilize intracellular Glt (Glt_i) to produce $GABA_i$ independently from the antiport; for this reason it has been proposed to divide the GAD system into extracellular (GAD_e) and intracellular (GAD_i) components (Karatzas et al., 2010; Karatzas et al., 2012). It has been showed that the GAD_i system is activated firstly (pH 4.5 to 5.0), than GAD_e (pH 4.0 to 4.5) system in response to acidic conditions (Karatzas et al., 2012).

Moreover, the results evidenced that several spot proteins involved in metabolite transport, such as PTS systems and ABC transporters (spots, 7402, 7801 and 8601) were synthetized by *L. innocua* in presence of stressLA.

The spot proteins 2101 and 3307 compatible respectively with Serine/Treonine Fosfatase stp and Phosphoserine aminotransferase were neo-expressed in stressLA. These proteins are involved in several metabolic vies including the production of glutamate. Glutamate is a substrate involved in the GAD system and its high intracellular concentration could stimulate the GAD intracellular activity (GAD_i).

The spot protein 5402, compatible with the arginine deaminase, a protein of the arginine deiminase (ADI) system was over-expressed. It is known that the ADI system is a metabolic process activated in *Listeria* to respond the acidic stress. In this process, several proteins (ArcA, ArcB, ArcC and ArcD) are involved for the conversion of arginine into ornithine and production of NH_3 (Ryan et al., 2009).

StressCFS-RTB caused several changes in the proteome of *L. innocua* during the exponential phase. 20 spot proteins were neo-expressed and 37 spots resulted over-expressed (Figure 4.3). The 20 spots neo-expressed with stressCFS-RTB resulted neo-expressed also when *L. innocua* was cultivated with stressLA. In Table 4.2 are reported the main proteins induced in *L. innocua* (exponential phase) by stressCFS-RTB.

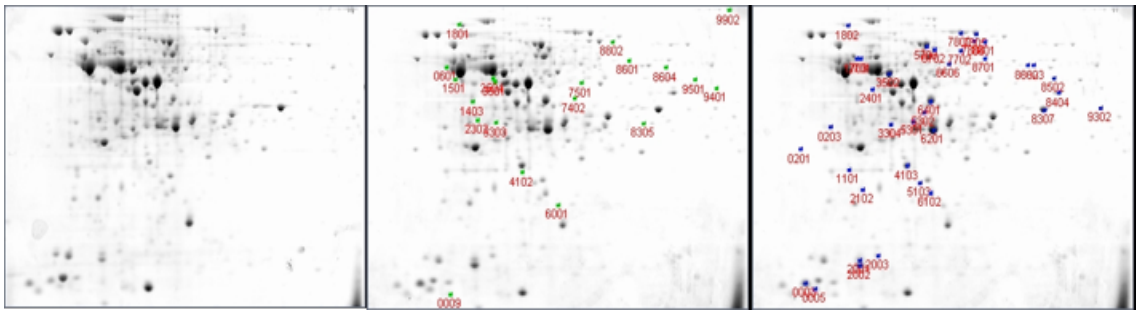

Figure 4.3 2D-E gels of cellular proteome of *L. innocua* cultivated without stress condition (gel left) and in presence of stressCFS-RTB (gel central and right). The proteins of neo-formation (gel central) and over-expressed (gel right) by the stressCFS-RTB are indicated by a dot and an identification number. The pI and molecular weight (MW) of the main proteins neo- and over-expressed are reported in the Table 4.2.

Table 4.2 Main proteins induced in *L. innocua* (log phase) by stressCFS-RTB.

Identification Number	Induction Ratio[a]	Molecular Mass (Kda)	Isoelectric Point (pI)	Putative Protein[b]
9	Novel	10.76	4.45	GroES
601	Novel	59.43	4.63	GroEL
1001	Novel	19.99	4.73	Ycel like family protein
3501	Novel	53.16	4.99	Probable glutamate decarboxylase gamma o GadB
4102	Novel	27.77	5.18	Oxidoreduttase Short chain dehydrogenase
7501	Novel	52.47	5.63	Ribonuclease Y
8601	Novel	61.44	5.98	Probable ABC trasporter
9401	Novel	50.12	6.63	Transketolase
5402	3.02	47.04	5.24	Arginine deaminase

[a] The induction ratios given are means of the values from several gels.
[b] Putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL

The map (Figure 4.4) relative to the proteins of *L. innocua* cultivated in stressCFS-RTB (exponential phase) showed the repression of 14 spot proteins. In Table 4.3 are reported the main proteins repressed in *L. innocua* (exponential phase) by stressCFS-RTB.

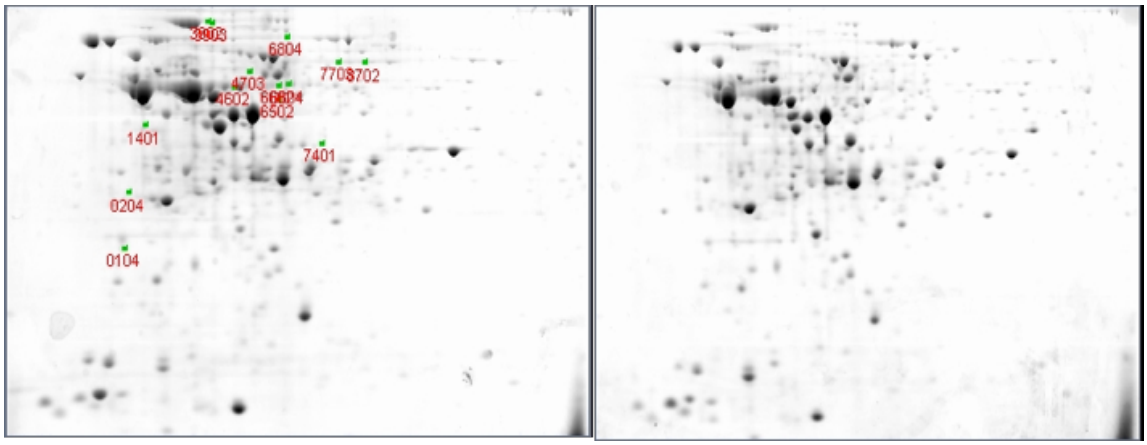

Figure 4.4 2D-E gels of cellular proteome of *L. innocua* cultivated without stress condition (gel left) and in presence of stressCFS-RTB (gel right). The proteins indicated by a dot with the identification number are absent in the gel regarding the stressCFS-RTB.

Table 4.3 Main proteins repressed in *L. innocua* (log phase) by stressCFS-RTB.

Identification Number	Molecular Mass (KDa)	Isoelectric Point (pI)	Putative Protein[b]
3902	93.31	5.09	Leucine-tRNA ligase
3903	93.14	5.11	Protein translocase subunit SecA 1
4602	65.84	5.21	Glutamine--fructose-6-phosphate aminotransferase
4703	71.58	5.28	Potassium-transporting ATPase B chain 1
6502	61.66	5.42	ABC transporter, substrate-binding protein, family 5
6602	66.72	5.42	PTS system, beta-glucoside-specific, IIABC component
6604	67.01	5.47	DNA mismatch repair protein MutL
7703	75.33	5.71	Fructose-1,6-bisphosphatase class 3

[b] Putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Several spot proteins repressed in *L. innocua* during the growth with stressCFS-RTB were compatible with membrane transporters. Of interest is the repression of proteins translocase (Protein translocase subunit SecA 1, spot 3903) associated to uptake of extracellular proteins, as well as the repression of ABC transporters and PTS system (spots 6502, 6602). ABC transporters and PTS system play an important role in the internalization of carbohydrates such as glucose, fructose and mannose (Mitchell et al., 1993; Vu-Khac & Miller, 2009). Other proteins repressed with stressCFS-RTB are ascribable to enzymes involved in the carbohydrate metabolism. For example the Fructose-1,6-bisphosphatase class 3 (spot 7703) and Glutamine--fructose-6-phosphate aminotransferase (spot 4602), involved into carbohydrate metabolism, were absent in the listeria proteome in stressCFS-RTB condition and expressed without stress (control).

The spot 6604 compatible with a MutL (DNA mismatch repair protein) was repressed in *L. innocua* when cultivated with stressCFS-RTB.

The repression of these proteins in *L. innocua* when cultivated in stressCFS-RTB could be responsible of decrement of grow rate of bacteria.

4.3.2.2 CELL PROTEIN IN STATIONARY PHASE

As regard to the stationary phase, the map (Figure 4.5) relative to the proteins of *L. innocua* cultivated in stressLA (stationary phase) showed the neo-formation of 15 spots and the over-expression of 29 spots (Table 4.4).

Table 4.4 Proteins induced in *L. innocua* (stationary phase) by stressLA.

Identification Number	Induction Ratio[a]	Molecular Mass (Kda)	Isoelectric Point (pI)
2	2.97	12.50	4.45
4	2.10	12.04	4.54
5	2.29	12.67	4.56
101	2.37	32.23	4.42
502	4.92	59.43	4.64
1501	3.66	58.15	4.68
1602	3.90	63.12	4.74
1902	3.44	85.21	4.71
2001	3.31	14.77	4.81
2002	2.45	14.30	4.81
2202	2.09	39.09	4.90
2401	2.49	53.24	4.81
2601	2.46	64.01	4.82
3303	1.93	46.67	5.03
3401	7.79	53.48	4.97
3704	2.09	73.59	5.02
4203	3.13	38.43	5.14
4403	0.70	51.43	5.13
4902	2.08	77.51	5.12
4904	2.14	94.66	5.13
5101	1.76	29.04	5.16
5202	1.75	38.36	5.23
5305	2.88	47.38	5.22
6402	1.62	53.49	5.33
6501	3.22	57.17	5.34
7402	2.48	53.14	5.63
7501	2.48	56.48	5.42
7703	2.67	71.46	5.76
1	2.9	12.27	4.31
8	Novel	14.00	4.30
501	Novel	57.32	4.58
703	Novel	70.57	4.64
1001	Novel	11.69	4.67
1002	Novel	19.71	4.73
1901	Novel	85.65	4.68
2103	Novel	16.6	4.97
3102	Novel	30.70	5.00
4101	Novel	25.42	5.06
5102	Novel	27.57	5.16
5601	Novel	68.17	5.23
6201	Novel	38.05	5.29
6601	Novel	63.70	5.28
7503	Novel	58.64	5.76
9201	Novel	36.70	6.98

[a] The induction ratios given are means of the values from several gels.

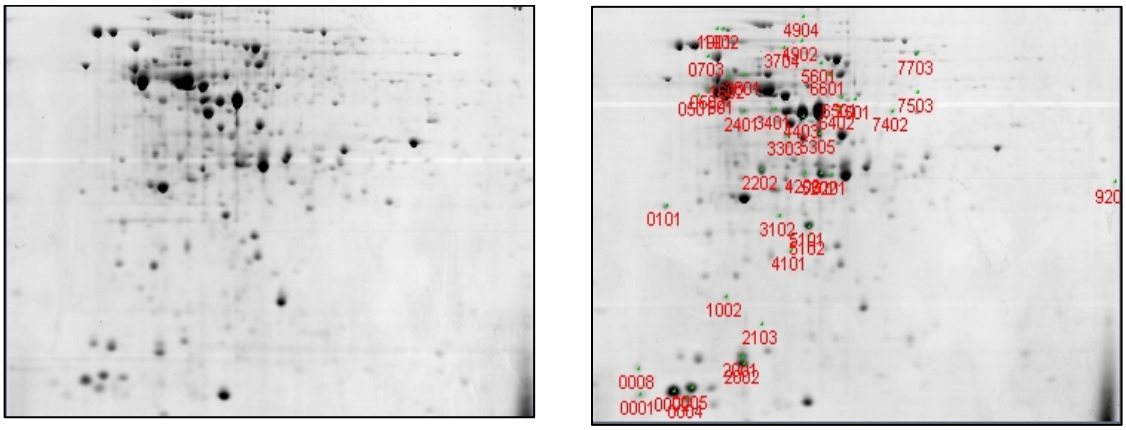

Figure 4.5 2D-E gels of cellular proteome of *L. innocua* cultivated without stress condition (gel left) and in presence of stressLA (gel right). The proteins induced by the stressLA are indicated by a dot and an identification number with pI and molecular weight (MW) given in Table 4.4.

Putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL are shown in the Table 4.5. The spot proteins identified with numbers 1501 and 4203 and compatible with the molecular chaperones GroEL and GroES where over-expressed when *L. innocua* was cultivated with stressLA. The induction of the synthesis of these proteins were observed in *Listeria* by other authors (Phan-Thanh & Mahouin, 1999) in presence of acidic stress, as well as in presence of other stress conditions, including salt and heat stress.

Table 4.5 Putative proteins with a high similarity in Mw and pI with proteins reported into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Identification Number	Induction Ratio[a]	Molecular Mass (KDa)	Isoelectric Point (pI)	Putative Protein
4	2.10	12.04	4.54	Cell division suppressor protein YneA
5	2.29	12.67	4.56	50S ribosomal protein L7/L12
101	2.37	32.23	4.42	Putative uncharacterized protein
1501	3.66	58.15	4.68	GroEL protein
1602	3.90	63.12	4.74	Phosphoenolpyruvate-protein phosphotransferase
2401	2.49	53.24	4.81	ABC transporter, permease protein
3401	7.79	53.48	4.97	Probable glutamate decarboxylase gamma o GadB
3704	2.09	73.59	5.02	DNA ligase (ligA)
4203	3.13	38.43	5.14	GroES-like protein
4902	2.08	77.51	5.12	Putative ATP-dependent Clp protease
5305	2.88	47.38	5.22	Arginine deiminase
7402	2.48	53.14	5.63	Ribonuclease Y
7501	2.48	56.48	5.42	Two-component sensor histidine kinase (LisK)
1	2.9	12.27	4.31	Negative regulation of transcription, DNA-dependent
1001	Novel	10.76	4.65	GroES
1002	Novel	19.71	4.73	YceI like family protein
2103	Novel	16.6	4.97	Putative universal stress protein
5102	Novel	27.57	5.16	Oxidoreductase, short chain dehydrogenase
5601	Novel	68.17	5.23	Sensor protein kinase WalK
6601	Novel	63.70	5.28	Adenine deaminase
7503	Novel	58.64	5.76	DNA-binding response regulator

[a] The induction ratios given are means of the values from several gels.

The spot protein 4902, compatible with a Putative ATP-dependent Clp protease, was also over-expressed. The Clp proteases degrade proteins damaged by acidic conditions or proteins not correctly fold by chaperones.

Of interest was the strong induction (induction ratio, 7.79) of spot 3401, compatible with a protein of the glutamate decarboxylase (GAD) system. As above evidenced, the GAD system is a complex of decarboxylases and transporters involved in *L. monocytogenes* acidic stress response (Cotter et al., 2001).

The spot protein 5305 is compatible with the arginine deaminase, a protein of the arginine deaminase (ADI) system. It is known that the ADI system is involved in a metabolic process activated in *Listeria* to respond the acidic stress. In this process, several proteins (ArcA, ArcB, ArcC and ArcD) are involved for the conversion of arginine into ornithine and production of NH_3 (Ryan et al., 2009).

The spot 4, compatible with the YneA, was also over-expressed in *L. innocua* in presence of stressLA. The YneA is a division suppressor protein that inhibits cell division during the SOS response (van der Veen et al., 2010). *L. monocytogenes* produces SOS response over the acidic stress, also when exposed to heat stress, oxidative stress and mitomycin C (van der Veen & Abee, 2011).

Other spots were induced in *L. innocua* in presence of stressLA, including spots compatible with solutes transporters (1602 and 2401) and spots compatible with stress regulators such as the two-component sensor histidine kinase (LisK) (spot 7501) and the negative regulation of transcription (spot 1).

Among spots of neo-formation (Table 4.5), the spot 2103 resulted compatible with a Universal stress protein. This family of proteins is involved in the response to several stress conditions, including acidic stress and oxidative stress. Their importance in acidic

stress response has been documented but the exact role still unknown (Seifart Gomes et al., 2011).

The spot 6601, compatible with the adenine deaminase, was expressed when *L. innocua* was cultivated with stressLA and it was not expressed without acidic stress. This enzyme could be responsible of the of the intracellular pH increase through the production of NH_3 by degradation of adenine.

These phenomena were not observed in the cell protein 2D-E map obtained by *L. innocua* cultured without stress conditions.

The map relative to the proteins of *L. innocua* cultivated in stressCFS-RTB (Figure 4.6) (stationary phase) showed that the phenomena of protein neo-formation or protein over-expression were less evident than those observed in the map relative to the proteins of the strain cultivated in stressLA. Moreover, in the presence of stressCFS-RTB a repression of several proteins was observed (Figure 4.7). In detail, the map relative to the proteins of the strain in stressCFS-RTB (Figure 4.6 and Figure 4.7) showed the neo-formation of 1 spot (Identification number 1001, M.M. 10.75 KDa; I.P. 4.65), the over-expression of 9 spots (Table 4.6) and the repression of 15 spots (Table 4.7).

The neo-expressed spot 1001, compatible with the GroES protein, was also neo-expressed in presence of stressLA. As reported in literature (Phan-Thanh & Mahouin, 1999) this protein is often expressed in *Listeria* spp. in response to several stress conditions including acidic, salt and heat stress. Among the 9 over-expressed proteins, the spots 1501, 5305 and 7402 were the most interesting. The spot 5305 was compatible with Arginine deaminase which contributes to both growth and survival of the *L. monocytogenes* under acidic conditions. The spot 7402 was compatible with ribonuclease Y, an enzyme that could recycle damaged or unnecessary macromolecules

to satisfy changing cellular needs (Price et al., 2001).

Remarkable results in presence of stressCFS-RTB were represented by the repression of 15 spot proteins (Table 4.7). The repressed spot 4102 was compatible with D-alanine aminotransferase, which is active in the synthesis of glutamate, the starting substrate of glutamate decarboxylase (GAD) system involved in the stress response of *L. innocua*. In presence of stressCFS-RTB the Phosphoenolpyruvate-protein phosphotransferase (compatible with spot 1602) resulted repressed too. The PTS system is essential in the bacteria metabolism, in fact it provides the transport of sugars into the cell and phosphorylates sugars during this transport process (Donaldson et al. 2009).

Moreover, the 2D-E map of *Listeria* in stressCFS-RTB evidenced the repression of five protein spots (4902, 4003, 5503, 7101, 7501) respectively compatible with Putative ATP-dependent Clp protease, Helicase, RecD/TraA family, ATP synthase subunit alpha 2, Beta-glucoside kinase, two-component sensor histidine kinase. As reported by several authors (Chaturongakul et al., 2008; Montague et al., 2009) these proteins are involved in stress response of *Listeria* to several conditions.

So, it is possible to hypothesize that the repression of a series of proteins does not allow the overcoming of adverse and/or lethal conditions produced by stressCFS-RTB. Therefore, the cell free supernatant of *Lb. plantarum* RTB (stressCFS-RTB) determines one or more stress conditions, different from acidic stress, and able to exert a lethal effect against the indicator strain.

Table 4.6 Proteins over-expressed in *L. innocua* (stationary phase) in presence of the stressCFS-RTB. Proteins identified on the basis of the high similarity in Mw and pI with proteins existent into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Identification Number	Induction Ratio[a]	Molecular Mass (KDa)	Isoelectric Point (pI)	Putative Protein
101	2.32	32.23	4.42	Putative uncharacterized protein
1501	3.3	58.15	4.68	GroEL protein
1902	3.16	85.21	4.71	-
2002	2.15	14.30	4.81	-
2202	1.94	39.09	4.9	-
5305	3.45	47.38	5.22	Arginine deiminase
6501	2.89	57.17	5.34	-
7402	1.98	53.14	5.63	Ribonuclease Y
7703	2.89	71.46	5.76	-

[a] The induction ratios given are means of the values from several gels.

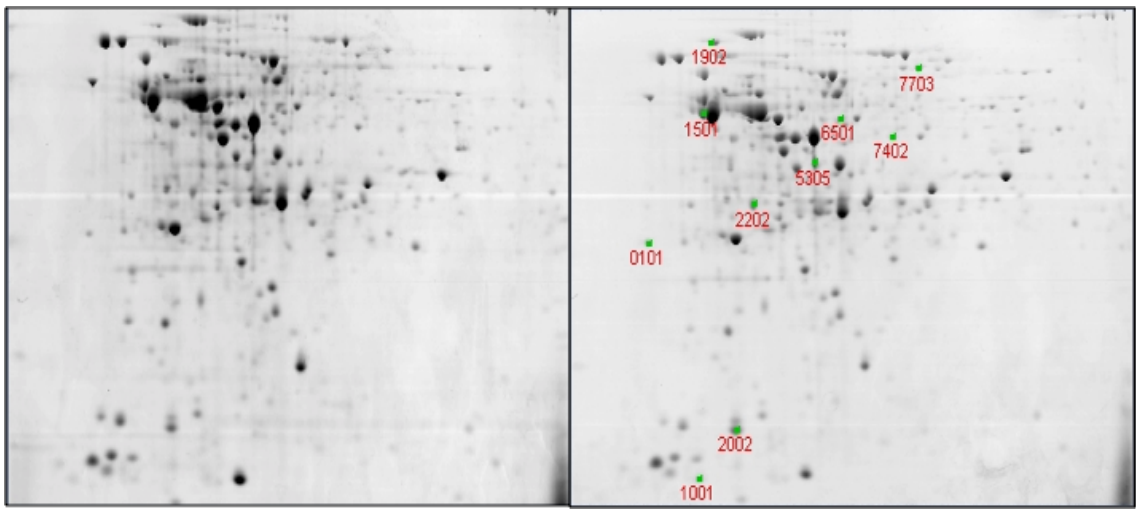

Figure 4.6 2D-E gels of cellular proteome of *L. innocua* (stationary phase) cultivated without stress condition (gel left) and in presence of stressCFS-RTB (gel right). The proteins induced by the stressCFS-RTB indicated by a dot and an identification number with pI and molecular weight (MW) given in Table 4.6.

Table 4.7 Proteins repressed in *L. innocua* (stationary phase) by stressCFS-RTB. Proteins identified on the basis of the high similarity in Mw and pI with proteins existent into databases UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Identification Number	Molecular Mass (KDa)	Isoelectric Point (pI)	Putative Protein
1401	54.80	4.69	-
1602	63.12	4.74	Phosphoenolpyruvate-protein phosphotransferase
2602	60.59	4.83	-
3505	57.49	4.97	-
3602	60.05	5.00	-
3703	68.72	4.96	-
3902	88.99	5.03	Glutathione biosynthesis (GshAB)
4102	32.56	5.13	D-alanine aminotransferase
4702	72.15	5.13	DNA primase
4902	77.51	5.12	Putative ATP-dependent Clp protease
4903	91.03	5.13	Helicase, RecD/TraA family
4904	94.66	5.13	-
5503	56.04	5.27	ATP synthase subunit alpha 2
7101	31.73	5.41	Beta-glucoside kinase
7501	56.48	5.42	Two-component sensor histidine kinase (LisK)

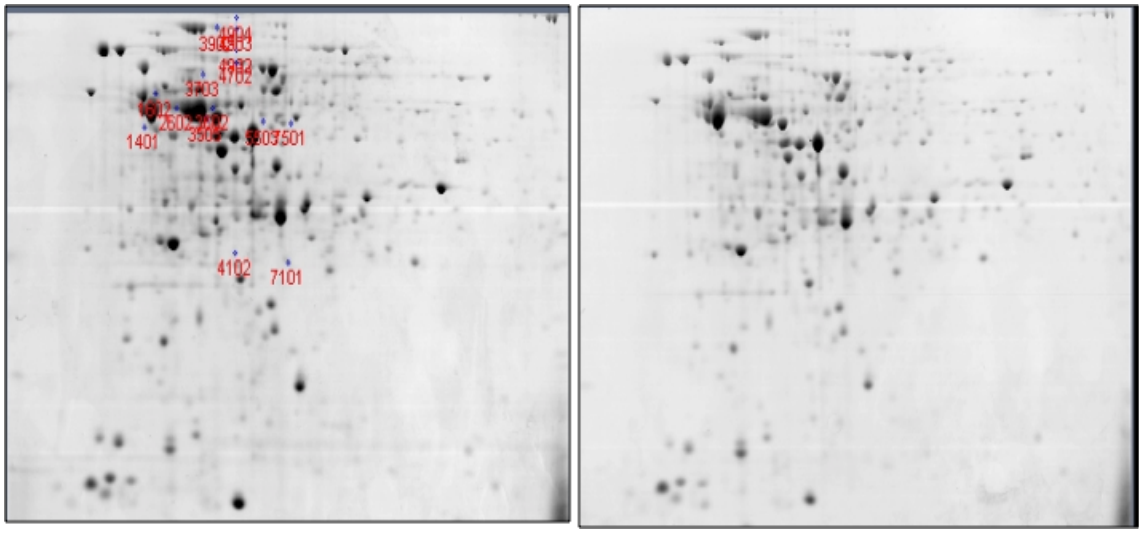

Figure 4.7 2D-E gels of cellular proteome of *L. innocua* (stationary phase) cultivated without stress condition (gel left) and in presence of stressCFS-RTB (gel right). The proteins indicated by a dot with the identification number are absent in the gel regarding the stressCFS-RTB.

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STRUCTURE PREDICTION OF A UNIVERSAL STRESS PROTEIN (USP) IN *LISTERIA INNOCUA*

5.1 INTRODUCTION

Universal stress proteins (Usps) are small cytoplasmic proteins that are found in bacteria, Archea, fungi and plants. The production of these proteins is stimulated when bacteria is exposed for a long time to stressors, such as stationary phase, starvation, exposure to heat, oxidants, uncouplers, ethanol, antibiotics, acid and osmotic stress (Kvint et al., 2003). Usps have been observed for the first time in *Escherichia coli* K-12 and subsequently have been described for several species, including *Haemophilus influenzae* (Fleischmann et al., 1995; Sousa & McKay, 2001), *Mycobacterium tuberculosis* (O'Toole & Williams, 2003; Drumm et al., 2009), *Pseudomonas aeruginosa* (Schreiber et al., 2006; Boes et al., 2008), Salmonella typhimurium (Liu et al., 2007; Sagurthi et al., 2007) and *Lactobacillus plantarum* (Licandro-Seraut et al., 2008; Gury et al., 2009). Recently, the importance of Usps in acid or oxidative stress response in *Listeria monocytogenes* has been reported (Seifart Gomes et al., 2011). Six Usps have been found in *E. coli*, five of witch (UspA, UspC, UspD, UspF, UspG) characterized by a single domain whilst one (UspE) presents two domains (Nystrom & Neidhardt, 1992; Gustavsson et al., 2002). On the basis of sequence similarity Usps may be divided in four groups: i) UspA-type proteins (UspA, UspC, UspD), ii) UspFG-type proteins (UspF, UspG), iii) UspE1 and, iv) UspE2 (Sousa & McKay, 2001;

Nachin et al., 2008). The two main groups of Usps (UspA-type and UspFG-type) may assume a dimeric and a tretrameric structure. This characteristic would seem to be related to the absence (dimer) or presence (tetramer) of ATP in the structure. Usually the UspA-type form a dimer, while the UspFG-type assumes a tetrameric structure where the ATP plays an important role in the stabilization of the molecule.

Few informations are available in literature about the biochemical function and 3D-structures of Usps in bacteria and there are no3D-structures for Usp of *Listeriae*. available.

Bioinformatics approach can help to get more information about the structure of Usps and the function of these proteins.

In the present study, the three-dimensional (3D) structure of a Universal stress protein (EHN60729.1) belonging to *L. innocua* was predicted on the basis of the available template (PDB code:3S3T ; structure deposited by Osipiuk et al., 2011) homologues from Protein Data Bank.

5.2 MATERIALS AND METHODS

5.2.1 TEMPLATE SELECTION AND SEQUENCE ALIGNMENT

The FASTA sequence (152 aa) of universal stress protein (number access EHN60729.1) from *Listeria innocua* was obtained from the NCBI database (National Center for Biotechnology Information). Comparative modeling (Notredame et al., 2000) was used to build the protein 3D structure. The target sequence was searched into the Protein Database (PDB) using BLASTp (protein-protein Basic Local Alignment Search Tool). The Usp (PDB code: 3S3T) from *Lactobacillus plantarum* showed the highest score with 31% of identity, therefore was used as template for the homology modeling analysis. This value is at the borderline of the so-called twilight region of protein

sequences (ref), but we decided to proceed to the modelling anyway and to perform more tests later on the accuracy of the model. The sequence alignment (Figure 5.1) between template (3S3T chains A, B, C, D) and the Usp of *Listeria* was performed with the T-COFFE program (Notredame et al., 2000).

5.2.2 COMPARATIVE MODELING

The theoretical structure of Universal stress protein (EHN60729.1) from *Lactobacillus plantarum* was generated using MODELLER v9.10. MODELLER implements comparative protein structure modeling by satisfaction of spatial restraints (Sali & Blundell, 1993). Two hundred models were generated and the best model (lower DOPE score, -0.443) was used for validation.

```
3s3t chain_A       1 N-ARYTNILVPVDSSDAAQAAFTEAVNIAQRHQANLTALYVVDDSAYHTPALDPVLSELLDAEAAH   65
USP                1 MLQQYERVLVAVDGSKEAERAFQKAIQVANRNDAALGLVHVIDTRAFSSVAN--YDTSMADKATEY   64
consenso           1    :* .:**.**.*. *: ** :*:::*:::* *  ::*:*  *: : *     :.: *  : :   66

3s3t chain_A      66 AKDAMRQRQQFVATTSAPNLKTEISYGIPKHTIEDYAKQHPEIDLIVLGATGTNSPHRVAVGSTTS  131
USP               65 ADELLSGYKEDALKAGVTKVESYIEYGSPKTAITKEAAKAFQADLIMCGATGLNAVERLLIGSVSE  130
conserved         67 *.: :   :: . .:...:::: *.** ** :* . * :  : ***: **** *: .*: :**.:.  132

3s3t chain_A     132 YVVDHAPCNVIVI--------R  145
USP              131 YIIRHSPCDVLVVRNDVPDYKE  152
conserved        133 *:: *:**:*:*:         .  154

3s3t chain_B       1 --ARYTNILVPVDSSDAAQAAFTEAVNIAQRHQANLTALYVVDDSAYHTPALDPVLSELLDAEAAH   64
USP                1 MLQQYERVLVAVDGSKEAERAFQKAIQVANRNDAALGLVHVIDTRAFSSVAN--YDTSMADKATEY   64
conserved          1    :* .:**.**.*. *: ** :*:::*:::* *  ::*:*  *: : *     :.: *  : :   66

3s3t chain_B      65 AKDAMRQRQQFVATTSAPNLKTEISYGIPKHTIEDYAKQHPEIDLIVLGATGTNSPHRVAVGSTTS  130
USP               65 ADELLSGYKEDALKAGVTKVESYIEYGSPKTAITKEAAKAFQADLIMCGATGLNAVERLLIGSVSE  130
conserved         67 *.: :   :: . .:...:::: *.** ** :* . * :  : ***: **** *: .*: :**.:.  132

3s3t chain_B     131 YVVDHAPCNVIVI--------R  144
USP              131 YIIRHSPCDVLVVRNDVPDYKE  152
conserved        133 *:: *:**:*:*:         .  154

3s3t chain_C       1 N-ARYTNILVPVDSSDAAQAAFTEAVNIAQRHQANLTALYVVDDSAYHTPALDPVLSELLDAEAAH   65
USP                1 MLQQYERVLVAVDGSKEAERAFQKAIQVANRNDAALGLVHVIDTRAFSSVAN--YDTSMADKATEY   64
conserved          1    :* .:**.**.*. *: ** :*:::*:::* *  ::*:*  *: : *     :.: *  : :   66

3s3t chain_C      66 AKDAMRQRQQFVATTSAPNLKTEISYGIPKHTIEDYAKQHPEIDLIVLGATGTNSPHRVAVGSTTS  131
USP               65 ADELLSGYKEDALKAGVTKVESYIEYGSPKTAITKEAAKAFQADLIMCGATGLNAVERLLIGSVSE  130
conserved         67 *.: :   :: . .:...:::: *.** ** :* . * :  : ***: **** *: .*: :**.:.  132

3s3t chain_C     132 YVVDHAPCNVIVI--------R  145
USP              131 YIIRHSPCDVLVVRNDVPDYKE  152
conserved        133 *:: *:**:*:*:         .  154

3s3t chain_D       1 N-ARYTNILVPVDSSDAAQAAFTEAVNIAQRHQANLTALYVVDDSAYHTPALDPVLSELLDAEAAH   65
USP                1 MLQQYERVLVAVDGSKEAERAFQKAIQVANRNDAALGLVHVIDTRAFSSVAN--YDTSMADKATEY   64
conserved          1    :* .:**.**.*. *: ** :*:::*:::* *  ::*:*  *: : *     :.: *  : :   66

3s3t chain_D      66 AKDAMRQRQQFVATTSAPNLKTEISYGIPKHTIEDYAKQHPEIDLIVLGATGTNSPHRVAVGSTTS  131
USP               65 ADELLSGYKEDALKAGVTKVESYIEYGSPKTAITKEAAKAFQADLIMCGATGLNAVERLLIGSVSE  130
conserved         67 *.: :   :: . .:...:::: *.** ** :* . * :  : ***: **** *: .*: :**.:.  132

3s3t chain_D     132 YVVDHAPCNVIVI--------R  145
USP              131 YIIRHSPCDVLVVRNDVPDYKE  152
conserved        133 *:: *:**:*:*:         .  154
```

Figure 5.1 Sequence alignment between the hypotetical Usp of *L. innocua* and the chains A, B, C and D of template (PDB code: 3S3T). The conserved regions are indicated by '*'.

5.2.3 MODEL VALIDATION

The best model of *L. innocua* Usp protein according to the DOPE score (Model_195) was validated with PROCHECK and QMEAN Servers. PROCHECK was used for a preliminary evaluation of the stereochemical quality of Model_195 (Laskowski et al.,

1993).

QMEAN server was used to evaluate the QMEAN Z-score of Model_195. The QMEAN Z-score provides an estimate of the absolute quality of a model by relating it to reference structures solved by X-ray crystallography (Benkert et al., 2011).

To evaluate the similar degree of three dimensional structure between the template and the Model_195, the Root Mean Squared Deviation (RMSD) was calculated using PyMol software. The lower the value, the more similar the structures are (Maiorov & Crippen, 1994).

5.2.4 INTERFACES ANALYSIS

The interfaces between monomer-monomer or dimer-dimer of Model_195 were evaluated using PISA WebServer. The surface and the energetic state of interfaces were evaluated. Furthermore the presence of residues with a key role in dimer formation was investigated through a multi structural alignment. For this aim MUSTANG software (Konagurthu et al., 2006) was used to perform a multiple structural alignment between a single chain of Model_195 and homology protein structures.

5.3 RESULTS

5.3.1 COMPARATIVE MODELING OF UNIVERSAL STRESS PROTEIN MODEL

Three dimensional structure of hypothetical Universal stress protein belonging to *L. innocua* was predicted by the homology modeling technique. The final 3D structures created with MODELLER were stored as PDB output file and the best model (Model_195) with a lower DOPE score (-0.443) was used for both validation and interfaces analysis. Model_195 was visualized by PyMol program and has been

represented in the figure 5.2. Model_195 seems to represent the quaternary structure of Usp with a tetrameric conformation. Four monomers (chain A, B, C and D) with a 3D structure similar to the Rossmann-like α/β fold have 5 parallel β-strands and 4 α-helices.

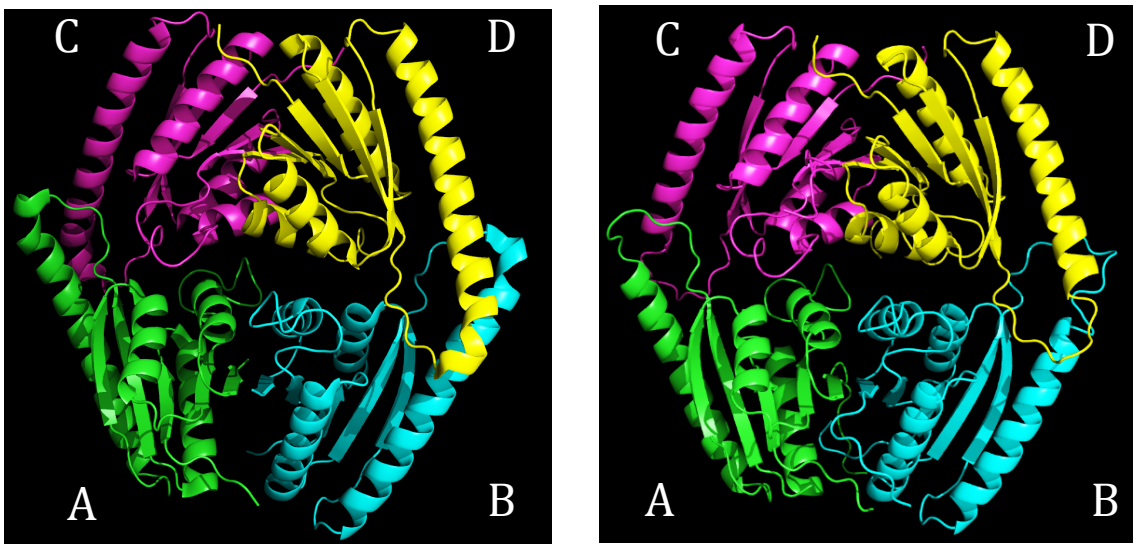


Figure 5.2 Cartoon representation of the final 3D structure of the Universal stress protein model from *L. innocua* (right); 3D structure of template (3S3T) from *Lb. plantarum* (left). The chain A (green), chain B (cyan), chain C (magenta) and chain D (yellow) are distinguish by different colours.

5.3.2 UNIVERSAL STRESS PROTEIN MODEL VALIDATION

Two approaches were used to evaluate the accuracy of the protein model generated. The first was based on the stereochemical properties of model, the second, more accurate method was based on the absolute quality of the structure.

To check the torsion of dihedral angles phi and psi of amino acid residue of Model_195 PROCHECK was used and the Ramachandran plot is reported in the figure 5.3.

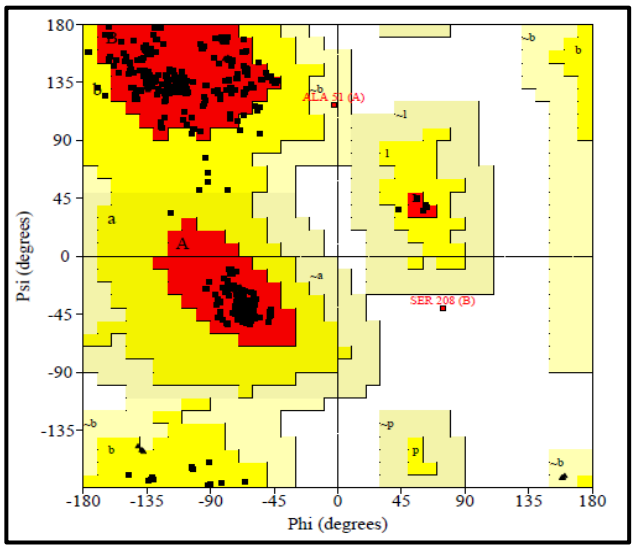


Figure 5.3 Ramachandran plot of hypothetical Universal stress protein (Model_195) of *L. innocua.*

Ramachandran plot analysis showed that main-chain conformations for 93.3% of amino acid residues are within the most favoured region only two residues (ALA 51 e SER 208) are in the disallowed region. In general, a score higher to 90% implies good stereochemical quality of the models.

The good quality of Model_195 was also showed by the good distribution of normalized QMEAN Z-score (Figure 5.4).

The circles of different shades of grey colour in the plot represent the QMEAN scores of the reference structures from the PDB. The model's QMEAN score is compared to the scores obtained for experimental structures of similar size (model size +/- 10%) and a Z-score is calculated (Benkert et al., 2011). The QMEAN Z-score of Model_195 is represented in the plot by the red dot.

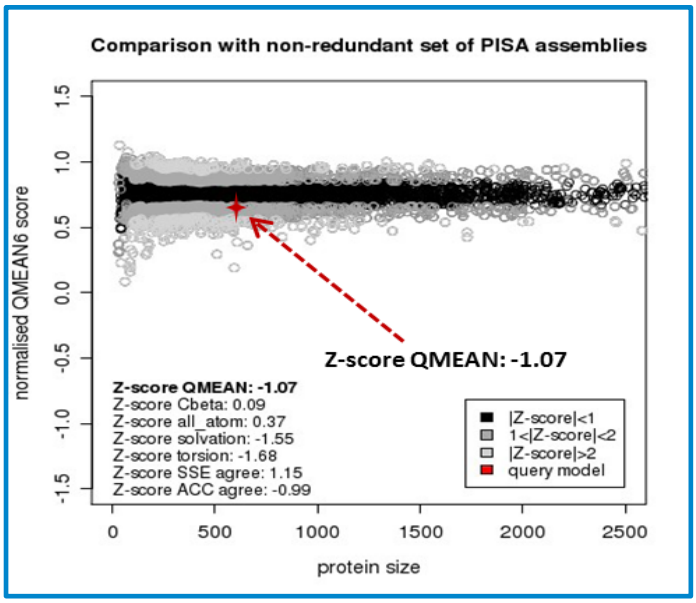


Figure 5.4 Distribution of normalized QMEAN Z-score. The red dot represents the QMEAN Z-score of Model_195.

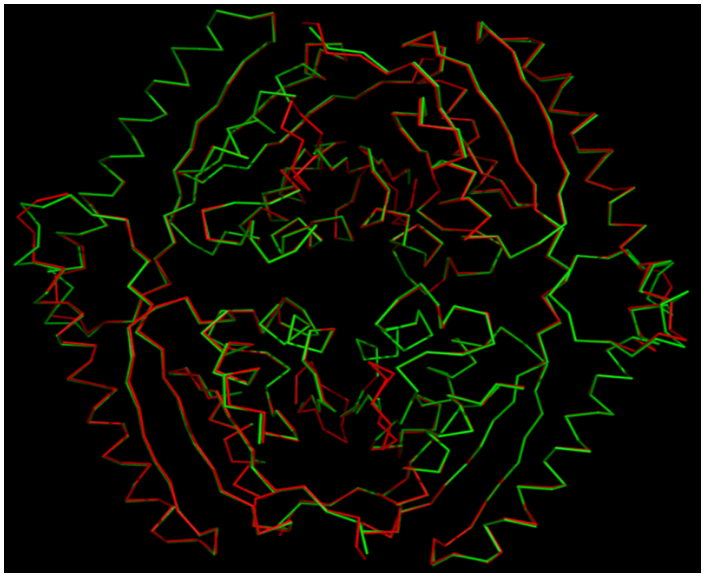
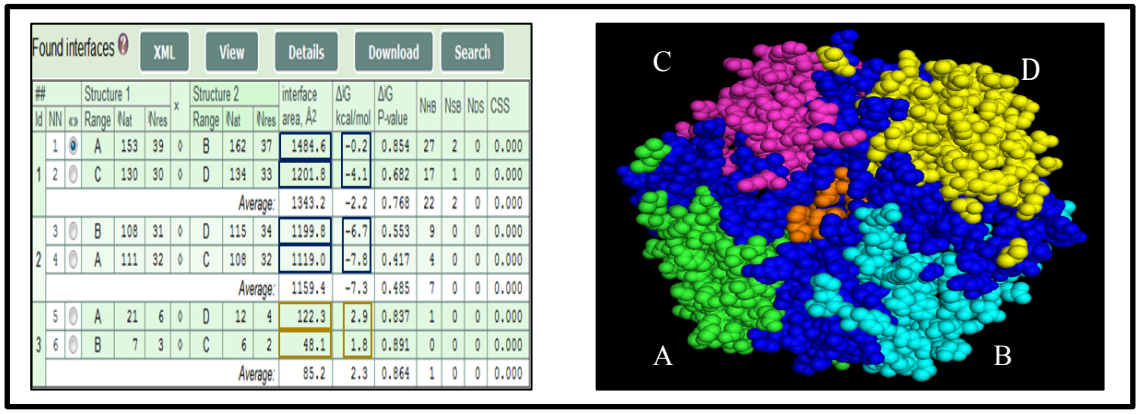


Figure 5.5 Superposition of Cα trace of Universal stress protein model (Model_195) from *L. innocua* (represented in red color) and the template 3S3T (represented in green color) from *Lb. plantarum*.

The close relationship between the predicted Model_195 and the template (3S3T) was evidenced by the low RMSD of 0.3 Å (Figure 5.5). Superposition of Cα trace of Universal stress protein model (Model_195) from *L. innocua* (red) and the template 3s3t (green) from *Lb. plantarum* was very close, small differences was observed between the interface of chains A and B as well as between the chains C and D.

5.3.3 INTERFACES ANALYSIS

To understand if the quaternary Model_195 structure may be significant from the biological point of view, the exploration of macromolecular interfaces of the quaternary structures predicted were investigated. In the figure 5.6 are reported the results of the interfaces analysis performed with PISA WebServer and the graphical representation of residues involved in the interfaces.



##	Id	NN	Structure 1 Range	Nat	Nres	x	Structure 2 Range	Nat	Nres	interface area, Å2	ΔG kcal/mol	ΔG P-value	Nhb	Nsb	Nds	CSS
1	1		A	153	39		B	162	37	1484.6	-0.2	0.854	27	2	0	0.000
	2		C	130	30		D	134	33	1201.8	-4.1	0.682	17	1	0	0.000
									Average:	1343.2	-2.2	0.768	22	2	0	0.000
2	3		B	108	31		D	115	34	1199.8	-6.7	0.553	9	0	0	0.000
	4		A	111	32		C	108	32	1119.0	-7.8	0.417	4	0	0	0.000
									Average:	1159.4	-7.3	0.485	7	0	0	0.000
3	5		A	21	6		D	12	4	122.3	2.9	0.837	1	0	0	0.000
	6		B	7	3		C	6	2	48.1	1.8	0.891	0	0	0	0.000
									Average:	85.2	2.3	0.864	1	0	0	0.000

Figure 5.6 Number and surface area (left) and graphical representation (right) of interfaces of Model_195. The interface residues with ΔG < 0 are represented in blue whilst the interface residues with ΔG > 0 are represented in orange.

Results show that six interfaces have been found in the Model_195. The interface area between the chains A and B was of 1484.6 $Å^2$ and the formation resulted energetically favorited (ΔG <0). Also the interface area between the chains C and D was energetically favorite (ΔG <0) with a surface area of 1201.8 $Å^2$. These data show that the monomers A and B as well as the monomers C and D may assume a dimeric structural conformation. Furthermore, the interfaces between the chains A and C even the interfaces between the chains B and D were also energetically favorited (ΔG <0), and the surface interfaces were of 1119.0 $Å^2$ and 1199.8 $Å^2$ respectively. These results show that the tetrameric assembly between the two hypothetical dimers structure of Model_195 is biologically possible. On the other hand, the two interfaces between the chains A-D and the chains B-C were not energetically favorited.

The presence of residues with a key role in dimer formation was investigated with the multiple structural alignment between the chain A of Model_195 and proteins of homologue structure (Figure 5.7).

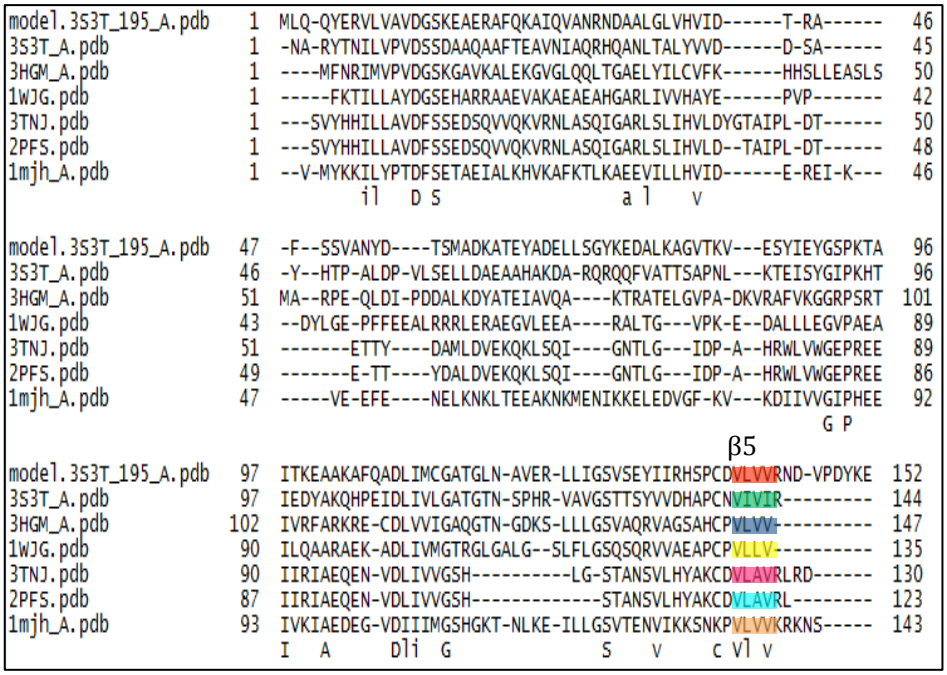


Figure 5.7 Multiple structural alignment of sequences between the chain A of Model_195 and proteins of homologue structure. Residues coloured are conserved in all the structures.

Residues involved in dimer formation take a β sheet conformation and are allocated in the β5 of Model_195. The β5 residues are strongly conserved in the structures of similar proteins and show an important role in dimer formation in the protein structures experimentally resolved.

Of interest are the two conserved residues (Figure 5.8) of VAL 140 and VAL 142 (in β5 sheet) of Model_195 that show an important role in dimer formation trough hydrogen bonds. This observation is in accord with previous studies for TeaD (Usp) protein by *H. elongata* (PDB: 3HGM) (Schweikhard et al., 2010).

Figure 5.8 Superposition of multiple structural alignment between the chain A of Model_195 and proteins of homologue structures. Conserved residues of β5 sheet are coloured (see Figure 5.7) and the two dots represents the position of VAL140 and VAL142.

By the multiple structural alignment between Model_195 and proteins of homologue structure (Figure 5.9) was showed the presence of the conserved ATP-binding motif [GXXGXXXXXXXXXG(S/T)] (O'Toole & Williams, 2003). The ATP-binding motif, is located in the interface between the two dimers of our model (Figure 5.10), suggesting the "capability" of Universal stress protein model of *L. innocua* to bind the ATP. It has in fact shown that the presence of ATP in the interface between the two dimers of Universal stress protein may be responsible of tetrameric assemblies whilst Usp without ATP-binding motif have a dimeric structure.

```
model.3S3T_195_A.pdb    1  MLQ-QYERVLVAVDGSKEAERAFQKAIQVANRNDAALGLVHVID------T-RA------   46
3S3T_A.pdb              1  -NA-RYTNILVPVDSSDAAQAAFTEAVNIAQRHQANLTALYVVD------D-SA------   45
3HGM_A.pdb              1  ----MFNRIMVPVDGSKGAVKALEKGVGLQQLTGAELYILCVFK------HHSLLEASLS   50
1WJG.pdb                1  -----FKTILLAYDGSEHARRAAEVAKAEAEAHGARLIVVHAYE------PVP-------   42
3TNJ.pdb                1  ---SVYHHILLAVDFSSEDSQVVQKVRNLASQIGARLSLIHVLDYGTAIPL-DT------   50
2PFS.pdb                1  ---SVYHHILLAVDFSSEDSQVVQKVRNLASQIGARLSLIHVLD--TAIPL-DT------   48
1mjh_A.pdb              1  --V-MYKKILYPTDFSETAEIALKHVKAFKTLKAEEVILLHVID------E-REI-K---   46
                                  il   D S                 a l    v

model.3S3T_195_A.pdb   47  -F--SSVANYD----TSMADKATEYADELLSGYKEDALKAGVTKV---ESYIEYGSPKTA   96
3S3T_A.pdb             46  -Y--HTP-ALDP-VLSELLDAEAAHAKDA-RQRQQFVATTSAPNL---KTEISYGIPKHT   96
3HGM_A.pdb             51  MA--RPE-QLDI-PDDALKDYATEIAVQA----KTRATELGVPA-DKVRAFVKGGRPSRT  101
1WJG.pdb               43  --DYLGE-PFFEEALRRRLERAEGVLEEA----RALTG---VPK-E--DALLLEGVPAEA   89
3TNJ.pdb               51  -------ETTY----DAMLDVEKQKLSQI----GNTLG---IDP-A--HRWLVWGEPREE   89
2PFS.pdb               49  -------E-TT----YDALDVEKQKLSQI----GNTLG---IDP-A--HRWLVWGEPREE   86
1mjh_A.pdb             47  -----VE-EFE----NELKNKLTEEAKNKMENIKKELEDVGF-KV---KDIIVVGIPHEE   92
                                                                              G P
                                       GXXGXXXXXXXXGS/T

model.3S3T_195_A.pdb   97  ITKEAAKAFQADLIMCGATGLN-AVER-LLIGSVSEYIIRHSPCDVLVVRND-VPDYKE  152
3S3T_A.pdb             97  IEDYAKQHPEIDLIVLGATGTN-SPHR-VAVGSTTSYVVDHAPCNVIVIR---------  144
3HGM_A.pdb            102  IVRFARKRE-CDLVVIGAQGTN-GDKS-LLLGSVAQRVAGSAHCPVLVV----------  147
1WJG.pdb               90  ILQAARAEK-ADLIVMGTRGLGALG--SLFLGSQSQRVVAEAPCPVLLV----------  135
3TNJ.pdb               90  IIRIAEQEN-VDLIVVGSH----------LG-STANSVLHYAKCDVLAVRLRD------  130
2PFS.pdb               87  IIRIAEQEN-VDLIVVGSH-------------STANSVLHYAKCDVLAVRL--------  123
1mjh_A.pdb             93  IVKIAEDEG-VDIIIMGSHGKT-NLKE-ILLGSVTENVIKKSNKPVLVVKRKNS-----  143
                               I   A      Dli  G              S    v     c Vl v
```

Figure 5.9 Sequences multiple structural alignments between the chain A of Model_195 and proteins of homologue structure. Residues coloured represents the ATB-binding motif.

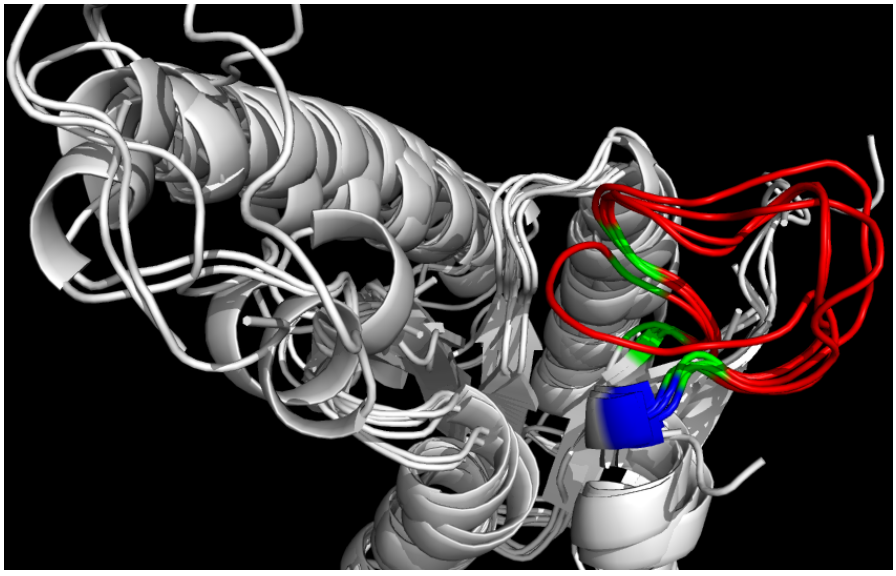


Figure 5.10 Superposition of multiple structural alignment between the chain A of Model_195 and the proteins of homologue structure. Conserved residues of the ATP-binding motif are coloured according to the alignment (see Figure 5.9).

5.4 DISCUSSION AND CONCLUSIONS

The Usps are proteins expressed under different stress conditions. The role of these proteins is still unknown and the literature is lacking of information about the structure

and function of the Usps. These proteins have about 150 amino acid and can assume a dimeric and/or a tetrameric structure. This characteristic may be associated with the capacity to bind or not the ATP. Several studies showed that some Usps are not able to bind ATP (UspA-Type), while other crystallize with ATP (UspFG-Type). Usually the UspA-Type form a dimer, while the UspFG-Type assume a structure where the tetrameric ATP plays an important role in the stabilization of the molecule (Schweikhard et al., 2010).

Currently the 3D structure of the Usp of *Listeria* is not known, but there is evidence that these proteins are involved in resistance to acid stress and oxidative stress. Gomes et al., (2010) have shown that mutants of *L. monocytogenes* devoid of genes (Δlmo0515, Δlmo1580 and Δlmo2673) which encode for the Usp, showed a reduced growth capacity and resistance in the presence of acid stress and oxidative stress both in vitro and in vivo. Due to the lack of data regarding the Usps, the bioinformatics approach can help to get more knowledge about the structure and function of these proteins. The Homology modeling uses the structure of proteins experimentally determined (template) to predict the 3D structure of a protein that has a similar amino acid sequence (target). The comparative homology modeling approach can be used when the template and target possess at least 30% identity. In the present study the hypothetical Usp of *L. innocua* shares

31% amino acid with the template 3S3T which corresponds to about 85% of the C-α with 3.5 Å from the correct position. The accuracy of the model is confirmed by the values of the torsion angles *phi* and *psi* showed in the Ramachandran plot as well as the QMEAN Z-score. The RMSD (0.3 Å) confirms the evolutionary relationship between the model and the template.

Of interest are the results regarding the analyses of the interfaces carried out with both PISA WebServer and with the multiple structural alignment (MUSTANG). The surface of the interfaces ($\Delta G < 0$, see Figure 5.6) is amongst the average values of expected for homologous proteins, but even more interesting is the presence of highly conserved residues in the region involved in the formation of the dimer and of residues that represent the ATP-binding motif, which is fundamental in the formation of the tetramer. This work suggests that *L. innocua* possesses a UspFG-Type and that this protein can assemble in a tetrameric structure. The results of this study, although to be confirmed experimentally, provide important information about a poorly studied protein and may stimulate experimental investigations.

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